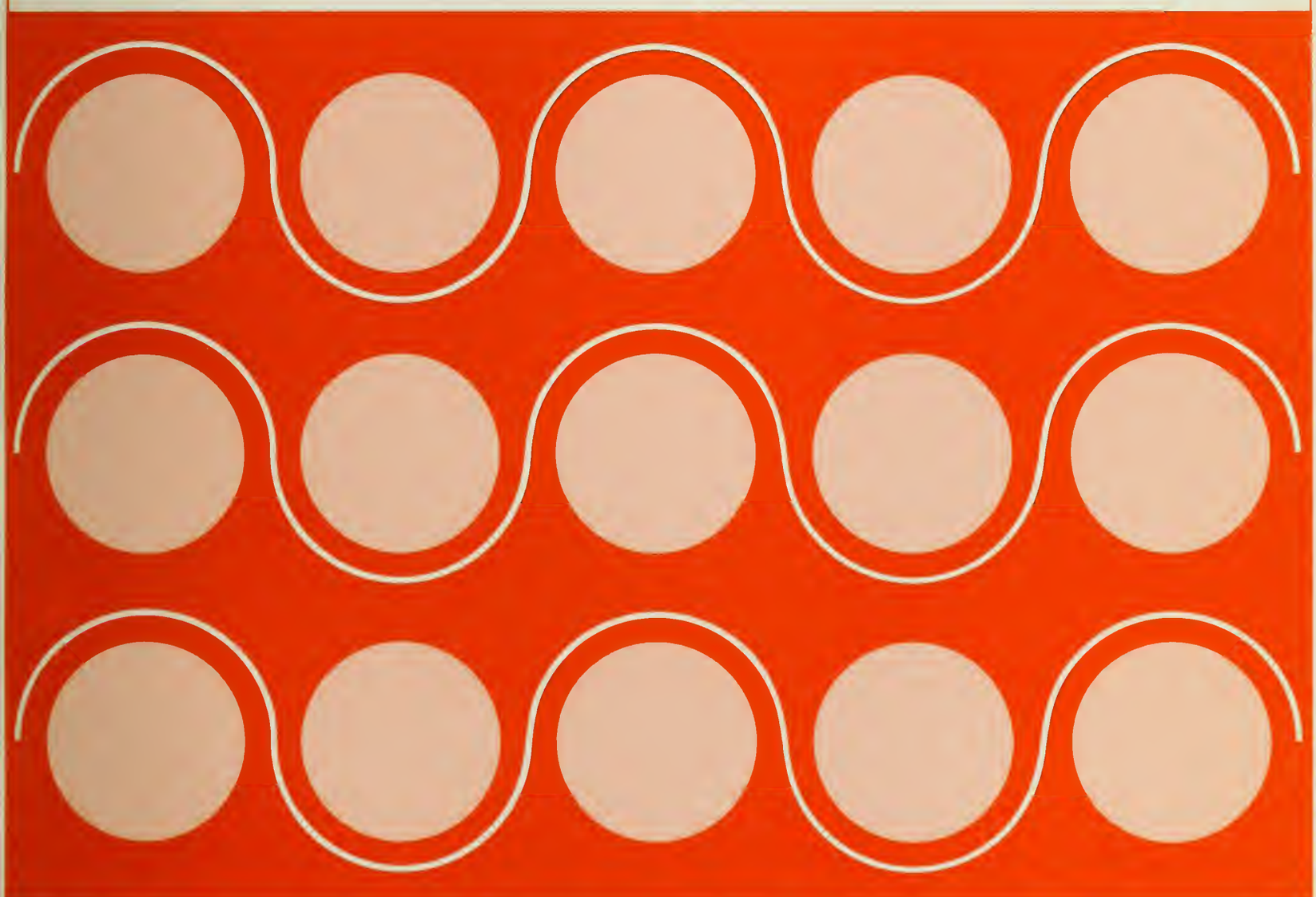
EMBRYO TRANSFER IN FARM ANIMALS

a review of techniques and applications

Monograph No.16 1977



Agriculture Canada

EMBRYO TRANSFER IN FARM ANIMALS

A Review of Techniques and Applications

edited by
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AGRICULTURE CANADA
Monograph 16
1977

Monograph No. 16, available from
Information Services, Agriculture Canada, Ottawa K1A 0C7

Cat. No. A54-3/16 ISBN: 0-662-00769-7
Printed 1977 Reprinted 1980 1M—6:80

THE FIRST EMBRYO TRANSFER

"On the 27th April, 1890, two ova were obtained from an Angora doe rabbit which had been fertilized by an Angora buck thirty-two hours previously; the ova were undergoing segmentation, being divided into four segments.

These ova were immediately transferred into the upper end of the fallopian tube of a Belgian hare doe rabbit which had been fertilized three hours before by a buck of the same breed as herself . . .

In due course this Belgian hare doe gave birth to six young — four of these resembled herself and her mate, while two of them were undoubted Angoras . . . characterized by the possession of the long silky hair peculiar to the breed, and . . . true albinoes, like their Angora parents.

As a proof of their parentage, I would add they inherit a habit which nearly all the Angoras I have kept affect . . . a habit of slowly swaying their head from side to side as they look at you . . .

The experiment described above was undertaken to determine in the first place what effect, if any, a uterine foster-mother would have upon her foster-children, and whether or not the presence and development of foreign ova in the uterus of a mother would affect the offspring of that mother born at the same time.

So far as this single case goes, the evidence is negative.

Before long, I propose to continue my experiments and to extend them . . ."

Preliminary Note on the Transplantation and Growth of Mammalian Ova within a Uterine Foster Mother. By Walter Heape, M.A., Balfour Student at the University of Cambridge. Proc. R. Soc., Lond. 48:457-458.

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PREFACE

The Canadian livestock industry, over the past few years, has become one of the principal commercial users of embryo transfer as a means of rapidly proliferating desirable cattle. For much of the resulting benefit, Canada is indebted to reproductive research and development in laboratories throughout the world. It is especially appropriate, therefore, that this monograph has been prepared by a panel of internationally respected research workers brought together under the chairmanship of a member of Agriculture Canada's own embryo transfer research group. It is hoped that this resulting review of the 'state of the art' in all the common farm species will be of correspondingly wide usefulness by helping those already engaged in embryo transfer, or those considering its use as an aid to world food production either directly or indirectly through related research.

To compile the review, contributors were asked to cover assigned topics. Each contributor then received the combined reviews for comment and revision and their opinions have been taken into account in editing the whole into monograph form. Thus, although the principal authorships are indicated, many segments include material from other contributors and the editor accepts responsibility for any errors caused by the amalgamation. Graphs, a drawing (Figure 7), quotations and photographs have been added editorially.

For simplicity, the term "embryo" has been used synonymously with the terms "fertilized egg," "fertilized ovum," "morula," "blastocyst" or "expanded blastodermic vesicle."

Unpublished data from the Animal Diseases Research Institute, Ottawa, have been described as being by ADRI (unpublished).

ACKNOWLEDGMENTS

The assembly of this panel of reviewers was occasioned by the 8th International Congress on Animal Reproduction and Artificial Insemination at Krakow, Poland, in July 1976 and the help of the organizers of that meeting has been invaluable in preparing this monograph. We also wish to thank colleagues who contributed to the panel in Krakow and thereby helped in the preparation of this text: Drs. N. Ayalon, M. P. Boland, C. Godard-Siour and J. Testart.

The editor is grateful to Drs. M. D. Eaglesome and G. C. B. Randall for constructive criticisms of the manuscript; to contributors who provided additional illustrations; to the Graphics Section of Agriculture Canada for preparing illustrations; to Mrs. Claire Barriger for tireless editorial assistance; and to Mrs. Evelyn Brown and Mrs. Lucille Dauda who did most of the typing.

ABBREVIATIONS

Abbreviation	*Explanation*
ADRI	Animal Diseases Research Institute (Eastern), Agriculture Canada
AI	Artificial insemination
ARC	Agricultural Research Council (United Kingdom)
Av	Average
BMOC-3	Brinster's medium for ovum culture
BSA	Bovine serum albumin
C	Centigrade, Celsius
CL	Corpus luteum, corpora lutea
cm	Centimetre
CO_2	Carbon dioxide
d	Day
DM	Dry matter
DMSO	Dimethyl sulfoxide
FCS	Fetal calf serum
FGA	Fluorogestone acetate
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
h	Hour
HAP	Horse anterior pituitary preparation
HCG	Human chorionic gonadotrophin
HEPES	N^1-2-hydroxyethylpiperazine-N^1-ethanesulfonic acid
Hg	Mercury
ICI	Imperial Chemical Industries Ltd.
ID	Inside diameter
IM	Intramuscular
INRA	Institut National de la Recherche Agronomique (France)
IU	International unit
IV	Intravenous
kg	Kilogram
ℓ	Litre
LH	Luteinizing hormone
m	Metre
M	Molar
MEM	Minimum essential medium
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mOs	Milliosmols
MRC	Medical Research Council (United Kingdom)
N_2	Nitrogen
ng	Nanogram
No.	Number (of)
O_2	Oxygen
OD	Outside diameter
PBS	Phosphate-buffered saline
PG	Prostaglandin
PMSG	Pregnant mare's serum gonadotrophin

SC	Subcutaneous
SE	Standard error
SERSIA	Société d'Etudes et de Recherches Scientifique sur l'Insémination Artificielle
SOF	Synthetic oviduct fluid
TCM	Tissue culture medium
USP	United States' pharmacopeia
μg	Microgram
vs.	Versus

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ILLUSTRATIONS

SECTION ONE

TECHNIQUES AND RESULTS OBTAINABLE IN EMBRYO TRANSFER

Contributors to this section review the technical difficulties involved in embryo transfer, the effects that they have on results and the efforts being made to overcome problems and improve results.

INTRODUCTION

Section one comprises successive reviews of the problems involved in embryo transfer arranged in the order that they are encountered in practice. The first requirement is a source of embryos to transfer. They then have to be collected and handled *in vitro* in supportive media so that they can be examined, perhaps transported and perhaps manipulated for procedures such as sexing. Finally, embryos that are considered viable must be selected and transferred to suitable recipients. This chain of events is considered species by species, followed by a general review of methods of preserving mammalian embryos for long periods at low temperatures.

