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EGG TRANSFER IN CATTLE

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EGG TRANSFER IN CATTLE

Edited by

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SEMINAR ON EGG TRANSFER IN CATTLE IN THE EEC PROGRAMME OF CO-ORDINATION OF RESEARCH ON BEEF PRODUCTION

Organised by

AGRICULTURAL RESEARCH COUNCIL
Unit of Reproductive Physiology and Biochemistry
Animal Research Station
307 Huntingdon Road
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PREFACE

This publication contains the Proceedings of a seminar held in the United Kingdom on December 9-12, 1975, under the auspices of the Commission of the European Communities, as part of the EEC programme of co-ordination of research on beef production.

The programme was drawn up by a scientific working group on "Physiology of Reproduction" on behalf of the Beef Production Committee. The working group comprised Dr. E.P. Cunningham (Chairman), Ireland; Mr. L.E.A. Rowson, United Kingdom; Professor C. Thibault, France; Dr. H. Karg, Germany (Fed. Rep.); and in the planning of the seminar they were joined by Dr. J.C. Tayler (temporarily seconded to the CEC during 1975) and Mr. P. L'Hermite, CEC.

The subject chosen for this seminar was drawn from the list of priorities in research objectives drawn up in 1973 by members of a Committee (now the Standing Committee on Agricultural Research, CPRA) in the form given in Appendix I. One of the functions of this series of seminars was to summarise and update the information available on the selected subjects and to discuss future needs for research, so as to assist the Commission in evaluating the probable impact of research on agricultural production within the Community.

The Commission wishes to thank those representatives of the Member States who took responsibility in the organisation and conduct of this seminar; notably Mr. L.E.A. Rowson (Chairman and local organiser) and his colleagues at the ARC Unit of Reproductive Physiology and Biochemistry, Cambridge.

Thanks are also accorded to the Chairmen of sessions, Mr. L.E.A. Rowson, Dr. D. Whittingham, Dr. C. Polge and Professor A. Robertson.

OBJECTIVES

The aims of the seminar were to review recent research on the techniques employed in the production, presentation, culture and transfer of ova in cattle; to consider the practical aspects of these methods in relation to livestock improvement and increased production of beef, and to discuss possible lines of future research.

BACKGROUND

The development of egg transfer could lead to a rapid improvement of beef cattle by increasing the genetic gain through selection. The possibilities of twinning require investigation, particularly as it affects the beef cow or dairy cow used for beef crossing. There is a necessity for developing techniques to obtain more eggs, either by superovulation or in vitro fertilisation, and also for the development of non surgical techniques both for the recovery and transfer of eggs. The problems of synchronisation of oestrus have been largely overcome, as will be indicated in this symposium, by the use of either progestogens or prostaglandin $F_{2\alpha}$. The question of prolonged storage of eggs is now a practical possibility and if this could be linked with sex determination it would greatly speed the rate of progress.

SESSION ONE

EGG PRODUCTION

Superovulation and Egg Removal

Chairman: L.E.A. Rowson

THE ENTRY OF SUPEROVULATED EGGS INTO THE UTERUS

R. Newcomb, L.E.A. Rowson and A.O. Trounson
ARC Unit of Reproductive Physiology and Biochemistry, Cambridge

ABSTRACT

A group of 123 heifers were superovulated with 2,000 i.u. PMSG and oestrus synchronised with PGF2a.

Laparotomy was performed between Days 3 to 8 after oestrus and the degree of stimulation, represented by numbers of follicles (> 10mm) and ovulations on each ovary, was estimated. In order to examine the position of the eggs within the tract methods for recovering them from the oviduct and sections of the uterus were devised. The degree of stimulation of the right and left ovary was similar and was not influenced by the situation of the regressing corpus luteum. The proportion of eggs recovered was not affected in a local manner by the degree that the right or left ovary was stimulated nor was the total recovery affected by the magnitude of the total ovarian response.

The proportion of eggs recovered from the oviduct was similarly unaffected by the ovarian picture, but there was a decrease in the proportion of oviduccal eggs with increasing time after oestrus. Most eggs (73%) on Days 6, 7 and 8 were recovered from the uterus within 10cm of the utero-tubal junction (26 animals). There was a significantly greater proportion of degenerate eggs recovered from the uterus than from the oviduct, but the cleavage stages of normal eggs from each section were similar.

The proportion of degenerate forms increased with time after oestrus and increased significantly between Days 7 and 8.

In a group of 14 heifers eggs were flushed from one oviduct into the uterus at Day 3 and the utero-tubal junctions ligated. When the eggs were later recovered 69.4% of uterine eggs were degenerate, but only 18.5% of oviduct eggs. It is concluded that premature entry into the superovulated uterus has a deleterious effect on eggs.

INTRODUCTION

Various factors have been shown to affect the success of surgical egg transfer in the bovine. These include the medium used and the degree of synchronisation of oestrus of the donor and recipient. (Rowson, Moor & Lawson, 1969; Rowson, Lawson, Moor & Baker, 1972; Sreenan & Beehan, 1974; Newcomb & Rowson, 1975_1). Provided that there is close synchronisation of oestrus and a suitable transfer medium is used then a high conception rate may be achieved by means of surgical transfer.

It has more recently been shown that the success of surgical egg transfer to the bovine uterus is affected also by the age of the eggs transferred. Where Day 3 eggs were transferred to a Day 3 recipient very low success rates were achieved, but a significantly higher success rate resulted from the transfer of Day 4 eggs to a Day 3 uterus ($P < 0.01$) even though these transfers were asynchronous (Newcomb & Rowson, 1975_1). Subsequently it has been demonstrated that the bench storage of Day 3 eggs in medium 199 impairs their development in the rabbit oviduct, but that the morula stage is more resistant and is unaffected (Trounson, Willadsen, Rowson & Newcomb, 1976). It is now well established that the resistance of bovine eggs to imposed external factors, including deleterious media, cooling and freezing, increases with age (Wilmut, Polge & Rowson, 1975; Trounson et al. 1976; Willadsen, Trounson, Polge & Rowson, 1976).

The subjection of young eggs to an early uterine environment which in some way is inhospitable to them, is unlikely to explain completely the low success rate after their transfer, since expulsion from the uterus may also be important.

The expulsion of microspheres transferred to the uterus at this time both surgically and non-surgically is very rapid (1½ hr after injection) (Harper, Bennett & Rowson, 1961; Rowson, Bennett & Harper, 1964). It has been found that this rapid ejection of microspheres only occurs in the early luteal phase, but that at later stages retention is good (Tervit, 1973; Lawson, Rowson, Moor & Tervit, 1975). Likewise the success rate of eggs transferred non-surgically on Days 3 to 5 is very low but improves with later stage transfers (Days 6 to 9) (Lawson et al. 1975).

After the normally expected time of entry of bovine eggs into the uterus, that is Day 4 after oestrus (Hamilton & Laing, 1946), a significantly lower surgical recovery of superovulated eggs is achieved compared with recovery before the fourth day (Newcomb & Rowson, 1975_2). Grossly abnormal blood steroid levels have been demonstrated in superovulated heifers and it has been suggested that higher levels of oestrogen in particular might be responsible for accelerated transport and expulsion of eggs in some cases (Booth, Newcomb, Strange, Rowson & Sacher, 1975).

For the purpose of egg transfer a high recovery rate of normal eggs from superovulated donors is critically important. It was considered that knowledge of factors adversely affecting egg quality and retention might be gained by the study of the distribution and morphological appearance of superovulated eggs throughout the tract at different times after oestrus and the experimental exposure of them to an early uterine environment. Such information would also be of importance to the efficient recovery of eggs by non-surgical means.

MATERIALS AND METHODS

Heifers of mixed breeding, but predominantly Hereford crosses, and of approximately 350-400 kg were superovulated by the intramuscular injection of 2,000 i.u. of a preparation of PMSG in 10 ml of physiological saline, followed 2 days later by Prostaglandin F2a (either 1 mg of ICI 80996 or 25 mg Prostin, Upjohn). At oestrus, 2 days after the prostaglandin injection, the heifers were inseminated with liquid semen. Oestrus was recorded as Day 0 and surgical recovery of eggs undertaken between Days 3 to 8 after oestrus. The medium used for recovery was TCM 199 or modified Dulbecco's Phosphate Buffered Saline (PBS) (Whittingham, 1971) and egg identification and morphological assessment was made using a stereo dissecting microscope. In Experiment 3 only, all abnormal eggs were stained and examined at higher magnification (X 400). Prior to the surgical repair of the laparotomy incision the number of corpora lutea and the number and size of follicles on each ovary were recorded.

Experiment 1. Heifers (123) were subjected to surgery and the tract flushed using the method described for the simultaneous flushing of oviduct and uterus described by Newcomb & Rowson (1975_3). The average volume of air

used to inflate the cuff of the urological catheter and thereby to occlude the uterus was 9.5 ml and was a distance of 25 cm from the utero-tubal junction. By first flushing eggs from the uterus (Fig. 1b) and then directing the flow of the medium through the oviduct (Fig. 1c), separation of eggs from the two sites was achieved. Sixty ml of medium was used for this procedure.

Experiment 2. In 26 donors umbilical tape was used to ligate each uterine horn 10 cm from the utero-tubal junction. The basal part of each uterine horn was first flushed with 30 ml of medium (Fig. 1a) and then the ligature removed and a double flush performed as in Experiment 1.

This method of flushing first removed eggs from the basal section of uterine horn, followed by those retained in the tip of the uterus by the ligature and also identified oviduccal eggs.

Experiment 3. 14 donors were subjected to double laparotomy. At the first laparotomy at Day 3 a small volume either of saline or PBS was infused into one oviduct via the fimbria expelling any eggs it contained into the uterus. Both oviducts were then ligated at the utero-tubal junction and the laparotomy incision repaired. After an interval of 4 or 5 days a further laparotomy was performed and a double flush performed as in Experiment 1. One uterine horn was first flushed and then the ligature at the UT junction on that side was removed and fluid directed through the oviduct. Although on one side all eggs should have been recovered from the oviduct only and on the other from the uterine horn, such an approach enabled this to be confirmed.

RESULTS

Experiment 1. The numbers of follicles ($>$ 10mm) and ovulations in relation to the right and left ovary and the situation of the regressing corpus luteum (corpus albicans) were examined in 37 animals. The raw data were transformed to square roots before analysis of variance. There was no evidence of an effect of side of ovary on the numbers of follicles and ovulations nor any unilateral effect of the corpus albicans, and there were no interactions of any combination of factors. In this series there were significantly more ovulations than follicles ($P \lesssim 0.026$).

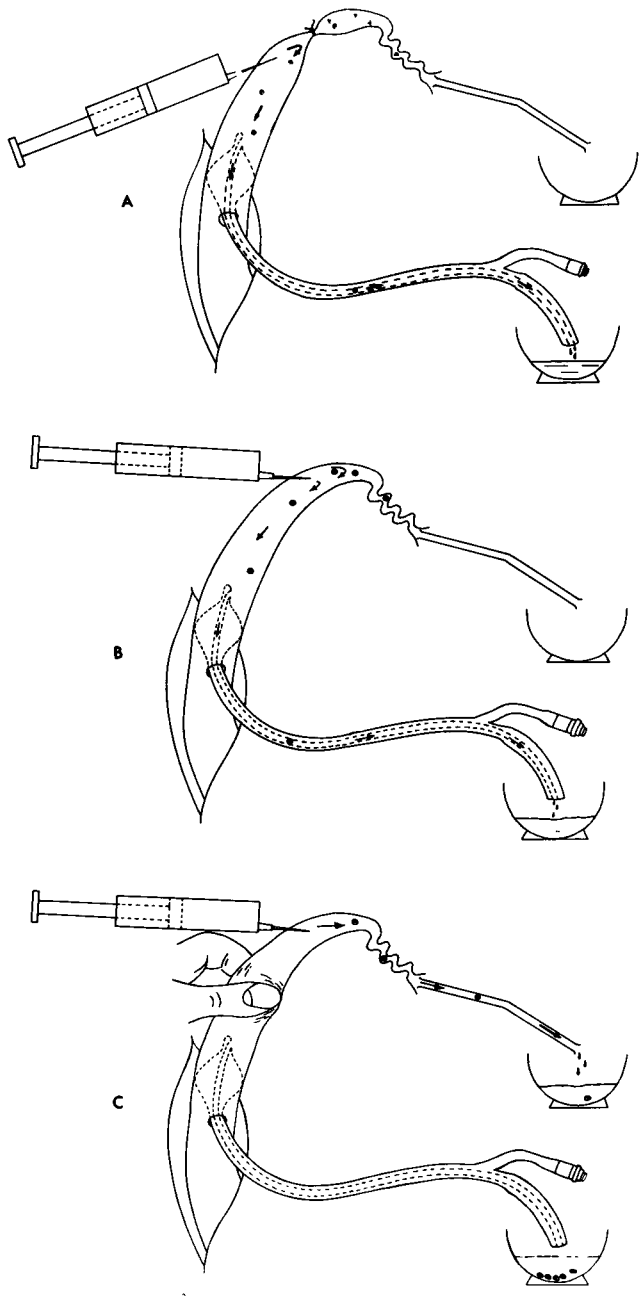

Fig. 1 The surgical recovery of eggs from the bovine genital tract a) from the uterine base, b) the uterus, c) from the oviduct.

Table 1. Cleavage stages of eggs and their location in relation to the interval from oestrus to surgical recovery

CELL CLEAVAGE		The number of eggs and their cleavage stage recovered on different days after oestrus				
Stage	Code	Day 3	Day 5	Day 6	Day 7	Day 8
2-cell	1	8	2	1	-	-
4-cell	2	11	2	-	-	-
8-cell	3	81	24	2	7	-
Morula (16-32 cell)	4	30	175	10	8	1
Morula (32-64 cell)	5	-	55	187	73	4
Blastocyst	6	-	-	5	94	37
Hatched blastocyst	7	-	-	-	17	30
Mean cell stage		3.0±0.1	4.1±0.1	4.9±0.1	5.5±0.1	6.3±0.2
Empty zona		-	13	10	11	4
1-cell (unfertilised)		13	28	3	22	8
Degenerate eggs		3	55	63	75	79
Total eggs recovered		146	354	281	307	163
Number of heifers		13	33	29	33	15
Oviduct eggs as a proportion of total eggs. Mean ± S.E.		96.5±5.3	17.4±3.0	14.2±3.9	7.9±3.6	6.7±4.8

To examine the relationship between egg recovery and numbers of follicles and ovulations a weighted covariance analysis was carried out (115 animals). There was no evidence that recovery from the left side differed from the right, or that follicle numbers or ovulations had any effect on recovery rate. The average percentage recovery of eggs from the left side was 60.0 $\pm$ 2.5% and 59.6 $\pm$ 2.5% from the right, and for both sides was 59.8 $\pm$ 1.8%.

The proportion of eggs recovered from the oviduct and the cleavage stages of eggs recovered on different days after oestrus are recorded in Table 1. Predictably, there was a very large effect of the interval from oestrus to the day of surgery on the proportion of eggs recovered from the oviduct but there was no difference due to the total number of follicles and ovulations.

The cleavage stages from 2-cell to hatched blastocysts were coded 1 - 7 and a weighted average egg stage was computed (109 animals) for each day after oestrus that surgical recovery was performed and appear in Table 1. A similar mean egg stage was computed for animals (30) from which eggs were recovered both from oviduct and uterus. There was no evidence of any difference between the uterus and oviduct in the stage of development of eggs implying a similar rate of cleavage and a similar population of eggs. It is therefore unlikely that the eggs recovered from the oviduct represent a more recently ovulated population.

The proportion of degenerate eggs from the oviduct were compared with those from the uterus for animals where eggs were recovered from both locations (40 animals). There was a greater proportion of degenerate eggs recovered from the uterus ($P \eqsim 0.05$). In this same group of animals there was an increase in the number of degenerate eggs with increasing time after oestrus, this effect of time being small except for the 8th day when there was a dramatic increase in the proportion of degenerate eggs. There was no evidence of an interaction between the uterine and oviduct effect with the day after oestrus.

The proportion of degenerate forms recovered from all animals (123) at various days after oestrus is recorded schematically in Fig. 2. The large increase in the proportion of degenerate eggs between Days 7 and 8 was significant ($P < 0.01$).

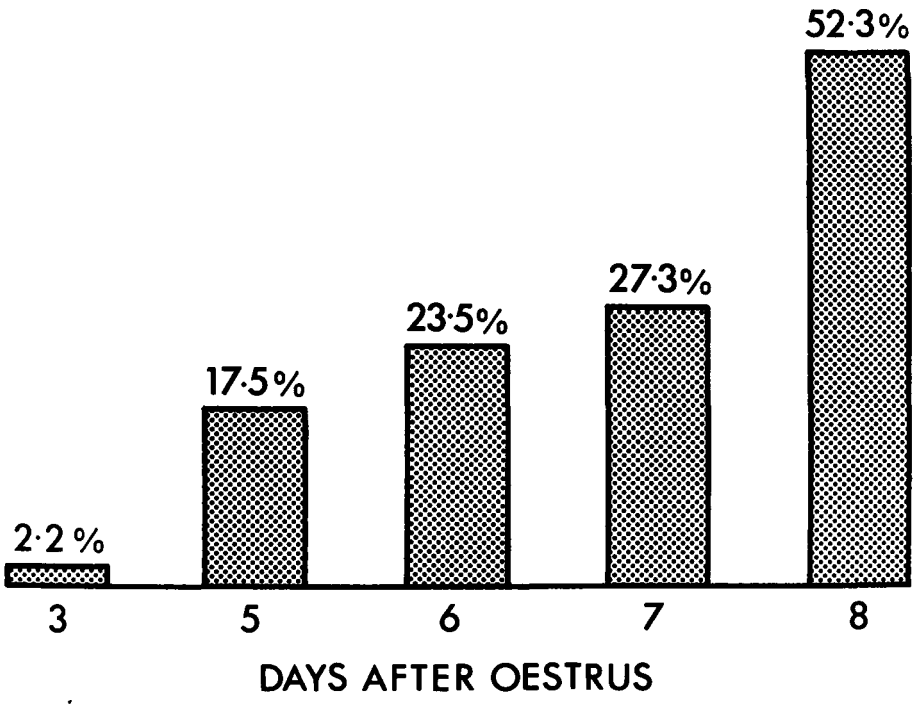

Fig. 2 Degenerate eggs as a proportion of morphologically normal and degenerate eggs recovered at various intervals after oestrus (Empty zonae and unfertilised eggs excluded from data)

Experiment 2. Combined data from Days 6, 7 and 8 are depicted in Fig. 3. The number of animals in each daily group was small (Day 6, 10 animals; Day 7, 6 animals; Day 8, 10 animals) but, as in Experiment 1, there was a greater proportion of eggs in the oviduct at Day 6 (11.4%) than Days 7 and 8.

Experiment 3. The results of Experiment 3 are recorded in Table 2. Where eggs were flushed into the uterus at Day 3, 69.4% were degenerate at recovery compared with 18.5% of eggs which had been ligated in the contralateral oviduct. After staining, the majority of degenerate eggs were found to be at the morula stage. Recovery of eggs was low, particularly from the uterus (32%), and it was not considered justified to subject the results of this experiment to statistical analysis. However a trend is

quite apparent in the results and in 6 of 9 animals where eggs were recovered from both uterus and oviduct, the proportion of morphologically normal eggs recovered from the oviduct was better than from the uterus, but in only 2 of the 9 animals was the converse true. In only one case (cow 08) were eggs retrieved from the wrong site, suggesting in this case that the ligature may have insufficiently occluded the utero-tubal junction, or that the eggs had entered the uterus prior to the ligation.

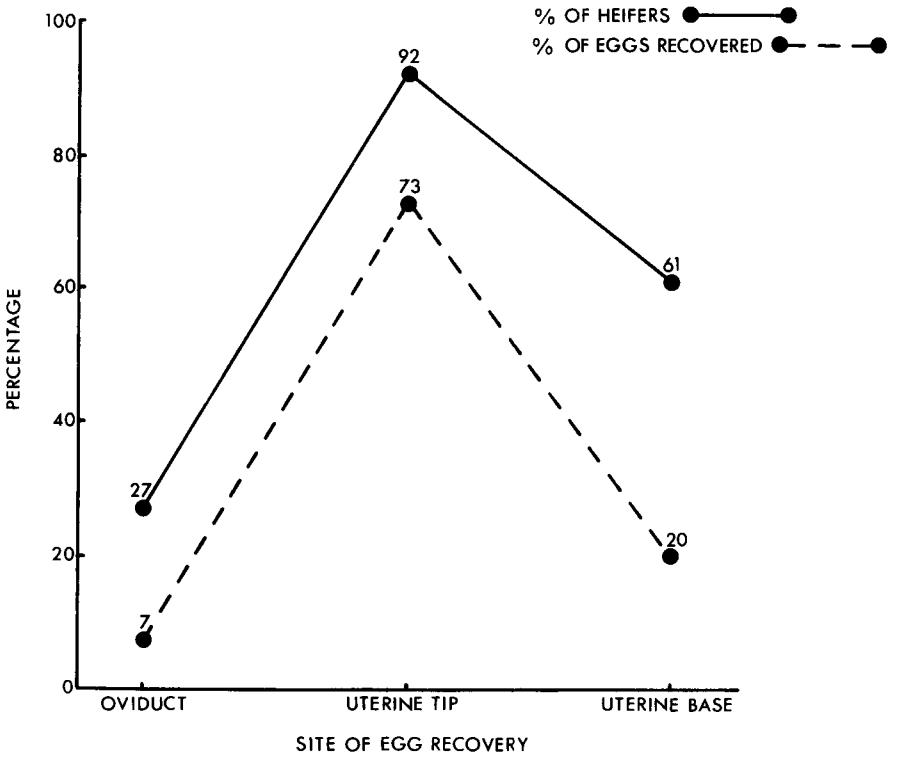

Fig. 3 The percentage of heifers with eggs in the oviduct, uterine tip (<10cm from the UT junction) or uterine base and the percentage of eggs recovered from these regions on days 6, 7 & 8 after oestrus

Table 2. The number and normality of eggs surgically recovered from one uterine horn and the contralateral oviduct. (Fluid was flushed through the ipsilateral oviduct and into the uterus at Day 3 after oestrus and both utero-tubal junctions ligated.

COW NO.	OVIDUCT (Flushed at Day 3 after oestrus) Side	Volume ml.	Medium used	EGG RECOVERY DAY AFTER OESTRUS	UTERINE SIDE No. of ovulations	Total* egg recovery	No. of normal eggs	No. of degenerate eggs	OVIDUCT SIDE No. of ovulations	Total* egg recovery	No. of normal eggs	No. of degenerate eggs
59	R	0.5	Dulbecco	7	2	1	0	1	8	7	6	1
42	R	0.5	Saline	7	3	3	2	1	5	1	1	0
60	R	0.5	Saline	7	4	2	1	0	7	5	4	1
53	L	0.5	Saline	7	6	0	-	-	9	5	3	1
61	R	0.5	Dulbecco	8	8	1	0	1	15	11	6	1
02	R	1.0	Dulbecco	7	25	5	0	4	22	11	9	2
22	L	1.0	Saline	7	27	12	0	12	25	6	1	3
35	R	1.0	Saline	7	8	1	0	0	11	2	2	0
30	R	1.0	Saline	7	5	3	1	2	3	2	2	0
36	L	2.0	Saline	7	4	0	-	-	7	6	5	1
111	L	0.5	Saline	8	8	3	3	0	2	3	2	0
25	L	0.5	Saline	8	2	1	1	0	5	2	0	2
+08	R	0.5	Dulbecco	8	5	4	0	4	7	0	-	-
43	R	0.5	Dulbecco	7	5	0	-	-	7	4	1	0
TOTALS					112	36	8	25	133	65	42	12
PERCENTAGES					-	32%	22%	69.4%	-	49%	64.6%	18.5%

+ 3 eggs recovered from oviduct side were in uterus and were included in uterine data

* Includes unfertilised eggs and empty zonae

CONCLUSIONS

The absence of any difference between the right and left ovaries in relation to numbers of ovulations and follicles is in agreement with earlier findings. Hafez, Sugie & Gordon (1963) found that the total number of follicles developed and the percentage of them which ovulated was almost identical for the right and left ovaries. They also found that super-ovulatory response in the ovary where corpus luteum enucleation was performed was similar to that in the other gonad.

The recovery of superovulated eggs does not appear to be influenced in any local manner by the numbers of follicles and ovulations in the ipsilateral ovary, nor does the total number of follicles and ovulations appear to influence egg recovery. Scanlon, Sreenan & Gordon (1968) did not find any difference in egg recovery rate when comparing animals with 1 - 12 ovulations (71% recovery) and animals with more than 24 ovulations (70%). However, Hafez et al. (1963) did find that the proportion of ova recovered tended to fall with increasing numbers of ovulations, although the factors associated with poor recovery were not necessarily related to high ovulation rates.

The rapid decline in the proportion of eggs in the oviduct between Days 3 and 5 (Table 1) coincides with the expected time of entry of bovine eggs into the uterus (Hamilton & Laing, 1946). However, the persistence of eggs within the oviduct after this period, also noted by Hafez et al. (1963), and the significantly lower proportion of degenerate forms recovered from this site has implications for both surgical and non-surgical methods of egg recovery. A surgical recovery method which does not also recover the apparently superior eggs from the oviduct, in addition to eggs from the uterus, will be less efficient. Likewise, since non-surgical methods of egg recovery involve flushing the uterus only, they will be less efficient than surgical methods. In this respect the findings of Experiment 2 emphasise the importance of flushing the tip of the uterus and indicate that acceptable non-surgical recovery of eggs should be possible using an apparatus with a fixed distance between fluid outlet and recovery, such as the Rowson-Dowling apparatus (1949) and that the use of a variable distance apparatus, such as the Sugie apparatus (Sugie, Soma, Fukumitsu & Otsuki, 1972) is not imperative to success.

Both factors affecting the proportion of degenerate eggs recovered, viz. oviduct cf. uterus, and time after oestrus, may not be equally important in the unsuperovulated animal. The lower proportion of degenerate eggs recovered from the oviduct in Experiments 1 & 3 were anticipated by the findings of Newcomb & Rowson (1975_1) of an age effect on conception rate after uterine transfer. However the increase in degenerate forms with increasing time from oestrus to egg recovery and the dramatic increase at Day 8 could be the result of the superovulated environment. The grossly abnormal hormonal pattern and, in particular, the massive blood levels of oestrogen at Day 6 (Booth et al. 1975) imply a progressive asynchrony between egg and uterus which could entirely explain these findings. Support for this conclusion is also provided by evidence indicating that the removal of superovulated Day 3 eggs and subsequent transfer to the rabbit oviduct results in 85% showing normal development at Day 7 (Trounson et al. 1976). The considerable increase in the proportion of degenerate eggs at Day 8 is most probably a real effect but could partly be explained by the subjective manner in which these degenerate forms are recognised. It is interesting that most of the eggs stained in Experiment 3 were degenerating at the morula stage. It would be expected that these eggs would not be readily recognised at the moment of degeneration, but that this degeneration would be more apparent with time.

These experiments suggest that early removal of eggs from the superovulated tract is desirable and although not directly applicable to the normal animal the results of Experiment 3 further support the idea that premature entry into the uterus has an adverse effect on eggs. Premature entry of superovulated eggs into the uterus has been shown to occur because 3.5% of Day 3 eggs were recovered from it (Experiment 1). Although this is a low proportion of the recovered eggs, since efficiency of egg recovery is 70% or less (Newcomb & Rowson, 1975_2; Scanlon, Sreenan & Gordon, 1968), it is open to speculation whether the eggs which were unaccounted for had been expelled from the tract after premature entry into the uterus or whether they were not recovered or were never picked up by the fimbria. The low recovery rate of eggs from the uterus in Experiment 3 suggests that expulsion had occurred. Although this is a difficult area in which to work it would seem desirable to study further egg transport in the superovulated and in the normal animal and in particular the time of entry of eggs into the normal uterus. Such information and a deeper knowledge of mechanisms involved in

synchrony of egg and uterus might have considerable application not only to egg transfer, but to raising the conception rate of cattle.

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DISCUSSION

R.Church (Canada)

To what do you attribute the low recovery rate, especially in your oviduct versus uterine recovery? These seem to be abnormally low and I wonder if this might not be interpreted within your data as finding them where you expected them to be.

R.Newcomb (UK)

Do you mean the last experiment which I reported where there were only 32% of eggs recovered from the uterus?

R.Church

Even the 50% I find a little bit low.

R.Newcomb

Well, certainly the 50% from the oviduct is low. Normally, as I pointed out, where we are recovering oviduccal eggs we would expect something like a 70% recovery rate. I really can't explain why it should have been low other than the possibility that eggs may have gone through the ligatures which we placed on the UT junctions or that these ligatures themselves had perhaps increased motility in the rejection of eggs and that this had been responsible for the loss. But I did also mention that we didn't find eggs, except in one case, in the wrong site - they were all recovered from the site where we would have expected them to be secured by the ligature. I think that the recovery from the uterus - 30% - was only to be expected because at Day 3 one would expect quite a reasonable degree of motility in the uterus and if one was flushing them in even with what we considered to be a small volume, usually 0.5 ml of fluid, this is still probably rather an unphysiological amount, so I would have expected that. What I can't explain is the low recovery from the oviduct itself.

A.O.Trounson (UK)

Could I make a comment. I think in the rabbit, unless we have a double ligature on the oviduct when we are transferring eggs, especially when Willadsen and myself were doing early transfers, we find that the eggs will pass very rapidly through from the oviduct into the uterus and end up in the vagina. I think this is probably the case in the cow - putting

enough pressure on the ligature at the utero-tubal junction. Those that do pass through are very likely to pass all the way out.

C.Polge (UK)

I am a bit perturbed Mr. Newcomb, that possibly you might be painting too black a picture if we are to interpret your figures correctly. It appears that on Day 5 or 6, which is the time most people are working at to collect and transfer eggs at the present, you are suggesting that one can only collect 50% of the eggs and that 50% of these will be degenerate. So presumably, working on superovulation in the cow, we might only expect 25% of the ovulated follicles to be represented by viable eggs which are worth transferring. Could you say whether this is correct or not?

R.Newcomb

Well, I think in practical terms it is probably fairly near the truth, but in actual fact the proportion of degenerate forms at Day 5 was 17%, at Day 6 it was 23%, at Day 7 it was 27% - not 50%. The recovery rate that we had in our first experiments in total approached 60%. It was only in the last experiment that the recovery rates were desperately low and I think one has got a very abnormal situation there. But if one has a 60% recovery and less than 20% degenerate forms then things are not quite as black as you paint them. Certainly, the results of transfers commercially aren't terribly good regarding the number of young which result from transfers.

B.Shea (Canada)

Mr. Newcomb mentioned that 17% of the eggs were recovered from the oviduct on Day 5. I just wonder, did this represent something which is fairly consistent across the board or does it represent the majority of animals where the majority were recovered from the uterus and some animals where the majority were recovered from the oviduct?

R.Newcomb

Certainly, that was the situation in some cases and this applied to Day 7 and Day 8 also. Sometimes one would come across an animal where the majority of eggs were still within the oviduct. I haven't looked at the proportion of animals, I have only looked at the proportion of eggs, but there were quite a number of animals with eggs within the oviduct - it wasn't just one or two animals which were producing this effect. Certainly, in some

cases you would recover ALL the eggs from the oviduct at Day 5. This was not just an odd animal effect which had biased these results. Of 123 animals totally, there were 13 at Day 3, about 30 at Days 5,6 and 7 and 15 at Day 8. Amongst that lot 40 had eggs within the oviduct and uterus. So it certainly wasn't just an odd animal, it was an overall effect.

A.O.Trounson

I wonder if I could make one more point. We quite often get normal eggs with degenerate eggs from the uterus and from the oviduct. If you think it is the effect of the uterus per se, or the uterine environment, one would think that this would be operative on all of the eggs whereas a proportion of the eggs are still normal. I think this does raise a question about the ovulated oocytes, especially with the very high ovulation rate that we get in the superovulated cow, whether they do, in fact, have the potential to develop into normal blastocysts. I think there is a definite effect of the uterus and I wonder if a proportion of this effect could be due to poor oocytes or non-normal follicles from which they come.

R.Newcomb

No doubt there could be a difference in sensitivity between different eggs but one would have expected that premature entry itself could have been a factor. One would also expect that superovulated eggs, in particular, would be at several different stages of progress. I understand from people who have done endoscopy work that you can have superovulation taking place over a period of about 30 hours which, in this case, would explain fairly well the increased resistance of some eggs. If one looks at the experiments which we have done on cooling and so forth, there is the progressive increase in resistance with age. If you have got a population of eggs which differs by as much as 30 hours one would have expected to find this sort of range, so it may not be quite so important.

S.M.Willadsen (UK)

I think both Mr. Newcomb and Dr. Trounson have got points there in their respective ways but I think the rabbit experiment on Day 3 eggs, where we removed isolated eggs on Day 3, transferred them to rabbits, and recovered them on Day 7, would tend to suggest that these superovulated eggs are all right. The proportion which went on in those experiments, which represents quite a fair number of cows, was quite high, and much higher than what we were getting from the Day 5 eggs.

METHODS OF INDUCTION OF SUPEROVULATION IN THE COW AND TRANSFER RESULTS

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INTRODUCTION

The application of egg transfer techniques will depend on a consistent supply of viable fertilised ova being available for use in such programmes. It has been established that the bovine neonatal female ovaries contain many thousands of oocytes (Erickson, 1966) and this has been the basis of many attempts to induce large numbers of these potential ova to be matured and fertilised both in vivo and in vitro.

Attempts to effect in vitro fertilisation of artifically matured ova have not however been successful in the bovine to date (Sreenan, 1970).

A number of studies involving the induction of superovulation in prepuberal calves have been reported (Onuma & Foote, 1969; Onuma et al. 1969; Onuma et al. 1970; Seidel et al. 1970, 1971). Both ovum recovery and fertilisation rates have generally been low following treatment in prepuberal animals.

Most of the reports dealing with the supply of fertilised ova for use in culture or transfer studies have concentrated on the hormonal induction of superovulation in sexually mature animals and have been reviewed (Foote & Onuma, 1970; Gordon, 1975). Basically, this involves the administration of compounds with follicle stimulating properties (FSH) to augment the endogenous levels of FSH, combined some 3 - 6 days later with pituitary luteinising hormone or compounds with similar properties. Many attempts to induce multiple ovulations in cattle have involved the use of pituitary extracts (Casida et al. 1943; Umbaugh, 1951; Avery et al. 1962; Avery & Graham 1962). Because of their rapid elimination from the body, however, pituitary extracts have to be administered daily or twice daily for periods of up to five days. One commonly used serum, gonadotrophin, pregnant mares' serum (PMSG) has the advantage of being both easily available and more slowly eliminated so that it is effective when administered as a single dose. With PMSG, the normal

TABLE 1 RESPONSE TO SUPEROVULATION TREATMENTS ADMINISTERED DURING THE FOLLICULAR PHASE OF THE CYCLE IN A NUMBER OF STUDIES

Gonadatrophin Treatment	No. Animals	Mean No. + (range) Follicles	Mean No. + (range) Ovulations	% Ova Recovered	% Ova Cleaved
Days 16-20 : 20,10,10,10 mg FSH + 5 mg LH each day Day 5 : 100 mg LH	32	-	21 (4 - 55)	53	61
Day 16 : 3,000 iu PMS Days 19, 20 : 20 mg 17 B oestradiol Day 21 : 2,000 iu HCG	14	59	33 (14 - 104)	50	54
Day 16 or 17 : 4,000-7,000 iu PMS Days 19, 20 : 20 mg 17 B oestradiol Day 21 : 2,000 iu HCG	-	-	12 (2 - 28)	47	-
Day 16 : 3,000 iu PMS Day 21 or oestrus 2,000 iu HCG	89	15	9 (0 - 55)	71	68
Progestogen synchronised + 2.5 mg FSH 2 x daily, Days 8 - 12	7	27	15 (1 - 32)	63	84

(From Foote & Onuma, 1970)

treatment regime in sexually mature animals has been to administer a single dose (usually between 1,500 and 3,500 i.u.) either subcutaneously or intramuscularly during the follicular phase of the cycle (Days 15 or 16), in many cases combined with an ovulating hormone preparation administered at or near oestrus to augment the endogenous levels of ovulating hormone (LH) at that time (Hammond Jnr. & Bhattacharya 1944; Hammond Jnr. 1949; Rowson, 1951; Hafez et al. 1963; Scanlon et al. 1968, and many others).

All of these reports have indicated that this method of superovulation leads to great individual variation in follicular development and ovulation rate following the use of standard doses of either PMSG or pituitary extracts during the follicular phase of the cycle. A summary of results from some studies are shown in Table 1 and indicate the range of responses encountered. Treatments have normally been initiated on days 15 or 16 of the cycle coinciding with the second wave of follicular growth (Rajakoski, 1960). However, a number of authors have administered follicle stimulating compounds during the luteal phase and results indicate that while the ovulatory response was increased egg recovery and fertilisation rates have been lowered. (Avery et al. 1962; Hafez et al. 1963).

FACTORS INVOLVED IN THE OVARIAN (FOLLICLES + OVULATIONS) RESPONSE TO SUPEROVULATION

Many of the factors affecting superovulation responses are not clearly understood. The following comments refer mainly to the use of PMSG during either the follicular or luteal phase in the sexually mature heifer.

GENETIC DIFFERENCES

There is evidence of a breed response relationship, with beef breeds showing a greater sensitivity to PMSG than dairy breeds. Scanlon (1969) and Sreenan (1969) have both reported higher mean ovulating responses in Hereford and Angus beef cattle as compared with Friesians, all treated with a standard dose of PMSG (3,000 i.u.) in the follicular phase of the cycle. Likewise, Mariana et al. (1970) reported a higher ovulation response in Charolais than in Friesian cattle following 1,600 i.u. PMSG (Table 2). In many other reports where breeds are not clearly specified, it is possible that this factor is responsible for some of the variation in response.

TABLE 2 BREED RESPONSE TO PMSG

Dose Level (iu)	Breed	No. Animals	Mean Ovulation rate	Author
3,000	Friesian	31	7.3	Scanlon (1969)
	Hereford/Angus	203	12.2	
3,000	Friesian	18	13.7	Sreenan (1969)
	Hereford/Angus	34	32.0	
1,600	Friesian	78	2.6	Mariana et al. (1970)
	Charolais	52	6.2	

DOSE-RESPONSE RELATIONSHIP

While among animals considerable variation exists in ovarian response to a standard dose of PMSG, nonetheless, a dose response relationship seems clear from some of the reports in the literature. Both the mean and variance of follicle and ovulation response seem to increase with dose of PMSG (Gordon et al. 1962; Henricks et al. 1973). Current work on dose response relationships within different batches of PMSG (Table 3) indicate that while mean ovarian development increases with dose, at the higher dose levels this increase may result from a relatively larger number of persistent follicles ($>$8 mm) than at low and intermediate doses. High dose levels of PMSG have been associated with a high proportion of persistent follicles (Dowling 1949; Folley & Malpress, 1944). This may be due in part to the short PMSG - oestrus interval associated with high dose levels of PMSG (Mauleon et al. 1970). Table 4 indicates that at the highest level of PMSG (2,500 iu) the lowest proportion of ovulations occurred.

TABLE 3 DOSE-RESPONSE RELATIONSHIP WITHIN TWO BATCHES OF PMSG

	Dose Level (iu)			Batch
	1,500	2,000	2,500	
No. animals	29	-	25	A
Mean follicles (±SE)	0.7±0.2	-	7.2±1.1	"
Mean ovulations (±SE)	11.5±1.3	-	16.8±2.1	"
Mean ovarian response (±SE)	12.2±1.3	-	24.1±2.5	"
No. animals	21	30	10	B
Mean follicles (±SE)	3.3±0.8	3.9±1.0	9.8±2.7	"
Mean ovulations (±SE)	8.0±1.7	14.7±1.6	9.4±2.1	"
Mean ovarian development (±SE)	11.1±1.6	18.6±2.2	19.2±1.8	"

Beehan & Sreenan (1975) unpublished

TABLE 4 THE EFFECT OF DOSE LEVEL OF PMSG ON THE RATIO OF OVULATION AS A PROPORTION OF TOTAL OVARIAN RESPONSE

	Dose Level PMSG (iu)		
	1,500	2,000	2,500
PMS-Prostaglandin	.75	.75	.64

Beehan & Sreenan (1974) unpublished

INTERVAL PMSG - OESTRUS

It has been reported that following the use of PMSG during the follicular phase of the cycle, a definite relationship exists between the mean percentage of follicles ovulating and the time interval separating PMSG and oestrus. The highest percentage of ovulations occur when intervals of about 5 days are achieved, while the lowest percentage occurs after 2 - 3 day intervals (Hafez et al. 1963; 1965; Scanlon et al. 1968). However one of the disadvantages of administering PMSG during the follicular phase is that normal cycle length variation makes it difficult to control the interval from PMSG to oestrus. Recent oestrous cycle control techniques open up the possibility of more consistent control of this interval and thus maximising ovulation for any particular dose level of PMSG. Recent reports have indicated that prostaglandin $F_{2\alpha}$ and analogues are effective in causing rapid luteolysis (between Days 5 - 15 of the cycle) resulting in oestrus within about 2 - 3 days (Tervit et al. 1973; Louis et al. 1973; Lamond et al. 1973, Cooper 1974, and many others). Some preliminary data on the use of prostaglandin in combination with PMSG have been reported by Elsden (1974). This data indicated results superior to those obtained with PMSG only, both in the proportion of animals ovulating and in higher ovulation rates achieved in the animals responding. Tervit et al. (1973) using PMSG in conjunction with prostaglandin reported a greater degree of synchronisation in those animals pre-treated with PMSG.

Many short term (9 - 10 day) progestogen treatments have recently been reported for oestrous cycle control in cattle (Wiltbank & Kasson, 1968; Wishart & Young, 1974, Sreenan, 1974; Sreenan 1975; Sreenan & Mulvehill 1975;

Roche, 1974). These short term treatments are based on administration of oestrogen-progestogen combinations to inhibit or regress C.L. development, followed by 9 - 10 days progestogen administration (subcutaneously or intravaginally) and sudden withdrawal. PMSG has been used with these synchronisation methods to study the effect on PMSG - oestrus intervals and also ovarian response. PMSG has been administered at the midluteal phase (Days 9-11) followed 48 and 72 hours later with prostaglandin (ICI-80996 analogue). PMSG has also been administered at 48 hours prior to removal of either progesterone intravaginal pessaries or SC-21009 subcutaneous implants (G.D. Searle & Co.). The effect of such treatments on PMSG-oestrus intervals are shown in Table 5.

TABLE 5 EFFECT OF TREATMENT ON THE PROPORTION (%) OF ANIMALS COMING INTO STANDING OESTRUS AT DIFFERENT INTERVALS AFTER PMSG

Treatment	Interval PMSG + Oestrus (Days)			
1,500 - 2,500 iu PMS, Batch A	3	4	5-7	Total No.
PMS - Prostaglandin*	8.8 (4)	84.8 (47)	9.6 (5)	56
PMS - Progesterone	54.4 (24)	35.9 (19)	14.5 (3)	46
PMS - SC-21009	45.8 (12)	34.3 (9)	19.9 (5)	26

Nos. in ( ) are no. of animals

Beehan (1975) unpublished

Clearly a high degree of synchronisation was achieved following the use of PMSG (midcycle) and prostaglandin. Approximately 85% of animals responding (oestrus) came into oestrus at a 4 day interval with less than 10% coming into oestrus at intervals of 3 or 5 days. The onset of oestrus was more variable following the progestogen treatments with a higher percentage of animals coming in oestrus earlier than progestogen synchronised animals without PMSG (Sreenan, 1975; Wishart & Young, 1975). PMSG in conjunction with prostaglandin or progestogens hastens the onset of oestrus.

Animals synchronised with 9-day progesterone intravaginal pessary treatments normally show a high degree of synchronisation with approximately 75% coming in oestrus on day 2 following pessary removal (Sreenan, 1975), however, when treated with PMSG, approximately 50% of animals have shown oestrus one day earlier than this, and a lesser degree of synchronisation has been achieved. Similarly with SC-21009 treated animals, Wishart & Young (1974) have reported 80% of animals show oestrus at day 2 following implant removal, however when treated with PMSG over 45% of animals came in oestrus one day earlier and the response was spread over three days. The overall oestrus response for prostaglandin, progesterone and SC-21009 treated animals when combined with PMSG (1,500 - 2,500 iu) was 85.2%, 78.6% and 63.3% respectively. The effect of the interval (PMSG-oestrus) for these treatments is shown in Table 6. The longer the interval, the higher the proportion of total ovarian response that is represented as ovulations. Prostaglandin treated animals have yielded a higher proportion of ovulations overall.

TABLE 6 EFFECT OF PMSG - OESTRUS INTERVALS ON THE RATIO OF OVULATION AS A PROPORTION OF TOTAL OVARIAN RESPONSE

	Interval PMSG - Oestrus (Days)		
Treatment	3	4	5 - 7
PMSG - Prostaglandin	.60 (4)	.77 (47)	.98 (5)
PMSG - Progesterone	.63 (24)	.76 (19)	.98 (3)
PMSG - SC-21009	.53 (12)	.86 (9)	.94 (5)

Nos. in ( ) = animal no.

Beehan (1975) unpublished

The effect of these prostaglandin and progestogen treatments on the ovulatory response at different levels of PMSG is shown in Table 7. The prostaglandin treated animals received the PMSG at Days 9 - 10 when progesterone levels would be at a maximum. The progestogen treated animals received their PMSG during a stage when the progesterone levels would be dependent on the release rate from the intravaginal sponge. While there is evidence that response is affected by the ovarian follicle population yet it has been shown that PMSG will cause growth in all follicle types in the

bovine. (Mariana & Nyugen - Huy, 1973). Animals treated in the luteal phase have shown a high ovulation response (Foote & Onuma, 1970) and the endogenous level of progesterone may be important at the time of PMSG administration, however, perhaps the rate of decline of endogenous progesterone prior to oestrus may be equally important. It is essential to determine the optimum conditions (ovarian follicle population, endogenous progesterone/oestrogen levels) at the time of PMSG administration for best response.

TABLE 7 EFFECT OF TREATMENT ON MEAN OVULATION RESPONSE AT THREE LEVELS OF PMSG

	PMSG Dose Level (iu)		
	1,500	2,000	2,500
PMSG - Prostaglandin	8.0 ± 1.7** (21)	14.7 ± 1.6 (30)	9.4 ± 2.1 (10)
PMSG - Progesterone	6.4 ± 1.1 (19)*	11.2 ± 1.8 (27)	17.1 ± 3.7 (10)
PMSG - SC-21009	9.1 ± 1.2 (8)	10.5 ± 1.7 (11)	12.7 ± 4.2 (11)

* Nos. in ( ) = animal no.

** ± S.E.

Beehan (1975) unpublished

FACTORS INVOLVED IN THE RECOVERY AND FERTILISATION OF SUPEROVULATED OVA

While the literature contains many reports on the induction of superovulation, few of these yield information on mean recovery rates of fertilised ova per animal. This however, is the most important result of any treatment routine. Where mean yields of fertilised ova are given, the data indicate a range of from about 3 - 5 fertilised ova per animal from the more successful treatments (Avery et al. 1969; Hafez et al. 1963, Scanlon et al. 1969; Sreenan 1969). The data in Table 1 (Foote & Onuma, 1970) indicate fertilisation rates of recovered ova ranging from 50 - 80%.

EFFECT OF OVULATION - RECOVERY INTERVAL ON RECOVERY RATE

The ova remain in the oviducts until approximately 96 hours after ovulation and then enter the uterine horns. Estimates of recovery (by surgical procedures) in the literature vary from 25 - 75% of the number of ovulations recorded (Foote & Onuma, 1970). Little data is available on recovery rate relative to the interval post oestrus or ovulation. For ova recovered surgically by cannulation of the oviduct (Sreenan & Beehan, 1974), data are given in Table 8 on recovery rates relative to day of recovery post oestrus. The proportion of ova recovered decreased with the length of interval.

TABLE 8 EFFECT OF DAY OF RECOVERY ON RECOVERY RATE

	Days post oestrus					
	3	4	5	6	7	8
No. animals	22	10	15	68	55	15
% ova recovered*	78.3	53.4	66.6	64.1	50.0	45.9

* based on number of ovulation recorded.

Beehan (1975) unpublished.

DEGREE OF SUPEROVULATION

Little data is available on the effect of degree of superovulation on recovery rate. Scanlon et al. (1968) reported no difference in recovery rate between two groups of cattle yielding either 1 - 3 or more than 10 ovulations respectively. Recovery rate relative to level of PMSG (degree of stimulation) is shown in Table 9. There was a tendency for a lower proportion of ova recovered at the high level of PMSG.

TABLE 9 OVUM RECOVERY RATE RELATIVE TO LEVEL OF PMSG

	PMSG - dose level (iu)		
	1,500	2,000	2,500
No. animals	29	52	22
% ova recovered	65	56	51

Beehan (1974) unpublished

FERTILISATION RATE OF RECOVERED OVA

As already stated the most important figure is the mean number of fertilised ova recovered per animal. This has been shown to range from 3 - 5 with a large degree of variation. Elsden et al. (1974) however combining PMSG and prostaglandin reported a mean of 8.5 fertilised ova recovered per cow in a group of 10. Data from current work is shown in Table 10 on the mean number of fertilised ova (morphologically normal for stage of cycle) recovered using two batches of PMSG and for prostaglandin and progestogen treatments.

TABLE 10 MEAN RATES OF FERTILISED OVA RECOVERED PER ANIMAL FROM VARIOUS TREATMENTS

	PMSG - dose level (iu)			
	1,500	2,000	2,500	Batch
PMSG - Prostaglandin	4.5 ± 1.1	4.9 ± 1.0	3.1 ± 1.0	B
PMSG - Progesterone	2.0 ± 0.6	4.3 ± 0.5	6.1 ± 1.6	"
PMSG - SC-21009	3.8 ± 0.8	6.0 ± 1.3	3.5 ± 1.8	"
PMSG - Prostaglandin	5.1 ± 0.7	-	8.0 ± 1.1	A

Beehan & Sreenan (1975) unpublished

Some treatments yielded a mean of 6 - 8 fertilised ova per animal. All of these animals were bred either with fresh or frozen semen. Studies are in progress in an attempt to determine the effectiveness of fresh or frozen

semen in effecting fertilisation of superovulated ova. Preliminary data is shown in Table 11. Using approximately twice the total number of sperm per insemination there was a tendency for a high fertilisation rate in fresh inseminated ova but the numbers at this stage are not adequate to draw final conclusions.

TABLE 11 EFFECT OF USING FRESH OR FROZEN SEMEN ON THE % OF OVA RECOVERED THAT WERE FERTILISED AND NORMAL

	Fresh Semen*	Frozen Semen**
No. animals	23	20
% ova fertilised	70.5	61.1

* Fresh Semen = 50 million sperm/straw (3 x straws)

** Frozen Semen = 30 million sperm/straw (3 x straws)

Beehan, 1975 unpublished

TRANSFER RESULTS

Many superovulations have been reported in the literature but little data is available on the viability of the ova in terms of survival following transfer. Surgical transfer techniques as developed by Rowson et al. (1969) have been used to determine the effect of level of PMSG and method of superovulation (PMSG $\pm$ Prostaglandins or Progestogens) on subsequent egg survival. Fertilised ova have been recovered and transferred from donors superovulated with either 1,500 or 2,500 iu PMSG and egg survival rate is indicated in Table 12.

TABLE 12 EFFECT OF LEVEL OF PMSG ON SURVIVAL OF FERTILISED OVA FOLLOWING TRANSFER

	PMSG dose level (iu)	
	1,500	2,500
No. ova transferred	70	30
No. surviving	49	17
% survival	70	56.7

A higher survival rate was recorded for ova released following 1,500 iu, however, the difference was not significant. Induction of multiple ovulations following either prostaglandin or progestogen treatments did not affect subsequent egg survival rates.

SUMMARY

Response to treatments for the induction of superovulation in the bovine is highly variable. This response, in terms of ovulations and follicular response is affected by breed, dose of PMSG, interval from PMSG - oestrus and method of superovulation. There is a tendency for ova recovery to be affected by day of recovery and level of PMSG. Fertilisation rate did not seem to be affected by either the use of fresh or frozen semen. Some treatments have yielded 6 - 8 normal fertilised ova per animal and survival rate was not affected by level of PMSG or method of superovulation.

The indications are, that within a particular breed of animal and particular dose level of PMSG, if the interval from PMSG - oestrus is controlled (at 5 days) that predictable responses could be obtained.

Early work indicated that treatment with PMSG during the luteal phase resulted in extremely low fertilisation rates however combined with rapid luteolysis of the CL following PMSG high mean numbers of fertilised ova have been recovered.

Work is needed to determine the optimum conditions (ovarian follicle population, stage of cycle, endogenous hormone levels) at the time of superovulation treatment on results. One important area perhaps would be to find an alternative compound to PMSG.

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DISCUSSION

M.J.Cooper (UK)

I would just like to ask Dr. Sreenan one question. It is interesting to me that the only good relationship between a dose of PMSG and the number of ovulations is occurring following the progesterone treatment and I wonder if you have extended this dose response curve in terms of PMSG dose or in terms of the amount of progesterone that you are giving these animals.

J.Sreenan (Ireland)

In fact, we are, but we don't have any data because we have had to set up a totally new system. In the dose response curve I showed that was the last of that particular batch of PMS and we do show a PMS batch difference. Now, when we use PMS we buy it off the shelf, we don't use particular batches, they are all commercially prepared batches. I started out with the idea that there probably wasn't a great batch difference in PMS anyway, but, at the same time, because there are so many variables involved in the business, we have had to attempt to use one batch of PMS for each range of treatments. Obviously it is interesting for us to increase the progesterone level, to increase the PMS level as well to above 2,500 units, with the progesterone treatment. But we have to set up the whole thing again with the separate batch of PMS. We have to do this anyway because we try, as far as possible, to have the treatments carried out within the same season as well because these things haven't been sorted out too well.

M.J.Cooper

It doesn't seem to tie up too well with the effects of the endogenous progesterone concentrations that you mentioned.

J.Sreenan

No, in fact we were quite surprised. The levels of progesterone after sponge removal were much lower than we had anticipated at any rate. (David Wishart may have some further data on the levels of progesterone after the implant removal). Having placed the sponge in position for ten days the

sponge would have 3 gm of progesterone at insertion - most of them would anyway - between 2 and 3 gm. The ones quoted would have started with about 3 gm of progesterone. I get three levels that were between 1 and 1.5 ng per ml and the third level would be at Day 7. So, in fact, those three levels would be on days 5, 6 and 7. In the cyclic animal the CL has been very effectively dealt with depending on exogenous progesterone which is at a very low level. Obviously, progesterone levels may have a big part to play in the response to superovulation. It just seems that with our two very different levels that we didn't get a marked effect. The other things that come into play here are the length of time between the time the CL regresses, in other words, the fall off in progesterone level. This may be very important as well in this interval. But certainly, if we could have standardised that progesterone interval between PMSG and oestrus, we were doing pretty well as far as ovulations, fertilisations and recovery of eggs were concerned.

I.Gordon (Ireland)

How was the problem of silent heat in relation to the different systems? It was always a problem - this business of the animals that didn't come into oestrus. Would you like to say a word or two on that and whether you are taking that silent heat situation into account when you are talking about fertilised eggs per animal?

J. Sreenan

In fact a lot of this is in the paper that was circulated and obviously you are not going to get time to look at this in a hurry. When we are talking about intervals between PMS and oestrus we are obviously talking only about those animals that came into oestrus, but when we are talking about eggs fertilised we are talking about every animal that went into the system. So the mean recovery of fertilised eggs is from every animal, silent heat or otherwise. The percentages of the animals that came in oestrus for the three different treatments were 85.2% for the PMS/prostaglandin, 78.6% for the PMS/progesterone, and 63.3% for the PMS/SC 21009. We had a system of inseminating on a fixed time basis in all animals whether they showed heat or not because we had started out to effect a synchronisation procedure by using PMS in conjunction with a synchronisation agent.

I am sure I have it somewhere but I can't tell you now what proportion of the animals not standing in oestrus had fertilised eggs although we certainly have the data. As far as silent heats are concerned, those are the percentages of heats. I have to relate that, of course, to the very last slide, which I should have thought of, and that is the fertilisation rates of 70% were fertilisation rates in animals that were in oestrus. To try to standardise the thing again we took only animals from both sides that stood in oestrus and were inseminated at comparable intervals from the start of oestrus.

S.M. Willadsen (UK)

Apparently there is a potential for more ovulations because there are some more follicles which have responded. Have you thought of any ways of trying to increase the ovulation rate proper, that is in terms of the follicles which are developing?

J.Sreenan

You are thinking, probably, in terms of using some of the releasing factors or something like this?

S.M.Willadsen

HCG would be a possibility that I would like you to comment on.

J. Sreenan

I can't really comment on that. We didn't want to confound what we were doing even further. Going back through the literature, and I have already mentioned it briefly, in superovulation one of the problems in trying even to pick a cumulative trend out of the literature is that everybody has used different breeds, different weights of animals, animals superovulating at different times of the year. Some of them make little reference to batch of PMS - there is a whole confusion there. I think the overriding factor is that the numbers of animals involved in many of these papers are too small to make any comment on. In fact, what we set out to do here was to get reasonable numbers of animals into set treatments and

answer some questions. We do have an interest in using releasing factors for a start, GnRH, LH or whatever, to see whether we could induce further ovulation of follicles that are growing. Obviously as we increase the interval this is one way. From the animals that came into oestrus between Days 5 and 7 from PMS we got virtually 100% of stimulation represented as ovulations. But we haven't used HCG or GnRH yet. In fact, in superovulation the only data that we have on that would be in sheep where there is a brief communication in JRF showing that following progestogen treatments in sheep they had three systems, a controlled progesterone sponge for 14 days and PMS prior to removal. Then they had that plus HCG and that plus GnRH. In fact, they ended up with more persistent follicles in the two groups having GnRH and HCG than they did in the control group. So they have got to think a little bit more about it. We would like to do two things: standardise the interval and increase it and maybe increase the ovulation percentage.

I. Wilmut (UK)

I wonder if you have looked to see whether or not the pre-treatment affects the survival of the eggs when they are transferred?

J. Sreenan

I must apologise. We have, and I have data on transfers and that kind of thing, but this didn't seem to be the place to discuss it because this subject is too involved. At the end of the paper we have one table showing the effect of level of PMS, for a start, on pregnancy rates following transferred eggs. I forget what the actual percentages were but following the transfer of 70 ova from 1,500 units of PMS and 30 from 2,500, we got no great difference. There was 70% survival versus 56% survival. I don't have the data in table form showing PMSG versus PMS/progesterone or PMS/SC 21009, but I can tell you that we have looked at it and there is no effect of treatment on survival after transfer.

I.Wilmut

I think Robin Tervit published some information recently where in one experiment they got quite a big difference between the prostaglandin pre-treatment and the progestogen pre-treatment, in favour of the progestogen

in fact. Of course, that was in only one experiment with not very large numbers of animals. But you find there is no major difference?

J. Sreenan

Well, when you come down to look at differences you have got to be very careful because many of the eggs here were actually recovered for culture purposes so the numbers of eggs that would have gone for good surgical transfers to Day 0 recipients is probably quite small. However, there are comparable numbers across the treatments and there is no effect.

A.O. Trounson (UK)

Have you ever examined a shorter half-life compound like HAP instead of PMS? I think some results show that PMS has a very long half-life in the cow and I think this is one reason why there are as many unruptured follicles. Have you ever used HAP in the cow?

J. Sreenan

No, we haven't. Possibly Professor Gordon could come in and say something about that. I would agree with you about the length of time the PMS hangs around. This is something we are trying to find out at the moment - how long does it hang around and whether, in fact, there is any way that we could come in with something and cut this off. The Australians were attempting to assay the PMS levels at various timings after administration and then to come in with an anti-PMS and cut it off. I think this is one of the problems.

L.E.A.Rowson (UK)

Thank you, Dr. Sreenan - I am afraid that we will have to stop at that point.

NON-SURGICAL EMBRYO TRANSFER IN CATTLE
I MYOMETRIAL ACTIVITY AS A POSSIBLE CAUSE OF EMBRYO EXPULSION

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ABSTRACT

The electrical activity of the bovine myometrium was recorded in two cycling heifers, by means of four bipolar silver electrodes implanted at four distinct sites. Myometrial activity started on Day -3, culminated on Days 0 and 1 and disappeared on Day 4 or 5 of the next cycle. Manipulations of the genital tract, such as those performed during non-surgical embryo transfer procedures or irrigation of the uterus by gravity flow, did not induce myometrial contractions which were previously held responsible for expulsion of the embryo. Only non-specific electrical activity of the myometrium was noted during manipulation, both before and after Day 5, which ceased immediately upon removal of the instruments. It is postulated that the spontaneous myometrial activity before Day 5 is responsible for low pregnancy rates when non-surgical embryo transfers are performed early (Days 3 - 5) in the cycle.

A single i.m. administration of 115mg Isoxsuprine [1] blocked electrical activity of the myometrium for 2 to 4.5 hours on Days 0, 1 and 2 of the normal cycle and in a heifer with cystic follicles. Intravenous administration of oxytocin[2] subsequent to Isoxsuprine immediately re-established myometrial activity.

It is concluded that manipulation of the genital tract during non-surgical embryo transfer after Day 5 does not elicit uterine contractions which result in embryo expulsion.

1) Isoxsuprine lactate (Duvadilan); Philips-Duphar, Holland.
2) Oxytocin-S; Intervet, Holland.

The intensity of the myometrial activity during the induced superovulatory oestrus was about two times greater than during a normal oestrus. The myometrial activity persisted until Day 4, when the progesterone concentration had already risen to 11 ng/ml. Peak levels of progesterone were 15 times greater during the induced superovulatory cycle than during the normal cycle.

INTRODUCTION

Until recently the approach to the concept of embryo transfer in cattle has been surgical. However, for both clinical and economic reasons it is apparent that efficient non-surgical techniques have to be developed. When compared to surgical methods, non-surgical transfer of bovine embryos has resulted in lower pregnancy rates (Sugie, 1965, Rowson & Moor, 1966; Vincent, Mills & Rundell, 1969; Sugie, Soma, Fukumitsu & Otsuki, 1972; Tervit, 1973; Sreenan, 1975; Lawson, Rowson, Moor & Tervit, 1975).

The reduced pregnancy rate may be caused by trauma or infection of the cervix and/or uterus, by alteration of the biochemical environment of the uterus, by expulsion of the embryo from the uterus, or by technical failures. Lawson, Rowson, Moor and Tervit (1975) stated that non-surgical transfer of fertilised ova might best be done after Day 6 rather than earlier. The purpose of this study was to determine whether spontaneous or induced myometrial activity could be held responsible for the reduced pregnancy rate after non-surgical transfer via the cervix.

The electrical activity of the myometrium was measured during the oestrous cycle, during manipulations of sham transfers such as the introduction of pipettes into the uterus, during irrigation of the uterus and after administration of isoxsuprine, xylocaine or oxytocin.

MATERIALS AND METHODS

Two heifers of the MRI (Maas- Rijn and IJssel) breed were subjected to mid-ventral laparotomy under general fluothane anaesthesia. Four silver electrodes (5mm in length) were positioned in the myometrium. The sterile bipolar electrodes were mounted at fixed distances on a small teflon plate, which was embedded in a silastic sheet to facilitate suturing to the myometrium. Three electrodes were placed on one uterine horn; one 3cm from the tip, one half way and one near the bifurcation. A fourth electrode was placed on the opposite uterine horn near the bifurcation. The electrodes were connected to wires embedded in silastic, which led to the pelvic cavity. From here the leads emerged through the sacrosciatic ligament and were connected with a gold-lined 8-pin plug to an Elema - Schönander Mingograf 81. The amplifiers EMT 12, were set at 30 Herz with

a time constant of 0.03 sec., which had been shown to give the best results. For detailed information see Naaktgeboren, van der Weyden, Klopper, Kroon, Schoof & Taverne, (1973).

The sensitivity was tested after the ink-jet recording had been started and the recorder was calibrated at 50, 100, 200 or more µV. Paper speeds of 2.5 or 5mm per sec. were used. Daily recordings were made for one to two or more hours depending on the stage of the oestrous cycle. Recording was started one week after placement of the electrodes and continued for more than three months. The electrical activity of the myometrium was measured during six normal oestrous cycles, during an induced and a superovulatory cycle and during a period when one animal was showing cystic follicular degeneration after superovulation.

Sham transfers were performed under and without epidural anaesthesia (5ml 2% xylocaine) using a set of three sterile pipettes. The first metal pipette (35cm long and 6mm in diameter with a piece of relatively non-absorbent paper over the end) served as a speculum for a second metal pipette, which had a length of 45cm and a diameter of 3mm. The second pipette was manipulated through the cervix 3 - 4cm into the uterine horn. A third flexible Rüschelit[1)] ureteric catheter, 70cm long and 1.65 or 1.98mm in diameter was then introduced via the second one as far as possible to the tip of the uterine horn. Finally 1 to 5ml TCM 199 were injected via the third catheter.

Irrigation of the uterus was done with two flexible disposable Rüsch catheters. The first of these catheters was a three-way balloon (30ml) catheter with a diameter of 6.6mm and a length of 40cm which was passed through the cervical canal with the aid of a stilette. The second, a ureteric catheter (2.6 - 2.74mm in diameter and 70cm long) was passed as far as possible through the three-way catheter into the tip of the uterine horn. Both catheters were connected by tubing to a bottle containing flushing medium (saline) and to a collecting bottle respectively. The uterus was flushed by gravity flow and was not manipulated per rectum during collection.

Isoxsuprine, a beta-mimetic adrenergic compound, was tested at a

1) Rüsch, West Germany.

dose level of 115mg, oxytocin at a level of 20 to 50 i.u. and 5ml of 2% xylocaine was adminstered either as an epidural injection or as a bolus in the vagina.

Analysis of the recordings focused on the time of onset of activity during pre-oestrus and the cessation of activity during metoestrus, the mean amplitude of the highest potentials and patterns of activity.

Peripheral blood samples were taken daily via an indwelling jugular catheter. Plasma was stored at -25°C and assayed for progesterone by radioimmunoassay, according to de Jong, Baird and van der Molen (1974), with minor modifications. Antiserum produced in rabbits against progesterone - IIα-BSA conjugates was used, with (1,2,6,7-^{3}H) progesterone (84 Ci/mmol) as the tracer, while dextrancoated charcoal was used for separation of bound and free fractions. The high progesterone concentration of some of the plasma samples was checked by GLC with FID.

RESULTS

CYCLE LENGTH

The post surgical cycle lengths of the two heifers are given in Table 1.

TABLE 1 CYCLE INTERVALS IN HEIFERS WITH ELECTRODES IN THE MYOMETRIUM

Animal no.	Cycle length in days	Remarks
10	25 - 21 - 19 - 19 - 11*	*PG treated cycle
11	24 - 21 - 22 - 12*- 28**	**cystic after superovulation

Both animals, operated on Day 13, showed a prolonged cycle of 25 and 24 days respectively, Subsequent cycles were of normal length. During the fifth cycle heifer 11 was injected i.m. with 2500 PMSG (Intervet batch 1491) on Day 8, followed by 500 µg PG (ICI, 80,996) on Day 10. Standing heat was observed on Day 12. Rectal examination 13 days later revealed 8 to 10 corpora lutea and one 25mm follicle on the right ovary and 3 corpora lutea on the left. Oestrus was observed 28 days after the induced oestrus, however the heifer subsequently developed cystic follicles.

ELECTROMYOGRAMS

The presence of electrical activity of the myometrium during the oestrous cycle is presented in Table 2.

TABLE 2 OCCURRENCE OF ELECTRICAL ACTIVITY IN THE MYOMETRIUM AROUND OESTRUS. 1) INDUCED CYCLE 2) INDUCED SUPEROVULATORY CYCLE

Oestrous cycle no.	Animal no.	Day of Cycle -4	-3	-2	-1	0	1	2	3	4	5	6
1	10	-	±	+	+	+	+	+	+	+	+	-
2	10	-	±	+	+	+	+	+	+	±	-	-
3	10	-	+	+	+	+	+	+	+	+	±	-
4[1)]	10	-	+	+	+	+	+	+	+	+	±	-
1	11	-	+	+	+	+	+	+	+	+	±	-
2	11	-	±	+	+	+	+	+	+	±	-	-
3	11	-	+	+	+	+	+	+	+	+	±	-
4[2)]	11	-	-	-	+	+	+	+	+	+	-	-

Electrical activity of the myometrium was observed from Day -3 to Day 4-5 (oestrus = Day 0). No distinct activity was detected during the remainder of the cycle. Occasionally minimal activity of short duration was noted at odd times during dioestrus, particularly at the electrodes near the body of the uterus and sometimes at the electrode near the tip of the uterine horn. The activity started very slowly with formation of isolated trains (groups of spikes). The frequency of occurrence and the intensity of the contractions gradually increased from Day -3 to Day 0 when individual trains merged into continuous periods of activity, synchronised between the different electrodes (Plate 1). At the end of such a period several individual spikes were seen. These concentrated periods of activity were particularly pronounced on Days 0 and 1 and, to a slightly lesser extent, on Days -1, -2, and 2. Most of these periods lasted for 6 minutes but showed considerable variation (1 to 16 minutes). The intervals between these periods were either essentially inactive or consisted of individual trains which at times tended to group. The intervals varied from 4 to 40 minutes but became shorter around oestrus itself. By Day 3 this pattern had reversed itself and truly synchronous trains could no longer be observed. On Day 4 or Day 5 only occasional

short and weak trains of very low amplitude were recorded. It appeared that the electrical activity of the myometrium as measured by the electrodes on the left and right uterine horn near the bifurcation, could occur independently of each other.

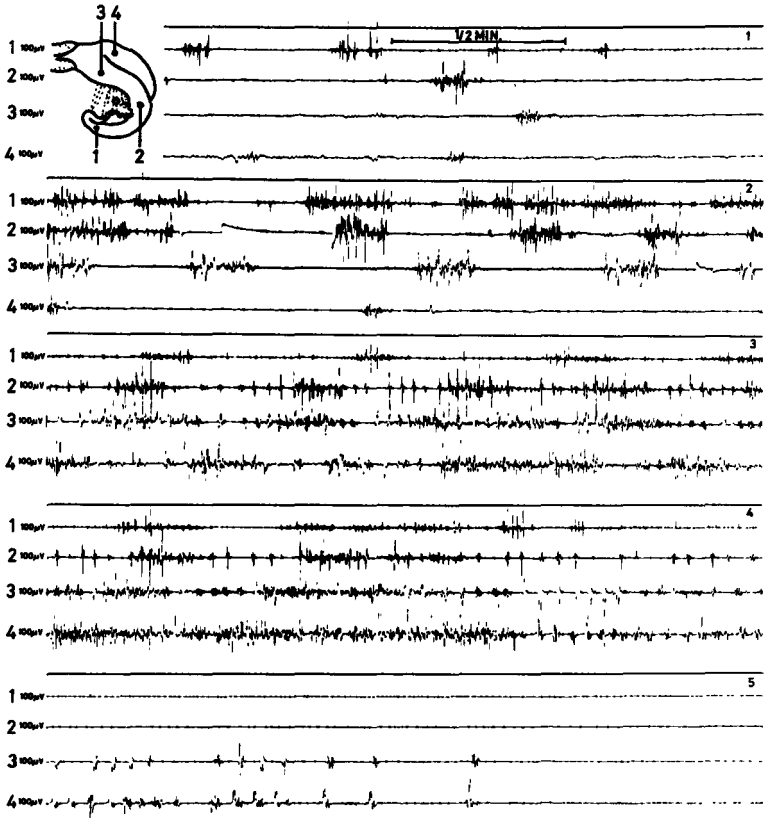

Plate 1. Representative excerpts of a concentrated period of myometrial activity on Day 0.

A diagram of the mean amplitudes during a spontaneous and the subsequent induced, superovulatory cycle of heifer 11 is given in Figure 1. The amplitude is expressed as the average of the maximal amplitudes of six or more successive 10 minute periods measured by four pairs of electrodes. The recording showed the greatest amplitudes during oestrus and the first two days after oestrus. During the spontaneous oestrous cycle of this heifer there was a distinct increase in the

amplitude on Day 2 which exceeded that of Day O. In comparison with the normal oestrous cycle, the induced superovulatory cycle showed a greatly increased amplitude on Days O and 1. Some early electrical activity could be observed after PMSG treatment.

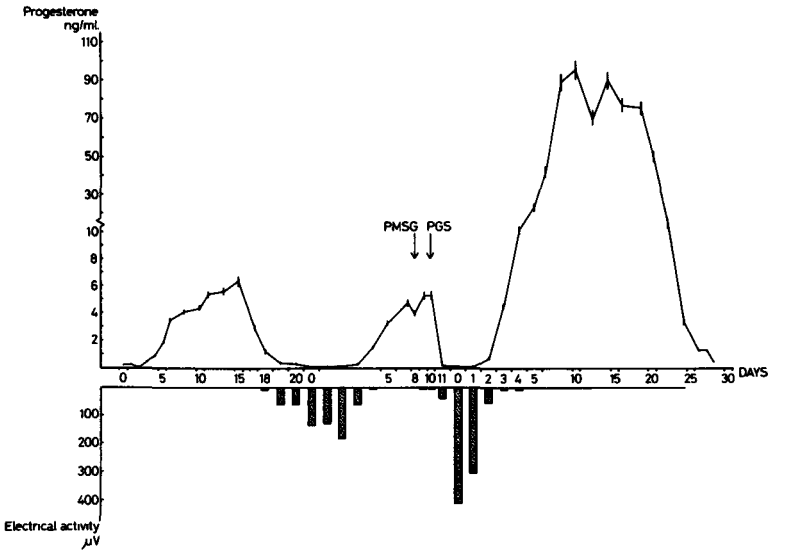

Fig. 1. Concentration of progesterone during a normal and an induced superovulatory cycle and the mean amplitude (μV) around oestrus.