There are some general points to make about sources of embryos. First, they need to be abundant for most applications of embryo transfer. The superovulation of potential donors, and, possibly in the long term, the use of follicular oocytes from specific donors or from the abattoir are, therefore, crucial to the agricultural practice of the procedure. Second, there are no indications that the progeny of superovulated farm animals are any more likely to be abnormal than are those resulting from normal ovulation.

Fears that superovulation might increase the incidence of abnormalities followed the report that such was the case in mice (Elbing, 1973), but this report has been contradicted (Smith and Chrisman, 1975) and there is further evidence that embryos from gonadotrophin-treated mice are normal, at least in pre-implantation stages (Spindle and Goldstein, 1975). Chromosomal analyses of rabbit blastocysts obtained by superovulation did not indicate any increased incidence of abnormalities in one study (Fechheimer and Beatty, 1974), but did so in another (Fujimoto et al, 1974). A direct comparison of the development of embryos from superovulated and non-superovulated rabbits showed no difference between them (Maurer et al, 1968). In practice, the large numbers of normal young that have been obtained from transferred embryos after superovulation also suggest that there is no major effect on embryos carried to term, but it would be interesting to know the incidence of chromosomal abnormalities in those that are lost at various stages of gestation.

Embryos can only be transferred during pre-attachment stages of development. Austin (1973) has described how this makes it extremely unlikely that any manipulations involved in the procedure could lead to abnormalities in offspring, because pre-attachment embryos are remarkably resistant to teratogenesis in contrast to embryos of slightly later stages undergoing organogenesis. The basis for the resistance is that any adverse effect on the relatively few cells of an early embryo will either be severe enough to kill the whole embryo or will spare enough undifferentiated cells to accomplish normal development.

TECHNIQUES AND RESULTS IN CATTLE

SUPEROVULATION

K. J. Betteridge

First, it is relevant to summarize Gordon's (1975) review of the literature up to 1974 in which he well described the notorious variability in response of adult cattle treated with gonadotrophins with special reference to his own experience in Ireland. Some parts of Gordon's review can then be commented on in the light of the more recent literature.

Gordon's (1975) conclusions — The most widely used gonadotrophin has been pregnant mare's serum gonadotrophin (PMSG). Although anterior pituitary preparations from pigs, sheep, cattle and horses have been tried, no critical comparative data on their efficacies are available except that earlier suggestions that the horse anterior pituitary preparation (HAP) might be better than PMSG have not been substantiated.

There is general agreement that PMSG has to be given during the transition from the luteal to the follicular phase of the estrous cycle, about day 16 (day 0 = first day of estrus) in animals undergoing natural luteolysis.

Doses have varied from 1500 to 3000 IU and both the mean response and variability increase with the dose. A few reports have suggested the use of higher doses of PMSG and divided doses as a series of injections. These treatments have not found use in practice.

The failure of some treated cattle to come into estrus has been a common problem which may be reduced by giving 10 mg estradiol-17β on each of days 19 and 20 to animals not in estrus by that time.

Superovulation rates can vary with different manufacturers' preparations of PMSG, but suggestions that there is real batch-to-batch variation could not be confirmed. Neither could it be confirmed that the variation in ovulation rates was attributable to the proportion of stimulated follicles that actually ovulated rather than variation in the number of follicles stimulated to grow, as has been suggested.

It seems best to give PMSG injections in a small volume as an intramuscular injection.

The interval from PMSG injection (for a given dose on a given day of the estrous cycle) to estrus affects ovulation rates (the longer the interval, the higher the average rate). The interval is itself affected by the PMSG treatment (the higher the dose, the shorter the interval). It can be added that there is a tendency for treated cattle to divide into two groups with short and long treatment-to-estrus intervals (Mauleon et al 1970; Betteridge and Mitchell 1974). Longer intervals and their associated high ovulation rates may depress fertilization rates by creating an endocrine environment hostile to the gametes or by disrupting normal transport of gametes and zygotes.

There are well defined breed differences in responsiveness to PMSG, Friesians responding more poorly than either Charolais or Hereford × Angus and Herefords being more responsive than Angus.

Seasonal variations in response have been demonstrated in Ireland with highest ovulation rates during February, March and April, perhaps because of an increase in the number of vesicular follicles capable of responding during that period.

Nutritional effects on response are only manifest after radical changes such as starvation.

Population sizes of ovarian follicles have a marked influence on response rates, raising the possibility that assessment of these might in the future allow responsiveness to be predicted.

Indications that repeated treatment with PMSG leads to a decline in response for immunological reasons are inconclusive and evidence from sheep work argues against such a possibility.

Prepuberal calves can be superovulated quite readily with PMSG but rates of fertilization and recovery of ova are both low, although at least one pregnancy has been produced by embryos obtained in this way.

Follicular oocytes are not yet a practical source of bovine embryos, but attempts at *in vitro* fertilization of cow oocytes have been reported and, in rabbits and mice, some young have resulted from the transfer of zygotes formed *in vitro*.

References and data supporting the above statements are cited by Gordon.

Prostaglandins were only just beginning to be used in conjunction with superovulation treatments at the time of the review and information on hormonal levels in treated cattle was also limited, so these two aspects provide useful topics with which to trace recent developments in superovulation as has also been done by Sreenan and Beehan (1976b).

The use of prostaglandins (PG) with PMSG — The interval between PMSG treatment and regression of the CL is subject to considerable variation in cows treated on day 16 of their natural cycle. The interval can now be controlled by using PG to bring about luteolysis at a set time after PMSG injection. $PGF_{2\alpha}$ and several synthetic 'analogs' (structurally related compounds) have been used to treat donors by intrauterine or intramuscular routes for this purpose. Neither the route of administration nor the preparation of PG has been shown to affect responses once working dose levels have been established, so their effects can be considered together. The only advantage of intrauterine treatment is economic, because doses are lower than those used intramuscularly.

Doses of $PGF_{2\alpha}$ by intrauterine infusion have been 1 mg or 0.5 mg for 2 successive days into the horn ipsilateral to the CL (Elsden et al 1974; Moore, 1975a) or 2 mg as a single infusion into the uterine body (Hill et al, 1973). Intramuscular doses of $PFG_{2\alpha}$ have generally been 25-30 mg as a single

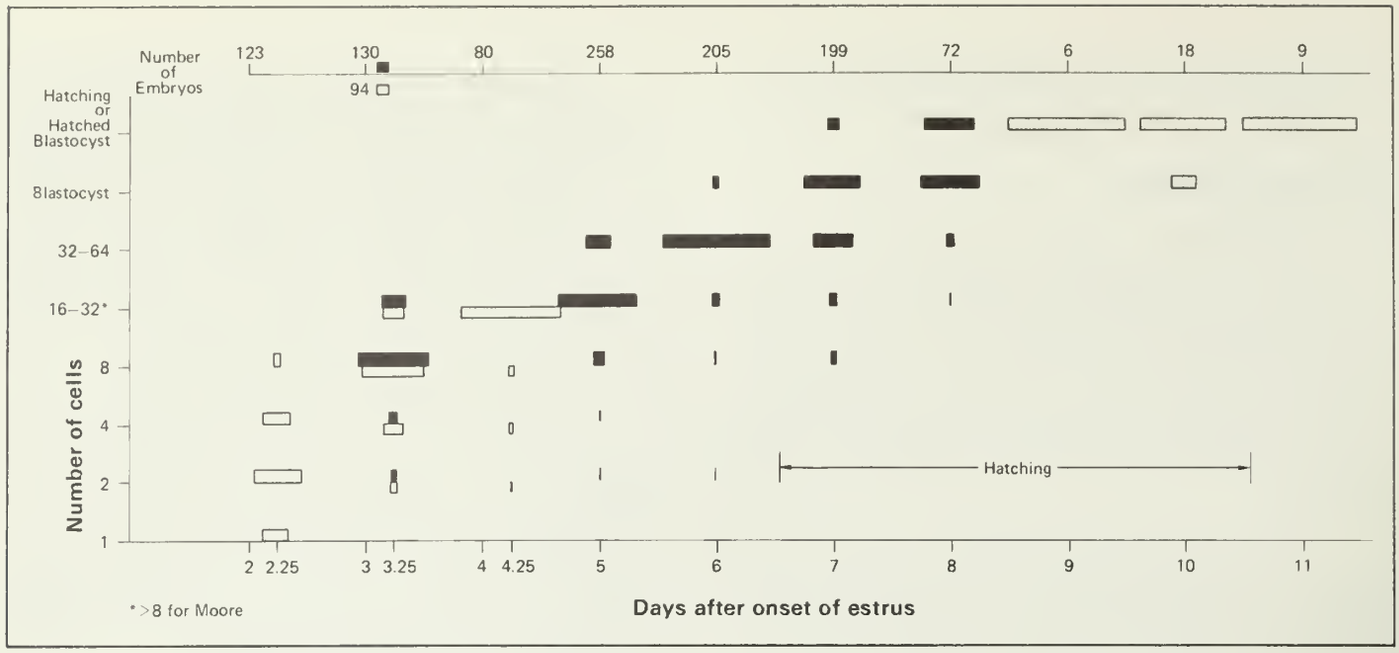


Figure 1. The cleavage and hatching of embryos in superovulated cattle. For each day, the proportion of recovered embryos in each developmental stage is represented by the length of the horizontal bar.

Data from Moore, 1975a (open bars); Newcomb, Rowson and Trounson, 1976 (solid bars); and ADRI, unpublished (stippled bars).