Plasma progesterone levels, several days before and after the induced superovulatory oestrus are also given in Figure 1. The peak levels of progesterone in the induced superovulatory cycle were about fifteen times greater than those of the normal oestrous cycle. Elevated progesterone levels were maintained longer than during the normal cycle; they declined to nearly undetectable levels on Day 28.

Electrical activity of the myometrium during the spontaneous oestrous cycle coincided with a peripheral plasma progesterone concentration of less than 2 ng/ml.

During the induced superovulatory cycle myometrial activity of low amplitude was also observed on Days 3 and 4 when the concentration of

progesterone was 4.5 and 11 ng/ml respectively. It was difficult to determine whether uterine contractions occurred in a peristaltic or anti-peristaltic direction, however most activity on Days 0 and 1 tended to be anti-peristaltic, which changed to peristaltic on Day 2.

THE EFFECT OF SHAM TRANSFER ON ELECTRICAL ACTIVITY OF THE MYOMETRIUM

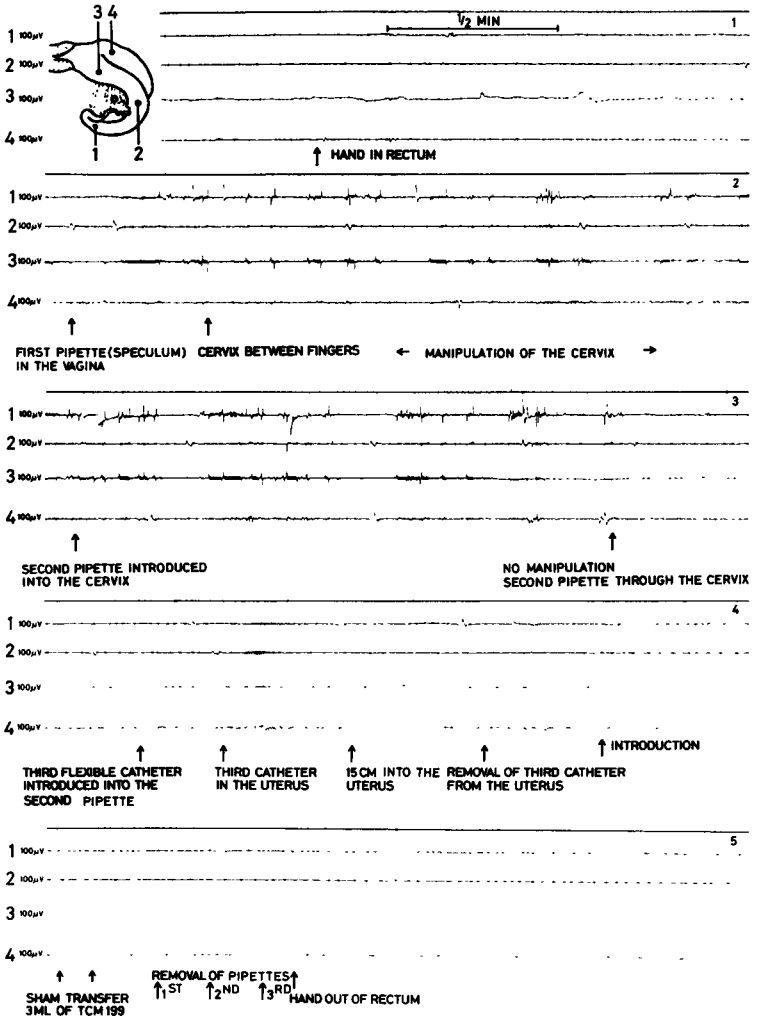

Plate 2. Myometrial activity during sham embryo transfer.

Sham embryo transfers were performed on Days 0, 3, 6 and 8. The effects of sham transfer on Day 6 on the electrical activity of the myometrium are shown in Plate 2. Sham transfers on Day 6 or Day 8, when normally no activity can be observed, did not induce electrical activity of the myometrium.

A few atypical spikes were recorded at the time of manipulation of the cervix and uterus per rectum, however, this activity ceased immediately when the manipulations were stopped. Even when the flexible catheter was advanced towards the tip of the uterine horn, no activity could be detected. Recording for more than one hour after the sham transfer did not reveal any activity. A sham transfer on Day 0 or Day 3, when there is spontaneous myometrial activity, intensified the electrical activity only during the manipulations but not for a prolonged period thereafter. Furthermore, when the internal uterine pressure was measured on Days 1, 3 and 6, rectal manipulation of the cervix or uterus did not induce extra internal uterine pressure beyond the time of manipulation (Taverne & Brand, unpublished data).

THE EFFECT OF IRRIGATION OF THE UTERUS ON ELECTRICAL ACTIVITY OF THE MYOMETRIUM

Irrigation of the uterus by gravity flow on Day 8 also did not stimulate myometrial activity (Plate 3). Introduction of the two disposable catheters into the uterus caused the same artificial spikes as were seen during sham transfer. 850 ml of 1 litre flushing medium were recovered in 15 minutes. During this time no activity could be recorded. Afterwards the uterus was massaged for one minute. This caused some non-specific myometrial activity which stopped immediately upon cessation of the massage. Furthermore, no delayed effect of stimulation could be recorded during the following hour.

THE EFFECT OF ISOXSUPRINE ON ELECTRICAL ACTIVITY OF THE MYOMETRIUM

The effect of the administration (i.m.) of 115mg isoxsuprine was studied on Days 0, 1 and 2, in heifer 10, and in heifer 11 which developed cystic follicles about 28 days after an induced superovulatory oestrus.. Administration of isoxsuprine had the same suppressing effect on the

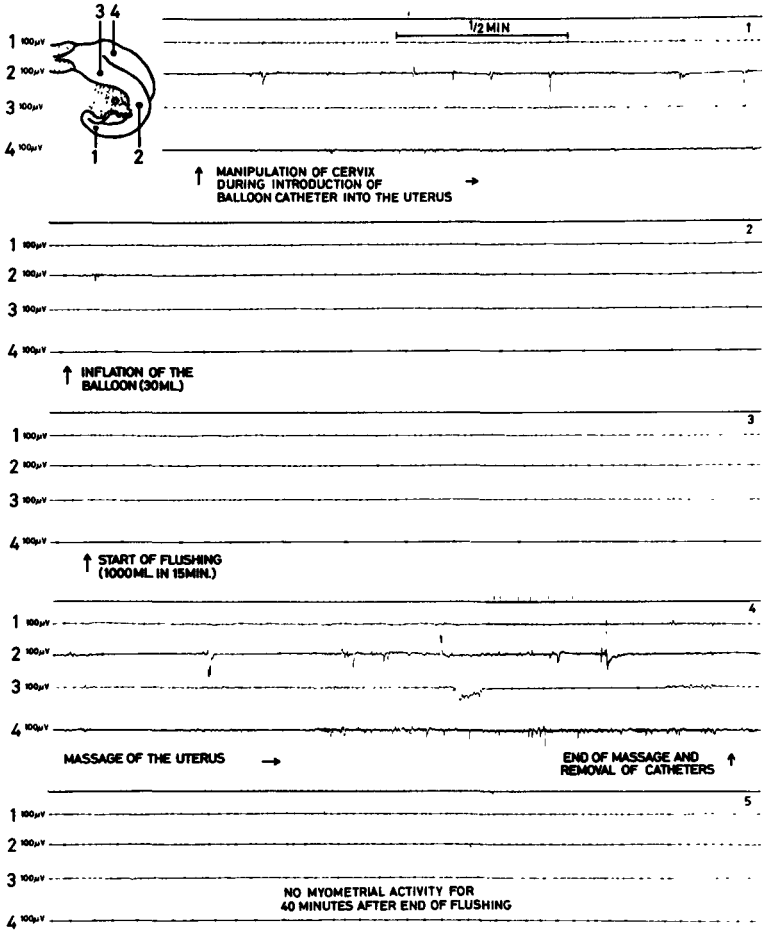

Plate 3. Myometrial activity during uterine irrigation.

electrical activity of the myometrium in both animals. The results obtained in heifer 11 with cystic follicles are shown in Plate 4. The patterns of myometrial activity of the animal with cystic follicles continued for a period of 5 weeks until slaughter and resembled the general pattern of the normal oestrus. However, the amplitude varied in intensity and duration, and might be related to fluctuations in activity of the cystic follicles.

Before treatment the electrical activity occurred in concentrated periods with a mean length of 10 minutes (6 - 18 minutes) alternating

with some solitary trains or periods of inactivity, averaging 34 minutes. Isoxsuprine inhibited electrical activity within 10 minutes and suspended the activity for 2 - 4.5 hours in both the heifer with cystic follicles and during the active period of a normal cycle. Intravenous administration of 50 i.u. oxytocin immediately stimulated electrical activity in the form of a concentrated period of distinctly separate trains. This period lasted 20 minutes and was followed by a 4 minute period of lesser activity after which the normal pattern of activity resumed. Intravenous administration of 20 i.u. oxytocin on Day 7 resulted in continuous activity of low amplitude for 20 minutes followed by individual trains for a period of 30 minutes. Administration of 5ml of 2% xylocaine either intravaginally or epidurally did not affect myometrial activity during the active period of the cycle.

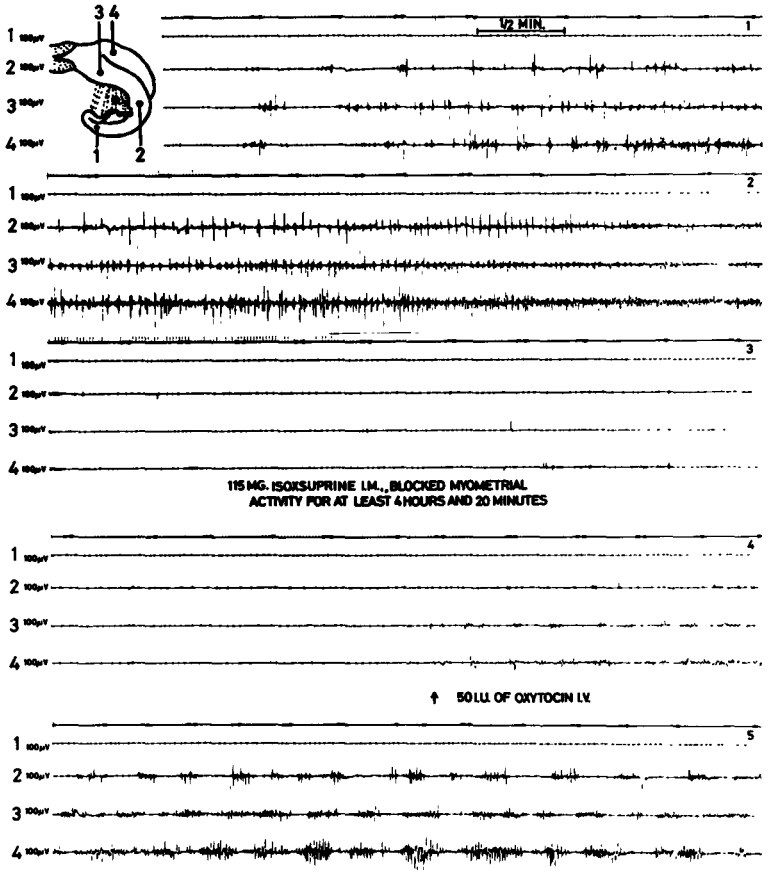

Plate 4. Myometrial activity before and after administration of Isoxsuprine and oxytocin in a heifer with cystic follicles.

CONCLUSIONS

The results of this study show that distinct electrical activity of the bovine myometrium occurs from 3 days before until 4 to 5 days after oestrus. This is in agreement with the findings of Ruckebusch & Bayard (1975) who found similar patterns of activity occurring over a period of approximately one week centred around oestrus. Lawson et al. (1975) found a low pregnancy rate after non-surgical transfer on Days 3 to 5. Distinct spontaneous electrical activity of the myometrium can still be seen during this period, but not on subsequent days of the cycle. Such spontaneous myometrial activity might be held responsible for the lowered pregnancy rates after embryo transfer early in the cycle. This is in support of the findings of Tervit (1973) that radioactive beads, when placed in the uterus via the cervix on Days 3 to 5, were expelled within hours. We were unable to confirm the generally held assumption that the introduction of pipettes into the uterus via the cervix would stimulate contractions of the uterus which in turn would lead to the expulsion of the embryo. Our results clearly show that manipulation of the cervix and uterus on Day 6 or 8 produced only atypical electrical activity which stopped immediately upon cessation of the manipulation. These findings might explain the improved pregnancy rates following embryo transfer on Days 6 to 9 (Sreenan, 1975; Lawson et al. 1975; Brand, unpublished data).

The cessation of spontaneous myometrial activity on Days 4 to 5 coincides with an increase in progesterone concentrations above 1.5 - 2ng/ml. Conversely, the resumption of the electrical activity of the myometrium could first be observed during pre-oestrus when progesterone levels had fallen below 1.5 - 2ng/ml. The occurrence of myometrial activity is dependent upon a rising oestrogen/progesterone ratio. Lemon, Pelletier, Saumande & Signoret (1975) found fluctuating peripheral plasma concentrations of oestradiol-17β with maximum levels up to 25pg/ml occurring around the onset of oestrus. This might explain the alternating periods of tonic myometrial activity (6 minute duration, range 1 - 16 minutes) and periods of reduced activity as they occur around oestrus.

Distinct myometrial activity could be observed during the induced superovulatory cycle in the face of elevated progesterone levels (4.5 ng/ml and 11ng/ml on Days 3 and 4 respectively). This as well as the low mean

recovery rate of 54% (Gordon, 1975) and the retention of embryos in the oviduct (Newcomb & Rowson, 1975) of superovulated animals might be related to a disturbed oestrogen/progesterone ratio.

Isoxsuprine effectively blocked the electrical activity of the myometrium. This effect could be reversed with oxytocin. The same effects have been registered in parturient cows (Taverne, unpublished data). It is doubtful whether the administration of Isoxsuprine or other uterine relaxants are indicated after Day 5 as no electrical activity could be recorded from this day on. Rowson, McNeilly & O'Brien (1972) were unable to find detectable levels of oxytocin in the peripheral blood of cows following vaginal and cervical stimulation during the early luteal phase.

Furthermore, Lawson et al. (1975), were unable to demonstrate a difference in pregnancy rates between animals treated with fluothane and control animals after Day 5. It is possible, however, that uterine relaxants, in addition to an inhibitory effect on the myometrium, also exert a suppressive effect on the metabolic processes of the endometrium which may influence subsequent conception in a positive way. In conclusion it can be stated that uterine contractions cannot be held responsible for the reduced pregnancy rates following non-surgical embryo transfer after Day 5. Furthermore, from the work of Lawson et al. (1975); Sreenan (1975) and Brand, Gunnink, Drost, Aarts & de Bois (1975), it appears unlikely that uterine infections must be regarded as a major cause if sufficient precautions have been taken. It is also possible that alterations in the endocrinological and/or biochemical environment of the uterus as well as technical factors play a major causative role in a low pregnancy rate following non-surgical embryo transfers.

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NON-SURGICAL EMBRYO TRANSFER IN CATTLE
II BACTERIOLOGICAL ASPECTS

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ABSTRACT

Bacteriological examination of the vulva, vagina and cervix as well as instruments used for non-surgical (sham) embryo transfer upon withdrawal from the uterus, was performed in 20 and 12 clinically healthy cows respectively. There was a decrease in the number of isolates obtained from the vulva to the external os of the cervix. Using three concentric pipettes more isolates were cultured from the outer than from the inner flexible catheter. Bacteriological examination of the uterus was positive in only 2 out of the 12 cows which were slaughtered 48 - 72 hours after a non-surgical sham embryo transfer.

Non-surgical sham embryo transfers did not seem to have a negative effect on the pregnancy rate of inseminated animals as 50% of the animals became pregnant.

It is concluded that contamination of the uterus by introduction of pipettes through the cervix is not a major contributing factor to the low pregnancy rate after non-surgical embryo transfer in the cow if adequate precautions have been taken.

INTRODUCTION

Transfer of an embryo via the cervix may be accompanied by the introduction of contamination from the vulva, vagina and cervix into the uterus. This may partly explain the lowered pregnancy rate after non-surgical embryo transfer as found by several authors.

According to Rowson, Lamming & Fry, 1953; Hawk, Brinsfield, Turner, Whitmore & Norcross, 1964; Gunnink, 1973, the uterus is much more susceptible to infection during dioestrus than during oestrus. A bacteriological survey was therefore made to investigate to what extent introduction of bacteria into the uterus leads to the development of a (localised) endometritis which might result in a lowered conception rate.

MATERIALS AND METHODS

10 heifers with normal cycle intervals were selected from an experimental herd for bacteriological examination of the vulva, vagina and external os of the cervix. 10 lactating cows (3 to 6 years old) were selected at the slaughterhouse for the same purpose. Non-surgical sham embryo transfers were performed on a farm in 12 cycling, lactating cows (3 to 7 years old) which were slaughtered 48 to 72 hours later. A group of 10 lactating animals (3 to 8 years old) were inseminated and a non-surgical sham transfer was made to the horn contralateral to the corpus luteum, 6 to 10 days after oestrus. All the instruments were examined bacteriologically immediately upon withdrawal from the genital tract.

The vulva was sampled with a sterile cotton swab. After sampling the vulva was cleaned with 70% alcohol. The lips of the vulva were then parted as far as possible and the vagina was sampled by introduction of a sterile cotton swab. In order to obtain a sample from the external os of the cervix a sterile tube speculum (diameter 4 to 5cm) with a built-in light source was introduced into the vagina and kept in position 3 to 5cm in front of the cervix. A sterile cotton swab on a thin wooden stick was then introduced via the speculum into the external os. Upon withdrawal the samples were placed in sterile test tubes and transported to the laboratory.

Sham transfers were performed by using two sterile metal concentric pipettes, 6 and 3mm in diameter, and an inner sterile flexible Rüschelit ureteric* catheter, 1.65 or 1.98mm in diameter, which were 35, 45 and 70cm long respectively.

The outer pipette was covered at one end with a piece of non-absorbent paper prior to autoclaving. An epidural injection of 5ml 2% xylocaine was administered to reduce straining and to suspend defaecation. At the time of transfer the vulva of the recipient was cleaned with 70% alcohol. While the vulva lips were parted, the first two sterile pipettes, one inside the other, were introduced into the vagina up to the external os of the cervix. The second pipette was then extended from the outer pipette (speculum) and introduced via the cervix 3 - 4cm into one of the uterine horns. The third flexible catheter was introduced under sterile precautions into the second pipette and advanced as far as possible into the uterine horn. One ml of TCM 199 was injected into the uterus through the flexible catheter.

The uteri of animals which were slaughtered 48 - 72 hours after the transfer, were flamed locally with a Bunsen burner and incised. A sample was taken with a wire loop from each uterine horn, the body of the uterus and the cervix. Cultures were prepared as soon as possible after collection by inoculation of horse blood agar, serum broth and liver broth, which were incubated both aerobically and anaerobically.

RESULTS

TABLE 1

BACTERIA ISOLATED FROM THE GENITAL TRACT OF CLINICALLY NORMAL CATTLE

A. Bacillus species (haemolytic and non-haemolytic)
B. Streptococci, α haemolytic
C. Streptococci, β haemolytic
D. Streptococci, non-haemolytic
E. Staphylococci, coagulase-positive
F. Staphylococci, coagulase-negative
G. Corynebacterium pyogenes
H. Diphteroids
I. Escherichia coli
J. Enterobacteria
K. Pasturella species
L. Streptomyces species

* Rüsch, West Germany

A list of the bacteriological isolates from the vulva, vagina and external os of the cervix of 20 clinically normal cattle is presented in Table 1.

Streptococci and Bacillus species were the most common bacteria found in the tract followed in numbers by staphylococci, enterobacteria, E. coli and C. pyogenes, while diphteroids were seen in only a few animals. More detailed information about the relative incidence of bacteria obtained from the vulva, vagina and cervix is presented in Table 2.

TABLE 2 INCIDENCE OF BACTERIA IN THE BOVINE VULVA, VAGINA AND CERVIX

(+) = sporadic; fewer than 5 colonies ++ = several; 10-30 colonies
+ = some; 5-10 colonies +++ = many; more than 30 colonies

ANIMAL NUMBER	VULVAR ISOLATES												VAGINAL ISOLATES												CERVICAL ISOLATES											
	A BAC. SP.	B STREP. α	C STREP. β	D STREP. N.H.	E STAPH. C+	F STAPH. C-	G C. PYO.	H DIPHTH.	I E. COLI.	J ENT. BAC.	K PAST. SP.	L STREPTOM.	A BAC. SP.	B STREP. α	C STREP. β	D STREP. N.H.	E STAPH. C+	F STAPH. C-	G C. PYO.	H DIPHTH.	I E. COLI.	J ENT. BAC.	K PAST. SP.	L STREPTOM.	A BAC. SP.	B STREP. α	C STREP. β	D STREP. N.H.	E STAPH. C+	F STAPH. C-	G C. PYO.	H DIPHTH.	I E. COLI.	J ENT. BAC.	K PAST. SP.	L STREPTOM.
1	+++				+++											+						++			(+)				+					++		
2	++	+										(+)		+++																						
3										+++			(+)			++																				
4	+++						+++						+			++				++												(+)				
5	+++								+++	+++			+++									+++														
6	+++				+++								+++				+++																			
7										+++			(+)			+++																				
8	+++			+++	+								+++												+											
9	+	++		+++										+++		+++										+										
10	+++			+++	+				+++							++	+++				++							+				+				
11	++	+										(+)		+++																						
12	+++				+++											+					++				(+)				+				++			
13	++								+	++			(+)			++																				
14	+++						+++						+			++				++								(+)				(+)				
15	+++								+++	+++			+++									+++														
16	+++				+++								++				++					+++												+		
17	+++								+	++			(+)			+++																				
18			+++	+++	+								+++												+											
19	+	++		+++										+++		+++										+										
20	+++			+++	+				+++							++	+++				+++											+				
NUMBER OF ISOLATES	52												41												17											

110 isolates were obtained from 20 animals. 52, (47%) originated from the vulva, 41 (37%) from the vagina, and 17 (16%) from the external os of the cervix. There were no negative findings in the vulva and vagina, but 9 (45%) in the external os of the cervix. In addition to a decrease in the number of isolates from the vulva to the cervix, there was also a decrease in the number of colonies of the different bacteria in the same direction (Table 2).

The results of the bacteriological examination of the instruments used for a non-surgical sham embryo transfer and of the uteri of recipient animals slaughtered 48 to 72 hours after sham transfer, are shown in Table 3.

TABLE 3 OCCURRENCE OF BACTERIA ON THE INSTRUMENTS BEFORE, AND IN THE GENITAL TRACT AFTER, A NON-SURGICAL SHAM BOVINE EMBRYO TRANSFER

Each dot represents one of the groups listed in Table 1
For meaning (+), +, ++, +++, see Table 2.

ANIMAL NUMBER	DAY OF CYCLE	AT TRANSFER												AFTER SLAUGHTER										INTERVAL TRANSFER TO SLAUGHTER (HRS)
		OUTER PIPETTE				MIDDLE PIPETTE				INNER CATHETER				CERVIX				BODY				L.HORN	R.HORN	
		(+)	+	++	+++	(+)	+	++	+++	(+)	+	++	+++	(+)	+	++	+++	(+)	+	++	+++	(+)	+	++	
1	1				••																				48
2	2			•	•	•				••	•			•											72
3	2	•		•										••	••			•••							48
4	5	•		•																					48
5	6				••																				48
6	6		•	•		••	•			•				•											72
7	8		••		••	•	•				•			•											48
8	10			•	••	•••				•	•														72
9	16	•		•			•			•															72
10	17	•	•	•	•	•		•			••														48
11	18				•••		•	•																	48
12	19		••	•				•	•					•					•	•		••	•	•	48
TOTALS		31				16				10				8				6				2	2		

57 isolates were obtained from the three pipettes used for sham transfer in 12 animals, 54%, 28% and 18% from the outer, middle, and flexible catheters respectively. The bacteriological examination of the first pipette was positive in all cases. The second pipette and third catheter were negative in 4 (33%) and 6 (50%) cases respectively.

Bacteriological examination of the cervix and uterus 48 - 72 hours post sham transfer showed that bacteria could be cultured from the cervix

of 5 out of 12 animals (8 isolates) and from the uterus of 2 out of 12 animals (10 isolates). Only one animal showed contamination of all three parts of the uterus. In a second animal only the body of the uterus was contaminated. 8 of 10 isolates obtained from the uterus yielded fewer than 10 colonies and 2 produced 10 to 30 colonies.

The pregnancy results of 10 cows which were inseminated and in which a subsequent sham transfer was done are presented in Table 4. Pregnancy diagnosis 50 days after insemination revealed 5 out of 10 animals pregnant.

TABLE 4

THE EFFECT OF SHAM TRANSFER ON PREGNANCY MAINTENANCE IN INSEMINATED CATTLE

Cow no.	Age (years)	Day of cycle at transfer	Bacteriological examination pipettes		Pregnant
			second	third	
1	3	7	-	-	+
2	5	7	-	-	+
3	5	9	-	-	-
4	8	7	-	-	-
5	7	7	Staph. aureus	-	+
6	4	10	-	-	+
7	5	8	Strept. lactis	Strept. lactis	+
8	5	8	-	-	-
9	3	6	-	-	-
10	5	8	-	-	-

CONCLUSIONS

The bacteriological findings in the vulva, vagina and external os of the cervix are comparable to those of Taubrich (1958) and of Gibbons, Attleberger, Kiesel and Dacres (1958) in cervical mucus, and of Khalil (1970) in the cervix. Streptococci and staphylococci were the most common bacteria in this part of the genital tract. Like Taubrich, we found a decrease in the number of isolates from the vulva to the cervix. Kampelmacher (1954) examined the external cervical os of 25 cows and found 17 (68%) of them to be culturally negative. This is in good agreement with

our negative findings of the external cervical os of 9 out of 20 (45%) animals.

A decrease in the number of isolates could also be observed when the two metal pipettes and the flexible catheter, used for sham transfer, were examined. Contamination of the third catheter occurred in only 50% of the cases and when contamination did take place, fewer than 10 colonies were cultured. In contrast, more than 10 colonies were cultured in 60% of the isolates from the outer pipette. Thus, the use of a set of three concentric pipettes offers good protection against contamination of the uterus by potential pathogenic bacteria from the vulva, vagina and cervix during dioestrus.

Kampelmacher (1954) demonstrated that healthy cows are able to eliminate haemolytic staphylococci introduced into the uterus within 48 hours. From biopsies taken after inoculation it appeared that the bacteria caused only a slight reaction of the endometrium together with small accumulations of inflammatory cells. The endometrium returned to normal in 5 to 6 days. This emphasises the possibility that bacteria introduced into the uterine lumen may alter the uterine environment. Even though bacteria do not produce an endometritis per se, they may nevertheless interfere with conception.

At slaughter, bacteria were found in the uterus of 2 out of 12 animals in which a non-surgical sham transfer was done. The question remains whether these bacteria were introduced into the uterus by the instruments or by post mortem contamination by vaginal fluids. Vaginal fluids may enter the uterus upon relaxation of the cervix after slaughter when the animals are suspended by the hind legs (Naksmura, et al. 1975). The same observation was made earlier by Kampelmacher (1954), who separated the uterine horns into two compartments with strong clamps, just after killing the animal and before the animal was suspended by the hind legs.

Griffin, Murphy, Nunn and Hartigan (1974), using a similar set of concentric tubes for sampling the bovine uterus, found that the sampling procedure yielded uncontaminated samples and that the technique did not lead to colonisation of the uterus by exogenous bacteria, even when cows were sampled repeatedly during the luteal phase of the oestrous cycle.

The effect of a non-surgical sham transfer in inseminated cattle in the horn contralateral to the corpus luteum resulted in pregnancy in 5 out of 10 animals. This is in agreement with the findings of Seidel, Bowen, Homan and Okun (1975) who performed sham transfers to the horn ipsilateral to the corpus luteum using the same technique. They achieved pregnancy rates of 48% and 54% respectively, in 25 treated and 26 control animals. Thus, contamination of the uterus after non-surgical embryo transfer using three concentric pipettes does not appear to be a major cause of the reduced pregnancy rates reported. Lawson, Rowson, Moor & Tervit (1975) obtained a promising pregnancy rate of 37.5% after late (Day 6 to Day 9) non-surgical transfers, by using a single glass insemination pipette. Sreenan (1975) obtained a pregnancy rate of 50% by using a normal bovine artificial insemination apparatus (Cassou apparatus) to introduce the embryo. However, it would appear to be safer to use more than one pipette, whereby the first one can serve as a speculum especially when non-surgical embryo transfer is done under field conditions.

As spontaneous uterine contractions do not occur after Day 4 to 5 (Brand et al. 1975) and better results have been obtained with late transfers on Day 6 to 9, Lawson et al. (1975), the optimal time to transfer embryos via the cervix may be Day 6 to 8 when the embryo is still surrounded by the zona pellucida, and therefore less vulnerable than the hatched embryos at Day 8.5.

In conclusion, it appears unlikely that contamination of the uterus is a major factor contributing to the lower pregnancy rate after non-surgical embryo transfer when compared to surgical methods in clinically healthy animals. It is possible that technical failures, such as lack of experience, are of greater importance. Trauma of the endometrium in combination with an induced leucocytosis, or a disturbance of the endocrinological and biochemical environment of the uterus may also play a role.

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DISCUSSION

C. Thibault (France)

Is the decrease or the increase of uterine activity related to progesterone level or to oestradiol level? In other words, if there is an increase in myometrial activity during the period of the luteal phase, is there a possibility of an increase in oestradiol by the growth of follicles during the first week?

A. Brand (Netherlands)

We haven't measured the oestradiol levels in this experiment but there was an increase in the amplitudes from Day -3 to Day 0, Day 1. So I don't know if this related to the oestrogen level or to a decrease in the progesterone level. I think it is a combination related to the ratio of oestrogen to progesterone, but I have not exact data.

J. Hahn (W. Germany)

Have you done non-surgical ova collection using the balloon catheter and the other instruments, and what are your results?

A. Brand

We are still in the experimental stage. First of all, we used several metal instruments. We have also used the Sugie apparatus. The most important thing, in our opinion, is the recovery rate of the medium - before you consider the recovery rate of the embryos. We started with a recovery rate of the medium of about 60% - 70% last year. Then a few months ago we increased that to 85% and now we have improved that to 95% -96%. So we are still in that stage; we are not looking at embryos at all at the moment. In the early stages, when we were using different instruments, we got very poor results and we had a recovery rate of about 12½% at that time. What we have done is to simplify the instruments. I don't like to use metal instruments because then you are damaging the endometrium and you cause bleeding etc. At the moment we are using two catheters - one balloon catheter and one urethral catheter. We are hoping to manage it with only one catheter. The diameter is still a problem. At the moment we are using a diameter of 6.6mm and we are using an open-ended balloon catheter. It is very difficult to introduce an open-end catheter into the uterus - I can show you later on how we solved that

problem - but the whole situation at the moment is that we are still improving the technique and we are not looking at eggs.

R. Newcomb (UK)

I would like to make two comments. Firstly, I believe you said you got a 50% conception rate when you transferred to the contralateral horn. We have the impression that conception rate is lower in cattle when one transfers to the contralateral horn. We did a series of 13 transfers to the ipsilateral horn and we got 6 pregnancies resulting. In a series of 13 transfers to the contralateral horn we got no pregnancies. So I am rather interested in your finding there and in any comments other people might have - I know Joe Sreenan has some evidence on this.

The other thing is that I was very interested in your results with stimulating the cervix on electrical activity in that it didn't cause massive increase in electrical amplitude but only an effect of short duration. We have been looking at the effects of injecting oxytocin with the same outcome as you were looking for in relationship to non-surgical transfers. We have been looking at the effect of oxytocin on prostaglandin $F_{2\alpha}$ release and up to Day 3 we had a release in prostaglandin $F_{2\alpha}$. After that time, in exactly the same way as your electrical activity drops off, we don't get any response to the oxytocin injection in terms of $F_{2\alpha}$ release. Where we have mimicked non-surgical transfers and exaggerated what would normally be done we have not found any release of $F_{2\alpha}$. I wonder if you could comment.

A. Brand

We performed a sham embryo transfer in the inseminated animals in the contralateral horn to the corpus luteum. So we didn't transfer an embryo in the contralateral horn but we just looked at the pregnancy rate in the inseminated animals and what the effect was of a sham embryo transfer in the contralateral horn to the corpus luteum. Then we couldn't find any difference - 50% maybe. Normal is 60% I think, so it was just by chance. However, I agree that at Day 3 there is a big change in the electrical activity. If you compare the situation of Day 3 to Day 4 the amplitude has decreased tremendously and you really can't compare the situation on Day 4 with the situation prior to Day 4.

A.O. Trounson (UK)

If you think that egg rejection is not important, and if you think you can protect the instruments by using this three way catheter, then do you think you will get very high pregnancy rates, or do you think there is something else?

A. Brand

We are very interested in this specific field - what is happening with the biochemical environment? If you look very carefully you are always damaging the uterine lining. Sometimes you get bleeding, sometimes other damage, but you are causing damage. That is also the reason that I like to use the third flexible catheter. Even then it is possible to cause damage. Another thing is that I don't know whether we deposit the egg at the correct site. I like to deposit my embryos at the tip of the uterine horn. I don't know if it is very important - there are other indications that it is not important at all. I think technical failures are very important because from the work of Sreenan, he shows that one of his best inseminators did the non-surgical transfers. Also, in artificial insemination there is a difference in pregnancy rates between inseminator (a) and (b) of 10 - 15%. It is my opinion that you have to do certain things before you can get good pregnancy rates. During the last few months the best result I got was 40% and then we changed the instruments and used the Cassou apparatus and so on. Then we didn't get any results at all; sometimes only one pregnancy out of ten transfers. So quite a lot of factors are involved I think and we have to start with examining one factor, then another.

R. Church (Canada)

I have two technical questions. Firstly, how long were these cows post partum and were they all checked previously for bacterial infection? Secondly, do you have the same type of recordings on virgin heifers?

A. Brand

In this experiment we used two heifers. They hadn't had a parturition before. We examined the vagina, the cervix and the vulva and we found the same isolates as we found in the other animals. We did this also in lactating animals and the same pattern of activity was obtained from these lactating animals. I can't remember how long the interval was

between parturition and the time of measuring the electrical activity. I think it was three months but I am not sure.

R. Church

Presumably then it was relatively easy to introduce the pipette into the virgin heifers, through the cervix?

A. Brand

Yes, but we selected these two heifers beforehand.

J. Sreenan (Ireland)

I would just like to make a couple of comments rather than ask a question. They are on the basis of what Alan Trounson was talking about a little earlier. Obviously from your work it would seem that egg ejection is not a major problem, especially when we are transferring after Day 5, or in the region of Day 7 and Day 9. Our suggestion would be that infection is probably not a problem. It's not that we have been doing bacteriological counts, but when eggs are transferred to the contralateral horn of bred animals it seems to me that you don't affect the pregnancy rate at all in the control animals, or, rather, the control eggs. I believe you have some data on this yourself and Dr. Gordon has data that I am aware of. In other words, the animal that sheds her own egg and is bred has the same pregnancy rate which would suggest that if there is infection it is not enough to upset that end of it.

I would like to clear one point, you don't have to use just one technician. We had one technician in that first period of transfer but we are using three people now and they are all able to obtain about equal pregnancy rates.

The problem lies somewhere in the damage effect on the lining of the uterine tissue - especially when we are using a rigid instrument like the Cassou gun. We have done a number of slaughterings to look at this problem and no matter how gently you go about it you are liable to create damage. I think the other problem is the point at which you deposit the egg. We are now going back to look at that as a surgical procedure where we can control the conditions a little bit more clearly and deposit eggs at two different stages from the same donors.

The only other comment I would make is just to agree with Ray Newcomb. We carried out a small trial during the summer where we transferred one egg to the contralateral horn of non-bred recipients, and another group carried out the same trial but transferred one egg to each side. This was surgical transfer. The pregnancy rates have been 70% in the two egg transfers but we are not sure yet what the egg survival is because it was a two egg transfer. The pregnancy rate in the animals that got one egg, contralateral to the CL, is about 20%. When we look back at our data in terms of single pregnancies following twin transfers the highest survival of those single pregnancies has always been on the side ipsilateral to the corpus luteum. I am not clear why there should be a relationship - I am not sure whether there is one or not, but when we get our embryo survival in the twin group relative to the site of survival, we should be able to see more clearly whether there is.

A. Brand

I agree with you except that I am a vet and I don't trust the vagina of a cow with regard to the bacteria that can occur in the vagina. There is bacteriological flora and the bacteria are not pathogenic. When you go to another cow, another herd, then the bacteria can be very pathogenic. In one or two animals we found coli, C. pyogenes. I think if you transfer an egg in such an animal you must be aware that you can introduce infection resulting in a lowered pregnancy rate.

J. Sreenan

I don't disagree with that of course. The only point I was making was that when you transfer you don't seem to upset the pregnancy rate that the animal has, irrespective of whether you use the pipette technique or the Cassou gun through cannula. I am not involved in measuring the bacteriological levels but my impression is that if there is infection there it is not enough to upset the pregnancy rate and therefore it can't be of great importance.

M.J. Cooper (UK)

Just a general question really: does anybody know what the incidence of short cycles is in these animals which do not get pregnant following non-surgical transfer? Are you, in fact, interfering with the cycle length or the maintenance of the corpus luteum?

J. Hahn (W. Germany)

I will present some data on that tomorrow in my paper.

L.E.A. Rowson (UK)

I think we will have to stop at this point and I thank Dr. Brand for his paper.

SOME ASPECTS OF BOVINE EMBRYO TRANSFER

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INTRODUCTION

The full impact of embryo transplants to cattle breeding programmes has not been recognised by many animal breeders as yet. Although technological improvements remain to be perfected, commercial application of the Cambridge (Rowson, 1971) embryo transplant procedures are in wide use (Church, 1974; Gordon, 1975). The successful use of embryo transplants is one of the most significant developments in cattle improvement programmes since it permits the proliferation of offspring of selected females. The short term utilisation of embryo transplants and the major reason for the rapid and often technically unsound development of transplant teams is due to the artifical gene pool "limitations" presented by health quarantine limitations. Much of the impetus for embryo implant centres has come from the economic demand to increase the number of certain newly imported breeds. In the long run however, the success of embryo transplant-genetics will be in the successful multiplication of offspring from genetically documented matings (Church, 1974). One can only expect four or five daughters, ranging in age, from an honour merit cow by natural reproduction compared to that many daughters the same age with a successful embryo transplant operation. Discussion is currently centred on the documentation of genetically superior females (Church, 1974_2). Some of the management parameters which might be considered in documenting prospective donor cow performance to maximise progress with embryo transplant-genetics include:

1. No more than two breeding services for initial conception.
2. Regular oestrous cycles commencing at a young age.
3. First three calves born within two calendar years.
4. Superior performance index in the traits selected for.
5. Previous matings with contemporary comparisons above average.
6. No parturition difficulties or reproductive irregularities.
7. No conformational or detectable genetic defects.

Genetic progress in selection programmes can be doubled through the

use of embryo transplants with no increase in herd inbreeding (Church,1974$_1$). A cow may meet all the above requirements, have a regular reproductive history and an unsuccessful donor in a transplant programme. Her past reproductive performance may be a reflection of good reproductive management and veterinary care as opposed to inherent reproductive superiority or her ability to respond to transplant technology. Conversely, an apparent non-breeder of considerable genetic merit may well have tubal blockage cleared, or low level infection resolved and subsequently become productive because of the tract flushing used in embryo transplant (Church, 1974). At Alberta Livestock Transplants the percent of transplant operations which have produced calves increased from $<$30% in 1971 to $>$80% in 1975. The cows which have been unsuccessful as donors will be characterised as follows:

1. Do not respond to superovulation, 5 to 10%
2. Stimulate but do not ovulate, 2 to 3%
3. Do not come into heat, 1 to 8%
4. Fertilisation does not occur, 1 to 8%
5. A failure of transferred embryos to implant, 1 to 11%

THE EFFECT OF THE SUPEROVULATING AGENT ON EGG PRODUCTION

One of the major problems faced in embryo transplantation is a biologically reliable method for superovulation. From October, 1972 until June, 1975, a number of pregnant mare serum gonadotrophin (PMSG) preparations were assayed in terms of their ability to superovulate donor cows successfully. The results of this work are presented in Table 1. Assay of PMSG indicates that the in vitro assay of FSH and LH is not correlated with embryo transplant success. Superovulation must stimulate ovaries without causing excessive ovarial oedema, abnormal follicular development, yield optimal follicular numbers, cause ovulations which result in normal corpora lutea and still leave the uterine environment receptive to sperm transport, subsequent fertilisation and cleavage development. The results presented utilise an experimentally chosen optimal dose, for each batch of PMSG, which ranged from 1500 to 3200 i.u. In Table 1 the average number of observations as well as the range (in brackets) is presented. It should be emphasised that the environmental and management conditions found in a particular transfer programme have a very profound effect on the results which can be obtained from any given preparation of PMSG.

TABLE 1 VARIANCE IN PMSG RESPONSE IN SUPEROVULATION

PMSG PREPARATION	NUMBER OF ANIMALS STIMULATED	NUMBER OF CORPUS LUTEUM OBSERVED	NUMBER OF FOLLICLES OBSERVED	TOTAL EGGS RECOVERED	NUMBER EGGS FERTILISED
1	13	133(6-24)	23(0-4)	104	71
2	12	142(0-37)	186(10-30)	80	51
3	12	136(3-21)	184(2-25)	54	0
4	8	74(2-16)	21(2-9)	68	27
5	9	104(0-14)	13(0-5)	98	8
6	19	217(3-17)	41(1-6)	206	194
7	23	197(4-16)	47(1-5)	183	101
8	17	214(1-25)	79(0-11)	150	117
9	16	236(2-12)	68(1-6)	162	136
10	287	3124(2-91)	294(0-12)	2975	2252

TABLE 2 EFFECT OF TIMING OF SUPEROVULATION ON EMBRYO TRANSFER

	Pregnant mare serum gonadotrophin	
	Day 10	Day 16
Number of animals	43	37
Ave number CL observed	13.4	7.2
Ave number follicles observed	2.1	0.4
% ova recovered	81.5%	75.3%
% recovered ova fertilised	87.4%	94.0%
Ave number fertilised ova recovered	9.3	5.05
Ave number of pregnancies	3.76	3.31

Table 2 presents the results of studies of the timing of superovulation within the oestrous cycle. Donors were superovulated with PMSG on Day 10 or

or Day 16 of the oestrous cycle. In each case 2000 i.u. of PMSG were injected intramuscularly. The Day 10 group received an intramuscular administration of 35mg of PGF_{2a} thirty-six hours later while the Day 16 group were followed to natural oestrus. The Day 10, PMSG and PGF_{2a}, routine has proven to be the most effective and avoids the problems of "silent" or heat failures in superovulated donors.

Effective superovulation yielding good quality fertilised embryos shows wide seasonal and geographic variation. For example, in the months of January, February and March, superovulation response rates drop to 80% of summer ovulation rates with much less predictability. The number of fertilised eggs and subsequent recipient pregnancy rates drop from near 90% and 60% to 40/50% and 30% respectively with an increased standard error. Very high levels of follicular stimulation usually produce low "quality" eggs with less biological capability of implantation than optimal ovulation rates of from 8 - 10 per ovary. In 1971, 1972 and 1973, the percentage of cows showing no stimulation or "silent" heats in natural oestrus, varied from 10 to 25% of total cows receiving PMSG. Different breeds of cattle differ significantly in their response to similar doses of PMSG. In Alberta, superovulation with the same dose and batch of PMSG can be modified dramatically by seasonal and nutritional variation. The most unreliable superovulatory response occurs during the months of January, February and March. The number of follicles stimulated would appear to be fairly similar over the year, however, the number of ovulations as judged by corpus lutea present is lower and less predictable during the winter months. Fertilisation is less complete in the January to March period and is highest between August 1 and October 31. In every year since 1972 a ten day to two week period has been encountered in late June or early July during which unusual variation has occurred in stimulation and up to 80% of the donors in which ovulation had occurred did not yield fertilised eggs. The effect of natural oestrogen in forage grown in this particular geographical area should be investigated.

RECIPIENT MANAGEMENT

The success of an embyro transfer programme is primarily dependent on the quality and reproductive capabilities of the recipient pool. Most programmes fail because of insufficient attention being given to the logistics

of maintaining a recipient pool of carefully chosen heifers. The rates of implantation in recipients displaying natural and PGF_{2a} induced oestrus is presented in Table 3.

TABLE 3 EMBRYO IMPLANTATION IN RECIPIENTS DISPLAYING NATURAL AND PGF_{2a} INDUCED OESTRUS

Number of animals in:	Natural oestrus	Single injection PGF_{2a}	Double injection PGF_{2a}
Group	365	60	40
Oestrus	109	37	26
Implantations	104	36	26
Pregnancies	65	16	12
% Implants	62	44	46

Under our pasture conditions we have seldom observed more than 65% of the non-PMSG stimulated animals in standing heat following PGF_{2a} treatment. In contrast Day 10 PMSG stimulated donor cows treated with PGF_{2a} show standing heat sometime between 45 and 95 hours in most instances. Our recipient herd is selected for the absence of reproductive tract irregularities, the presence of regular oestrous cycles, good reproductive backgrounds and an absence of disease. Management is optimal with animals gaining about ¼ to ½ pound per day. The importance of a continuous management and nutritional regime prior to and after implantation cannot be overstressed if maximum implantation rates are to be obtained. Under our conditions implantation rates vary from a low of 35% to a high of 80% for large uniform groups of yearling recipient animals. We prefer Hereford/Angus or Jersey/Cross yearling heifers weighing about 800 pounds at the time of implantation.

EVALUATION OF EGGS

In most embryo transfer programmes the pregnancy rate success is at first rather discouraging. It is not unusual for initial implantation rates to be from 10 to 30 percent. Some of the factors which determine the implantation rate include superovulation, the management of the recipient

herd, the success in recovery, storage and handling of eggs, and the evaluation of the eggs to be implanted. In other species 80% implantation of transferred embryos is easily achieved. In the bovine, using closely synchronised recipients, and critical egg selection, single implantation rates of as high as 80% can be achieved with an average between 50 to 60%.

Ova are recovered from donors by the surgical procedures developed by Rowson (1971). A midline laparotomy is performed on donors anaesthetised with Fluothane. The surgical recovery of eggs is done via a glass cannula placed in the infundibulum of the oviduct. Approximately 75cc of flushing media (Tissue Culture Medium (TCM) 199 with serum, 20mg/ml, and phenol red 20mg/ml) is then injected into the ligated ipsilateral horn and gently forced out through the oviduct and cannula to be collected in a small watch glass. The eggs are located under a Wild M7 stereodissection microscope equipped with camera and stored until implantation in TCM 199, with Hank's salts, glutamine, 10mg/l phenol red, 25mM Hepes buffer, 0.35gm/l $NaHCO_3$ at pH 7.1 (Grand Island Biological No.235). Implantation is done with surgical midline exposure since higher rates of implantation have been achieved than via the surgical flank route (Church, 1974_2).

To achieve high implantation rates it is essential that a meaningful criteria for the morphological evaluation of biological capacity of embryos be developed. It is easy to distinguish between unfertilised, eight or more cell cleavage stages and well developed morula, etc., however, it is more difficult to detail the multitude of morphological types found in most tract flushes. Detailed biological and morphological characterisation of cattle eggs has not been the subject of many investigations (Gordon, 1975: Shea, 1975).

The superovulation regime seems to interfere with the uterine environment in such a way as to have direct effects on the processes involved in egg maturation, sperm transport, fertilisation, egg transport and normal cleavage division. Our data supports the concept that there is a higher incidence of abnormalities in eggs from superovulated compared to normal donors. Also, ovulation in the superovulated donor can occur over a fairly wide time span since it is not unusual five days after standing heat to recover normal appearing unfertilised eggs (Fig. 1). In contrast to other species in which fragmentation of unfertilised eggs occurs, complicating

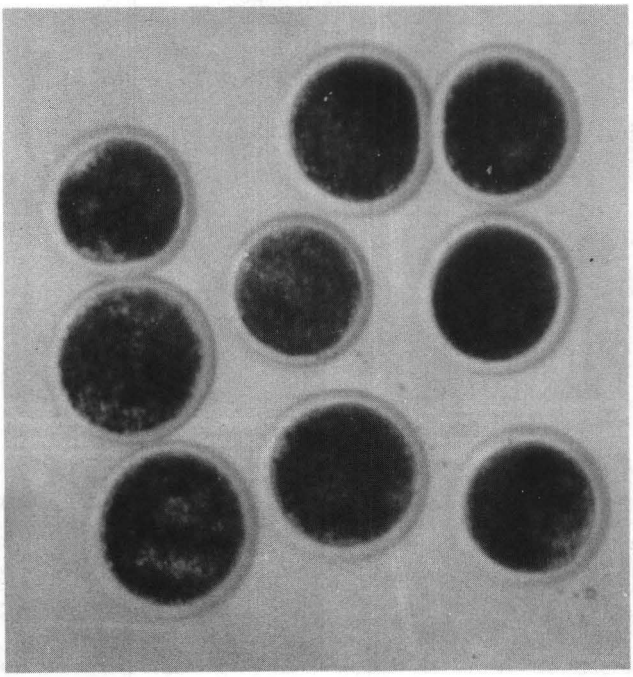

Fig. 1 Nine unfertilised bovine ova recovered from a Fimbrial cannulated uterine flush 5 days after standing heat of a superovulated donor cow. Very little fragmentation can be detected. All eggs recovered from the uterine horn were unfertilised. Ten corpora lutea were observed on the ipsilateral ovary. All photographs of eggs were taken directly in flushing or storage media with an automatic camera attachment mounted on a Wild M7 stereo microscope.

egg evaluation, deterioration in cattle eggs usually results in "doughnut" formation (Fig. 2). In the stimulated bovine well developed morula can be found above the utero-tubal junction five days after oestrus and conversely fertilised eggs undergoing cleavage can be found in the uterus soon after oestrus (Neufeld, unpublished). This erratic transport is in direct contrast to the rather orderly and defined movement of fertilised embryos in the naturally cycling cow (Hamilton and Laing 1946). In some animals debris and flocculent mucus can be seen surrounding eggs which were flushed out (Fig. 3). Batches of eggs recovered from superovulated donor tracts can include a mixture of unfertilised eggs, eggs in early cleavage stages and late morula (Fig. 4). Some of the morphologically abnormal eggs which have been observed are shown in Figure 5.

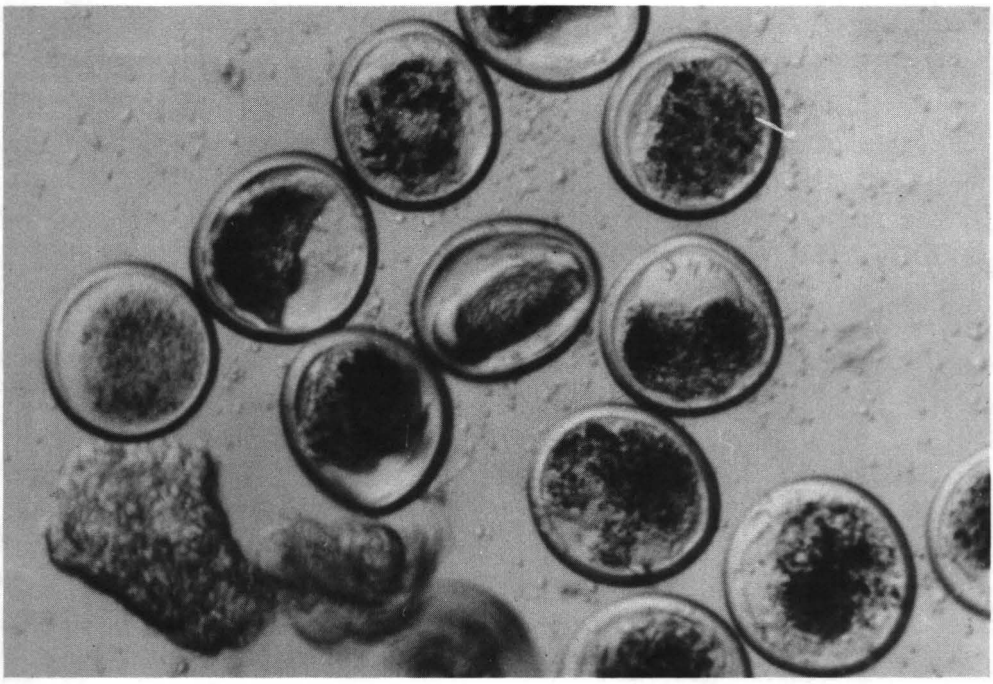

Fig. 2 Twelve degenerating bovine ova in flushing media. Unfertilised bovine ova degeneration usually results in "doughnut" formation rather than the type of fragmentation seen in other species.

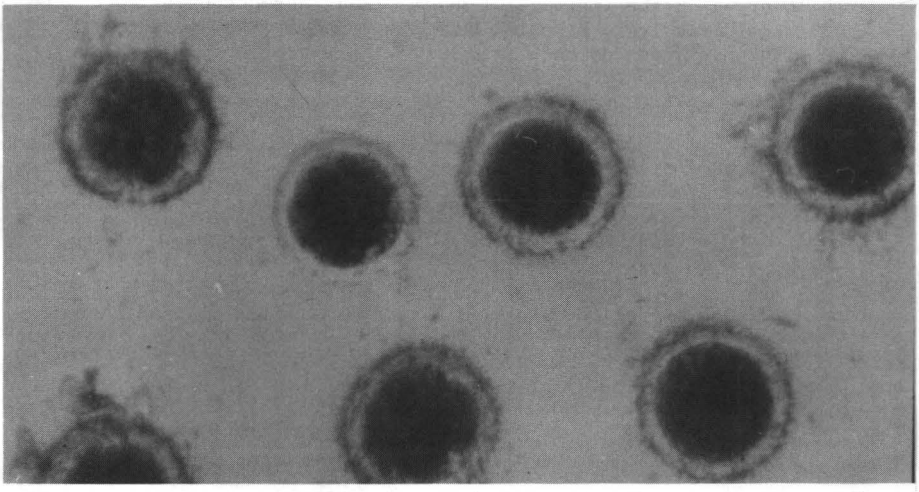

Fig. 3 Seven unfertilised ova in flushing media in which each egg is surrounded by flocculent debris from the infected reproductive tract. No external or cervical signs of infection were evident.

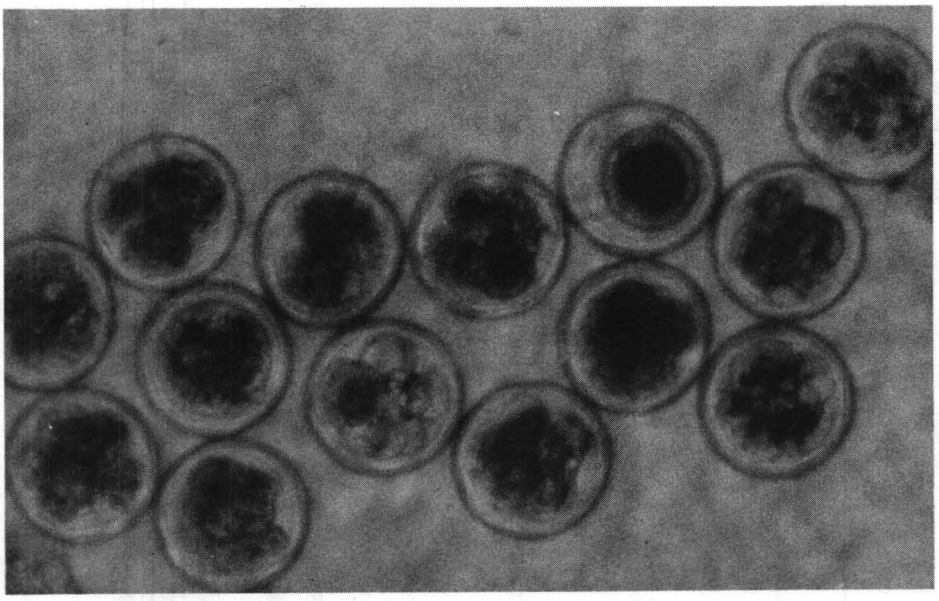

Fig. 4 Fifteen eggs showing a range of development. Three are unfertilised, 6 have less than 12 cells, and 6 are in various morula stages. Of the uterine flush shown 12 embryos were implanted into synchronous recipients resulting in 8 pregnancies.

It is a common occurrence for a single blastomere to be excluded from further development of an embryo. There is no apparent difference in implantation rates of these morula in which a blastomere is excluded versus those in which all blastomeres are included in a tight morula.

The morphological evaluation of embryos is a difficult and frustrating process. It is almost impossible to delineate with any certainty the possibility of a particular egg implanting in a recipient uterus. Evaluation criteria have been developed which serve as a general guideline for egg evaluation and potential pregnancy (Shea, 1975).

The ultrastructure and morphological characterisation of the bovine ova has not received very much attention in the past. Hamilton and Laing (1946) have delineated the cleavage process in the bovine embryo in naturally ovulating animals. The ultrastructure of bovine follicular oocytes has been examined by Fleming and Saacke (1972).

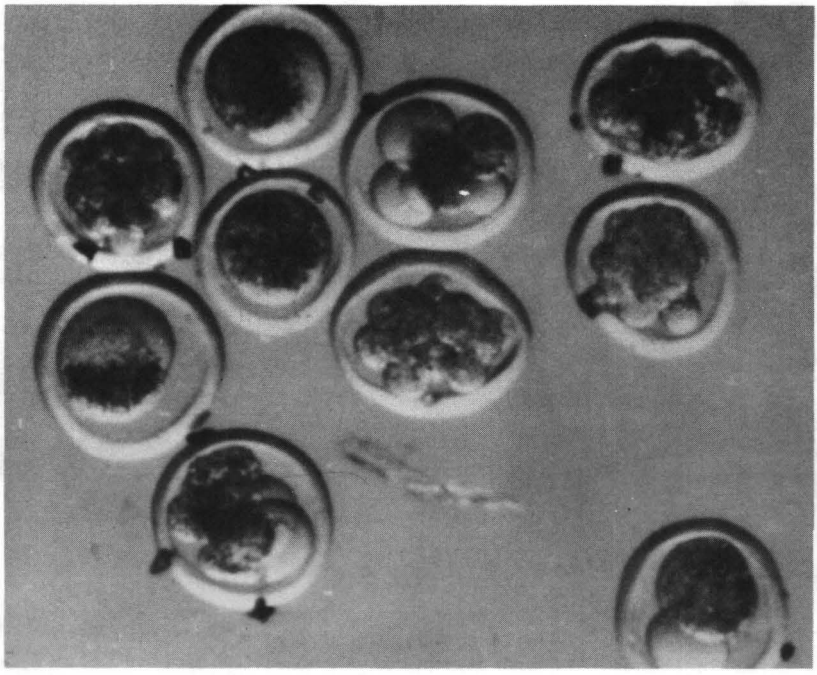

Fig. 5a

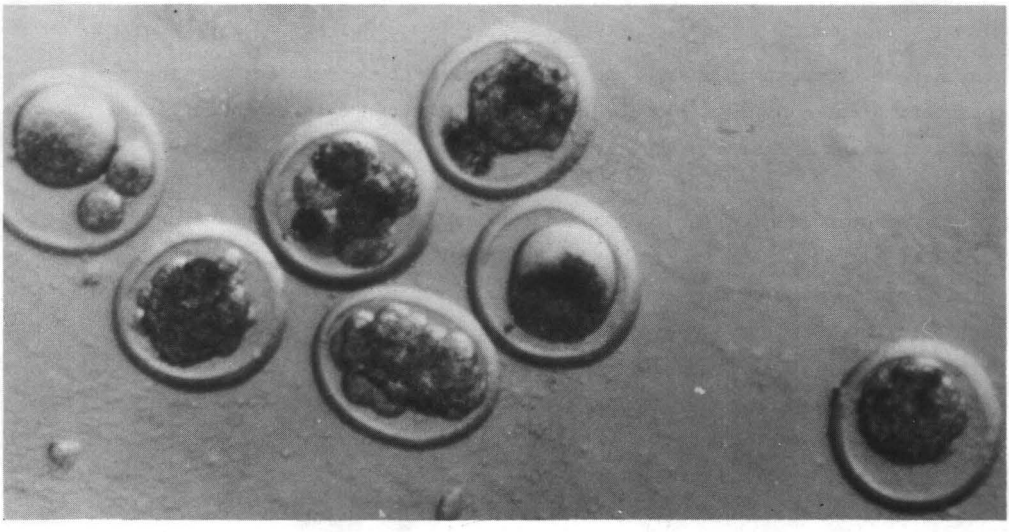

Fig. 5b

Fig. 5 Examples of some of the abnormal types of Day 5 development observed

Fig. 5a (opposite) Ten embryos in flushing media illustrating: (i) 4 normal morula, (ii) one 'hormal' unfertilised ova, (iii) 2 unfertilised ova with 'incomplete' ooplasm, (iv) 2 early cleavage stage embryos with 'clear' blastomeres. At least one of the morula has an excluded blastomere.

Fig. 5b (opposite) Seven embryos in flushing media illustrating: (i) unfertilised ova with 'incomplete' ooplasm, (ii) unequal polar body like vacuole exclusions, (iii) elongated morula, (iv) granular cytoplasm in a 6 cell embryo, (v) 3 'tight' morula which can easily be mistaken for immature unfertilised eggs.

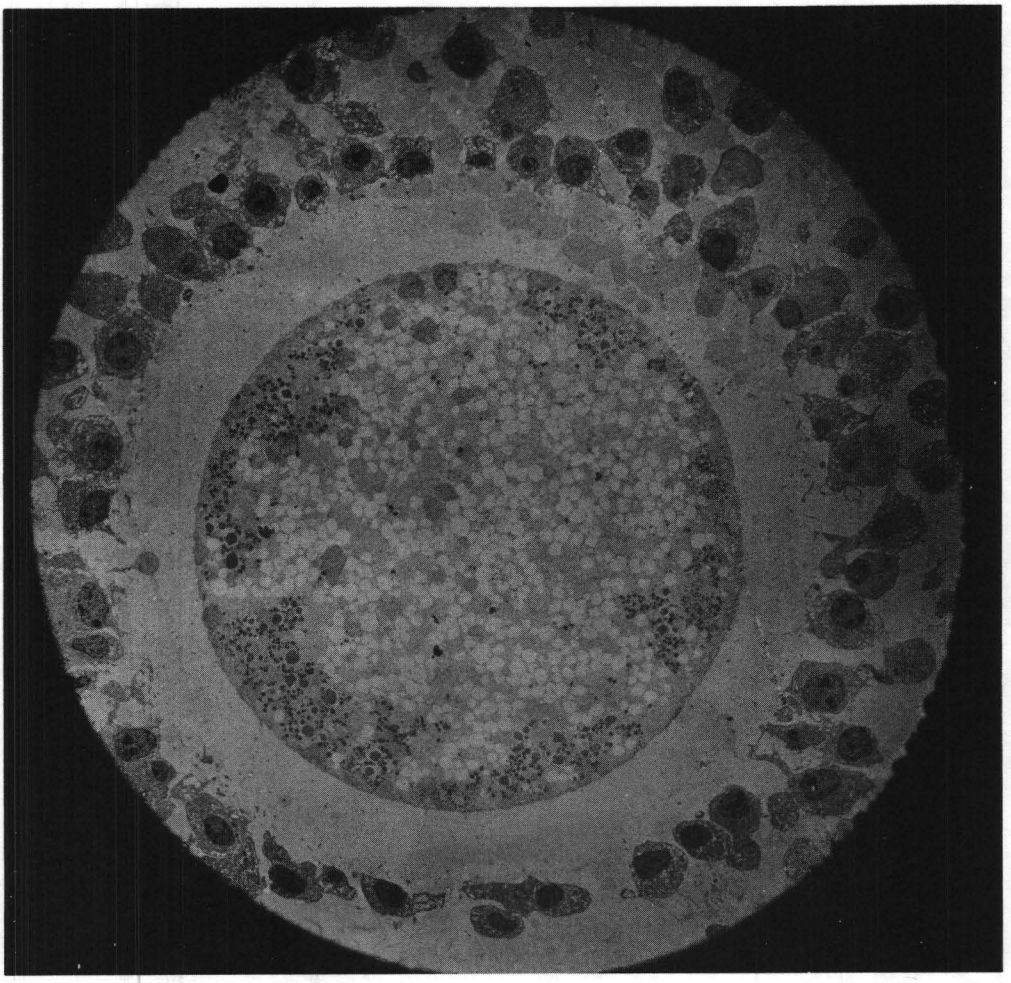

Fig. 6 A whole mount oocyte fixed in gluteraldehyde and osmium at 300 X magnification showing excessive granulation.

Bovine ova are approximately 100 to 130 μ in diameter and have not been available in very great numbers for research purposes. While whole mounts are readily obtained the detail which can be ascertained by this route is limited requiring the development of refined techniques for ultrastructural examination. A whole mount oocyte fixed in gluteraldehyde and osmium is shown in Figure 6 at 300 X magnification. Numerous vacuoles and dense granular structures are evident. The spherical oocyte is surrounded by the zona pellucida within which small cumulus cells can be detected. At higher powers of magnification considerable ultrastructure is easily discernible (Watters, unpublished). It is particularly interesting to find hooded mitochondria, densely staining vacuoles and bodies with several whirls of membrane throughout the ooplasm. A great deal of research remains to be done on the delineation of the morphological, physiological and ultrastructural indicators of biological potential for implantation of bovine embryos (Fig.7).

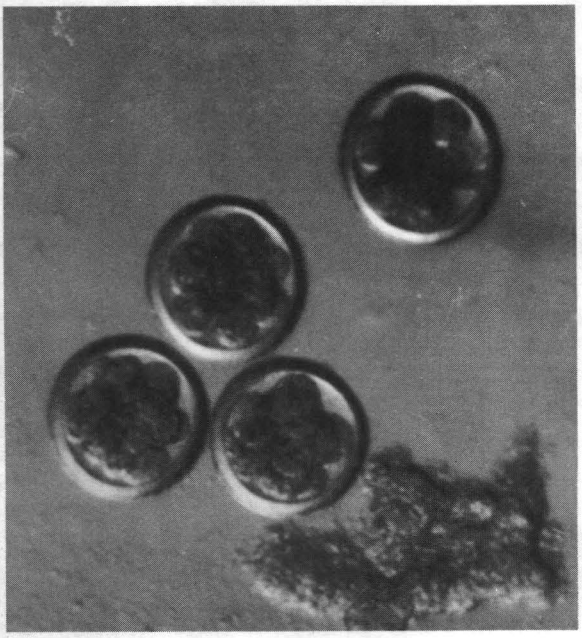

Fig. 7

Four Day 5 morula in flushing media. The superovulated donor had 5 copora lutea on the ipsilateral ovary, 4 morula were recovered and implanted resulting in three pregnancies. This donor cow has been transplanted 3 times resulting in a total of 21 pregnancies, and is now safe in calf.

TRANSPLANT SUMMARY

A summary of the results of embryo transplantation operations carried out at Alberta Livestock Transplants from 1971 to September of 1975 is presented in Table 4. Due to the fact that pregnancy diagnosis is not done until 90 days post-transfer, the 1975 results are only to September.

TABLE 4 SUMMARY OF BOVINE EMBRYO TRANSPLANT RESULTS AT ALBERTA LIVESTOCK TRANSPLANTS FROM 1971 - 1975

Year	1971	1972	1973	1974	1975
Number of donors accepted	11	114	142	291	281*
Number of successful donors	4	86	94	233	234
Ave. number eggs recovered - range	8.1(0-70)	8.3(0-41)	7.9(0-43)	10.6(0-35)	10.34(0-44)
Ave. number embryos implanted - range	11.4(0-34)	5.7(0-28)	5.6(0-21)	8.7(0-34)	8.23(0-37)
Ave. number pregnancies per successful donor - range	2.1(1-6)	2.9(1-8)	3.4(1-15)	4.5(1-20)	4.53(1-20)

* Operations which have pregnancy diagnosis to September 1, 1975

The number of animals which have been unsuccessful donors, for the reasons previously discussed (Church, 1974_2) has declined from over 50% to less than 20% of those stimulated in the four year period. The average number of pregnancies per operation has increased, as has the maximum number of pregnancies obtained in the best super litter. The best donor cow production in 1974 was 32 live calves; one dropped prior to transplant, two transplant operations resulting in 15 calves each, and a natural pregnancy with a calf born 35 days after the last transplant litter. This type of success in embyro transfer adds significant new possibilities in the establishment of genetically superior maternal bloodlines. An animal breeder can establish which of his cows have the proven performance based on past matings on which to base a genetic selection and breeding programme.

ACKNOWLEDGEMENTS

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DISCUSSION

I. Gordon (Ireland)

Professor Church, I would like to ask if you found an association between the age of the donor animal and its superovulatory response? From the point of view of making the fullest possible use of the good dairy cow or whatever, if you leave it until it is getting towards the end of its reproductive career, whether you have any problems there.

R.Church (Canada)

Well, in this particular regard Dr. Shea will be presenting some breed differences - and we believe there are some very significant breed differences in superovulation. Unfortunately, with regard to age, our current bias (and I think that is the correct way of saying it) is that the old Friesian cow is a damned tough animal to stimulate successfully. This is in contrast to a number of "old" - 13 - 15 year old - Red and Black Angus which have been stimulated and transferred with considerable success. It would appear that Herefords are more variable in that particular category in that they either don't stimulate at all or they are quite successful. There doesn't seem to be any intermediate range with them. I think this means that we may have to follow the hormone profiles over a couple of cycles in these older animals in an attempt to delineate what stimulating conditions are best for them. I don't think that there has been any proper kind of work done at all in this particular regard.

H.Karg (W. Germany)

According to your observations on seasonal effects, it is not just a matter of the development stage of the eggs recovered but also of the numbers.

R.Church

Dr. Shea will present some broken-down data on this particular aspect. Two things occur. You will appreciate that in January, February and March, it is not unusual for the temperature to be -30 or -40°F in our particular environment and there are three phenomena - these animals are all outside, by the way - one is that cycling tends to deviate a little bit both in donors and recipients, secondly, I think under this type of environmental and nutritional stress there is less predictable response to any particular

given stimulating regime. This results in a wider range of stimulation which has a consequence in a wider range of eggs recovered and also a range of fertilisation. But, as you will see in Dr. Shea's data later, the percentage implantation of those eggs that are developing is higher. It will be above 50% in contrast to other times of the year when it appears that stimulation is more predictable but, because in our system anything that has a chance is implanted, there are more implantations and hence the number of pregnancies per operation holds up higher than in January to March but the implantation percentage will drop below 50%. If you look at the October to December period of the year you may be at a 45% implantation. In contrast to a research situation where you discard any egg that is degenerate and just implant the good eggs and twin them to obtain maximum implantation rates, in our kind of circumstances we are after the maximum number of pregnancies, and that is inversely related to implantation.

M.T.Kane (Ireland)

Have you, in your morphological studies of bovine eggs, considered the reason why bovine eggs have this very black appearance in contrast to laboratory animals like rabbit and mouse? I wonder if this might have something to do with different culture requirements?

R.Church

That's an interesting question; we haven't explored that to the extent that we should but we are just starting to do that on three bases - one is using the Welles type of microanalytical biochemical techniques to assay biochemical parameters, secondly to assay hormone receptor properties; however, all I can say is that I don't have any data that substantiates anything one way or the other.

L.E.A. Rowson (UK)

I may add that the horse egg is even blacker.

M.T.Kane

I would suspect that it might be due to lipid content.

R.Church

Well, I can't comment on that. Initial analyses would say not - which is surprising.

A.O. Trounson (UK)

Your classification of embryos differs very much from our classification so it's very difficult to put the two together. What I did want to say is that when we take Day 5 eggs which is the morula stage, or Day 3 eggs, if we classify them as retarded, and we are saying that they are of the divisional stage 24 or 48 hours behind what they should be, when we put them in the rabbit oviduct they all come out the same. So, in fact, they can be retarded at some stage but still have the potential to develop to exactly the same point as eggs which are at a cleavage stage much more in advance. The other thing is we very often see single blastomeres on the outside of morulae and, in fact, they are 'never included' but this doesn't have any relationship to their ability to go on in culture, deep freezing, and so on. The other thing is, these clear areas in blastomeres - this is very typical of what we got with parthenogenic development of cow oocytes. So, if you stain and look at the nuclei you will find that they are, in fact, very abnormal, as you said. We would only accept this as degenerate eggs - eggs in which the cytoplasm or cell membranes have dispersed. Some of the eggs which you said were abnormal we would, in fact, call normal. You can have a single large cell fracture on the 4 cell stage not going on at all but a very compact area of the morulae going on in others and they will turn into quite nice blastocysts.

R.Church

The question you can't differentiate between is, are they retarded or were they late ovulates?

A.O. Trounson

You see, in the sheep, it's a very short period that superovulation occurs over. I think it is something less than 6 hours. We have been doing quite a lot of experiments around the time of ovulation in superovulated animals to collect oocytes and freshly ovulated eggs. I wouldn't say ovulation goes any further than 6 hours. Now, it would surprise me that the cow would ovulate a much longer period than about 12 hours. I am willing to be surprised but I don't think the ovulation time would be anything like 24 hours and I don't think then you would pick up the differences in the cleavage division just during a 24 hour ovulation period. Now, I might be surprised in this but I don't think so.

R. Church

Well, our bias would be that ovulation in the superovulated cow extends over a longer range of time than that and I think Dr. Shea will show some evidence on this later today. We have some endoscopic evidence that suggests that it may be as long as 30 hours over which a superovulated cow will ovulate. I'm not saying they all do that - all I am saying is that it is possible and when you are running through the types of donors and the variety of reproductive backgrounds that we have,as opposed to mostly cross-bred donors which you are working with, it has got to the point where nothing surprises us.

B. Shea (Canada)

I would just like to mention a point here. We prefer not to talk about normal and abnormal embryos in our rating. We prefer to talk about average, above average, below average. That is more meaningful for us. If I could just bring up one other point, Professor Church - maybe we could hear from some of the other people present - and that is with regard to the recovery of unfertilised eggs. We have looked at several hundred of these that we have recovered and sperm attachment is not something that is seen commonly. Secondly, I have never seen (and I imagine the same holds for Professor Church), we have never seen a vesicular nucleus in an ovulated egg and I have never observed polyspermy in an ovulated bovine egg. Is this something that is commonly found and have other people observed these phenomena?

A.O.Trounson

It's not common but we have found it. We have found polyspermy.

B. Shea

How soon after ovulation?

A.O.Trounson

We collect the eggs from Day 3 to Day 7 and I have got some photographs of polyspermic eggs. It's not common though.

B. Shea

When were these animals inseminated?

A.O. Trounson

At oestrus.

B. Shea

This is something we have never really observed.

R.Church

It is interesting that our insemination routine is basically every 12 hours until the cervix closes.

S.M. Willadsen (UK)

I think you are right in saying that we are less stressed by the temptation to transfer eggs which are not really considered good. I think it is also true to say that considering the assessment of eggs is a very subjective thing. In our case there would be minor differences in the assessments that we would make of eggs. It is only that we have been working so closely now in the assessment for a period of a year or two and that is probably why we assess eggs very much the same. On top of that, just to bring the results that we will be presenting later into the proper context, I think you have got a rather rosy picture of our possibilities of choosing and not kicking out that many eggs. We will kick out eggs which are obviously degenerate in our system but we are using quite a lot of eggs which we don't consider particularly good.

R. Church

There is one other question I would like to ask which is relative to what Ray Newcomb talked about this morning. If there is a consistent degeneracy of about 50 - 55% at, say, Day 8 or Day 7, it seems surprising that we can get substantial numbers at certain times of the year with 80% success in single transfers. That's an interesting correlation and this may be related to that superovulated uterine environment.

A.O.Trounson

I think the problem here is in the 55% being an average. I think the proportion degenerate varies very considerably from 0% to 90 or 100%. The average is a little misleading in that respect. I think you can get 80% normal eggs, very rarely 100%, but certainly quite high percentages.

RECOVERY OF UTERINE EGGS IN COW BY TRANSVAGINAL ROUTE

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ABSTRACT

Using a technique of fertilised egg recovery by transvaginal route, we flush the uterus 5 to 6 days after insemination in 6 naturally ovulated cows and 6 superovulated animals. The amount of liquid recovered after uterine flushing with 20 to 50ml of solution is 82% of the volume injected. Eggs are recovered in half of the cows having a single ovulation and in 5 of the 6 superovulated animals. Recovery rate is 43% of ovulated eggs (20/46).

INTRODUCTION

Attempts to recover uterine eggs by non-surgical means in super-ovulated cows have not usually given very satisfactory results. (Foote & Onuma, 1970). Only Sugie et al. (Sugie, 1965 & Sugie et al. 1972), recovered about 40% of the ovulated eggs.

All studies modify somewhat Dowling's technique, (Dowling, 1949): a 2 or 3-way catheter is introduced in utero through the cervix; it has a balloon which is inflated in the uterine lumen. The uterus is flushed with the medium which is returned as it is injected.

We experimented with a transvaginal, transperitoneal technique using the same route as the embyro transfer technique (Testart and Leglise, 1971)

MATERIAL AND METHODS

Animals

Twelve cows were used of which 6 ovulated naturally and 6 were super-ovulated with PMSG. At 5 - 6 days after insemination, the ipsilateral horn was flushed in the non-superovulated cows, while both uterine horns were successively flushed in superovulated cows.

A few days after recovery, donor cows were slaughtered in order to count the number of corpora lutea.

Technique

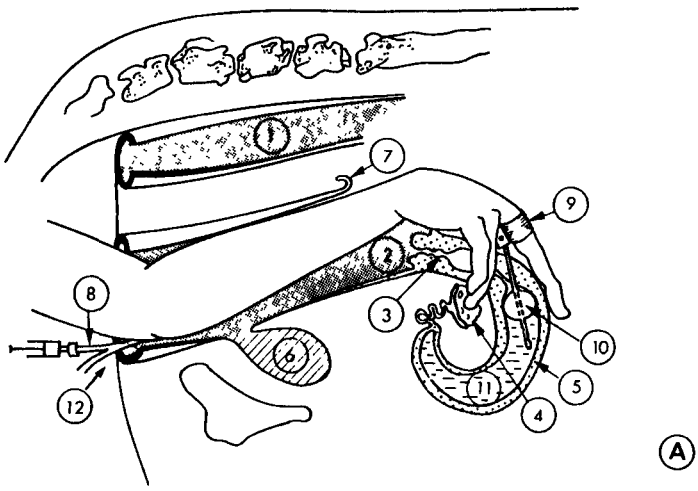
Animals were prepared as for transplantation (epidural anaesthesia, sanitised vagina and vulva, vaginal incision).

The apparatus used for flushing the uterus is composed of a metallic teat cannula (Morin) 2mm in diameter, having 2 lateral openings, attached to a latex catheter with a balloon (Porges, Foley's catheter, ch 9); only the last 15mm of the metallic cannula are exposed and constitute the distal extremity of the assembly. Another latex balloon, which can stand more pressure, is substituted for the original one on the catheter.

After the apparatus, mounted on a ring on the middle finger, has

penetrated the lower third of the horn and is inserted into the uterine lumen up to and including the balloon, the balloon is inflated with 30ml of air. This has a twofold purpose: to ensure the position of the cannula and to prevent a back flow of the liquid injected towards the cervix (Fig.A)

Figure A



Egg recovery by transvaginal route

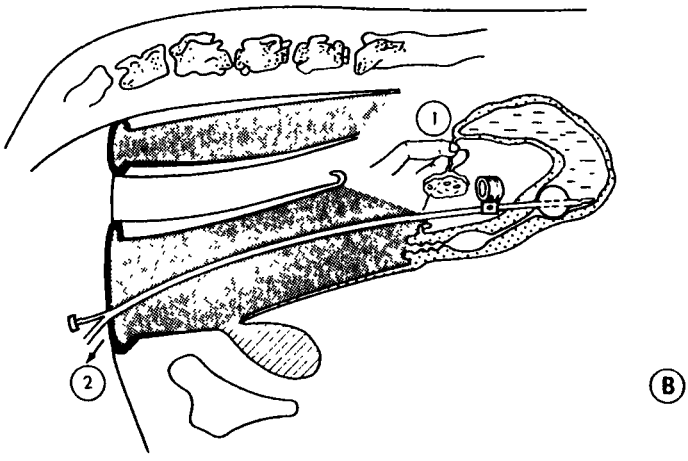
Injection of flushing medium in utero:
1 rectum, 2 vagina, 3 cervix, 4 ovary, 5 uterus, 6 bladder, 7 vaginal opening, 8 inflation of balloon, 9 ring, 10 balloon, 11 uterine cavity, 12 medium injection.

20 to 50ml of solution (TCM 199, Gibco-Biocult) are injected in the uterine cavity through the catheter. The supporting ring remains in position on the apparatus, but the pin-hinge ring permits it to be put off the middle finger, thus completely freeing the operator's hand. Return flow is permitted through the same catheter by a 3-way valve placed on the exterior circuit.

The tip of the uterine horn is usually situated lower than the puncture point, and is pushed still further down by the weight of the flushing medium. Thus, the utero-tubal junction must be lifted by the free hand to

drain off all the medium (Fig.B)

Figure B



Egg recovery by transvaginal route

Return of the flushing medium:

1 pinching the oviduct and raising the uterine horn

2 medium recovery

The utero-tubal junction is pinched with the fingers at the same time as it is raised to prevent the liquid from flowing towards the oviduct. The flushing medium is recovered in a graduated test-tube and after sedimentation for several minutes, the supernatant is drawn off by aspiration. The remaining 15ml at the bottom of the test-tube are distributed in watch glasses so that eggs can be looked for under a binocular microscope.

RESULTS

Most (82%) of the medium injected in utero is recovered. Examination of the sedimented fraction of the flushing medium recovered revealed one egg from 3 of the 6 non-superovulated donors (Table 1). Seventeen eggs were recovered from superovulated donors, or 42% of the number of corpora lutea present on the ovaries.

TABLE 1 RESULTS OF EGG RECOVERY BY TRANSVAGINAL ROUTE

Treatment of donor cow	Superovulation	No superovulation
Number of cows	6	6
Total number of ovulations	40	6
Number of cows with recovered embryo(%)	5(83%)	3(50%)
Number of embryos recovered(%)	17(42%)	3(50%)

The vaginal wound is generally closed after 4 days; scarring does not impede several operations on the same animal. There are few or no adhesions, if both peritoneal and vaginal muscular layers are cut together.

When compared to non-surgical techniques of egg recovery, which use the cervical route, the technique described here appears to have several advantages:

The position of the operator's hand permits him to pinch the utero-tubal junction to prevent escape of the flushing medium through the oviduct; it further insures drainage of the medium by gravity when the tip of the horn is raised.

The chance of cell fragments or contamination being introduced into the uterus is reduced because the catheter does not pass through the cervical canal during the luteal phase of the cycle.

Scraping of the endometrium wall is reduced to a minimum since the flushing catheter passes through only a few centimetres of the uterine lumen.

So far, this technique is limited to parous cows because the particular anatomy of heifers presents difficulties, such as inserting the arm into the heifer's vagina (this could be solved by episiotomy) and introducing the cannula into the lumen when the uterine horn is too small.

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DISCUSSION

L.E.A.Rowson (UK)

Well, this is a unique method of extracting eggs. Perhaps I can start the ball rolling on the questions. How are you certain, when you have the probe on the finger ring, that you are in the lumen of the uterus?

J. Testart (France)

The probe is not sharp, the end is rounded and the penetration of the round probe, 2mm in diameter, is so difficult during the insertion in the wall that it is easy to perceive when you attain the lumen.

R. Newcomb (UK)

Two questions: firstly, what was the size of the balloon on your Foley catheter? Secondly, have you looked at these animals post mortem because our surgical technique is quite similar to this in that we pass a Foley catheter through the uterine wall and in two of six cases which I looked at a month after surgery, there was a fistula?

J. Testart

The original balloon on the catheter had only a capacity of 5 to 10cc of air so we changed it to a balloon with a capacity of about 30cc of air. We slaughtered some cows after this manipulation or we used the same cow for other manipulations and sometimes we also have some adhesions but it is very rare and is mostly when we cut the peritoneum in the process of the manipulation.

I. Wilmut (UK)

Does the wound in the uterus heal over very readily when you put the cannula through the uterine wall?

J. Testart

In recovery or transfer?

I. Wilmut

After recovery.

J. Testart

I don't know exactly. For transfer I know sometimes two days after it is not possible to recognise the place of the operation but for recovery I can't really tell you.

L.E.A. Rowson

Thank you, Dr. Testart, for that fascinating paper.

SESSION TWO

EGG PRESERVATION

Freezing, Culture, in vitro Fertilisation

Chairman: D. Whittingham

GENERAL ASPECTS OF EGG CULTURE AND PRESERVATION

D.G. Whittingham

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INTRODUCTION

The recent reviews of fertilisation in vitro and egg culture (Whittingham, 1975_1) and the low temperature preservation of mammalian embryos (Whittingham, 1975_2) provide an outline of the progress achieved in these areas to date. The most detailed studies of the nutrient requirements and metabolic activity have been made with mouse oocytes and embryos (Brinster, 1972; 1973; Biggers and Stern, 1973; Whitten, 1971; Whittingham, 1971). At present, the specific requirements for other species except the rabbit (Brinster, 1970; Kane and Foote, 1971, and Kane, 1972) are ill-defined, limited partly by their refractoriness to culture, illustrating the necessity to find the particular in vitro requirements for each species. The mammalian embryo unlike many other cell types is unable to adapt easily to conditions imposed upon in vitro, however simple they may be. If it is assumed that the developmental sequence through pre-implantation is pre-programmed in the oocyte, then these events can only take place when the necessary key factors are provided by the environment of the oviduccal and uterine lumen or the culture medium. Only by increasing our knowledge of the biochemistry of the different mammalian embryos together with their oviduccal and uterine secretions can development in vitro be adequately achieved.

Success in all egg and embryo manipulations in vitro is ultimately determined by the progeny resulting from their transfer to foster mothers. The numbers of mammalian eggs and embryos available are extremely limited, even with techniques for superovulation, and therefore, manipulative procedures in vitro e.g. oocyte maturation, fertilisation, embryo culture and storage, must have high survival rates if they are to have any practical application. Also, the development of non-surgical procedures for the collection and transfer of embryos is essential for the exploitation of these in vitro techniques in the cattle breeding industry. My task, in this symposium, is to outline briefly the pertinent areas of research in egg culture and storage which are most applicable to cattle egg transplantation

and which will be discussed in further detail by some of the later speakers.

OOCYTE MATURATION IN VITRO

The oocytes of many mammals, when released from ovarian follicles, at the germinal vesicle stage, will undergo maturation in vitro to the metaphase of the second meiotic division (Donahue, 1972). Maturation in vitro will only occur after the oocyte has attained a certain size and this appears to be independent of the actual size of the follicle (Iwamatzu and Yanagimachi, 1975). Fertilisation of oocytes matured in vitro is usually low and extremely few of these have resulted in liveborn young after transfer (Thibault, 1972 - rabbit; Cross and Brinster, 1970 - mouse). Although nuclear maturation can be attained relatively easily in vitro it would appear that certain maturational changes have to occur in the cytoplasm before the oocyte becomes fully competent. Thibault (1972) has observed that the sperm head fails to decondense to form the male pronucleus in rabbit oocytes previously matured in vitro. He postulates that a substance (male pronuclear growth factor - MPGF) is being produced by the granulosa or thecal cells and this is transferred to the maturing oocyte in vivo shortly before ovulation. From studies in the human (Edwards, 1973), fertilisation in vitro is more readily achieved with oocytes released from follicles shortly before ovulation, confirming the earlier findings of Chang (1955) on rabbit follicular oocytes.

In the first instance, an approach to obtaining normal maturation of the oocyte in vitro both of the nuclear and cytoplasmic components would be the culture of whole follicles (Moor, Hay, McIntosh and Caldwell, 1973 - sheep; Thibault, Gerard, Menezo, 1976 - rabbit and calf). Further analysis of the contributions made by the follicular and thecal cells may lead to the development of culture media capable of supporting complete maturation of the oocyte in vitro.

FERTILISATION IN VITRO

In comparatively few mammals has fertilisation in vitro been achieved with the birth of live young following transfer (rabbit, mouse and rat - see review by Whittingham, 1975[1]). The most important factors in achieving fertilisation in vitro are sperm capacitation, fertilisable life of the

oocyte and a knowledge of the nutritional requirements of both sperm and oocyte. The use of complex culture media has made an analysis of the conditions for capacitation and fertilisation in vitro virtually impossible. I think the main analysis of the system will be obtained with the rat, mouse and rabbit upon which future work on bovine fertilisation in vitro will be based.

In the mouse the best survival rates for oocytes fertilised in vitro are below the values obtained after transfer of embryos obtained after fertilisation and development in vivo (49% versus 70% - see Whittingham, 1975_1). In the rat and rabbit approximately 20% of oocytes fertilised in vitro developed to term and this represents 10% or less of the original population of oocytes with which fertilisation in vitro was attempted (Toyoda and Chang, 1974 - the rat; Frazer and Dandekar, 1973;Brackett, Mills and Jeitles, 1972 - the rabbit). Clearly these results indicate some inadequacies in the fertilisation and/or culture techniques which warrant further detailed investigation.

EGG CULTURE

Except for the mouse, the exposure of most mammalian embryos to prolonged periods in culture during pre-implantation development appears to have a detrimental effect on further development after transfer (Whittingham, 1975_1). This is an indication that, so far, we have not defined the specific requirements for early development in vitro. Cattle embryos develop over short periods in culture but as the period is extended, the viability rapidly decreases (Tervit, Whittingham and Rowson, 1972). If media fail to support development in vitro the adverse effects upon the embryos can only be assessed by subsequent transplantation. The use of a defined medium such as PBl (Whittingham and Wales, 1969 - see Table 1) enables the pH, osmolarity and ingredients to be controlled. This has recently been found to be an effective medium for flushing cattle and sheep embryos and for their subsequent storage (Tervit, Whittingham and Rowson, 1972; Trounson, Willadsen, Rowson and Newcomb, 1976; Willadsen, Trounson, Polge and Rowson, 1976). However, this medium, unless supplemented with serum does not support development probably due to its lack of bicarbonate ions. It is recommended for all manipulations in vitro that reproducibility of conditions are best controlled with chemically defined media with stable pH in air used in large

TABLE 1 COMPOSITION OF MEDIA FOR RECOVERY, STORAGE AND CULTURE OF MOUSE EMBRYOS

COMPONENT	MEDIUM PBl FOR RECOVERY & STORAGE*		MEDIUM NO. 16 FOR MOUSE EMBRYO CULTURE**	
	g/l	mM	g/l	mM
Nacl	8.00	136.87	5.534	94.59
Kcl	0.20	2.68	0.356	4.78
$Cacl_2$	0.10	0.90	0.189	1.71
KH_2PO_4	0.20	1.47	0.162	1.19
$MgCl_2\ 6H_2O$	0.10	0.49	-	-
$Mg\ SO_4\ 7H_2O$	-	-	0.294	1.19
$Na_2\ HPO_4$	1.15	8.09	-	-
$NaHCO_3$	-	-	2.106	25.07
Na Lactate	-	-	2.608	23.28
Na Pyruvate	0.036	0.33	0.036	0.33
Glucose	1.00	5.56	1.00	5.56
Bovine Serum Albumen (BSA)	3.00	-	4.00	-
Penicillin	100U/ml	-	100U/ml	-
Streptomycin	-	-	50µg/ml	-
Dist. H_2O	up to 1 litre		up to 1 litre	
Phenol Red	0.010		0.010	

* Source: Whittingham & Wales, 1969

** Source: Whittingham, 1971

enough volumes to safeguard against changes in osmolarity due to evaporation. If the nutrient requirements of the bovine oocyte for maturation in vitro were defined this could form the basis for a suitable egg culture medium for the later embryo. From the mouse studies, the requirements for the later embryo are less stringent but the media designed to support the early stages will support the whole pre-implantation development (see Table 1). The use of egg culture in cattle egg transplantation would be firstly to determine viability after storage or other manipulations in vitro e.g. fertilisation and secondly to obtain development to the stage most optimal for transplantation.

EMBRYO STORAGE

Low temperature preservation of mammalian eggs and embryos has interested the reproductive physiologist since the techniques for the freeze preservation of spermatozoa were first discovered by Polge, Smith and Parkes (1949). The embryo, unlike the spermatozoan, contains the complete genome of the individual to which it will give rise and therefore it can be propagated in a foster mother of known or unknown genetic background.

Initial attempts to preserve mammalian embryos met with limited success (see reviews by Hafez, 1969; Smith, 1961; Whittingham, 1973) but in the past few years a successful and reproducible technique has been developed for the low temperature storage of mouse embryos (Whittingham, Leibo and Mazur, 1972; Wilmut, 1972). This technique has now been adapted to preserve successfully the embryos of the rat (Whittingham, 1975_3, rabbit (Whittingham and Adams, 1974, 1976) sheep (Willadsen, Polge, Rowson and Moor, 1974) and cow (Wilmut and Rowson, 1973) - see Table 2. The extent of the work carried out to date in mammals other than the mouse has been reviewed recently (Whittingham, 1975_2).

The major discovery in the freezing technique was the extreme sensitivity of the embryos to the rates of freezing and thawing. Successful survival was achieved with relatively slow rates of cooling (0.2 to 2.0°C/minute) and warming (4 to 25°C/minute). The general technique for freezing mouse embryos has been described previously (Whittingham et al. 1972; Whittingham, 1972, 1974_1). So far, only in the mouse have all the pre-implantation stages been shown to survive freezing and thawing (Whittingham

et al. 1972; Whittingham, 1974_2). In contrast to these results, 8-cell pig embryos do not survive cooling below approximately $15^{o}C$ (Polge, Wilmut and Rowson, 1974), and cooling of Day 5 and Day 6 cattle embryos to $7.5^{o}C$ and below caused degeneration of most of the embryos (Trounson, et al. 1976). These examples emphasise the differences in responses of various mammalian embryos both to storage and development in vitro.

TABLE 2 LOW TEMPERATURE STORAGE OF MAMMALIAN EMBRYOS AT - $196^{o}C$

SPECIES	STAGES DEVELOPING TO BIRTH AFTER FREEZING AND THAWING	REFERENCES
Mouse	1-cell, 2-cell, 8-cell	Whittingham, Leibo and Mazur (1972)
	Blastocyst	Whittingham (1974_1)
Rat	2-cell, 4-cell, 8-cell	Whittingham (1975_3)
Rabbit	4-cell, 8-cell, Morula	Whittingham and Adams (1974)
Sheep	Morula	Willadsen, Polge, Rowson and
	Blastocyst	Moor (1974)
Cow	Blastocyst	Wilmut and Rowson (1973)

The tissue culture medium most extensively used for collecting mouse embryos prior to freezing is a modified Dulbecco's phosphate buffered medium - PBl - (Whittingham and Wales, 1969, see also Table 1). This medium has also been used successfully for recovering sheep (Tervit, et al. 1972) and cattle embryos (Trounson, et al. 1976). The advantages of a phosphate buffered medium are its constant pH in air and that the viability of embryos is not decreased during exposure to this medium for periods up to 48 hours at $0^{o}C$, (Whittingham, 1974_1). A modified Krebs-Ringer bicarbonate medium for mouse embryo culture is used for cultivating the embryos recovered after freezing and thawing - Table 1 (Whittingham, 1971).

The basic freezing technique can be carried out with relatively simple equipment (Leibo, Farrant, Mazur, Hanna and Smith, 1970) or with the use of an automatically programmed freezing apparatus (Whittingham, unpublished). Several major modifications to the original procedure have been made to facilitate long term storage and are outlined below:

(a) Two millilitre glass borosilicate ampoules are washed, dried, coded with suitable glass marking ink which is baked on during subsequent sterilisation.

(b) 0.15ml of PBl medium is placed into each ampoule and the embryos transferred in approximately 1µl PBl. The ampoules are placed in an icebath at $0^{o}C$ for 10 minutes before adding a further 0.15ml PBl containing 3M dimethylsulphoxide (DMSO). After another 10 minutes the ampoules are transferred to a seeding bath at $-5^{o}C$ where each is seeded with an ice crystal 2 minutes later. The ampoules are sealed with an oxygen/butane flame, returned to the seeding bath for 5-10 minutes before transferring them to the cooling bath at $-6^{o}C$. Since the ampoules float it is not necessary to place them on holders. After cooling to $-80^{o}C$ at approximately $0.5^{o}C$/minute, the ampoules are immersed in liquid nitrogen ($-196^{o}C$) and finally placed on canes before storing in a liquid nitrogen refrigerator.

(c) The ampoules are warmed at between 6 to $8^{o}C$/minute to $0^{o}C$. The seal is broken and 0.3ml, 0.3ml and 0.6ml PBl medium added at 1 minute intervals. The contents of the ampoule are drawn up into a 1 ml plastic pipette and dispensed into a solid embryological watchglass. The ampoule is rinsed with 2 x 1ml PBl and the embryos collected, washed in a further 2 changes of PBl medium before culture in No. 16 medium.

TABLE 3A COMPARISON OF MOUSE EMBRYO SURVIVAL AFTER FREEZING AND THAWING IN 2ml AMPOULES CONTAINING VARYING VOLUMES OF MEDIUM

VOLUME OF MEDIUM IN AMPOULE (ml)	No 8-cell embryos recovered/total no frozen	No Blastocyst after 48 hours culture %
0.2	78/80[1]	47/78 (60)
0.3	79/80	76/79 (96)
0.4	73/80	53/73 (73)

1 Each experiment replicated 4 times (20 embyros/replicate)

In Table 3A, a comparison is made of the volumes of PBl used in the 2ml ampoule and the final volume of 0.3ml achieved the best recovery and survival. In comparing the ampoules, (sealed and unsealed) with unsealed test tubes (Table 3B) no significant difference was observed in any of the 3 treatments. The main advantage of storing in sealed ampoules is that they can be stored sterile and there is no risk of contamination from the liquid nitrogen.

TABLE 3B COMPARISON OF MOUSE EMBRYO SURVIVAL AFTER FREEZING AND THAWING IN UNSEALED TEST TUBES AND AMPOULES AND SEALED AMPOULES

TYPE OF FREEZING TUBE	No 8-cell embryos recovered/total no frozen	No Blastocyst after 48 hours culture %
unsealed test tubes	43/45[2]	37/43 (88)
unsealed ampoules	43/45	40/43 (93)
sealed ampoules	42/45	39/42 (93)

2 Each experiment replicated 3 times (15 embryos/replicate)

The importance of inducing ice formation (seeding) in embryo samples before slow cooling, cannot be over-emphasised. Seeding below $-7^{o}C$ significantly reduces survival (83% at $-6^{o}C$ and 54% at $-8^{o}C$) and there is no survival when seeding takes place below $-10^{o}C$ (Whittingham, 1976). The mouse embryos are not killed by the rapid rise in temperature during ice formation but by the rapid decrease in temperature as the sample returns to the temperature of the cooling bath (this rate is greater than $2^{o}C$/minute - Whittingham, 1976).

Survival of mouse embryos after freezing and thawing has been assessed in two ways - development to the blastocyst stage in vitro and development to full term foetuses and liveborn after transfer. Both methods of assessment produce similar results unlike the rabbit where the viability of frozen-thawed embryos was high in vitro but low in vivo (Whittingham and Adams, 1976). However, when frozen-thawed 8-cell mouse embryos are transferred directly upon thawing to the uterus of a recipient only 26% survived compared with 73% of the controls (Whittingham, 1975_{2}). Examination of frozen-thawed 8-cell mouse embryos at the ultrastructural level failed to reveal any changes which may have caused delay in the resumption of normal

development observed in the transfer experiments (Whittingham and Anderson, 1976). This aspect clearly warrants further investigation as it may also be applicable to the frozen-thawed embryos of other mammals such as the cow.

In a recent study of the effect of background radiation on mouse embryos during storage at -196°C (Whittingham and Lyon, 1976) the overall survival to foetuses and liveborn from the embryos originally frozen was approximately 36% (479/1330). This is somewhat lower than in an earlier study (Whittingham, 1975_2) but this was due to the use of plastic freezing ampoules which significantly reduced survival in controls and all levels of background radiation. Nevertheless, we can expect an overall survival of between 36 and 48% in a species where embryos are easily obtained in greater numbers than the cow and where the freezing techniques are well defined. To apply the embryo storage technique to the cattle breeding industry survival rates as high as those for mouse will have to be obtained or the procedure will not be economically viable.

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DISCUSSION

L.E.A.Rowson (UK)

Do you think that in your 8-cell frozen, non-cultured embryos, you are virtually throwing them out of synchronisation?

D. G. Whittingham (UK)

Yes, I think so, because although you can put the fresh egg into the uterus it normally is only just passing into the uterus at that point of transfer. I think what it is reflecting is that the embryo is just that much out of synchrony, delayed - because implantation in the mouse occurs over such a short period, it isn't ready to implant.

T.R.R. Mann (UK)

I was very interested in what you said about eggs that were first frozen, next unfrozen, and which had then to be incubated for a period of time in order to regain their viability. I wonder if that incubation period is needed in order to induce a full rate of metabolism. It is known from studies on other cells that in certain instances an induction period is needed in order to restore the metabolism in previously inactivated cells. For example, immobile epididymal spermatozoa require a certain induction period in order to become mobile and metabolically active. This induction period is probably needed for the synthesis of nucleotide coenzymes which are necessary for glycolysis or respiration. The coenzymes most likely to be involved are adenosine triphosphate and cyclic adenylic acid. It has been shown experimentally that the activation of epididymal spermatozoa involves first, the synthesis of the cyclic adenylic acid, with an increase in glycolysis following only later. It would be interesting if one could demonstrate that it is necessary for unfrozen eggs to build-up their coenzyme reserve in order to regain full viability and metabolic activity.

D.G. Whittingham

Yes, I'd like to be able to get a system to measure this - to measure some form of metabolic parameters in the embryos to show this.

T.R.R. Mann

Picogram quantities of cyclic adenylic acid are required for the determination of this nucleotide in cells. A determination of this kind

in spermatozoa requires about 10^8 cells. An alternative would be to measure adenyl cyclase activity. I can see that this may be very difficult to carry out with the small number of eggs at your disposal.

C. Polge (UK)

My question was much on the same lines in that your results refer only to 8-cell embryos that have been frozen. It is possible that at this time there is some critical change in relation to metabolism and so forth which is being possibly slowed down by the freezing and thawing process. It would be interesting to examine the effect of culture on later stage embryos that have been frozen and thawed, and whether, in fact, the culture before transfer was necessary in relation to the state of development of the embryo.

D.G.Whittingham

Yes, fine - point taken.

M.T. Kane (Ireland)

You mentioned that in some work done by George Seidel in Colorado State, rabbit embryos were fertilised in vitro and cultured for three days and implanted and live young were born. I don't have the percentage figures with me but there was one thing that was interesting about this that Seidel found. It may have been just a fluke of a preliminary experiment. The very early blastocysts were transferred to the oviduct but when he transferred them to the uterus there was absolutely zero implantation. It is possible that that passage through the oviduct might be necessary.

There is another point I would like to make, and it is a criticism in a way but it applies to myself as much as anybody. I would say that the ova of no species have been cultured in what could be called a chemically defined medium; I think almost all of them have contained albumen. I think people should be aware of this fact that one of the major functions of albumen is as a carrier for other molecules, particularly fat soluble molecules such as fatty acids, steroid hormones and various other molecules as well. One thing we have seen recently with albumen and rabbit embryos is that the fatty acids on the albumen can affect the development of the embryos.

D.G. Whittingham

I agree with you on that. I did some earlier work with Ray Wales on

the ability of albumen to support egg cells - mouse eggs to blastocysts. If you use fatty acid-free albumen you get very, very poor development.

M.T. Kane

There is another point: it strikes me that in culturing embryos there are two sets of problems. There are the initial problems associated with 1-cell to 2-cell, to 4-cell, to 8-cell, which are quite common even in certain strains of mouse, in the rat, in cattle, in sheep etc. There are a second set of problems which probably attend the growth of the blastocyst. This would apply particularly to the domestic animals because of the rapid growth of blastocysts that takes place, whereas in the mouse there is almost zero blastocyst growth before implantation.

D.G. Whittingham

Well, perhaps we had better move on; I think more discussion will come out with the later papers this afternoon.

LOW TEMPERATURE PRESERVATION OF COW EGGS

S. Willadsen, A.O. Trounson, C. Polge, L.E.A. Rowson & R. Newcomb
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Reliable methods for storage of embryos in vitro must be developed if the full potential of embryo transplantation is to be realised in cattle breeding. Major steps towards achieving this were the demonstration that mouse embryos can survive deep-freezing (Whittingham, 1971; Whittingham et al. 1972; Wilmut, 1972) and the birth of a normal calf following transplantation of a frozen-thawed cow embryo (Wilmut and Rowson, 1973). However, the method could not assume any immediate practical importance for preservation of cow embryos because 1) the survival rates obtained were very low and 2), relatively advanced embryos, i.e. Day 10-12 blastocysts, were used in these early experiments whereas younger embryos, particularly Day 5-7 late morulae and early blastocysts are generally considered more suitable for transplantation. Finally, although long term storage at -196^{o}C would probably not have reduced embryo viability significantly, this remained to be proved.

The deep-freezing of mouse embryos has now progressed to the stage at which it can be used as a routine method for long term storage, and the technique has also been applied to embryos of other species, notably the sheep (Willadsen, Polge, Rowson & Moor, 1974) and the rabbit (Whittingham & Adams, 1974; Bank & Maurer, 1974). Although the cow embryo was among the very first to be successfully frozen, and workers in various countries have attempted to follow up the initial success, no further developments have been reported in the literature. During the last two years, experiments on deep-freezing of cow embryos have been continued at our Unit, but only within the last year has any real progress been made. It is fair to say, that experiments on deep-freezing of sheep embryos have played an important role in the subsequent improvement of survival rates of cow embryos. Indeed, no survival of early cow embryos was obtained before the sheep work was well advanced. Therefore I shall include experiments on sheep embryo freezing in this presentation. I shall not attempt any detailed discussion, but briefly describe the method employed, and thereafter concentrate on the main results obtained.

TABLE 1

1. Flushing of embryos from genital tracts of superovulated donor ewes after laparotomy. Flushing medium: phosphate buffered saline (PBS, Whittingham 1971)

2. Storage of embryos in PBS at room temperature for up to 3 hrs.

3. Addition of Dimethylsulphoxide (DMSO) to the medium at room temperature to a final concentration of 1.5M DMSO in PBS.
   Four steps: 0.25M DMSO in PBS : 5 min
   0.5M " " " : 5 "
   1.0M " " " : 10 min
   1.5M " " " : 20 "

4. Cooling at 1°C/min to -7°C in test-tubes (0.5 x 5.5cm) containing ~0.2ml 1.5M DMSO in PBS.

5. Initiation of crystallisation through "seeding" with crystal of frozen medium.

6. Freezing at 0.3°C/min to -65°C; from -65°C freezing at ~1°C/min to ~-120°C.

7. Transfer of test-tubes to LN_2
   Storage for 2 - 3 months in LN_2

8. Transfer to ~-100°C and thawing at 10°C/min to -10°C.

9. Rapid rewarming to room temperature.

10. Removal of DMSO from the medium at room temperature,
    Six steps: 1.5M DMSO in PBS : 5 min
    1.25M " " " : 10 min
    1.0M " " " : 10 min
    0.75M " " " : 10 min
    0.5M " " " : 10 min
    0.25M " " " : 10 min
    PBS

11. In vitro culture for 24hr in PBS + 20% sheep serum under atm. air at 38°C.

Table 1 summarises the standard method used for deep-freezing sheep embryos and, with minor alterations, this is the method employed in deep-freezing cow embryos as well. The various points represent steps into which the technique can conveniently be broken down, and the rather rigid standardisation has been introduced mainly to render the procedure amenable to analysis and controlled experimentation. The main effort at present is concentrated on examining the effect and adequacy of the successive treatments and conditions to which the embryos are exposed with a view to introducing alterations which may further improve survival rates.

The central problem in these investigations concerns the optimal combination of DMSO concentration, freezing rate and thawing rate. In Table 2 an experiment with early sheep blastocysts is presented in which two DMSO concentrations, two freezing rates and three thawing rates were examined.

TABLE 2 EFFECT OF DMSO CONCENTRATION, FREEZING RATE AND THAWING RATE ON THE SURVIVAL OF SHEEP EARLY BLASTOCYSTS STORED AT -196°C

DMSO Conc.	1.5M					
Freezing rate	0.3°C/min			1.0°C/min		
Thawing rate	4°C/min	10°C/min	360°C/min	4°C/min	10°C/min	360°C/min
N. frozen	11	10	10	10	10	10
N. surviving	4	7	6	0	0	4
DMSO Conc.	1.0M					
Freezing rate	0.3°C/min			1.0°C/min		
Thawing rate	4°C/min	10°C/min	360°C/min	4°C/min	10°C/min	360°C/min
N. frozen	10	10	10	10	10	10
N. surviving	2	3	0	2	3	0

The primary survival rates were assessed after in vitro culture for 20 - 24 hours, but representative surviving embryos from seven of the eight effective combinations were subsequently transferred to ewes, and lambs have now been produced from embryos frozen by five of these combinations. However, survival rates were highest with the original combination of 1.5M DMSO in the medium, a freezing rate of 0.3°C/min, and a thawing rate of 10°C/min. Interestingly, in these as in earlier experiments rapid thawing was found to be less damaging than has been reported for mouse embryos,

(Whittingham et al. 1972; Wilmut, 1972). Indeed, embryos frozen in 1.5M DMSO at 1.0°C/min did not survive unless thawed rapidly.

Obviously it would be advantageous if surviving embryos could be recognised immediately after thawing and be transferred directly to ewes with minimal waste of recipients on non-viable embryos. Table 3 presents the results of two such experiments: one, involving transplantation of frozen thawed Day 7 and 8 hatched blastocysts, to Day 7 ewes, carried out in February 1974, and the other involving transplantation of Day 6 late morulae to Day 5 recipients, carried out in February 1975.

TABLE 3 VIABILITY OF SHEEP EMBRYOS STORED AT -196°C

Stage of Development	Morulae (Day 6)	Hatched blastocysts (Day 7 & 8)
No. frozen	24	10
No transferred	12 x 2	4 x 2
No. of lambs born	2 x 2) 4 x 1) = 8	3 x 1 = 3

Although the embryos were transferred irrespectively of their post-thawing morphology, care was exercised to ensure that each ewe received at least one embryo which was considered to have survived storage at -196°C. Incidentally, the embryos used in the 1975 experiment had been stored for 2½ - 3 months. The overall lambing rate in these experiments was relatively low, indicating a lower viability of the frozen-thawed embryos than suggested by the results of experiments involving in vitro culture. However, it should be noted, that the number of lambs born corresponded precisely to the number of "surviving" embryos transferred to their uterine foster mothers. More trivial causes, e.g. poor quality of recipients may have adversely influenced the final result. Also the synchronisation requirements in transplantations where frozen-thawed embryos are used, has not been determined precisely. In an attempt to counteract the possible effect of the initially slow development of frozen-thawed embryos as compared to freshly collected embryos, recipients which were about one day earlier in the oestrous cycle than the donors were arbitrarily chosen.

Nevertheless, from the data presented it is obvious that sheep late

morulae and early blastocysts can survive deep-freezing even for a relatively extended period, and that the survival rates which may be expected are approaching a practically acceptable level. Although these particular stages of development may for various reasons be considered the most suitable for freezing, survival of younger embryos, e.g. the 1-cell fertilised egg, has also been obtained with the method described.

Early sheep embryos may be considered valuable models for the corresponding developmental stages in cattle, but there are important differences. Attempts to deep-freeze Day 5 cow morulae by the same general method as used for sheep embryos failed almost completely. Only one out of 105 embryos survived, and the vast majority of the remainder were totally degenerate. Experiments with Day 2 - 4 embryos were equally unsuccessful. By contrast, early blastocysts, collected Day 7 or 8 survive almost if not quite as well as sheep embryos (Table 4).

TABLE 4 THE EFFECT OF DMSO CONCENTRATION, FREEZING RATE AND THAWING RATE ON THE SURVIVAL OF COW BLASTOCYSTS STORED AT -196°C

DMSO concentration	1.0M	1.0M	1.5M	1.5M
Freezing rate	1.0°C/min	0.3°C/min	0.3°C/min	0.3°C/min
Thawing rate	10°C/min	10°C/min	10°C/min	360°C/min
No.frozen	25	20	35	12
No.recovered after thawing	24	19	31	12
No.cultured in rabbit oviduct or in vitro	19	19	29	12
No.recovered after culture	17	18	29	12
No.completely degenerate	3	3	6	2
No.partially surviving	12	10	13	6
No.considered normal	2	5	10	4
No.transferred to heifers	0	2 x 2	1 x 2 1 x 1	0
No.developing normally		0	1	

It should be noted that the majority of these particular blastocysts derived from Day 3 and Day 5 embryos which were cultured in ligated rabbit oviducts for 4 and 2 days respectively before being frozen. The distinct difference in survival rates between morulae and early blastocysts barely

two days older was subsequently explained by the results of experiments where cow embryos were cooled to various low temperatures. Some of these experiments are summarised in Table 5 from which it is quite clear that even cooling for short periods to a temperature well above the freezing point of PBS containing 1.5M DMSO damages the majority of embryos younger than the early blastocyst irreversibly whereas older embryos survive. The cause of this damage is as yet unknown, though a similar phenomenon has been found to occur in all stages of development from 1-cell egg to late blastocyst in the pig.

TABLE 5 SURVIVAL OF COW EMBRYOS AFTER STORAGE AT $0^{\circ}C$ FOR 30 Min

Stage of development	Morulae > 24 cells (Day 5)	Blastocysts (Day 7)
No. cooled	17	14
No. degenerate	12	1
No. normal	5	13

After it had been established that early cow blastocysts could survive freezing with the method employed for sheep embryos a relatively large experiment was carried out to determine the viability of surviving cow embryos. The embryos used were all blastocysts collected from heifers on Day 7 or Day 8. After 1 - 2 months at $-196^{\circ}C$ they were thawed and cultured in vitro for 6 - 12 hours. Those which had re-expanded during this brief culture period were subsequently transferred to heifers synchronous with the original donors. The events of this experiment are presented in Table 6A. A third of the heifers to which frozen and thawed embryos were transferred became pregnant. Of the 10 non-pregnant recipients 2 returned to oestrus on Day 30 and Day 33 respectively, suggesting early embryonic death. Of the 6 recipients which were diagnosed pregnant by rectal palpation 5 weeks after oestrus, one returned to oestrus on Day 50. Two of the remaining 5 were slaughtered when they were 3 months pregnant and each was found to be carrying one apparently normal foetus. The last 3 recipients in this group are also pregnant with singles. However, the discrepancy between the primary survival rate and the pregnancy rate suggested that omission of in vitro culture and more careful selection of recipients might improve the results. Hence an experiment was carried out in which embryos that were judged to be

survivors were transferred immediately after thawing. This experiment is summarised in Table 6B. Two of the 3 recipients were slaughtered on Day 42 and Day 56, respectively. The first was carrying one normal and one partially resorbed foetus and the second was carrying one normal foetus. The third recipient is believed to be carrying twins. The two embryos transferred to this animal had been stored at -196°C for 9½ months.

TABLE 6 THE VIABILITY OF COW BLASTOCYSTS TRANSFERRED TO RECIPIENT HEIFERS AFTER STORAGE FOR 2 - 9½ MONTHS AT -196°C

Blastocysts were frozen in 1.5M DMSO at 0.3°C/min and thawed at 10°C/min.

	A		B	
	Embryos cultured in vitro for 6 - 10 hrs before transfer		Embryos transferred directly after thawing and removal of DMSO	
No. frozen & thawed	51		10	
No. considered normal	28		7	
No. transferred to heifers	9 x 2	7 x 1	1 x 3	2 x 2
No. developing	4*x 1	2 x 1	1 x 2**	1 x 2 1 x 1

* 1 recipient returning to oestrus Day 50 ** 1 conceptus dead and partly resorbed

The results of the present experiments provide further evidence that early-stage cow embryos can survive freezing and storage at liquid nitrogen temperature and develop normally following thawing and transfer to recipients.

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DISCUSSION

J-P. Renard (France)

Using seven day old blastocysts and cooling them, how long do you culture them afterwards and what kind of criteria do you use to check the viability of those blastocysts?

S.M. Willadsen (UK)

As I have already pointed out there has been a slight shift in the emphasis in our work so far as our Unit is concerned; we are working mostly with Day 6 and Day 7 rather than Day 3 and Day 5 embryos and this is for a number of reasons. One of the reasons is that the freezing work goes better with these later stages. Another reason is that these stages are probably better suited for non-surgical transfer, which is really what we are most interested in at the moment. On top of this, it seems that these relatively late stages are much more hardy in almost every respect. Therefore, we have started building up information on these particular stages. We have done in vitro culture, not just as part of the freezing work, but we have done separate experiments where we have been culturing embryos. We have done experiments on storage - short term storage and here I should point out that although the deep freezing is probably the most promising way for streamlining the technique of embryo transplantation (if it is to be streamlined), short term storage, say for a couple of days, could probably be done more effectively by other methods. Now, as I said, we have been building up information on this and we have done transfers both from embryos which have been stored by cooling to zero degrees and kept there for 48 hours, and the equivalent stages of embryos, slightly earlier embryos, cultured for 48 hours and then transferred, so we do have some indication that the basically morphological criteria that we are using in relation to the in vitro culture does, in fact, have a good correlation with the ability of the embryos to develop when they are transferred - except in the case of the deep frozen embryos - they don't seem to be doing too well when they are transferred. Then again, one wouldn't know whether the optimal conditions for deep frozen embryos during in vitro culture would be the same. Certainly the results we have been getting so far suggest that this is not so because we have got quite a high survival rate in this group - in the order of 50+% in one of the sheep experiments - but even so we don't get the same transfer results as we would be expecting when using fresh embryos.

R. Church (Canada)

What would you say is the major influence in the success of your blastocyst freezing in the cow? Is it cell size, cell number, cavity etc?

S.M. Willadsen

The major influence is the choice of stage and I think this has got a biochemical side to it rather than any morphological or physical implication. What finally got us worried was that we were getting quite good survival rates with sheep embryos which are morphologically quite similar to cattle embryos. A Day 5, Day 6, sheep embryo would correspond very closely to a Day 5½ to 6 cow embryo, so one wouldn't think that the morphological aspects have not been taken proper care of. In other words, the physical set-up was probably O.K. Then , of course, we did some rather inconclusive experiments on the cooling, or interrupted freezing, of cow morulae but later on, when we did the straight cooling work, it was quite obvious that although there was no immediately apparent effect -you can't tell whether the embryo has been damaged by cooling if you just look at it - but if you culture it, or transfer it, then you get your results. So I think this has a biochemical side to it rather than anything. On top of that, there is a gradual trend in the work - or there has been so far - that the results are improving even with the stages that we could freeze successfully in the first place. I should mention that we are also freezing earlier stages of sheep embryos and we think we can freeze the 1-cell egg for instance, quite effectively. However, we have restricted the main work to the later stages for various reasons.

T.R.R. Mann (UK)

Is it possible that some of these stages of embryonic development differ in sensitivity to cold shock, that is to cooling from body temperature (or whatever temperature you are starting with), to temperatures above zero?

S.M. Willadsen

It's not just possible - it is definitely the case. We have been wondering whether this is so because although there is definitely a possibility that this is basically the same biochemical damage which is occurring, cold shock, as I understand it, can be avoided by certain measures, for instance, if you cool very slowly or you leave the sperm at room temperature for some time and then you cool gradually. So far we haven't been able to

show any effects of slow cooling as opposed to rapid cooling. So, although it could be the same phenomenon, I don't think shock is the proper word for it.

D.G. Whittingham (UK) *(to Dr. Trounson)*

Did you do the cooling of the cow eggs below zero or above zero? Did you hold them as you were taking them down, at certain temperatures, or were they always taken down at a rate to the final temperature?

A.O. Trounson (UK)

No, they were always taken down to a fixed point. We looked at 0^{o}, 2^{o}, 5^{o} and 7.5^{o}C as the final temperatures. We also looked at the length of time at which we held them at these temperatures - 2 minutes, 30 minutes, and in some cases a longer period of time. None of these factors had any particular effect. We looked at the inclusion of serum in the cooling medium and a few other things. It just seemed to be rather the stage of the development. If we classified the eggs as 32-cells and above, or 8 to 24-cells, (and Ian Wilmut also showed this with 8-cells), it showed that the earlier the cleavage stage the more susceptible they are to cooling.

S.M. Willadsen

It looks as if there may be individual variations although we don't really know. In some experiments we have been getting relatively good survival; this has been one of the intriguing problems involved in this work. We haven't really done any extensive cooling. We have just been able to pick up the differences and we know now that the morula is no good for cooling and so, for the moment, we are leaving it there without knowing what is behind it.

D.G. Whittingham

Well, if there are no more questions we will pass on to the next paper.

POTENTIAL APPLICATIONS OF TECHNIQUES FOR EMBRYO STORAGE

I. Wilmut

A.R.C. Animal Breeding Research Organisation, Edinburgh

Techniques of embryo storage are only now being developed to the stage at which they can be used routinely (Willadsen, Trounson, Polge, Rowson and Newcomb, 1975) although the survival of embryos is already very much higher than it was in the first successful experiments (Wilmut and Rowson, 1973_2). The purpose of this paper is to describe briefly the storage techniques which have been developed and to discuss the way in which these techniques can be utilised by commercial and research organisations. In this way I hope to be able to demonstrate some limitations of the storage techniques and so stimulate different lines of research.

TECHNIQUES OF STORAGE

Almost all research has been concentrated on the possibility of developing methods of freezing and thawing embryos. Essentially, the techniques which have been successful with embryos of cattle (Wilmut and Rowson, 1973_2) as well as sheep, (Willadsen, Polge, Rowson and Moor, 1974) mice, (Whittingham, Leibo and Mazur, 1972, Wilmut 1972) and rabbits (Whittingham and Adams, 1974) depend upon very slow cooling of embryos in the presence of the low molecular weight protective compound dimethyl sulphoxide (DMSO). The medium containing DMSO is added slowly at room temperature, and after a brief period of equilibration the samples are cooled in ice ($0^{o}C$). During the subsequent slow cooling to the temperature of liquid nitrogen ($-196^{o}C$) at 0.2 to $0.3^{o}C$/min. it is necessary to "seed" the medium with an ice crystal to prevent supercooling and it should be remembered that the present cooling equipment does not do this effectively. The embryos are also warmed slowly (4 - $20^{o}C$/min.) and the medium containing DMSO diluted out gradually at room temperature. Although only 2 of 47 late cow blastocysts survived this treatment (Wilmut and Rowson 1973_2; unpublished results) the survival rate of earlier stages is very much higher (Willadsen, et al. 1975).

In contrast to the effort devoted to the development of methods of freezing, techniques of storage at temperatures between $0^{o}C$ and $37^{o}C$ have received very little attention. Eight-cell cattle embryos and morulae are

known to be killed when cooled in ice (Wilmut and Rowson, 1973$_1$; Wilmut, Polge and Rowson, 1975) but little else is known of the survival of any stage of development at any temperature between 0°C and 20°C. It is, however-encouraging to note that mouse and sheep embryos will survive some storage at these temperatures (Whittingham and Wales, 1969; Kardymowicz and Kremer, 1971; Kardymowicz 1972).

The potential value of such techniques will clearly depend upon the uses which are to be made of the transfer techniques.

APPLICATIONS OF EMBRYO TRANSFER

The surgical techniques of embryo recovery and transfer which were largely developed by Rowson, Moor and Lawson (1969) have been utilised in research projects and to multiply "exotic" breeds of cattle (see Graham, 1974 for review). This multiplication has been a lucrative business in many parts of the world but it clearly is becoming less profitable as sufficient representatives of each breed are now available. In contrast, these techniques have not been incorporated into any long term breeding scheme. Analysis has shown that application of embryo transfer could almost double the increase in daily weight gain if applied in a closed beef herd (Land and Hill, 1975), but provide only a 10% to 15% improvement in the efficiency of an effective dairy selection scheme (Skjervold 1974). At present prices the value of the extra milk or meat produced by any one animal is only a few pounds. (The extra milk produced if embryo transfer is utilised to breed replacements from 10% of females, instead of the usual 60% or more, would be of the order of £20/cow/year (Hume and Wilmut, 1975).

In contrast, in elite beef herds where the livestock produced will have a high price, particularly if bulls can be sold for A.I., it seems that application of the technique can certainly be justified.

Non-surgical techniques of embryo transfer would permit us to produce large numbers of calves which obtain their genes and their succour from different "mothers", and Skjervold (1974) has drawn attention to the possibilities of establishing specialised dam and growth lines. Selection in the one line would be for such characteristics as ease of calving, milk yield and small size, while in the other it would be for a growth characteristic. In this way it is possible to overcome negative genetic correlations

between "dam" and "growth" characteristics, and maximum heterosis is exploited as there need be no relationship between dam and calf. This is all very intriguing, but a great deal remains to be learned about the efficiency of such selection schemes. As with the possibility of producing twin pregnancies by embryo transfer (Rowson, Lawson and Moor, 1971) the considerable capital investment as well as the inevitable costs of drugs to synchronise heat and of skilled personnel all make it far from certain that the increase in biological efficiency will be sufficient to create the profit which producers will require.

I would conclude that the embryos which are likely to be transferred in the near future will almost certainly be from selected donors and that they will, therefore, be valuable. An exception to this generalisation might arise if large numbers of embryos recovered from beef heifers at the time of slaughter were being transferred in the hope of establishing twin pregnancies; such embryos would relatively be far less valuable.

VALUE OF STORAGE PROCEDURES

Four particular advantages have been suggested for storage procedures (Wilmut and Rowson 1973_2).

a) to create embryo banks
b) to facilitate export
c) to create genetic controls
d) to conserve rare breeds.

EMBRYO BANKS

Conception following embryo transfer is maximal if the oestrous cycles of donors and recipient are closely synchronised (Rowson et al. 1969; Rowson, Lawson, Moor and Baker, 1972). This fact, along with the well-known individual variation in the number of embryos recovered from donor cows, causes considerable managerial problems when planning embryo transfer. It is tempting to dream of a time when all embryos can be frozen and stored until suitable recipients become available, but a number of factors make it unlikely that this will occur with present freezing techniques.

First, the effects of a difference in cycles of one day may be a little less than was originally observed (cf. Rowson et al. 1972; with Newcomb and Rowson, 1975; Sreenan, Beehan and Mulvehill, 1975). Second, some embryos will always be killed by freezing techniques and, since it is likely that the embryos will be valuable, it seems foolhardy to waste them. Third, as it is not known before transfer which embryos have survived, the conception rate will fall and the expense of all unsuccessful operations, as well as that of the freezing, will be borne by the successful transfers. Finally, effective methods of synchronisation are now available at low cost so that the cycles of groups of animals can be controlled. I believe that the use of such synchronisation techniques along with the development of methods of storage for 24 - 72 hr at temperatures above $0^{o}C$ will offer more successful and cheaper solutions to the problem, until survival of freezing and thawing is very much higher than at present.

EXPORT

An international trade in intensively selected, well-characterised types of cattle seems certain to continue and increase, although movement of mature animals is very expensive because of shipping and quarantine costs. Furthermore, the animal may not adapt and may in fact succumb to local diseases, particularly when a major change in climate is being made. Many of these problems would be reduced or overcome by use of freezing techniques, and present survival rates are quite adequate for use in export.

GENETIC CONTROLS

The value of genetic controls and the relative merits of the different methods by which they can be maintained were discussed fully by Hill ($1972_{1/2}$) before methods of mammalian embryo freezing were available. Methods of embryo freezing offer a new form of control with the particular advantages that the population would be stable and cheap to maintain. However, the effect of the recipient must be considered and moreover in using freezing it has to be assumed that the freezing itself does not cause selection among embryos. It can be stressed that the present survival rates are adequate for the application, and that the genetic controls have great value for improving commercial production.

CONSERVATION OF RARE BREEDS

Biological research and commercial production will both benefit from the conservation of a great variety of genotypes (Bowman and Aindow, 1973). In recent years a thriving trade has developed for these breeds which are now low in number, and this may provide the reserve of genetic variation which is required, although such "pools" are liable to genetic change. Frozen embryos would be stable and are cheaper to maintain.

CONCLUSIONS

Techniques of freezing already offer unique advantages for exporting embryos, when establishing genetic controls or protecting rare breeds. Present techniques are, however, laborious and are difficult to automate so that further research should be designed to overcome these limitations. In particular it may be possible to simplify methods of adding and removing DMSO, and the utilisation of a "two-step" method of cooling (Luyet and Keane, 1955) might remove the necessity to seed.

A technique of storage without freezing could have very great value and it is to be hoped that more studies will be made in this area. In particular, the ability to hold embryos for a few days would greatly facilitate all transfer operations.

Finally, I would conclude that even if these techniques are not used on a very large scale their use for export and for creating genetic controls certainly justify further research.

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DISCUSSION

D.G.Whittingham (UK)

May I make a comment first. I'd like to comment on your genetic control because I feel that although it would be a valuable thing to do in cattle, the most genetically defined mammal we have is the mouse and the genetic drift is going to be looked at by frozen stored embryos much more effectively in the mouse embryo than it can ever be done in the cattle breeding system. So I would not put the greatest stress on that as an application to cattle breeding. If you were going to look at genetic drift you would look at it in mice and not in any other mammal.

I. Wilmut (UK)

I think I am probably thinking in far more mundane terms in the main. It simply is not possible to know what the breeding programmes are achieving without some kind of genetic control. These have not frequently been available in ordinary breeding experiments, particularly in Britain, and embryo freezing may well offer a method of achieving this. But I am thinking in terms of far more commercially oriented projects rather than achieving basic information. I agree that it is of great value with the laboratory animal but I think the application in cattle, or even in pigs and sheep, if you could do it, would be quite useful in itself, just simply to demonstrate that the breeding schemes that are being followed are being effective.

J. King (UK)

Am I allowed to disgree with the Chairman? I think mice are probably the prime example where you don't require a genetic control because you have very good in-bred lines .....

D.G. Whittingham

But after 20 generations they have drifted far enough apart to donate or mark them down as a separate sub line.

J. King

I think not in very many cases - in a few isolated cases .....

D.G. Whittingham

Oh, in many cases, especially in cases like Bar Harbor, every sub line

is separated because immunologically it is already different from the parent stock.

J. King

Well, I think this is matter of degree but certainly the amount of change you get there is minimal compared with what you would get in an out-bred cattle population so unless there is selection of the genotypes which you prefer by the freezing and thawing process, then this would be an in-valuable kind of control to have and one which is not open to us.

D.G. Whittingham

Yes, but I still maintain that if we are going to look at genetic drifts per se, the in-bred mouse is the one to study. You have got a defined in-bred mouse in generation 1 which you can freeze and then compare it with generation 20 or 30. This is something you have never been able to do before; you can do it in bacteria.

J. King

I think we are in trouble with nomenclature here. You are talking about mutation are you not?

D.G. Whittingham

Well, that could be occurring .....

W.G. Hill (UK)

A comment which I think is relevant to this. I am not sure about mice but certainly in cattle one can freeze semen and a lot of advantages in terms of conservation of breeds or genetic controls that Ian Wilmut has talked about, most of this can be achieved by storage of semen. I would like to ask a question also.

I believe it is a problem with pigs that - I quote - 'some boars don't freeze'. Is there enough information yet, or is it known to be a problem with embryos, that you cannot, in fact, store embryos from some donors?

S.M. Willadsen (UK)

This is something we don't know but I think Whittingham has some

material to suggest that there would be differences between strains of mice for instance.

D.G. Whittingham

There are differences more in strains of mice in being able to obtain sufficient numbers of embryos but so far we haven't found any major difference between freezing the embryos of specific mutant or multiple recessives and various in-bred lines. We haven't picked up any that have low viability.

I. Wilmut

I thought I remembered reading in one of your papers that there was one donor, for instance, which gave very high survival rate?

S.M. Willadsen

Well, this is true so far as cooling is concerned.

I. Wilmut

This is more what Bill Hill was referring to.

S.M. Willadsen

We haven't pinned this variation down to any particular thing. The eggs could, in fact, be infected with a virus, so there you are - we don't know anything about that. We just happen to observe this now and then. We have observed this in freezing as well.

I. Wilmut

So I think you could conclude that it is very likely that there would be individual variations.

W.G. Hill

So you are not finding strains which you can't freeze in the mouse work? This would obviously be a problem if you could.

A.O. Trounson (UK)

I wonder if I could just make a comment on that last statement. Even though we don't have perfect systems for short term storage of embryos, I think we are getting towards them. Firstly, there is the one that Steen Willadsen spoke about - we have cultured embryos for periods of 24 hours,

48 hours, 72 hours, and we get above 80% developing normally at 24 hours at 37°C. There is a reduction to about 65 or 67% of them still going on at 48 hours and it's about 73 or 74% at three days. We have transferred these embryos after two days culture and obtained 8 of 13 cows pregnant and an overall embryo survival rate of 50% which is only 9% lower than our results with direct transfer of embryos under the same conditions. We have also kept embryos at 0° in iced water for 48 hours and transferred them. They look the same when they come out of the cooling; you can't tell if there is any damage. We have transferred them directly to recipients and we have had 6 out of the 10 cows to which eggs were transferred, pregnant and a 40% embryo survival. So, although they are not perfect systems, I think there is a definite trend to acceptable short term storage. If we extend the cold storage for any longer than two days the embryo survival rates seem to go down.

I. Wilmut

One comment I would have about that is that the culture isn't strictly what I would refer to as storage because it doesn't help you to overcome the synchronisation requirements.

A.O. Trounson

But it does allow you to transport the embryos and I think that is the only important thing about it.

I. Wilmut

I would accept that but the one limitation to that is whether or not the animal health people, whatever they are called, will actually allow you to do that; whether you will be allowed to transport eggs which have not been held in quarantine, from one place to another.

A.O. Trounson

It is true that under Australian conditions you have to keep them for two years so obviously freezing is the only way.

D.G. Whittingham

But into this country you overcome the rabies order and you can bring in embryos which I did from Bar Harbor. They don't have to be in quarantine; embryos haven't been written into the Order yet.

R. Newcomb (UK)

This is moreorless along the same lines as the question I was going to ask Ian Wilmut. He envisaged that one of the potential applications was for the large scale twinning of cattle. How do you foresee that this can be done unless you have freezing of embryos and do you think that freezing of embryos will ever reach a sufficiently high success rate to be able to do this on a large scale?

I. Wilmut

The system I was alluding to would achieve only twinning with fairly nondescript material. We are relying on embryos recovered at slaughter from heifers which were on their way to slaughter anyway. In these circumstances the embryos don't have sufficient value to be worth freezing. I am envisaging a system where you synchronise both donors and groups of recipients, recover the embryos at the slaughterhouse and take them out and transfer them on a pre-arranged date.

R. Newcomb

Do you envisage that restrictions will ever be sufficiently relaxed for anything like this to be done?

I. Wilmut

I've no idea - probably not!

S.M. Willadsen

I think at this stage one must keep the various problems separate from the possible applications. We are the people who are concerned with trying to develop the techniques and I think you will agree, (Whittingham will agree), that although it doesn't look as if this is going to be automated within the next five months, it certainly will be automated if there is a soundly based decision to use frozen embryos. I can see various ways in which this could be done and there is no reason to go into any details now. The second thing is considering the problems in superovulating the cow, considering the widely differing results which people are getting and the variation which people are getting, you can't really say what the price of an embryo is going to be finally. Some people who are specialists in cattle breeding come in saying, "We want embryo transplantation" and if they say they want long term storage of embryos to be part of this, then one would start looking

at, say, superovulation along with it and it could be that the price of embryos would be brought down considerably. So one can't, at this stage, say that we can't use the technique because embryos are too expensive. We don't know what the price of embryos is going to be.

I. Wilmut

I think it is only fair to say that I disagree.

L.E.A. Rowson (UK)

I think you have rather underplayed the part of the dairy animal in this. A lot of our beef comes from crossing the beef bull on the poor yielding dairy cow. Now, if instead of using that cow for crossing one can use eggs of beef crosses, possibly as singles, possibly as twins, into these animals you are going to get a tremendous advantage.

I. Wilmut

Are you?

L.E.A. Rowson

Yes, you must do.

I. Wilmut

Allowing for the fact that there is a far greater cost of actual egg storage and egg transfer than there is of A.I., is the greater value of the carefully chosen beef calf which you are going to get out going to justify all these costs - if you can already achieve a dairy cross beef calf by use of A.I?

L.E.A. Rowson

Yes, well I think the costs at the moment are hopelessly inflated of course; I agree with Steen Willadsen that the cost of production and freezing of eggs and so on is going to drop very much over the next few years.

I. Wilmut

Clearly this is very, very difficult to give any precise figures on but my impression was that the difference between, let's say, a Friesian calf which has been sired by one of the big beef breeds and a pure beef calf, of whatever type it is going to be, the difference in performance of those

animals is going to be so little that it won't really carry the cost of actual embryo transfer.

L.E.A. Rowson

I was really thinking of a beef cross - a cross between two beef breeds. If one introduced twinning on top of that one has got a tremendous advantage. I know some farmers don't like twinning but if you look at the work carried out recently in Israel, where they obtained a percentage of 170 offspring per cow with only a delay, compared with a single offspring, of one week of getting her in calf, then the advantages seem to me to be pretty great.

I. Wilmut

I think the only way I can hide behind this is that I don't dispute there would be an increase in, if you like, biological efficiency. The guess that I was making was that that increase in efficiency would just not be enough to carry the cost of the transfer. Even if you are relying on non-surgical transfer, for each donor you are going to have to carry the cost of keeping her on the way to slaughter or whatever is happening to her; you are going to have to pay for the cost of the drugs which are going to be used to treat her - the prostaglandins, the PMS, the inseminations for her, and the actual transfer technique - the medium and so on.

L.E.A. Rowson

This would only work for frozen embryos but with frozen embryos you don't need to worry about synchronisation at all.

I. Wilmut

Particularly if you throw in twinning. I don't think it could possibly work if you used freezing because the survival rates have got to be almost 100% in order for it actually to produce what you are aiming for.

S.M. Willadsen

I still think that you have got to keep these things separate because I still don't think that we know what the costs are going to be. We don't know whether we can get 100% survival; I think that might be a possibility. As you say, we can disagree on all this - it's just unsettled, isn't it?

I. Wilmut

Yes, I would agree with that.

D.G. Whittingham

Perhaps you can continue the discussion afterwards but we have to stop at this point.

THE TRANSFER OF BOVINE EMBRYOS

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With the advent of bovine embryo transfer companies, a source of data derived from a multitude of donor and recipient operations has been made available. This discussion will encompass transfers undertaken at Alberta Livestock Transplants Ltd. in Calgary, Alberta, Canada, involving exotic beef cattle and will include principally animals of the Simmental, Maine Anjou, Limousin and Chianina breeds, with fewer numbers of the Charolais, Blonde d'Aquitaine, Gelbvieh, Pinzgauer, Murray Grey, Tarentaise, Romagnola and Marchigiana breeds. From October 1973 to May 1975, a total of 582 donors were stimulated and 3625 recipients implanted. When this data is tabulated and analysed, trends appear which might reflect upon various aspects of the reproductive physiology in the bovine species.

Annual results were categorised into 3 month intervals, that is, January to March, April to June, July to September, and October to December. (Table 1)

TABLE 1 SEASONAL INFLUENCE ON EMBRYO TRANSFER SUCCESSES

Month of Stimulation*	No. of Donors	% Passed**	Corpora Lutea per Stimulation	% Recovered	% Fertilised	% Pregnant
Oct - Dec '73	51	14	12.6	65	58	45
Jan - Mar '74	31	6	9.5	59	74	55
Apr - Jun '74	82	9	12.4	67	77	56
Jul - Sep '74	104	9	14.9	63	78	51
Oct - Dec '74	133	20	12.4	64	75	45
Jan - Mar '75	74	15	13.8	65	78	49
Apr - May '75	107	14	13.0	64	74	53

* 1700-2500 i.u. Pregnant Mare's Serum Gonadotrophin

** Less that 4-5 Corpora Lutea Palpated

In the periods October to December of both 1973 and 1974, the pregnancy rates obtained with embryo transfer were lower than at other times of the year. No conclusive evidence was obtained to suggest that other factors were similarly affected during this period, such as stimulation rate, recovery of embryos, or fertilisation. Definitive explanations for a lower successful implantation rate at this time of year are not known. Possibilities are generally attributed to environment and/or nutrition, which are inter-related. Winters in Western Canada commence in the period October to December and this season undoubtedly poses some stress to the recipients. Hours of daylight are considerably reduced in winter and temperatures can fall well below $0^{o}C$. What effects these parameters have on nidations are not clearly understood but are felt to result in a lowered fertility. These effects might be direct if one supposes the environment affects hypothalmic activity, or perhaps indirect, as through access to feed and water, known to affect reproduction (Hill et al. 1970).

When results were categorised by breeds (Table 2), one significant point appeared.

TABLE 2 BREED DIFFERENCES IN RESPONSE TO PMSG

Breed	No. of Donors	% Passed	Corpora Lutea per Stimulation	% Recovered	% Fertilised	% Pregnant
Simmental	178	8	15.2	64	75	54
Maine-Anjou	93	17	9.9	69	82	54
Limousin	79	16	13.6	60	79	43
Chianina	54	15	12.4	74	63	65
Others	76	13	12.2	63	77	43
Average		13	13.1	65	77	50

The number of corpora lutea present per stimulation (determined at surgery) were 15.2, 13.6, 12.4 and 9.9 for the Simmental, Limousin, Chianina and Maine Anjou breeds, respectively. The response to pregnant mare's serum gonadotrophin (PMSG) of the Maine Anjou animals presented for transfer was significantly ($p < 0.05$) poorer than for the other breeds. By contrast, the percentage of ova recovered, the fertilisation rate and pregnancy rate following transfer, were all at least equal if not superior for the Maine

Anjou than for the other breeds. As the factors which might affect response to hormonal treatment are numerous, no single factor can be attributed as the cause of this poorer response.

An important factor determining if a transfer resulted in a pregnancy was the embryo itself. Attempts were made to rate the embryos on the basis of compactness, symmetry and density of the blastomeres. This rating, by its very nature, was subjective and hence any rigid analysis of the results would not be truly scientific. Both morulae and 8-12 celled embryos were rated on a scale of 1 to 5 with 3 being an average appearing embryo, 1 very poor appearing, and 5 an excellent appearing one. A general pattern emerged in the pregnancy rates (Table 3), morulae rated 5 displaying the highest success rate and so on down to 8-12 celled embryos rated 1.

TABLE 3 EMBRYO QUALITY

Rating	Jan '74 - May '75 No. Transferred	%Pregnant	Sept '74 No. Transferred	%Pregnant
Morulae				
5	59	64	8	38
4	409	61	30	43
3	1709	56	122	52
2	350	44	51	55
1	14	29	4	50
8-12 celled				
5	1	0	1	0
4	28	43	3	67
3	330	43	8	0
2	281	36	13	38
1	44	18	1	0
4-7 celled	112	13	10	20
1-3 celled	54	4	1	0

However, this pattern was not consistent as is shown in the results for September, 1974. At any one time, a poorer rated group of embryos might have a high pregnancy rate and vice versa. It appeared that when overall

pregnancy rates were higher than average, embryo quality was an important determinant of success, but when pregnancy rates were low (October to December), embryo quality was not as consistent a determinant of pregnancy, some other factor or group of factors perhaps being more important. The most consistent indicator of pregnancy potential was stage of development. The majority of recoveries and transfers were done 5 days after the donor showed heat. If the recovered embryos were morulae, they had a high possibility of implanting, 8-12 celled embryos implanted less often, 4-7 celled embryos lesser yet, and 1-3 celled embryos rarely (2 out of 54) when implanted into recipients synchronised with the donor.

Synchronisation has been recognised as essential for successful embryo transfer for many years (Chang, 1950). Rowson et al. (1972) examined this phenomenon in cattle. If the recipients were in heat a day before or a day after the donor, pregnancy rates were considerably reduced. Due to the large numbers of recipients required with commercial bovine transfers, many of these animals were not perfectly synchronous with the donors. Examination of the effects of this lack of synchrony provided no evidence to support these previous observations (Table 4).

TABLE 4 SYNCHRONISATION REQUIREMENTS FOR TRANSFERRING MORULAE RATED 3 OR 4

Month of Stimulation	-1/2 to -1 1/2 days No. Trans	% Preg.	Synchronisation 0 No. Trans.	% Preg.	+1/2 to +1 1/2 days No. Trans.	% Preg.
Jan - Mar '74	10	60	45	76	11	64
Apr - Jun '74	43	67	227	65	50	72
Jul - Sep '74	99	52	266	58	57	47
Oct - Dec '74	35	60	289	55	201	41
Jan - Mar '75	60	65	182	62	114	46
Apr - May '75	87	60	117	73	123	54

When considering the transfers of only the best embryos, if the recipients showed heat before the donors, there was perhaps a poorer pregnancy rate, although this was not observed consistently. When the recipients and donors showed heat at the same time or when the recipients showed heat shortly after the donors, there was no significant difference in pregnancy rates. The major discrepancy between our data and that of Rowson et al. (1972) was in the pregnancy rate of the 0 synchrony group. As a commercial enterprise

embryos with a reduced probability of successfully implanting must be transferred if there is a reasonable chance of success. Consequently it might be unrealistic to expect a 91% pregnancy rate commercially. Another uncertainty arises if ovulation time is erratic or spread over a lengthy period following superovulation. If the donor is in heat at a specific time but the ova are ovulated over a 24 - 48 hour period, then sychronisation must be somewhat uncertain.

The fact that the cow normally ovulates only a single ovum, whereas with superovulation this is increased, seems to have many effects physiologically, including the problem of synchronisation already discussed. Rowson (1971) discussed an optimal number of ovulations, since when more than 20 ovulations were present, the physiology of the reproductive tract was upset and poorer results obtained. Our results (Table 5) confirm that with increased superovulation, fertilisation tends to decrease, although not being an absolute relationship.

TABLE 5 EFFECTS OF NUMBER OF CORPORA LUTEA ON SUCCESS RATE

No. of CL at surgery	1 - 10	11 - 20	21 or more
No. of donors	164	193	100
% of ova recovered	63	66	61
% fertilised	84	78	71
% pregnant after transfer	49	50	51
Pregnancies per surgery	1.8	4.0	6.4

One donor ovulated 37 ova and 34 were recovered, all fertilised. When transferred, 19 recipients were pregnant. Neither recovery nor pregnancy rate after transfer were affected by the number of corpora lutea. When donors were grouped according to number of corpora lutea (CL), those with 1 - 10 CL, 11 -20 CL, and 21 or more CL, produced an average of 1.8, 4.0, and 6.4 pregnancies per donor. Since more calves are obtained with more CL, then the important factor becomes whether the cost, both financially and physiologically, to produce the extra calves is greater than their value. Also important in determining the optimum dosages of stimulating agents is the effect these have on ovulation. If one stimulates too many follicles, will these simply fail to ovulate? Perhaps the number of follicles stimulated is more important than the number of CL, that is, the degree of

superstimulation as opposed to superovulation.

As the ratio of follicles to corpora lutea at surgery increased, recovery of ova also increased but the rate of fertilisation decreased (Table 6). The differences were not large but perhaps with a higher follicle to CL ratio, the oestrogen to progesterone ratio was also elevated. This might delay entry of the ova into the uterus and simplify recovery. Possible effects on fertilisation are unknown, as fertilisation presumably occurred as much as 4 days prior to determining the follicle to CL ratio.