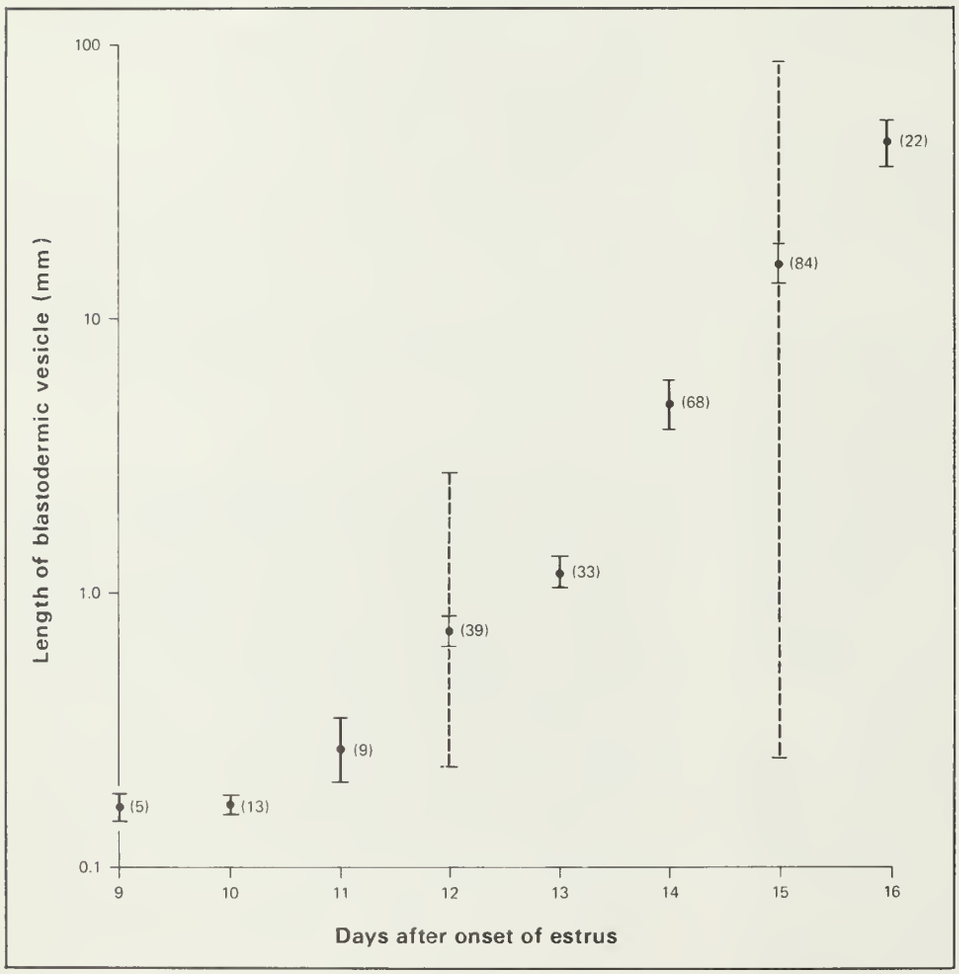


Figure 2. The growth of hatched embryos in superovulated cattle. Mean lengths (± SE, solid bars) are indicated on a logarithmic scale with the number of embryos for each point in parentheses. The range of sizes (broken bar) is given for 2 days to illustrate the enormous variability encountered.

Data from ADRI, unpublished.

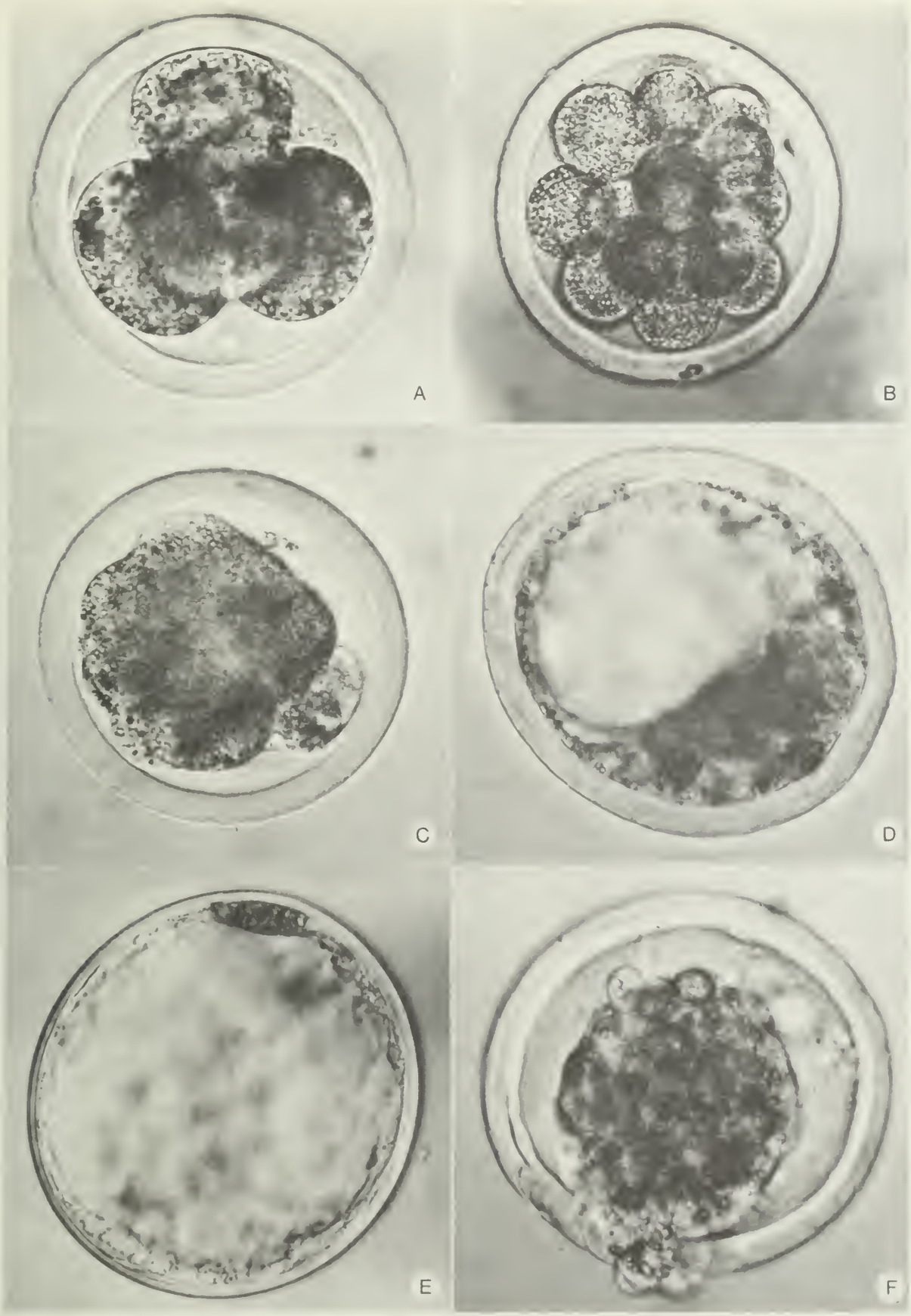


Figure 3. Cattle embryos at various transferable stages of development.

A. 4-cell egg, day 3. (X 350)
B. 16-cell egg, day 5. (X 360)
C. Morula, day 6. Cells have compacted and have lost their visible individual outlines. (X 330)
D. Early blastocyst, day 7. (X 350)
E. Blastocyst, fully expanded within the zona pellucida, day 10. Note the prominent inner cell mass. (X 350)
F. Hatching blastocyst, day 10. Note the cells extruding through the zona pellucida. (X 350)

Photos from ADRI.

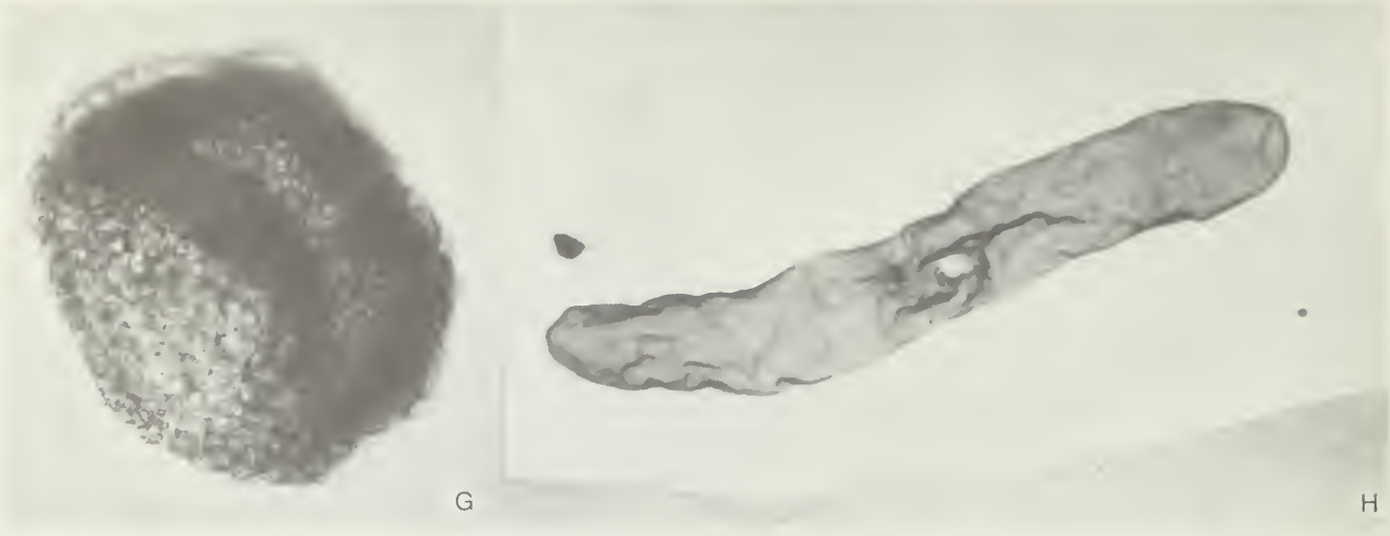


Figure 3 (Cont'd). Cattle embryos at various transferable stages of development.

G. Hatched blastocyst, day 12, with typical wrinkled appearance. (X 165)
H. Elongating blastodermic vesicle, day 14, with a prominent embryonic disc. Part of a much larger embryo from the same donor can be seen at the bottom of the photograph. (X 9)

Photos from ADRI.

injection, but some have used 30-40 mg in divided doses 4-10 h apart (Seidel, Bowen, Nelson and Homan, 1975; Elsden et al, 1976; Nelson et al, 1976) and a lower total amount as two consecutive daily injections of 8 mg and 4 mg has also been used (Anderson and Parker, 1976). Of the PG analogs, ICI 79939 is effective by the intrauterine route as a single infusion of 350 μg or, intramuscularly, at doses of 800 μg and 200 μg on successive days (Tervit et al, 1973) or similar treatments totaling 900 or 1000 μg over the 2 days (Sreenan et al, 1975); ICI 80996 ('Estrumate') is used as a single IM injection of 500 μg (Phillippo and Rowson, 1975) or 1000 μg (Booth et al, 1975) or as IM injections totaling 900 or 1000 μg over 2 days (Sreenan et al, 1975); AY 24655 (Ayerst) has been effective as a single IM injection of 100 mg (Betteridge et al, 1977). The time interval between PMSG treatment and the PG injection that follows has generally been 40-48 h, but published data comparing the effects of different intervals is sparse. Little change in results is produced by reducing the interval to 24 h (Tervit et al, 1973; Anderson and Parker, 1976); but giving PG on the same day as PMSG reduces the ovulation rate and its variability and increases the incidence of 'split-estrus', compared with donors treated with a 48 h interval (J. R. Hill et al, 1973, 1976).

Compared with donors treated with PMSG on day 16 of natural cycles, animals brought into estrus by PG treatment after PMSG appear to have higher superovulation rates (Elsden et al, 1974; Seidel, Bowen, Nelson and Homan, 1975; Nelson et al 1976) and it has been shown that treatment initiated during the mid-luteal phase (days 8-12) gives higher ovulation rates and yields of embryos than treatment begun earlier (Table 1). Newcomb and Rowson (1976) found that PMSG treatment resulted in significantly more ovulations when given on day 9 than it did when used on day 8. Using PG after PMSG given late in the luteal phase may be less effective (Table 1), although it does improve estrus synchronization compared with that shown in animals treated with PMSG on day 16 without PG (Phillippo and Rowson, 1975). It also improves the ovulation rate in such animals. Elsden (personal communication) obtained a mean ovulation rate of eight in 34 donors given 2000 IU PMSG alone on day 16, and a mean ovulation rate of 13 in 26 donors that were similarly treated with PMSG but received PG 48 h later.

The interval between PG treatment and estrus is shorter in PMSG-superovulated animals than in others (Tervit et al, 1973), estrus usually beginning on the second rather than the third day after PG. The initiation of ovulation also seems to be accelerated, beginning as early as 52 h instead of 80-90 h after PG although the time span involved in multiple ovulations remains uncertain (Phillippo and Rowson, 1975). On the other hand, PMSG at a dose level too low to induce superovulation (1000 IU) has been found to increase the time elapsing between subsequent PG treatment and estrus and to reduce the precision of synchronization (Moore, 1975b).

At least 66-80% of animals treated with PG and PMSG come into estrus and some of the remainder ovulate silently. Tervit et al (1973) detected estrus in 76/79 animals receiving an optimum dose; Elsden et al (1974) detected estrus in only 18/24 treated donors, but all 24 ovulated; Phillippo and Rowson (1975) detected estrus in 28 and ovulation in 29/35 treated animals; Moore (1975a) detected

TABLE 1. OVARIAN RESPONSES IN CATTLE TREATED WITH PMSG AT VARIOUS TIMES OF THE ESTROUS CYCLE AND WITH PG 2 DAYS LATER

Criterion of response and/or additional treatment	Day of cycle at time of PMSG treatment						Reference
	3-8		8-12		13-16		
	No. animals	% responding	No. animals	% responding	No. animals	% responding	
8 or more ovulations	32	31.3	58	74.1			Phillippo and Rowson 1975
6 or more eggs recovered	32	34.4	58	60.3			"
6 or more fertilized eggs recovered	32	6.3	58	34.5			"
3 or more ovulations	16	37.5	58	77.6	9	55.5	ADRI, unpublished
		Av ovulation rate[1]		Av ovulation rate[1]		Av ovulation rate	
Group A, controls	10	6.9	16	11.9			Sreenan, 1976a
Group B, progesterone treated	10	6.8	17	15.3			"
	15	5.0	84	12.0-12.5			"
	32	7.4	58	12.3	12	7.0	Phillippo and Rowson 1975

[1]Least square mean for data of Sreenan.

estrus in 48 and ovulation in 65/71 mature cows treated with PG after either PMSG or HAP.

The reason for improved superovulation as a result of mid-cycle stimulation may not be related simply to elevated progesterone levels at the time (Sreenan, 1976a; cf Groups A and B, Table 1). Perhaps variations in response are linked to the way in which the time of treatment relates to waves of follicular growth or atresia and their endocrinological background.

Endocrinology of superovulation with PMSG — Knowledge of the endocrinology of superovulation is fragmentary and disputed. The distribution and metabolism of the injected PMSG itself has not been described in cattle but a long half-life can be anticipated from studies in other species. Endogenous PMSG in mares has a half-life of about 6 days after hysterectomy (Cole et al, 1967). Injected PMSG has a half-life of about 6 days in horses and 26 h in rabbits (Catchpole et al, 1935), 26 h in rats (Parlow and Ward, 1961) and about 21 h in sheep (McIntosh et al, 1975). In sheep, McIntosh et al found no evidence that the ovaries removed PMSG from the circulation; but, in cattle, low doses of PMSG injected directly into the ovary appear to bind to receptors there and produce a local effect (Betteridge, 1974). The half-life of different preparations of PMSG can be expected to vary according to the extent to which sialic acid is removed from the native molecule (McIntosh et al, 1975).

Systemic PMSG injections initially exert a luteotrophic effect (Henricks et al, 1973; Hallford, Turman, Wetteman, Pope and Meyerhoeffer, 1975; Moore, 1975b), which has been related to its luteinizing hormone (LH)-like activity (Newcomb and Rowson, 1976). This property is also presumed to be responsible for the occasional premature ovulation of a large follicle present at the time of PMSG injection (Newcomb and Rowson, 1976). The precocious CL generally inhibits further ovulation, perhaps because it is not old enough to be lysed by PG given 2 days later and yet secretes enough progesterone to block LH release.

Spilman et al (1973) state that PMSG leads to release of endogenous LH within 24 h (according to the text) or 48 h (from the graph) of injection, but this was not the experience of others (Henricks et al, 1973; Lemon and Saumande, 1974; Saumande and Pelletier, 1975; Hallford, Turman, Wetteman and Pope, 1975; Barbella et al, 1976) who describe no LH peaks before the one coinciding with estrus. Pre-ovulatory release of LH bore no relationship to ovulation rates when expressed as maximum plasma LH concentrations (Henricks et al, 1973) but did so when expressed as the total quantity released (Saumande and Pelletier, 1975).

Circulating estrogen levels rise prodigiously before estrus in superovulated compared with untreated cattle (Lemon and Saumande, 1972; Henricks et al, 1973; Saumande and Pelletier, 1975; Booth et al, 1975; Hallford, Turman, Wetteman and Pope, 1975) and at estrus they may be four times as high as in untreated animals. After estrus and/or ovulation induced by PMSG, Booth et al (1975) found a temporary trough in circulating estrogen levels followed by a secondary rise around days 5 and 6 when levels could be eight times those found in normally cycling animals. They fell to normal levels by day 12.

Progesterone levels also often rise to extremely high levels after superovulation (60-100 ng/ml and

higher), with consequent extension of cycle length in non-pregnant animals (Spilman et al, 1973; Booth et al, 1975). Although most of the progesterone normally comes from induced CL, some may originate from luteinized and atretic follicles (Booth et al, 1975). Numbers of CL are positively related to levels of both progesterone and estrogen and negatively related to day-6 ovum recovery rates (Booth et al, 1975).

Although the correlation between estrogen production and subsequent ovulation rate has been confirmed in several studies (Lemon and Saumande, 1972; Henricks et al, 1973; Saumande and Pelletier, 1975; Hallford, Turman, Wetteman and Pope, 1975) there have, as yet, been no reports of using this to monitor the individual animal's responsiveness to PMSG with a view to adjusting the dose to produce controlled stimulation as is tried in human medicine. Neither does there appear to have been further investigation of the suggestion of Lamond et al (1971) that breed and individual variations in the length of the pro-estrus progesterone trough in untreated cycles may affect the dose of gonadotrophin required for superovulation. In considering PMSG doses in different breeds and individuals, it might be relevant that, in rats, there is a depression of ovulation rate within a defined intermediate dose range (Wilson et al, 1974).

In a small proportion of superovulated, inseminated donors, premature CL regression has been noticed by several investigators (Booth et al, 1975; Brand et al, 1977; ADRI, unpublished) and presumably also occurs in the considerable proportion of untreated animals that undergo short cycles after failing to conceive (MacMillan, 1970; Boyd, 1973).

A great deal has been learned of the anatomy and endocrinology of superovulation of prepuberal calves from extensive studies in France. These are best described in the thesis of Testart (1975) with various portions of the work and some supplementary information in the publications of Testart (1972a, b), Testart and Kann (1973), Testart and Arrau (1973), Arrau (1974), and Saumande and Mariana (1976). Building on the American work reviewed by Gordon, these workers used 332 calves, about 3-4 months of age, in evolving a reliable means of superovulating them and collecting fertilized ova, although the ultimate viability of the resulting embryos has remained disappointing (see page 9).

Given alone, PMSG on day 0 does not induce ovulation in calves at doses below 660 IU. Increasing the dose to 1500-3000 IU leads to estrous behavior in 3-5 days, ovulation in about 50% of the treated animals but only a single ovulation in most of these. Supplementing an injection of 1800 IU PMSG with an HCG injection on day 5 increases both the proportion of calves that ovulate (31/37) and the number of ovulations per calf. It also reduces the amount of PMSG that is necessary to induce ovulation, but a major problem that remains is the prolonged time span over which ovulations occur. This can be largely overcome by inserting vaginal sponges impregnated with 60 mg fluorogestone acetate (FGA, Searle) at the time of PMSG treatment and leaving them in place for 4 days. The 4 days of FGA blocks ovulation until day 6 but, beginning 41 h after sponge withdrawal, 75.3% of 93 calves averaged 13.9 ovulations each and they were grouped within a 20 h ovulatory period. Giving 1500 IU HCG IV on the day after FGA withdrawal further improved ovulation rates, 97.5% of 120 calves having an average of 18.1 ovulations each. The benefit of HCG, however, is in doubt for two reasons. First, the time span for the ovulations again lengthened, resulting in a wider range of developmental stages among the ova recovered. Second, the abnormally high levels of ovarian steroids resulting were felt to be detrimental to embryos.

In contrast to the findings of Spilman et al (1973) the French workers found no evidence that PMSG alone induced an immediate release of endogenous LH into the circulation. Instead, it resulted in a short-lived peak of 3-4 ng/ml for up to 8 h between 108 and 132 h after PMSG. This LH peak is insufficient to bring about ovulation over and above the single one provoked by the exogenous PMSG, which explains why exogenous HCG becomes necessary. The use of FGA improves results by leading to a much greater LH release for 8-16 h with peak values of 11-72 ng/ml 12-20 h after sponge withdrawal. This matches LH release in normally cycling adult cows and is sufficient to lead to multiple ovulation some 20 h later. There is also an FSH peak coincident with the major LH peak.