TABLE 6 EFFECTS OF FOLLICLE TO CORPUS LUTEUM RATIO ON SUCCESS RATE

F : CL	0.67 or less	0.68 - 1.50	1.51 or more
No. of donors	192	80	31
No. of CL/Surgery	16.6	13.8	10.8
% Recovered	63	66	69
% Fertilised	78	75	70
% Pregnant	48	51	50

Another observation of Rowson et al. (1972) was that within a limited period, the time over which the embryos were stored had little effect on subsequent development. Our results are in agreement (Table 7). When our better appearing morulae were transferred (a) within approximately one hour, (b) between 2 - 3 hours, or (c) after 5 hours following recovery, 58%, 63% and 58% respectively, resulted in pregnancies. The embryos were stored at 37°C in medium 199 containing Hepes buffer and 10% foetal calf serum.

TABLE 7 EFFECT OF TIME FROM RECOVERY TO TRANSFER*

Storage Time (Hr)	No. of Recipients	% Pregnant
1 or less	579	58
1 - 2	278	62
2 - 3	110	63
3 - 4	48	56
4 - 5	40	55
5 or more	73	58

* includes only morulae 3 + 4

Two factors which appeared to be related were fertilisation and recovery. Analysis of the data (Table 8) indicated that when the fertilisation rate of recovered ova was low, so were the recovery rate (as a percentage of the number of CL) and pregnancy rate (pregnancies per 100 embryos transferred). It can be speculated that fertilised ova are more readily recovered than unfertilised ova. Perhaps in the absence of fertilisation, ovum transport and/or ovum integrity are altered. Or, perhaps the poorer fertilisation is due to a tubal or uterine factor which also affects ovum recovery.

TABLE 8 EFFECT OF RATE OF FERTILISATION ON SUCCESS

Fertilisation rate	0 - 34%	35 - 67%	68 - 100%
No. of donors	33	55	212
% of ova recovered	42	59	70
% of recipients pregnant	28	43	51

One final point is well taken with bovine research. Our average number of calves per donor surgery is between 3 and 4. However, only 11% of the surgeries produced 3 calves and 9% produced 4 calves. Thus, while about 20% of the donors produced an average result, 80% produced either more or less pregnancies. This results in a Poisson type of distribution. When one plots the number of surgeries that produce 0, 1, 2, 3, 4, 5... calves, one observes that as the number of calves increases, the percentage of surgeries producing that result decreases. The vast majority (75%) of donor surgeries produce between 0 and 5 calves. If one could obtain just one more calf from each of these donors the number of pregnancies per surgery would rise from 3.7 to 4.5. Thus, emphasis in research perhaps should not be aimed at obtaining a large number of calves per donor but rather at obtaining calves more consistently from donors. However, consistency seems to be one quality which the cow has, to the present time, avoided.

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DISCUSSION

A.O. Trounson (UK)

I want to ask the same question I asked Professor Church this morning. You said that you believed that ovulation occurs over a period of 24 hours. What is your evidence for this? In unsuperovulated animals ovulation occurs at a precise time after the LH peak, and again, in the superovulated sheep, total ovulation occurs within six hours.

B. Shea (Canada)

I believe there is published data - it was mentioned to me this morning - I believe Hafez may be one of the co-authors - to indicate that in the superovulated bovine ovulation is spread out over a longer interval than six hours for sure. We have no direct evidence; we haven't looked, for example, at successive 12 hour intervals to check on ovulation. We have done a fair amount of work with recovering eggs for in vitro fertilisation, operating at periods of 24 - 36 hours, and we have observed ovulation occurring with these cases.

A.O. Trounson

This would be different animals?

B. Shea

Yes, this is the problem we run into. There is other indirect evidence: you have probably observed yourself that if you go in on Day 3 the corpora lutea structure can be extremely variable. You can have something which resembles a haemorrhagic follicle, a recently ovulated follicle, all the way to a well formed corpus luteum.

A.O. Trounson

But I don't accept that as evidence ....

B. Shea

No, it's not conclusive; it's all indirect evidence.

A.O. Trounson

Professor Thibault, have you any evidence on the time of ovulation in superovulated cattle?

C. Thibault (France)

We have not sufficient experience on superovulation in cattle in order to answer but many years ago when trying to obtain superovulation we observed that there is the egg at five different places in the fallopian tube, but I am not sure that it corresponds to the time of ovulation.

D. Whittingham (UK)

In the mouse at least, the spread of ovulation in superovulation is shorter than in the natural ovulation and sometimes they ovulate up to 100 eggs; it occurs over three hours.

C. Thibault

I am not sure that the stimulated follicle cannot ovulate 20 hours later and give a normal oocyte.

R. Newcomb (UK)

The only work I know of is the work that Michael Phillippo has done and, if I remember correctly, he found a range of between 7 and 30 hours for the extent of the ovulation period in the superovulated animal, by endoscopy.

I would just like to make one comment on your recovery at Day 3. We also observed corpora lutea which appear very much older at Day 3 than we would imagine them to be. In this particular case we think one has probably had a premature ovulation of a pre-ovulatory size follicle, so the prostaglandin wouldn't be given at a stage when it would cause luteolysis when the injection was given. So it would be something like 4 days older than the other corpora lutea which you see in the ovary. We recognise this particularly when PMS has been injected very early in the cycle, at Day 6 particularly.

J. Sreenan (Ireland)

I would just like to make a comment first, then ask a question. The comment is in respect of what has been said about Michael Phillippo's data on laparoscopy. I was involved in some of that work with him and the suggestion was that there was a spread of approximately 24 hours at that stage. However, subsequent laparoscopy work in control animals shows that continuous observation by laparoscopy seems, in some unexplained way, to inhibit ovulation occurring in those follicles, and that if we had been

able to open up the animal and observe the counts, rather than continuously going in with a laparoscope, we would have had a more accurate picture. I suspect that we got a spread in ovulation actually caused by repeated observations by laparoscopy. I think Jim Roche has some data published on this.

A.O. Trounson

We had the same results in the sheep when we were doing continuous endoscopy and from the LH peak we put off ovulation some 64 hours.

J. Sreenan

This is probably one of the dangers of the method by which you measure the technique.

I would like to ask Dr. Shea a question on the breed effects. It is very hard to be definite about it but looking back through the literature there is a suggestion that there are breed effects in terms of the commercial breeds - and these will be more breed types than pure breeds. The suggestion would seem to be that these breeds are more sensitive to PMSG than, for example, dairy breeds. This is not very definite but it seemed to be fairly definite this morning that the Friesian is much harder to superovulate. Following on from that, may I ask whether you have also got data on age effects because I am not aware of any good data where people have set out to look at breed and age effects with these particular breeds in mind.

B. Shea

No, we haven't really got good data on age effects. I would say that the majority of animals we treat are heifers and not too old. As far as the dairy versus beef goes, again, commercially we have done virtually no beef animals at Alberta Livestock Transplants. The problem with stimulation in the dairy breeds, in particular, is the age of the animal being stimulated. I have reason to believe from a worker who is working primarily with Holsteins in Ontario that younger Holsteins don't present a big problem with stimulation but up until this time the majority of the Holsteins that I am aware of that were stimulated were going out of production. That is the reason they were being stimulated, because they were no longer producing calves themselves. So it is probably not fair to say, on the basis of that, that Holsteins are harder to stimulate.

J. Sreenan

The only reason I make that comment is that it is reasonably important from some of the discussion that went on this morning; the reasons why one would use egg transfer at all, why one would superovulate a dairy breed to spread genetic merit. In the course of looking at breed effects ourselves, we are trying to gather some information from other sources on definite breed effects and we don't seem able to pick up anything. Neither breed nor age effects would seem to be in the literature.

B. Shea

No, the only difference we can see with breed is in the stimulation of Maine Anjou. Obviously we didn't stimulate all the Maine Anjou but the ones we did responded significantly more poorly than the others.

C. Thibault

We have made some experiments on the Charolais breed and among five families we observed a difference from one family to the other - even in the same breed. We have not got the precise data here but I remember the five families and the different results.

D. Whittingham

Coming back to mice again, I would make a comment about sensitivity to gonadotrophin. Certainly in-bred lines are particularly insensitive to stimulation by gonadotrophin. Some you can't stimulate to superovulate at all. The amount of hormone you give varies.

Another point, with regard to what Ray Newcomb said, the first ovulation that occurs is the phenomenon that is observed in mice. When you give the PMSG you get an ovulation of a normal clutch of eggs within the first 20 hours, irrespective of the state of the cycle in which you start the injection. Actually, you can get it with HCG, PMSG, or pure ovine FSH. These eggs are viable; I have collected them, fertilised them and obtained young.

R. Newcomb

In respect of this premature ovulation which you do get sometimes, this would be one of the phenomena which might possibly be associated with PMS batch variation. When we have looked at batch variation within the Unit, quite honestly, I don't think we can see any, but one could hypothesise

that a batch with a high LH potency, compared with another batch, might more frequently cause a premature ovulation, in which case, we have found the subsequent crop of ovulations is generally disturbed and fertilisation can be disturbed also.

D. Whittingham

In mice there is a specific age effect in sensitivity to gonadotrophin. Immatures are very sensitive, they go through a period when they are definitely unresponsive, and then go up again, subsequently they dwindle again in old age. There is quite a lot of data on this.

B. Shea

I would like to mention a point that was mentioned by Professor Thibault earlier. Recently, for some reason, we had three donors that showed heat 3 days late; this was 5 days after prostaglandin and 7 days after PMS was administered. These animals were stimulated very well and fertilisation appeared to be normal. We can, of course, suppose the follicles had been there for an extra 3 days but it is possible that they were there for 3 days longer than normal, yet fertilisation still appeared to be normal. We haven't got the results of the transfers yet because it occurred a fairly short time ago.

M.T. Kane (Ireland)

Do you have any actual data on transfer of in vitro cultured embryos?

B. Shea

The number of transfers that we have actually done is limited. We did this in our initial work. We transferred into 17 recipients a collection of 8-cell embryos that we had cultured through to the morula stage. Of those 17, 2 became pregnant. After that we abandoned the transfer aspect. I feel we can do better now but we just felt it was more significant at this stage to culture them and try to get morphologically appearing embryos. We haven't carried on with the transfer of the cultured embryos yet.

S.M. Willadsen (UK)

In relation to synchronisation, you are getting a wide spread of stages in these animals. Our embryos have been exposed to all sorts of

treatments and some of them still have the same morphology when they come out two days later, or a month later. Our general policy has been not to be too worried about the donor but rather, once we have got the embryos, to consider the stage of development which the embryo has attained at that particular point and then synchronise related to that. What is your general policy in your experiments? In other words, if you have them at 8-cells, do you transfer to a Day 5 recipient, and do you transfer to the uterus?

B. Shea

We treat Day 8 to Day 12 embryos the same as we do morulae. When we have attempted to analyse the data we expected to observe that when we transferred 8 to 12 cell embryos we would get higher pregnancy rates in those animals that were in heat a day after the donor, but in actual fact this wasn't true. The relationship that was depicted with the morulae 3 and 4 was fairly consistent in the sense that if the recipient was in heat before the donor she didn't seem to do as well. With 8 to 12 cells, when the recipient came into heat the day before the donor and when you would expect the donor to produce morulae, and she is producing 8 to 12 cells, so that perhaps you are out by as much as 2 to 2½ days, they did equally well in terms of pregnancy rate as the 8 to 12 cell eggs that were transferred into Day 4 recipients that came into heat a day after the donor. Consequently the pregnancy rate is lowered and we spread the number of actually viable 8 to 12 cells over the three groups. It has resulted in a not really significant difference between the three groups.

D. Whittingham

Thank you very much Dr. Shea.

IN VITRO CULTURE OF COW EMBRYOS FROM DAY 6 AND DAY 7

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ABSTRACT

Cow eggs, recovered at Day 6 and Day 7, were cultured 2 to 4 days in different conditions. The culture was done in a closed tube under oil; the atmosphere in the tube was composed of a mixture of 5% of O_2, 5% of CO_2 and 90% of N_2. 135 eggs were observed after culture.

Two different media were compared for their fitness for developing eggs cultured from Day 6:

- *Brinster's medium, $BMOC_3$ with 5g/l of BSA added (Pos 290 mos. pH 7.4)*
- *Menezo's medium, B_2 (1975) (Pos 290 mos. pH 7.6)*

The percentage of embryos hatching from the zona pellucida after 4 days of culture was significantly higher in medium B_2 (65%) than in medium $BMOC_3$ (18%).

In medium B_2, 80.1% of the eggs cultured from Day 7 hatched from the zona pellucida after 3 days.

After transfer, two eggs collected at Day 6 stage and cultivated 4 days in medium B_2 gave embryonic development when examined at Day 20 of gestation.

Culture in vitro in these conditions may constitute a technique for controlling egg viability at different stages of storage or deep-freezing.

* With technical assistance from J. Pont, P. Chesne, Marie-Claude Naule and Claude Rebours.

INTRODUCTION

The first successful in vitro culture of cow eggs was reported by Tervit, Whittingham & Rowson (1972). These authors described a medium in which eggs could develop from the one cell to the morula stage, and even to early blastocyst. Wilmut & Rowson (1973) cultivated cow blastocysts for 24h: these were recovered from day 10 - 13 of pregnancy in order to examine their survival after deep-freezing.

As with the latter authors, we have tried to define a criterion of the viability of eggs used by observing development at the end of culture.

MATERIAL AND METHODS

Recovery of embryos

The embryos were recovered at days 6 and 7 of pregnancy (Day 0 is the first day of heat) after 36 cows or heifers* were slaughtered. These were treated at a 14 day interval with two injections of prostaglandins** and given an injection of 2400 i.u. of PMSG 2 days before the second prostaglandin injection.

The recovery and observation of eggs was done at 26°C in Brinster's medium $BMOC_3$ (1971); 1g/l of BSA was added to this medium

Embryo culture

Embryos appearing normal were cultivated at 37°C, either in Brinster's medium, $BMOC_3$ with 5g/l of BSA (Pos 290 mos, pH 7.4), or in Menezo's medium, B_2 (Pos 290 mos, pH 7.6)***

* Most of the animals were Holstein-Normande or Charolais-FFPH crosses. Pure breed cows were either Normande or FFPN. Some of the animals were supplied by the Department of Genetics. We wish to thank MM Frebling, Poutous, Colleau, Daburon and Marchand, and the personnel at Le Pin and La Minière who assisted in this study.

** Eleven cows were treated with 2 x 50mg of Upjohn prostaglandin and 25 cows were treated with 500 µg ICI 80,996. We wish to thank these two firms which gave us the products without charge.

*** Institut Pasteur in Lyon, France.

Embryos were put into glass tubes (9 x 75mm) containing 1ml of medium under paraffin oil. The atmosphere was gassed, before closing, with a mixture of 5% of O_2, 5% of CO_2, 90% of N_2 (Tervit et al. 1972). The mean time elapsing between slaughter of the female and placing the eggs in the tube was 40 minutes.

After culture, the embryos were observed with a binocular microscope (X 40) and their developmental state noted, using two morphological criteria: the development of well-formed blastocysts and the rupture of the zona pellucida and blastocyst release.

The embryos were then fixed for histological study.

RESULTS

Recovery of eggs

Only the eggs of 25 females (69.4%) were used for the culture and storage experiment (Fig. 1).

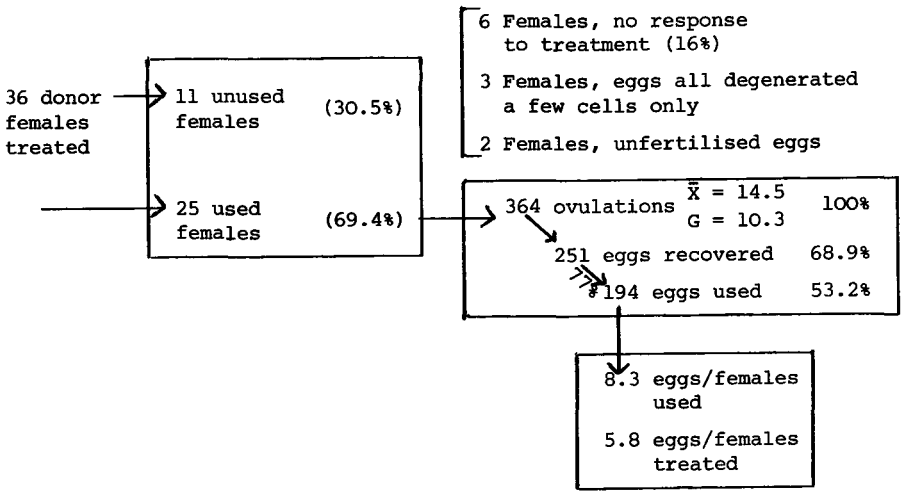

Fig. 1 Diagram - Production and Collection of Embryos

Two hundred and fifty one (68.9%) of the 364 eggs produced (estimated from the number of corpora lutea) were recovered and observed under a binocular microscope. Fifty seven eggs (22.6%) were very late or

degenerating and were not used. The others (194 eggs) appeared normal; 128 eggs at stage Day 6 came from 18 donor females, 66 eggs at stage Day 7 were from 7 donor females. 135 eggs were used in this experiment and observed at the end of culture.

Embryo culture

The percentage of embryos cultured from Day 6 and which were hatched from the zona pellucida membrane after 4 days of culture, was significantly higher ($P < 0.025$) in medium B_2 (65%) than in medium $BMOC_3$ (18%) (Table 1).

TABLE 1 DEVELOPMENT OF EMBRYOS CULTURED FROM STAGE DAY 6

(Day O = heat day)

Comparison of two mediums

Medium	Culture time (days)	No. of Embryos	Stage of development after culture			% of blastocysts hatching from zona pellucida membrane
			Degenerated embryos	Well-formed blastocysts	Blastocysts hatching from zona pellucida membrane	
$BMOC_3$	2	9	3	2	4	44
	3	16	8	3	5	31
	4	16	11	2	3	18
Total		41	22	7	12	29
B_2	2	9	8	O	1	11
	3	18	9	3	6	33
	4	20	7	O	13	65
Total		47	24	3	20	42

In medium B_2, the percentage of cultured eggs which hatched from their zona pellucida was high. It was the same after 4 days of culture for eggs put in a tube at stage Day 7 (80.1%). In both cases, the same stage of theoretical Day 10 development was reached (Table 2).

46.6% of the eggs of stage Day 7 hatched from the zona pellucida after 2 days of culture. There was a significant difference with the 80.1% obtained after one more day of culture ($P < 0.05$).

Two eggs taken at stage Day 6, cultivated 4 days in medium B_2 and transferred by the surgical route to a recipient on Day 10 of the cycle, after oestrus synchronisation, gave apparently normal embryonic development which was examined by slaughter of the recipient on Day 20 of pregnancy.

TABLE 2 IN VITRO CULTURE IN MEDIUM B_2

COMPARISON OF DEVELOPMENT OF EGGS CULTURED FROM STAGE DAY 6 AND FROM STAGE DAY 7

CULTURE TIME	2 D		3 D		4 D
Eggs cultured from Stage Day 6					
Number of Embryos	1 (9 eggs)	NS	6 (18 eggs)	NS	13 (20 eggs)
%	11.8		33.3		65.0
		NS		NS	
Eggs cultured from Stage Day 7					
Number of Embryos	7 (15 eggs)		26 (32 eggs)		
%	46.6		80.1		

NS = Insignificant S = Significant (α = 0.05)

CONCLUSIONS

Utilising medium B_2 and our culture conditions, we could use blastocysts release from the zona pellucida, a simple morphological criterion, to estimate embryo quality at stages Day 6 and Day 7. The developmental conditions of these cultured embryos may be considered as normal because a high proportion are released from the zona pellucida membrane by the tenth day; this is the exact day of zona pellucida rupture at the beginning of gestation (Chang, 1952).

Furthermore, these embryos may develop normally after transfer, at least up to Day 20. However, the number of transfer trials is too small to determine the proportion of eggs cultivated which will develop regularly

after transfer. Also, the recipients were slaughtered too early to guarantee that an absolutely normal development would be maintained in utero. This point must be confirmed by observing more advanced pregnancy or calving.

However, we hope from this first result to obtain a slower drop in in vitro viability than that reported by Sreenan et al. (1975) after culture at 37°C with TC 199 (percentage of development dropping from 70% for 1 to 2 hours of storage to 30% for 4 to 8 hours of storage.

We think that the in vitro culture method proposed here could constitute a technique for egg viability control, especially at different stages of storage and deep-freezing. This method could be substituted for the system of "culture in vivo" in the rabbit fallopian tube.

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STORAGE OF EWE AND COW EGGS AT + 10°C

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ABSTRACT

Ewe and cow eggs were recovered after slaughter 4 days (Day 4) and 6 days (Day 6) respectively after mating; Day 0 is the day of oestrus. Both ewes and cows were given a superovulation treatment.

Flushing was done with the medium of O. Kardymowicz (modified Locke) at room temperature. Eggs were stored in the same medium in closed tubes at +10°C. Depending on the species studied, 50% of heated ewe or calf serum was added to the medium. Cooling or heating rate never exceeded 0.9°C/mn.

EWE

67.8% of eggs stored in tubes 4 and 5 days or 9 and 10 days were transferred into the uterine horn of ewes at Day 4 of their own cycle; one egg was placed in each uterine horn. The other eggs were eliminated because of their poor appearance after 9 or 10 days of storage (46.2%) or after 4 or 5 days (20.9%).

Donor and recipient ewes were of the Ile-de France breed.

We found the following results:

- egg storage in vitro for 4 or 5 days: 20 recipient ewes, 6 ewes lambed, 18.4% of eggs transferred produced one lamb,

- egg storage in vitro for 9 or 10 days: 11 recipient ewes, 4 ewes lambed, 23.8% of eggs transferred produced one lamb.

* With technical assistance from P. Chesne, M. Marcel, Marie-Claude Naule and Claude Rebours.

COW

59 eggs recovered at Day 6 and Day 7 were stored 24h in the same conditions.

The development of eggs stored and then cultivated 3 or 4 days in vitro in medium B_2 (Menezo) was taken as a criterion of their viability at the end of storage. 17.5% of the eggs developed normally when they were stored 24h at 10°C at stage Day 6 (65% of control eggs not stored developed). None of the 19 eggs stored at stage Day 7 developed further (80.1% of control eggs not stored developed). The medium conditions which we tried were thus not very satisfactory for storage of the bovine blastocyst, even when limited to 24h.

INTRODUCTION

Ewe eggs can be stored in good conditions for 6 to 9 hours at 5° or 8°C. At the end of this time Avrill and Rowson (1959) obtained 75% of development after transfer. However, prolonged storage at the same temperatures or at 10°C gives poor or no results (Harper & Rowson, 1963).

M. Kardymowicz et al. (1966) observed 18% development after transfer of ewe eggs previously stored 5 days at 10°C. Two lambs were born after egg storage at 7°C for 10 days.

The results obtained in storage trials at 0°C on cow eggs at stage 8-16 cell (Day 4) were disappointing on the whole (Wilmut et al. 1975). The survival of eggs at a more advanced stage (Day 6) after cooling to 0°C seems better. Slow cooling rates are beneficial (Trounson, personal communication).

The storage trial at + 10°C which we report here was done on both species, using the medium of Olga Kardymowicz as indicated. Ewe eggs were collected at the beginning of segmentation (Day 4) and those of the cow were recovered in the form of morulae or small blastocysts (Day 6 or Day 7) during the storage period.

EWE

101 eggs of 8-16 cells were recovered from Ile-de-France ewes more than 4 years old, slaughtered at Day 4 of the cycle (Day 0 is the first day of oestrus) during their normal sexual season. All animals were given an injection of 800 i.u. of PMSG on days 13 or 14 of the cycle. They were mated to Ile-de-France rams after bi-daily detection of heat.

The genital tract was taken and flushed immediately after slaughter. Eggs were recovered at room temperature in a solution of modified Locke, according to the technique of Kardymowicz (1966, 1971). They were then observed with a binocular microscope and put into tubes at 2 eggs per tube in 1ml of a solution of 50% modified Locke, 50% homologous ewe serum (Kardymowicz, 1972). The tubes were gassed according to Whitten's technique (1970), then stored in a cold room at 10°C.

After 4-5 and 9-10 days of storage, the eggs were reheated to room temperature and observed before transfer.

Those eggs which had ill-defined limits and blastomeres of very unequal size, were not used.

The remaining 59 eggs were transferred, according to the technique of Hunter et al. (1955) into ewes of the same breed (Ile-de-France) which were in Day 4 of their cycle and had not mated at the time of oestrus. One egg per uterine horn was transferred (except for 2 ewes which were given one egg each).

The 28 donor ewes treated with PMSG had a mean ovulation rate of 4.8 per animal and a recovery rate of 75.3% (101/134).

10% of the eggs recovered were unfertilised (9 eggs) or presented a burst zona pellucida (2 eggs). The 90 eggs used for storage were all at the 8-16 cell stage.

67.8% of stored eggs were used for transfer. The proportion of eggs eliminated because of their morphological aspect was higher for eggs stored 9 and 10 days (46.2%) than for those stored 4 and 5 days (20.9%).

The results obtained after transfer are shown in Table 1.

Ten out of the 31 ewes (32%) receiving transferred eggs gave birth to 12 lambs.

Seven lambs (18.4%) were obtained with 38 eggs transferred after 4 days (11 eggs) and 5 days (27 eggs) of in vitro storage.

Five lambs (23.8%) were obtained with 21 eggs transferred after 9 days (15 eggs) and 10 days (6 eggs) of in vitro storage.

No lambs were born after 10 days of egg storage at 10°C. The results obtained after 9 days of storage seem better than those obtained after only 4 and 5 days, but the difference is not significant.

Among the 11 lambs, 6 females weighed 3.9kg and 5 males weighed 4.9kg.

TABLE 1 TRANSFER RESULTS OF STORED EWE EGGS

Length of Storage (Days)	Number of Recipients	Number of Pregnant Females	%	Number of Eggs Transplanted	Number of Lambs Born	%
4	6	2	33	11	2	18
5	14	4	28	27	5	18
4 & 5	20	6	30	38	7	18.4
9	8	4	50	15	5	33
10	3	0		6	0	
9 & 10	11	4	36	21	5	23.8
Total	31	10	30	59	12	20

COW

Fifty nine cow eggs were recovered from Day 6 - Day 7 and stored 24h in the same conditions (ewe serum was replaced by calf serum) maximum cooling or heating rate never exceeded 0.9°C/mn. The state of the eggs at the end of storage was judged by their fitness for development in culture medium B_2 of Menezo (Renard et al. 1975).

TABLE 2 COW EGG STORAGE FOR 24H AT 10°C

Stage of Eggs at Storage Time	Number of Eggs Stored 24h then Cultivated	Results at End of Culture	
		Number of Eggs Hatching from the Zona Pellucida M.	%
6 Days	40	7*	17.5
7 Days	19	0**	-

* Eggs cultivated 4 days: 65% development for control eggs not stored

** Eggs cultivated 3 days: 80% development for control eggs not stored

The results shown in Table 2 are low because after 4 days of culture only 17% of eggs stored 24h hatched from the zona pellucida, while 80% of the control eggs developed in the same culture conditions.

CONCLUSIONS

Cow and ewe eggs cannot be compared for their fitness for storage at + 10°C because of the differences between the stages of egg development in the two species at the time of their storage.

The observation of the development of cow blastocysts recovered from Day 6 only permits us to say that after 24h of storage in a very simple medium, 17% of the eggs were still alive.

After cooling the eggs from Day 6, Wilmut et al. (1975) obtained excellent results but eggs were maintained at 0°C for 15mn after cooling at 10°C/mn.

No survival was observed at Day 7 in our trials. The good results obtained by Trounson (1975) with cow eggs recovered from Day 7 and stored at 0°C indicate that our storage conditions were bad.

The high percentage of development obtained by Kardymowicz et al. (1966) on 8-16 cell ewe eggs after storage for 24h and transfer, prove that medium conditions are relatively satisfactory at the beginning of storage.

Our results indicate that ewe egg metabolism is sufficiently slowed down so that its survival may be prolonged to 9 days.

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DISCUSSION

D. Whittingham (UK)

With the culture of the cattle embryos to expanded, or hatching, blastocysts, did you test the viability of those after transfer? When you had cultured them to the blastocyst or hatched stage, did you transfer them to a recipient?

J.P. Renard (France)

Yes, just in one recipient; we transferred two eggs and we are now doing more recipients. Four weeks after transfer those two eggs looked apparently normal.

S.M. Willadsen (UK)

As you know, we are using the in vitro culture of embryos for the assessment of various treatments we are trying. We have reason to believe that the culture, in itself, is a bit dangerous to these embryos. We would not claim that our culture conditions are really adequate for routine use and, indeed, so far as freezing is concerned, I think we have produced evidence that the viability of cultured embryos is not too good. Have you any comments as far as your embryos are concerned? I realise that your culture periods are quite long.

J.P. Renard

Yes, we do quite long culture periods to check the bio-chemical potentiality of the eggs. At Day 7 the metabolism of the eggs is quite rapid and during this hatching period eggs must be in very good condition to maintain a rapid and complete metabolism. We believe that culturing for two or three days can give good information of the metabolic activity of the eggs. Of course, it is not precise metabolism.

S.M. Willadsen

What I was getting at really was that you may be killing some embryos during culture and that you may, in fact, be getting considerably better results from your cooling than you are able to get out of your culture system.

J.P. Renard

Yes, but we have quite a large number of eggs that in these in vitro conditions are still alive.

A.O. Trounson (UK)

I wasn't quite clear; are you cooling in this complex culture medium? What is your medium for cooling the eggs?

J.P. Renard

We have done two things. First we used a very simple medium, PBS, the same as you use. Afterwards we cultured the eggs in the B_2 medium. Another thing: cooling the eggs in the complex medium and going down to $4^{o}C$ we have also good results when taking the eggs and putting them in culture for three days. We have about 70 or 75% of eggs hatching from the zona pellucida.

A.O. Trounson

These results were with Dulbecco's phosphate buffered medium were they not?

J.P. Renard

Yes.

A. O. Trounson

Why do you think your results were so bad since we do very well?

J.P. Renard

I think it may be because we use Day 7 blastocysts and just stay at $10^{o}C$ so the metabolism of the eggs is not stopped enough and it can go on under bad conditions.

C. Polge (UK)

When do you think that the cow egg normally hatches from the zona pellucida.? It seems to me that under your culture conditions they are hatching very late. I noticed that in one of your experiments where you were comparing Brinster's medium with the medium developed in your own laboratory, that taking Day 6 eggs, cultured for two days, you had 44% hatched from the zona pellucida, whereas in the other medium you had, I

think, 65% hatched from the zona pellucida at four days of culture. It would seem to me that possibly you are getting slower development in the second type of medium.

J.P. Renard

Yes, we have significant differences between the Day 9 and Day 10 theoretical stages. That means that most of the blastocysts are hatching between the ninth and tenth day of age. By most of them I mean about 70%, or in the late stage, 80%.

C. Polge

This is much later than in vivo?

J.P. Renard

There are few differences from in vivo conditions of hatching of cow eggs. What is known, and it has also been reported this morning, is that from Day 8 hatching is just beginning. This morning it was reported that at Day 8 there was 20% of hatching blastocysts; but the hatching is going on and in our in vitro conditions hatching is complete on the tenth day of age.

A.O. Trounson

But they have all hatched in vivo by Day 9 - it's very rapid.

J.P. Renard

Yes, from Day 8 to Day 9.

M.T. Kane (Ireland)

What is the major difference between your complex Menezo's, B_2 medium and Brinster's medium?

Y. Menezo (France)

There is the double concentration of BSA. That is one point. The second point is in the sodium and potassium ratio, it is lower than in Brinster. There is pyruvate lactate but also a very high level of glycine - 8 millimolar. In fact, we have put in all the free amino acids we have found in tubal secretions. We are not sure that all are necessary but it has been shown by Epstein and Wales that there is a high incorporation of free amino acids from Day 3.

ATTEMPTS TO MATURE CALF OOCYTES IN CULTURE

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In vitro studies of maturation of rabbit follicular oocytes have shown that while meiosis resumes normally, cytoplasmic maturation is not achieved outside the graafian follicle. After sperm penetration, the oocyte is unable to assume sperm nucleus swelling, i.e. the rupture of nuclear S-S bands and the substitution of somatic histones to sperm nucleoproteins. We have called the cytoplasmic elements inducing these processes male pronucleus growth factors (MPGF). Experiments with pigs (Motlik and Fulka, 1974), cattle (Thibault, Gerard and Menezo, 1975) and humans (Soupart, 1973; 1974; 1975) oocytes confirm the absence of MPGF when oocytes are matured in culture outside their follicle.

However, Mukherjee (1972) has drawn contradictory conclusions, obtaining the birth of five young from 325 mouse oocytes matured and fertilised in vitro. Yanagimachi (1974) described the presence of a swollen sperm head in the cytoplasm of guinea pig oocytes matured in vitro 1.30 to 2 hours after sperm penetration. Hunter, Lawson and Rowson (1972) obtained 11 eggs with two pronuclei 22 hours after transplanting, into inseminated heifers, 55 ovarian oocytes matured in vitro for 24 hours.

In fact, in the in vitro matured rabbit egg, the sperm head remained unchanged for some hours, but finally enlarged moreorless completely; 50 p. 100 of the fertilised eggs cleaved (Thibault, 1973). Niwa and Chang (1975) also described a failure or a delay in male pronucleus formation following fertilisation of rat eggs matured in vitro.

Thus, it seems possible that some embryos overpass this initial delay of sperm nucleus swelling and develop normally.

ATTEMPTS TO INDUCE THE PRESENCE OF MPGF IN VITRO BY STEROIDS OR GONADOTROPHINS

In lower vertebrates both nuclear and cytoplasmic maturation are steroid-dependent; progestin potency only varies with species differences.

Resumption of meiosis in mammals is not steroid-dependent, since the denuded rat oocyte matures normally in culture after progesterone-secreting cumulus cells are removed (Nicosia and Mikhail, 1975, Zeilmaker and Verhamme, 1974). Thus, it is fascinating to postulate that cytoplasmic maturation in mammals remains under the control of ovarian steroids.

Rabbit oocytes collected from the largest follicles of oestrous does were cultured with steroids alone or in combination. Gonadotrophins and prolactin were added in some trials because these hormones are present in the follicular fluid during the pre-ovulatory period, and may also stimulate steroid synthesis by the cumulus cells. (Table 1).

TABLE 1 ATTEMPTS TO INDUCE MPGF IN RABBIT OOCYTE IN CULTURE

STAGES OF SPERM NUCLEUS SWELLING 4-5 HRS AFTER FERTILISATION

Hormones in the culture medium	Number of oocytes	Fertilised oocytes (%)	Stages of sperm nucleus (%) 1	2	3	4
FSH ± LH	119	63	96	4	0	0
Progesterone	24	50	100	0	0	0
20 α OH Pg	20	80	100	0	0	0
E_2 ± 20 OH Pg	80	48	100	0	0	0
Cortisol ± (FSH+LH)	26	70	100	0	0	0
E_2 + testosterone	30	57	60	30	10	0
Prolactin ± FSH+LH	117	60	50	50	0	0
E_2 +test. + prolact. +FSH + LH	16	94	60	15	0	25
Follicular fluid	5	100	100	0	0	0
c AMP	5	60	100	0	0	0

1. Sperm nucleus unchanged
2. Beginning of swelling
3. Male pronucleus with fragments of condensed chromatin
4. Normal pronucleus

Oestradiol, progesterone, 20β-OH progesterone, cortisol, dibutyril cyclic AMP are without effect; FSH and LH, although very active on the separation of the corona cells, are also inefficient. On the other hand,

after maturation in the presence of testosterone or prolactin, some advanced stages of pronucleus formation are shown. When gonadotrophins, prolactin, testosterone and oestradiol are used together or in sequence which imitates intrafollicular evolution of these hormones, a subnormal male pronucleus is present in 25% of the oocytes 5 hr after sperm penetration. We are not sure, however, of the physiological significance of the effect of this hormonal cocktail, since it also induces parthenogenetic activation with regular cleavage in one third of the maturing oocytes, perhaps by changing egg permeability.

However, Soupart, (1975) obtained full human oocyte maturation by combining a sequential steroid treatment (oestradiol and then 17α-OH Pg) with the presence of gonadotrophins in the culture medium, oocytes being surrounded by cumulus cells.

Thus, follicular layers seem to participate in oocyte maturation in a more complicated way than by the well-known change in steroid secretion after LH and FSH pre-ovulatory surge.

Therefore the culture of the entire follicle appears as the most general approach to the study of oocyte maturation. We have shown at least in rabbit, that all follicular functions can be preserved in culture since in oocytes matured in vitro in their follicle in the presence of gonadotrophins, the sperm nucleus swells as rapidly as in naturally ovulated eggs (Thibault and Gerard, 1973). The birth of young is the proof of the complete oocyte maturation (Thibault, Gerard and Menezo, 1975).

Although complete development has not yet been reported in the rat, normal resumption of meiosis occurred when rat preovulatory follicles were cultured with LH or injected with cAMP; all steroids tested were completely ineffective (Tsafriri et al. 1972).

IN VITRO ATTEMPTS TO OBTAIN NUCLEAR MATURATION OF CALF OOCYTES IN THEIR FOLLICLE

Ovaries of prepuberal mammals generally contain a higher number of graafian follicles than do adult ovaries. Half a million calves are slaughtered every year; this number represents a vast oocyte source. We have shown that progestogen pre-treatment (FGA or progesterone) of 3 to 4-

month old commercial calves for a 4-day period, followed by PMSG injection 24 hr before slaughter, stimulates the differentiation of 20-50 middle size follicles; most of these show no atresia. In studying physiological maturation of oocyte it is imperative to preserve perfectly all granulosa, theca and corona cells.

Using the same rich media as for the rabbit, it appears that the gonadotrophic increase of follicular metabolism is rapidly followed by a drop in follicular fluid pH and then by lysis of granulosa cells, very similar to that occurring during natural atresia. This phenomenon has been prevented by impoverishing the medium, increasing its buffer capacity and raising the pH to 7.8. Culture was done under 57p. 100 O_2, 38p. 100 N_2 and 5p. 100 CO_2.

We use the following media:

Medium A: 70 parts TC 199 (Earle), enriched with 5 parts Na pyruvate at 15g/l, 10 parts potassium (bicarbonate) at 14g/l, and with the addition of 10 parts of calf serum and foetal calf serum in equal quantity.

Pyruvate and potassium are added in consideration of the composition of cow pre-ovulatory follicle fluid. The Na/K ratio is lower than in usual media where it resembles that of the serum. The pH of this medium is about 7.8 when it is put under a gas mixture containing either 20 or 57 p. 100 oxygen, 75 or 38 p. 100 nitrogen and 5 p. 100 carbonic dioxide; osmolarity is 285-290 milliosmoles.

TABLE 2 MEDIUM A

TC 199 (Earle)	70 parts
Na pyruvate 15g/l	5 parts
K bicarbonate 14g/l	10 parts
Foetal and calf serum (5/5)	10 parts
pH 7.8	
Osmol. 0.285	

Medium B. Aminoacid composition is identical to that of follicular fluid (Menezo and Testart, 1975). With the exception of glutamine and glycine, aminoacid concentration is lower than in medium A.

Medium A proteins are replaced by bovine serum albumen at 10g/l, containing no protein or mineral contaminant.

There is no vitamin in the medium except vitamin C, and there are no nucleotides or puric and pyrimidic bases.

Glucose, pyruvate, lactate concentrations and the Na/K ratio are the same as those in Medium A. Osmolarity and buffering potency are similar.

TABLE 2 (1) MEDIUM B (µg/100ml)

BSA	1000	Na acetate	5	Glutamine	15
NaCl	525	Na pyruvate	5	Glycine	60
$NaHCO_3$	250	Ca lactate	50	Tween	20
K Cl	80	Glucose	100	Cholesterol	12.5
		Vitamin C	5	a.a. (Cf Menezo and Testart (1975).	

Na_2HPO_4 , KH_2PO_4 , $MgSO_4$ (id. : TC 199)
pH 7.6
Osmol.0.288

When cultured for 42-48 hr without gonadotrophins, follicles show varying numbers of pyknotic cells scattered in the granulosa and cumulus.

As for the rabbit follicle, the presence of FSH and LH prevents pyknosis and induces physiological resumption of meiosis. Insulin seems to have a beneficial effect on both mitotic activity of follicular cells and the percentage of oocytes reaching metaphase 1 and 2 (85-95% vs 62%). However, the percentage of oocytes reaching metaphase 2 is, in any case, relatively low (between 32 and 45%) if we compare these values with those obtained after culture of free oocytes. (Table 3).

In the follicles studied, we found no relation between size and the moreorless complete resumption of meiosis; follicle size ranged between 1.2 and 4.9mm (70 p. 100 were between 2 and 3mm). However, oocytes of similar follicles practically all resumed meiosis (Table 4) when cultivated outside their follicle.

TABLE 3

CALF FOLLICLES CULTURED FOR 46 - 48 HOURS

Culture Media	Follicle Number	Granulosa cells		Cumulus cells		Nuclear stages			$M_1 + M_2$ %	M_2 %
		pyknos.	mitos	pyknos.	mitos	D.-RM	M_1	M_2		
A or B	10	0-3/2	+/0	0-3/2	+/0	6	3	1	40	10
A or B +I	25	0-2/1	+	0	+	21	3	0	14	0
AE $\frac{+}{-}$ I	10	0-2-3	+/0	0 - 1	+/0	6	4	0	40	0
A or B + GE	37	0 - 3	+/0	0 - 1	+/0	14	11	12	62	32
A or B + GI	15	0	+	0	+	1	9	5	95	33
A or B + GEI	47	0 - 1	+	0	+	7	19	21	85	45

Media A or B: see Thibault, Gerard and Menezo (1975)

G = O FSH and O LH : 1 µg/ml each; E = oestradiol-17β : 100 ng/ml; I = insulin : 1 µg/ml:

Gas : 57 p. 100 O2 + 38 p. 100 N_2 + 5 p. 100 CO2.

Stages of pyknosis : 0 = no pyknotic cells; 1 = some pyknotic cells scattered in the tissue; 2 = many pyknotic cells amongst healthy cells; 3 = all cells are pyknotic at least in an area of the follicle

TABLE 4 IN VITRO MATURATION OF RABBIT AND CALF OOCYTE

Species	Duration of culture (hrs)	Oocyte number	Nuclear stages M_1	M_2
Rabbit	9 - 15	145	0	145
Calf	24 - 30	79	5	74

This lack of response indicates that the immature follicle is impervious to gonadotrophins or that the granulosa regulates cumulus cell activity in the immature follicle. In fact, the characteristic reaction of cumulus cell dissociation under gonadotrophic effect, which is observed if they are cultured outside the follicle, hardly ever occurs inside the follicle. While the non-dissociation of cumulus cells may account for the fact that meiosis does not resume, it cannot explain why reductional division stops in metaphase 1. This arrest in metaphase 1 was reported for cultured mouse oocytes (Donahue, 1968); Haidri, Miller and Gwatkin (1971) and Gwatkin and Haidri (1974) showed that the proportion of mouse and hamster oocytes reaching metaphase 2 in vitro decreased when oxygen tension exceeded 5-10 p. 100. It may be that the proportion of 57 p. 100 oxygen in the gas mixture, which was necessary to insure ganulosa and cumulus cell survival, may be too high for complete nuclear maturation of the oocyte.

Recent experiments with a higher level of gonadotrophins in the culture medium (10 µg of FSH and LH vs 1 µg) seems to increase the percentage of oocytes reaching metaphase 2 after 42 - 48 hours of culture (6 out of 9 oocytes) (Table 5).

TABLE 5 TIMING OF NUCLEAR MATURATION OF INTRAFOLLICULAR CALF OOCYTE

Culture in hours	Follicle number	Granulosa		Cumulus		Nuclear stages		
		Pykno.	Mit.	Pykno.	Mit.	D.-RM	M_1	M_2
42 - 48	11	0 - 2	+	0 -0.5	+	2	3	6
24 - 36	5	0 - 1	+	0	+	0	4	1

Medium B = Gonadotrophins : 10 µg oFSH + 10 µg oLH + Insulin 1 µg/ml

Gas : 57 p. 100 O_2 + 38 p. 100 N_2 + 5 p. 100 CO_2

Similar conclusions have been drawn by Tsafriri et al. (1972) with rat pre-ovulatory follicles: 84 to 95 p. 100 of oocytes reach metaphase 2, when levels of LH in the medium are 5 or 10 µg/ml whereas only 39 p. 100 of the oocytes extrude the first polar body with 0.5 or 1 µg of LH/ml.

Four transfer trials of in vitro intrafollicularly matured calf oocytes into inseminated recipient cows have been unsuccessful: oocytes are not fertilised even when sperm sticks on the zona.

The main differences between rabbit and cattle are: 1. the physiological stage of the cultured follicle, i.e. pre-ovulatory follicles in the rabbit and middle size graafian follicles in the calf: 2. the steroid environment during follicle growth. In calves follicles differentiate under the influence of PMS in the presence of exogenous progesterone.

Better understanding of gonadotrophin penetration into the follicle and knowledge of the amount of hormone receptors depending on the size of the follicle and the presence or absence of progesterone, appear as a prerequisite to normal oocyte production in vitro.

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DISCUSSION

A.O. Trounson (UK)

Steen Willadsen and myself carried out a very large programme on cow oocytes which were obtained from ovaries of slaughterhouse cattle. We were aspirating the oocytes culturing them in vitro, and we got to metaphase 2 development in 75% as you describe. We did some transfers to rabbits inseminated with bull semen and could never get sperm penetration but we observed a lot of parthenogenic development to 8 cells. We also put some cultured oocytes into recipient cows. Some 40% of them were penetrated by sperm and I think 25 or 30% of them had male pronuclei. In fact, they would develop in the cow oviduct, or the cow tract, all the way through from morulae up to blastocyst but if they developed to blastocysts they never looked as good as the cow's own egg, but, in this case, the culture medium was just foetal calf serum and we did get pronuclei formation and as far as we could judge, normal nuclei in the dividing eggs.

C. Thibault (France)

Yes, yes, I agree and I indicated in the first part of my presentation that even in the rabbit we can obtain formation of male pronuclei but this formation in the rabbit is not immediate as in normally ovulated oocytes. In normally ovulated oocytes we have immediately - in 20 minutes or 30 minutes after fertilisation - swelling of the sperm head. In the rabbit, when we check for the first hours of the penetration we have no swelling but we have swelling later and many times this swelling is abnormal at the beginning. A part of the chromatin will swell, the other part does not, it remains as a black point. In fact, after 10 or 12 hours from fertilisation in the rabbit we often have two pronuclei and we have 50% cleavage. However, if we consider just the beginning of fertilisation it is the moment when we observe the difference between in vitro or in vivo matured oocytes. I agree with you that there is a possibility of a sperm nucleus formation, but not normally, not at the same time. Do you check at the beginning of fertilisation if there is a swelling or not?

A.O. Trounson

No, we recovered them 24 hours after transfer so we could not tell. However, they did develop to 40 or 50 cells with normal looking nuclei.

Professor Thibault showed three slides here to clarify what he was saying

S.M. Willadsen (UK)

We made some experiments as well with a slightly different system and maybe you would like to comment on that. We did some experiments in super-ovulation of prepuberal lambs and these experiments were fairly successful. Your work with progesterone served as the inspiration there. These lambs were put on progesterone and later treated with PMS and, in fact, we got them to ovulate and we had the eggs fertilised. We tried to repeat these experiments in calves with what we considered to be relatively speaking the same conditions, i.e. we pre-treated with progesterone and we treated with PMS, however, we never got these calves to ovulate. What I am getting at is, do you think the calf ovary will respond properly to progesterone and PMS treatments? In other words, do you think your follicles are normal? Would you have got the same results if you had been using mature cows?

C. Thibault

Testart has done some work on this question and he can answer himself but I can summarise. When he treated the calf by this technique, that is FGA by vagina sponge for four days and injected PMS, he regularly observed an LH surge - I don't remember how long ....

J. Testart (France)

20 hours.

C. Thibault

..... 20 hours after the withdrawal of the vagina sponge. These LH surges were apparently normal in size and duration and there was ovulation in most cases but ovulation was not so limited in time as in a normal ovulation and this is perhaps a partial response to the answer in the preceding discussion. The duration of ovulation is sometimes one day and they have an increase in oestrogen before the LH surge as in the natural oestrous cycle of the cow, but, the percentage of fertilisation of these oocytes is not as good as expected for many reasons, particularly the difficulty of introducing semen in the calf and obtaining capacitation of the semen or the passage of the cervix and so on.

Y. Menezo (France)

May I add something? When we have studied the follicle of the cow and the calf, there is a very important difference either in sodium and potassium, in free amino acids, in dehydrogenase and enzymatic patterns between normal follicular fluid and the stimulated calf.

S.M. Willadsen

There's no difference?

Y. Menezo

There is an important difference.

D.G. Whittingham (UK)

Well, thank you , Professor Thibault.

ATTEMPTS AT IN VITRO FERTILISATION IN CALF

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Twin birth pregnancy rate in cattle, even in the best conditions and taking into account age and breed, is never higher than 5%. The induction of superovulation is highly variable.

Egg transfer technique, as such, was begun as soon as it was shown that twin pregnancy could be sustained by a single CL. This technique confirmed, in its experimental stage, that it could provide a high rate of twin pregnancy.

Since Thibault et al. (in press), have demonstrated that calf oocytes can be matured in vitro in their entire follicles, and Tervit et al. (1972) and Renard et al. (1975) have succeeded in cattle ova culture, these methods can provide a great number of viable embryos after in vitro fertilisation.

The aim of our preliminary trials was to fertilise calf eggs in defined media using freshly ejaculated or frozen sperm, which are the only reasonable sources of bovine spermatozoa. We have tested different chemical or biochemical treatments described in the literature as being able to capacitate spermatozoa of different species.

MATERIAL AND METHODS

OOCYTES

495 oocytes were collected from 2-5 mm graafian follicles of the pretreated calf, according to Thibault et al. (in press). They were cultured for 30 hours in the presence of gonadotrophins in order to induce corona radiata cell reaction. In such an in vitro system, 95% of the oocytes resumed meiosis up to the second metaphase spindle.

SPERMATOZOA

For sperm manipulation and incubation we used an entirely chemical, defined medium which is more similar to female genital tract secretions than to serum (medium B). This medium allows great motility and survival of seminal plasma-free spermatozoa for at least 24 hours (Menezo, in press). The seminal plasma has to be removed, first, because of its deleterious effect on the oocyte (Masaki, Sugie and Otsuki, 1968), and secondly, to displace components such as enzyme inhibitors, decapacitation factor and seminal plasma antigens normally eliminated from the sperm head during passage through the female tract.

The entire ejaculate was first diluted with the medium (V/V) and centrifuged at 1500 rpm for 10 minutes. The supernatant was then discarded. The spermatozoa were resuspended with a volume equal to the one used for the first dilution and centrifuged again. A sperm suspension was then prepared (1.2×10^6 sp/ml).

For frozen sperm we thawed 5 pellets in 10 ml of physiological serum. After centrifugation, spermatozoa were resuspended in 2 ml of the medium and centrifuged again. The spermatozoa were then placed in 1 ml of the medium. Capacitating treatments used are all described in the literature on in vitro capacitation, (Zaneveld, 1975). They were performed at $37^{\circ}C$.

A) single incubation of sperm in the medium with gonadotrophins and oestradiol for different lengths of time ranging from 5 to 17 hours;

B) release of plasma antigens from the sperm head by means of:

1. reducing agent (S-S bond rupture); β mercaptoethanol, dithiothreitol, glutathion, Na ascorbate (2 mM) (60 mn).

2. proteolysis with chymotrypsin or pronase ($10^{-4} \frac{\text{Weight}}{\text{Volume}}$) for 10 mn

3. hypertonicity (up to 400 milliosmols with ClNa - urea - glycine) for 10 mn.

C) sperm membrane lysis

1. detergents: hyamine ($10^{-4} \frac{W}{V}$) for 10 seconds

2. phospholipase and lysolecithin (0.5×10^{-4} $\frac{W}{V}$) 5 mn.

D) addition of exogenous activators

1. EDTA (2 mM) for 30 mn, Ca ionophores (10^{-5} $\frac{W}{V}$) for 10 seconds

2. dibutyril cAMP (2mM), ATP (1 mM) for 2 hours.

At the end of short-time treatments, the sperm suspension was diluted and centrifuged; for incubations of half an hour or more, it was directly centrifuged.

MIXING THE GAMETES

Ten oocytes were placed in a 5 ml Petri dish with 3.5 ml of medium and then inseminated with 2 to 5×10^5 spermatozoa in 0.5 ml of the same medium. This was covered with paraffin oil and gassed with 5% CO_2 in air.

The oocytes were fixed 24 hours later and embedded in paraffin according to Thibault (1949), cut in serial 10 µ sections, stained with hematoxylin and examined under light microscope.

RESULTS (See Table 1)

In group A there was a constant, considerable percentage of parthenogenesis, but no spermatozoa were found in the vicinity of the zona. The addition of follicular fluid had no effect.

RELEASE OF SPERM ANTIGENS FROM SPERM HEAD (Group B)

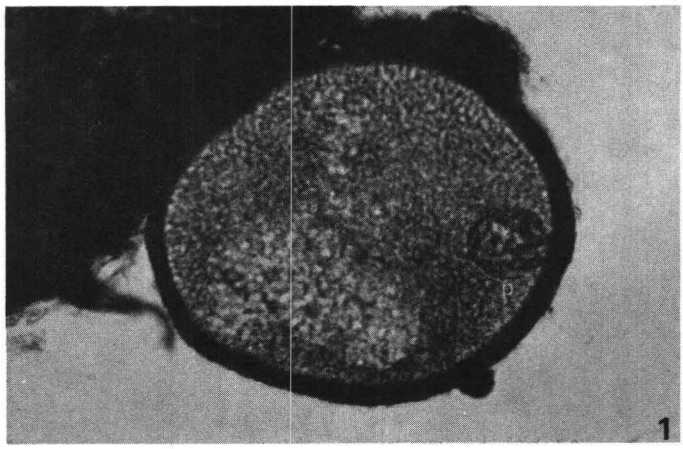
The spermatozoa treated with reducing agent generally adhere head to head and sometimes to the zona pellucida. mercaptoethanol and dithiothreitol generally enhance sperm motility.

Hypertonicity and proteolysis are generally unfavourable to sperm motility and survival. However, sperm treated this way adhere to the zona; the zona is really attacked only when spermatozoa are treated with pronase. The percentage of parthenogenesis was lower than in group A.

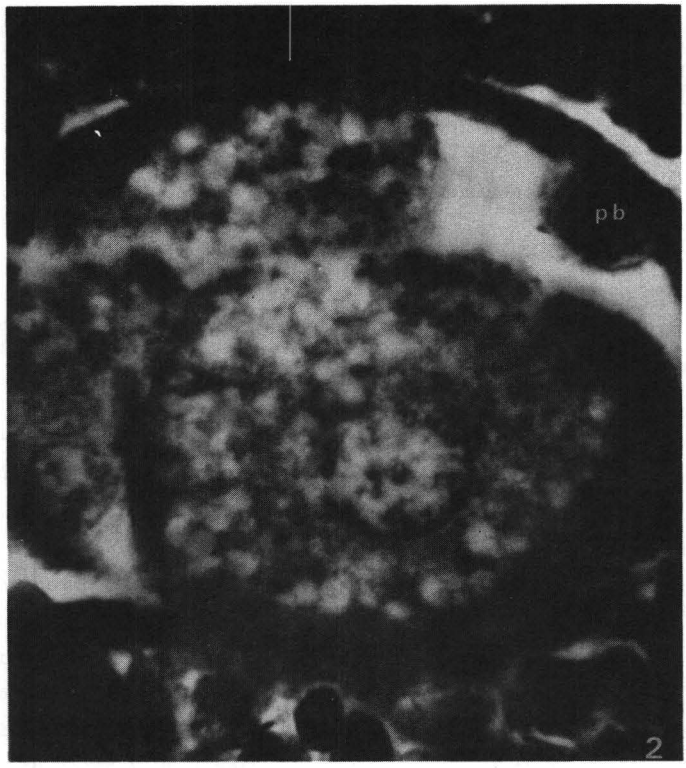
TABLE 1 EFFECT OF "CAPACITATING AGENTS" ON IN VITRO FERTILISATION

	Sp	No.of eggs	No. of partheno-geneses	Fertil-isation	Observations
A. Control group	O	12	O		
(FSH-LH-oestradiol)	E	64	25		
	C	17	2		
+ Follicular Fluid	E	15	2		
+ EDTA	E	6	3		
+ Ca++	E	8	1		
B. Seminal plasma compound release from sperm head					
- mercaptoethanol	E	26	1		
dithiothreitol	E	26	2		Sperm attach-
Na ascorbate	E	19	1		ment to zona.
glutathion	E	21	2		
- chymotrypsin	E	7	2		Sperm attachment
	C	18	1		and partial zona
pronase	E	18	4		lysis (E)
	C	7	O		
- hypertonicity	E	12	1		Sperm attach-
CINa	C	23	5		ment to zona
urea	E	25	O		
glycine	E	15	O		
C. Sperm membrane lysis					
hyamine	E	18	O		
lysolecithin	E	10	O		
phospholipase	E	14	O		
D. Exogenous activators					
- ionophores (Ca)	E	8	1		
	C	6	O		
- EDTA	E	16	3		
- dibutyril cAMP	E	16	9		
	C	4	O		
- ATP	E	7	O	1	Sperm attach-
	C	10	O		ment to zona
+ hypertonicity	E	11	O	1	and
	C	10	O		Eggs denuded
+ Ca^{++}	E	8	O		(E)

E : ejaculated sperm C : frozen sperm



Parthenogenesis (X 340) 24 hours after in vitro insemination p female pronucleus



Parthenogenesis (X 640) 48 hours after in vitro insemination. Note the two blastomeres and only one polar body (pb)

Membrane lysis (Group C)

The treatments have a deleterious effect on sperm survival. Spermatozoa were not found in the vicinity of the egg, and the percentage of parthenogenesis was nil.

Exogenous substrates (Group D)

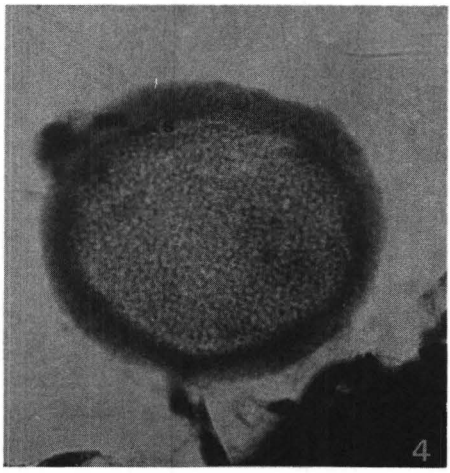
Good spermatozoa survival was not maintained with ionophores, and we did not try it on a large number of eggs.

ATP has a more stimulatory effect on sperm motility than cAMP. There was no parthenogenesis with ATP and eggs were generally denuded of corona cells. When freshly ejaculated sperm was used some spermatozoa adhered to the zona. Two eggs were fertilised without any evolution of the sperm head, since the oocytes were matured in vitro outside their follicles and MPGF was missing (Thibault, Gerard and Menezo, in press). Very few frozen sperm were found close to the zona, even with ATP treatment.

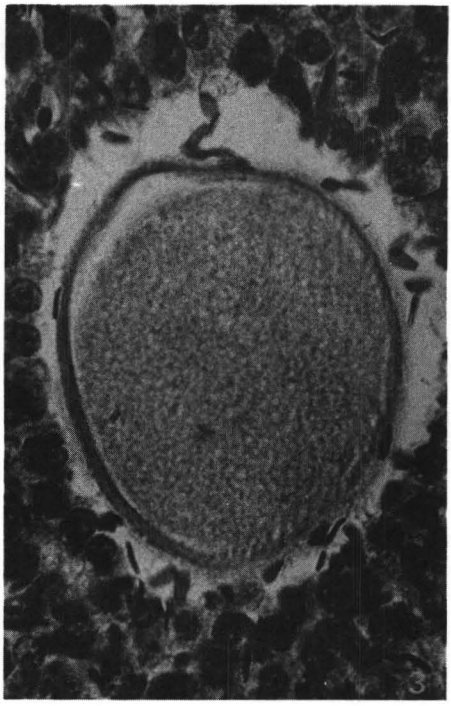
CONCLUSIONS

Parthenogenetic activation was never observed in group A oocytes cultured without spermatozoa. This phenomenon may be attributed to sperm hyaluronidase, (Kauffman, 1973), and throws some doubt on many conclusions in the literature on in vitro fertilisation experiments.

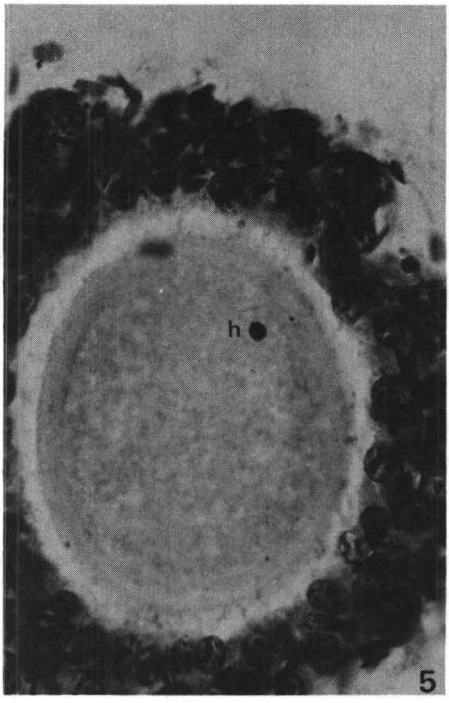
A long sperm incubation in the medium or in follicular fluid does not seem to have "capacitating efficiency", as observed in rodents and in non-human primates (Dukelow and Kuehl, 1975). However, for rodents, the problem seems to be simplified by use of epididymal sperm. Neither gonadotrophins nor steroids had any effect, even by way of the corona cells as observed in the human (Soupart, 1973). If we consider sperm head attachment to the zona to be a good indication of capacitation, as in in vivo fertilisation, proteolysis and hypertonicity seemed to be efficient for capacitating bull sperm in vitro. ATP was more effective, in this regard, than cAMP with fresh ATP-treated sperm. We obtained 2 cases of fertilisation in 18 of the oocytes used. Moreover, spermatozoa denuded other oocytes and some of them stuck to the zona. It seems that ATP can be a capacitating



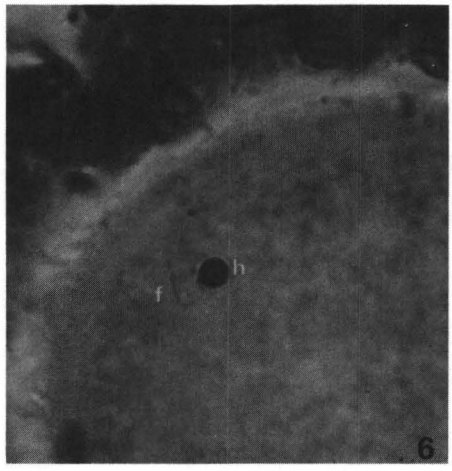
Spermatozoon (oa) under the zona
Sperm treated with chymotrypsine



Sperm adherence to the zona (X 340)
Sperm treated with pronase



In vitro fertilisation (X 340) 24 hours after insemination; sperm suspended in the medium with 1 mM ATP (h sperm head)



The same egg (X 1000) as in 5
h : sperm head
f : flagellum

agent, if only a "pro parte" one. Furthermore, sperm penetration in cattle seems to require a longer time than generally described for other mammalian species, since the two cases of fertilisation were observed 24 hours after sperm-egg mixing.

In conclusion, it seems that sequential treatment combining pronase and ATP can be sufficient to fully capacitate bull sperm and prevent undesirable parthenogenetic activation.

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DISCUSSION

D. Whittingham (UK)

In the cow egg, can you recognise cortical granules?

Y. Menezo (France)

Oh yes, but we have not checked because the success of fertilisation is not encouraging.

D. Whittingham

When you get the sperm penetration and the head doesn't swell, does that cause normal cortical granule release?

Y. Menezo

Oh yes, always, because in these experiments in calves or in rabbits, after in vitro maturation there is no polyspermy. That means the cortical granules release normally.

B. Hoffmann (W. Germany)

Did you ever use spermatozoa after physiological capacitation?

Y. Menezo

No, we have not tried; it is difficult to get bovine sperm capacitated.

B. Shea (Canada)

We have recovered embryos about 30 hours after the animal has first shown heat and we also have observed that pronuclei will form. We are not convinced that it is normal pronuclei formation either. We did a series of experiments to find out whether the eggs were fertilisable. At 30 hours some of the eggs were recovered and put with semen in vitro and, at the same time, the animal was inseminated. Fertilisation was normal when insemination occurred so the eggs themselves, when placed with semen in vivo at the same stage, are fertilisable but, of course, fertilisation didn't take place in vitro. We allowed the eggs to remain with the sperm for 2 to 3 hours and 5 to 6 hours after insemination, then recovered them and attempted to culture them. We got what appeared to be normal fertilisation after a minimum of 4 hours. The sperm had to be with the ova at least 4 hours in vivo before there was any type of cleavage at all. It appeared normal but we didn't check at intervals to determine whether sperm head swelling was normal. After 24 hours culture we did get some normal appearing 3-cell embryos.

SESSION THREE

EGG TRANSFER

Oestrus Synchronisation

Chairman: C. Polge

EXPERIENCES WITH NON-SURGICAL TRANSFER TECHNIQUES

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** Besamungsverein Neustadt a. d. Aisch e.V.

Ever since the first successful surgical ova transfer in a cow was performed 25 years ago (Willett et al., 1951) several attempts have been made to develop a non-surgical technique especially by Rowson and co-workers in Cambridge in the beginning and middle years of the sixties. They reported several reasons for the failure or poor results of non-surgical ova transfer. Interested investigators will know of these papers (Harper et al., 1961; Bennet and Rowson, 1961; Rowson et al., 1964 and Rowson and Moor, 1966), therefore no further discussion will be included here.

All of the reports showed that the effect of introducing a transplant instrument through the cervix of a standing cow produces uterine contractions and the eventual ejection of the transplanted oocyte. Various attempts to relax the uterus with medications or CO_2 had a poor or no effect on the transfer result. It should be mentioned that, to my knowledge, all transfer experiments were performed on recipients 3 - 4 days after ovulation. Investigators were discouraged by the poor results of non-surgical ova transfer, so that by the end of the sixties and the beginning of the seventies little work was done. This is understandable since the transfer-boom of the United States and Canada led to further developments of surgical techniques and successful pregnancies resulted from these methods.

When we started experiments of ova collection and transfer in cattle in early 1974 at a farm near the A.I. centre, Neustadt/Aisch, it became clear that in the German situation the practical application for breeding purposes of ova transfer is only useful when collection and transfer can be performed non-surgically.

Professor Kräusslich will point out at this symposium that only those cows with proven milk production can be used as donors in a breeding programme. When high production animals are used as donors for surgical ova collection certain problems arise. Among these, the procurement of these

cows is difficult because of the owners' dislike of extraneous surgery. Also, the donors' large udder and blood vessels and the surgeon's displacement of the uterus from the abdomen may cause complications.

At this time in the BRD attempts are being made to solve the problem of non-surgical ova collection in producing cows. The team of investigators from the Tierärztliche Hochschule Hannover and from the Besamungsverein Neustadt/Aisch has as its primary goal, the development of a non-surgical transfer technique to overcome the problems which arise when keeping a large herd of recipients.

After successful surgical ova transfers were performed in 1974, the first non-surgical transfer was attempted in Spring, 1975. At the time that the problems of non-surgical operations were being considered, the team received a medication from a pharmaceutical company which was capable of relaxing the uterine tonus for several hours.

Non-surgical transfer operations on the first two standing recipients led to their pregnancies when this medication was used. Encouraged by such immediate success, more than 40 non-surgical ova transfers were performed on different farms affiliated with A.I. centre Neustadt/Aisch, this past summer. The 3 month pregnancy diagnosis was done and the results of the operations are now available.

In connection with these experiments it must be said that once the superovulation treatment at the donor was started synchronous recipients were sought on different farms. The A.I. technicians who routinely worked with the herds were in the best position to find suitable recipients and to request the owners' permission.

After ova collection the transplant team travelled to the farm of the selected recipient to perform the non-surgical transfer.

The transportation of the eggs was done in siliconised tubes filled either with 0.25ml TCM 199 with Hepes Buffer + 10% FCS or 0.25ml Hams F 10 + 10% FCS and covered with rubber stoppers. Afterwards the tubes were gassed with 5% O_2, 5% CO_2 in nitrogen. During transportation the tubes were stored in a thermos with warm water to maintain 35^{o}C.

Upon arrival at the farm an epidural anaesthesia was administered to the recipient. In the beginning every recipient received the uterine relaxant, however, in later experiments this was reduced to every second animal. Afterwards a rectal examination was performed to determine the location and quality of the corpus luteum. The next step was meticulous cleansing of the external genital area. Before inserting the metallic transplant instrument precautions were taken to prevent cervical mucus entering the instrument by filling the tube with a smaller rod.

The introduction of the instrument into the proper uterine horn was performed under rectal control. Once the correct position was established the internal rod was removed and a polyethylene tube was inserted into the uterine horn through the remaining tube. Precaution must be taken during this manipulation to prevent violation of the mucosa. In order to manipulate the ova a glass pipette was connected to a 1ml syringe. Air was aspirated prior to picking up the egg from the tube, and the entire contents were injected through the polyethylene tubing. 0.25ml medium was a sufficient vehicle to transport the oocyte. The entire procedure lasted 15 min. The results of these experiments can be found in the following table.

TABLE 1 RESULTS OF NON-SURGICAL OVA TRANSFER (Summer 1975)

Age of postovulatory ova at time of transfer in days (developmental stage)	No. of recipients (No. of pregnant animals)		Abortions after 6 weeks	
	HEIFERS	COWS	OBSERVED	POSSIBLE
4 (16 cell)	5 (0)	9 (1)	1	1
5 (morula)	8 (2)	16 (5)	2	4
6 (early blast.)	2 (2)	2 (0)	1	0

Only four, five or six day postovulatory ova were used for transfer. The corresponding developmental stages are 16 cell, morulae and early blastocysts. Heifers and cows of different ages acted as recipients. One can see from the table that of the 14 transplanted ova in the 16 cell stage, only one was successful but from 28 in the morula and blastocyst stage, 9 = 31% pregnancies were achieved. There was a remarkable rate of abortions. Four abortions were observed 6 weeks after transfer, and possibly another 5 abortions occurred as determined by the irregular cycle of the recipients

observed 2 months later.

The experimental treatment of every other cow with the uterine relaxant showed that when 5 or 6 day ova were used for the transfer no difference on the results was observed.

Fifty percent of the non-successful animals came into heat between 14-18 days after the transfer, and another 15% after 35-40 days. Thus, non-successful transfer seems to have no effect on the following oestrous cycle of two-thirds of the animals. These had been inseminated artificially with an average of two inseminations per individual. No genital diseases were observed. Looking at the results it is obvious that they are in accordance with the published results of Lawson et al. (1975). They reported that with non-surgical transfer of ova unto 20 recipients who were between day 3 to 5 of their oestrous cycle 2 pregnancies resulted and those ova transferred on day 6 - 9 of their oestrous cycle in 40 recipients gave 15 pregnancies. In the latter group no difference could be seen between an untreated group and a group using Fluothane anaesthesia. Sreenan (1975) also confirmed these results, when he reported a pregnancy rate of 50% with the use of a simple insemination instrument for non-surgical transfer with ova 5 days after ovulation. Lawson et al. (1975) and Sreenan (1975) did not observe any genital infections after non-surgical transfer. Unfortunately they did not report the abortion rate.

Considering these results the indication is given that with the beginning of dioestrus (5 - 6 days after ovulation) the increasing progesterone levels cause a relaxation of uterine musculature which makes non-surgical transfer possible. It is not clear if ova in later stages of development (morulae and blastocysts) show more resistance to the negative influences of the uterine environment than earlier stages.

Because of these preliminary results a new procedure is being introduced. Prior to every transfer 2 progesterone determinations are made in the recipient, one at the time of oestrus and the second 5 days later. The progesterone assay is taken from a milk sample which is immediately sent to an appropriate institute. They, in turn, relay the results by telephone 24 hours later. Only those animals with an observed progesterone increase are used for transplantation. It is hoped that with this new

selection method for recipients better results will be achieved. Another outcome of this procedure may be the better understanding of the relationships between progesterone levels and transfer results.

SUMMARY

Non-surgical ova transfers through the cervix were performed on 42 recipients (heifers and cows) under field conditions. Fertilised ova 4, 5 and 6 days after ovulation were used for transfer and thereafter recipients were observed carefully by the owners. In animals showing no heat signs 3 months after transplantation pregnancy was determined by rectal examination. Only one pregnancy was obtained from 14 recipients using 16 cell stage ova, in comparison to 9 pregnancies out of 28 recipients when transfer was performed with morulae and early blastocysts. The results suggest that non-surgical transfer in early dioestrus of recipients (5 - 6 days after ovulation) could be done successfully without externally applied medication to relax the uterus.

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DISCUSSION

B. Shea (Canada)

At what stage are your pregnancy checks done and how are they done? By palpation?

J. Hahn (W. Germany)

Yes, by rectal palpation three months after transfer.

B. Shea

You mentioned your abortion rate as being after six weeks.

J. Hahn

Yes, I differentiated between observed and possible abortions but the observed abortions occurred six weeks after transfer.

A. Brand (Netherlands)

I am wondering what might be the cause of the abortions that occurred. It is possible that sub-clinical infections play a major role and I am a little bit afraid of your methods - I have the same problems with my instruments, that is, if you withdraw the rod I think you may contaminate the whole inner surface of the instrument and then you go in with the flexible catheter - it is possible that the catheter is already contaminated before you have deposited the egg in the uterine horn.