Circulating estrogen levels are greatly elevated before ovulation, levels being well correlated with the degree of follicular development but not with the number of ovulations. There appear to be two populations of follicles in the ovaries of stimulated calves at the time of the LH peak. The first (averaging about 45 follicles) consists of residual ones that differentiated before FGA withdrawal and are destined to undergo atresia. The second is the group of follicles that mature after sponge withdrawal and are responsive to the ovulatory stimulus. After ovulation, very high levels of progesterone and secondary peaks of estrogen occur, as in the superovulated adult cow.

Other regimens for superovulation — There have been some reports on attempts to use pituitary FSH in addition to those covered in Gordon's review. The half-life of sheep FSH in cattle is about 5 h, slightly shorter during the follicular than during the luteal phase (Laster, 1972 b). This necessitates either frequent injections or alternative means of maintaining biologically active levels. Infusing a constant level of FSH totaling up to 9.75 mg into the systemic circulation for 3 days during late pro-estrus or during progestogen feeding did not decrease the variation in ovarian response (follicular growth), compared with studies in which FSH was injected intramuscularly twice daily for 5 days (Laster, 1972a). Infusion of 45 mg FSH over 5 days in gradually

increasing amounts from day 16 had an initial luteotrophic effect, led to estrus in less than one third of treated animals and, following injection of 200 μg of gonadotrophin-releasing hormone (GnRH) on day 21, resulted in ovulation rates averaging between three and five in various groups (Warren et al, 1975). Attempts to prolong the effect of single injections of FSH by slowing its absorption using vehicles such as carboxy-methyl-cellulose and poly-vinyl-pyrolidone have not been very successful (Smith et al, 1973). Discrepancies of up to 350% between labeled and assayed potencies of commercial preparations of FSH have been recorded by Laster (1973), who also found that FSH produced more variable results than PMSG given in divided doses in a study aimed at producing *limited* superovulation for multiple pregnancies. Laster's data (1973) and those of Moore (1975a) also confirm that an exogenous LH source is not necessary to bring about ovulation in PMSG- or FSH-stimulated cattle. This view is shared by most workers, but some continue to use HCG at the time of estrus and others have reported satisfactory use of GnRH to ensure the release of endogenous LH (Cupps et al, 1974; Warren et al, 1975). Colorado workers have used an FSH:LH mixture (5:1) given as 10 injections over 5 days commencing on days 9-11 of the estrous cycle. The dose required is 5, 4, 3, 2 and 2 mg twice daily. Prostaglandin is used on the 3rd day. Numbers of follicles and rates of ovulation, recovery and fertilization have not differed from those following PMSG stimulation in concurrent experiments (Seidel, Bowen, Nelson and Homa, 1975; Nelson et al, 1976; Elsden et al, 1976; Bowen et al, 1977). Similar results with pituitary FSH:LH have been experienced in commercial units (Mauer, unpublished). Moore (1975a), in a controlled comparison of PMSG and HAP, found both gonadotrophins equally effective in superovulating both cows and heifers. In heifers, however, HAP produced more unruptured large follicles, than did PMSG.

As an alternative to relying on natural or PG-induced luteolysis soon after PMSG administration, it is possible to time the PMSG to precede withdrawal of progestogen treatment. Laster (1973) used 16 days feeding with 6-chloro-$\triangle^6$-acetoxy-progesterone (CAP) as the synchronizing agent and found that ovulatory responses to PMSG and FSH were 36% and 47% higher, respectively, than to analogous treatments in normally cycling animals. However, the progestogen depressed fertility in the FSH-treated animals. Testart, Godard-Siour and du Mesnil du Buisson (1975) obtained an average ovulation rate of eight in eight cows synchronized with two 6 mg subcutaneous implants of the synthetic progestational steroid SC 21009 (Norethandrolone, Searle) for 8 or 10 days beginning during the luteal phase of the cycle and treated with PMSG 2 days before removal. The CL present at the time the implants were inserted were lysed with the aid of an injection of 5 mg estradiol valerate.

Sreenan and Beehan (1976b) have compared PG, SC 21009 and intravaginal pessaries containing progesterone as means of synchronizing donors being treated with PMSG. Estrus began earlier after each of synchronizing treatments than in animals not treated with PMSG. With the progestogens, synchronization was also less precise after PMSG treatment than without it and less precise than that produced by PG. Ovulation and fertilization rates seem similarly variable in all groups and pregnancy rates were not affected by the different synchronization treatments.

In mature cows, Moore (1975a) found that injected progesterone (40 mg/day, IM, for 17-30 days) produced more precise synchronization of estrus after PMSG or HAP than did intrauterine PG, although the proportion of cows exhibiting estrus (92% of 50 progesterone treated and 69% of 48 PG treated) did not differ significantly. Injected progesterone was similarly effective in synchronizing superovulated heifers.

Sources of variation in response to PMSG — Little can be added to Gordon's review, but there is now more evidence against seasonal variations in responsiveness to PMSG as judged by ovulation rates. Remarkably similar responses to comparable treatments have been found in all quarters of the year (Table 2), although Shea et al (1976) found

TABLE 2. RESPONSES OF CATTLE TO PMSG DURING DIFFERENT SEASONS

	Jan.-Mar. or winter[1]	Apr.-Jun. or spring[1]	Jul.-Sep. or summer[1]	Oct.-Dec. or autumn[1]	Author
No. donors	32	32	26	28	ADRI, unpublished
Ovulations/*treated* donor	9.5	9.7	8.8	10.5	
% donors with ⩾3 ovulations	68.8	65.6	61.5	64.3	
No. donors	56	55	93	81	Beehan and Sreenan, unpublished
Ovulations/donor	10.2	9.9	8.4	8.6	
No. donors	105	189	104	184	Shea et al, 1976
Ovulation/*responding* donor	12.5	12.7	14.9	12.5	
% donors with ⩾4-5 ovulations by palpation	87.6	88.3	91.0	81.5	

[1]For data of Beehan and Sreenan that may not match the months exactly.

that pregnancy rates did fall during October-December. Similarly, Elsden (personal communication) found no seasonal variation in ovulation rates in response to PMSG in Australia, but collection, fertilization and pregnancy rates were all significantly depressed in the winter months. However, Church and Shea (1976) found that responses to PMSG are most irregular between January and March and a seasonal variation in the twinning rate has been recorded in Charolais cattle, with lower rates in winter (Ortavant, 1974). It may also be noted that seasonal differences in response to PMSG have been recorded in pigs (Webel et al, 1970b) and hamsters (Moore and Greenwald, 1974).

Further breed differences in responsiveness to PMSG are recorded by Shea et al (1976), 178 Simmental, 79 Limousin, 54 Chianina and 93 Maine-Anjou donors averaging 15.2, 13.6, 12.4 and 9.9 ovulations, respectively. The Maine-Anjou ovulation rate was significantly ($p<0.05$) poorer than for other breeds. In calves, dairy breeds have more small follicles than beef breeds have and dairy × beef hybrids tend to have still more (Testart 1975).

Batch-to-batch variation in the effectiveness of PMSG has been reiterated, with unpublished evidence variously suggesting that varying FSH:LH ratios are involved (Newcomb and Rowson, 1976) or are not involved (Church and Shea, 1976). Newcomb and Rowson (1976) cite Louwerens as suggesting that PMSG has, in fact, relatively little FSH-like activity, its FSH:LH ratio being 1:30 rather than the 5:1 ratio documented in the literature. It should be noted that these ratios are determined in assays in mice or rabbits and so their relevance to biological effects in cattle may be questioned. Recent analyses of the FSH:LH activity of PMSG with radio-receptor assays has cast interesting new light on batch-to-batch variation. Stewart et al (1976) showed the FSH:LH ratio to remain constant (about 1:5) in unextracted serum from six different mares and throughout the period between days 40 and 80 of gestation. This implies that any batch-to-batch variation in this ratio in commercial preparations can be ascribed to extraction and purification procedures. Testing several batches from one manufacturer, they found the ratio ranged between 0.9 and 1.3, a variation that was not significant. Further, using the same assayed batches to superovulate cattle and sheep, their colleagues found no significant variation in mean ovulation rates between groups. They conclude that the variation between animals in response to PMSG is unlikely to be due to differences in the FSH:LH ratio of the preparations used.

There are recent indications (Sreenan and Beehan, personal communication) that the interval between PMSG treatment and estrus in heifers affects the proportion of stimulated follicles that ovulate (the longer the interval, the higher the proportion) rather than the mean ovulation rate itself. In mature cows, however, the interval between treatment with PMSG or HAP and estrus had no effect on ovarian response (Moore, 1975a).

The lactating dairy cow, especially in mid-lactation, is often unresponsive to PMSG. Brand et al (1977) found that 21/62 given 3000 IU PMSG failed to respond with three or more ovulations. Moore (1975a; Table 5) has also noted lower ovulation rates in mature cows than in heifers and reasons that the difference may be due to a decline with age in the number of oocytes.

The use of estrogens at the time of expected estrus after PMSG is said to improve fertilization rates mainly by increasing the incidence of overt estrus (Gordon, 1975). Despite the high yields of embryos that Gordon et al continue to obtain (see Table 5), estrogen injection does not seem to be widely practiced, although some workers use it at the much lower (and more physiological) dose of 400 μg (Drost, personal communication).

Fertilization, recovery and quality of ova and embryos following superovulation — The length of time over which multiple ovulations are spread in superovulated adult cattle is not known, but it is generally assumed to be long enough to necessitate repeated inseminations at about 12 h intervals during, and immediately after, estrus to ensure good fertilization rates. Gordon (1975) has reviewed the usual practices and the evidence for the superiority of fresh over frozen semen, but work in progress suggests that this difference may not be clear-cut (Sreenan and Beehan, 1976b). Trounson, Willadsen, Rowson and Newcomb (1976) found that a single insemination with fresh semen gave good fertilization rates. Nelson et al (1976) detected no consistent differences between fertilization rates in heifers inseminated at 12 h intervals either twice with frozen semen, four times with frozen semen or twice with fresh semen. Moore (1975a; Table 5) obtained very high fertilization rates in superovulated cows and heifers running free with bulls. In cattle treated with both PMSG and PG, it is said to be advisable to inseminate before estrus (Anon., 1976), but reasons for this view are not given.