J. Hahn

I heard this yesterday and I agree with you but we have no evidence that there is an infection; there might be some kind of sub-clinical infection but we don't know. On the other hand, the inseminations done in the non-successful recipients - they are quite normal. As I said, we had a rate of two inseminations per recipient and they conceived normally I would say. So there is no real indication of infection by introducing this instrument.

J. Sreenan (Ireland)

I would like to make a comment in respect of what has just been said. We have been doing non-surgical transfers to the side opposite to the corpus luteum - contralateral. This is with the bred animal. However, in

one of the groups that we have recently slaughtered, a reasonably large group of over 40 animals, we have a pregnancy rate of 58% and this is slaughtering between days 27 or 30, up to 50 or 60. We have an overall pregnancy rate of 58% In fact, what it has meant is that 58% of the times the animal ovulated has resulted in a pregnancy. For this reason, first of all, we would have assumed that we weren't introducing any great level of infection. However, this may not be quite so - from talking to people here there may be a localised infection, something sub-clinical. The level of normal embryo development on the contralateral side in this particular group has turned out to be 21%. In other words, at various slaughter stages, 21% of our embryos transferred survived, but we also had a level of exactly 20% of resorptions on the transfer side which is something that we have only encountered in much smaller percentages in the surgical transfers. We are doing surgical transfers at the same time from some of these donors as well and we have been slaughtering these animals and we are getting pregnancy rates in the region of 60%. It seems to tie in somehow with your abortion rate because your abortion rate has come at about the same time as we have been slaughtering some of these animals. Now, if we had slaughtered all of these animals at 27 days, the possibility is that we are showing about 41 or 42% pregnancy rate to the transferred embryo. When we go up from about 27 days to 50 days we have 21% normal and the rest of them are resorptions. So I am just wondering whether some of that is because of the placement of the egg further down the uterine horn and it does take for a while but maybe it is not in the best environment, or whether it is because in the transfer we do introduce, possibly, some uterine infection which maybe comes into play later on - I am not sure.

N.O. Rasbech (Denmark)

I am very interested in the question of infection and I would like to mention that we take uterine biopsies of about 400 heifers per year, that is the test heifer method for vibriosis. Now, what we do is that we inseminate each heifer with the semen that we wish to test and then we start to take uterine biopsies 5 days, 9 days and 15 days later. We push the instruments through the cervical canal and take the biopsy in the corpus, in the body. We practically never see infections in all these heifers. We have done this for many years now, involving thousands of heifers. Another interesting thing is that most of these heifers became pregnant although this test is really only done as the official vibrio test. However,

it is very difficult to set up a uterine infection by pushing instruments through.

J. Hahn

I think the problem is not solved yet and we have to make further experiments and to look for infections of sub-clinical signs in these recipients. One of the reasons for the abortions may be if you do not pay enough attention when you are introducing the instruments into the uterine horn that may cause a bleeding of the mucosa of the endometrium and so you have to be very careful. This also applies when you are introducing the polyethylene tubing into the uterine horn. You have to withdraw the instrument when you have the feeling that you are at the surface of the endometrium. Maybe we can increase results by improving our technique as well. So far there is no convincing evidence that there is an infection after non-surgical transfer.

C. Polge (UK)

Well, I think we have to wind up the discussion at that point.

CYCLE SYNCHRONISATION UNDER HORMONAL CONTROL

B. Hoffmann, D. Schams and H. Karg
Technische Universität, Weihenstephan, W. Germany

A key-position in regulation of the oestrous cycle is held by the corpus luteum. Success in bioengineering of reproduction in the female mammal therefore depends on the possibilities to manipulate this gland. For cycle synchronisation in cattle both stimulation and inhibition of corpus luteum function have been used with various success. Similarly, the functional state of the corpus luteum in individual animals is decisive for the success of superovulation (egg-recovery) and of egg-transplantation. The status of the corpus luteum in the recipient animal has to be adjusted to the developmental stage of the egg transferred which is, concerning the 16-cell, morula and early blastocyst stages, the 4th, 5th and 6th days after ovulation respectively. According to Hahn (1975) a tolerance of maximal $\pm$ 0.5 days in synchrony is allowed. The course of the occurrence of progesterone in peripheral plasma 10 days before and after ovulation is shown in Figure 1.

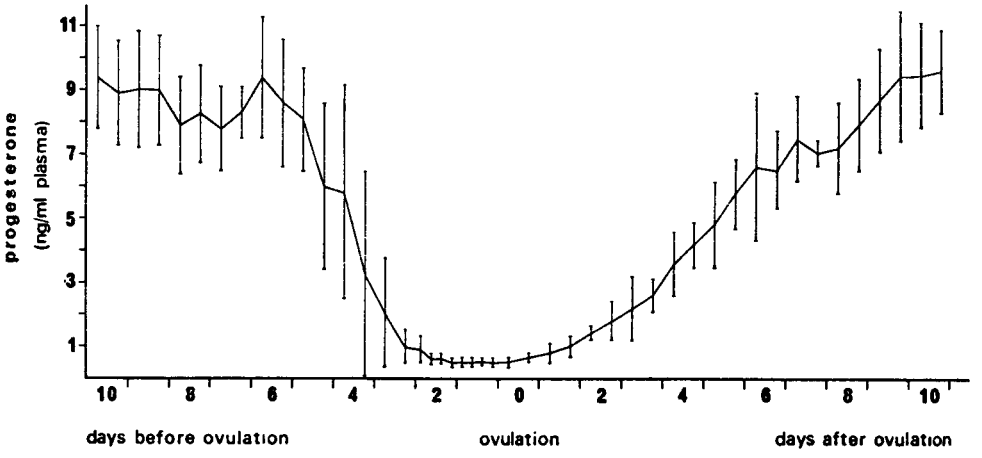

Figure 1: Progesterone in bovine peripheral plasma 10 days before and after ovulation (4 heifers; mean values $\pm$ s)

The effectiveness of prostaglandin $F_{2\alpha}$ and of the analogue ICI 80 996 in respect to induction of luteolysis and oestrous-synchronisation in the cow has been demonstrated (Cooper et al. 1974).

In the following paper some results obtained from our group (Hoffmann et al. 1974, Schmidt et al. 1975, Karg et al. 1976) after application of the analogue ICI 80 996 (0.5mg i.m. per animal) will be discussed, especially to the effect of the occurrence of synchrony and fertility.

SMALL SCALE EXPERIMENTS

In a first series of experiments animals accessible on our experimental farm which showed regular oestrous cycles were treated on the 10th or 11th day of cycle with the analogue and changes in progesterone and LH-plasma concentrations, the electrical resistance of the vaginal mucus and the occurrence of ovulation were registered. As can be seen from the results summarised in Table 1, in all 6 animals, oestrus, with the characteristic decrease in the electrical resistance of the vaginal mucus, preovulatory LH-peaks (except 1 animal) and ovulations, were observed.

While luteolysis (progesterone $<$ 1ng/ml plasma) was completed within 18.0 hours showing a very low variation between animals with S = $\pm$ 3.3 hours, considerable variations were observed as to the occurrence of the preovulatory LH-peak and ovulation. With standard deviations of S = $\pm$18.3 hours (LH-peak) and $\pm$ 17.6 hours (ovulation) synchrony within half a day was not achieved.

In a similar experiment 10 animals were artificially inseminated and simultaneously injected (i.m.) with 1500 µg GnRH (LRH-Hoe 471) 75 hours after treatment with ICI 80 996. In all animals 3 hours later (78 hours after treatment) an induced LH-peak could be determined. In 6 out of the 10 animals an endogenous preovulatory LH-peak occurring earlier was observed. Ovulations occurred 82 hours after application of the analogue and the variation with S = $\pm$ 5.4 hours indicates a synchrony well within 0.5 days. All animals conceived except the one where ovulation had occurred before insemination. (Table 2).

LARGE SCALE EXPERIMENTS

Altogether in 11 experiments each synchronisation was attempted in

TABLE 1 OBSERVED ALTERATIONS AND TIME-RELATIONSHIPS AFTER TREATMENT WITH THE PROSTAGLANDIN-ANALOGUE ICI 80 996 (0.5mg i.m.)

Animal	Treatment		Time (hours) from treatment until			Electrical resistance of vaginal mucus	
	Day of cycle	Prog. (ng/ml)	Luteolysis (finished)	LH.peak (preovulatory)	Ovulation	at treatment	at oestrus
Rita	11	4.40	21	100	120	50	28
Zilla	11	4.50	21	-x	120	48	33
Uta	11	4.30	21	96	120	56	32
Vroni	11	3.70	15	57	85	46	32
Xanta	10	4.60	15	69	84	45	34
Gilla	11	5.70	15	87	99	52	37
n		6	6	5	6	6	6
$\bar{x}$		4.5	18.0	81.8	104.6	49.4	33.6
$\pm$ s		0.6	3.3	18.3	17.6	4.6	2.1

[x] LH.peak not observed

TABLE 2 OBSERVED EFFECTS ON LUTEOLYSIS, OVULATION AND FERTILITY AFTER TREATMENT WITH THE PROSTAGLANDIN ANALOGUE ICI 80 996 (0.5 mg i.m.) AND SIMULTANEOUS INJECTION OF GnRH (1500 µg i.m.) AT THE TIME OF INSEMINATION

Animal	treatment day of cycle	treatment prog. (ng/ml)	luteolysis (finished)	time (hours) from treatment until LH-peak (preovulat.)	AI GnRH	LH-peak (induced)	ovulation	hours from AI to ovulation	conception
Uta	12	5.25	12	-x	75	78	87	12	+
Sepia	12	3.24	12	54	75	78	69	(-6)xx	-
Wanda	10	3.59	18	66	75	78	84	9	+
Gilla	10	6.90	24	72	75	78	84	9	+
Zilla	12	2.60	18	-x	75	78	85	10	+
Xanta	13	2.60	24	72	75	78	84	9	+
Peppa	13	3.43	18	-x	75	78	88	13	+
Rosa	12	3.08	18	-x	75	78	82	7	+
Tina	12	6.70	18	69	75	78	84	9	+
Ypsilon	8	2.50	18	51	75	78	78	3	+
n		10	10	6			10		
$\bar{x}$		4.0	17.0	64.0			82.0		
$\pm s$		1.8	5.0	9.2			5.4		

[x] LH-peak not observed

[xx] ovulation before AI

herds of 18 to 30 animals (heifers and cows) under field conditions by giving 2 injections of the PG analogue 10 or 11 days apart (Cooper 1974, Cooper et al. 1974, Stelflug et al. 1973, Sequin et al. 1974). Insemination was performed independently of oestrus observations 63 or 75 hours or two times 75 and 99 hours after the second treatment. In some experiments GnRH (1000/1500 µg) was given at the time of insemination.

Progesterone was determined in the milk of cows and in the plasma of heifers at the 2 treatment days and at the day of insemination. In cows, milk samples for hormone analysis were then collected for another 20 days, in heifers, a single plasma sample was taken on day 20 after insemination (= Day 0). By rectal palpation ovarian control on the day of insemination and pregnancy diagnosis 6 - 8 weeks later were performed.

Results of the progesterone analysis allowed classification of the animals' responses into 3 groups:

a) synchronisation negative (progesterone $>$ 2ng/ml milk or $>$ 1ng/ml plasma)

b) synchronisation positive, animal pregnant

c) synchronisation positive, animal not pregnant.

As far as progesterone was analysed in milk, group c could be further subdivided into:

c_1) synchronisation positive, animal not pregnant but cycle after synchronisation normal

c_2 synchronisation positive, animal not pregnant and cycle after synchronisation abnormal

An example for each category is shown in Figure 2. From altogether 257 animals 10% fell into category a). In these animals, possibly due to prolonged luteolysis, synchrony at the time of insemination was not obtained making conception impossible.

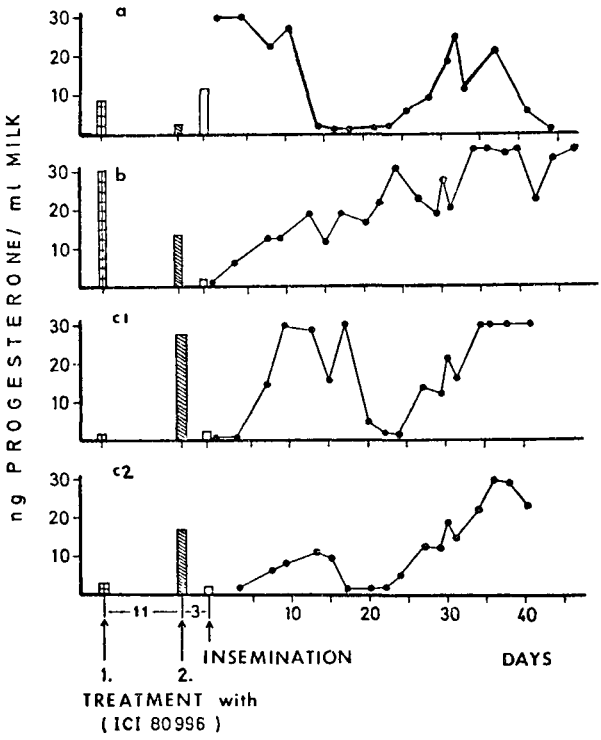

Figure 2: Examples for the 3 categories of progesterone profiles observed after oestrus synchronisation.

However, based on the number of experiments performed, total conception rate (51.7%) in "treated animals" was statistically not different from the conception rate in control animals (60.3%). With 57.2% conception rate in synchronised animals it was practically identical with the one in the control groups.

Ovarian control at the time of insemination revealed that the conception rate was higher (65.0%) in those animals having large follicles close to rupture than in animals having smaller follicles (conception rate 50.8%). Only 15.8% of the animals conceived where follicular rupture had occurred before insemination. The three groups yielded the following ratio: 10.2/4.6/1.

Though the two variables in the experimental design, i.e. 1 or 2

inseminations and treatment with GnRH at the time of insemination, did not affect the general result in respect of conception rate, it is interesting to note, that the highest conception rate within a group of animals was obtained, where two inseminations (75 and 99 hours) after treatment were performed. Similarly, in paired experiments conception was increased by more than 10% when GnRH was given with the insemination 75 hours after the second treatment with ICI 80 996. Based on the results obtained by rectal palpation these data would indicate that the observed increase in conception is a result of better control of the population of smaller follicles. However, animal numbers are still too low to make final statements.

CONCLUSIONS

The results obtained have confirmed previous observations, that the prostaglandin analogue ICI 80 996 is an adequate compound for manipulation of corpus luteum function in the bovine species. It has been demonstrated that fertility after oestrus synchronisation is not impaired, especially when only those animals are concerned which exhibit "synchrony" at the time of insemination. If it is considered that after superovulations and multiple inseminations only a maximum of 65 - 70% of the eggs recovered are fertilised (Hahn 1975), a conception rate of more than 70% - as obtained in some experiments performed - must be classified as "optimal".

The basic problem in respect of egg transplantation is the status of synchrony between the developmental stage of the egg and the uterine milieu, which strongly depends on the functional status of the corpus luteum. In this respect the experiments discussed have shown that synchrony within the required $\pm$ 0.5 days (Hahn 1975) and within a group of animals was not obtained when the animals were treated with the prostaglandin analogue alone. Additional treatment with GnRH at the time of insemination yielded this effect (small scale experiments), probably due to stimulation of growth and ovulation of follicles showing a delayed development (large scale experiment). It should be possible to further optimise the treatment schedule. However, the results obtained so far already clearly indicate that the use of the prostaglandin analogue ICI 80 996 - alone or in combination with other drugs - may be a useful tool for oestrus synchronisation in both herd management and egg transplantation. A most proper and effective method of monitoring the regularity of the bovine oestrus cycle, the effect of luteolytic agents and of the status of recipient cows in egg transplantation is

the evaluation of the progesterone profile. The determination of progesterone by radioimmunoassay in plasma or milk samples (Heap et al. 1973, Hoffmann and Hamburger 1973) for that purpose has been established.

SUMMARY

After induction of luteolysis and oestrus synchronisation with the prostaglandin analogue ICI 80 996 (2 injections 10 to 11 days apart) the induced changes leading to ovulation resemble the physiological situation and fertility is not impaired ($p > 0.2$: conception rate: 51.7% of all animals, 57.2% properly synchronised animals, 60.3% control animals). Hormonal and clinical examinations further revealed that about 10% of the animals are not synchronised at the time of insemination (63 or 75 hours after the last treatment) and that conception is higher (65%) in those animals showing large follicles at the time of insemination compared to animals showing smaller follicles (50.8%). Application of GnRH (1000 - 1500 µg i.m.) with the insemination gave indications of a better synchrony and higher yield in conception.

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DISCUSSION

C. Thibault (France)

I would like to comment on Table 1 and Table 2 in your paper. In Table 1 you estimate the LH peak approximately 80 hours after the prostaglandin treatment and ovulation 104 hours, that is normal time between the LH peak and ovulation - approximately 24 hours. However, in Table 2 you observe that after the same treatment you have the natural LH peak 64 hours after the prostaglandin treatment and when you inject GnRH the LH peak is approximately 78 hours later, and ovulation occurs 4 hours later. I cannot understand why you can estimate that GnRH can act on ovulation since ovulation occurs 4 hours later.

B. Hoffmann, (W. Germany)

Well, it is an observation we have made in these experiments and prior to the physiological, preovulatory LH peak, you may see that we have not observed this peak in our animals .....

C. Thibault

It's O.K. The relation between the natural peak and the estimated time of ovulation is correct - it is approximately one day in the cow, but the LH peak after the GnRH treatment is 78 hours after, then ovulation four hours later.

B. Hoffmann

Well, it depends, it varies between animals, in some animals it was even before the insemination and in some animals it was six, seven, ten hours - four hours in one and then six again. Ovulation was checked by rectal palpation .....

C. Thibault

No, my precise question is whether you think the LH induced peak has an effect on ovulation?

B. Hoffmann

We would speculate this, that the follicle is already there and that the additional release of LH due to the treatment with GnRH may enhance ovulation.

C. Polge (UK)

I think in all these situations, when you are trying to control ovulation time, it is, of course, important that either your exogenous LH or GnRH must be given before an endogenous release of LH otherwise it won't be controlled precisely by your treatment, and, of course, there will be individual animal variations.

J.F. Roche (Ireland)

We have timed ovulation following the double injection of prostaglandin, the ICI analogue, by slaughtering animals at various intervals and in some of these animals we have given GnRH, 100 micrograms, 48 hours after the second injection. We then get all of the animals ovulating 78 hours after the second injection. In the control animals that have not received GnRH, about 30% have ovulated at 78 hours. So giving GnRH 48 hours after the second injection of prostaglandin is an extremely good way of controlling the time of ovulation. However, in trials where we have compared the double insemination regime as recommended by ICI, that is, AI at 72 and 96 hours after the second injection of prostaglandin, with the single insemination at 72 hours, where we have controlled ovulation with GnRH we get no benefit on fertility by synchronising the time of ovulation. Now, that is with GnRH at 48 hours and AI at 72 hours. Perhaps other times would give an effect. It did control ovulation but it had no effect on fertility.

T.R.R. Mann (UK)

Can you tell us why double insemination should be so much more effective? Is it just additional spermatozoa or is there something else?

B. Hoffmann

This is just one experiment and I don't want to overstress this observation. However, follicular evaluation of the ovaries reveals that we have to deal with two populations of follicles and that an animal which is not treated with GnRH shows quite a variation in the time of ovulation. If we accept the importance of the time relation between insemination and

ovulation, a second insemination which is closer to the follicular ruptures may be very effective.

M.J. Cooper (UK)

We have got quite a lot of experience of comparing these two inseminations with one and there is little doubt in my mind that the distribution of ovulation time is what makes the two inseminations a better bet. Without using GnRH you have got two choices, as Dr. Roche has said, you either give them another insemination or you give them an injection of something to control the timing of ovulation. It does seem to me that the use of GnRH leaves you with two rather difficult alternatives. You have either got to try to anticipate the endogenous LH release or you have got to allow that to occur and then use your GnRH to pick up the stragglers, as it were, which are ovulating at a later time. In the field, as Dr. Roche has said, this does not seem to be particularly beneficial. I would like to ask Dr. Hoffmann one question. On your last slide you showed a lot of treated animals and follicular sizes which had been assessed. Were these done at the time of AI by rectal palpation and did you examine your control animals by rectal palpation at the time of AI?

B. Hoffmann

The answer to the first question is 'yes' and the second one, 'no'. The control animals were inseminated according to oestrus observation of the animal keepers and they didn't call us.

C.D. Nancarrow (Australia)

Dr. Hoffmann, when you give GnRH to an animal that has not yet appeared in heat, you of course, produce an endogenous LH peak and ovulate the follicle. This has the effect of limiting the amount of oestrogen produced by the follicle and, in many cases, the animal will not appear in oestrus because of this effect. Do you think this would affect the endocrine environment of the animals and make them unsuitable for use as the recipient cattle in egg transfer?

B. Hoffmann

We have heard so much about endocrinogical environment involved with egg transplantation and we have seen that at least from the donor animals there is such an enormous output in oestrogens which does not affect the

fertility of the transferred eggs, that I cannot imagine that a little bit less endogenous oestrogens in the recipient animal will effectively inhibit fertility. I think it is really a matter of time - when you give it, how long the follicle may last and have a chance to produce oestrogens.

C. Polge

I would add another comment there. If fertility and pregnancy rate is the same in animals inseminated, whether they come on heat or not, I would assume that the fact of whether or not they show oestrus is not important in relation to pregnancy and embryo transfers.

C.D. Nancarrow

I'm not certain that we have the data yet to show that.

A.O. Trounson (UK)

I would just like to add to that. In the sheep we have been using prostaglandin after PMS and we have been giving GnRH 24 hours after prostaglandin. The animals very rarely come into oestrus. We have been inseminating the ewes and doing various other manipulations; the embryos have become fertilised and they develop normally to blastocysts. We haven't transferred any from the blastocyst stage but at least in that initial period they developed quite normally.

C. Polge

Well, I think we must stop there.

PREGNANCY RATE IN CATTLE IN RELATION TO OESTRUS SYNCHRONISATION AND CELL STAGES

H. Breth Hansen
East Jutland Animal Hospital, Laurbjerg, Denmark

The practical aspects of ova transfer are well known and surgical recoveries of fertilised bovine eggs are now performed all over the world. Since summer 1974, transplantation of bovine ova has been carried out at East Jutland Animal Hospital and we have in several ways tried to increase the number of calves per donor operation. In these efforts the synchronisation time, i.e. the difference between the donor and recipient oestrus, has been minimised. However, very little is known concerning the relation between oestrus synchronisation, cell stages, and the pregnancy rate. The objective of this brief study was to investigate these relations. The presented data have been achieved by surgical recoveries and surgical transfers.

MATERIAL

The donor group consisted of gynaecologically normal heifers and cows of the Belgian Blue-White breed. Prior to use they experienced two or three normal oestrous cycles.

Jersey heifers comprised the recipient group. There were always at least 250 animals available. The recipients had all had one or two regular oestrous cycles.

METHODS

The donors received an average of 2700 i.u. PMSG on day 10 to 13 of their oestrous cycle and 48 hrs later they were treated with 25 mg prostaglandin $F_{2\alpha}$ (Upjohn). Heat occurred within 48 hours after this last injection. Two or three inseminations were made with Cassou medium straws containing approximately 120 mill. live sperms per straw. Surgical recoveries were carried out 5 - 5½ days after the onset of standing heat. General anaesthesia and ventral midline approach was employed. The uterus was flushed with 3 x 20ml Brinster's medium, and eggs were identified under

a stereomicroscope. The cell stages were carefully defined as 8, 8-16, 16-32, 32-64 cells. Rarely did blastocysts occur.

The donors and recipients were checked for heat every 6 hours. The recipients experienced spontaneous heat. When they were in heat before the donor the synchronisation time was defined positive (ST+).

Conversely, when the recipients were in heat after the donor the synchronisation time was negative (ST-).

All recipients were chosen at random within +12 and -24 hrs synchronisation.

Two hundred and thirty nine transfers were done via flank approach under paravertebral analgesia. The eggs were inserted into the uterus 2cm from the utero-tubal junction. The recipients were examined for pregnancy 5-6 weeks after the transfers.

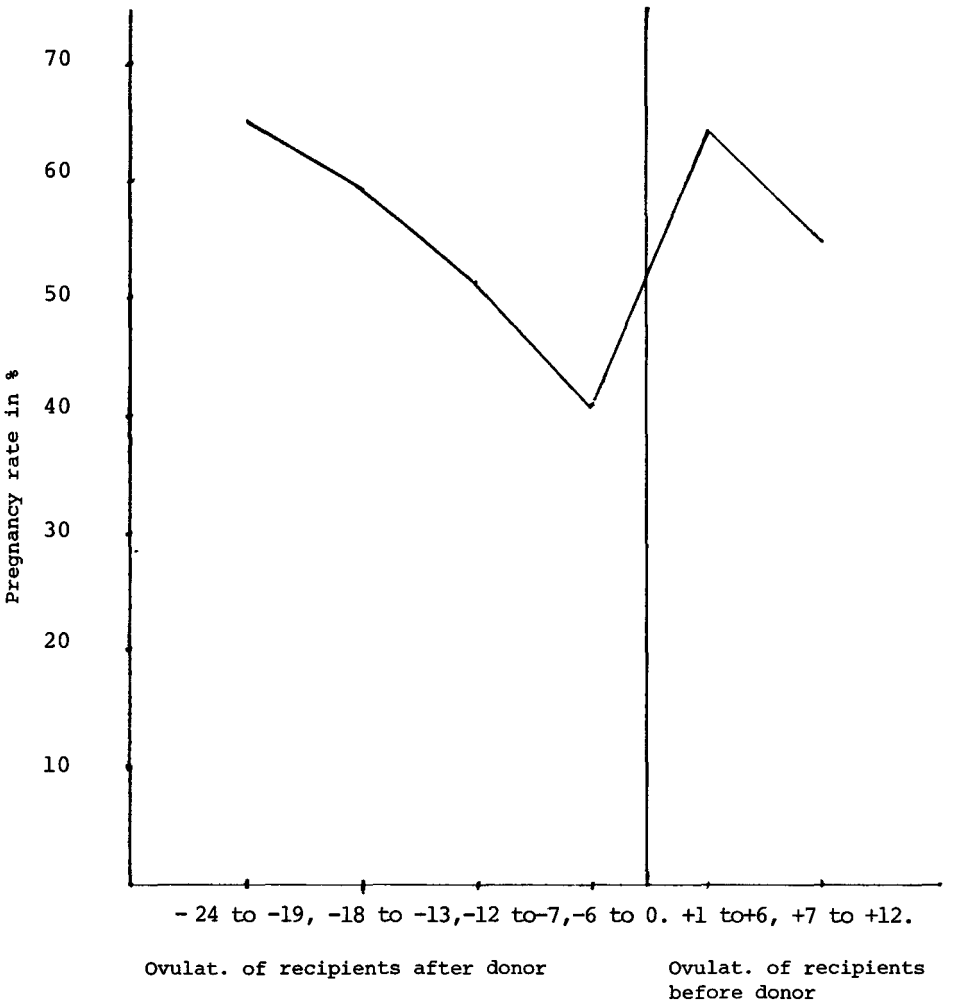
The data was analysed using one way analysis of variable (F-test).

RESULTS

Major results are given in Table 1 and Fig. 1. It must be emphasised that the exact determination of developmental stage can be very difficult. Thus, there may be some overlap between the subgroups.

The overall pregnancy rate (PR) was 54.7% and this is lower than the data presented by Rowson et al. (1972) and Sreenan & Beehan (1974). These groups were using general anaesthesia of the recipient and approaching the uterus via midline incision, whereas we have been transferring the ova via the flank. This same technique has been employed by Drost et al. (1975) and they achieved a pregnancy rate of 56% in beef cattle. When Rowson et al. (1972) and Sreenan & Beehan (1974) used a $\pm$ one day synchronisation the pregnancy rate dropped significantly. This study aimed to examine the effects of smaller variations in synchronisation time and was enabled through very frequent heat observations (4 times daily). It is surprising that the highest pregnancy rate occurred at -19 to -24 hours and +1 to +6 (significant $P < 0.05$) and not at the 0 to -6 hours synchronisation, which might have

FIG. 1.



been expected. Several Canadian groups also have experienced this. (Wilson, 1975). There was no significant difference between the pregnancy rates at -13 to -18, -7 to -12, and +7 to +12 hours, but the lowest rate occurred at the 0 to -6 hours synchronisation. The results of these pregnancy rate determinations are presented graphically (Fig. 1).

The cell stages, as previously stated, can be extremely difficult to

TABLE 1

	- Synchronization (hrs)												+ Synchronization (hrs)						Total		
	-19 to -24			-13 to -18			-7 to -12			0 to -6			+1 to +6			+7 to +12					
Cell Stage	no eggs tr	no anm. pr	% pregn.	no eggs tr	no anm. pr	% pregn.	no eggs tr	no anm. pr	% pregn.	no eggs tr	no anm. pr	% pregn.	no eggs tr	no anm. pr	% pregn.	no eggs tr	no anm. pr	% pregn.	no eggs tr	no anm. pr	% pregn.
8	6	3	50.0	12	5	41.7	16	9	56.3	6	4	66.6	2	1	50.0	2	1	50.0	44	23	52.3
9-16	6	4	66.6	13	8	61.5	42	21	50.0	18	8	44.4	14	7	50.0	4	2	50.0	97	50	51.5
17-32	9	6	66.6	14	8	57.1	18	11	61.1	16	5	31.3	9	7	77.8	3	2	66.6	69	39	56.5
33-64	2	2	100.0	7	6	85.7	5	2	40.0	5	2	40.0	3	3	100.0	2	1	50.0	24	16	66.6
Total	23	15	65	46	27	58.6	81	43	53	45	19	42	28	18	64.2	11	6	54.6	234	128	54.7

differentiate, but very careful analysis was done in each case. It was surprising that there was an extremely big spread of developmental stages in the same donor animal. This may indicate that the ovulations occurred at various times after oestrus. Nevertheless, there was no marked difference in pregnancy rate following transfer of eggs at different stages of development. The highest pregnancy rate was recorded at the 33-64 cell stages (66.6%). The PR was significantly higher than in any other groups ($P < 0.05$). The pregnancy rate of the 17-32 developmental stage was 56.5% and the second highest of all groups.

It can be added that the recovery rate of eggs was 63% and the ovulation rate 73%. The transfer rate was 83%, and the eggs that were not transferred were either degenerate or of very poor quality.

In an attempt to determine if egg quality would influence the pregnancy rate all the fertilised, recovered ova, were graded A B C, based on structure, circumference, etc. The results gave no support for the concept that A and B eggs yielded higher conception rates than C eggs and it is suggested that very little attention should be paid to the grades of eggs. Several Canadian groups believe that this criterion is valid (Wilson, 1975).

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Wilson (1975) Personal communication.

DISCUSSION

L.E.A. Rowson (UK)

When you did these flank transplants did you take notice of which side the corpus luteum was on and always transplant to the corpus luteum side?

H. Breth Hansen (Denmark)

The egg was always transferred to the ipsilateral side.

C. Polge (UK)

You mentioned quite a wide variation in ovulation time, what is your feeling regarding the detection of heat? Do you think it is fair, if there is this wide variation, to classify your synchronisation into such small groups as 0 to 6 hours, 7 to 12 hours, and so forth?

H. Breth Hansen

No, but because we had a feeling from the beginning that the synchronisation time should be on the minus side, we decided to investigate more of these criteria. I agree the group is very small. On the other hand, there should be a way to minimise the synchronisation time.

C. Polge

Looking at these results superficially, one might say that apart from the slight drop you get in your -12 to -7 hour group, there is not really a great deal of significant difference between animals to which transplants were made within half a day after or a day before. I think this would agree with Mr. Rowson's results.

B. Shea (Canada)

Dr. Hansen, I am one of those 'Canadian groups' and I wonder, on the basis of the limited number of eggs that your group has attempted to grade, whether your ability to do this indicates that perhaps eggs are capable of being graded, or perhaps criteria might one day be found where they can be graded, or perhaps do exist.

H. Breth Hansen

Yes, that's right; we discussed this with Wilson from Modern Ova Trends.

B. Shea

I might mention that Dr. Wilson is not an embryologist.

H. Breth Hansen

No, that's right, but others who are attached to Modern Ova Trends are, and they had the feeling too that the way of setting embryos up in several grades is not so important as it seemed because they have very often found that eggs which they considered to be of poor quality did have high conception rates.

B. Shea

I get the impression from your statement though that you feel this is an area which should just be ignored.

H. Breth Hansen

Not at all, but I think you should not regard that as so important as the synchronisation time.

J. King (UK)

I would be interested to know of the calving experience of the Belgian breeder and the Jersey cows. Can you tell us please?

H. Breth Hansen

Yes, of course, I can tell you it is Caesarian in about 97% of cases.

C. Polge

Thank you Dr. Hansen.

SYNCHRONISATION OF OESTRUS IN HEIFERS AND COWS USING A 12-DAY TREATMENT WITH PROGESTERONE COILS WITH OR WITHOUT GnRH

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INTRODUCTION

There are two basic approaches to synchronise oestrus in cattle. The first involves administration of progesterone or one of its synthetic analogues for the period of an oestrous cycle, i.e. 18 to 21 days. This treatment has little or no effect on the normal life span of the corpus luteum but the exogenous progesterone prevents animals coming into oestrus and allows all the animals to reach the follicular stage at the end of treatment. Following withdrawal of the progesterone, oestrus occurs 2 to 6 days later but fertility is low to natural or artificial mating (Robinson, 1968; Jochle, 1972; Mauleon and Chupin, 1971, Roche, 1974_1; Lamming et al. 1975; Sreenan and Mulvehill, 1975). The second basic approach is to shorten the life span of the corpus luteum by using an oestrogen in conjunction with a short-term progesterone treatment (Wiltbank and Kasson, 1968) or more recently by the administration of prostaglandin $F2_\alpha$ or one of its synthetic analogues, (Lauderdale, 1972; Cooper, 1974; Hafs, Manns and Drew, 1975). Fertility has been reported to be normal after the short-term progesterone or prostaglandin treatments (Wishart and Young, 1974; Roche, 1974_2; Cooper and Jackson, 1975; Sreenan and Mulvehill, 1975; Roche, 1976_1).

The aim of this paper is to describe the use of intravaginal silastic coils impregnated with progesterone as a practical method of administering progesterone to cattle and the development of a 12-day progesterone treatment to synchronise oestrus which also results in normal fertility.

EXPERIMENTAL APPROACH & RESULTS

DEVELOPMENT OF SHORT-TERM PROGESTERONE TREATMENT

The development of a 12-day progesterone regimen to synchronise oestrus has already been described using progesterone implants (Roche, $1974_{1,2}$). When using a 9-day treatment with progesterone implants and injecting 5 mg

TABLE 1 RETENTION RATE OF PROGESTERONE IMPREGNATED SILASTIC COILS IN CATTLE AT PASTURE

Animal	Length of Insertion (days)	No. Treated	No. Coils Removed	% Retention Rate
ANIMALS AT PASTURE:				
Dairy Cows	12	670	620	92.5
	18	70	67	95.7
Suckler Cows	12	114	104	91.2
	7	88	86	97.7
Heifers	18	60	58	96.6
	12	499	480	96.2
	7	37	34	92.0
		1,538	1,449	94.2
ANIMALS HOUSED:				
Dairy Cows	12	309	271	87.7
Suckler Cows	12	167	160	95.8
	7	65	64	98.5
Heifers	12	128	117	91.4
	7	19	19	100.0
		688	631	91.7

TABLE 2 RETENTION RATE AND ONSET OF HEAT FOLLOWING REMOVAL OF PROGESTERONE COILS FOR 18 DAYS

Animals	Coils Inserted No.	Coils Removed No.	Onset of Oestrus (days after removal) 2	3	4	Total
Cows	32	31	24	3	1	28
Heifers	20	20	15	-	3	18

TABLE 3 PROGESTERONE LEVELS IN HEIFERS BEFORE AND AFTER INSERTION OF THE COILS

Heifer No.	Before Insertion	Days after Insertion 1	2	3	4	5	6	7	8	9	10	11	12	Oestrus after Removal of coils
16	1.0	4.6	1.7	2.1	2.3	2.6	1.9	1.4	1.7	2.6	1.9	1.2	2.8	Day 2
147	1.1	5.8	5.9	8.7	4.0	2.3	2.0	3.7	3.2	3.0	3.3	3.4	3.4	-
149	0.2	8.3	6.4	6.9	3.2	2.6	2.4	1.6	2.3	2.0	1.6	1.5	1.5	Day 2
223	3.1	8.5	7.2	5.7	4.6	0.8	0.8	0.9	0.4	0.8	0.2	1.2	0.8	Day 2

oestradiol benzoate at the start of treatment to cause premature regression of the corpus luteum, the subsequent synchronised oestrous response was found to be dependent on the stage of the oestrous cycle at the start of treatment (Roche, 1974_2). Animals treated shortly after ovulation or during the follicular stage of the oestrous cycle had a lower oestrous response after treatment than those during the luteal phase of the oestrous cycle at the start of treatment. Incorporating 50 mg progesterone with the oestradiol benzoate and removing the coils after 12 rather than 9 days gave a high synchronised oestrous response in all animals except those between day 0 and 3 at the start of treatment.

ADMINISTRATION OF PROGESTERONE

A practical method of administering progesterone to cattle has hindered widespread use of progesterone for controlled breeding in cattle. A number of different types of silastic rings and coils were made and after intravaginal insertion, were tested for retention rate and ability to synchronise oestrus in heifers and cows (Roche, 1976_2). The most promising system consists of intravaginal silastic coils. These are made using strips of stainless steel (EN 58A 400/450 VPN: Knight Strip Metal, Herefordshire, UK), 30 cm long, 3.2 cm wide and 0.0203 mm thick and these strips were coated with silastic rubber, containing 10% or 6.6% progesterone, to a final thickness of 3 mm. They were coiled by hand to a final diameter of approximately 5.0 cm and a piece of nylon cord was attached to one end. Following steam sterilisation, the coils were placed into the anterior vagina with the aid of a plastic speculum and removed by pulling on the string. The retention rate of these coils is shown in Table 1 and their ability to synchronise oestrus after an 18-day treatment period in Table 2. The progesterone levels in blood during a 12-day insertion period for 4 heifers treated between day 0 and 3 of the oestrous cycle are shown in Table 3. The levels the day after insertion increased 4 to 8 fold and remained high in 3/4 animals until day 4 and the levels then fluctuated between 0.3 and 3.8 ng/ml plasma until the coils were removed. In animal No. 16, progesterone declined after the initial rise and fluctuated between 1.4 and 2.5 ng/ml. Heifers 223, 149 and 16 were in oestrus on the second day after removal of the coils while heifer 147 was not detected in oestrus within 6 days of removal of the coil.

FERTILITY FOLLOWING THE 12-DAY PROGESTERONE TREATMENT

Farm trials were carried out using the intravaginal silastic coils to administer progesterone. These were inserted into the vagina for 12 days and an injection of 5 mg oestradiol benzoate and 50 mg progesterone was also given at the start of the treatment.

Three hundred and fifteen Friesian dairy cows in three herds which had calved at least 40 days were randomised according to the post-partum interval and lactation number and allocated to a 12-day progesterone treated or control groups. Normal oestrus detection was carried out in the control group and cows were inseminated once with frozen semen stored in straws towards the end of, or shortly after, standing oestrus. Cows treated with progesterone were observed three times daily for oestrus 1 to 6 days after removal of the coils and were inseminated with semen from the same bulls at the synchronised oestrus. Cows that lost coils were deemed not to have responded to treatment irrespective of when they were observed in oestrus. Animals that returned to oestrus were re-inseminated and all animals were allowed to calve.

Next, 253 Friesian dairy cows at least 40 days post-partum were allocated to a 12-day progesterone treatment, inseminated and allowed to calve as just described. To determine the effect of this treatment on fertility, the calving rate for 228 control cows in the same herds which were inseminated as they showed oestrus either 10 days before or during treatment was determined and compared to the calving rate in the progesterone treated group.

Finally, the oestrous cycle of 156 mainly Friesian maiden heifers on farms were synchronised with the 12-day treatment and inseminated at the oestrus following removal of the coils. These heifers were then allowed to calve.

ONSET OF OESTRUS

The occurrence of oestrus for all treated animals that retained coils is shown in Table 4. Ninety-three and 90% of the heifers and cows were detected in oestrus two to six days after removal of the coils. Of the heifers and cows detected in oestrus 71, 17, 7, 4 and 1% were detected in

in oestrus the second, third, fourth, fifth and sixth days respectively after removal of the coils. The majority of animals not detected in oestrus within six days of removal of the coils were detected in oestrus 18 to 26 days after treatment.

TABLE 4 ONSET OF OESTRUS FOLLOWING REMOVAL OF THE INTRAVAGINAL SILASTIC COILS CONTAINING PROGESTERONE

	Heifers	Dairy Cows
Coils inserted .... No.	156	412
Coils removed ..... No.	153	378
Animals in oestrus	143	342
Day 2 after removal	104	242
3 " "	20	62
4 " "	16	16
5 " "	1	18
6 " "	2	4

FERTILITY

There was no significant difference in calving rate to first or to the repeat insemination for treated and control cows (Tables 5 and 6). Eighty-three of the 143 heifers on farms which were bred calved to the first insemination and 40 of the 53 remaining heifers calved to the first repeat insemination (Table 7). The indications from this work was that synchronisation was precise following this progesterone treatment and that calving rate was normal following a single insemination at the synchronised oestrus.

CONTROL OF TIME OF OVULATION AND FIXED TIME AI

In order to determine the time of ovulation following the 12-day progesterone treatment, mature Hereford cross heifers received intravaginal coils for 12 days and an injection of 5 mg oestradiol benzoate and 50 mg progesterone at the start of treatment. Thirty one heifers received 100 µg gonadotrophin releasing factor (GnRH, Abbott Labs.) intramuscularly 30 hr after removal of the coils. The time of ovulation is shown in Table 8. Heifers were slaughtered at various times after treatment and the reproductive tracts recovered and examined for recent ovulation points.

TABLE 5 CALVING RATE IN DAIRY COWS RANDOMISED TO CONTROL AND PROGESTERONE-TREATED GROUPS ON THE BASIS OF POST-PARTUM INTERVAL

		Control	Treated
Cows treated	No.	156	159
Cows that retained coils	No.	-	150
Cows inseminated	No.	137	137
Non-return rate	No.	76	76*
	%	56	56
Cows sold with no repeat	No.	6	12
Cows aborted with no repeat	No.	3	4
Cows present at calving	No.	128	121
Cows calved to first AI	No.	67	62
Cows calved to repeat AI	No.	28	30
Percent treated that calved		43	39
Percent AI'd that calved		49	45
Percentage of cows present at calving that calved to first AI		52	51

* Two treated cows that returned to oestrus calved to the first insemination

TABLE 6 CALVING RATE IN DAIRY COWS FOLLOWING A 12-DAY PROGESTERONE TREATMENT IN COMPARISON TO CONTROL COWS IN THE SAME HERDS INSEMINATED ABOUT THE SAME TIME

		Control	Treated
Animals	No.	228	253
Cows inseminated	No.	228	205
Non-return rate	No.	123	124
	%	54	60
Cows sold - no repeat	No.	15	9
Cows aborted - no repeat	No.	2	2
Cows present at calving	No.	211	191
Cows calved to first AI	No.	102	109
Cows calved to repeat AI	No.	39	41
Percent treated that calved		45	43
Percent AI'd that calved		45	53
Percentage of cows present at calving that calved to first AI		48	57

TABLE 7 CALVING RATE FOR HEIFERS ON FARMS

	Number of animals
Heifers treated	156
Heifers Inseminated	143
Non-return rate	90
Aborted - no repeat	4
Calved to first AI	83
Calved to repeat AI	40
Percent treated that calved	53
Percent inseminated that calved	58
Percentage present at calving that calved	60

TABLE 8 TIME OF OVULATION IN HEIFERS FOLLOWING A 12-DAY PROGESTERONE TREATMENT WITH OR WITHOUT GnRH

Treatment	No. of heifers injected	Heifers ovulating No.	%	Time of slaughter after removal of coils (hr)
Control	4	-	-	60
Control	11	5	45	65
GnRH*	9	2	22	55
GnRH	12	8	67	60
GnRH	10	9	90	65

* Time of ovulation significantly different ($P < 0.10$) from controls

Ovulation had not begun in control animals slaughtered 60 hr after removal of the silastic coils following the 12-day progesterone treatment while five of eleven had ovulated at 65 hr after removal of the coils (Table 7). When GnRH was given, ovulation had started at 25 hr after GnRH and eight of twelve had ovulated at 30 hr after GnRH (i.e. 60 hr after removal of the silastic coils). Ovulation was virtually complete at 35 hr after GnRH (i.e. 65 hr after removal of the silastic coils). The differences between the heifers treated with GnRH and the controls in the proportion of animals ovulating at 60 and 65 hr was significant ($P < 0.10$).

In order to determine if fixed time insemination schedules could be developed to give normal fertility, two further experiments were conducted.

In the first experiment, mature Hereford cross heifers at pasture were randomised and allocated to a control group which were inseminated as they showed oestrus; or to one of three 12-day progesterone treated groups where animals were inseminated (i) as they showed oestrus after treatment; (ii) 48 hr after the end of treatment or (iii) as (ii) but animals were also given an intramuscular injection of 100 μg GnRH 30 hr after the end of treatment. Heifers were inseminated with frozen semen from pooled ejaculates from the same bull and all animals were slaughtered 30 to 40 days later. The reproductive tracts were recovered and the number of animals with viable embryos was recorded.

In the second experiment, similar heifers at pasture were randomised and allocated to a control group and were inseminated at oestrus; or to one of three 12-day progesterone treated groups where animals were inseminated at (i) 56 hr; (ii) 74 hr, or (iii) 56 + 74 hr after the end of treatment. Frozen semen from the same pool as in the previous experiment was used and animals were slaughtered 30 to 40 days later to determine the number pregnant.

In the first experiment there were no significant differences in pregnancy rate between control and treated heifers bred at oestrus or 18 hr following GnRH injection (Table 9).

TABLE 9 PREGNANCY RATE AT SLAUGHTER OF HEIFERS TO DIFFERENT BREEDING SCHEDULES FOLLOWING THE 12-DAY PROGESTERONE TREATMENT

	Control		Progesterone treated	
	AI at oestrus	AI at oestrus	GnRH at 30 hr - AI at 48 hr	AI at 48 hr
Heifers ---------- No.	14	16	23	14
Inseminated ---------- No.	14	16	23	14
Repeat oestrus ---------- No.	4	3	9	8
Pregnant ---------- No.	10	11	12	3
Percent pregnant to AI	71	69	52	21*

* Significantly lower ($P < .05$) pregnancy rate

A single insemination 48 hr after treatment without GnRH treatment resulted in a significant ($P < .05$) lower pregnancy rate. In the second experiment, there were no differences in pregnancy rate between control and treated heifers inseminated at 56 hr only or at 56 and 74 hr after removal of the coils without recourse to oestrous detection (Table 10). However, a single insemination at 74 hr resulted in significantly ($P < .10$) lower pregnancy rate in comparison to inseminations at 56 hr or at 56 + 74 hr after the 12-day progesterone treatment.

TABLE 10 PREGNANCY RATE AT SLAUGHTER OF HEIFERS TO FIXED-TIME AI FOLLOWING THE 12-DAY PROGESTERONE TREATMENT

	Control	Progesterone treated		
	AI at oestrus	AI at 56 hr	AI at 74 hr	AI at 56+74 hr
Heifers ---------- No.	24	26	25	25
Inseminated ---------- No.	24	26	24	25
Repeat oestrus ---------- No.	10	4	6	4
Pregnant ---------- No.	14	17	11	17
Percent pregnant to AI	58	65	46*	68

* Significantly lower ($P < .10$) than AI at 56 hr or 56 + 74 hr

MODIFICATION OF 12-DAY TREATMENT

Using the 12-day progesterone treatment described in previous experiments, the synchronised oestrous response is dependent on the stage of the cycle of the heifers at the start of treatment; animals between day 0 and 3 being most difficult to control (Roche, 1974_2). An experiment was conducted to determine if extra injected progesterone was required with the oestrogen at the start of treatment when using the intravaginal coil, to obtain good synchronisation of oestrus in animals between day 0 and 3 at the start of treatment. The silastic coils used have a larger surface area than the progesterone implants and result in a rapid 4 to 6 fold increase in progesterone in blood within 90 min of insertion (Mauer et al., 1975).

Mature Hereford cross heifers, run with vasectomised bulls at pasture, were checked twice daily for the occurrence of oestrus and the day of oestrus

was taken as day 0 of the oestrous cycle. Intravaginal silastic coils (Roche, 1976$_2$), containing 6.6% progesterone by weight (2.1 g), were inserted into 73 heifers between day 0 and 3; 4 heifers which lost coils were not used. The heifers were allocated at random to 4 groups and received an intramuscular injection of corn oil at the time of insertion of the coils containing either (i) 5 mg oestradiol benzoate; (ii) 5 mg oestradiol benzoate + 50 mg progesterone; (iii) 5 mg oestradiol benzoate + 200 mg progesterone and (iv) 200 mg progesterone. The coils were removed after 12 days and each animal was individually checked for oestrus at 08.30, 16.30 and 20.30 hr with a vasectomised bull. Heifers not in oestrus were run with another vasectomised bull during the night.

Blood samples were obtained from all animals by jugular veni-puncture before insertion of coils and then daily while the coils were in the vagina. Plasma was obtained by centrifugation at 4°C and stored at -20°C until assayed by radioimmunoassay for progesterone as described previously, (Gosling et al., 1975).

TABLE 11 OESTROUS RESPONSE AND OCCURRENCE OF OESTRUS FOLLOWING A 12-DAY TREATMENT WITH PROGESTERONE COILS WHEN HEIFERS WERE BETWEEN DAY 0 AND 3 OF THE CYCLE AT THE START OF TREATMENT

	Treatment at Insertion of Coil			
	5 mg OB	5 mg OB + 50 mg P	5 mg OB + 200 mg P	200 mg P
Heifers ---------- No.	18	18	17	16
In Oestrus ------- No.	17	16	14	12
Onset of Oestrus				
Day 1				
" 2	16	8	14	6
" 3		3		2
" 4		4		3
" 5	1	1		1

OESTROUS RESPONSE

There were no differences between groups in the number of heifers observed in oestrus from days 1 to 6 following removal of the coils (Table 11). However, there were significant differences in the temporal pattern of the onset of oestrus. Significantly ($P < 0.05$) more heifers were in oestrus within 3 days of removal of the coils in groups 1 and 3 compared to groups 2 and 4.

PROGESTERONE LEVELS

The mean daily plasma levels of progesterone for animals in oestrus within 2 days of removal of the coils, did not differ significantly between groups. However, the mean progesterone levels for animals in oestrus 3 or more days after coil removal differed between groups: the average progesterone level for the first 2 days after coil insertion was significantly lower ($P < 0.005$) for groups 1 and 2 compared to groups 3 and 4. The average progesterone level over the last 3 days of treatment was lower ($2.13 \pm .14$ ng/ml) for animals in oestrus within 2 days of the end of treatment in comparison to animals in oestrus later than this ($3.61 \pm .25$ ng/ml). The results of this experiment indicated that either 5 mg oestradiol benzoate alone or with 200 mg progesterone injected at the start of a 12-day progesterone treatment with intravaginal coils resulted in more precise onset of oestrus after treatment than either of the other two treatments used.

CONCLUSIONS

From the results presented, intravaginal silastic coils are a practical and effective method to administer progesterone to cattle. Following the 12-day treatment described, there is a precise onset of oestrus and calving rate to insemination at this oestrus is not different to that in contemporary control cows in the same herds. Preliminary results indicate that a single fixed time insemination at 56 hr, with or without GnRH injection, or two inseminations at 56 and 74 hr after removal of the coils also result in normal fertility. Using the 12-day treatment, the exact combination of oestrogen and progesterone required at the start of treatment to shorten the cycle sufficiently to result in a high synchronised oestrous response is still not clear. The indications are that an injection of 5 mg oestradiol

benzoate alone or with 200 mg progesterone is superior to the use of 5 mg oestradiol benzoate and 50 mg progesterone.

At present the factors that affect fertility on farms such as level of managment, plane of nutrition, inseminator stress, differential fertility effects of individual bulls, and post partum interval need further study in synchronised animals. The calving rates obtained in control and treated cows, although not different, were lower than the non-return rates used by artificial insemination stations indicating the need for clear definition of what the exact calving rate to a single insemination at farm level is expected to be.

*

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DISCUSSION

I. Gordon (Ireland)

I would like to ask Dr. Roche if he did have trouble with infections in the vagina? Did 'things' crawl up the draw-string under some conditions?

J.F. Roche (Ireland)

In the majority of animals - 60 to 70% - there is no great discharge following removal of the coil. In some animals, however, there is a whitish discharge and we can't explain why this appears in some animals and not in others. The one point that is obvious is that some animals have the capability of sucking the string back into the vagina - I don't know how they do this but where that happens there is a mess at removal. This is presumably due to the fact that the string had been out for a few days and there is plenty of faecal material and plenty of bacteria on it and so when it is sucked in it sets up an infection in the vagina.

M.J. Cooper (UK)

Is it the size of these coils which is critical or the springiness - the tension, on them?

J.F. Roche

We have only used one type of steel on the coil that we are using so that we have always had a constant elasticity of the steel. It is the diameter that is important with this particular coil. We haven't looked at different types of steel with different elasticity so I can't really answer on that part of the question.

J. Sreenan (Ireland)

I would just like to make a comment in relation to retention rates and the questions from the last two speakers. We have just published data in JRF on the use of 10-day progesterone sponges intravaginally and one of the problems with this method of synchronisation of oestrus in cattle in the past, has been retention rate. Jim Roche will be aware of what I am talking about. Over long-term treatments, that is, 20 day treatments, we had very poor retention rates in both heifers and cows. When we reduced the interval, which we have done for quite a large number of animals now, something in the region of 2,000 on farm trials, when we reduced the interval

to a 10 day period and incorporated 5 mg of ODB and 250 of progesterone on the day of insertion, we find we are having heifers with 98% retention. We don't think that we are going to get much better than this.

We have come up against some of the same problems that Jim Roche talked about with regard to strings which suddenly disappear into the animal and you get varying sorts of discharge. There doesn't seem to be (when we categorise this), any relation between the type of discharge and fertility afterwards. Retention rate in cows over a similar period is just about 90% in respect of some 1500 animals. (This is with a 10 day progesterone sponge in the vagina). The point Dr. Roche was making was that he increased the diameter of the coil rather than increasing the tension. As we increased the density of the sponge material we reduced retention. As we reduced the density we increased retention, until we came to an optimum.

R. Church (Canada)

I am interested in your synchronous group versus your less synchronous group. Have you ever analysed the amount of progesterone that's left in your implant in these two groups to determine whether or not there is an uptake difference?

J.F. Roche

No, we haven't looked at the levels of progesterone in the coils in the synchronised and unsynchronised groups.

H. Karg (W. Germany)

Are there any calculations about the price of this method compared to others? I would also like to remark that using oestradiol benzoate there is quite a long clearance time which would be involved also with the withdrawing time.

J.F. Roche

Yes, we are acutely aware of the problems of using oestradiol benzoate with the synchronising treatment. To answer your first question: I have no idea of the price really.

As far as oestradiol benzoate is concerned we have experiments planned and some preliminary data that would indicate that we can get away with a

coil only without any oestradiol benzoate by leaving them in for 14 days. The question now, of course, that remains to be answered, is what will the fertility be?

A.O. Trounson (UK)

I am a little bit concerned about the pregnancy rate in your controls - 45% - bearing in mind that Dr. Brand says that he can get 40% from non-surgical transfers.

The other question is that I noticed on your Tables that you say that the differences are significant at point one. I would not think that this would be accepted as a significant difference.

J.F. Roche

Well, you can decide on that whether it is significant to you or not. That is the only comment I would make on that.

As regards the low calving rate in trials - yes - I think this is a problem and I think that in the paper handed out the final sentence is that there is need for clearer definition of what the exact calving rate to a single insemination is. I think this is one of the big problems of synchronisation; people are quoting non-return rates of 70 - 75%, particularly in the AI stations. When you come in and do trials and allocate control animals and treated animals, our calving rates were varying from 35% up to 65%. In general they average out at about 50%. It is a problem but these are the facts as we have come across them in our trials - that's all I can say.

M.J. Cooper

First of all, I would like to support Dr. Roche on the last point. I think his figures are very realistic and they are certainly very similar to the figures we have got from 'on farm' trials.

The question I want to ask may be a little naive - I don't know. You have talked about the need for oestradiol benzoate which is presumably the luteolytic agent at the beginning of this treatment. Now, the addition of progesterone to shorten the cycle confuses me a little. Isn't it possible that in the absence of progesterone, animals treated towards the end of the

cycle, after spontaneous regression has occurred, are likely to ovulate in response to the oestradiol unless you were putting a high blood level of progesterone in simultaneously?

J.F. Roche

Yes. The need for injected progesterone arose in our hands from the use of progesterone implants which have a surface area about 50% smaller than the progesterone coil. We have not measured the plasma levels of progesterone using these implants but with implants we showed a definite necessity to inject extra progesterone to prevent the oestradiol causing ovulation in animals between Day 17 and Day 20. However, using the progesterone coil, due to its large surface area, the levels of progesterone in the blood increase four to six fold within one hour after insertion. The inference here is that this initial rapid increase in progesterone from the coil is sufficient and no extra injected progesterone is required when using this particular method for administering progesterone.

L.E.A. Rowson (UK)

I think my question has been partly answered. I think the conception rates quoted in this country and abroad are extremely misleading and, in fact, they are really nothing more than a preliminary guide. If one takes actual calving rate you will find it is somewhere between 50 and 55%. The figures which you quote are not too far out I think.

C. Polge (UK)

That's an interesting comment from the Director of an AI centre! However, we must move on.

THE USE OF PROSTAGLANDINS IN THE CONTROL OF THE BOVINE OESTROUS CYCLE

M.J. Cooper and B.J.A. Furr

INTRODUCTION

For many years a simple, safe and inexpensive method of controlling the bovine oestrous cycle has been sought. Such a method would allow the widespread use of artificial insemination and thus the exploitation of its many well established benefits. It is also clear that a number of managemental advantages would accrue from the application of oestrus control on the farm. The harvesting and transfer of viable embryos from high quality parent stock has also been recognised as potentially the best method of distributing good genetic material in its most concentrated form. However, it has been shown unequivocally that best results can only be obtained when there is a high degree of cycle synchronisation between donor and recipient animals (Rowson et al. 1972_1). This factor has been important during the development of the technique and will be essential for successful commercial exploitation.

It is now well established that natural prostaglandin $PGF_{2\alpha}$, or more particularly, its Tham salt, is luteolytic in cattle, and that its administration during the luteal phase of the cycle is followed by a fertile oestrus 3 or 4 days later (Rowson et al. 1972_2; Louis et al. 1973; Lauderdale, 1975; Oxender et al. 1974). The corpus luteum of the cow is insensitive to this luteolytic effect until it is about 4-5 days old. Moreover, the administration of prostaglandin around the time of spontaneous luteal regression does not appear to influence the course of events. Thus, a single administration of PG to a group of randomly cycling cattle will be followed by a predictable response in only a proportion of them. A method of overcoming this problem, using $PGF_{2\alpha}$ and its analogues has now been described (Cooper, 1974) and consists of a regime of 2 injections of PG separated by 10-12 days. This is followed by a well synchronised heat 48-72 hours after the second injection, which provides a very promising method of controlling the cycle.

'Estrumate' (cloprostenol; ICI 80,996) is a synthetic prostaglandin

analogue, structurally related to $PGF_{2\alpha}$ (Binder et al. 1974). It is a highly potent luteolytic agent (Dukes et al. 1974), effective in cattle at a single intramuscular dose of 500 µg (Cooper and Furr, 1974). Cooper (1974) and Cooper and Rowson (1975) have already shown that the compound can be successfully used to control the oestrous cycle in Friesian heifers. It is the intention of this paper to consider briefly some of this work and the investigations which have been carried out, under both laboratory and field conditions, to confirm the normal fertility of the synchronised heat. Some consideration is also given to aspects of this work which have implications for the technique of embryo transfer.

MATERIALS AND METHODS

The work fell into two main areas:-

1. Examination of the efficacy of 'Estrumate' in controlling the oestrous cycle in Friesian heifers.

2. Investigation of some of the endocrine changes occurring during a treatment regime of 2 injections of 'Estrumate', separated by 11 days.

One hundred and seventy five Friesian heifers were used, all known to have no pathological abnormalities of the reproductive tract and known to be cycling normally prior to the experiments. The heifers were treated in groups, at different times, with 2 intramuscular injections of 500 µg 'Estrumate' separated by 11 days.

Oestrus responses following each injection were closely monitored, the animals being under regular observation for at least 12 hours daily, and for longer periods at critical stages of the work. Detection of oestrus was aided by vasectomised teaser bulls wearing 'chin ball' marking devices. In addition, many animals were examined clinically, involving rectal palpation of the uterus and ovaries and examination of samples of cervical mucus (Cooper, 1975).

In about 50 animals, cycle lengths following treatment were also monitored, but the remainder were inseminated in various experiments to examine

the fertility of the synchronised heat.

Seven heifers, selected to be in the mid-luteal phase of the cycle at the commencement of the experiment were also treated with 2 intramuscular injections of 500 µg 'Estrumate' separated by 11 days. These animals were housed and restrained, and their jugular veins cannulated to facilitate regular bleeding. Blood samples were taken into heparinised syringes, centrifuged at 5°C. and the plasma separated, frozen and stored for hormone assay. Samples were taken twice before the first injection, every 2 hours for 96 hours after each injection, daily or twice daily during the remainder of the experiment and every other day throughout the next oestrous cycle. Clinical changes were monitored by regular rectal palpation and mucus sampling as described in Experiment 1. In this way the onset of oestrus and the time of ovulation were determined.

Plasma concentrations of LH, oestradiol - 17β , and progesterone were measured by radioimmunoassay. LH was assayed by a modification of the double antibody procedure of Niswender et al. (1969), using a rabbit anti-ovine LH, a highly purified ovine LH (LER-1056-C_2) for iodination and ovine LH (NIH-LH-S_{14}) as a standard.

Measurement of both oestradiol-17β and progesterone was carried out by Dr. Hilary Dobson at Liverpool University. Oestradiol-17β was estimated in ether extracts of plasma without chromatography using a specific rabbit antiserum prepared against oestradiol-17β -6-carboxymethyloxime - bovine serum albumen (Dobson and Dean, 1974).

Progesterone was measured by radioimmunoassay in hexane extracts of plasma using goat antiserum prepared against progesterone 11-α-succinyl - bovine serum albumen (Furr, 1973).

RESULTS

The histogram in Figure 1 shows the oestrus response obtained in 175 Friesian heifers. The broken line represents the small number of animals (in this sample, 14%) showing heat between 36 and 48 hours after the 2nd injection of 'Estrumate'. Reference to more detailed data and those from other groups suggest that the earliest time that animals are likely to show

heat is around 44 hours after the 2nd injection of prostaglandin. The majority of animals show oestrus between 48 and 72 hours after treatment while a small number do not come into heat until after 72 hours. It should be emphasised that this pattern of response was obtained in cycling Friesian heifers, on a high level of nutrition, treated during the spring and early summer. However, a very similar pattern can be obtained in heifers and lactating cows under field conditions on commercial farms.

FIGURE 1

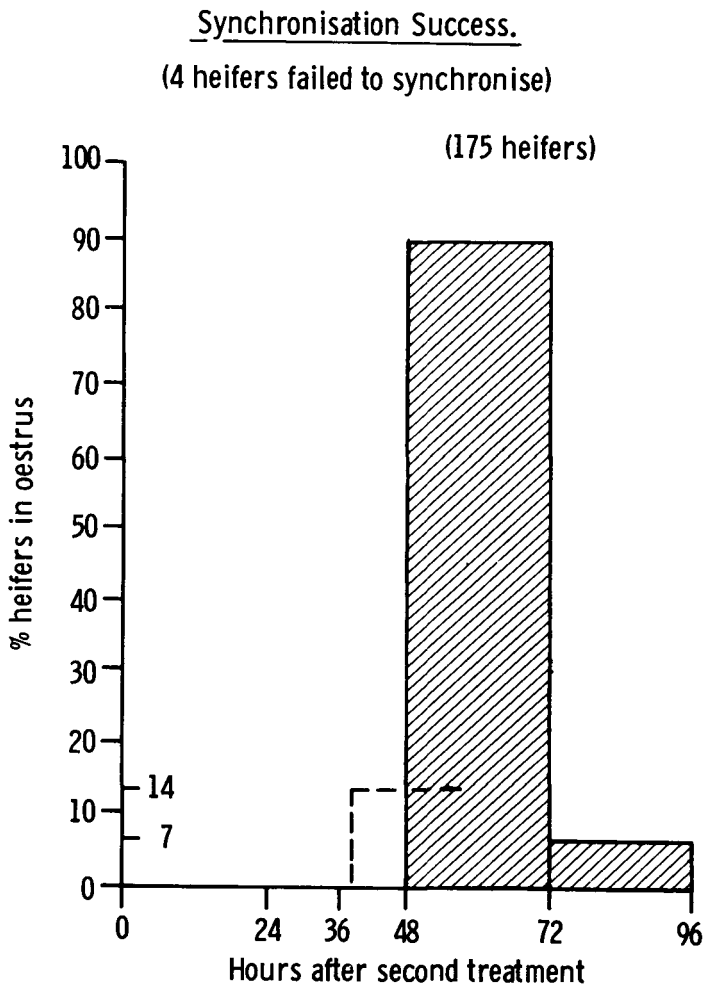


Figure 2 shows the alterations in peripheral plasma concentrations of progesterone, oestradiol-17β and LH occurring in one of the 7 heifers,

while Figure 3 shows the changes in progesterone in all 7 heifers, together with the standard error of the mean. The fall in plasma progesterone is followed by a rise in the concentration of oestradiol-17β which reaches peak values at about the time of the onset of oestrus, coinciding with (and provoking) the release of the preovulatory surge of LH. These changes follow both injections of 'Estrumate'. It is interesting to note the rapidity with which 'Estrumate' causes functional regression of the corpus luteum. Plasma progesterone is significantly reduced ($P < 0.05$) within 2 hours, and is down to half the pre-treatment value within 6 hours of the injection. Basal concentrations (below 1 ng/ml) are reached within 24 hours of treatment.

FIGURE 2

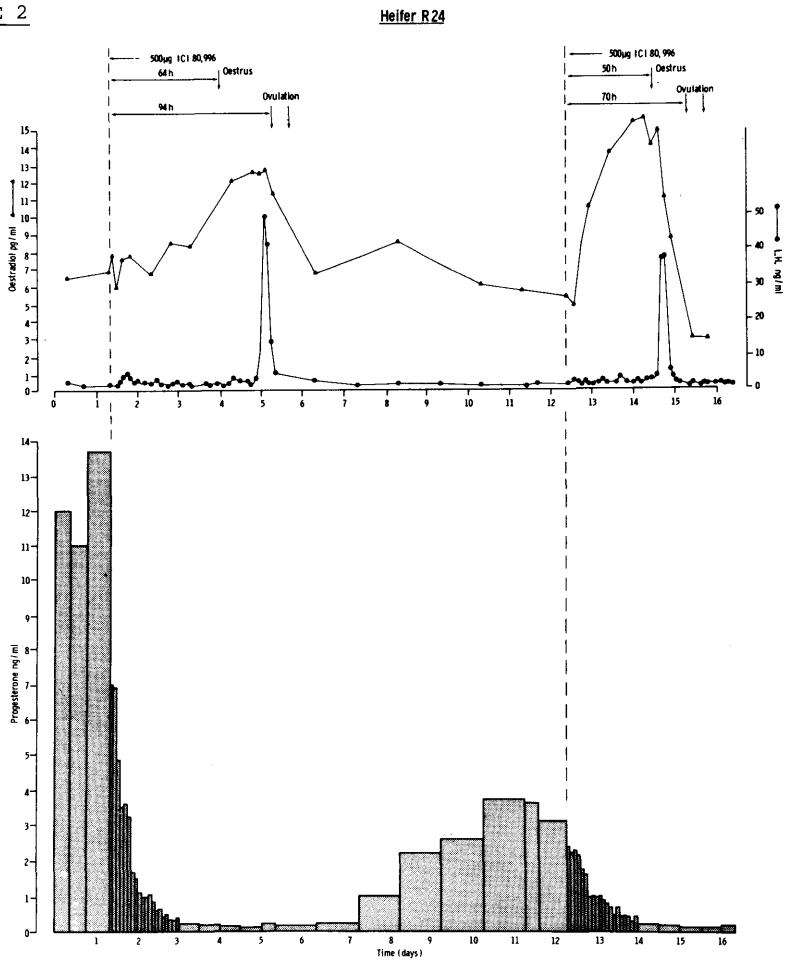


FIGURE 3

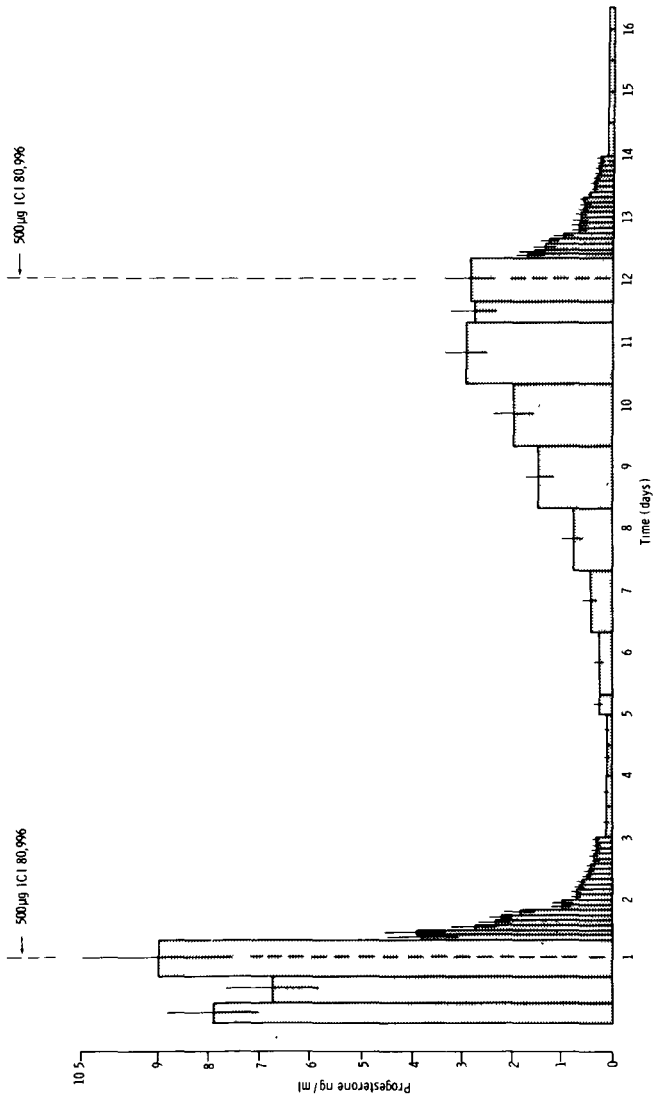
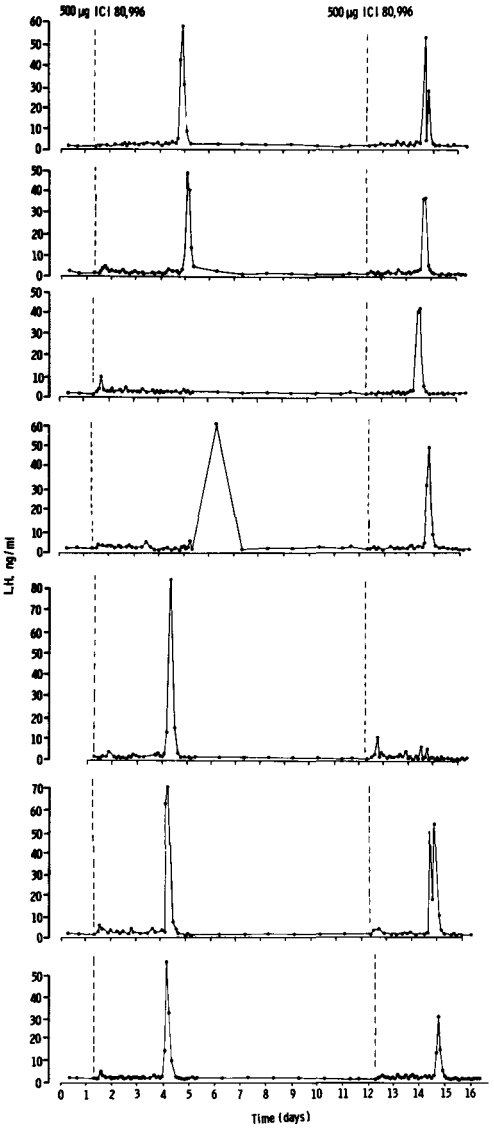

500µg ICI 80,996
500µg ICI 80,996
Progesterone ng/ml
Time (days)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Figure 4 shows the changes in LH concentration in all 7 heifers. It can be seen that the preovulatory release of this hormone occurs much more promptly and in a better synchrony following the 2nd injection than following the first. This release precedes ovulation by about 24 hours and is an excellent indicator of the degree of oestrus control which has been achieved.

FIGURE 4



The preovulatory release of LH did not occur following the 2nd injection of 'Estrumate' in one heifer (Figure 4) and in this animal reference to the clinical findings shows that no ovulation was detected. The remaining 6 heifers, however, ovulated normally, developed a new corpus luteum and showed a post-treatment cycle of normal length. Similarly, during Experiment 1, animals observed after the controlled heat also developed morphologically normal corpora lutea and showed cycles of normal length (18-24 days).

CONCLUSIONS

The onset of oestrus, particularly in heifers, following 2 spaced injections of 'Estrumate' consistently falls into the pattern described in these results and has been confirmed by other groups (Leaver et al. 1975; Thimonier et al. 1975) and also by workers using $PGF_{2\alpha}$ (Lauderdale, 1975). The distribution may vary somewhat in lactating cattle under field conditions, although good data are not available on this point, but the basic pattern of response is consistent. The most variable figure reported is the proportion of animals actually showing heat after treatment, and it is likely that this is dependent not only on the proportion of animals known to be cycling normally prior to treatment, but, even more, upon the efficiency of the various methods of oestrus detection used to monitor response. Detailed information on this latter point is essential for adequate interpretation of results.

The endocrine changes described here are reassuringly similar to those which have been reported to occur around a spontaneous heat (Glencross et al. 1972; Nancarrow et al. 1973), and are also similar to those occurring at oestrus following luteal regression induced by prostaglandin $F_{2\alpha}$ (Oxender et al. 1974; Louis et al. 1975). In addition, according to clinical evidence, ovulation appears to occur at a similar and consistent time (in relation to the onset of heat and the LH surge) as at a spontaneous heat (Schams and Butz, 1972). Measurement of plasma LH, therefore, provides the most satisfactory endocrine parameter for assessment of the precision of any synchronisation regime.

The data shown in Figure 4 have two important implications. The first being that in these particular animals, all of which were selected to be at

the same cycle stage before treatment, LH release occurred between 55 and 65 hours after the second injection of 'Estrumate'. Reference to the oestrous response (Fig.1) suggests that this is the earliest time after treatment that LH release will occur. Other animals in a randomly cycling group are therefore likely to release their LH after, rather than before, this time. This means that the earliest time after treatment at which ovulation will occur is approximately 79 hours. It is widely accepted that the optimum time for artificial insemination is approximately 12 hours before ovulation. Consequently, these data suggest that 2 inseminations would be required to produce good fertility, and that 72 and 96 hours after treatment would be appropriate and practical times for these.

The second implication, and the more important one with respect to embryo transfer, is the more rapid and precise response seen after the second treatment with 'Estrumate' as compared to the first. This is important since a single injection of prostaglandin will frequently be used to synchronise recipient animals. This phenomenon has already been reported by Cooper (1974) and confirmed by both Leaver et al. (1975) and Thimonier et al. (1975). It is tempting to suppose, as suggested by Thimonier et al. (1975) and Cooper (1975), that this treatment regime exploits the inherent rhythmical activity of the ovary. The 2nd injection, in a large proportion of animals which undergo luteolysis following the first, is given at a time when follicular growth is known to be actively occurring in the ovary (Rajakoski, 1960). This may lead to rapid maturation and ovulation of follicles which would otherwise become atretic. Some of the earliest published results on the prostaglandin analogue ICI 79,939 would seem to be compatible with this idea. Tervit et al. (1973) showed clearly that cattle responded more rapidly following luteal regression when their ovaries were primed, prior to treatment, with PMSG. This has been confirmed in superovulated donor animals by many groups and a clear difference in response in donor animals when compared to unprimed recipients has frequently been observed. However, an initial attempt to investigate this theory has failed to substantiate it. Baischya et al. (1975) synchronised heat in a group of heifers with 2 injections of 'Estrumate' and then treated half the animals on the 7th day and half on the 14th day of the new cycle with a third injection. The oestrous response in both these groups was identical and was very similar to the response at the controlled heat. It may be, therefore, that persistent interruption of the progestational influence on the pituitary

and hypothalamus is more important, and may perhaps cause an altered gonadotrophin output.

Clearly, a number of factors affect the speed with which follicular development, oestrus, and ovulation occur after luteolysis, and these will require elucidation if optimum results are to be obtained from embryo transfer.

FERTILITY OF THE CONTROLLED HEAT

The fertility of the heat following 2 injections of 'Estrumate' separated by 11 days has now been examined in some detail and although it is beyond the scope of this paper to discuss this in depth, some general comments are relevant.

In a closely controlled study involving 180 animals under experimental circumstances, Cooper (1975) obtained identical fertility in treated and control Friesian heifers. The treated heifers were inseminated once on detection of heat following treatment, and the controls were inseminated once at spontaneous heat during the experimental period. In a further trial, Cooper (1975), also obtained excellent fertility in 30 Friesian heifers treated with 'Estrumate' and inseminated twice, at 72 and 96 hours after the second injection, without reference to heat.

Normal fertility (i.e. identical with that of control animals) has also been obtained following 'Estrumate' treatment by Leaver et al. (1975). Again Friesian heifers were used and in this case optimum results were obtained from a single insemination at 72 hours following treatment.

In a large multi-centre field trial in beef animals, involving 3,810 cattle, Cooper (1975_1) has shown that the fertility of the controlled heat is unaffected by 'Estrumate'. These trials confirmed the efficacy of the treatment regime under field conditions, and that for optimum fertility cattle should be inseminated twice at 72 and 96 hours following the second injection of prostaglandin. They also showed clearly, that best results could only be obtained when appropriate attention was paid to the management and nutrition of the cattle before the trial. Inadequately fed and badly supervised cattle consistently yielded poor results. Similar conclusions

have now been drawn by other groups involved in controlled breeding field trial work.

In spite of these constraints, prostaglandins, and in particular 'Estrumate', offer a safe, simple and inexpensive method of controlling ovulation in cattle, and one which will have an important part to play in the continued development of embryo transfer.

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DISCUSSION

D. Wishart (UK)

What happened to the LH peak in the fifth heifer in the last slide?

M.J. Cooper (UK)

She didn't ovulate.

D.Wishart

Why? Do you know?

M.J. Cooper

No idea! The only thing I can associate it with is that we have found that putting jugular cannulae in these animals is quite a stressful procedure and this particular animal got an infection. She developed what I suppose you might call polyarthritis, anyway she had infected joints and she was in rough shape at the end of the experiment and she didn't ovulate. My guess is that is the reason for it.

D. Wishart

Did you monitor that through and are you sure that over a number of days she didn't ovulate or was it just at that particular time? What was the base line in fact?

M.J. Cooper

Well, we measured progesterone every other day subsequent to the period of intensive bleeding and she never developed more than 1½ nanograms and she was still at that level when we stopped bleeding 22 days later.

J.F. Roche (Ireland)

As I mentioned before, we have timed ovulation following a double injection of ICI 80,996 and essentially 30% have ovulated 78 hours after the second injection and 60% of the animals have ovulated at 92 hours after the second injection. The interesting thing is that essentially all of the animals have ovulated at 96 hours. We have repeated this in two different batches of animals and apparently 35 to 40% of the heifers are ovulating between 92 and 96 hours. This emphasises, as Dr. Cooper has said, the need for two inseminations. My question is, do you think that 96 hours is the

right time for the second insemination if 30% of the animals are ovulating between 92 and 96 hours?

M.J. Cooper

Well, I have answered this question before to you. I am convinced that 72 hours is the right time for the first insemination but I'm not convinced that 96 hours is the right time for the second one, but it is a practical time - it fits in nicely with any sort of managemental regime and we find that, certainly in suckling beef cows and in lactating dairy cows, it is not uncommon to find animals still in heat at 96 hours. So, although you may be getting this pattern of ovulation in heifers, this isn't necessarily repeated in lactating cows. One of the problems that we have identified, and I don't know whether Dr. Nancarrow wants to make any comments on this, is that there is a variability in the response. You can treat one group of animals and check ovulation or the onset of oestrus, and treat them again two or three months later and get a different result. So for the time being we are going to stick with our 96 hour insemination.

R. Church (Canada)

I assume that you are working under optimal environmental conditions and nutritional status. Do you get a wider variation if you do it at a different time of the year, in terms of day length or nutritional status?

M.J. Cooper

I don't think we have got any data on that, quite honestly. These animals were obviously housed and I wasn't trying really to show that LH data to prove a point but just to demonstrate it. In terms of the onset of heat, and in terms of the difference between a single injection and a double injection, we have repeated that over quite a large number of animals and that certainly occurs and has been confirmed, I think, by Dr. Thimonier's group at Nouzilly and also by Dr. Leaver and Dr. Pope. So that is really just a demonstration.

R. Church

The suggestion has been made that animals in a transfer situation that have been stimulated show a much better response to one injection of prostaglandin in coming on to heat than animals that have not been stimulated.

M.J. Cooper

That supports the idea that the second injection in the two injection regime is given at a time when follicular development is occurring. There is no question that if you prime the ovaries you get a quicker response.

I. Gordon (Ireland)

I would like to make a general comment on fixed time AI and the need or otherwise to use two inseminations rather than one. I often wonder how much more could be done from the point of view of the semen quality. In other words, if you were using fresh instead of frozen semen whether you could get away better with the single insemination than with the double. I just wonder whether enough thought is being given to the oestrus synchronisation or the fixed time AI in cattle, whether enough thought is being given to the question of semen quality. I just throw that thought out to you because it is something over which I have often pondered on but never done anything about.

M.J. Cooper

It does seem to be a great unknown area; the viability of the semen, the viability of the ovum and the need, or otherwise, for precision timing of insemination and ovulation.

C. Polge (UK)

Is there anybody who would care to comment on the possibilities who has had experience of using prostaglandin for synchronising recipient animals in transfer? It seems to me that a single injection, although it gives some slight spread in LH release and ovulation time, might be quite acceptable in what we know in plus and minus hours necessary for synchronisation of the recipient.

R. Church

We have had some experience with this and to try to avoid the problem of variability on the second injection (I showed some data yesterday), it is more satisfactory from the recipient point of view to use a single injection of prostaglandin in recipients in which you know what their cycle periocity is and to utilise mid-cycle.

R. Newcomb (UK)

Could I make one or two comments. Firstly, relating to your comment about double injections one day after the other, I believe it is true to say that in Mr. Rowson's original experiments he found that 0.5 mg into the ipsilateral horn on two occasions was more efficient than getting 1 mg on one occasion. I think this was the basic theory behind giving a double injection. I think subsequently Inskeep has also shown that injecting a very tiny quantity of prostaglandin $F_{2\alpha}$ into the largest follicle in the ovary on two occasions is again more efficient in causing luteal regression than giving one injection of the double quantity. In view of this it might be rather interesting, although not commercially very profitable, to look at two daily injections to see whether dosage could be reduced somewhat - not an advantage to the commercial firm but possibly to the user.

The other comment that I would like to make, and perhaps other people could come in on this, I know that you and I have had disputes in the past over the use of your product for synchronisation of oestrus in superovulated donors. Now, these are clinical observations which we make and we don't care to get too many failures in our donor programme because we are using eggs for various other procedures. However, where we use what would normally be a sufficient quantity of $PGF_{2\alpha}$ to cause luteal regression in recipients, if we use the same quantity in donors we do not get the degree of synchronisation that we would like. I would submit that one possibility - and I would like your comments also - is that the PMS itself is acting in a luteotrophic manner and the amount of prostaglandin that may then be necessary might be slightly more than one would normally have to give.

The other comment I would make: I would think one should be rather hesitant in using the double injection routine for donor animals. Certainly I would concede that it would be very useful to use it in recipient animals (and we have done this very successfully) but for the donor animals - and I have no experience in the use of the double injection technique - we have shown that where one injects PMSG prior to Day 9 of the oestrous cycle that we get a significantly lower superovulation response. Now, it is just possible that this may be important if one used the double injection technique with prostaglandin because presumably quite a few of the animals coming up for the second injection would, in fact, be at an earlier stage than Day 9 of the oestrous cycle.

M.J. Cooper

The only comment I have got to make really is about the donor animals. I guess that the definitive way of looking at this would be to examine progesterone changes in these sorts of animals and we may be pre-empting Dr. Nancarrow.

C. Polge

I am afraid that I have got to close the discussion there. Thank you, Dr. Cooper.

CONTROLLED BREEDING OF PROGESTIN TREATED CATTLE

D.F. Wishart
Searle Research Laboratories, High Wycombe

Ever since the demonstration in the 1930s that progesterone would synchronise the oestrous cycle of laboratory rodents, research workers throughout the world have attempted to do the same for farm animals using both progesterone and synthetic progestins. Until recently these steroids have been administered by a variety of routes for approximately 21 days. Such lengthy treatments have been associated with a reduction in fertility at the controlled breeding period. The following abnormalities have been reported as being associated with long-term progestin treatment.

1. Suppression of dioestrus follicular activity.

2. Increased rate of atresia.

3. Growth of large preovulatory follicles devoid of oocytes.

4. Interference with the normal expression of oestrus.

5. Abnormal time relationships of oestrus and ovulation.

6. Altered physicochemical properties of cervical mucus.

7. Failure of fertilisation.

8. Abnormal cleavage rate of the early embryo.

Two potent progestins, SC9880 (17α-acetoxy-9a-fluoro-11B-hydroxy-4-pregnene-3,20 dione) and SC21009 (17α-acetoxy-11β-methyl-19-norpreg-4-ene-20, dione) have been studied extensively in this laboratory. These steroids cause oestrus and ovulation suppression by daily intramuscular injection at dose rates of 2.4 mg and 0.14 mg respectively (Wishart 1972). In a study involving 150 oestrous heifers inseminated by one inseminator using semen from one ejaculate from a Hereford bull (25×10^6 total cell count in 1.0 ml Cassou straws) a surgical attempt was made to collect ova.

TABLE 1 OVUM RECOVERY RATE - BY DRUG, BY DAY OF COLLECTION AND BY OVARY

Drug	Day of Collection.	Successful Recovery			Failure to Recover			Grand Total
		Left Side	Right Side	Total	Left Side	Right Side	Total	
SC9880	3	9	10	19	3	3	6	25
	4	7	15	22	1	2	3	25
	Total	16	25	41 (82.0%)	4	5	9	50
SC21009	3	6	15	21	1	3	4	25
	4	11	10	21	2	2	4	25
	Total	17	25	42 (84.0%)	3	5	8	50
Control	3	14	7	21	-	4	4	25
	4	9	12	21	2	2	4	25
	Total	23	19	42 (84.0%)	2	6	8	50
Grand Total		56	69	125 (83.3%)	9	16	25	150

TABLE 2 FERTILISATION RATE - BY DRUG AND BY DAY OF COLLECTION

Drug	Day of Collection	Number of Ova		Total
		Fertilised	Unfertilised	
SC9880	3	18	1	19
	4	21	1	22
	Total	39 (95.1%)	2	41
SC21009	3	18	3	21
	4	20	1	21
	Total	38 (90.5%)	4	42
Control	3	20	1	21
	4	21	-	21
	Total	41 (97.6%)	1	42
Grand Total		118 (94.4%)	7	125

TABLE 3 CLEAVAGE STAGE OF FERTILISED OVA - BY DAY OF COLLECTION AND BY DRUG

Day of Collection	Drug	No. of Fertilised Ova: Cleavage Stage (No. of Cells) 1	2	4	8	Total
3	SC9880	3	3	10	2	18
	SC21009	-	3	14	1	18
	Control	-	-	14	5	19*
	Total	3	6	38	8	55
4	SC9880	3	1	2	14	20*
	SC21009	-	1	4	14	19*
	Control	-	-	-	20	20*
	Total	3	2	6	48	59
Grand Total		6	8	44	56	114

* indicates 1 fertilised ovum, the cleavage of which could not be classified due to blastomere breakdown

Fifty animals were in oestrus without treatment, 50 after 21 daily injections of 2.4 mg SC9880 and 50 after 21 daily injections of 0.2 mg SC21009. On Day 3 and 4 after oestrus equal numbers were subjected to surgery. The results are presented in Tables 1,2 and 3. It can be seen that treatment did not affect the ovum collection rate nor the fertilisation rate. It appeared that the progestin related subfertility was not caused by failure of fertilisation as has been reported for ewes treated intravaginally with SC9880 (Quinlivan and Robinson, 1967). Treatment with both SC9880 and SC21009 were associated with early cleavage forms which were absent in embryos collected from untreated controls. It is difficult to evaluate the biological significance of this finding other than by relating it to embryo transfer. If the degree of asynchrony (24-72 hours) in development of the embryos from the progestin treated heifers and the maternal endometrium was duplicated in an embryo transfer situation then very low pregnancy rates could be expected to result (Rowson et al. 1972).

The development of a successful progestin based method of synchronising oestrus without adversely affecting pregnancy rates to insemination at the controlled breeding period has been due to three main findings:

1. That short term progestin treatments (9-12 days) do not reduce fertility although many fewer animals exhibit oestrus.

2. That a combination of a luteolytic substance such as $PGF_{2\alpha}$ or oestradiol valerate with a short progestin treatment increases the proportion of animals in oestrus.

3. That 3.0mg·SC21009 administered intravaginally would:
   a) inhibit luteal development in animals in the first few days of the cycle,
   b) potentiate the luteolytic action of oestradiol valerate (Lemon, 1974).

A convenient and simple method of administration of SC21009 and oestradiol has now been developed and is in final stages of evaluation. The treatment consists of inserting a small implant subcutaneously in the ear, superficial to the conchal cartilage. The implant measures 3 x 18 mm and weighs approximately 0.125 gm. Each implant contains 6.0 mg SC21009. At the time of implantation a 2.0 ml injection is given intramuscularly. The injection contains 3.0 mg SC21009 and 5 mg oestradiol valerate. After 9 days in situ the implant is removed by nicking the ear and expressing the implant with the thumb (Plates 1,2,3,4 & 5).

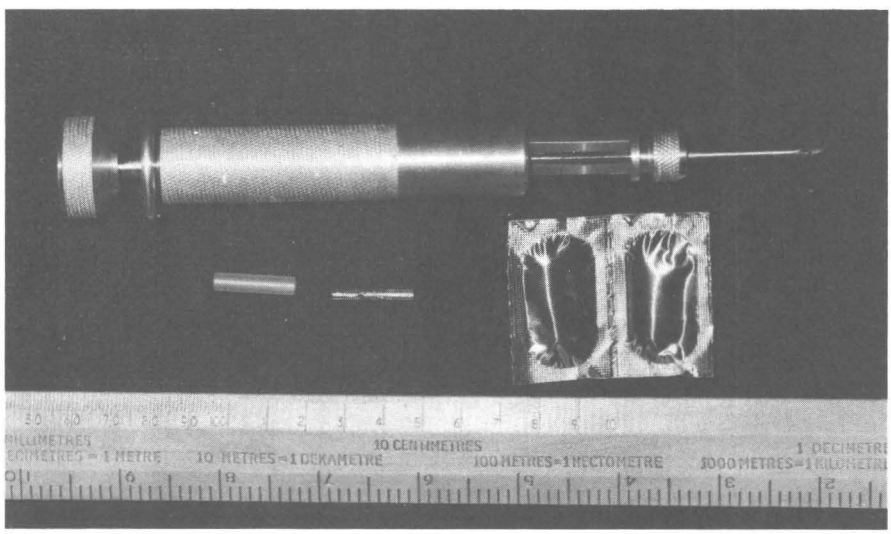

PLATE 1 Implant, protective sheath and implanter

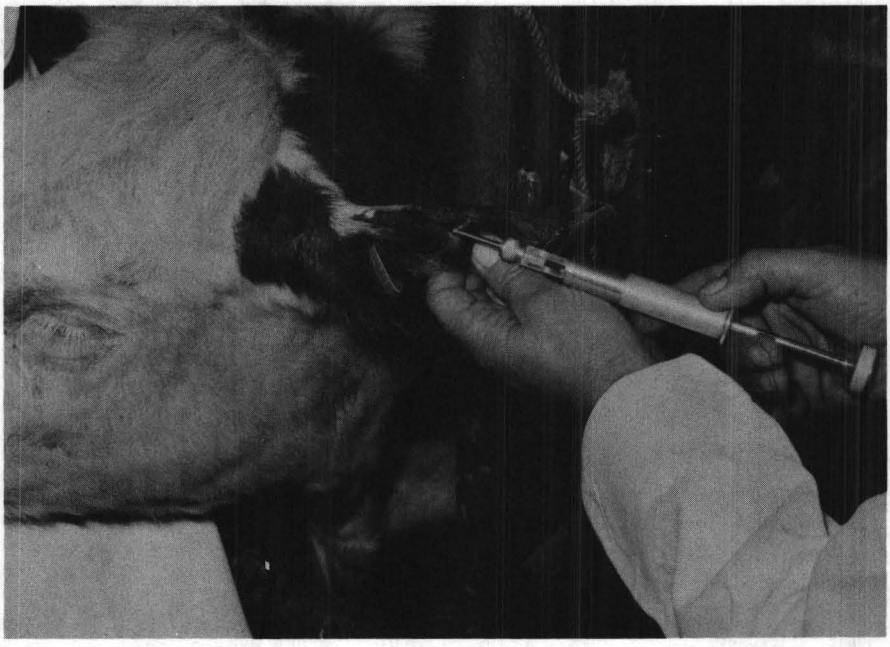

PLATE 2 Loaded applicator - showing the method of needle insertion

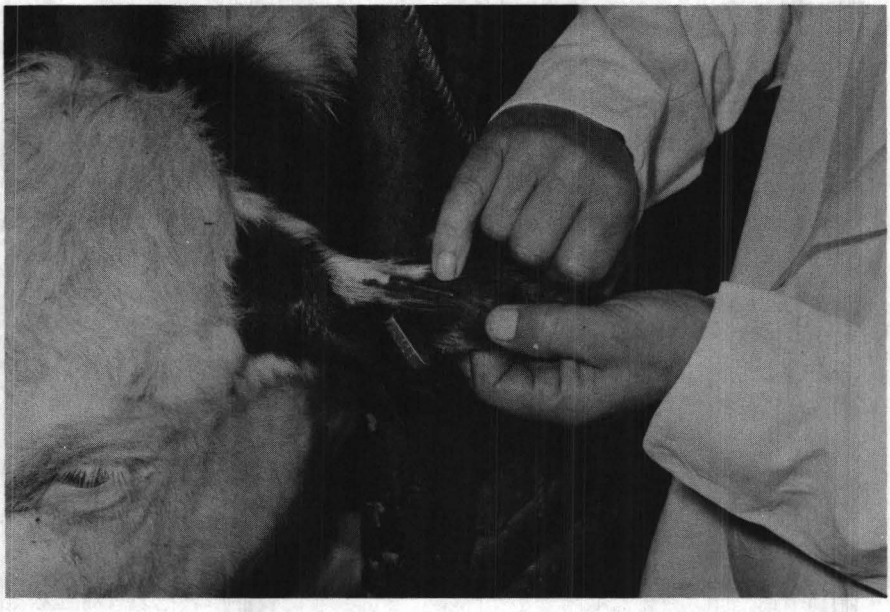

PLATE 3 Correct subcutaneous position of the implant superficial to the conchal cartilage

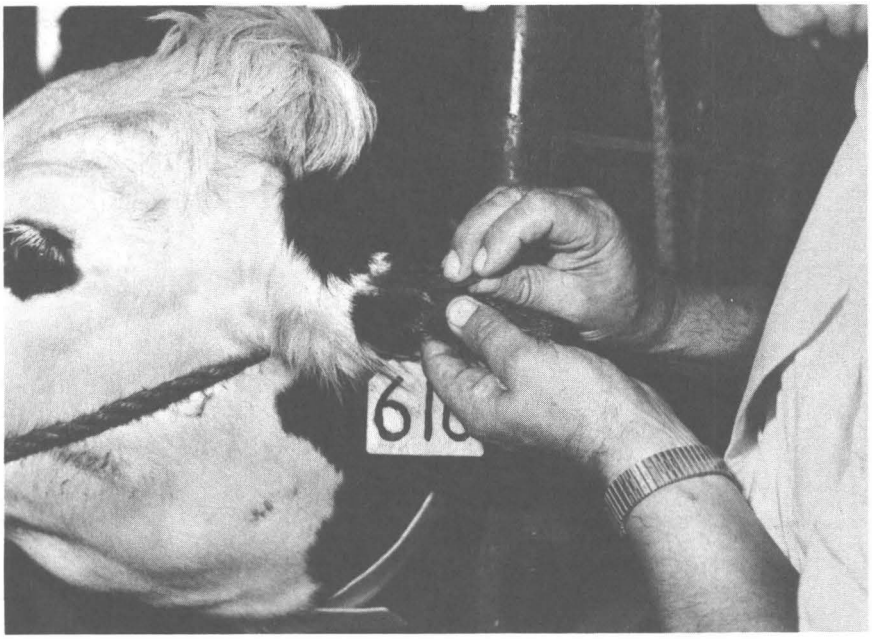

PLATE 4 Nicking the ear at the distal end of the implant

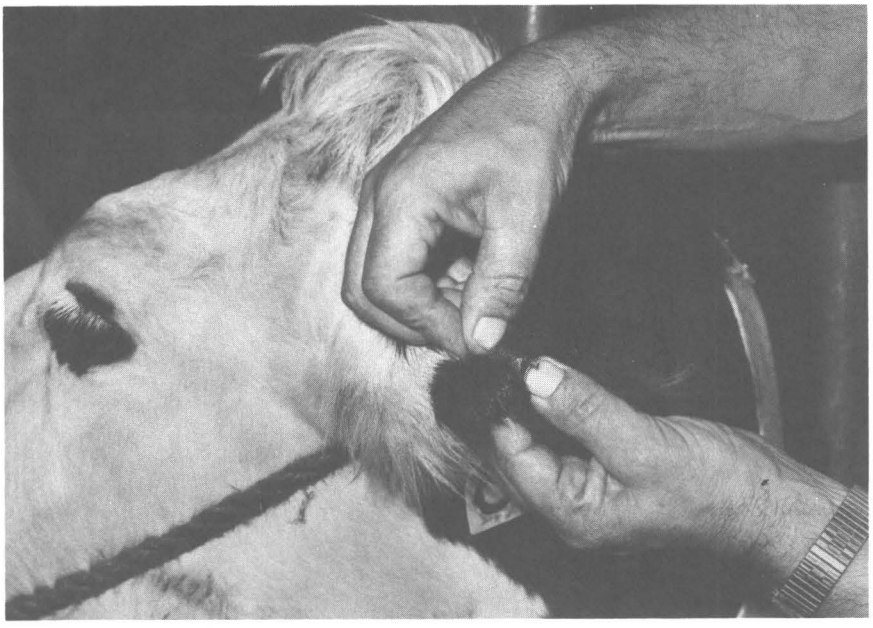

PLATE 5 Expression of the implant between the thumbs

It has been demonstrated (Wishart and Young 1974) that this treatment is not associated with delayed embryo cleavage or with a reduction of fertility at the controlled oestrus. Further, it is known from endoscopic studies that the mean interval from implant removal to ovulation is 68.5 hours (SD 9.7 hours). The precision of ovulatory response follows a precise oestrous response. In two studies involving 350 treated heifers observed every 4 hours, day and night for five days, the numbers in oestrus were: 1; 249; 40; 1 at 0-24; 24-48; 48-72 and 72-96 hours respectively from implant removal. Pregnancy rates have been unaffected by treatment whether the comparison is made with untreated control animals or with the pregnancy rate of those inseminated at the return to service period or with the non-return to service rate of the bull.

A number of different fixed AI timings have been investigated. Insemination in this way avoids errors in oestrus detection which becomes more difficult when the majority of animals are showing marked sexual activity, it also affords the non oestrus ovulating animals the opportunity of becoming pregnant. From a practical viewpoint fixed time insemination at 48 and 72 hours after implant removal provides acceptable pregnancy rates. In field trials in the British Isles involving 5391 heifers and single suckler beef cows, 48.2% of 2242 cows and 58.2% of 2602 heifers became pregnant. Calving results are awaited for a further 547 cattle. Under strictly controlled conditions artificial insemination at 48 and 60 hours after implant removal has resulted in superior pregnancy rates than at 48 and 72 hours. However, the differences in fertility from farm-to-farm are much greater than the differences between the AI timings chosen.

The pregnancy rates obtained by the farmer will depend upon a number of factors, some of which are shown in Table 4. In my view it is essential that an oestrus synchroniser is properly applied at farm level if full benefit is to be obtained by the farmer and consumer alike. This can only be achieved if the synchroniser is a part of a comprehensive controlled breeding programme. There are now a number of effective methods of synchronising oestrus in cattle but there are still no comprehensive farm management systems to accompany their use. Without such systems low pregnancy rates and financial loss are inescapable. In many cases disillusionment with oestrus synchronisation as a managment technique may follow.

TABLE 4 FACTORS AFFECTING ATTAINMENT OF PREGNANCY RATE TARGETS

A. To "By the Clock" Insemination

I. Heifers	II. Suckler Cows	III. Both
Age	Interval from calving	Cyclicity
Weight	Genital Health	Breed
Maturity		Weight Change
AI Timings		Condition Change
		Nutrition (energy)
		Animal to insem-inator ratio
		Bull fertility
		Inseminator Skill
		Acclimatisation
		Mineral Status
		AI Bull Fertility

B. To Remating 18-28 Days After Implant Removal

AI or natural service

Efficiency of oestrus detection

If natural mating - bull health
condition
bull: cow ratio
diet

All factors in A. above

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DISCUSSION

C. Polge (UK)

Well, here we have got another synchronisation technique to discuss. May I start by asking you if you have had any experience of using this type of treatment in anoestrus animals?

D.F. Wishart (UK)

I haven't but there is some data on heifers - not suckler cows - and it is claimed that one can induce oestrus in anoestrus heifers, what one calls prepubertal heifers, and a proportion of these will conceive. I don't know what proportion.

L.E.A. Rowson (UK)

Do you get any infection of the site where you actually puncture it and put in the progesterone implant?

D.F. Wishart

We have lost approximately 0.2% of the implants we have inserted and fewer than 0.1% of the animals have had any infection. The polymer which is used is also used for contact lenses and it absorbs about 30% of its weight of water; it equilibrates quite well with the body fluids.

I. Wilmut (UK)

Could you tell me how long after calving you are treating the suckler animals?

D.F. Wishart

We wouldn't recommend that they be inseminated earlier than 50 to 60 days. Some have been treated a lot earlier than that and had passable conception rates but really the closer one comes to calving the better.

B. Hoffmann (W. Germany)

What kind of plasma levels are you getting with your compound? What is the clearance, and did you try to treat the animals without oestrogen?

D.F. Wishart

I have never tried to treat them without oestrogen with a short

treatment period. As far as the levels are concerned, I am not sure of the absolute level, it is a few nanograms, within three days of the injection, following the base line by about four days.

S.M. Willadsen (UK)

A completely practical thing - why the ear?

D.F. Wishart

Well, I think there are a number of reasons for that, it is easy to get at and also it isn't a part that is commonly consumed.

S.M. Willadsen

It takes two people though.

D.F. Wishart

Well, it certainly takes two people, yes. When we use 9880 in an implant, one requires an implant measuring about half the size of a pencil and we inserted it in the anal fold. Of course, the problem was that we no sooner let the tail go than half the implant was showing out of the incision. It then required a suture and it was quite impractical. From start to finish, when one gets on the farm, one has to insert these at the rate of about one a minute.

C. Polge

Would you care to comment Dr. Sreenan?

J. Sreenan (Ireland)

Yes, David mentioned that approximately 700 of the animals involved were treated in trials with us and that he is awaiting calving data. I have some information here: There were two groups of animals, heifers and suckling cows approximately 45 days interval from post partum. With the heifers we have a calving rate of 54% in control animals. With the SC implants we have a calving rate of 56% and with the 10 day progesterone sponge (with 238 animals involved in this trial) we have a calving rate of 57.5%. So there was no difference between the 9 day implants - in fact, both of them were slightly above the control animals. With regard to the suckler cows, 53% of those treated have calved to a fixed time insemination. This is probably relevant to whether it is possible to induce oestrus in non-cyclic animals.

The suckler cows involved here are not pre-selected in any way so some proportion of them must be non-cyclic. With the progesterone sponges the calving rates to the 10 day treatment was 48%. I think David Wishart was producing figures on pregnancy diagnosis at 6 weeks, of maybe 70% and our calving rates are comparable with that. We are talking about 56% in the heifers and 53% in the cows.

D.F. Wishart

Well, actually I would dispute that because there is a big difference between the 6 week pregnancy data and the calving data. I think if you had examined yours for pregnancy at 6 weeks you would have found maybe 55 or 56% pregnant.

J. Sreenan

Yes, but we are not in the habit of doing PDs because first of all, it is very costly and time consuming, and we also feel that you may do some damage if you are in a hurry and you have got to do a large-scale trial and keep moving round on different farms. The other thing is that when it comes to suckler herds, non return rates and PDs that are passed to us have not always borne a great relation to the calving data. It takes time to collect the calving data but it is the end result of what the exercise is about. In both cases we are doing that much better. We don't have control data for suckler cows; we don't have enough animals that are inseminated without some form of treatment. I wasn't taking issue with your PD figures but the calving figures are 53% for the cows and 56% for the heifers.

D.F. Wishart

Very close to our averages in fact?

J. Sreenan

Yes.

C. Polge

That seems to tie in with many people's figures. Well, I think we must stop there. Thank you very much Dr. Wishart.

SYNCHRONISATION OF OESTRUS IN THE COW WITH PROGESTOGENS AND PROSTAGLANDINS

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I.N.R.A. - Station de Physiologie de la Reproduction
Nouzilly, France

With egg transfer, the necessity for close synchronisation between 'donor' and 'recipient' to achieve high pregnancy rates has been well demonstrated by Rowson et al. (1972). They reported a pregnancy rate of 91% following transfers where the donor and the recipient came into oestrus on the same day. When the donor and the recipient were out of phase by one day, the pregnancy rate decreased to nearly 50%. More recently, Sreenan, Beehan and Mulvehill (1975) confirmed these results with a pregnancy rate of 82% observed when recipient and donor were well synchronised, compared to 65% when the variation was $\pm$ one day from the exact synchronisation. When the donor and recipient are out of phase by one day, not only the pregnancy rate is affected but also the egg survival and twinning rate following bilateral transfer in the cow.

For the method of egg transfer to be efficient, it is thus necessary to have techniques available for the synchronisation of 'donor' and 'receiver' females which are as perfect as possible.

This report deals with methods of synchronisation which might be used in receiver females (methods studied at the Station de Physiologie de la Reproduction of INRA at Nouzilly). The reported experiments concern only cyclic adult and yearling females.

The desired synchronisation of oestrus in receiver females should conform to two criteria:

- a maximum of females in oestrus within a 24 hour (two consecutive detections of oestrus at a 12 hour interval) or 48 hour (four consecutive detections of oestrus) period,

- a constant interval between end of treatment and onset of oestrus.

In addition, after a treatment for synchronisation, the uterus should be in a state which allows development of fertilised eggs.

The control of the sexual cycle in domestic animals is related, in general, to the control of the corpus luteum. The lifespan of the corpus luteum and its secretory activity, are the result of trophic and lytic factors (Denamur, 1973; Goding, 1973). Thus it is necessary either to prevent the formation of a new corpus luteum, or to induce luteolysis in an already existing one.

SYNCHRONISATION OF OESTRUS USING PROSTAGLANDINS

Prostaglandins, in particular $PGF_{2\alpha}$ and its analogues, induce luteolysis in an existing corpus luteum but have no effect on the corpus luteum during the first five days of the cycle (Rowson, Tervit and Brand, 1972; Lauderdale, 1972; Thimonier et al. 1974). To overcome this difficulty, a treatment has been described by Cooper (1974) and Thimonier et al. (1974) consisting of two injections of a prostaglandin analogue (ICI 80996) separated by a period of 10 to 12 days.

In an experiment to control the efficiency of such a treatment, 79 FFPN cyclic cows and heifers received two intra-muscular injections of 500µg of the analogue ICI 80996, 10 days apart. 31 females received the first injection between days 1 and 5 of the cycle and 48 between days 6 and 19 (Table 1).

Detection of oestrus was carried out twice a day with vasectomised bulls and androgenised cows (Signoret, 1975).

43% of the treated females were in oestrus during a given period of 24 hours and 70% during a 48 hour period. In total, 82% of the treated females came into oestrus within 5 days after the second prostaglandin analogue injection. Silent ovulations, inefficiency of the second injection because of a too short interval, long follicular phase, were the reasons for the lack of synchronisation in some cows.

The onset of oestrus after the second prostaglandin analogue injection depended on the efficiency of the first injection to induce luteolysis. When the first was ineffective (days 1 to 5), only 16% of treated females came into oestrus during the first 60 hours after the second injection, compared to 66.6% ($P < 0.01$) when the first injection induced luteolysis (days 6 to 19).

Thus, the relatively low degree of synchronisation observed with this treatment can be explained by variations in the interval from the second injection to the onset of oestrus depending on the time at which treatment was applied. Follicular growth waves (Mariana and Nguyen Huy, 1973) during the cycle are probably responsible for the variation in the onset of oestrus.

Such variations in the onset of oestrus have not been found by other workers (Cooper and Rowson, 1975). This may be due in part to a different interval between the two injections and to differences in the management of the herd or in methods of detection of oestrus,

TABLE 1 SYNCHRONISATION OF OESTRUS AFTER THE SECOND PROSTAGLANDIN ANALOGUE INJECTION

Treatment 2 x 500µg ICI 80996 - 10D	No. of Females	Maximum in oestrus % in a period of		% in oestrus after the end of treatment	
		24h	48h	during the first 60h	within 5 days
First injection Days 1 & 5	31	48.4	71.0	16.1+	90.3
between Days 6 & 19	48	64.6	77.1	66.6+	77.1
TOTAL	79	43.0	68.4	46.8	82.3

+ $P < 0.01$

SYNCHRONISATION OF OESTRUS IN CYCLIC COWS AND HEIFERS WITH PROGESTOGENS

Long progestogen treatments have been shown (Thimonier, Chupin, Pelot, 1975) to be associated with high synchronisation rate but low fertility. This may be due partly to modification of the uterine environment. So it seems necessary to avoid them for use in egg transfer experiments.

Short progestogen treatment in association with an oestrogen administration

The administration of one of a number of synthetic progestogens, or progesterone for a short period (8 to 10 days) in association with a single injection of the oestrogen, oestradiol valerate, has become a classical treatment for the synchronisation of oestrus in cyclic cattle. The 'luteolytic effect' of oestradiol valerate was mentioned by Wiltbank (1966).

In our laboratory, the most widely used progestogens are Norethandrolone (Nilevar) and SC 21009 (Norgestomet, Searle). A short treatment with one of these two progestogens in association with an intramuscular injection of 5mg of oestradiol valerate at the beginning of treatment gives a variable synchronisation. It seems (Lemon, 1975) that the effect of the oestrogen injection is not the same in combination with norethandrolone and norgestomet as shown in Table 2. With SC 21009, the inhibitory or anti luteotrophic effect of oestradiol valerate is more pronounced and cycle lengths are markedly reduced. (Lemon, 1975).

In small groups of cyclic females treated with SC 21009 implants plus an injection of oestradiol valerate on the first day of treatment, the synchronisation of oestrus varies from about 55% females in oestrus within 48 hours to nearly 100%. These variations can be explained by variations in the proportions of females treated during the first days of the cycle from one experiment to another one.

The extreme variability of the percentage of females in oestrus within a 24 or 48 hour period does not allow a prediction of the number of receiver females which will be synchronised for a given number of donors.

TABLE 2 SYNCHRONISATION OF OESTRUS IN COWS AND HEIFERS RECEIVING A SHORT PROGESTOGEN TREATMENT BEGINNING ON DAYS 1-4 OF THE CYCLE

Treatment	No. of Females	In oestrus within 6 days after the end of treatment	
		No.	%
Norethandrolone: 7mg/day i.m. for 10 days + 5mg E.V. (1)	25	9	36+
SC 21009 Implants 12mg for 10 days + 5mg E.V. (1)	18	13	72+

+ $P < 0.05$ (1) On the first day of treatment

Short progestogen treatment in combination with a prostaglandin injection

The objective is to obtain synchronised oestrus during short and predetermined intervals without taking into account the stage of the cycle

at which treatment begins and also to obtain fertility rates similar to normal. A prostaglandin injection at the end of a short progestogen treatment may result in luteal regression in those females with an active corpus luteum, thus allowing a high degree of synchronisation (Thimonier et al. 1974; Lauderdale, 1975).

In the first experiment 79 cyclic cows and heifers received a subcutaneous implant of 12mg of SC 21009 for 10 days plus an intramuscular injection of the prostaglandin analogue (ICI 80996) at implant removal.

Detection of oestrus was carried out in the same manner as the experiment with the two prostaglandin analogue injections.

The results obtained with this treatment are comparable to those obtained with the 2 injections of prostaglandin $F_{2\alpha}$ analogue at an interval of 10 days. In fact, 40% of treated females were in oestrus during a given period of 24 hours and 73% during a 24 hour period. In total, 94% of the treated females came into oestrus within 5 days after the end of treatment.

However, when treatment began during the first half of the cycle, the interval from the end of treatment to the onset of oestrus was long (72 to 96 hours). It was shorter (48 hours) when the treatment started during the second half of the cycle : analysis of progesterone shows that luteal regression in the latter case is complete before the end of the progestogen treatment.

Thus, when the treatment began during the first half of the cycle, only 28% of cows and heifers were in oestrus within 60 hours after removal of SC 21009 implants, compared to 80% (P 0.01) when the treatment commenced during the second half of the cycle (Table 3). This variation in the interval from the end of treatment to the onset of oestrus is thus an unfavourable aspect for obtaining a high degree of synchronisation.

For an increase in the degree of synchronisation two methods have been tested:

(i) Use of PMSG at the end of treatment : in sheep an intramuscular injection of PMSG at the end of a progestogen treatment increases the degree

TABLE 3 SYNCHRONISATION OF OESTRUS AFTER REMOVAL OF SC 21009 IMPLANTS AND INJECTION OF 500 µg OF ICI 80996 (DURATION OF TREATMENT - 10 DAYS)

Treatment 12 mg SC21009 Implants + 500 µg ICI 80996		No. of Females	Maximum in oestrus % in a period of		% in oestrus after the end of treatment	
			24h	48h	During the first 60h	within 5 days
Implants inserted between	Days 1 & 9	44	50.0	65.9	27.7+	90.9
	Days 10 & 19	35	71.4	82.9	80.0+	97.1
TOTAL		79	40.5	73.4	48.1	93.7

+P < 0.01

TABLE 4 EFFECT OF PMSG AT THE END OF A SHORT PROGESTOGEN TREATMENT IN COMBINATION WITH A PROSTAGLANDIN ANALOGUE INJECTION ON THE SYNCHRONISATION OF OESTRUS IN CYCLIC HEIFERS

Treatment	PMSG (iu)	No. of females	Maximum in oestrus % in a period of		% in oestrus within 84h after the end of treatment
			24h	48h	
6mg SC 21009 for 9 days + 500 µg ICI 80996 at implant removal	0	38	42.1	76.3	86.8
	600	37	70.3	97.3	97.3

TABLE 5 ONSET OF OESTRUS IN CYCLIC COWS AND HEIFERS RECEIVING A PROSTAGLANDIN ANALOGUE INJECTION (ICI 80996), 2 DAYS BEFORE (R-2) OR AT THE END (Ro) OF A 9 DAY PROGESTOGEN TREATMENT (SC 21009)

Time of Prostaglandin Injection	No. of Females	Maximum in oestrus % in a period of		% in oestrus after the end of treatment	
		24h	48h	During the first 60h	within 5 days
R - 2	76	65.8	86.8	86.8	93.4
Ro	89	57.3	74.2	59.5	96.6

of synchronisation and reduces the interval end of treatment. Onset of oestrus, allowing only one AI at a predetermined time without detection of oestrus (Colas, 1975).

75 cyclic heifers of the FFPN breed received 6mg of Norgestomet for 9 days and 500µg of the ICI 80996 compound at implant removal.

37 of them received also 600 i.u. of PMSG at the time of prostaglandin analogue injection.

Detection of oestrus was then done twice a day for 4 days.

As already observed in the sheep, a PMSG injection at the end of the progestogen treatment in association with a PG injection, reduces the interval to the onset of oestrus and increases the synchronisation : 70% of treated females were in oestrus within a given 24 hour period (36 and 48 hours after the end of treatment) (Table 4).

Such a treatment was applied to several breeds: 63% and 69% respectively of Friesian and Norman cows receiving this treatment were in oestrus during the same 24 hour period (36 and 48 hours after the end of treatment).

This treatment thus appears to conform to the two criteria mentioned earlier: a high percentage of females in oestrus in a 24 hour period, and a constant interval between the end of treatment and the onset of oestrus.

(ii) Modification of the time of injection of the PG analogue injection to increase the degree of synchronisation. The observation that the delay between the end of the progestogen treatment and the onset of oestrus was shorter when no endogenous progesterone was detectable at the end of the progestogen treatment (Thimonier, Chupin, Pelot, 1975) led us to administer the PG analogue before the end of the progestogen treatment.

165 FFPN cows and heifers, regardless of the stage of their cycle, received a progestogen treatment of SC 21009 (12mg or 6mg plus 3mg at the time of implant insertion) for 10 days; 76 of them received 500 µg of the analogue ICI 80996 2 days before the end of treatment, the 89 others at the time of implant removal. Oestrus was checked twice daily.

Synchronisation of oestrus for the two groups was different. 87% of the females receiving the PG analogue 2 days before the end of treatment were in oestrus during the first 60 hours compared to 59.5% for the other group ($P < 0.01$) (Table 5) and the percentage of females in oestrus during a 24 hour period were respectively 66% and 57%.

Thus, a prostaglandin analogue injection before the end of the progestogen treatment increases the degree of synchronisation. This treatment appears also to conform to the two criteria mentioned earlier.

CONCLUSIONS

The aim of the different experiments we have reported here was to find a method of giving a high degree of synchronisation in herds of cyclic cows. This would allow a single artificial insemination without detection of oestrus, or egg transfer, in groups of females. Our purpose here was not to check the fertility following such treatments. Different treatments have been checked for synchronisation of oestrus, and although the pregnancy rates are unknown after egg transfer in recipient cows prepared by these treatments, fertility is known after artificial insemination with detection of oestrus. Fertility was similar in all groups, and nearly the same as in controls when comparing the same herds in the same environment. It varied from 40% of females pregnant after AI at the synchronised oestrus, to 80%, depending on age. The fertility in heifers is generally higher than in multiparous milking cows.

In conclusion, it appears that efficient techniques are now available for synchronisation of oestrus, but they must be tested for fertility in experiments involving egg transfer.

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DISCUSSION

C. Polge (UK)

Further permutations to discuss - Dr. Roche.

J.F. Roche (Ireland)

Dr. Thimonier, is there an advantage in using the treatment described by Dr. Wishart, that is the oestradiol valerate plus progestogen at the beginning, and the nine day implant period, in comparison to the treatment that you have described - the nine or ten day implant period with prostaglandin added two days before the end of treatment or at the end of treatment?

J. Thimonier (France)

In France we prefer using the Dr. Wishart treatment in nursing cows. We have only a small number of females suckling and oestradiol valerate induces luteolysis in those cows - and also has an effect on fertility. We have shown it increases fertility, but in suckling cows it is not sufficient to have a high degree of synchronisation on big numbers of females and to be able to do artificial insemination without detection of oestrus. So we prefer to do prostaglandin analogue injection two days before the end of treatment.

D.Wishart (UK)

Dr. Thimonier, have you ever found, as we have here, that the cows of the Charolais breed, or the cross bred Charolais animals, conceive less readily in a synchronised situation using a progestin?

J. Thimonier

We are just beginning our experiments with the Charolais this year and we have a fertility, at the induced oestrus, without the detection of oestrus, with two AI 48 hours and 72 hours after implant removal of less than 50% and I think it is lower than in other breeds but we don't know the reason. Perhaps we can increase the fertility by other experiments.

B. Hoffmann (W. Germany)

Did you quantify the luteolytic effect of the oestrogen by measuring progesterone and peripheral plasma?

J. Thimonier

Oh yes. When you inject the oestradiol valerate it depends on the stage of the cycle. When you inject at the beginning of the cycle in combination with SC 21 009, in most of the cows you have an abnormal pattern of endogenous progesterone for a short time - perhaps five days - and then no endogenous progesterone but it does not always work in the same manner.

R.A. Bouters (Belgium)

Does the addition of 600 units of PMSG have any effect on the number of ovulations?

J. Thimonier

No, in that experiment we injected 600 international units but the twinning rate was not increased. It is well-known in sheep that a small quantity of PMSG increases the degree of synchronisation without having an effect on twinning rates. We tried the same in the cow.

C. Polge

Would Dr. Cooper care to comment on the rather long delay in the onset of oestrus in the two prostaglandin injections reported by Dr. Thimonier when the animals were treated between Days 1 to 5 to begin with?

M.J. Cooper (UK)

Well, I suppose this relates back to the other data - that these animals are, presumably, going to be anything up to Day 15 of the cycle at the time of the second injection of prostaglandin. The majority of animals are going to show heat between the two injections and if there is a benefit in injecting them early in the cycle when they have got this follicular development that is going to be lost in this particular group. But, as I say, although that is a very nice theory it seems to be difficult to substantiate.

C. Polge

Thank you very much Dr. Thimonier.

FACTORS INFLUENCING OESTRUS SYNCHRONISATION RELATIVE TO SUPEROVULATION AND EGG TRANSFER

C.D. Nancarrow and W.J.B. Miller

C.S.I.R.O. Division of Animal Production
Ian Clunies Ross Animal Research Laboratory, Prospect
and Department of Veterinary Surgery, University of Sydney

INTRODUCTION

Transplantation of fertilised ova from genetically superior donor cows to herd recipients necessitates several skilful manoeuvres involving both anatomical and physiological techniques. For success, it is necessary for the donor to be superovulated, ova to be fertilised, embryos to be collected and transferred to recipients, and the hormonal status of the donor and recipient cows to be matched. In this paper, methods of synchronising the hormonal status in both donor and recipient cattle are investigated.

A reasonable degree of synchronisation of oestrus can be obtained by shortening the luteal phase of the cycle with prostaglandin $PGF_{2\alpha}$ (Rowson Tervit and Brand, 1972) or its analogues (Tervit, Rowson and Brand, 1973; Hearnshaw, Restall, Nancarrow and Mattner, 1974; Cooper, 1974) or by extending the length of the cycle and inhibiting ovulation by implants of progestational steroids (Roche, 1974; Wishart and Young, 1974). Although useful in controlling oestrous cycles for artificial insemination purposes, neither of these methods allows a predictable oestrous response for individual animals. Despite a rapid and synchronous decline in circulating progesterone (P) concentration following PG administration, (Nancarrow, Radford, Connell and Mattner, 1974), oestrus may occur up to 5 days later. As oestrus and the preovulatory LH release are both functions of endogenous oestrogen and related normally in PG treated cows (Nancarrow et al., 1974), it is reasonable to suggest that asynchrony in the onset of oestrus arises from variations in the mechanism controlling follicular growth and oestrogen synthesis. The question therefore arises as to how growth of the definitive follicle(s) can be manipulated to enable oestrus in both the donor and recipient cows to be accurately predicted.

Figure 1 indicates 6 points at which the end response, ovulation, may

be controlled upon removal of the source of P. It is our philosophy that the more remote from ovulation the event at which manipulation is achieved, the greater the chance of normal hormonal status at the time of ovulation and fertilisation (donor) or at transplantation (recipient). We have chosen to investigate points 3,4 and 5 at the present time.

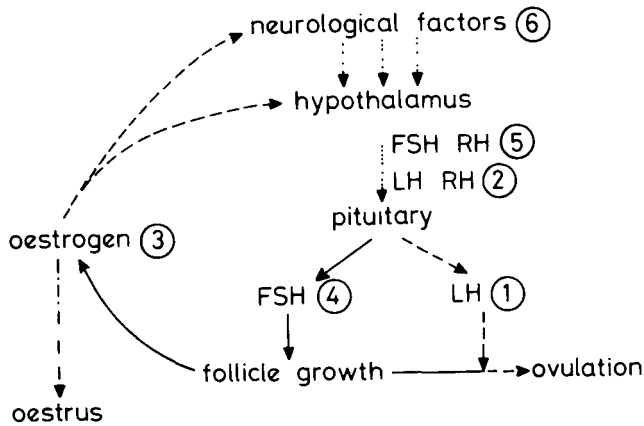

Figure 1 Neural and hormonal control of oestrus and ovulation

METHODS

Two series of experiments have been carried out. In the first, oestrous response and ovulation rate (O.R.) were determined in donor Hereford cows given 2500 I.U. PMSG (Folligon, Organon Laboratories Ltd.) on either day 10 or 16 of the oestrous cycle, followed by PG analogue injection (ICI 80996, 800 μg) either 24 (see Table 2) or 48 hours later (Treatment 1, T). The same regime was followed during either the next cycle or that succeeding an intervening cycle (Treatment 2, T). P concentrations were assayed by the CPB method (Thorburn and Schneider, 1972) in plasma collected at the time of PMSG and PG treatments, 24 and 48 hours following PG, and on Days 2 and 5 following the induced oestrus. Ova were collected via midline laparotomy under general anaesthesia on Day 6.

In the second series of experiments aimed at synchronising the recipients, attempts were made to stimulate follicular growth by administration of either PMSG (765 I.U.) or GnRH (ICI Ltd., Aust., 250 ug) simultaneously with PG, or to stimulate oestrus and LH release by oestrogen (oestradiol benzoate, ODB, 500 µg) or testosterone (380 µg) injected 28 hours after PG treatment. The effect of TRH (250 ug) on luteal function was also considered.

Animals were checked constantly for their ability to stand in heat, while critical blood samples were collected for P, LH and prolactin assays.

RESULTS AND DISCUSSION

Treatment of donor cattle.

The data presented in Table 1 show that circulating P concentrations increased during the period between PMSG injection on Day 10 of a normal cycle and PG injection. This also occurred when 3 cows with multiple corpora lutea (CL) (mean O.R. = 7.3) were treated although the group (T_{2-}*) response was not significant ($P > 0.1$). The data suggest that this particular preparation of PMSG was mildly luteotrophic, however the lack of specific controls and the possibility that the CL may not be functioning maximally at Day 10 denies a firm conclusion.

TABLE 1 EFFECT OF PMSG ON CIRCULATING PROGESTERONE CONCENTRATION (mean ng/ml $\pm$ S.E.).

Day of treatment	Intervening cycle*	Treatment at which blood sampled		Significance level
		PMSG	PG	
10 (13)	+	6.4 $\pm$ 0.7	9.7 $\pm$ 1.2	$P < .01$
16 (18)	+	5.7 $\pm$ 0.7	6.4 $\pm$ 1.1	$P > 0.5$
10 (3)	-	40.8 $\pm$ 13.2	74.9 $\pm$ 31.0	$P > 0.1$
16 (6)	-	20.8 $\pm$ 9.8	33.5 $\pm$ 20.5	$P > 0.2$

Figures in parentheses are number of cows.

* In this and other tables, and in the text, (-) indicates that T_2 was given during the cycle immediately following T_1, and (+) indicates that the cows experienced one untreated cycle between T_1 and T_2 (e.g. T_{2-} and T_{2+}).

Table 2 contains data relating to the O.R. response to PMSG given on Days 10 or 16, and the timing of the oestrous response to PG administered 1 or 2 days later. P concentrations in most animals bled 48 hours after PG and which had been in oestrus by 120 hours were 0.86 $\pm$ 0.09 (n = 21) which satisfies the criterion of PG efficacy (Nancarrow et al., 1975). The exceptions were 5 animals (T_{2-}) whose O.R.s varied between 3 and 27. The mean O.R.s for cows treated a second time on Days 10 or 16 (T_{2-}) were 5.3 and 6.7, respectively and for the remainder (T_1 plus T_{2+}) they were 7.1 (Day 10) and 10.2 (Day 16). These means did not differ, nor was there difference ($P > .1$) between the treatment means (for Days 10 + 16) of 9.8 $\pm$ 1.9 (T_1) and 6.5 $\pm$ 1.9 (T_{2+}). The low correlation co-efficient ($r = -0.055$) between the peripheral concentration of P at the time of PMSG injection and O.R. indicated that neither the day of the cycle nor the P status of the animals were critical.

TABLE 2 OCCURRENCE OF OESTRUS, AND OVULATION RATE IN COWS TREATED FOR TWO CYCLES ON DAYS 10 OR 16 WITH PMSG AND FOLLOWED BY PROSTAGLANDIN

	Cow	Day of PG administration	Intervening cycle	Treatment 1 OES*	Treatment 1 O.R.	Treatment 2 OES*	Treatment 2 O.R.
Day 10	49	11	-	36	8	120	0
	23	12	-	60	8	48	2
	24	12	-	48	6	72	14
	2	12	+	48	3	56	1
	18	12	+	48	6	46	7
	43	12	+	36	22	56	1
	47	12	+	36	20	-	1
	37	12		60	2		
	45	12		48	7		
Day 16	4	17(T_1),18	-	36	3	43	11
	42	17(T_1),18	-	36	1	43	8
	1	18	-	56	2	120	4
	14	18	-	48	27	120	4
	48	18	-	56	1	120	1
	50	18	-	48	1	41	12
	8	18	+	24	23	-	-
	32	18	+	48	3	47	9
	35	18	+	48	22	40	33
	41	18	+	24	17	54	4
	61	18	+	36	22	24	4
	44	18	+	90	4	24	1
	36	17		45	7		

* Time (hours) from administration of PG (ICI 80996, 800 ug) until oestrus was detected.

PMSG 2500 I.U.

When the onset of oestrus was considered (Table 3) it appeared that greater synchrony, hence predictability, was achieved if an intervening cycle was included between successive treatments ($T_1 + T_{2+}$ vs T_{2-}, $P < .001$). This fact suggested that the concentration of progesterone present at the time of PG injection may influence the time to the detection of oestrus. The regression equation was $y = 42.338 + 0.595\ x$ ($r = 0.692$, $P < .001$). It must be emphasised, however, that these cattle were treated with PMSG prior to PG and this relationship may not hold for recipient cows.

TABLE 3 EFFECT OF INTERVENING CYCLE ON TIME (HOURS) BETWEEN PROSTAGLANDIN INJECTION AND OESTRUS DETECTION

Day of treatment		Intervening Cycle +	Intervening Cycle -
10	mean ± SE (n)	48.2 ± 2.5 (12)	80.0 ± 21.2 (3)
	range	30 - 60	48 - 120
16	mean ± SE (n)	43.6 ± 3.8 (18)	81.2 ± 17.4 (6)
	range	24 - 90	41 - 120
Total	mean ± SE (n)	45.4 ± 2.5 (30)	80.8 ± 12.8 (9)
	95% C.I.	40.5 - 50.3	55.7 - 105.9
Data groups		$T_1 + T_{2+}$	T_{2-}

TREATMENT OF RECIPIENT CATTLE

The lack of knowledge of the neural factors which may regulate follicular growth, thus controlling oestrogen synthesis, oestrus and ovulation at present prevents manipulation of hypothalamic function as an aid in synchronisation of oestrus. Follicular growth may be directly stimulated as demonstrated above and 2500 I.U. PMSG resulted in satisfactory synchronisation of oestrus induced by injection of PG. The data presented in Table 4 show that a closer synchronisation was also obtained ($P > 0.1$) when 765 I.U. PMSG was injected simultaneously with the PG. Unfortunately, the range in onset of oestrus is still too large to enable reliable prediction to be made and this technique is not adequate for synchronising cattle for commercial AI purposes. However, it does indicate that if and when a suitable FSH releasing hormone becomes available (see below), treatment concurrent with PG may be of great practical significance.

TABLE 4 EFFECT OF PMSG ON TIME (HOURS) FROM PROSTAGLANDIN INJECTION TO ONSET OF OESTRUS IN CATTLE

Treatment	PG	PG + PMSG
n	14	14
range	51 - 130	47 - 93
mean $\pm$ S.E.	78.9 $\pm$ 5.5	67.2 $\pm$ 3.0

PMSG, 765 I.U., administered simultaneously with PG (ICI 80996, 500 µg).

At present, preparations of GnRH are unsuitable for use in stimulating growth of the definitive follicle. Administration of 250 ug simultaneously with PG in mid-cycle caused an increase in the mean time of onset of oestrus (Table 5).

TABLE 5 EFFECT OF GnRH OR TRH ADMINISTERED CONCURRENTLY WITH PROSTAGLANDIN ON TIME OF OCCURRENCE OF OESTRUS

Hours from PG	PG + GnRH	PG	PG + TRH
44 - 48	0*	2	2
49 - 56	0	4	3
68 - 72	0	5	6
73 - 80	1	5	7
94 - 96	3	7	8
120	5	9	8
144	7	9	8
Mean (hours) $\pm$ S.E.	114.1 $\pm$ 9.4	79.1 $\pm$ 9.8	68.0 $\pm$ 5.9

* Cumulative number of animals detected in heat following PG injection.

PG ICI 80996, 500 µg; GnRH 250 µg; TRH 250 µg

The most likely explanation of this observation is that the released LH luteinised all growing follicles, preventing the action of released FSH, thus delaying the development of a definitive follicle. Alternatively, an injection of TRH which released prolactin in these animals (A.L. Wallace, personal communication), did not affect the normal expression of oestrus. Neither the LH released by GnRH, not the prolactin (and TSH) released by TRH supported the CL against the luteolytic action of PG (Figure 2).

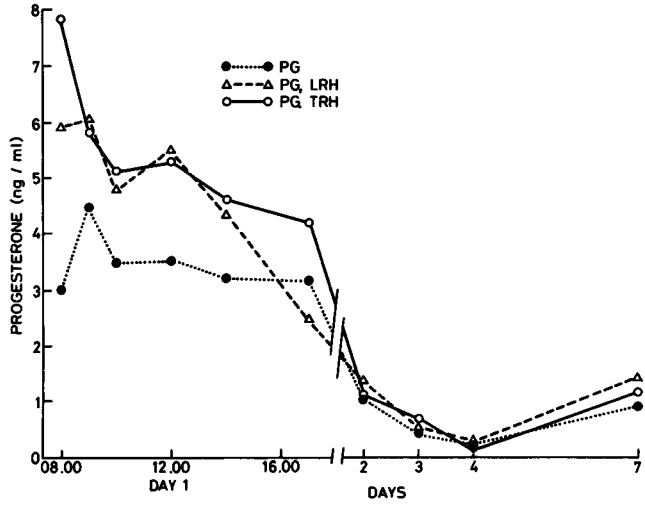

Figure 2 Progesterone concentration in peripheral plasma following injection of PG ± GnRH (LRH) or TRH

The last method used to regulate the synchronisation of oestrus involves the induction of the LH peak, and of oestrus itself, with ODB. This steroid was administered precisely 28 hours following PG to produce a synchronised series of LH peaks (Nancarrow and Radford, 1975) as shown by the data summarised in Table 6. This method has been repeated in this laboratory many times, always with similar results, which enables prediction of oestrus in most of the animals to begin between 48 and 56 hours following PG. On the other hand, stoichiometric quantities of testosterone administered 28 hours after PG produced no effect on the onset of oestrus.

TABLE 6 SYNCHRONISATION OF OESTRUS WITH OESTRADIOL BENZOATE FOLLOWING PROSTAGLANDIN INJECTION

Parameter	PG	PG + ODB	P
Onset of oestrus	74.6 ± 7.0	52.9 ± 2.4	$< .01$
range	44 - 100	47 - 67	
LH peak	77.3 ± 7.0	54.3 ± 1.6	$< .005$
range	48 - 104	48 - 60	

Mean hours following PG injection ± S.E. : PG ICI 80996, 500 µg.
ODB 500 µg administered 28 hours after PG
(Data from Nancarrow and Radford (1975) with permission).

CONCLUSIONS

Follicular growth can be stimulated in mid-cycle by PMSG and successful collection of ova can be made provided the oestrous cycle is interrupted with PG.

Superovulation of cattle is possible in 2 subsequent cycles with or without an intervening cycle. If consequent cycles are employed, oestrus synchronisation induced by PG following the second treatment is more variable, apparently due to the higher P production by the multiple CLs.

Synchronisation of oestrus in recipient cows is best achieved by injection of PG followed 28 hours later by ODB. This produces a full oestral response at a predictable time. Methods are not yet available for precise synchronisation following a single hormonal treatment.

ACKNOWLEDGEMENTS

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APPENDIX

PROGESTERONE CONCENTRATION (ng/ml) IN PERIPHERAL PLASMA DURING TREATMENT AND FOLLOWING OESTRUS

Cow	TREATMENT 1 PMS	PG	D2	D5	O.R.	TREATMENT 2 PMS	PG	D2	D5	O.R.
Day 10										
49 } No	10.2	16.0*	1.4	14.6	8	67.2	136.8	1.3	1.1	(0)
23 } cycle	7.6	16.8	1.9	24.4(4)	8	28.8	45.6			2
24 }	5.6	6.7	0.6	6.7	6	26.4	42.2	1.8(3)	26.6(6)	14
2	2.7	3.6	0.3	1.9	3	5.1	7.2	0.5	1.4	1
18	6.0	4.7	0.9	4.2	6	4.1	10.5	1.3	17.7	7
43	7.6	14.9	2.7	26.0	22	4.0	6.0	-	3.2(6)	1
47	4.6	7.6	1.2	3.2	20	6.3	9.4	2.1	5.4	1
37	7.6	8.2	3.2	4.7	2					
45	11.4	14.8	1.9	13.4	7					
Day 16										
4 } No	3.0	3.4*	2.6(3)	4.3	3	5.3	6.7	2.9	11.9	11
42 } cycle	4.8	2.1*	0.8(3)	1.4	1	5.5	7.6	0.8	8.0	8
1 }	4.9	7.8	0.6	2.9	2	25.0	31.0	1.2	9.8	4
14 }	9.4	13.3	2.2	36.0	27	67.2	134.4	2.8	14.9	4
48 }	6.4	3.7	0.2	1.5	1	7.4	8.0	2.2	7.0	1
50 }	6.0	13.3	0.3	5.5	1	14.4	13.3	0.8	14.4	12
8	3.6	1.8	0.9	16.5	23	1.3	1.3			
32	3.3	5.6	1.7	40.0	3	3.2	10.5	2.3	13.6	9
35	3.2	5.5	0.3	1.4	22	6.8	13.8	2.9	60	33
41	10.3	3.4	1.4(3)	10.5(6)	17	7.8	13.2	2.1(3)	12.4(6)	4
61	12.6	2.0	1.9	5.2(6)	22	4.9	7.4	1.4(3)	8.4(6)	4
44						3.3	1.0			1
36	7.0	5.3*	-	3.8	17					

* PG given one day after PMSG

DISCUSSION

J.R. Roche (Ireland)

Does the oestradiol benzoate administered after the prostaglandin have an effect on fertility?

C.D. Nancarrow (Australia)

It depends. We're in the act of assessing a lot of data at the moment so I can't give you a positive answer. Looking at some of the data, provided the animals are inseminated with respect to oestrus the same as a normal group, it does not appear to - but if we utilise this technique in the field for the normal insemination of beef herds we get a very variable type of pregnancy rate. Sometimes it is as good as a control group, sometimes it is not quite as good. At the moment we are assessing that to see how useful this technique would be in the large-scale field situation. However, in relation to this Symposium, I would say, yes, they appear to be able to become pregnant if they are inseminated at the correct time and therefore if one is transferring an embryo to them I suggest there is no problem.

B. Hoffmann (W. Germany)

I think the time differences you have observed concerning the onset of oestrus in normal and superovulated animals after using prostaglandin are to be expected. There is a dose dependent relationship. Secondly, the superovulated animals are loaded with progesterone. In our experience quite a bit of progesterone is stored in fat and it takes over to release this progesterone from fat so you have somewhat like an artificial corpus luteum and I think oestrogens need receptor sites to exhibit their action. If an animal is so massively loaded with progesterone for quite a while, it takes some time to replenish the receptor sites.

M.J. Cooper (UK)

Dr Nancarrow, can you just elucidate, I am not quite clear whether you answered Ray Newcomb's earlier question - does luteal regression occur as quickly following PMSG administration in animals which have not previously been superovulated, that is, in cattle with one corpus luteum presumably?

C.D. Nancarrow

I guess so - I can't answer that because we haven't followed progesterone closely.

M.J. Cooper

You did say you had got progesterone say at 24 and 48 hours?

C.D. Nancarrow

At 48 hours, yes - we don't bother about 24 hours now - we use a straight 48 hour because if luteolysis has not occurred the progesterones start rising by 48 hours and therefore they average around 2.5 ng/ml which is quite different from averaging around .6 to 1 ng/ml. We merely use that to make sure that luteolysis has occurred and certainly in our intensively looked-after animals in the lab this is 100% accurate. I certainly haven't monitored animals treated with PMSG in the way that you have done which I think would be necessary to have done in order to answer your question.

R. Newcomb (UK)

I wasn't interested in the speed with which luteolysis takes place but more in the quantity of prostaglandin which is required in order to cause luteolysis. Our results are exactly the same in that we find that there is a much closer synchronisation at oestrus and that oestrus is brought forward by approximately 20 hours compared with recipient animals. I see from the methods here that instead of using the usual 500 micrograms which are recommended for recipients you are using something like 750 to 800.

C.D. Nancarrow

Yes, this is fairly early, Mr. Newcomb, and we suspected this may be a problem with superovulated animals that we may need more prostaglandin but now I suspect we don't.

R. Newcomb

The other question is, you found there was no relationship between the level of progesterone at the time of PMS injection and the ovulation rate, did this apply only to those animals which were being superovulated a second time or did it apply to animals in their initial superovulation as well?

C.D. Nancarrow

We considered all animals, both treatments.

R. Newcomb

Did you consider them separately at all?

C.D. Nancarrow

No, but the data is there, I think. The progesterone levels are given in the appendix.

A.O. Trounson (UK)

In relation to what Dr. Wishart said earlier about high levels of progesterone, do you have the data on fertilisation rates in the eggs collected from the donors and do you have any idea whether there is some delaying of the cleavage in the animals which have been superovulated while they still have a large number of corpora lutea?

C.D. Nancarrow

There are two problems here. One is that I was out of the room during part of that talk! More detailed information on the donor work here is the subject of Wendy Miller's thesis and I am not completely familiar with it. I think if you want more information you should write to her. I can't give you an answer. I believe she told me that fertilisation was normal as reported in the literature but on your second point, I can't give you any information. Wendy Miller was looking at them, I know.

C. Polge (UK)

Thank you very much, Dr. Nancarrow.

SESSION FOUR

PRACTICAL ASPECTS

National Livestock Improvement and Cost of Egg Production

Chairman: A. Robertson

CATTLE TWINNING BY THE EGG TRANSFER APPROACH

I. Gordon
Faculty of Agriculture, University of Dublin

ABSTRACT

Research in controlled breeding in farm animals, including cattle twinning by the egg transfer approach, has been pursued in University College Dublin for a decade. In the past two years, work has involved about 775 beef cattle, including 120 animals in which non-surgical transfers have been attempted. Although much remains to be done, data suggest that it may be possible to achieve cattle twinning on a farm scale by a simple trans-cervical transfer technique.

INTRODUCTION

Gordon, Williams and Edwards (1962), in the light of data from their 3-year MMB cattle twinning project, suggested that twin induction might be approached by introducing a second egg into the cow's uterus in the first few days of pregnancy. In following the egg transfer approach, one major objective is to limit the number of embryos to no more than two at the outset, so that the cow in pregnancy is not placed at risk of carrying calves in excess of two; the transfer technique also permits the distribution of the eggs within the uterus to be appropriately controlled. Research in cattle reproduction subsequently initiated in University College, Dublin, (UCD) in the mid-sixties had this objective in mind and was supported by funds from the Irish Department of Agriculture & Fisheries and An Foras Taluntais. Reports dealing with aspects such as superovulation/oocyte culture (Scanlon, 1968; Scanlon, Sreenan and Gordon, 1968; Sreenan, 1968, 1970), cattle egg storage in vitro and in the rabbit (Sreenan and Scanlon, 1968; Sreenan, Scanlon and Gordon, 1968; Sreenan, Scanlon and Gordon, 1970), synchronisation of oestrus (Scanlon, Sreenan and Gordon, 1972) and the incidence, distribution and orientation of bovine multiple foetuses (Scanlon, 1972; Scanlon, Gordon and Sreenan, 1973; Scanlon, 1975) came from that programme.

A second twinning project was initiated in autumn 1973; in the two years of its operation, about 775 Hereford crossbred heifers have been utilised, the majority acting either as recipients or donors. Some mention of this work has already been made in reviews (Gordon, 1974, 1975); the successful induction of twin-pregnancy by non-surgical egg transfer has also been reported (Boland, Crosby and Gordon, 1975[1]). The present paper deals with data generated in the current programme and looks at evidence which has emerged elsewhere that appears relevant to twinning by egg transfer.

TWINNING BY TWO-EGG TRANSFER

An unknown factor in the nineteen-sixties was whether bilateral twin-pregnancy could be successfully maintained in the absence of a second corpus luteum. For that reason, it was especially encouraging to learn of the work of Rowson, Lawson and Moor (1971) in which a 73% incidence of twins was reported when a fertilised egg was placed in each uterine horn of cattle possessing the one corpus luteum; these results have now been amply confirmed

elsewhere (Sreenan and Beehan, 1974; Sreenan, Beehan and Mulvehill, 1975).

Although Rowson et al. (1971), in reporting the successful production of twins by two-egg transfer, refer to the alternative possibility of transferring a second egg to a pregnant animal, such studies have not apparently been reported. Rowson (1971) briefly mentions Cambridge work which attempted to produce twins of different breeds by egg transfer to an animal already pregnant; results, both in sheep and cattle, suggested a physiological or immunological effect operating in the uterus which led to the loss of either the native or donated embryo in a higher than usual percentage. Experience at UCD in the first year of the present programme indicated, however, that it is possible to induce a substantial percentage of twin-pregnancies in beef heifers by the surgical transfer of one egg to the 'empty' horn of the bred recipient (Boland, Crosby and Gordon, 1975_2). Trials in non-surgical transfer commenced in summer 1974, using the cattle (Cassou) inseminating instrument; results so far indicate that an acceptable level of twinning can follow the one-egg transfer by this route also.