The data of Shea et al (1976) illustrate that fertilization rates tend to decrease as rates of ovulation increase (Table 3) and that both recovery and pregnancy rates are reduced as fertilization rates fall (Table 4). Paradoxically, though, no decrease of recovery and pregnancy rates is apparent with increasing superovulation (Table 3). The same authors point out that individual exceptions to these tendencies can be striking: one donor provided 34 embryos from 37 ovulations, and 19 of these gave rise to pregnancies. Using smaller numbers of donors, Newcomb, Rowson and Trounson (1976) also found no effect of ovulation rate on recovery rate, but others have found recovery rates to fall with increasing ovulation rates. Testart (1975) records recovery rates of 72, 63 and 56% in groups of 37, 50 and 3 animals having 1-3, 4-20 and >20 ovulations, respectively. Similarly, Sreenan, Beehan and MacDonagh (1974) recovered only 32% of eggs shed in a group with an average ovulation rate of

TABLE 3. EFFECTS OF RATES OF SUPEROVULATION ON RATES OF RECOVERY AND FERTILIZATION OF OVA AND OF PREGNANCY FOLLOWING TRANSFER IN CATTLE

Number of CL	1-10	11-20	21+
Number of donors	164	193	100
% ova recovered	63	66	61
% recovered ova fertilized	84	78	71
% recipients pregnant	49	50	51
Pregnancies per donor surgery	1.8	4.0	6.4

From Shea et al (1976).

TABLE 4. RELATIONSHIPS BETWEEN RATES OF FERTILIZATION, RECOVERY OF OVA AND PREGNANCY FOLLOWING TRANSFER IN CATTLE

% recovered ova fertilized	0-34	35-67	68-100
Number of donors	33	55	212
% ova recovered	42	59	70
% recipients pregnant	28	43	51

From Shea et al (1976).

23.2, as against 50% when the average ovulation rate was 13.6. Moore (1975a) and Sreenan and Beehan (1976b) noted a tendency for egg recovery rates to fall in cows and heifers, respectively, as the dose of PMSG or HAP rose. There was a similar (non-significant) trend for lower pregnancy rates to be given by transferred embryos after higher doses of PMSG (Sreenan and Beehan, 1976b).

It is often assumed that the unusual endocrine environment following superovulation must adversely affect embryo recovery and viability and so it might be expected that embryo recovery rates would decline with increasing time after ovulation. Such has not been our experience (ADRI, unpublished, in Table 5) and these recovery rates are very similar to those reported from Cambridge for unspecified numbers of donors after day 5 by Newcomb and Rowson (1976), although their recovery before day 5 was considerably higher (69%). Other evidence, however (see Table 9 and pages 24-26), indicates a decline in embryo quality (i.e., its potential for further development) between days 5 and 7 and a marked increase in the incidence of degenerate eggs between days 7 and 8. Sreenan (personal communication) has found that the incidence of abnormalities in recovered embryos increases with time but at a much slower rate than in other reports. Brand et al (1977) found that days of recovery had no effect on the proportion of abnormal embryos among 165 recovered between days 7 and 12. Obviously, relationships between recovery rates, embryo quality and hormone levels in individual animals need further study. Hormone levels may also be suitable parameters to try to relate to the widespread, but undocumented, experience that individual donors providing apparently similar embryos may give very different pregnancy rates (see also page 26).

Shea et al (1976) found that their Maine Anjou animals with relatively low responses to superovulation treatments had rates of fertilization, embryo recovery and pregnancy in recipients at least equal to results given by other breeds. This illustrates the fact that the criterion of success in superovulation techniques must always be the yield of viable embryos, not merely the ovulation rate. Factors affecting egg and embryo quality are discussed on pages 24-26 of this monograph.

In superovulated calves, vaginal insemination is unsatisfactory. The French workers have confirmed that best results can be obtained by surgical, intrauterine insemination which led to fertilization occurring in 8/12 calves and 28/66 (42.4%) ova. A more practical method that gives almost as good results (fertilization in 68% of 50 calves and 33.3% of 387 ova) involves intra-abdominal deposition of sperm over the reproductive tract after perforating the dorsal fornix of the vagina through a special speculum (Testart, 1975). Satisfactory insemination can also be accomplished through the cervix with the aid of a speculum (Onuma et al, 1970). Recovery of ova is best (42%) 2 days after the LH peak (7 days after PMSG). Recovery rates decrease thereafter and also decrease as the number of ovulations increases. Calves having more than 10 ovulations can be expected to provide 2-5 fertilized ova, but their quality remains in doubt having only been tested in transfers to two cows (without success) and by further cleavage in rabbit Fallopian tubes (37.5% of 24 ova developed). Thus, the only recorded pregnancies produced from superovulated calves are the one obtained by Seidel et al (1971), after transferring 72 embryos in 14 cows, and two (unpublished) at the University of California, Davis (Drost, personal communication). Part of the reason for low success rates may lie in the site of transfer, since transfers to the oviduct give poor results even with embryos from mature cattle.

IN VITRO FERTILIZATION AND USE OF FOLLICULAR OOCYTES

K. J. Betteridge

In 1959, M. C. Chang obtained living rabbits from ovulated oocytes that had been collected from donors' Fallopian tubes, fertilized with capacitated spermatozoa *in vitro* and then transferred to recipients. Similar experiments since then have resulted in births or pregnancies following *in vitro* fertilization of ovulated oocytes in more rabbits (Thibault and Dauzier, 1961; Bedford and Chang, 1962; Fraser and Dandekar, 1973; Seidel et al, 1976) and in mice (Pavlok, 1967; Whittingham, 1968; Mukherjee and Cohen, 1970; Hoppe and Pitts, 1973) and rats (Toyoda and Chang, 1974). There is some concern over the normality of progeny resulting from *in vitro* fertilization because, in the very small numbers so far studied, increased incidences of microphthalmia

in rats (Toyoda and Chang, 1974) and of splayleg in rabbits (Fraser and Dandekar, 1973) have been noted, although the rabbits had normal chromosomes (Fraser et al, 1975) and others have obtained quite normal rabbits in this way (Seidel et al, 1976). From the practical standpoint, few applications warrant subjecting unmated donors to the flushing procedures necessary to recover ovulated oocytes when the same techniques after mating could provide fertilized embryos, but the techniques of *in vitro* fertilization that have since been used to obtain embryos from follicular oocytes are based on the above studies.

Follicular oocytes can be collected from two sources: either from mature follicles that have been naturally stimulated in females that are cycling normally or have been artificially stimulated in females treated with gonadotrophins; or from immature or atretic follicles found at random in ovaries, notably in abattoir material. Maturation of the oocyte to the stage at which it can be fertilized will have occurred, or have been initiated, *in vivo* in the first case but will have to be accomplished *in vitro* in the second. Fertilization of the matured oocytes can be brought about *in vitro* before transfer, or *in vivo* by transferring mature oocytes to the Fallopian tubes of recipients just before or after insemination.

To date, follicular oocytes have given rise to pregnancies in mice (Cross and Brinster, 1970; Mukherjee, 1972), rabbits (Brackett, 1973) and man (Steptoe and Edwards, 1976) after collection from artificially stimulated follicles, *in vitro* fertilization and culture for up to 4¼ days before transfer or reintroduction.

Normal young have been born to rabbits (Thibault et al, 1975b) and sheep (Cran et al, 1976; Moor and Trounson, 1977) after transfer of matured oocytes and fertilization in the recipients. These two studies are of especial interest in that oocytes were matured within follicles cultured under hyperbaric conditions to ensure adequate oxygenation (Thibault et al, 1975a) and in the presence of gonadotrophins and estradiol-17β (Moor and Trounson, 1977). Even follicles beginning to undergo atresia could be 'rescued' and it seems that any sheep follicle over 3 mm in diameter can give rise to fertilizable oocytes. The proportion of transferred oocytes that produced young reached 60% in rabbits and 63% in sheep but similar work with oocytes from cultured calf follicles has, so far, produced disappointing results (Thibault et al, 1976; Menezo et al, 1976). Taken together, these facts promise that abattoir material may eventually provide a useful source of oocytes and thence of embryos.

Work on this topic in pigs is fully discussed on page 43.

EMBRYO COLLECTION BY SURGICAL METHODS

R. P. Elsden

Once the decision has been made to undertake large numbers of elective laparotomies on normal, healthy cows, there is a moral obligation to provide adequate facilities and observe the principles of good surgery.

The operating room should be designed so that there is a logical flow or sequence of events from premedication to induction of anesthesia, followed by scrubbing up and surgery. If necessary, all these activities can be completed in one room without losing efficiency, but some workers have stressed what they consider the advantages of separating preparation, surgical and recovery areas (Reid and Betteridge, 1974). Many different facilities have been designed with varying degrees of success (e.g., Fig. 4). Generally, cows should walk into the building via a passage and be held there in a chute for premedication (if used) and induction of anesthesia. The floor of this holding area can serve as a wheeled surgical table, which is pulled out into the room and raised by a jack at one end to a height suitable

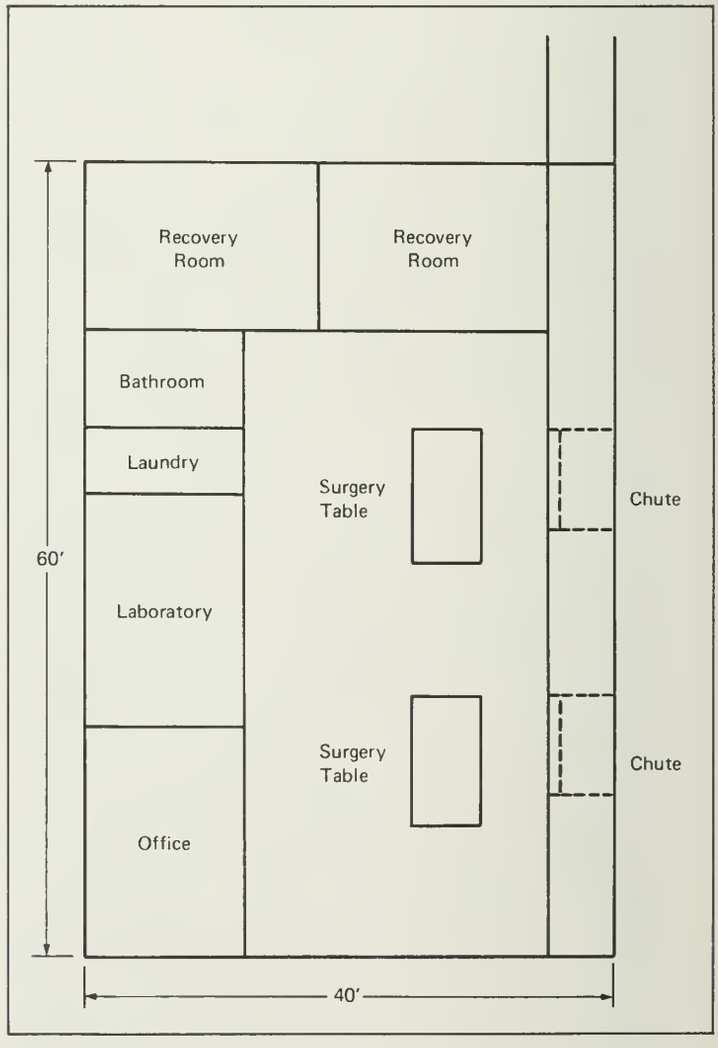


Figure 4. A design for a surgical embryo transfer unit.

for surgery. An alternative is to have one side of the chute double as a hydraulic table to which the pre-medicated cow can be strapped and then tilted into a horizontal position. From there the cow can be rolled onto a wheeled, hydraulic surgical table. In either system, when surgery is completed the table is lowered to the ground and the cow is wheeled out to a recovery room.

An efficient facility can be made from a modified farm building lined with materials that are easily cleaned. If possible, it is advantageous to be close to cattle yards to which access can be designed via a passageway. Cattle may then be selected and starved in pens close to the surgical unit.

Although embryo recovery through a flank incision under local anesthesia has been attempted, collection rates are lower and formation of scar tissue is greater than when it is done by the midline approach, which has been by far the most widely used surgical method for collection since first described by Rowson et al in 1969. As this method requires general anesthesia, cows should be starved and deprived of water for a minimum of 24 hours. Pre-medication with sedatives such as xylazine or acetyl promazine may be given about 20 min before surgery. Phenobarbitol sodium or thiamylal sodium is used to induce general anesthesia, which is then maintained by standard inhalation techniques with halothane in oxygen or by intravenous drip of an agent such as glycerol guaiacolate. All cows must be intubated with a correctly fitting endotracheal tube, a simple procedure provided the animal is induced to a sufficient depth of anesthesia.

With the cow secured on her back, clipping or shaving and a surgical scrub for a minimum of 5 min is followed by draping in the usual manner. A 15 cm incision is made just anterior to the udder in the midline. Care is taken to control hemorrhage and a dry field is attained before entering the abdomen. Wherever possible, blunt dissection is used and vessels are avoided rather than incised. To help maintain asepsis, skin towels are used or, when the abdomen is opened, further drapes are applied around the incision. All flushing equipment and extra personnel are organized at this point. It is imperative that the uterus be exposed for as little time as possible, in order to depress the tendency for scar tissue to form.

Once the uterus is located, gentle traction is applied to the horns. When they are just below the incision, the broad and mesovarian ligaments are grasped and tension is applied to exteriorize the uterine horn, oviduct and ovary one side at a time, taking care to handle the latter structures as little as possible. The mesovarian ligament is pierced at a suitable site with a blunt instrument, such as a pair of needle holders, which is pushed through so that the two ends rest on either side of the incision. Thus the fimbria and ovary are fixed in an accessible position, and the oviduct is easily cannulated (Fig. 5A). An alternative means of anchoring the reproductive tract, advocated by Rowson and used by many others, is by umbilical tape passed through the mesometrium, ligated around the body of the uterus and tied to a stainless steel rod placed across the incision. Flushing and ovum collection can then proceed as described by Rowson (1969) from the oviduct, or by tubal and uterine flushing methods described by Newcomb and Rowson (1975b), or as shown in Fig. 5. Exposed tissues are continually moistened with saline and, after returning the exposed organ to the abdomen, it is the author's practice to place 500 to 1000 ml of sterile saline in the abdomen. Some surgeons use heparin (10 USP units/ml) in the moistening saline in the belief that it helps reduce fibrin formation and consequent adhesions.

To close the incision, peritoneum and linea alba are sutured together with No. 4 chromic gut, or a synthetic material such as Vetafil or Polydek, using simple interrupted sutures. If there is more than a 2 cm depth of subcutaneous fat, it is often sutured with continuous No. 1 plain gut. Finally, the skin is closed with interrupted sutures using synthetic materials. Some cows appear to undergo severe reactions to these and too many sutures tend to cause large serotomas, which can be a temporary problem if they become infected. An alternative method of closing the abdomen is to place tension sutures of braided silk in the abdominal tunic, followed by a continuous line of chromic gut. However, this technique occasionally delays healing and causes extensive tissue reactions.

Surgical cases are held in small yards post-operatively and observed closely for a few days. Problems such as serotomas (10%), infected suture lines (0.5%) and herniations (1%), occur but under good management systems are of little significance. Some surgeons attempt to break down early adhesions by gentle rectal palpation every few days following the operation. In the author's opinion this practice is of little value. Skin sutures are removed after about 10 days.

Fibrin deposition and organization into fibrous tissue follows all operations, its significance varying from incidental to levels affecting fertility. The incidence of adhesions can be reduced by skilful surgery and speed. Besides length of exposure time, another major reason for scar tissue formation is the degree of tension applied to the uterus and ligaments. There are individual variations in the amount of scar tissue that results from surgery. In a small proportion of cases (1%) it can lead to infertility, in others (10%) to subfertility and, at the other end of the scale, some cows undergo the surgical operation for recovery of embryos up to five times and still remain fertile. There are four areas in which scar tissue is a problem:

1. The broad ligament itself, which can lose its limited elasticity as its fibrous tissue content increases. This leads to greater difficulty in exposing the uterus during future operations.

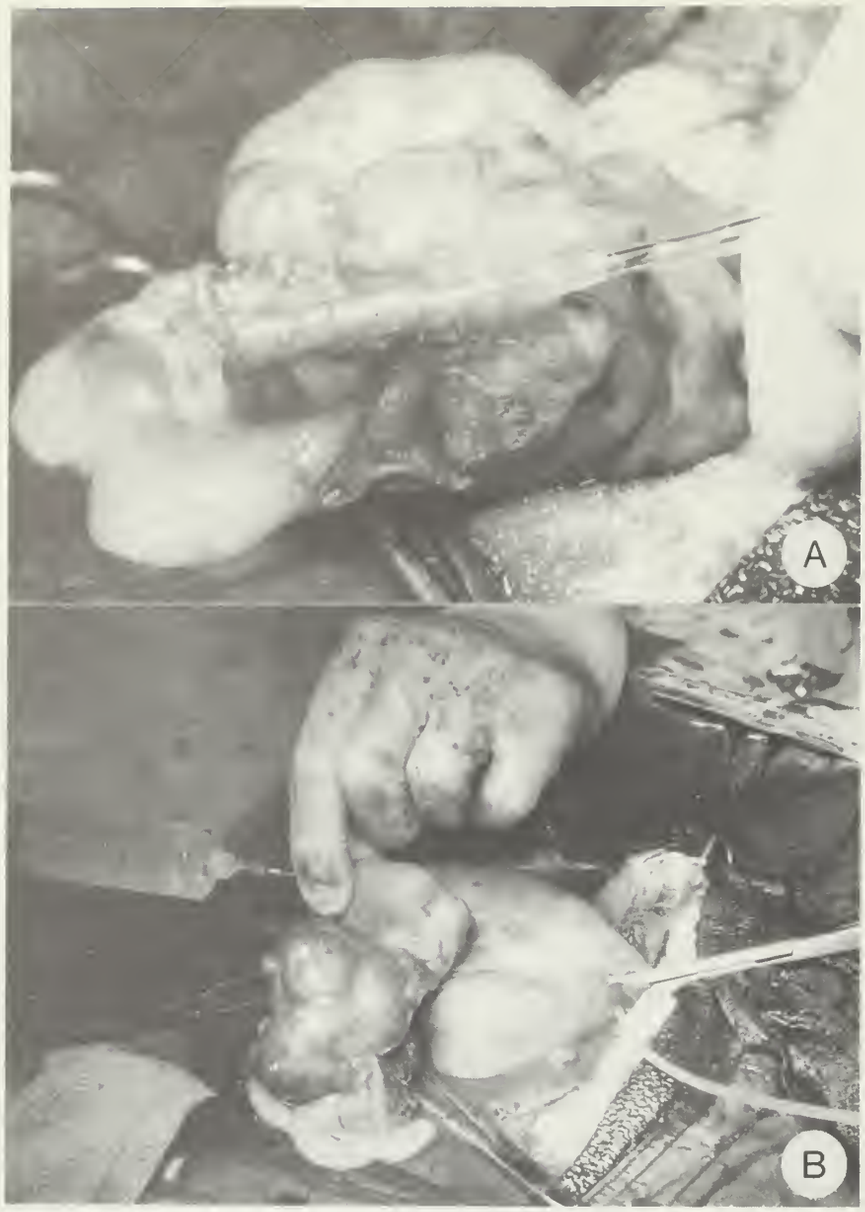


Figure 5. Steps in the surgical recovery of embryos from cattle.

A. A glass cannula inserted through the fimbria in preparation for collection of a flush of the oviduct. Note also the method of anchoring the reproductive tract by means of needle holders piercing the mesovarian ligament and traversing the abdominal incision.
B. A uterine flush in progress. Medium is being flushed into the uterine horn below the tip which is pinched closed. A prepared teflon tube with a fenestrated, flame-smoothed inner end is seen clamped into the uterine lumen with bowel clamps, ready to collect the flush.

2. Fimbrial, oviduct and horn adhesions often cause serious infertility problems. The fimbria may adhere to the ovary (especially after multiple ovulation), to the tip of the uterine horn and occasionally to the omentum. The oviduct itself sometimes becomes occluded by fibrous constrictions. These lesions can be avoided by uterine flushing techniques which do not involve manipulation and cannulation of the oviduct.

3. Adhesions of the omentum to the peritoneum around the abdominal incision line. At subsequent operations these have to be divided to gain access to the uterus. Minor hemorrhage results and the omentum becomes increasingly difficult to handle each time the animal undergoes surgery.

4. The skin and abdominal tunic. Usually, this is not a significant problem in relation to fertility,

Figure 5 (Cont'd). Steps in the surgical recovery of embryos from cattle.

C. Collecting a flush in a round-bottomed dish.
D. Handling collected embryos beneath the dissecting stereomicroscope. Aspiration into the Pasteur pipette is controlled by syringe or mouth.