Attention has been drawn to some problems in cattle egg transfer (Gordon, 1975). Although many of these considerations are common to twinning by transfer, the particular approach currently followed in UCD fortunately excludes certain of the questions which remain a major concern to those employing egg transfer to multiply the number of young in valuable females.

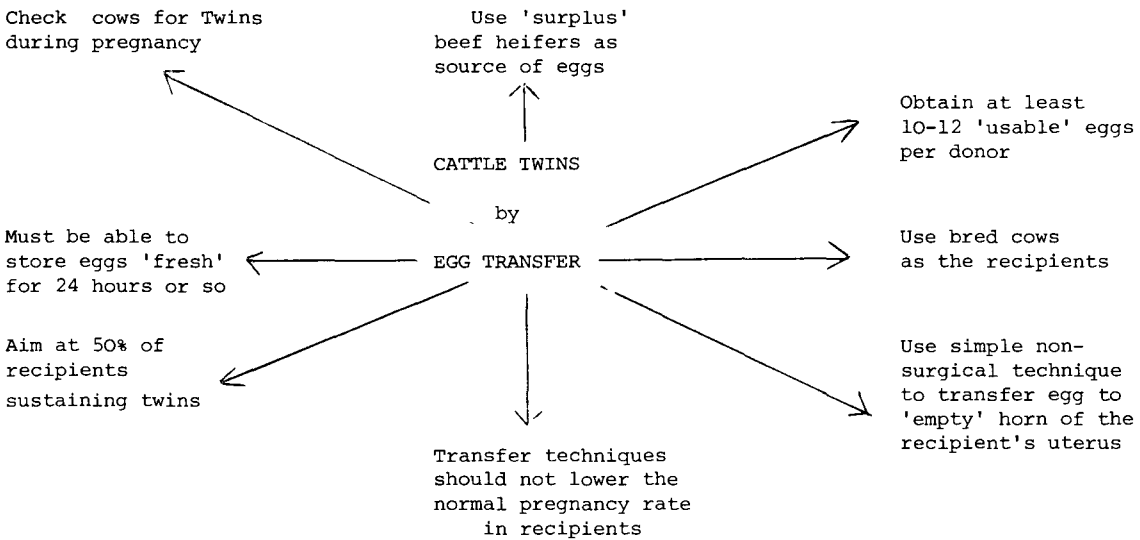
TWINNING BY ONE-EGG TRANSFER

In a previous review (Gordon, 1974), the needs of a twinning programme, based on one-egg transfer were summarised; this information is given again in Figure 1 as the basis of the present discussion.

SOURCE OF EGGS

The approach in UCD is based on what currently seems feasible in Ireland; it may not, however, always be relevant to conditions that obtain in other countries. The programme assumes, for instance, that a reasonably constant flow of fertilised eggs can be obtained from the beef heifers which are despatched daily in the large meat plants that operate in the Dublin area. After the induction of superovulation, using an inexpensive gonadotrophin

FIGURE 1 INDUCING TWINS BY EGG TRANSFER APPROACH



treatment, fertilised eggs would need to be recovered immediately after slaughter or by non-surgical flushing of the uterus of donor heifers; whichever way, the otherwise prohibitive costs involved in surgical recovery would need to be avoided. Eventually, as techniques for the maturation of bovine oocytes and in vitro fertilisation can be relied upon to yield viable embryos, the crude superovulation approach could be phased out. Several years ago, in discussing prospects for the once-bred heifer system as a means of producing additional calves for beef rearing, Crowley, (1971) estimated an annual surplus of 350,000 heifers in the Republic, i.e., heifers not required as herd replacements. The twins by egg transfer approach could be looked upon as a modification of the once-bred heifer system, the essential difference being that the heifer's calves are carried by competent adult uterine foster mothers rather than by the young beef animal itself.

YIELD OF FERTILISED EGGS PER DONOR

Although a recent survey of egg transfer clinics in North America revealed an average yield of less than 4 'usable' eggs per donor (Graham, 1974), it is not unreasonable to think in terms of getting 10-12 such eggs with increasing refinement of superovulation techniques. The standard UCD treatment involves an intramuscular dose of 2,500 i.u. PMSG (Intervet) in 2.5 ml. volume, given on Day 16, with i/m doses of 10 mg oestradiol - 17β on Days 19 and 20 and 2,000 i.u. HCG i/v at time of breeding on Day 21. The oestradiol is used on the understanding that it will facilitate oestrus and accelerate the regression of the current corpus luteum. Donors are bred twice by artificial insemination (12 hour interval) during oestrus, using freshly collected semen in milk diluent (200 million sperm/0.5 ml dose). Data for 147 heifers treated in a ten-month period in 1975 show a mean ovulation rate of 15 7, 61 per cent of eggs recovered (surgery 3-6 days after ovulation) and 87% of these fertilised; this gives a mean yield of 8.4 fertilised eggs per donor. In using heifers rather than older cattle, the possible problem of low superovulatory response to gonadotrophin, indicated in the report of Moore, (1975), can be avoided.

However, when eggs remain in the superovulated donor beyond 3 days, there is a substantial increase in the incidence of a well-defined abnormality, characterised by an apparent breakdown in the membrane of blastomeres; the possibility of adverse changes in cattle eggs because of undue exposure to high concentrations of ovarian steroids has been mentioned

elsewhere by Booth, Newcomb, Strange, Rowson and Sacher, (1975). For various reasons, 2 to 3-day old eggs have often been taken at UCD and stored for several days in the rabbit rather than used directly in transfers between donors and recipients. Whether using surgical or non-surgical transfer, the survival rate of eggs has been higher in those that have spent 2 to 3 days in the rabbit oviduct, even though the 'fresh' eggs appear to be morphologically normal (Table 1). It is not clear whether the greater viability of 'rabbit' eggs is the result of the oviduccal location conferring a form of protection or whether it is simply that eggs are inevitably screened more intensively.

TABLE 1 PREGNANCY, TWINNING AND EMBRYO SURVIVAL RATE ACCORDING TO METHOD OF TRANSFER AND ORIGIN OF 5-DAY EGG TRANSFERRED

		Origin of egg		
Method of Transfer		Direct Transfer	Rabbit Stored	Total
Surgical	No. Recipients	19	21	40
	No. (%) Pregnant	11 (57.9)	14 (66.3)	25 (62.5)
	No. (%) with Twins	3 (27.3)	9 (64.3)	12 (48.0)
	Survival of transferred eggs & %	5 (26.3)	11 (52.4)	16 (40.0
Non-Surgical	No. Recipients	26	22	48
	No. (%) Pregnant	14 (53.8)	15 (68.0)	29 (60.4)
	No. (%) with Twins	3 (21.4)	8 (53.3)	11 (37.9)
	Survival of transferred eggs & %	3 (11.5)	9 (40.9)	12 (25.0)

Boland, Crosby and Gordon (unpublished data)

THE BRED COW AS THE RECIPIENT

If the approach to twins is by way of one-egg transfer, it is essential that the recipient's own egg should be fertilised, so that recipients start pregnancy with two embryos (native and donated) developing. The literature shows comforting evidence of high fertilisation rates (96 to 100%), among heifers after normal breeding procedures (Laing, 1949; Bearden, Hansel and Bratton, 1956; Wishart and Young, 1974); further evidence on this comes from control cattle bred in the present programme (Table 2).

TABLE 2 FERTILISATION AND PREGNANCY RATES IN CATTLE IN NON SURGICAL TRANSFER PROGRAMME

	ALL HEIFERS BRED BY AI			
	CONTROLS (49)		NON-SURGICAL TRANSFER (48)	
	Checked for Fertilisation	Checked for Pregnancy	'Easy' Transfer	'Difficult' Transfer
Heifers	25	24	23	25
Yielded Egg/ Embryo	20 (80.0%)	18	16	13
With Fertilised Egg or Viable Embryo	19 (95.0%)	18 (75.0%)	16 (70.0%)	13 (52.0%)

Boland, Crosby & Gordon (unpublished data)

Even so, it may be desirable to ensure that potential recipients have a satisfactory breeding record. An advantage of the bred animal is in permitting the recipient to make full use of its own ovulated egg, thus enabling the highest number of twinning attempts to be made from the available supply of eggs.

In the first year of the present programme, when transfers were by surgery, some UCD results showed evidence of a much higher embryo survival rate in bred than in unbred recipients (Table 3). The suggestion here is that conditions for embryo survival may be more favourable among recipients that are carrying forward their own embryo into pregnancy.

TABLE 3 PREGNANCIES IN RECIPIENT CATTLE AFTER ONE OR TWO-EGG TRANSFER

Recipient Category	Heifers	Pregnant		Embryo Survival
		Twins	Singles	
Two Eggs — un-bred	23	2	2	6/46 (13.0%)
One Egg — Bred	21	4	6	5/21 (23.8%)

Boland, Crosby & Gordon (unpublished data)

Such data might also be interpreted as evidence that donated eggs in the

unbred recipients are less able to prevent the onset of luteolysis than native eggs in the bred recipients.

NON-SURGICAL TECHNIQUE

Attempts to induce pregnancy in cattle by the non-surgical transfer of eggs go back more than 25 years; despite the use of techniques requiring considerable manipulative expertise and/or special instruments designed to by-pass the cervix, such attempts have yielded negative or unacceptable results (see Foote and Onuma, 1970; Gordon, 1975). In carrying out non-surgical transfers in UCD, the simplest approach was taken, i.e. by way of the readily available cattle inseminating device. Although the instrument (45 cm long; 0.5 ml. straw) is introduced as far as possible into the 'empty' horn of the uterus, (generally some 16 to 20 cm from the os uteri) this is never further than about the mid-horn position. Evidence obtained in a continuation of surgical transfers in the second year of the UCD programme tends to suggest that the extremely low embryo survival rates found in earlier work may have been the result of young cattle eggs being deposited in a mid-horn location rather than near the uterotubal junction (Table 4); substantially more 5 day old eggs survived when placed in the tip of the horn. There is perhaps a suggestion in the data that eggs which pass through the rabbit survive the mid-horn site of deposition rather better than 'fresh' eggs.

TABLE 4 EFFECT OF SITE OF TRANSFER ON PREGNANCY, TWINNING AND EMBRYO SURVIVAL FOLLOWING SURGICAL TRANSFERS

Type of Egg	Site of Transfer					
	Mid-Horn			Tip of Horn		
	Direct Transfer	Rabbit Stored	Total	Direct Transfer	Rabbit Stored	Total
No. Recipients	9	10	19	10	11	21
No.(%) Pregnant	5 (55.6)	5 (50.0)	10(52.6)	6 (60.0)	9 (81.8)	15(71.4)
No.(%) with twins	-	3 (60.0)	3(30.0)	3 (50.0)	6 (66.7)	9(60.0)
Survival of transferred eggs	1 (11.1)	3 (30.0)	4(21.0)	4 (40.0)	8 (72.7)	12(57.1)

Boland, Crosby & Gordon (unpublished data)

There would seem to be the possibility of achieving increased egg survival after application of the non-surgical technique by using a form of

instrumentation capable of depositing the egg much closer to the uterotubal junction. It should be noted that the present data are derived from nulliparous heifers; it does not necessarily follow that similar results can be expected in adult cattle. On the one hand, entry through the cervix in the parous animal will be much easier than in the maiden heifer; on the other, the larger tract of the cow makes penetration with the inseminating intrument to any distance up the uterine horn towards the uterotubal junction that much more difficult.

PREGNANCY RATE IN RECIPIENTS

Although attempts have been made in UCD and elsewhere in previous years to transfer eggs by way of the cervix, it was always felt that this approach faced two major obstacles: firstly, that the uterus of the cow is highly susceptible to infection in the luteal phase (Rowson, Lamming and Fry, 1953); secondly, that the transcervical passage of a catheter results in greatly increased uterine contractility which leads to the expulsion of the egg (Harper, Bennett and Rowson, 1961). Somewhat surprisingly, the present work reveals the incidence of the first problem to be minimal and egg ejection, assuming that increased uterine motility does occur, is not of such nature as to disturb the majority of 'native' eggs. Recent reports from workers elsewhere would seem to agree with this (Seidel, Bowen, Homun and Okun, 1975; Sreenan, 1975). There is, however, evidence that the pregnancy rate in bred recipients can be markedly affected by the ease or otherwise of the actual transfer (see Table 2); e.g., when the transfer takes longer than about 3 minutes to complete, there is a substantial decrease in the pregnancy rate in recipients. Far from thinking in terms of the transcervical technique depressing pregnancy rate, an increase could occur when the donated egg remains viable in the face of the demise of the cow's native egg.

TWINNING PERCENTAGE

It is now well enough established from research in England and Ireland that egg transfer techniques can lead to a high incidence (70%) of twinning in cattle when the transfers are done by surgical procedures (Rowson et al., 1971; Sreenan et al., 1975). The essential fact is that the egg transferred to the horn contralateral to the ovulating ovary does remain viable in most instances. However, the embryo survival rate will be determined by factors such as age of egg, degree of synchrony between donor and recipient, site of

deposition and several others. Data from some of the UCD work suggest that transfers on Days 6 and 7 of the oestrous cycle result in a higher egg survival rate than transfers before or after that period. This may be chance, due to the limited number of cattle presently involved, but is of interest, however, in view of similar evidence in recent Cambridge work (Lawson, Rowson, Moor and Tervit, 1975); the suggestion is that an optimum time in the cycle exists for attempting non-surgical transfers.

In a paper dealing with age of egg and pregnancy rate, Newcomb and Rowson(1975$_1$) show the age factor to be especially important; 3-day old eggs survived poorly and the highest survival rates were evident in the 6-day old eggs. The general consensus among commercial clinics seems to be to transfer eggs at Day 5 or later (Graham, 1974). In the non-surgical work in UCD, transfers are currently timed for five days or later after breeding, when the corpus luteum in the recipient's ovulating ovary can readily be detected (Greve and Kendrick 1973). On synchronisation, the most commonly quoted data are those of the Cambridge group (Rowson, Lawson, Moor and Baker 1972) which showed a substantial reduction in pregnancy rates when donor and recipient cattle were other than in exact synchrony; the more recent evidence of Newcomb and Rowson (1975$_2$) indicates, however, that differences in pregnancy rates between cattle in exact synchrony and those one day out-of-phase may be no greater than about ten per cent.

STORAGE OF EGGS

In carrying out transfers, the bred recipients are currently held in a crush in an area which can be maintained at a temperature of 24^{o}C. It has yet to be shown that the cow egg will survive a rapid and substantial fall in temperature, such as would occur under farm conditions unless special precautions are taken. The medium employed in the recovery, temporary storage and transfer of cattle eggs in the twinning work is either a commercially prepared TCM 199 formulation or a laboratory prepared phosphate buffered saline (PBS) supplemented with 15 per cent foetal calf serum and trace amounts of penicillin; the PBS is a formulation based on an Australian report in sheep (Trounson and Moore, 1974). Preliminary comparative studies in the laboratory with these two media have shown that most young fertilised cattle eggs continue regular cleavage (albeit to a limited degree) in the PBS medium at 30^{o}C whereas such cleavage occurs but rarely in TCM 199 (Table 5)

there is also evidence in non-surgical transfers of a higher pregnancy rate (68%) when PBS is used rather than TCM 199 (44%) pregnancy rate. For such reasons, it may be that the PBS formulation is the more appropriate medium. Sreenan et al. (1975) show that TCM 199 may not be a satisfactory medium when eggs are stored beyond about two hours.

TABLE 5 EFFECT OF MEDIUM ON CONTINUED DEVELOPMENT OF FERTILISED CATTLE EGGS IN VITRO

Tissue culture medium used	Cleavage stage of cattle eggs prior to culture	Eggs cultured for 24 hr and longer	Continued development	Percentage showing continued development
TCM 199	2 - cell to morula	27	1	3.7%
PBS + supple-mentals	2 - cell to morula	50	30	60.0%

Boland, Crosby & Gordon (unpublished data)

CHECKING FOR TWINS

For several reasons, it is desirable that farmers become aware of cows carrying twins, in thinking towards any commercial application of induced twinning in cattle. Although American studies did indicate that the presence of twin calves can be accurately established by foetal electro-cardiography (Lindahl, Reynolds and Allman, 1968), French efforts in this direction have been less optimistic (Bosc and Chupin 1975). In view of the great strides that have been made in the recent decade in hormone assay techniques, there is the obvious possibility that this approach might be explored in the matter of identifying twin-bearing cows by the detection of foetal hormones somewhere about the seventh month of pregnancy. In the meantime, recipient cattle that hold to service can be booked down for checking by palpation per rectum at the 6-8 week stage; bilateral twins can be expected to be much easier to diagnose with safety at that time than unilateral twins.

TWINS ON THE FARM

From the data now available, some of which are detailed in this report,

there is reasonable justification for continuing to explore the induction of twinning by the transcervical transfer of a single egg into the bred recipient. Twins induced by the envisaged technique could hold four important advantages over naturally occurring twins. In the first place, having one foetus in each horn of the uterus can give a more desirable form of twin-pregnancy than occasionally occurs naturally, when both calves may be confined to the one uterine horn; secondly, it is possible to prepare the cow for the twinning event by appropriate feeding and management; thirdly, the herdsman can be ready, at the time of calving, to provide assistance; and fourthly, the farmer can confine twinning to those cattle which he feels have the constitution and ability to deal with two calves.

Lamond (1974), in a comprehensive review dealing with multiple births in cattle, observes that one of the important aspects of twinning work may be in facilitating the development of new systems of beef production which would be required in coping with the greater flow of calves. As a means of increasing the efficiency of beef production, the availability of a commercially feasible twinning technique could be of interest, especially in Ireland, in which beef is the single most important product of farming.

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DISCUSSION

R. Church (Canada)

I was wondering if you had ever let any of your twin-bearing cows go through pregnancy. There is a little bit of evidence around that the loss of one of a pair of twins may occur after your slaughter period of, as I understand it, about 30 days. Do you have any evidence of post 30 day slippage of calves?

I. Gordon (Ireland)

Well, certainly I am sure that does occur. It's a question of just what percentage of loss you are going to get. All work up to the present has been based on cattle which have been slaughtered between 30 and 45 days. We realise this is a weakness with the figures that we have currently got to present. We have got a group of 20 well grown heifers which are in the process of going through to term. At least, transfers were done to 20 well grown heifers and approximately two thirds of those are pregnant and hopefully will eventually calve singles of twins and we shall see what the story is at that point.

L.E.A. Rowson (UK)

We have let quite a number of animals go through to term or actual twinning and the loss doesn't seem to be very great in a comparison between slaughtering at three months and going to term. The actual detection of twinning is usually done at about six weeks.

A.O. Trounson (UK)

I wonder how long you keep eggs in the rabbit? Lawson showed that if you keep eggs for more than four days in the rabbit oviduct, even though they have normal development, they don't go on after transfer. The other thing is that I think before recommending the rabbit you should have some idea of what your recovery rate is, and what your selection rate of the final eggs are in terms of the number you put into the rabbit.

I. Gordon

None of the eggs I was talking about have gone more than three days in the rabbit. We are mindful of the evidence that four days or beyond could be a source of problems. It is difficult to arrive at a net figure of usable

eggs coming out of the rabbit in relation to the fertilised eggs recovered from the donors. That is a matter of the percentage of eggs recovered from the oviduct which is in the mid-eighties. Of those recovered, again, the proportion regarded as suitable for using in transfers is somewhere up in the eighties. So you have these two figures of 80%. Eighty percent recovered back from the rabbit and then 80% of those again that are useful for transfer.

A.O. Trounson

So you finish with 60% or thereabouts?

I. Gordon

Yes, that would be it.

M. Hansen (Denmark)

If you use bred cows as recipients, what will be the average loss at calving of your twins? Do you have, say,170 calves per 100 cows after calving or do you have 200?

I. Gordon

Well, you certainly don't have 200. On the basis of the most optimistic figures there is the possibility of 150 calves per 100 cows calving.

R. Newcomb (UK)

Can you tell me how you ensure that your placement of eggs is as you say, and what sort of volume of medium you are using when you are comparing middle horn and the tip of the uterine horn?

I. Gordon

Yes, well I forgot to mention at the beginning that it is not myself who is doing the transfers. There are two hardworking colleagues back in Ireland who have their noses to the grindstone most of the time on the actual work. On the transfer, the pipette is withdrawn for about ½" or so once the resistance against the uterine wall is evident to try to take some account that you don't deposit the egg into the uterine wall. As to the exact location of the egg, it is difficult to be certain about that. I may say we did a considerable number of dummy transfers either using excised tracts of slaughterhouse cattle, or dummy runs at the local slaughterhouses and checking for the recovery of the eggs after the animals were slaughtered

There were quite a few preliminaries before the Cassou gun was used in the actual living recipient.

R. Newcomb

I was thinking more in terms of the surgical comparison between tip and middle; what sort of volume you used and how you ensured the placing was as you say it was.

I. Gordon

Well, the placing was an estimate. The volume of fluid is based on the principle of minimal volume used in the transfer. With the non-surgical transfer there is, in fact, almost 0.5 ml of transfer medium.

A. Robertson (UK)

I am sorry but we have run out of time. There may be some opportunity later on in the meeting to ask further questions. Thank you Dr. Gordon.

MANAGEMENT AND ECONOMIC ASPECTS OF TWINNING

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INTRODUCTION

In this paper I examine some aspects of the management and economics of increasing twinning either by selection or by ova transplantation in beef and dairy cattle.

Under present circumstances, and for sound economic reasons, the farmer has no desire or incentive to increase the number of twin calvings. Such calvings cause herd management to be more complicated, higher culling rates of cows, lower birth rates, poorer quality calves, and the need for more veterinary attention. Beef and dairy farmers consider that these disbenefits more than outweight the benefits of any increased income which derives from the extra calves. In addition dairy farmers are under the impression that increased twinning is at the expense of increased milk production per cow. Examination of several years' records of the relative values of milk and calves to the individual herd owner, indicates that at all times it has been more profitable to increase by selection or management the output of milk per cow per unit time, rather than the number of calves per calving in dairy cows.

This seems to be a reasonable assessment of the present situation by the farmer. However, I wish to argue that this assessment should not be used as an argument against the development of more twin calvings. Recent research indicates that changes in management may overcome the difficulties and reproductive problems associated with twinning, that there is no inherent incompatibility between increases in calves per calving and milk unit per unit time, and that genetic change will be easier to achieve in the Friesian than in some beef breeds.

THE NEED FOR MANAGEMENT RESEARCH

The effects of naturally occurring twin births in dairy cattle have been reviewed elsewhere (e.g. Hendy and Bowman, 1970). In summary, twin births are associated with increases in dystocia, stillbirths, early

postnatal calf mortality, retained placentae, infertility, calving to conception intervals and lactation length. There is also consistent evidence that, when corrections are made for the effects of differences in lactation length, the phenotypic and genetic correlations between the incidence of twinning and milk yield are positive (Bowman and Hendy, 1970; Auran, 1974; Bar-Anan and Bowman, 1974; Syrstad, 1974; Wood, 1975). However, these are the conclusions under the present conditions of management of farms, which are designed essentially to cope with a cow carrying a single calf approximately once per year.

It is essential, if the farmer is to accept and benefit from increased twinning in cattle, that the research worker solves several management problems. The farmer must be given a production system which is suitable for cows which regularly produce twin calves without any deleterious effects on other aspects of reproduction and milk production. In developing such a system at least two areas of research require simultaneous attention. These are, firstly, the development of a simple diagnostic method for distinguishing the number of foetuses being carried by each cow not later than six months of gestation. Further modification of the electrocardiographic technique reported to be successful by Bosc and Chupin (1975) may be one solution to this problem. The second area of research concerns the estimation of the nutritional requirements during pregnancy (particularly late pregnancy) and lactation to maintain milk yield, reproduction and health over several lactations of the dairy cow regularly carrying twins. There is limited evidence that the adverse effects of twin births in dairy cattle can be overcome by appropriate modification of present feeding levels during late gestation (Gordon, Williams and Edwards, 1962) though there is an urgent need for this possibility to be thoroughly tested in both dairy and beef cattle.

GENETIC CHANGE

Land and Hill (1975) have considered methods of selection for twinning and emphasised that the heritability and the rate of genetic change are related to the level of incidence. There are marked breed differences in the mean incidence (e.g. Johansson, Lindhé and Pirchner, 1974) and the Friesian has a higher incidence than the major British beef breeds, the Hereford and Aberdeen Angus used as beef breeding cows (Johansson, 1932; Johansson et al. 1974; Ortavant and Thibault, 1970). It should be possible

to make genetic change by selection for ovulation rate or twinning in Friesians more rapidly than in these beef breeds. However, it also is relevant to note that some of the European beef breeds, particularly the Charolais, have a mean incidence as high or higher than the Friesian.

The genetic correlation between milk yield and twinning has been estimated to be of the order of +0.3 (Bar-Anan and Bowman 1974) in Israeli Friesians. All available evidence suggests the correlation is positive in other strains of Friesians and other breeds. Therefore, selection for increased twinning is not likely to be deleterious to, and may allow some positive correlated response for, milk yield. The genetic correlations of twinning with growth rate or body weight have not been estimated. Assuming the following parameter values for milk production and twinning, it is possible to predict the direct and correlated responses to selection for the two traits.

Trait	Mean	h^2	σ_p^2	r_G
Milk production - 305 day mature yield kg.	4500	0.25	360,000	+0.3
Twinning calves per calving	1.03	0.04	0.025	

For similar levels of selection intensity i.e. $i_1 = i_2 = 1$
Direct selection for milk yield = 1 x 0.25 x 600 = 150kg per generation, and the correlated response for twinning = 1 x 0.5 x 0.03 x 0.3 = 0.0045 calves per generation.

Direct selection for twinning = 1 x 0.04 x 0.16 = 0.0064 calves per generation and the correlated response for milk yield = 1 x 0.2 x 300 x 0.3 = 18kg per generation.

SOME ECONOMIC CONSEQUENCES OF TWINNING

Recognition of the difficulties presently associated with twin calvings, coupled with the relative values of calves and milk, has led to the conclusion that selection for twinning is not worthwhile in dairy breeds even if male calves are used for beef, but may be beneficial in beef breeds. The relative values are based on the comparative returns to the farmer for increases in milk and calves. At present UK prices the value of direct selection for milk production or twinning in dairy cows can be calculated

as follows:

Assuming prices to be
1kg milk valued at 8p, 1 calf valued at 3000p
1kg feed costs 9p
1kg milk requires 0.4 kg feed
1 calf requires 100kg feed to dam during pregnancy

Therefore per generation the response to selection for milk yield is worth 150 (8 - 3.6) + 0.0045 (3000 - 900) = 669p. By similar calculations, selection for twinning is worth 0.0064 (3000 - 900) + 18 (8 - 3.6) = 93p.

The equivalent levels of selection intensity applied for milk production or twinning, yield an increased output per cow per generation of 669p and 93p respectively. No estimate has been made of the comparative costs of selection programmes or generation intervals for the two types of selection.

This comparison does not change if the national or European Community interest is considered rather than the individual farmer. If the Community is assumed to be self sufficient in milk and beef then the comparison is different. The consequence of increasing milk production per cow is to reduce the number of cows required so releasing capital, land and other resources for the production of something else. The consequence of increasing calf production (twinning) in dairy cows, assuming their pure or cross-bred calves are suitable for beef, is to reduce the number of beef breeding cows so releasing land for the production of something else. The consequences can be summarised as follows:

	Gains	Losses
1. ΔG_1 = 150kg milk + 0.0045 calves per cow per generation	.03 dairy cow .015h for alternative product - e.g. barley	60kg feed 0.015 calf loss
2. ΔG_2 = 0.0064 calves + 18kg milk per cow per generation	.004 dairy cow .002h for alternative product - e.g. barley .001 calf gain	7kg feed (milk)

It is clear that the balance of advantage is still in favour of selection for milk production in the dairy cow.

As an alternative approach, selection for twinning might be undertaken in a beef breed. The possibilities for selection response for twinning

have been outlined by Land and Hill (1975). The difficulty which would arise in British beef breeds is that the level of twinning is low at present and there is little possibility of establishing a base population with a twinning incidence of about 10%, which is probably a prerequisite of selection for twinning. There is a better chance of achieving such an objective using Charolais cattle.

A third approach would be the selection for twinning of a strain of beef or dual purpose cattle which would be used only for the production of ova for transplantation. Superovulation rate is probably related to natural ovulation rate. Thus the development of strains of cattle with a high natural ovulation rate might reduce the number of donor cows required to provide ova for transfer. However, the cost per ovum from cows kept solely for ova production would be greater than the cost per ovum obtained from cull cows. Therefore this approach is unlikely to be economically attractive.

CONCLUSIONS

There is an urgent need to solve the management problems associated with twinning arising naturally or as a result of ovum transplant in beef and dairy cows. There appears to be no economic benefit from selection for twinning in dairy cows, or in a special strain of cows to be used as ova donors. The most promising approach to selection for twinning would be in a beef breed such as the Charolais.

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DISCUSSION

L.E.A. Rowson (UK)

I would like to raise two points. First of all, you mentioned that Dr. Gordon's work was the only work on commercial twinning. There is an Israeli paper which I saw recently, I can't remember who it was by, but there they produced 170% calving rate and they only lost one week in the delay in getting the animals pregnant compared with the animals with singles. The other point is that you mentioned the question of nutrition in animals carrying twins. This is being looked at by the Rowett Institute at the moment. We have produced quite a lot of twinned cattle for them in the past summer and they are working on it.

J. Bowman (UK)

I think you may have misunderstood - or I may have stated it wrongly. What I thought there was only one report about was, in fact, the nutritional aspect. As far as I know, that is the only published evidence of what happens when you give cows carrying twins a rather higher level of nutrition during late pregnancy. I am aware of the Rowett work but it isn't really off the ground yet and I believe they have concentrated on beef cattle whereas it seems to me we need to do this in dairy cows.

L.E.A. Rowson

Well, they have concentrated on beef out of dairy cows.

E.P. Cunningham (Ireland)

There is another factor which affects the relative economic value of what you do with twinning and that is the variation in calf price, both laterally between countries, and vertically, through time. For example, in Canada I know that the value of a surplus calf in a dairy herd is maybe one twelfth of the value of the milk output of the cow. By comparison, in Ireland, in normal circumstances, the value would be perhaps one quarter to one third. On the other hand, here in Northern Europe, in the last two or three years, we have seen calf prices vary by several hundred percent. So what might make good economics in one year might be totally uneconomic in another year. This fluctuation in the profitability of the enterprise is something else which has to be contended with.

J. Bowman

Yes, I think you have to alter the calf price by factors of something like six even to make the equation equal, never mind to change the order of advantage.

I. Gordon

Well, either I was drunk or it was certainly before inflation hit us that I was talking about 10p per egg! I'll have to deny that in public!

The other point I wanted to make was that in trying to detect twin pregnancy in cattle, I wasn't really thinking of the progesterone possibility because the cow is different from the sheep inasmuch as progesterone isn't produced in the uterus, it is mainly ovarian until quite late in gestation.. I was thinking that somebody like Brian Heap might come up with some other hormone of foetal origin which could be checked out at the sixth or seventh month.

J. Bowman

I might just say about the 10p that it is in print. I'll tell Dr. Gordon where and then he can make sure he knows what he is being exposed to!

J. Thimonier (France)

For the detection of twins in France, we are now measuring the level of total oestrogens at 220 days; the first measures are very promising.

J. King (UK)

Professor Bowman, you suggest there may be some connection between the ovulation of an egg under a superovulation regime and a natural one, but I wonder if there is any evidence for that. I believe there is very little evidence of the incidence of multiple ovulations under non-superovulation conditions - under spontaneous conditions.

J. Bowman

I make this statement on the relation between natural ovulation rate and superovulation rate on the basis of evidence of other species, which is quite good I think. The natural ovulation rate in the cow is slightly difficult to come by but certainly I would have put it as high as 1.1 ova per ovulation, and possibly higher.

R. Newcomb (UK)

If anything, the evidence would seem to be the converse of what you have said in the cow. I believe it is true to say, for example, that the Friesian has been compared to other breeds in several studies now, and has been found to superovulate less well. Certainly I think Joe Sreenan has shown the difference between this breed and the Hereford, and this would tend to be opposite to what you would regard as the natural ovulation rate in these breeds.

I. Wilmut (UK)

May I ask Dr. Gordon if he does have an accurate estimate of the cost of an ovum?

I. Gordon

Well, certainly you may ask but I am afraid you won't get an answer because I would have to do some fresh sums on that.

A. Robertson (UK)

It depends how much vets value their time per hour, no doubt.

J. Bowman

May I just put in a comment on the use of culled cows as a source of ova. It does seem to me that there are problems likely to be associated with actually collecting the ova in the commercial situation. I would have thought the veterinary profession would have been very hesitant about taking ova from culled cows, particularly in the abbatoir and so on, as a source of ova for commercial herds. I throw that one out to the veterinary profession.

I. Gordon

Well, just in case I am being connected with culled cows, I never suggested culled cows as a source of eggs. It is the beef heifer which I regard in a slightly different light from culled cows.

J. Bowman

If that is the point then it does seem to me that there is more value in considering the development of a strain specifically as a donor strain rather than finding the necessary ova from other animals.

W. Hill (UK)

Unless one could get very large numbers of eggs from a donor strain it doesn't seem likely to be an efficient technique relative to superovulation of cows whether they be from a donor strain or not. If by twenty years you can get the rate up to 1½ eggs it doesn't make it very much of a donor strain. I would question the value of it.

J. Bowman

Then it comes back to what is the relationship between natural ovulation rate and superovulation.

A.O. Trounson (UK)

I would like to take issue with what Ray Newcomb has said. There is a very clear indication in Helen Turner's work in Australia, that animals which have been selected for multiple births in fact had a very much higher response to superovulation and I don't think at this stage, in the cow, we can really say that the animals have not yet been selected. I think it would be very surprising if the relationship doesn't hold because it holds in the mouse; it holds in very nearly every other species that has been looked at.

S.M. Willadsen (UK)

I think there is too much of a mixing of intentions; that is in the establishment of the twin pregnancy and what one could eventually get out of that. One must consider the two things separately; we don't know what the final cost of cow embryos is going to be but we could possibly at this stage decide whether twins would be a useful thing in cattle production. What we are considering now are several techniques for inducing twins and somebody might come up with a wonder drug at some point whereby it would be quite easy to induce these.

J. King

It is rather a vicious circle. Until such time as we can have large numbers of cows with a high frequency of twinning to evolve management systems then we can't really know how economically desirable it might be.

J. Bowman

I am not at all convinced of that actually. I think we can certainly solve the management problems without having methods for inducing twinning by getting information from animals where twinning occurs naturally.

APPLICATION OF SUPEROVULATION AND EGG TRANSPLANTATION IN AI BREEDING PROGRAMMES FOR DUAL PURPOSE CATTLE

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INTRODUCTION

In most European countries, the dairy cow population is the major source of beef animals. To improve the genetic capacity of the population for both beef and dairy traits, many countries select AI bulls on a dual purpose basis. In dual purpose programmes selection on dairy and beef traits is made simultaneously. After this definition Friesian programmes without selection on growth rate, such as the British Friesian programme, are dairy programmes, and Friesian programmes with selection on growth rate, such as the Dutch programme, are dual purpose programmes. In dairy programmes, the beef objective is further pursued by crossing a proportion of the dairy cows to beef breed bulls. McClintock and Cunninghan (1972) have shown that increasing the percentage of cows bred to beef bulls from 0% to 33% and 50%, increases the genetic value of each dual purpose insemination by approximately 30% and 80%, respectively. We can apply these conclusions, however, only to populations where the progeny of beef breed bulls are at least as good for beef production as those of dual purpose bulls. This assumption does not hold true for beefy dual purpose breeds such as Fleckvieh (Simmental).

In addition, the dual purpose concept has general advantages as well, such as:

1. The dual purpose concept can adapt beef production quicker to changing market conditions than the crossbreeding strategy. This has a balancing effect on price cycles.

2. In dual purpose cattle the size of national or regional populations is more adaptable to changing market conditions.

3. The crossbreeding strategy is less suitable for smaller herds. But smaller herds will still play an important role in the foreseeable future.

4. Cow beef is an important part of beef supply.

Thus, dual purpose populations can be an important stabilising factor in the cattle industry of the EEC. It seems, therefore, worthwhile to improve them by efficient breeding programmes. The purpose of the present study is to examine how the effectiveness of selection programmes for dual purpose AI bulls can be affected by superovulation and embryo transfer.

CONVENTIONAL SCHEME

An important step in deciding on a breeding programme for meat and milk in a given economic situation, is to estimate the economic value of the traits. Therefore, we need an estimation of the additional income that can be obtained by way of a unit genetic increase in live-weight at a given age (live-weight for age: LFA), as compared to additional income produced by way of a unit genetic increase in milk production (M). The ratio M/LFA does not vary greatly in the European populations, and the values 1 : 6, 1: 7, 1 : 8, cover most conceivable European situations. The value 1 : 8 is used for the following calculations. Once the relative economic value of LFA : M has been determined, it becomes possible to evaluate the relative economic effect of various breeding strategies. Such evaluations provide a basis for determining whether the costs of a particular breeding operation, for example, the use of superovulation and egg transfer, are economically justifiable.

The population is presumed to consist of dual purpose cows, all of which are milked commercially. Population size is constant. All male calves are reared as beef animals for sale at 550kg liveweight. 80 progeny are reared per hundred cows per year. All cows are bred artificially, 85% with proven sires and 15% with test bulls. Bull selection is based on progeny testing in milk yield, and on performance testing up to 420 days on liveweight -for-age. The sum of generation intervals over the four paths of gene transmission amounts to 25 years in the proven sire programme, and to 20 years in the young sire programme. The genetic correlation between milk yield and growth rate is assumed to be zero. Selected proven bulls are used for two years at a fixed number of inseminations per year. In the young sire programme all young bulls are used for one year for a fixed number of inseminations. 90% of all inseminations are carried out with semen from young bulls. Thus, selection of sires to breed dams in the young sire schemes is based on pedigree information (planned matings). Selection procedures are shown in Table 1.

TABLE 1 AI BREEDING PROGRAMME WITHOUT SUPEROVULATION

	Number
Population size (cows)	100,000
Active population	30,000
Inseminations by proven bulls (85%)	85,000
Inseminations by test bulls (15% of total population, 50% of active population)	15,000
Test bulls per year	30
First inseminations per test bull	500
Proven bulls	10
Replacement of proven bulls per year	5
First inseminations per proven bull per year	8,500
Planned matings necessary to produce one test bull (pregnancy with recommended bulls 0.60; male calves per pregnancy 0.40; bulls available for selection after rearing period 0.83)	5
Elite cows necessary per year for planned matings	150
h^2 milk = 0.25 σ_P milk = 700kg	
h^2 LFA = 0.40 σ_P LFA = 45kg	

SUPEROVULATION SCHEME

Like Land and Hill (1975), we assume the following numbers of progeny per donor cow available for selection: 4, 6, 8, 10. It is further assumed that one out of two elite cows can be superovulated successfully, and the eggs transferred to recipient cows with each receiving one egg. Cows with at least one lactation are suitable as donors. In the conventional scheme, one out of 200 cows in the active population (Table 1) is needed for planned matings, if there is no selection on LFA. The number of elite cows required, however, is increasing with an increasing selection intensity in LFA after performance testing. In the superovulation scheme only one out of 500 cows in the active population is needed for planned matings. If we require two elite cows to get one donor cow for the production of test bulls, we require 60 elite cows for 30 test bulls.

In the superovulation scheme the selection intensity in milk yield is not reduced as long as the number of performance tested bulls per donor cow is sufficiently large for selection on LFA.

In the superovulation scheme an additional selection on carcass characteristics is possible by estimating breeding values on the performance of full brothers. However, some loss of selection intensity for LFA has to be accounted for because at least 2 full sibs are required to get a satisfactory accuracy. Assuming a heritability of $h^2 = 0.60$ for carcass characteristics, a full sib family size of two, and a half sib family size of 32, accuracy amounts to $r_{IG} = 0.54$. Since full sibs are reared by different dams in the superovulation scheme there should be no common environmental effects between full sibs.

SUPEROVULATION SCHEME WITH TEMPORARY INBREEDING

Dickerson (1973) proposed sire-daughter or full sib inbreeding in alternate generations to increase genetic variation and accuracy of optimum combined family and individual selection, without changing type of selection or generation interval. Thus, he suggests temporary inbreeding of the best AI progeny tested sires to a few of their highest producing daughters. According to Dickerson's (1973) estimation this procedure should increase accuracy by about 18% and response by about 30% from young sire selection in alternate generations, or average annual rate of improvement by over 10%. Superovulation offers several advantages in an AI programme with temporary inbreeding of the best proven AI bulls.

Selection intensity in milk yield on the path Dam-Sire will be higher. In progeny groups of 50 daughters only the best daughter is required (2%) to produce between 2 and 5 sons. This is approximately the same selection intensity as in conventional AI breeding programmes without inbreeding where about 2% of the active population are selected as elite cows. In a conventional scheme with inbreeding, between 10 and 25 daughters are needed to produce between 2 and 5 sons.

In a superovulation scheme full sisters are additionally available for inbreeding.

COMPARISON OF THE VARIOUS SCHEMES

Genes are transmitted from one generation to the next via four paths:

from sire to son (SS)
from sire to daughter (SD)
from dam to son (DS)
from dam to daughter (DD)

If $G = r_{IG} \cdot \sigma_G \cdot i$ and L = the length of the generation interval, then G year can be estimated from the following formula (Rendel and Robertson, 1950).

$$G \text{ year} = \frac{G_{SS} + G_{SD} + G_{DS} + G_{DD}}{L_{SS} + L_{SD} + L_{DS} + L_{DD}}$$

For the comparison of the various schemes the following parameters are assumed.

Breeding Scheme	Path D D		D S		S D		S S	
	i	r	i	r	i	r	i	r
Conventional	-	-	2.56*	0.5	1.5	0.85	2.0	0.85
Superovulation	-	-	3.17	0.5	1.5	0.85	2.0	0.85
Superovulation with temporary inbreeding	-	-	3.17	0.5	1.5	0.93**	2.0	0.93**

* Selection intensity on the path DS is reduced with increasing selection intensity after performance testing.

** Breeding values are estimated on the bulls' own progeny and on the progeny of full brothers according to Dickerson (1973).

The parameters required for the calculation of genetic gain on liveweight for age are shown in Table 1. The increase of genetic variance in the temporary inbreeding scheme is assumed to be proportional to:

$$1 + F \quad (\sigma_G^2 I = 1/2\, \sigma_G^2 + \frac{1+F}{2}\, \sigma_G^2).$$

Genetic gain in milk equivalents (G_{ME}) is calculated as follows:

$$(\sum G_M + 8 \sum G_L) \Big/ \sum L,$$

where G_M and G_L are the genetic superiority of selected individuals for milk production in kg and for liveweight in kg respectively.

The yearly genetic gain of the various schemes in percent of the maximum value is calculated separately for the young sire schemes and for the proven sire schemes (Table 2).

TABLE 2 PREDICTED GENETIC GAIN PER YEAR IN % OF MAXIMUM

Breeding scheme	Selection after performance test 1 : 2			1 : 3			1 : 4			1 : 5		
	M	LFA	ME	M	LFA	ME	M	LFA	ME	M	LFA	ME
1. Young Sire Programme												
Conventional	84	45	73	82	61	76	81	70	78	80	79	80
Superovulation	91	47	79	91	66	84	91	79	88	91	88	91
Superovulation and temporary inbreeding	100	53	84	100	75	92	100	89	97	100	100	100
									= 63kg	3.21kg		88.68
2. Proven Sire Programme												
Conventional	84	45	75	83	61	78	82	70	79	81	79	80
Superovulation	89	47	80	89	66	84	89	79	87	89	88	89
Superovulation with temporary inbreeding	100	53	89	100	75	94	100	89	98	100	100	100
									= 68.36kg	2.52kg		87.67

M = Milk LFA = Liveweight-for-age ME = Milk equivalents

The economic evaluation is based on cash return over feed costs (milk (DM/kg) = 0.30; LFA (DM/kg) = 2.90). The economic value of genetic improvement is calculated by a method developed by Brascamp (1975). In this method the time lag between investment in AI or superovulation and expression of genetic improvement in the population has been accounted for. The assumptions are a time period of 25 years and an interest rate of 10%. The fraction of inseminations from young bulls are assumed to be 0.20 in the proven sire schemes, and 0.90 in the young sire schemes. The economic evaluation of the different schemes is shown in Table 3.

TABLE 3 ECONOMIC DIFFERENCES BETWEEN VARIOUS BREEDING SCHEMES
(Economic evaluation according to Brascamp 1975)

		M (kg)	LFA (kg)	Economic value (DM) per cow	Population DM
Conventional proven sire programme	1:2	--	--	--	--
Superovulation proven sire programme	1:2	14.34	0.36	5.35	535,000
Superovulation proven sire programme with temporary inbreeding	1:2	41.68	1.02	15.45	1,545,000
Conventional young sire programme	1:4	- 39.64	4.44	0.99	99,000
Superovulation young sire programme	1:4	- 10.38	5.82	13.76	1,376,000
Superovulation young sire programme with temporary inbreeding	1:4	6.54	7.86	24.76	2,476,000

CONCLUSIONS

The incorporation of superovulation and embryo transfer into an AI breeding programme for dual purpose cattle enables an increasing selection intensity for liveweight-for-age without decreasing the selection intensity for milk yield on the path Dam-Sire. Assuming the same selection intensity for liveweight-for-age in the conventional schemes and in the superovulation

schemes, the additional genetic gain achieved by superovulation amounts to between 5 and 10%.

Superovulation offers further advantages in combination with a system of cyclical inbreeding proposed by Dickerson (1973). In this system sire-daughter or full-sib inbreeding is carried out in alternate generations to increase genetic variation and accuracy of selection, without changing type of selection or generation interval. The combination of superovulation and cyclical inbreeding shows an increase of genetic gain per year between 20 and 25% if the genetic variance can be increased as assumed by Dickerson (1973).

However, the advantage of the temporary inbreeding scheme will be reduced considerably if the genetic variation has been much reduced by selection in the previous generation (selection of sires to breed sires) as is expected by Robertson (personal communication).

Table 3 shows the differences in genetic gain per year between the conventional system which is mainly used at present, and various combinations of superovulation and inbreeding.

TABLE 4 ECONOMIC COMPARISON OF THE CONVENTIONAL PROVEN SIRE PROGRAMME AND THE YOUNG SIRE PROGRAMMES
(without costs of superovulation and embryo transfer)

	Difference in economic value per cow	Additional costs of the young sire programme per cow	Net difference per cow	Total population
	DM	DM	DM	DM
Conventional proven sire programme (1:2) to:				
a. young sire programme 1:4	0.99	1.24	- 0.25	- 25,000
b. superovulation young sire programme 1:4	13.76	1.24	12.52	1,252,000
Gottschalk (1975) Costs for bull sampling (1:2)			1.39 DM per cow	
Costs for waiting bulls			0.15 DM " "	

On Table 4 the conventional proven sire programme is compared to a superovulation young sire programme with a selection intensity on growth

rate of 1:4. The additional costs for the performance testing of a greater number of bulls are estimated from figures from the Bavarian performance testing stations (Gottschalk, 1975). Thus, the expected net return per round of selection is estimated for a period of 25 years at 1,252,000 DM for a population of 100,000 cows. If we assume that it is justified to invest 20% of the expected net return as suggested by Cunningham at the EEC seminar on optimisation of cattle breeding schemes in Dublin in November 1975, costs for superovulation and egg transfer of approximately 250,000 DM per year seem to be justified for a 100,000 cow population. Since 30 donor cows and approximately 250 recipients are required per year, the economic limit for additional costs in the superovulation schemes as compared to the conventional schemes amounts to approximately 8,300 DM per test bull. The rate of inbreeding per year amounts up to 0.31% when exclusively estimated over the paths sire to breed sires and dams to breed sires. The 30 young test bulls per year are sons of two bull fathers and 8 bull mothers in the most extreme case. The young sire superovulation scheme has the advantage of almost doubling the genetic gain for liveweight-for-age without decreasing genetic gain in milk yield considerably. However, this holds true only as long as the genetic correlation between milk yield and growth rate does not become negative. At this stage rotational crossing programmes could be applied.

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DISCUSSION

A. Robertson (UK)

May I just get the basis of your last calculations clear; I believe you said they were for a population of 100,000 cows for a total benefit of just over 1,000,000 DM?

H. Krausslich (W. Germany)

Yes.

A. Robertson

And for that 100,000 cows, this is a total benefit ... well, its a discounted benefit.

H. Krausslich

Yes, a discounted period; it took 25 years.

A. Robertson

To give us a figure for comparison, how many eggs would have to be transferred per year in such a programme?

H. Krausslich

About 250 eggs per year.

A. Robertson

Yes ..... so we begin to ask how many discounted eggs there are!

E.P. Cunningham (Ireland)

It is interesting that you are getting as big, if not bigger, benefit from adding this temporary inbreeding to your structure as you are from adding a superovulation element. I wonder whether in practice, since you are doing sire/daughter matings, that you might lose a lot of the benefit because inbreeding to that extent might give you much poorer egg survival. This is something we don't know about but in general one would expect it - so the difference here might tend to be over estimated.

H. Krausslich

I have asked the experts in Germany and I couldn't get any answer

about the egg survival. That is the reason why I compared the two schemes separately.

M. Hansen (Denmark)

In connection with Dr. Cunningham's question, I would like to know if a temporary inbreeding scheme is easy to run in practice. Would it be accepted and adapted by the AI associations? What do you think about that?

H. Krausslich

I think if you use a superovulation scheme the AI station has to change from buying bulls to buying cows. If they buy cows they can do the inbreeding themselves. They don't use very many cows - it is a small herd.

A. Robertson

Does this then involve them in having a proportion of their bulls inbred all the time?

H. Krausslich

It means that you use 50% of your inbred bulls with an inbreeding coefficient of 25%.

A. Robertson

This could conceivably also result in some extra loss in terms of semen production?

H. Krausslich

That's open to question - I don't know.

J. King (UK)

I would like to be clear on that. I think the last table you showed was very important but as an investment strategy could one say, or could one not say, "First of all we will try the inbreeding ploy and then superovulation"?

H. Krausslich

That is very difficult to answer; I think we can only employ the inbreeding scheme if we use superovulation because in our country it is very difficult to use an inbreeding scheme with the farmers. The farmer has to do the mating. The farmer is very reluctant to give his cows for inbreeding.

THE USE OF EGG-TRANSFER TECHNIQUES IN GENETIC IMPROVEMENT

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INTRODUCTION

The technical prospects for egg-transplantation have been reviewed in a general way by Foote and Onuma (1970), and by Gordon (1975). The genetic implications for cattle breeding have been considered by Beilharz (1970), Miller (1973), Vissac (1974), Cunningham (1974) and Land and Hill (1975). Various aspects of the subject have been covered in the papers presented at a symposium in Sweden (SHS,1974). My purpose is to examine some of the technical possibilities for accelerating genetic improvement by the use of egg-transplantation techniques. I will be concerned primarily with their application in dairy cattle.

There are three ways in which superovulation and egg-transplantation can be used to accelerate genetic improvement:

1. By permitting the rapid expansion of rare breeds or stocks,
2. By increasing the selection intensity among females,
3. By increasing the twinning rate.

The genetic consequences of these possibilities are somewhat difficult to quantify. The reason for this is that we do not yet know the limit to the physical possibilities for superovulation and transfer of eggs. In theory, the possibilities are practically limitless. The average number of follicles in the ovaries of a three month old heifer is about 75,000 (Salisbury and Van Demark, 1961). Although this number may drop considerably with age, the pool of potentially fertilisable eggs per female is many thousands of times greater than we have as yet been able to use. In due course, it may be possible to recover a high proportion of these, to fertilise them, and to grow them to maturity in cows other than the donor. However, the practical limits at the present time are that without assistance five of these eggs may result in live births, and with the assistance of current techniques of superovulation and egg-transfer, perhaps a hundred of them could result in a live calf. The technique of egg-transfer must therefore wait for the further development of methods of superovulation, egg collection,

culture and storage before its full potential can be exploited. If and when these techniques reach a stage of commercial utility and can be harnessed to a programme of sex determination, we will have arrived at the ultimate situation in which the commercial cattle user could have his cow inseminated with a fertilised egg (or preferably two) of known sex, sired by an elite bull, and whose 'genetic mother' is an elite cow.

RAPID EXPANSION OF RARE GENETIC STOCKS

Of the four factors which change the genetic constitution of a population, mutation, migration, selection and chance, migration has probably been the most potent force for the improvement of domestic livestock. Most of the cattle populations, both dairy and beef, currently used in the developed countries are not natives of the country in which they are used. Even in a large population such as the Holstein/Friesian, or the Hereford, the different goals which these strains have had in different countries and the different rates at which they have progressed towards these goals, have created differences between sub-populations of the same breed. The exploitation of these differences then becomes the fastest method for the slower moving sections of the population to catch up with the rest. With the increase in world trade and the ease of communication and transport, the pace of this international and inter-population exchange of genetic material has greatly increased.

However, this exchange must take place within the constraints set by the veterinary and livestock regulations which control the movement of stock from one country to another. This situation, therefore, creates bottlenecks through which stock introductions must pass. For example, in Ireland we have a quarantine station through which all imported stock, other than those from the UK are processed. It has a capacity of about 500 head per annum. With several breeds involved, this means that the numbers of animals of any one breed which can be introduced to the country in one year is quite limited. The same sort of situation applies to importations into the United States, Australia and New Zealand. In all of these countries there is at the present time a great demand for the larger European beef breeds. In this kind of situation, the transfer of eggs can be used to increase the reproductive rate of the animals which are imported by a factor of perhaps up to 500%. The genetic consequences of this are simply to speed up the rate at which the introduced breed becomes available in the introducing country.

The extent to which this is genetically a benefit is impossible to quantify since the subsequent use of the breed will affect the issue. However, this scarcity situation does create commercial opportunities in the sale of imported livestock, and it is the drive to exploit these opportunities which has been the main impulse to the development of practical egg-transfer techniques in recent years.

In the longer term it is difficult to see the continued use of egg-transfer for this purpose, unless the cost involved is greatly reduced. The high prices currently paid for rare exotic breeds must eventually subside to a level related to the commercial use of their offspring for beef or dairy production. In addition, it is likely that the veterinary status of different countries will tend to become more equal and that most of the exchanges of genetic material will tend to take place from countries with higher veterinary status to those with lower status. These developments will eliminate the physical bottlenecks in the exchange of genetic material which at present exist, and would therefore reduce the need for recourse to egg-transfer techniques.

INCREASING THE INTENSITY OF SELECTION IN FEMALES

Cows can be selected with two aims: as dams for producing bulls, or as dams for producing females. The first of these aims is overwhelmingly more important than the other. The reason for this is that through her son, a cow can effect the genetic merit of many tens and thousands of other cows. Through her daughters, even allowing for a five-fold increase in her reproductive potential by egg-transfer, her genetic merit finds its way into only some dozens of animals. So from the point of view of benefit to the population as a whole, the return for effort spent on selecting and increasing the reproductive rate of selected females comes entirely from their contribution as potential AI bull dams.

SELECTION OF DAMS OF FEMALES

If cows have on average four lactations, and produce four calves in a lifetime, then for every hundred cows, 25 heifer calves are required per annum as replacements. With a net reproductive rate of 85%, and a normal sex ratio, this means that 59% of all cows are required as dams of

replacement heifers. If these dams are selected strictly on milk for butterfat production, they will have an average phenotypic superiority of 0.66 standard deviations. The average genetic superiority of their offspring is then simply a half of this multiplied by the genetic standard deviation in the population and the correlation between a cow's genotype and the criterion on which this selection is based. Milk production has a heritability of about 0.25 and a coefficient variation of about 20%, so that the genetic standard deviation is about 10% of the mean. With selection based on three lactation records, the correlation of genotype and selection criterion will be about 0.65. The average genetic merit of the offspring of these cows should then be about 2.15% above the mean of the population. A similar calculation can be made assuming that each cow can have two, five, ten or twenty times as many progeny as she would under normal reproduction. The results of these calculations are given in Table 1.

TABLE 1 SELECTION EFFECT OF INCREASING NUMBER OF OFFSPRING PER COW

Offspring per cow lifetime	Percent of cows selected as dams of replacements	Genetic superiority of offspring (in % of mean)
4	59.0	2.15
8	29.5	3.80
20	14.8	5.07
40	7.4	6.14
80	3.7	7.15

It can be seen that increasing the net reproductive rate of cows 20-fold trebles the genetic superiority of their offspring. Since selection of dams of replacement heifers accounts for 5-10% of total improvement in the population, the effect of universal egg-transfers in this scale would be to increase the total rate of improvement in the population by something between 15 and 30%. It should be added that in these calculations it is assumed that cow selection is done with full effectiveness. This is not the case at present because of limited recording and inefficient use of whatever information is available, and so the assumptions involved are over optimistic. The achievement of these gains will also require a very extensive programme of egg collection and transplantation with 3.7% of all

cows being egg donors and more than half of the remainder being recipients. If sex control could be combined with egg-transfer, then the scale of the opération becomes much more feasible. It therefore appears that egg-transfers on a population wide basis are unlikely to be worthwhile unless the cost can be brought down to something approaching the cost of a normal insemination. The major technical problems in the storage and handling of eggs will also need to be overcome.

SELECTION OF BULL DAMS

The effectiveness of selection, whether of cows or bulls, is the product of two things, accuracy and intensity. In the case of bull selection based on progeny test, it is possible to get very high accuracy by increasing the number of progeny up to say 100 or more (accuracy 93%). The problem here is usually to get the intensity high, as it is very expensive to test a large number of bulls. With cows on the other hand, the problem is quite the reverse. It is easy enough to pick one cow in a thousand, or indeed, one in ten thousand. The difficulty is to pick them with sufficient accuracy. If selection is on the basis of milk, butterfat or protein production, the accuracy is approximately as follows: 50% (1 lactation), 58% (2 lactations), 61% (3 lactations), 63% (4 lactations).

Taking into account information on the cow's sire's progeny (that is her half-sisters) can improve this accuracy somewhat. However, the basic limitation is that the amount of information we can bring to bear on a single cow sets an upper limit to the accuracy with which we can identify superior cows.

Recognising this limitation, it is still well worthwhile to try to achieve maximum accuracy and intensity in the selection of cows which are potential bull dams, and which are therefore going to have a large genetic effect on the population. Current techniques of superovulation and egg-transfer might make it possible to increase the normal reproductive potential of a cow from five progeny to 25 progeny, 50 or, if we are very optimistic, 75. What contribution can this make to the efficiency of bull dam selection? It has next to nothing to offer on the question of accuracy. It could, however, increase the intensity of selection. If a 100 cows are currently used as bull dams, and if their reproductive

potential is increased five times, we could produce all the young bulls from the best 20 of these 100. The gain, therefore, is simply the average increase in merit of the dams achieved by using these 20 rather than the full 100.

This effect is quantified in Table 2. Bull dam selection schemes operated with normal efficiency in European countries use approximately the best 3% of the cows available. This gives cows which are phenotypically about 40% above average in production, but genetically perhaps 10% above average. The genetic merit passed on by these dams to their sons which get into service and subsequently transmitted by the sons into the population at large accounts for 20 to 25% of all the genetic gain achieved in the population. Table 2 shows the relative genetic merit which might be expected in bull dams selected in normal (3%) relaxed (5%) and exceptionally effective (1%) bull dam selection programmes, and using egg-transfer techniques which give 5, 10 or 15 calves per cow per year. The gain from using egg transplantation ranges from an improvement of 17% to an improvement of 47%. It should be noted that most of the improvement is achieved by moving from one calf to five calves per cow per year, so that even moderately successful egg transplantation is giving most of the benefits achievable.

TABLE 2 EFFECT OF EGG-TRANSFER ON MEAN VALUES OF BULL DAMS

Effectiveness of present bull dam selection	Calves/bull dam/year			
	1	5	10	15
	Relative Phenotypic Merit			
Moderate (5%)	100	131	141	147
Normal (3%)	100	127	136	141
Exceptional (1%)	100	117	125	125

To quantify this 'added value' in economic terms, let us take the situation where a normally effective bull dam selection programme is in operation, and egg-transfers enable each bull dam to produce 10 calves per year. The result of this is to improve the average merit of the bull dams by 36%. Since their merit contributes 20-25% of the total genetic gain, the net effect is to improve the rate of genetic gain in the integrated programme by about 8%. Assuming the average rate of genetic change

in the population is about 1% per year (which implies a conventionally effective progeny testing and selection programme) then 8% of this annual improvement is equal to 8% of (1% of 3,000 kg) = 2.4 kg in Irish conditions. This improvement is delivered through the insemination, and each insemination gives rise to about 1.3 discounted progeny lactation equivalents (McLintock and Cunningham, 1974). Assuming that two-thirds of the gross revenue for marginal improvements in milk production is profit, the value per insemination of the genetic merit added is therefore about 2.4 kg x 1.3 lactation equivalents x 4.4 p. per kg. profit = 13.7 p.

It is obvious that quite a few assumptions have to be built into these calculations. Most of them are quite defensible. The two most questionable are as follows. The calculation of this increased superiority of bull dams selected after superovulation and egg-transfer is based on expectations in the extreme tail of a normal distribution. Although these are likely to be true on the phenotypic scale (Syrstad, 1971), one must say that we are not quite sure how much of this increased superiority carries through onto the genetic scale. The reason for this doubt is that in dealing with cows at the extreme of the population, we are dealing with a group which are more likely to be subject to conscious attempts by their owners to modify their production, and that the heritability of production among these is therefore possibly less than in the population at large. The net result of this is that the conventional assumption will probably overstate the real benefit. The second assumption which must be discussed is that which assumes that we are simply adding the benefits of egg transplantation to a conventional bull dam selection scheme. In fact, bulls are at present selected in a variety of ways, and a minority, perhaps one-third, of those currently going into AI centres, come out of formal planned matings. This assumption does not directly affect the calculations, but affects the scale, that is the number of bulls to which the results might have some relevance.

Is egg transplantation then justifiable as an adjunct to the bull dam selection scheme in a dairy cattle population? The total genetic benefit obviously depends on the number of inseminations involved. In the reasonably feasible example I have chosen, it amounts to something over 10 p. per insemination. In a population with half a million dairy inseminations per year, it could therefore contribute about £50,000 of 'net added value' to the genetic merit of the inseminations carried out. As with all aspects of genetic improvement achieved through AI, the programme must be funded

centrally while the benefits are spread almost imperceptibly over the whole population. To make such an operation economically convincing one would therefore need a very high return for money expended. If we take a ratio of returns/expenditure of 5/1 as the minimum acceptable, then one would need to be able to replace a conventional bull dam selection programme with one involving egg transplantation for an additional expenditure of not more than £10,000 in the population specified. The whole question of the utility of egg-transfers in bull dam selection therefore becomes very largely one concerning the relative costs of providing young bulls for testing through a conventional planned mating programme or through a more concentrated programme involving egg transplantations.I believe that egg-transfers will play a part in dairy cattle breeding operations in the future, and that this involvement will be largely based on the grounds of operational and financial economies.

INCREASING THE TWINNING RATE

One of the possibilities which is opened by the use of egg-transfers is the implantation of 2 eggs at a time in each recipient cow. In this way it may be feasible to greatly increase the twinning level in a cattle population. Production of twin calves is not totally advantageous. They tend to have a higher mortality and poorer performance than singles and to cause longer calving to conception intervals and lower milk production in dairy dams, while in beef dams the strain of rearing 2 calves can significantly reduce the success of re-breeding. Even with these disadvantages, there is a net gain in the output of the population.

All of this increased gain comes on the beef side, since a population producing singles is already producing enough replacement females. The genetic consequences of an increase in the twinning rate are very similar to those which follow from an alteration in the sex ratio. In both cases, fewer cows are needed as the dams of both males and females of the next generation. These dams can therefore be selected with a greater intensity. In addition, since fewer cows are required as dams of replacement heifers, a higher proportion are available for crossing to beef bulls with all the consequences that this entails. The general tendency therefore would be to drive the population into specialised dairy and specialised beef strains, as would be the case with an altered sex ratio.

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DISCUSSION

M. Hansen (Denmark)

I have a question related to the expensive and rare breeds. You said there was a decline in interest here but isn't there a possibility in exports of embryos to developing or semi-developing countries in the future?

E.P. Cunningham (Ireland)

Yes, that is a possibility but it is obviously going to take a lot of organisation unless somebody thinks up a way of keeping eggs for rather longer than we are able to do at the present time. I should mention that earlier this year we did try to set up a system whereby we would fly eggs from France and implant them in cows in Ireland. After long negotiation with the veterinary people in our Department of Agriculture, it turned out that the conditions they would impose were so expensive that it just wasn't on. Technically, I believe, it was on in that case but whether one can envisage that on a trans-continental export basis, I don't know.

A. Robertson (UK)

Perhaps I will ask you a direct question now and ask you to put your calculations in parallel with Professor Kräusslich's. He said, one million DM profit for the selection operations per year in a population of 100,000 cows. How can that be paralleled by your statement that you could afford to spend £10,000 per year on a population of 500,000 cows? There seems to be a discrepancy.

E.P. Cunningham

I have introduced an arbitrary element and said that we need a return of 5 : 1. Dr. Kräusslich calculated solely the benefits so if you talk about it as benefit to benefit, the benefit in my case was about 10 p. per insemination. In his case it was about 10 times that - about DM10. I am not sure where the paths diverge.

J. King (UK)

I can't balance these accounts but surely the main gain on Dr. Kräusslich's system is on the beef side, in contrast to the dairy situation. Paddy Cunningham is producing 5 male calves from a selected bull dam and he is not discriminating between them; they are all of equal dairy merit. Obviously you could select between them on growth rates and get extra gains.

SUPEROVULATION AND OVUM TRANSPLANTATION IN GENETIC IMPROVEMENT PROGRAMMES

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INTRODUCTION

Genetic improvement of livestock may be carried out in two phases, firstly the most suitable breed, strain or cross available is identified, and subsequently gradual and continued improvement is made in the best population. In cattle, progress in both phases is limited by the low rate of female reproduction. An increase in the reproductivity of selected females by superovulation and subsequent ovum transplantation could therefore contribute to improvement, by allowing both a more rapid multiplication of superior stocks and more intense selection.

The current commercial use of superovulation and transplantation ("ovum transfer") to expand the population of exotic breeds illustrates their use in the first of these phases, where the scarcity value of the progeny carries the high cost of the procedure. In this paper we discuss the possible advantages of using ovum transfer in the phase of continual improvement. Much of the analysis for beef cattle and reproductive performance has been taken from our earlier paper (Land and Hill, 1975), where the assumptions and methodology are given in more detail, and we merely summarise the results. An analysis for dairy cattle has also been given by Skjervold (1974); like Skjervold we shall conclude that ovum transfer is unlikely to be of much benefit in dairy cattle improvement.

Incorporation of ovum transfer into a breeding programme will not in itself turn an inefficient programme into an efficient one, indeed a much higher level of organisation will be required to utilise the more sophisticated technology. We restrict the present discussion to the potential benefit of adding ovum transfer to an already efficient programme using conventional reproduction.

Several important assumptions are made: on technology that:

1. a scheme can be operated successfully year after year, with donor cows reliably producing sufficient embryos which,

2. can be implanted in recipient animals to give an average of at least six offspring, and, on the genetic side, that,

3. facilities for testing and recording animals are available, and,

4. the improvement programme has a clear objective.

This last may be contentious, for simplicity we assume that the objective in a dairy breed is solely milk yield and in a beef breed solely rate of gain of progeny, and defer aspects of genetic improvement of reproductive rate itself to later.

DAIRY CATTLE

In national dairy cattle breeding programmes bulls are largely selected on their progeny test and selection intensities among bulls are not dependent on the rate of female reproduction. The lower limit to the number of bulls used as sires of bulls for the next generation is set by considerations of inbreeding.

The majority of cows are needed to breed female replacements, so that the selection intensity among cows is low. Whilst an increase in reproductive rate would clearly permit more intense selection and increased genetic progress this would require, in a national breeding programme, that the majority of the national herd were recipients in a transfer scheme. Even if the cost of the recipient operation could be kept low, considerable technological improvements would be necessary, especially in the supply of embryos, before this could be contemplated on a national scale.

The remaining source of improvement is in the selection of bull dams, i.e. of cows to be mated to the best proven sires with sons of these matings subsequently progeny tested. Since only of the order of 100 young bulls may be required for testing in a national scheme, e.g. 135 Friesians by the English Milk Marketing Board in 1973/4 (MMB, 1973-74), ovum transfer may be feasible and perhaps worthwhile. Thus we consider bull dam selection in more detail.

Currently, potential bull dams are selected on their milk record over several lactations, perhaps with data on their half sibs, (i.e. sire's progeny test), dam record, and, if available, daughter records included. This scheme might be made more efficient by use of ovum transfer because fewer animals would need to be selected to produce enough young bulls for testing. Firstly, let us consider the effect of increasing selection intensity. In an efficiently run programme, such as in Norway, about 2% of cows are selected as bull dams (Skjervold, 1974) and this could be reduced to 1% with superovulation and not more than one son chosen per dam to reduce subsequent inbreeding and testing of full sibs, or to about 1/3% if six calves could be reared per donor and full sibs considered. These correspond to selection differentials in the ratio 100 : 110 : 125, and if about one quarter of the total improvement comes from selection of bull dams the improvements in response with the two increases in selection intensity could only be about 2½% and 6%. In Britain, selection differentials for milk yield of potential bull dams of less than one standard deviation, equivalent to the selection of the best 40% (MMB, 1973-74) suggest that considerable improvement can be made before resorting to ovum transfer.

Ovum transfer also facilitates progeny testing of cows, but only if they have many daughters is this an accurate procedure. It is clearly not feasible to progeny test a high proportion of the national herd, so assuming cows are selected for progeny testing on the basis of their first lactation record, they will have completed 5 lactations by the time their progeny have completed one. Depending on the genetic parameters it would take around 10-20 progeny with one record to give as much information as 5 of a cow's own records, and even more would be required to match the information available from her and her half sibs, although an exact figure can not be computed without information on the variation due to additive gene effects and to maternal environment with natural and transfer reproduction. With records on only 3 daughters (a more feasible possibility), the extra accuracy over using the cows own records and those of ther half sibs (i.e. sire's progeny test) is only about 1%, and the availability of only 2 full sibs would not add greatly to the accuracy of cow selection.

Therefore the effect of using ovum transfer on the rate of genetic gain seems trivial and not worthwhile, but depending upon the relevant merit of new and tested bulls, a reduction in the proportion, or an increase in

the merit of young bulls may lead to a once and for all increase in the mean level of performance of a national herd.

Superovulation and embryo transfer, although inappropriate for the improvement of present schemes based upon a single national herd, allows us to consider the possibility of using a section of the population as a nucleus and source of breeding animals for the whole breed. Hinks (unpublished) has discussed the use of such nucleus herds. Their important feature is that ovum transfer could be used to increase the selection intensity among dams of female replacements, for example, if only 30% rather than 75% of cows were used to select heifer replacements, the corresponding selection intensity would more than double, and the response increase by 20% or so. Bulls could be tested in the national herd thus maintaining selection. Whilst at first sight this seems an attractive proposition. if the nucleus is to contribute significantly, to progress in the national herd it must be of sufficient size to supply most of the young bulls required for progeny testing each year and still enable intense selection to be practised among bull mothers. With an annual requirement of 100 bulls for testing, and with the restriction that none should be full sibs, even with superovulation over 100 selected cows would have to be available each year. With current selection differentials among bull dams in Britain this might require 200-300 nucleus cows in all; to enable 2% selection, comparable with optimum field structure, some 5000 would be required. It may be possible to reduce these intensities if there is more environmental control and thus more accurate selection in the nucleus, and further detailed analysis might show how to compensate decreased selection of bull mothers by that on replacement cow mothers. At present, however, nucleus herds using ovum transfer do not seem economically justified.

BEEF CATTLE

In an improvement programme for traits of the growing animal replacements of beef cattle of both sexes can be selected on their own performance before reaching reproductive age in contrast to dairy cattle. But because the reproductive rate of females is low, selection amongst them is at the expense of an increase in the generation interval. Thus an ovum transfer scheme could potentially double response by enabling selection among females as well as males. This is a very large increase and we felt justified in

studying it in more detail (Land and Hill, 1975).

As a basis of comparison we considered an efficient programme run with a closed herd but traditional reproduction, although such a programme is more efficient than most or any run in commercial practice (Meat and Livestock Commission 1971). In the ovum transfer scheme, animals are selected on growth rate up to a year or so of age. Selected females are superovulated, mated to selected bulls and the ova transferred to a recipient herd so it should be possible to run the herd with a generation interval of 2 to 2½ years. The limits to selection intensity among males are imposed by inbreeding and among females by their superovulated reproductive rate. Results are summarised for the conventional and ovum transfer schemes in Figure 1. With reasonable assumptions of parameters, the rate of improvement in 400 day weight might be increased from 9 to about 16 kg per year, and is little affected by the number of offspring per donor.

A subsidiary problem is calving difficulties. Testing for this could be incorporated in the scheme by mating bulls to commercial animals as well as the nucleus cows. Replacement animals could then be chosen on their own growth rate and their purebred full sibs and pure and crossbred half sibs calving difficulties. Inclusion of family information would increase the rates of inbreeding shown in Figure 1.

The use of superovulation and ovum transplantation thus has great potential when selection objectives are clear, for example, the improvement of terminal sires in beef schemes where the associated increase in mature size may not be disadvantageous, but first there remains a need to bring current programmes up to optimum levels.

GENETIC IMPROVEMENT OF REPRODUCTIVE RATE

In addition to increasing the rate of response to genetic selection of traits expressed in both sexes, and of sex limited traits in conjunction with progeny testing, ovum transfer may make it possible to consider direct selection for female traits such as reproductive rate. In dairy cattle, or a dual-purpose breed such as the Friesian, increases in reproductive rate from selection can be used to increase beef output but have to be balanced against the reduced selection pressure applied, and less rapid improvement,

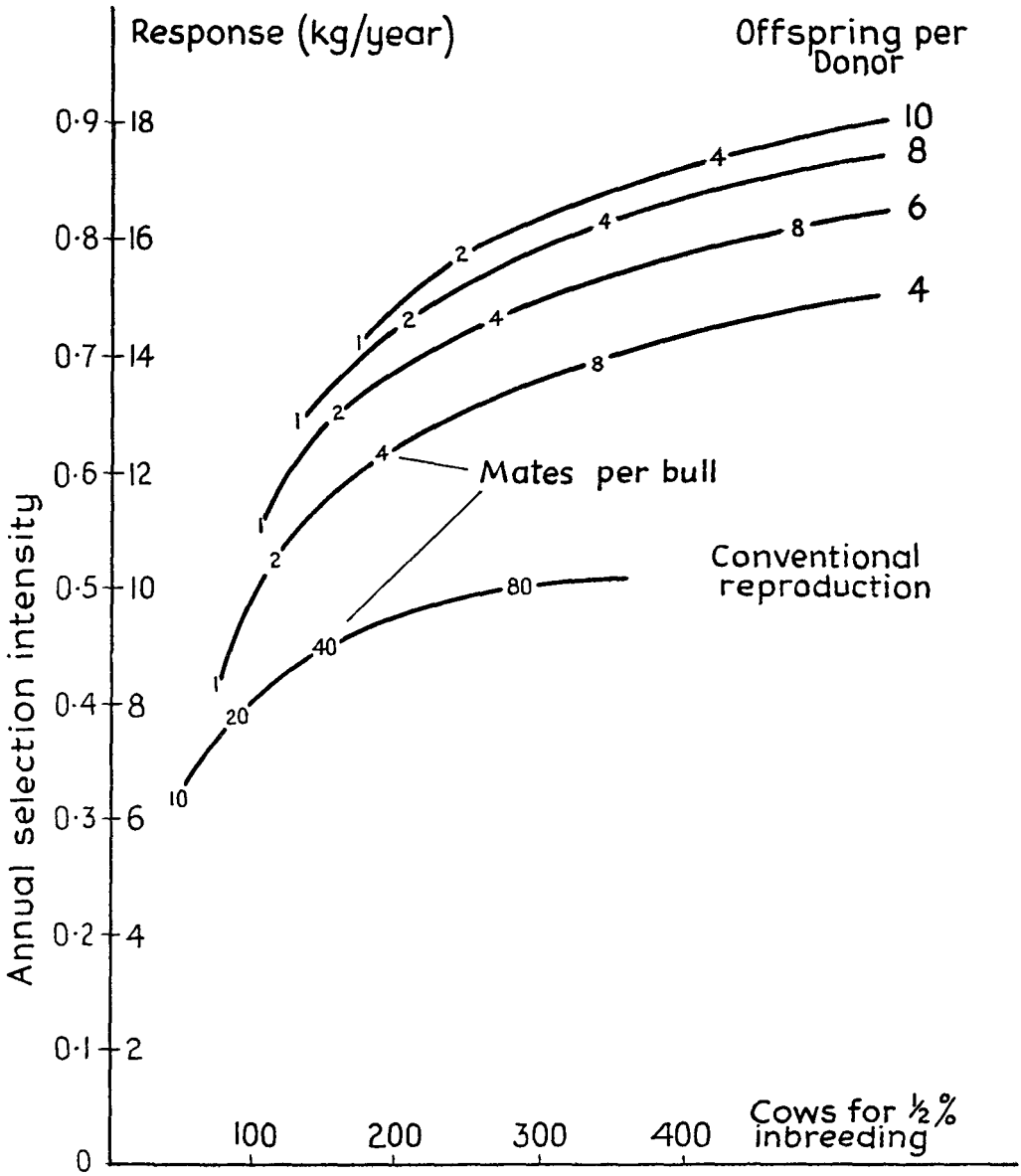

Fig. 1. Comparison between conventional and transfer schemes of selection for traits in the growing animal. Response is shown in relative terms as the annual selection intensity (mean selection differential among cows and bulls/generation interval) and as the predicted response in 400 day weight assuming h^2 = 0.5 and σ = 40kg. The rate of inbreeding is in terms of the total number of donor and recipient cows.

in milk yield. In breeds of beef cattle for suckling the objectives are less clear-cut, for an increase in progeny growth rate confers the disadvantage of an increase in mature size and cow costs. Thus the benefits of selection for increased reproductive rate in such animals may have less associated disadvantages.

Ovum transfer would facilitate intense selection among cows which have calved several times. This could be used to establish a herd from only animals with several multiple births; but with a low heritability the twinning rate in the next generation is likely to remain so low that few animals could be selected on twinning within it. By use of laparoscopy, however, several successive natural ovulations can be recorded on the same animal in the space of a few months, and available evidence suggest that in cattle an increase in ovulation rate would lead to an increase in twinning rate so that selection on the basis of ovulation rate should be effective. The animals selected on the basis of ovulation rate would then be superovulated and their fertilised ọva transferred. Bulls could be selected on their full sibs' performance, or on some other trait.

The rate of response depends on the basic parameters of initial incidence, number of ovulations recorded and heritability and repeatability of the trait, and more detailed results are given in our earlier paper (Land and Hill, 1975). Taking a heritability of 4%, a repeatibility of successive ovulations of 6% and families of size 6, the response is predicted to be about ½%/year if the current twin incidence is 4% and rather more than 1%/year if the incidence is 16%. The incidence in our breeds is typically nearer the first figure (at least in twinning rate), but by selection of an initial nucleus herd this value could be increased.

Although the advantages from increased twinning rate could, with adequate management, be worthwhile in either a suckler cow or dairy cow situation, in the latter it is unlikely to compensate for reduced selection for milk yield. In gross terms, with milk at, say £0.09/kg, an annual response of £6.0/cow is feasible (70 kg/year), whereas ½%/year in twinning rate is worth only ½% of the calf price of say £30 allowing for reduced size, i.e. £0.15/cow. It would be difficult to superimpose a twinning programme onto existing dairy improvement schemes in which the national herd is taken as one unit.

DISSEMINATION OF GENETIC IMPROVEMENT

If genetic progress is being made in a nucleus population it is important to minimise the lag in improvement between the nucleus and commercial populations, in other words, to rapidly disseminate the genetic improvement to commercial stock. Bichard (1971) discussed factors affecting this lag and how it could be computed. Traditionally in cattle most improvement is disseminated by movement of bulls, but this is largely by-passed in dairy breeds by use of AI. With only homebred female replacements and little selection practised among their dams, the average merit of new-born animals got by AI is one generation of response behind the AI bulls, that of lactating animals about two generations. This gap could not be closed substantially nationally by use of ovum transfer, for the supply of ova from cows of comparable genetic merit to the best bulls is necessarily small. It would require large technological improvement in the supply of eggs for this to change, even if cost were not prohibitive, for the national benefit would depend on the superiority of the nucleus herds and the proportion of ova deriving from them. In beef cattle where less use is currently made of AI, the multiplication of numbers from a nucleus could be more rapid by use of ovum transfer, but AI offers a cheaper alternative.

CONCLUSIONS

We consider that superovulation and ovum transfer could have a useful role in the improvement of characters of the growing animal where there are clearly defined objectives. The technique may also be useful in improvement of female reproductive rate where repeat examinations can be made in a short time on individuals using laparoscopy, and the best selected. With dairy cattle the advantages appear to be small, at least in schemes where intense and accurate selection is already practised among cows used to breed young bulls.

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DISCUSSION

A. Robertson (UK)

We seem then, to have focussed on two situations in which egg transplantation and superovulation are possibly of value, that is to say, in dairy cows, concentrating on those cows from which we could possibly breed bulls and select those more intensively. Having said this, what about the logistics of this situation - how would you do it? The other situation, in a pure beef breed in which one is concerned to increase growth, Dr. Hill is thinking in terms of a single herd of maybe a few hundred cows in which there is a superovulation and transplantation unit attached to that herd, carrying out several hundred transplantations per year. This is what seems to me to come out of the last three papers. Any comments and questions?

J. King (UK)

I wonder if it would be possible to have that slide back on the screen again because I would like to confirm one thing and make one comment. (Fig.1) The figures which are points on the graph are, in fact, mates per bull?

W. Hill (UK)

Yes.

J. King

I wanted to clear that up because I thought you said the opposite at one stage which might have been confusing.

W. Hill

If I did I didn't mean to. The figure 8, for example, the structure is that there are eight cows mated to every bull. In other words, if there are 8 bulls it would imply a herd of 64 cows.

J. King

Now the number of cows is the total number of donors plus recipients; I suggest that is misleading because once you have used the donors to provide the eggs you don't want them any more. So the number of cows on the ground which you have to maintain is, for a short period, that total number but in terms of long-term accommodation .....

W. Hill

Yes, except I would comment that if you are operating at this sort of level of offspring to donor, then the number of cows in the donor herd is a very small proportion of the herd. In other words, if it was 10 offspring per donor, that means you are going to have something like 15 - 20 cows per donor around. Probably you are right, but I don't think it makes any difference. You could argue the opposite way, that you shouldn't count the recipients because the recipients are put off somewhere else. It doesn't matter very much how the recipients are being looked after during the period from when they got in calf. They could be on somebody else's farm somewhere else provided you had access to the progeny.

I. Wilmut (UK)

Following up on the same question - did you assume that you would get every recipient pregnant?

W. Hill

No, I don't remember the exact figure, it's about 70% I think. I could tell you exactly if I looked at the original calculations. It should be borne in mind, in terms of this, we used the recipients again three weeks later if they re-cycled so there isn't a phenomenal excess of recipients. Obviously, recipients in which the embryo dies post three weeks is counted in but not ones which return infertile at three weeks. That sort of thing only affects the rate of inbreeding calculations, it doesn't really affect the rate of progress calculations so that all one does, if one assumes differential rates of take is to spread out or condense the graph accordingly.

C. Polge (UK)

If I understand correctly, although you might be doubling your rate of gain, if you work it out in cash value, it might only represent about 6 or 8 kg per year, mightn't it - if you transfer it into terms of the value of 1 kg of beef?

W. Hill

Well, I have carefully avoided doing calculations like that actually!

R. Church (Canada)

I would like to look at this from the point of view of the operator who has this particular transferred propagated herd. One of the reasons that most of the selection programmes in beef have got nowhere is because it is easier to make progress in a cross breeding situation. Really we are talking here about an elite breeder of a pure line of stock and in this particular regard, using Dr. Cunningham's figures, by the time he has looked after his replacements he has essentially the bottom half of his females for sale. To stay in business he is selling breeding stock. The implications of what you have presented here are that he will be able to keep the same number of replacement animals and have, depending on which curve you set yourself on, from two to three times as many replacement females for sale, and these are three to four times as many as he would normally have of those top animals. Hence his return on these animals would almost balance, I would suspect, the cost of the transfer itself. This is in contrast to the considerations we have had with the last three speakers where we have talked about the national herd. In the beef industry in Germany, and, to a lesser extent in Ireland, this national documentation is a reality. In most places elsewhere in the world you have individual herds which are operated as breeding stock establishments in the beef business. These same individuals have the most effective performance test programmes and would probably benefit from their performance programmes in using embryo transfers in the method you have suggested. The cost recovery to these breeders will be in the female replacements they will be able to offer for sale. That's another way of looking at the gene pool expansion - we have talked about gene pool expansion as being on a national basis really due to health requirements, but, from the individual operator's point of view, the gene pool expansion is the number of replacement females he has to offer for sale and that kind of gene pool would certainly be an ongoing type of demand.

W. Hill

Of course, I have talked of this with regard to breeding bulls for use in AI but there is no reason why an individual operator couldn't do such a thing providing they had the capital and providing they could be fairly sure of a market. The problem in all these things is whether you can be fairly sure that the animals are going to be bought by somebody at the end of the day. What Professor Church is saying is that if they are really

superior then you are bound to find a market for them and this, of course, would cover a large part of the cost. Certainly in Britain there is nothing operating in this sort of way. I guess in the States and in Canada there are people who are running quite large performance testing programmes who might well ask themselves if it is worth their while using superovulation to increase the rate of response.

A. Robertston

There is a sense in which, in a programme like this, such a breeder could have his cake and sell it at the same time because the animals which he has chosen for his breeding programme are selected young bulls which, after he has used them for one year, he sells, and heifers which he has got one lot of eggs out of which after this he sells.

R. Church

I am involved personally with 13 beef breeding herds which are actually using this programme, or are at various stages of implementing this programme.

A. Robertson

Well, we have run a little over time - thank you very much Dr. Hill.

TECHNICAL AND ECONOMICAL ASPECTS IN RELATION TO EGG TRANSFER IN CATTLE

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INTRODUCTION

For many years breeding plans have been accepted as tools for achieving the maximal genetic gain (and economical profit) in cattle breeding. However, breeding plans must not be the goal itself - they must be subject to change, if new conquests prove successful.

The application of embryo transfer and the related techniques of oestrus synchronisation and embryo storing might be an aid in further improvement. During the very last years the development of non-surgical egg transfer has been very rapid (Foote and Onuma, 1970; Sreenan, 1975) and further development might allow operation on farm level in the near future.

In cattle breeding genes are transmitted from one generation to another through the four paths bull-bull (bull sires), bull-cow (cow sires), cow-bull (bull dams) and cow-cow (cow dams).

Further improvement from a given breeding scheme when applying the egg transfer technique will obviously come from the bull dams and/or the cow dams. According to Skjervold (1974) and Hansen and Neimann-Sørensen (1974) the greatest technical gain will occur in the cow dam path. However, Hansen and Neimann-Sørensen (1975) showed that application of egg transplantation in the cow dam path does not pay. In the bull dam path the results were more promising.

The aim of the present paper is firstly to investigate further the technical and economical output from breeding plans, utilising embryo transfers in the bull dam path, secondly to discuss in which fields the geneticist would be interested in further improvements in the technical-biological field.

BREEDING PLANS UTILISING EGG TRANSFER

In a simulation study the effect of applying egg transfer on bull-dams

was examined. A description of the general principles in the breeding schemes in question is given by Petersen et al. (1974). These schemes are now moreorless adapted by the Danish AI societies. A survey of the factors used in the simulations is given in the appendix. The attention is drawn to the fact that a dual-purpose unit of 500,000 cows is assumed and that performance testing of potential breeding bulls is applied. In all alternatives a fixed ratio of 60% of the calves is rejected after the performance test. To ensure selection for management traits too, 10 cows selected on basis of yield are required per recruited bull calf. This proportion is kept throughout the simulations and seems to be a good approach in conventional breeding plans.

RESULTS

The results of the simulations of increasing number of bull-calves per bull dam per year is shown in Table 1.

TABLE 1 THE EFFECT ON GENETIC GAIN IN BUTTERFAT AND DAILY GAIN OF INCREASED NUMBER OF BULL-CALVES BØRN PER BULL DAM EACH YEAR. (POPULATION SIZE:500,000 COWS)

No. of bull-calves per dam	No. of bull-calves recruited per year	Genetic gain per year		Net return	
		Fat, kg	Daily gain, g.	Abs. D.Kr.	Relative
0.5 (Basis)	470	3.14	3.43	232.1	100
1.5	460	3.28	3.60	246.4	106
2.5	500	3.40	3.61	253.8	109
5.0	550	3.50	3.64	261.5	113
7.5	600	3.56	3.66	264.6	114
10.0	600	3.58	3.66	266.2	115

The basic plan is the one recommended by Petersen et al. (1974).
The scheme is supposed to have been working for a sufficient length of time to enable reaching a relatively steady rate.

A further evaluation of the economic result is shown in Table 2.

TABLE 2 MARGINAL NET RETURN AND MAXIMUM PRICE TO PAY PER RECIPIENT

(POPULATION SIZE: 500,000 COWS)

No. of bull-calves per dam	No. of recipients	Marginal net return, D. Kr. per cow	total (1000)	per recipient
0.5 (basis)	-	-	-	-
1.5	920	14.3	7150	7750
2.5	1000	21.7	10850	10850
5.0	1100	29.4	14700	13350
7.5	1200	32.5	16250	13550
10.0	1200	34.1	17050	14200

From the results in Table 1 it is obvious that the greatest improvement will be on the dairy production. This is partly due to the fixation of the ratio of discarded bulls after performance testing. Earlier results, reported by Petersen et al. (1974), show a pretty constant improvement ratio of 90% dairy to 10% beef.

There is a very great long term effect incorporated in the economic results. The price, which can be paid per recipient by the greater genetic gain, is from 7750 D.Kr. to 14200 D.Kr. assuming that a bull dam is able to give birth to 3-20 calves per year. Not taking the accumulated effect of earlier obtained genetic gain into account, Hansen and Neimann-Sørensen, (1975) found corresponding values of about 2000 D.Kr.

DISCUSSION

At the present stage of non-surgical egg transfer, the price to pay for a pregnant recipient is lower than the above mentioned range of 7750 - 14200 D.Kr. With a further developed technique the costs will decline still more. Though it seems, as if the economic background is present for applying egg transfer to common breeding plans.

However, the technical staff-veterinarians, cytologists and other necessary experts are as yet insufficiently trained as a whole to enable egg transfer programmes on a commercial cattle breeding scale to be set up. Many practical problems have still to be solved. Is the cattle breeder himself able to support the economical responsibilities involved or should it

be the AI associations leasing the outstanding bull dams? Will the "egg-transfer-team" be run as a private business or should it be an AI controlled operation? Which of these systems will prove most suitable in the long run? These questions, together with numerous others, will limit the speed of expansion in the field of egg transfer, but nevertheless, my opinion is that the field in question will be conquered within a relatively short time.

FIELDS OF IMPORTANCE IN RESEARCH

Though the technical problems closely related to egg transfer seem to be elucidated very well through research, there are still areas in which further research and development is needed.

Fertilisation of eggs in vitro

The possibility of fertilising eggs in vitro will be of importance in at least two fields:

The increase of inbreeding may be considerable, when egg transfer is utilised, especially in small populations (Land and Hill, 1975). In larger populations part of this increase could be avoided using several bulls as sires of a litter from a single bull dam.

Progeny testing of females is of minor value when performed on full sibs as the efficiency of progeny testing on full sibs compared to half sibs is equal to $\frac{4 + (n-1)\ h^2}{4 + 2\ (n-1)\ h^2}$. This proportion will never exceed 1, so also in this case fertilising of eggs in vitro will be a step forward.

Sex control

Many attempts to separate male and female spermatozoa have been made , e.g. Joakimsen, (1975). The results have so far proven rather poor. However, Voisin et al. (1974), suggests a new method which might show a greater degree of success.

Sex control might ensure bull-calves from bull dams and would be a powerful tool too in the selection work on the females within herds.

The latter is probably of greater importance than the former.

Field methods for oestrus synchronisation

The synchronisation of oestrus between donor and recipient is one of the main points in the egg transfer schedule. Recipients in natural heat can be used in large herds, PGF_{2a} is used too, its effectiveness is obvious, but it is expensive and very difficult to operate on "farm level". A system using other drugs such as, for example, progesterone would be preferable under field conditions.

Egg/embryo preservation

Successful freezing of cattle embryos has been reported (Polge and Rowson, 1974) and a development in this field may be expected. I think that I am not going too far in saying that when this problem is solved, the time might be within range when the AI technician, instead of using frozen semen, has frozen embryos of specific genetic potential to offer to the cattleman. The present problem ought to be seen in connection with the abovementioned possibility of fertilising eggs in vitro.

CONCLUSIONS

From the literature cited and the simulations reported in the present paper, it seems reasonable to conclude that the background for applying egg transfer in the bull dam path of conventional breeding plans is substantiated but, nonetheless, there are many practical problems to be solved before utilisation in commercial cattle breeding on a wide scale can be undertaken for this practice.

ACKNOWLEDGEMENTS

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APPENDIX (Mod. after Petersen et al. 1974)

BIOLOGICAL FACTORS

Size of cow population	500,000*
Active breeding unit, %	50*
Number fattened calves per year	250,000*
Average butterfat yield, kg	200
Average daily gain, g	1,150
Average gain, live weight per beef animal, kg	300
Regression of feed consumption per kg gain on growth rate, s.f.u./g daily gain	- 0.0045
Marginal feed consumption per kg gain over 340 kg live weight, s.f.u./kg.	6.0
Phenotypic S.D. for butterfat, kg	35
Phenotypic S.D. for daily gain, g	70
Heritability for butterfat	0.25
Heritability for daily gain	0.60
Genetic correlation between butterfat and daily gain	0
Accuracy of selection for bull dams (r_{IA})	0.6
Inbreeding depression per percent increase of inbreeding, %	1.0
Selection intensity for CC, %	90
Recovery rate for CC, %	33
Inseminations per cow	2.0
Test insemination per daughter in progeny testing	7.4
Number calves born per cow	1.1
Age at first calving, years	2.3
Calving interval, years	1.04
Wasted bull calves during performance testing, %	20*
Wasted bulls during period of semen storage, %	10
Number of collected semen doses per bull per year	12,000
Generation interval for bull sires, years	6.6*
Generation interval for proved bulls, years	6.1*
Generation interval for young bulls, years	2.0*
Generation interval for bull dams, years	6.5*
Generation interval for cow dams, years	4.5

MARKET FACTORS

Discount rate, %	10
Price per kg milk protein, D.Kr.	8.3
Price per kg butterfat, D.Kr.	14.2
Feed cost per kg butterfat, 11.1 s.f.u. of 0.8 D.Kr.	8.9
Transport cost of 25 kg milk, D.Kr.	0.9
Price per kg live weight, D.Kr.	6.5
Marginal net return per kg butterfat, D.Kr.	10
Marginal net return per g daily gain, D.Kr.	1.2
Cost per s.f.u. for marginal gain in live weight, D.Kr.	0.75
Purchase cost including transport etc. per bull calf, D.Kr.	2000
Cost of feeding, labour and housing for performance testing, D.Kr.	5400
Annual cost of feeding, labour and housing per bull, D.Kr.	6500
Carcass value of bull calf at 12 months age, D.Kr.	4500
Carcass value of bull after semen collection, D.Kr.	5400
Cost of semen processing per deep-frozen dose, D.Kr.	0.20
Cost of semen storage per year dose, D.Kr.	0.10
Recording cost per cow, D.Kr.	21
Insemination cost per cow, D.Kr.	20

FIXED FACTORS OF BREEDING POLICY

Number bull sires selected on yield	4
Number cows selected on yield per bull calf	10
Number of lactations for bull dams	2
Number cows from which bull dams are selected	31 500
Period of performance testing, days	335
Maximum stored semen per bull, doses	50 000

* Factors partly determined by the breeding policy

DISCUSSION

A. Robertson (UK)

Dr. Hansen has brought out several different points. Let me return to one of them: the question of sex control. As he said, 'It has a brilliant future behind it'. Is anybody, as far as the audience is concerned, working seriously on trying to separate sperm into male and female?

L.E.A. Rowson (UK)

The East Germans are supposed to be.

W.G. Hill (UK)

The related question there is the sex control of ova. When and will it be possible to sex fertilised ova before putting them back into recipients? That would seem much more likely to be feasible than ever to sex sperm.

M. Hansen (Denmark)

I think so too. HOwever, it depends on where you can recognise the different cleavage stages and you can moreorless have the chromosomes under the microscope just at the right point.

A.O. Trounson (UK)

I think at the present time it takes something like 200 cells from an embryo to get enough metaphase figures to decide on whether you can locate the Y chromosome and I think that approach has got very severe limitations because you have to take a very advanced embryo to take that many cells for a squash preparation. I think there are many other aspects that could be looked into. Perhaps trying to find out whether there is protein in the chromosome which could be used in an immunological method - or some other method like this. I don't think the squash preparation, looking for the Y chromosome, although it is successful and you can predict definitely if you can find the Y chromosome, has any merit at this stage.

C. Polge (UK)

I think that could be qualified again because at the present moment you only get something like 4 or 5% of the cells in metaphase, this is why you need so many - but, if you take a few cells and put them in tissue culture

and then keep them going and treat them with colchicine, you should be able to get a high proportion in metaphase. Meanwhile, you have frozen the embryo and you can put a sex on it afterwards.

S.M. Willadsen (UK)

Among the other problems you brought up was the question of in vitro fertilisation and I am afraid I didn't quite understand why this would be so important because I think the chance that you could produce half sibs is there as it is. You don't have to use semen from just one bull - you could mix semen and you would be transferring all the embryos anyway so that would not necessitate in vitro fertilisation.

M. Hansen

No, I think you are quite right.

S.M. Willadsen

There is one other point I think I should make and this refers to embryo preservation. One might think of preserving the unfertilised eggs but from the results that we have been having so far in the cow, as opposed to the results that Whittingham is presenting in the mouse, the effect of cooling is not restricted to the fertilised eggs but will probably also affect the non-fertilised eggs. In other words, all stages. This is shown in the pig definitely, we are not quite sure about the cow but we think it is the cow as well. So attempts to preserve the unfertilised cow eggs will probably fail at the present stage.

M. Hansen

That means it cannot be done as easily as you can with sperm now.

S.M. Willadsen

No, no chance.

W.G. Hill

A comment about the half sibs: it is worth bearing in mind that if one has a bull mated to several superovulated cows, you then have, if everything works fine, a full and half sib structure much as one has in, say, chickens and pigs. To some extent if you wanted to one could progeny test the cow in the sense of the deviation of the full sib family mean from the

half sib family mean - with the advantage that you don't get, using pigs for example, that the progeny will come out of a different dam and therefore presumably there are not maternal effect problems.

M. Hansen

Yes, I agree.

S.M. Willadsen

This is a question to the geneticists: would the fact that you could transfer your embryos to standardised recipients say, affect the heritability of certain characteristics in the offspring?

M. Hansen

Presumably not.

W.H. Hill

At the moment heritability in assessing cattle is a correlation of half sibs which all come from different dams anyway - we don't know what the full sib one is.

M. Hansen

The effect, I should say, would be mostly on the traits related to the calf at birth, the size and so on, the weight of the calf. As far as I know, nothing but that - but I dare not say more.

A. Robertson

Can I take up a further point in this. There has been mention in several of the papers that groups of full sibs would be produced inevitably in a programme like this but nobody mentioned using this information. Now, can we ask Professor Kräusslich or Dr. Cunningham, what difference is this going to make?

H. Kräusslich (W. Germany)

I have used information in the estimation of breeding values on growth rate. I used the performance of the bull and performance of full sibs in my calculations. You could also use, though I didn't try it, information on carcass value; if you have slaughtered some full sibs you can get the information on the carcass value as is done in pig breeding programmes now.

E.P. Cunningham (Ireland)

You can draw some inferences from the same situation in pigs and some calculations as we did a few years ago. The same type of calculations have been done elsewhere. Say that if you are already doing performance testing for growth and what carcass traits you can measure in the live animal, primarily back-fat measurement, most of the information that is useful to you is already there and adding slaughter information on, for example, two or four full sibs or a greater number of half sibs, is adding very little to the nett effect. The same sort of situation is likely to apply here, that is that there is undoubtedly a gain in accuracy of ranking breeding animals but that the gain is probably going to be quite marginal.

J. King (UK)

I would dispute that because the pig situation is confused by the common litter environment which reduces the value of full sibs. I think full sibs are potentially very valuable, particularly if you could increase the full sib family to say, 20 or 30, lactating females, then you could devise alternative breeding plans which would look very attractive. You will be aware of John Owen's alternative breeding plan using half sib families; I think if you could get full sib families which are big enough then you could have an even more attractive programme.

W.G. Hill

Whilst I agree there is a problem that if you start using full sibs or family information, you are bound to increase rates of inbreeding. Obviously one is not always concerned about rates of inbreeding but if we are trying to operate with not too many animals because of the expense of doing the superovulation and transfer, then there is going to be a cost involved in using the family information. I should point out that in these calculations that I have shown I ignored family information, partly through idleness but also because of the problems of inbreeding.

A. Robertson

Thank you very much.

EGG TRANSFER IN BELGIUM

Luc Henriet
Université Catholique, Louvain-la-Neuve

Egg transfer does not exist in Belgium. I can only tell you about some results of a long series of experiments on in vitro fertilisations and isolated trials on non fertilised ova transplantation. (These experiments took place between 1952 and 1974).

The non-surgical technique arose, over a long period, against the sensitivity of the cow uterus to infections; attempts performed outside the oestrus period are confounded by infections. During the past few years it seems that this disadvantage has been overcome but it was not always so and the trials which we made took place during the oestrus period when the sensitivity to infection is reduced.

Unfertilised ova were used throughout these experiments.

FIRST EXPERIMENT

3 ova plus spermatozoa were suspended in the medium (follicle fluid extended with citrate-glucose solution) and included in a small gelule of gelatine (0.5 x 7 cm). This was inserted at the end of the heat into the cervix and pushed forward with the help of a metallic catheter. Pregnancy did not occur. However, the inserted ova were not in a compatible stage with the donor, furthermore, fertilisation could happen within the uterus.

The trials were performed with 2 lactating cows, respectively 6 and 8 weeks after their calving, and consequently, it was impossible to slaughter these animals or to remove the uterus and to recover the gametes.

It was easy to verify the innocuousness of the method at the following heat and it appeared clearly then that infections were absent.

SECOND EXPERIMENT

A second attempt with 2 cows was made as follows: a plastic catheter

was inserted through the cervix during the oestrus period. The tip of the cannula was peeping out of the vulva and was closed by the aid of a plastic membrane and an elastic band. The cannula remained fixed by a silk thread embedded in the thickness of the cannula and stuck to the skin . The cows were in the grazing period and one cow lost the cannula. The other cow was thus the only animal available for the experiment.

Three days after inserting the cannula, 3 ova were recovered from culture and suspended in the same medium with sperm; these were injected into the uterus along the cannula which was then removed and discarded.

The result was similar to the first experiment; no pregnancy and no infection were apparent from the examination of the cows 3 and 6 weeks later.

These 3 cows became normally pregnant at the first service of artificial breeding.

THIRD EXPERIMENT

The third trial was more outside the classical line; in fact, it was a variant of the first experiment. The ova were then suspended in the same medium but the spermatozoa were not free. They were mixed with the gelatine at 37°C and they became embedded in the wall of the gelule when the temperature lowered. Why was this so? To get a certain delay of fertilisation and to take full advantage of the possibilities brought about by this delay to reach closer synchronisation between donors and recipients.

The spermatozoa do not die in gelatine when the operator does not prolong the interval for several hours. Milovanov inseminated mares with stallion sperm embedded in this manner. The advantage is this: the movement of the spermatozoon is slowed down and the energy reserves are stored for a further progression. The results were unchanged, no pregnancy and no infection.

A fourth experiment did not succeed. The purpose was to substitute thrombine for gelatine. Why use thrombine in place of gelatine? Because the thrombine requires a longer time to be destroyed and the ova are later liberated into the uterus. We were prevented from success by the brittleness

of the thrombine.

It is easy to explain the failure of pregnancy. This is linked with the use of unfertilised ova which cannot survive in the uterus. This seems to be an injurious medium for young ova. It would probably give better success with fertilised ova but it did not happen at this time as we were unable to get systematically fecondations in vitro. This also applies today .

In short, those trials most serve to demonstrate that the work could be performed easily without the risk of infection during the oestrus period, and also to carry out such transfers outside this period.

What kind of ova were transferred? The assays were made only with "slaughterhouse ova" - that is, with ova recovered from slaughtered cows immediately after they died.

It seems that the mortality of ova would be important, in fact, after a few weeks of "miscarriage lessons", when the slaughterman was converted and had learned the manner of helping, it was possible to come to the laboratory with a good batch of living cells. However, it oftens happens after years of practice that you get no ova at all on occasions.

I do not insist on the necessity that each handling phase should be at body temperature and I also discard the risk of evaporation of follicular fluid and medium. All the harvested ova are kept in warmed sterile vessels.

Why unfertilised ova? It is very simple to understand: as stated, the impossibility of accomplishing in vivo fertilisations and recovery of fertilised ova, there remain only two ways - to give up or to start from fertilisation in vitro. In the cow, in vitro fertilisation does not easily occur and for a long time, therefore, we did not get fertilisation. More than two thousand ova were examined, after discarding numerous cells for various reasons - namely immature ova surrounded by corona cells, though we selected the cows for oestrus.

The choice of the donors was made by the characteristics of the cervical mucus (see publication on this point) and each time one finds one or more ripe follicles, and if the mucus has the oestral characteristics, the recovery took place at the slaughterhouse immediately after the opening of

the abdomen, the follicular fluid was expressed in a glass tube. At the laboratory, the ova were cultivated in a tube or between glass and coverglass.

It was possible to maintain a correct temperature, to discard variations of osmotic pressure and infections in culture under the microscope. It even enabled us to renew the medium but it was practically impossible to transfer the eggs. We got a very poor number of fertilising figures - only penetration of spermatozoa and retractions.

I saw only once one spermatozoon just at the phase of penetration, through the zona pellucida into the cell membrane. Better results were attained with cultures in tubes. Finally, we obtained 3 first stages of 2 cells, several at the male pronucleus stage, many retractions involving likely fecondation and not osmotic changes, and numerous fragmentations, the final stage of unfertilised ova. These results were obtained on about 700 ova. The recovery from glass tubes for the transplantation is not difficult, the ova remains in the under layer of the medium, the supernatant is discarded and microscopically examined. Finally, the ova are suspended in a small volume of medium and they are examined also before transfer.

The results were very poor but the harvest of information was considerable for us. We had a better view of the phases of fecondation. We can first forsake and even contradict a lot of these like the moving of the ovum in "a clockwise direction" when a swarm of spermatozoa surround it or the thesis of the strongest spermatozoon which fertilises the ovum and other similar opinions which are credited in the medical world. The choice of the sex arises in animal and human medicines and when we started this research, many opinions of this kind were claimed. It is more interesting to observe the behaviour of the spermatozoon around the ovum, the absence of any tactism between the gametes, the reaction of the zona pellucida when spermatozoa are passing through, the behaviour of coated or denuded ova, the chemical and physical properties of the cervical mucus involving biological effects.

It is important to say that the poor results lead to the conclusion that: temperature of the body, osmotic pressure, pH levels of CO_2 and O_2, washing of the ova, discarding of antibiotics and mycostatics and renewal of the medium are linked to the success. The discarding of antibiotics

is not easy and on the other hand, they implicate the strong and quick development of mycoses and yeast. But the removing of the medium offers great possibilities against invasions of foreign bodies (note: mycoses are very often inconvenient in the cultures).

On the other side, it is impossible for me to say if the addition of hormones, tubal extracts, blood serum (reacting with follicular fluid), a lot of ionic mediums and spermine and spermidine are of any help.

At last, I may say that I remain perplexed by the fact that I have never seen one spermatozoon passing into another cell such as into an ovum (that explains the culture with ova coated by corona cells).

In conclusion, it is a small contribution which can be added to the knowledge of the present-day. It means, however, that contrary to the situation prevailing during the years during which these experiments were taking place, the information available today permits us to undertake far larger practical tests.

Two words now about the planes of research.

This research is of considerable interest for Belgium. The Authorities wish to exploit the possibilities of egg transfer and we realise that, we in Belgium, have much ground to catch up. We believe that egg transfer is of first importance in view of meat production. It is a "verité de La Pallice" to say that the world is in want of meat although this opinion is not unanimous. Each one, here, I think, believes that we must increase meat production and this means an enlargement of the meat breeds. In Belgium we have exploited this postulation at the upper level - the genetic potentialities of one of our breeds. This exploitation resulted in the detriment of the heritable health of this particular breed; more young mortality, bone malformations, joint diseases, dermatosparoxis, bursting of lungs' alveoli and infertility ensued. This infertility condemns this breed to a vanishing point sooner or later. We could well forsake this breed but from an economic standpoint it is the butchers who decide.

Egg transfer allows us to recreate a new blood for breeding supported by fertility and selection for health. This breed should serve as a receptor for the meat breeds. This purpose is supported by the following calculation:

One cow, according to our information, lives an average of 5 years. In order to maintain the livestock at the same level we must breed a number of female calves equal to a fifth of the female livestock. The other calves, roughly estimated to 80%, furnish meat. In beef breeds females have a lower efficiency in the same way that males have a lower efficiency in dairy breeds. Egg transfer of meat-breed ova into dairy cows, or in robust cows, offers a part of the solution. It should be performed on 60% of the cows and the remaining 40% should serve for continued breeding of the strain with or without transplantation.

THE EGG TRANSFER COSTS

We are still at an early stage and unable to produce an accurate costing of the process.

We did an estimation of the sterility cost and found that 6.5% of the cows are sterile when the non-return results of artificial breeding fluctuates between 60 and 65%. Such a sterility level means a loss of 1300 Belgian francs for each cow, and for all the cows 1.3 milliards or 26 million EEC units. Each loss of one percent of non-return, when the insemination techniques do not interfere, signifies a loss of fertility and when a further drop of 1% is incurred it amounts to a loss of 4 million EEC units. In the breed with which we were experimenting the level of non-returns fell 5 to 10% lower in the inseminating centres and the economic loss can be estimated between 10 and 30 million EEC units due entirely to the exploitation of a meat breed but happily compensated by the price of meat.

The help of the EEC research plan is concerned with improvement in meat production. If this plan is successful more meat will be offered on the market, but what will be the effect on the price level? In absolute data, the gain in cost price is bound to an increase of the cow's efficiency, and this justifies twin transplantations. However, from the birth of the calf to the butcher's stall the cost remains unchanged. In economic terms it is no wonder that the price escapes the purpose of the economists and it is not a truism to predict that the gain that we could realise by means of this research could be concealed by unexpected factors outside our control. (For example, the price of fatted geese livers remains very high,

not because of the scarcity of geese but principally because the time spent fattening the geese remains as long as before).

We are heading for serious trouble in our economy which can nullify our results and our efforts. I think that this must be recognised in order to refute doubtful arguments which are put forward when economic difficulties arise.

DISCUSSION

J. King (UK)

You chose not to mention the breed which is falling into low numbers but I wonder if you could tell us? I think it is one of the functions in which the reproductive physiologist can really help - to preserve breeds where there is a real case.

Luc Henriet (Belgium)

The name of the breed? Belgian Blue.

C. Polge (UK)

I would like to make a comment. I think it was very brave of you, Professor, to describe some of your earlier experiments in the present environment today. Of course, when you were taking eggs and sperm and putting them into the uterus with the hopes of obtaining a pregnancy, we probably realise that the chances of obtaining a pregnancy are virtually nil. On the other hand, there is, possibly, a use for such a technique in that you might be able to obtain fertilisation. We were interested in this in another species - to see whether one could obtain full capacitation of the sperm in the uterine environment alone and so we transferred unfertilised eggs into a uterus containing sperm and under these circumstances we were able to obtain fertilisation. Obviously, we wouldn't have got continued normal development in the uterine environment of very early stages of eggs, but, if we are to consider the situations where we can't obtain in vitro fertilisations to date but we could mature a large number of oocytes, it might be useful, in the cow, to obtain fertilisation in the uterine environment which might not involve a surgical insemination of putting such eggs in the oviduct. So, although, possibly, we might smile at some of these early attempts of yours I would like to congratulate you on having made them.

Luc Henriet

Thank you.

A. Robertson (UK)

Thank you very much, Professor Henriet.

GENERAL DISCUSSION

A. Robertson (UK)

Now, that brings us to the end. There is on the programme a slightly extended period for discussion at the end - are there any points that have arisen during the meeting that you wish to take up again? For example, there were two questions to Dr. Gordon which I had to stop because we ran out of time. Would the questioners like to take them up again now? Dr. Willadsen was one.

S.M. Willadsen (UK)

That's right, yes, I had a question. This concerns twinning in cattle. People at our Unit (and also Joe Sreenan) found that transplanting a second egg to a cow that was already thought to be pregnant - this is surgical transfer - did lower the pregnancy rate and the twinning rate quite considerably. I would like Joe Sreenan to comment on this to tell us whether he has changed his mind on this particular subject. It has some importance in relation to the non-surgical technique as well.

J. Sreenan (Ireland)

I think the data you referred to is probably not my data because what we do is to transfer surgically one egg each side, or one egg to the ipsilateral or one egg to the contralateral horn in a series of transfer experiments. Not in any case did we transfer contralateral eggs to a bred recipient surgically.

S.M. Willadsen

I am sorry if I have involved you then but would you comment on it? Would you think it a sham operation?

J. Sreenan

Well, the only comment I could make would be on the lines of the comment I made yesterday. We have a series of experiments going on at the moment where we are doing transfers to the contralateral side of the bred animals - this is non-surgical. We are doing transfers singly, and we are doing transfers on each side in non-bred animals as well. We have a fair amount of data on the contralateral horn to the bred animal in terms of pregnancy rates. We have just slaughtered one group of these and we get a consistent level of about 25% of embryo survival in the contralateral horn. Now, this

is a little bit lower than what Dr. Gordon was talking about having put the eggs into the rabbit. In fact, as it turns out, just listening and talking about the percentage of eggs going through the rabbit, it works out about the same pregnancy rate from eggs collected originally. I don't have any other data at the moment apart from that. First of all, we haven't upset the pregnancy rate in the bred animal herself - that's on the ovulated side. This is the comment I was making before. Now, on the side we transferred to, we had a 58% egg survival with the bred cow. On the other side we had a 25% egg survival. But we did have something crop up that hadn't cropped up in our surgical transfers - this was that we got a 20% level of embryo mortality in the form of resorptions following non-surgical transfers - and we haven't experienced this in surgical transfers. Now, we've slaughtered animals following surgical transfers at about four and five periods of gestation - 27 - 30 days, 31 - 40, 41 - 50, up to 117, - and we have calved a group to see, in fact, whether there is a uterine factor which might limit, in other words, that you might get a lot of twins at an early stage of gestation and that as you come towards term you might get a resorption of one of the embryos along the line. We have data in publication on this at the moment. There isn't a fall-off and it is relevant to some of the questions that came up this morning. It seems that if you get two viable embryos at 27 days and 30 days, the chances are high that you calve two normal calves from the animal. The point that does interest us at the moment is that we are obviously getting about a 45% pregnancy level from non-surgical transfers and if we slaughter our recipients to determine embryo survival at a late stage, about 20% of these have turned into resorptions. I suggest there may be one or two reasons for this. One may be that by transferring the egg non-surgically, you are placing it just above the common body. We are not able to get very far up the horn with the apparatus we use. Is it a vague possibility that because you are placing it so far down, presumably relative to the egg on the other side, (and some of the Cambridge people may have more information than I have on this), that it develops for a while and then doesn't survive?

The other point is this. We have presumed always that there is no infection. The basis for this presumption is rather a naive one. It is simply that we don't upset the normal controlled pregnancy rate in the animal herself c the bred side, but perhaps there is a sub-clinical infection that we do introduce here and possibly, more likely, the damage caused to the uterus is

caused when we introduce the pipette. So I think it is somewhere in this region that some of the problems arise.

S.M. Willadsen

I'll ask Dr. Gordon then whether the original data was his and whether there was some lowering when the second embryo was transferred surgically to a pregnant cow?

I. Gordon (Ireland)

I'm not sure I understand the data to which you refer.

A.O. Trounson (UK)

I wonder if I could help you out. I think one of your students visited us and showed us some results when your surgical transfers were not operating very well. There was a sham operation involved. What I would like to do is add further that this experiment has been done in the sheep by Mr. Rowson and Dr. Moor. They haven't published it because, in fact, the more they do the smaller the difference is. Looking at their data I don't think there is a very big difference but perhaps Mr. Rowson should comment.

L.E.A. Rowson (UK)

Well, the interesting thing in the sheep, of course, is that you do get migration between the two horns. We find if we put an alien egg in the contralateral horn to the native egg that either one or the other tends to go on. In something like 30 to 40 transfers where we have done this we have only had one set of twins where they are both native and alien. One can't help but wonder if there is some immunological effect within the uterus of the two different types of eggs.

I. Gordon

Well, only time will tell. I can't go any further than the data that I presently have.

E.P. Cunningham (Ireland)

Professor Robertson drew attention earlier to the apparent conflict between Professor Kräusslich's estimate of the nett benefit of adding transplantation to a bull dam selection programme, and my own. We have

attempted to reconcile the figures! As far as I can see they are fairly reconcilable when you compare like with like. That is, I was taking, very simply, the addition of transplantation to bull dam selection, considering simply the advantage in the selection programme for milk. Professor Kräusslich is including beef selection. So that accounts for some of it. Some of it is also accounted for by the fact that I was relating everything in mine to a mean population production of 3,000 kg and his is 60% higher than that. There is also the question of the price of milk. So the nett economic benefit for the same exercise in the two populations will differ by an order of 40% simply because of the scale and price. If you add in the meat element, I think that accounts for most of the rest. So, I am satisfied myself, at any rate, that there isn't any great conflict.

R. Newcomb (UK)

I wonder if Dr. Hansen could convert his kroner into pounds in terms of the cost of one recipient? The other question I would like to ask Dr. Gordon. Does he now discount completely any possibility of twinning by injection? I refer to one piece of data which he produced in his original work whereby those cows where he managed to get two ovulations he found that in most of them - I think it was something like 60% - one ovulation occurred on each ovary, and so would make the technique not too unpromising.

M. Hansen (Denmark)

Yes, I should be able to convert to pounds the price we were able to pay per recipient. It would be somewhere between £600 and nearly £1,000. That is per recipient which calved down with a calf.

A. Robertson

Before we leave the point, have either Professor Kräusslich or Dr. Cunningham thought in these terms? I'm sorry to face you with this question but how much would you be prepared to pay for a recipient? .....

M. Hansen

It will not be much less.

I. Gordon

One certainly can't discount the possibility of so precisely controlling ovulation in the cow that you could approach the twinning from that angle but again, it is a matter of waiting to see the technique appear.

SESSION FIVE

CONSIDERATION OF FUTURE RESEARCH REQUIREMENTS

Chairman: L.E.A. Rowson

CONSIDERATION OF FUTURE RESEARCH REQUIREMENTS

DISCUSSION

L.E.A. Rowson (UK)

The purpose of this session is really to look at the papers which have been delivered over the past two days and to make some form of recommendation to the EEC regarding future research. Obviously, we don't want too much overlapping of research within the EEC, many Institutes doing exactly the same type of work. We don't want to provide grants for Institutes within countries, or even in different countries, for more or less identical work. I believe a certain amount of overlapping is of value because it provides a check on what is happening in any one particular Institute and can confirm, or otherwise, its findings.

There are, to my mind, three outstanding problems which exist. The first of these is the supply of eggs for transplantation. Before I mention this I should say that the picture painted by the geneticists yesterday afternoon was, to me, a little disappointing. It was not the picture itself but the fact that the genetic gain in dairy cattle was going to be rather lower than those of us working in practice anticipated.

So far as the problems are concerned, the first one is obviously going to be the supply of eggs. Here one has two factors to take into consideration. First of all the question of better and more reliable methods of superovulation and, alternatively, the in vitro fertilisation of eggs of either the cow or the calf. Now, one might say that a certain Institute should concentrate on this type of work. For example, the French have a great deal of experience of this aspect of the problem. I think, in the long term, in vitro fertilisation must be the answer. Superovulation won't provide sufficient eggs for a national programme although it will be of considerable value for the points brought up by the geneticists yesterday, of producing superior animals for breeding.

The second problem which arises is that at the moment we haven't a completely reliable method of storing the eggs. This is going to be an absolutely essential part of it. The storage in culture medium is not going to be satisfactory for any length of time, nor in the rabbit. It really

comes down to the question of deep freezing of eggs. Very much more attention should be paid to this over the next year or so. As you heard from Dr. Willadsen's paper, the results are now getting to a very reasonable level and it probably won't need much more research before it is on a sound footing.

The third point we have got to consider is the non-surgical transfer of eggs. I can't see this technique ever being used on a large scale unless non-surgical transfer of eggs is a practical proposition. We shall, later on this morning, be seeing a non-surgical transfer of eggs demonstrated by Dr. Brand and also a non-surgical recovery of eggs.

It is also a little bit difficult to consider the question of distribution of research on a national basis. One country may feel it should have additional funds from the EEC for particular research and may feel, on national grounds, that it is being left out if the research is allocated to another Institute in another country. This can present certain national difficulties and could result in a certain amount of international friction.

That is the background to the discussion this morning and I would like to hear from people in the hall what they feel about the position, particularly covering the three points I mentioned: superovulation, in vitro fertilisation, the storage of eggs - deep freezing, culture, the rabbit, and so on - and the non-surgical transfers. We must consider what recommendations we should make to the EEC on these particular aspects of the work and what additional recommendations one might consider putting before them. I would remind you that our next meeting is in March.

J. King (UK)

I would like to make a comment at this stage. There is one topic not directly covered by the title of this Symposium which has arisen in the course of these papers and it is important. I refer to the effect of synchronisation, on its own, and the means that this provides of introducing AI and semen from beef bulls into suckler herds. I think it is a potential means of increasing the supply of beef and is something which could be discussed in its own right.

L.E.A. Rowson

Yes, I think this is something which should be done internationally.

J. King

I wondered if it might be useful to use this as a fourth topic under which to summarise the discussions.

L.E.A. Rowson

Yes, I think you are right

J. King

We could consider oestrus synchronisation without, necessarily, any egg transfer.

C. Polge (UK)

Yes, although we have had symposia specifically on synchronisation within different species fairly recently. There was one in France about a year ago.

J.F. Roche (Ireland)

I think the point John King has made is very relevant. Apart from discussing the actual mechanisms of control of the oestrous cycle, we need to discuss factors influencing the use of AI in beef cows, apart from synchronisation, the effects of nutrition and reproductive activity in these cows. I think we also need to get down to discussing the mechanisms - how is synchronisation going to be worked in the field, what are the factors? Everybody was agreed yesterday morning that the level of nutrition is most important irrespective of treatment used, so I certainly feel that this is the kind of topic that needs to be covered.

L.E.A. Rowson

Certainly in this country, synchronisation is already being used on a fairly considerable scale now by individual farmers for AI, particularly with heifers. Where they normally run a bull with their heifers, they are now considering synchronisation procedures. This is developing quite rapidly. We have run into slight trouble here over the double inseminations which Mike Cooper mentioned the other day - that one has to inseminate the animals two days running to get the best results. Organisations like the Milk Marketing Board have reduced the charge of the insemination fee simply because they are doing perhaps 20 or 30 heifers on a farm on any given day. So far as synchronisation is concerned, I don't think this is a thing for

a particular unit or one or two units, I think this is much more of an international feature.

M.J. Cooper (UK)

Perhaps what we are really saying, Mr. Rowson, is that it would be very useful in about a year's time to have another meeting like this on controlled breeding.

L.E.A. Rowson

Yes ... although I think one wants a little bit of a time lag before doing this.

M.J. Cooper

Yes, about twelve months to give us some commercial experience.

L.E.A Rowson

Well, it is the policy of the EEC that there shall be periodic symposia of this nature and also an interchange of scientists between one organisation and another, so I think your suggestion would be quite a valuable one.

C. Polge

I agree with Mr. Rowson that after listening to the geneticists yesterday we might tend to be rather depressed at the present moment. However, I was quite impressed by the way in which Dr. Cunningham introduced his talk. He said that when they were creating their plans they had to put in certain factors and at the present moment they can only work on the basis of the possibility of perhaps producing four or eight calves per donor cow. So I think it is very important that on the practical side the physiologists should continue research to try to make the horizon much greater for genetic improvement. One's ideas would change very radically if, for instance, we were able to show that we could obtain a large number of oocytes from calves or from individual donors, and that these could be matured and fertilised in vitro, as you suggest. So, although, at the present moment, the picture for application is not particularly rosy, the future depends upon how we can develop these techniques. One wouldn't want to discourage development, in any way, because it doesn't look too good at the present moment.

L.E.A. Rowson

I must say I was a little bit depressed after hearing the geneticists.

J. King

I am sorry if the impression is one of depression after yesterday afternoon's meeting because in the beef field it should be one of great jubilation in that the existing level of the transplantation technology is such as to transform the prospects. The 80% improvement in the rate of progress which Bill Hill showed us is surely a stupendous change. It certainly is in terms of the changes which most geneticists have been able to suggest in other improvement programmes. It is usually possible to suggest alternative dispositions of activities to give perhaps 10 to 20% improvement in programmes. Rarely does one get a situation where one can ultimately expect an 80% improvement. So I think the situation with beef is highly encouraging and we would like to carry on and hopefully, in the future, with greater numbers of successful transfers, there are potential applications in the dairy field.

L.E.A. Rowson

Yes, I agree that the beef side did look very much more encouraging than the dairy side.

R. Church (Canada)

As a privileged guest and non-EEC member, I would just like to echo what John King has said. It seems to me that this particular group is in a unique position in the world, basically, in the application of the egg transfer technique itself. I think the technique is adequately, though not ideally, developed for its application in a genetic sense. In some of the EEC member countries there are genetic programmes in existence where there is documentation and ideal selection programmes. Now is the time to start implementing these in these programmes, actually to get the hard data regarding the inseminating role in a genetic programme in both dairy and beef, although, on paper, the beef certainly looks the most promising. I think you have the opportunity in some of your member countries to utilise egg transfers in a unique way because of your national AI stud and performance and progeny testing programmes.

The other aspect that I think is shown very well at this particular meeting is that there has been a period of rapid proliferation of preliminary results which basically do very little more than substantiate work that has

come out of your own laboratory, the Cambridge laboratory, during the last fifteen years. It is very encouraging here to see programmes starting to involve proper numbers of animals in experiments and proper controls. Because we are working with animals this costs money but I think one of the priorities here must be to say that there are certain aspects where numbers are going to have to be increased, controls are going to have to be increased. There are programmes emerging, such as the Irish programme that Joe Sreenan is involved in, that do have this. I see this occurring in other laboratories in Germany and elsewhere. I think this particular meeting allows you to delineate which of these projects are going to be undertaken where and with a degree of emphasis that for once and for all we are not saying, "Those animals are done under a particular set of conditions, these animals are done under a different set of conditions and our results don't agree but we can rationalise them". I don't think that is good enough. The technique is established, now we are ready to take the next step and improve on it.

L.E.A. Rowson

I agree we are in a good position in this particular country. One has organisations like the Milk Marketing Board which can obviously do the breeding programmes which we talked about yesterday on a national scale for the production of bulls for our artificial insemination programme.

Incidentally, there are two or three people here who are not from EEC countries but I hope they will not hesitate to stand up and give their views. I did mention this to Professor Church earlier and I mention it now to Dr. Shea and Dr. Nancarrow.

E.P. Cunningham (Ireland)

I am one of the prophets of doom who so affected you yesterday. I should say that I over ran my time a little and the Chairman didn't quite give me time to get to the silver lining, but there is one! It seems to me that on the dairy side we are already, if we are doing a decent programme, selecting our bull dams so carefully that the marginal extra selection intensity we can get from adding egg transplantation doesn't offer much genetically, but it can offer quite a bit economically. This is something which depends on the circumstances of a particular country, how expensive it is to arrange contracts in the population at large and collect bull dams in that sort of way. I am rather surprised that our French colleagues have

not referred to some of the developments that are going on in France at the present time. I understand that Sersia, which is the centre of AI organisations in France, is now establishing a mobile egg transplant laboratory. If I understand the rationale behind this correctly, it is that there is something in the order of 5,000 planned matings going on in the various breeds, and that they can reduce that number to maybe 1,000, and that just the cost effectiveness of doing it in the latter way makes it well worthwhile. In addition, as some of you may be aware, a project was proposed for EEC funding which we hope to get under way in the next year. This is essentially to establish a gene pool based on several of the French beef breeds and using as a sort of launching platform something which has already been done by the Medatest organisation in the south of France. As originally proposed this involved bringing a large number of animals purchased in France through quarantine into Ireland and it was quite expensive. We have recast the whole thing now using egg transplantation and we have brought down the estimated cost of establishing the project to about a quarter of the original. So I think there is a very significant part for egg transplantation to play in practical breeding programmes.

J. Sreenan (Ireland)

I think it is relevant to mention here that one of the major effects would be twinning in the single suckling herds as opposed to the genetic change that may be gained in either the beef or dairy herd. As well as synchronisation which we realise would have great import to bring AI bulls into the single suckling herd where output is pretty low, there might also be a good possibility just for inducing straight twinning with normal beef embryos. So one could reduce the overhead of cow maintenance and the cost of a second calf. Obviously there are lots of problems involved but the picture, just in this short session this morning, sounds a little bit better than we were beginning to think yesterday.

One of the problems in a session like this is that we tend to look at the techniques developed as they are now which may not be a very bright picture, and possibly the cost involved in development is pretty high. However, as Chris Polge was saying, we don't yet know the ultimate potential of the techniques or how much we may be able to reduce the price of them. As we begin to understand a little bit more about the processes involved it may be that we can turn up methods of in vitro fertilisation that are not

expensive. It may be, as Willadsen was saying, quite possible to automate embryo freezing. These things look terribly complicated from preliminary data but I think in the long term we have to think that perhaps the price structure could be a little bit different. If we accept what John King and Paddy Cunningham have said, that there is a good possibility for improvement, we should be thinking about whether we are asking and answering the right questions within the total physiology programme. It comes back to the points raised: egg supply, egg storage, and non-surgical transfers.

J. Bowman (UK)

I would like to remove some of the gloom! It seemed to me that yesterday's discussion in the afternoon was mostly concerned with the improvement that would accrue from using the transfer technique as a genetic tool, whereas in fact, as Sreenan has just said, it is the direct effect on both beef and dairy production which will probably provide a greater improvement in the agricultural sector than the improvement on the genetic improvement programme. So I think getting the technique to work and using it in commercial practice will give you direct effects which will be beneficial and probably larger than the gain you will get by modifying the selection programme.

J.F. Roche

Mr. Chairman, perhaps at this point we could accept that the gloom has been somewhat lifted from yesterday afternoon and go on to discuss some of the problems. Joe Sreenan has outlined the three problems, as you have yourself. One of the things that amazes me in relation to egg supply is that the people working on superovulation do not seem to know how much FSH and/or how much LH, and a proper ratio to grow a follicle in the bovine. I think these are some of the fundamental questions we must start thinking about and seeking answers to. We have been using PMS for 15 or 20 years and we have accepted that there is nothing else around. Perhaps we should be a little more basic and find out what is required to grow a follicle in the bovine and then take it from there. Likewise, we know very little about what happens during the actual implantation process of the embryo, yet we are attempting to do this with non-surgical transfer. We don't know the requirements. Does the developing blastocyst produce hormones? Does it have hormone receptors?. What are the exact requirements? I think these are some of the things we need to start discussing.

J. Sreenan

I agree we must look forward. Of course there are great problems in response to superovulation and they will probably remain until the money comes to solve them; this is one of the areas of research that requires an awful lot of money - a big facility and a lot of people. One of the biggest problems with the whole business of stimulating has been the use of PMS but it was a readily available, cheap product. I agree that we don't understand all the problems; I mentioned this when I talked about the work which we have been starting on superovulation. There is a confusion of breeds and crosses involved; there is a confusion of everything. But as well as trying to pin-point some of the factors affecting follicular growth we have got to look at other products apart from PMS. In the meantime we have to stay with PMS and see within the animal at which stage of the cycle it can best be applied, under what conditions it should be applied. We must decide on the best method of fertilisation, and whether we should go for high crops of eggs, get them fertilised and remove them and put them into some of the culture systems that Alan Trounson was talking about. There is a whole area here which hasn't been deeply researched.

C. Polge

As a recommendation to the EEC my feeling is that progress in this field, particularly in relation to superovulation, will not come through large scale programmes of injecting hormones into animals in the field. It has got to come through a better understanding of the basic research, of factors influencing follicular development. We may even get down to supporting work on some basic chemistry on the units within PMS or within protein hormones and how these affect ovarian structures, as Jim Roche was saying. It might seem that the EEC, in their beef programme, could be having to support some very basic research which to the farmer might seem very remote from his immediate needs. However, I think we should stress here that until this basic research is done we are going to get nowhere in the field.

L.E.A. Rowson

I think there is one firm interested in this particular angle. They are the people who supply most of the PMS and they are, in fact, providing money for this very purpose. Their eyes are open to the situation.

A. Brand (Netherlands)

I think the supply of eggs is very important. We are trying to control

superovulation, we are trying to control synchronisation between the donor and the recipient. I think you can save quite a lot of eggs. Newcomb told us that a number of eggs are lost especially during the first 6 or 7 days after the ovulation. Now, what we have forgotten is that we must try to synchronise the endometrium of the superovulated animal with the development of the embryo. I think that is possible if you investigate the biochemical environment of the uterus of the normal animal, and then compare that situation with the uterine environment biochemically, histologically, and maybe immunologically also, of the superovulated animal. Perhaps that is the way to recover more fertilised eggs which can be transferred.

L.E.A. Rowson

I am sure this side is important. There is no doubt that the eggs being lost, which Newcomb spoke about, or rather the high percentage of abnormal eggs, provided these are removed from the particular uterine environment of the superovulated animal, they will go on perfectly happily. So there is a very great need for a look at the uterine environment both in the normal cow and in the superovulated cow. I agree entirely and I think this is something which should be supported.

A. Brand

I can tell you that we hope to start at the beginning of next year with that investigation. We have already done some work in this field.

I. Gordon (Ireland)

I would have regarded superovulation for egg transfer purposes as being something we have just got to live with for the moment. It's crude, it's never going to be anything great no matter what you do, from the very point of view of the problems of the eggs getting into the oviduct. I would have thought that for egg transfer purposes the thing to go for is the in vitro maturation of the oocytes and fertilisation, to concentrate attention very much on that area, whether that involves basic research or whatever is required. There is also the need, as Jim Roche was saying, for work on factors affecting follicle growth, the number of ovulations from the point of view of mild superovulation, the sort of thing which could possibly come in on cattle for twinning by superovulation. It could also be very useful on the sheep side to have much more precise information on controlling ovulation rates in controlled breeding situations. I can see there is a need for fundamental

work on mild superovulation with a view to cattle, and also applications on sheep, but on the out and out superovulation for egg transfer purposes, in vitro fertilisation would be the thing that really ought to be concentrated on.

L.E.A. Rowson

Yes, I think the main difficulty in the cow over mild superovulation is the one you pointed out on the blackboard, that one is never sure whether one is getting a corpus luteum on each side. Unless you do you are going to get a much lower twinning rate.

I. Gordon

Yes, I accept that all right. But if you could get a high percentage of the animals starting off with twin ovulations whether they are on the one ovary or between the two, you could end up with a respectable twinning rate. That side of it would, I think, justify more work.

B. Hoffmann (W. Germany)

I think Mr. Chairman, there are two ways to go on from this discussion. The first way is to enhance more rapidly genetic improvement in various countries and I think it has become obvious that there are different breeding programmes in the various countries in the Common Market. These matters have to be applied in the different countries. It is impossible to have a common breeding programme for the whole Common Market. As Professor Church has mentioned, there was a quick proliferation of preliminary results. I think in all countries the techniques available now can be used and I would suggest that the Common Market should support this development and application of these techniques in the various countries. The other way that has been shown is the more biochemical way, to study basic changes of the early blastocyst and of the uterine environment. This is really a large programme and if you screen the literature there is quite a bit of information available on this already - the work of Shelesniac, the early implantation work of Baier in Germany on the uterine factor. I think only those laboratories that have really been working with this and have some experience already will take this up.

A. Robertson (UK)

I would like to make a point referring back to yesterday. What came out of the discussion was that as geneticists we were going to want eggs from

two completely different kinds of animals. Firstly, animals of pure beef breeds aged between, say, 15 months and 2 years, and also animals from lactating cows of specialised dairy breeds at a time perhaps when they are under the considerable physiological stress of milk production. I would ask you to bear this in mind, the techniques required for these two kinds of animals may well be very different.

J. King

Might I, from my ignorance, ask a question and make a suggestion. I think that despite the desirability of moving on from this witch's brew that is called PMS this is still going to be used in the future. What co-operation is there between different laboratories in the assessment of which batches of PMS are to be used, for example, in field situations? It seems to me that many laboratories make their own assessment of this by quite empirical methods. Would there not be merit in trying to get some co-ordination in this field using batches with particular characteristics much more widely in different laboratories.

L.E.A. Rowson

This is actually being done. The Intervet Laboratories are alive to this and are providing funds for this very purpose.

I. Wilmut (UK)

May I move on to the second problem raised, that of storing the eggs. While clearly you wouldn't expect me to say that freezing is an unimportant technique, I would like to add the question of trying to store at temperatures above zero alongside the technique of freezing. As I tried to show the other day, I don't think you should underestimate the potential value of this technique to enable you to hold eggs for short periods of time and facilitate the general transfer technique. If you are transferring eggs as part of a genetic improvement programme, that might be much more effective than freezing which for many years to come would probably cost you a few eggs just as a result of the freezing stress itself. So, although I don't in any way suggest that freezing doesn't have an important role, I would suggest that for quite a few years to come storage at, say, 0° or $10^{\circ}C$ could be very useful and that a comparable amount of effort should be put into trying to develop these techniques.

L.E.A. Rowson

This is fine providing one doesn't get any development of the eggs during the process of cooling. Otherwise one is running into problems of synchronisation with the recipient animal. Normally, one doesn't need to synchronise animals with deep frozen eggs, you merely label the egg 'Day 8' or 'Day 9' and that is put into a cow which has been notified as being in oestrus the appropriate number of days beforehand. So synchronisation is a simple matter in that case. If you are culturing, for example, you have got the problem of synchronisation popping up all the time.

I. Wilmut

One potential disadvantage of the freezing technique which perhaps isn't allowed for is that if you are contemplating freezing in a straw and then going on to the farm to transfer that egg, at the current success rate, you are only going to get a success in a maximum of 50% of the animals. It is quite an expensive loss for the farmer to carry the 50% which failed for a further 3 week period. There are two problems here. You could try to alternate the freezing procedure in a simple way to enable you to go directly to the farm and transfer and then have a large proportion of animals returning, or, you could concentrate on the possibility of trying to assess the embryo in the laboratory before you go out and transfer it, thus hoping to transfer a smaller proportion of embryos which you have in fact killed.

L.E.A. Rowson

In other words, culture after freezing?

I. Wilmut

Or some such assessment like that, yes.

L.E.A. Rowson

Have any of our continental colleagues got anything to say on this? It has been practically all comment from Britain so far.

A. Brand

I think in our country the farmers are not so interested in egg transfer on a large scale. What a lot of farmers are interested in is transferring one egg from one specific excellent cow to another if it can be done without risk to the animal. He will say, "OK, if I can have two

calves in one year I am satisfied". That doesn't give you the problem of superovulation. Especially in dairy cattle, it is so disappointing to superovulate Friesian animals with PMS. In most cases 35% or 40% of the animals will give you a response but that is all. So there is a big need, especially in our country, for recovering that specific single egg from one cow. I think the situation is quite different in Great Britain and the United States. I would like to hear how the situation is in Germany, in France, and so on. I am very interested.

J. Hahn (W. Germany)

In Germany now there is a demand for ova transfer but I think we can overcome this problem only by developing methods of non-surgical collection and transfer. By your Cambridge standards, and by American and Canadian standards, our numbers given yesterday in my talk here are pretty small but you should remember that these experiments need a lot of money. It is easy to increase the number of non-surgical transfers when there is enough money but I think that these special techniques of non-surgical collection and transfer should be supported in the future a little bit more than in the past.

L.E.A. Rowson

I agree entirely.

A. Brand

With or without superovulation?

J. Hahn

Combined with superovulation.

J. Sreenan

One of the problems with superovulation would seem to be that, as far as I am aware, superovulation studies in most laboratories were carried out simply to provide eggs for transfer purposes. Until now I don't think people have been looking effectively at the response to superovulation in terms of the animals, the endogenous progesterone/oestrogen profiles, prior to, during and after superovulation. It is fair to say that most of the laboratories involved in transfer have, for a number of years, been trying to perfect transfer techniques, first and foremost. Everybody realised that if you

used PMS on enough animals at a reasonable dose level you would get eggs, not efficiently, but you would get eggs for transfer. Likewise PMS has been widely used just to produce lots of eggs for culture and for storage and for techniques like these. I think the problems Dr. Brand is talking about and the problems we are talking about of not understanding the processes involved, have to be considered. We have got a number of publications that suggest that we can transfer eggs efficiently by surgical means. In other words, the bovine is able to maintain pregnancy after single egg transfer, after twin egg transfer, in a high proportion of cases. We then move on to the non-surgical technique. Up until this last year or two there were very few instances of successful non-surgical transfers. Even though it is a proliferation of preliminary results, cumulatively a lot of people are getting some success rate non-surgically. Now, people are beginning to say that if efficient non-surgical transfer is practical, we have to go back and decide whether we can make the superovulation process more efficient. I think that ultimately superovulation won't be that important but, at the same time, it may continue to be important with particular breeds. In vitro fertilisation can be carried out on calves that are going for veal; it can be carried out on slaughter material. This may not necessarily be important in a particular breeding programme where you are selecting specific animals, specific lines, or specific strains. You need to be able to stimulate further development and either superovulate and take your oocytes away from the follicles - which is not too different from superovulating and inducing ovulation. I think one of the problems is that we don't know enough about breed response. This is the point I was trying to make yesterday. We don't know enough about cycle stage response. There has been a problem of PMS batches but work is under way to resolve some of that. I think work has to come under way to understand follicular growth and development and maybe look at different systems, different compounds, for superovulation. This is the kind of thing the EEC must support rather than going out into the field and superovulating a lot of animals with PMS just to get enough eggs for short term work.

H. Karg (W. Germany)

I would like to stress this point and perhaps, in a short synopsis, give you what was said just now and what Dr. Polge has said. I think it is a matter of the basic endocrinology of the responsiveness of the ovary. It concerns the different gonadotrophins and the different conditions, perhaps

they may be involved in some instances in immuno reactions and so on. I would also agree with Dr. Polge that basic research should be supported.

I. Wilmut

I would like to make one point as far as the value of the techniques is concerned. From what the geneticists were saying yesterday I think you should be happy that we can at least walk confidently in one area and not hope to run and effect a whole national programme in one go. Nobody has mentioned the fact that the techniques are of very great value in investigating a whole heap of other important factors: factors affecting fertilisation, embryonic mortality, foetal physiology, and so on. From a genetic point of view the techniques are very, very powerful when you are interested in maternal effects, either in terms of birth weight or performance in suckler herds, this sort of context. So I think it may be very important to see that the techniques are utilised in research programmes in this area as well as in the commercial production of superior animals. I don't think we should underestimate the value of this to the farmer in the long run.

L.E.A. Rowson

The spin-off from the technique is obviously extremely valuable - as valuable as the technique itself.

C.D. Nancarrow (Australia)

I would like to pick up one point that Joe Sreenan mentioned a while ago, that is obtaining unfertilised ova directly from the follicle. Certainly in the human there is some work being carried out on this - at least in Australia, and perhaps here in England - I'm not sure. This is involved where the human female is sterile due to the fact that the eggs can't get through the tube. Workers are removing the follicles and attempting to fertilise them in vitro. They are using micro-endoscopy, micro-laparotomy techniques which don't really harm the woman too much. Maybe for a start these could be developed for the cow which may overcome one of the problems discussed the other day on the length of time an animal takes to ovulate after it has been stimulated by PMS. It appears to be several hours, I think even more than a day occasionally. This of course means that in your collection of fertilised, or even non-fertilised, eggs you are getting embryos at various stages. Maybe the other important thing here is that there is a possibility that these unfertilised ova may be able to be stored in a frozen

state subsequent to being fertilised with semen from a particular donor. Maybe there should be some work done on this.

L.E.A. Rowson

I think this is a promising aspect because Dr. Willadsen mentioned yesterday that the single cell egg seems to freeze reasonably well.

B. Shea (Canada)

I would like to bring up just one further point with regard to the stimulation of animals and that is that we should perhaps be aiming, as you mentioned earlier, Mr. Rowson, for a far more consistent response from our animals to the stimulating agents. It would be interesting to hear from the geneticists in this regard. The way present techniques are developing, what we are actually doing is to place a selection pressure on those animals that will respond to PMS. Is this perhaps losing for us some aspects of the gene pool?

J.F. Roche

Mr. Chairman, I feel that this has been a very useful meeting and we should thank our hosts, the EEC, for organising the meeting. We should also thank the local committee for organising what has been, to me at any rate, a very useful and informative meeting. All I can say is that we look forward to further ones in the same area.

L.E.A. Rowson

Thank you very much.

APPENDIX I

RESEARCH OBJECTIVES DRAWN UP BY THE BEEF PRODUCTION COMMITTEE, JUNE 1973, AND

I. To obtain a greater number of viable calves through:

1. better control of reproduction:
   - interval between calvings
   - heat synchronisation
   - sex determination
   - production of twins
   - egg transplantation
2. a reduction in calf mortality during and after birth
3. early breeding and the use of once bred heifers for slaughter

II. To improve quality of meat and increase the weight of the carcass through a better understanding of the genetic, physiological and nutritional factors influencing body development

To improve the utilisation of the carcass by technological means

III. To achieve a better understanding, possibly by co-operative programmes, of the comparative suitability of the major cattle breeds used as pure breeds or in crossing (including beef and dairy breeds) for:

- fertility
- ease of calving
- maternal ability
- growth
- characteristics of carcass meat
- feed utilisation

Close co-operation was recommended with the agencies studying the economics of beef production and possibly special studies integrating biological and economic approaches with a view to improving the balance between milk and beef within the Community

APPENDIX I

LIST OF SEMINARS PROPOSED IN 1975-1976 ON SELECTED TOPICS

SEMINAR	PLACE	DATE
Egg Transplantation	Cambridge	Dec. 10, 11, 12, 1975
Perinatal ill-health in calves	Compton	Sept. 22, 23, 24, 1975
The early calving of heifers and its impact on beef production	Copenhagen	June, 4, 5, 6, 1975
Improving nutritional efficiency of beef production	Theix	Oct. 14, 15, 16, 17, 1975
Criteria and methods for the assessment of carcass and meat characteristics in beef production experiments	Zeist	Nov. 10, 11, 12, 1975
Optimisation of cattle breeding schemes	Dublin	Nov. 26, 27, 28, 1975
Cross-breeding experiments and strategy of breed utilisation to increase beef production	Verden	Feb. 9, 10, 11, 1976

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