Photos from Colorado State University, courtesy G. E. Seidel, Jr.

but the increased blood supply delays subsequent entries into the abdomen and increases suturing difficulties and, thus, the incidence of serotoma and hernia formation.

EFFECTS OF DIFFERENT SURGICAL COLLECTION METHODS ON YIELD OF OVA

R. P. Elsden and K. J. Betteridge

Recovery rates of ova and embryos that have been obtained by surgical methods are shown in Table 5. It should be noted that these data refer to animals that were considered to have responded to superovulation treatment. Many reports do not specify the proportion of treated animals that failed to respond and were therefore not flushed. These figures indicate considerable inefficiency in collection of ova after superovulation but they may well reflect the unknown effects of several other factors besides difficulties in the collection technique itself. Even counting CL and differentiating them from luteinized follicles can be inaccurate, especially when their numbers are excessive. Effects of the superovulation treatment itself on the yield of ova collected have already been considered (page 26). It is not known whether many ova remain trapped in apparently normal CL after superovulation in cattle, but this

TABLE 5. RECENT RECOVERIES OF EMBRYOS FROM SUPEROVULATED CATTLE AT SURGERY OR SLAUGHTER[1]

No. donors flushed	Collection		Ovulations		Embryos and unfertilized ova recovered			Embryos recovered				References
	Day	Method	Total	Av/ flushed donor	Total	% ovulations	Av/ flushed donor	Total	% total recovered	% ovulations	Av/ flushed donor	
436			7892	18.1	4261	54	9.8	1875	44	24	4.3	Gordon's 1975 summary of earlier reports
44	2-11	Surgery and slaughter	454	10.3	210	46.3	4.8	154	73.3	33.9	3.5	Betteridge and Mitchell, 1974
40	3-7	Slaughter	412	10.3	211	51.2	5.3	81	38.4	19.7	2.0	McGaugh et al, 1974
10	3-6	Surgery	141	14.1	97	68.9	9.7	85	87.6	60.3	8.5	Elsden et al, 1974
14	5	Surgery	126	9.0	82	65.1	5.9	—	—	—	—	Elsden, unpublished
39	7	Surgery	326	8.4	172	52.8	4.4	—	—	—	—	Elsden, unpublished
29	—	—	164	5.7	121	74	4.2	58	48	35	2.0	Cupps et al, 1974
25	3-6	Surgery	462	18.5	184	39.8	7.4	166	90.2	35.9	6.6	Booth et al, 1975
90	2-9	Slaughter	549	6.1	364	66.3	4.0	243	66.8	44.3	2.7	Testart, 1975
98[2] (cows)	2-5	Slaughter	347	3.5	254	73.2	2.6	201	79.1	57.9	2.1	Moore, 1975a
23[2] (heifers)	2-5	Slaughter	157	6.8	122	77.7	5.3	116	95.1	73.9	5.0	Moore, 1975a
147	—	—	2308	15.7	1411	61.1	9.6	1235	87.5	53.5	8.4	Gordon, 1976b
27	5-6	Surgery	—	—	331	—	12.3	260	78.5	—	9.6	Trounson, Willadsen, Rowson and Newcomb, 1976
34[3]	2-7	Surgery	373	11.0	186	49.9	5.5	104	55.9	27.9	3.7[4]	ADRI, unpublished
21[3]	2-7	Slaughter	182	8.7	91	50.0	4.3	38	41.8	20.9	2.3[4]	" "
34	10-16	Surgery	331	9.7	122	36.9	3.6	85	69.7	25.7	2.5	" "
34	10-16	Slaughter	384	11.3	226	58.9	6.6	124	54.9	32.3	3.6	" "
582	Usually 5	Surgery	—	10-15	—	59-67	—	—	58-78	—	—	Shea et al, 1976
25[5]	6-7	Slaughter	364	14.6	251	68.9	10.0	194	77.3	53.3	7.8	Renard, du Mesnil du Buisson, Wintenburger-Torres and Menezo, 1976
27	7-12	Slaughter	264	9.8	165	62.5	6.1	114	69.1	43.2	4.2	Brand et al, 1977

[1]Some data have been calculated from other figures given in the references cited.
[2]Estrous donors only.
[3]Includes six not superovulated.
[4]Excluding non-superovulated donors.
[5]Excludes six non-responding animals and five producing unfertilized or degenerate eggs.

certainly occurs in mice (Beaumont and Smith, 1975). Losses may occur after premature entry of ova into the uterus, leading to their expulsion (Newcomb, Rowson and Trounson, 1976). Finally, it seems reasonable to expect that some ova will not be picked up by the fimbriae when ovaries are greatly enlarged.

Yields are probably more affected by the timing of the flush after ovulation than by details of the method itself. Newcomb and Rowson (1976), for example, record higher rates of recovery within 4 days of estrus (69%) than on day 5 or later (49%), though the numbers of donors on which these figures are based are not given. Nelson et al (1976), also with unspecified numbers, recovered 57.4% ova on day 3, 30.5% on day 6. More detailed evidence of a decline in recovery rate with time up to day 8 is given in Table 6. In a series of 27 animals flushed at slaughter between days 7 and 12, Brand et al (1977) found no decline in total embryo recovery rates with time, the overall rate being 62.5% from 264 ovulations. There is no evidence that, before day 10, these relatively low collection rates are caused by surgical difficulties, because flushing excised tracts after slaughter gives similar results (Betteridge and Mitchell, 1974; ADRI, unpublished, in Table 5). After day 10, collection tends to be less efficient at surgery than at slaughter (ADRI, unpublished, in Table 5) possibly because it is difficult to place Foley catheters low enough in the horns and body of the uterus to avoid missing some of the more widely distributed embryos.

TABLE 6. EFFECT OF DAY OF RECOVERY ON RECOVERY RATE BY SURGICAL METHODS IN CATTLE

	Days after estrus					
	3	4	5	6	7	8
No. animals	22	10	15	68	55	15
Ova recovered (as % ovulations)	78.3	53.4	66.6	64.1	50.0	45.9

From Sreenan and Beehan (1976b).

The distribution of ova and embryos in the reproductive tract at various times after superovulation is depicted in Fig. 6. Passage into the uterus is largely completed between days 3 and 5, but up to 10% may remain in the oviduct until at least day 8. In 26 heifers on days 6, 7 and 8 combined, 7% of recovered ova were found in the oviduct, 73% in the anterior 10 cm of the uterine horn and 20% in the base of the horn (Newcomb, Rowson and Trounson, 1976). In superovulated dairy cows, on the other hand, no eggs were found in the oviducts in

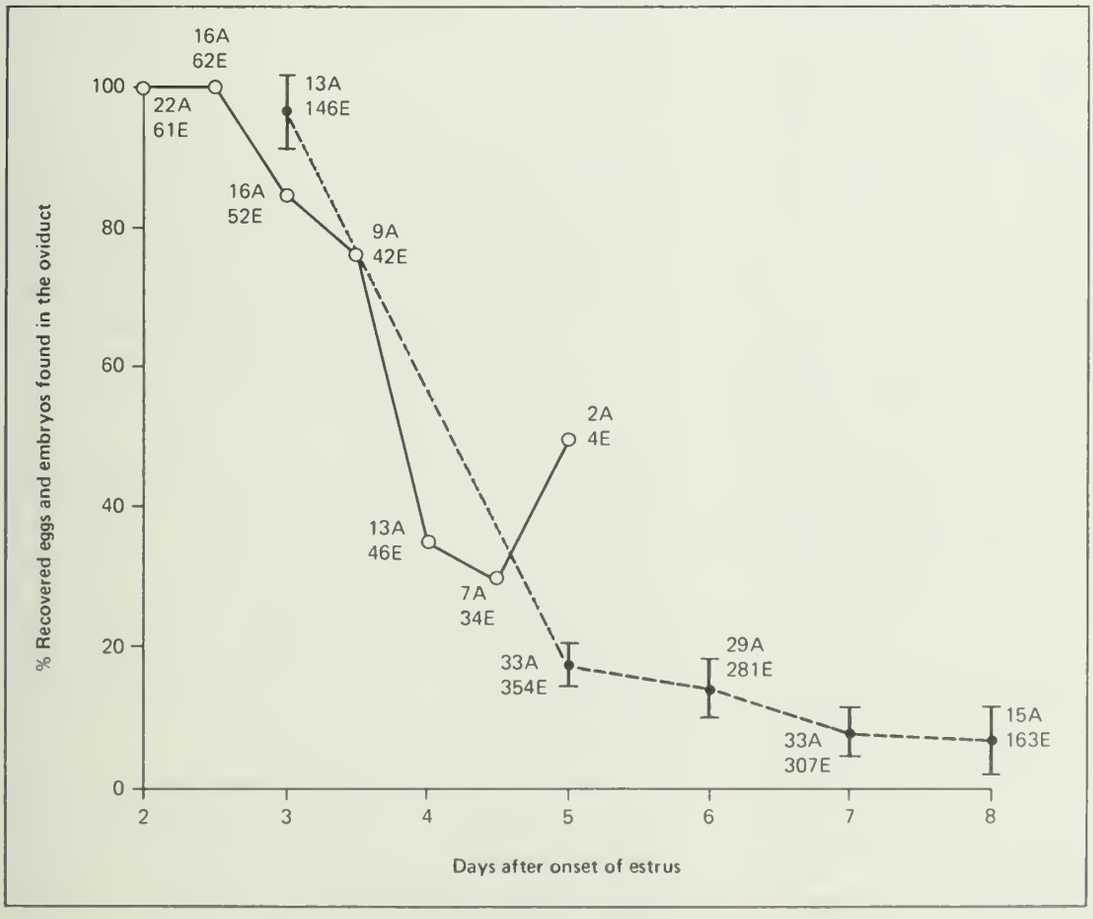


Figure 6. The location of eggs and embryos flushed from cattle 2-8 days after estrus.

Data from Moore, 1975a (○——○); and Newcomb, Rowson and Trounson, 1976 (●-------●). Numbers of animals (A) and eggs/embryos (E) from which each point is derived are indicated. Vertical bars indicate SE of mean.

27 animals slaughtered between days 7 and 12 (Brand et al, 1977). Very exceptionally, a normal embryo may remain in the oviduct until day 13 (ADRI, unpublished).

The effects of two methods and times of collection on yield of collected ova have been compared at Colorado; one at 5 days after estrus when ova were flushed by cannulating the oviduct, the other by flushing the uterus at 7 days. Collection from 14 donors by the first method resulted in a 65% recovery rate, compared with 53% from 39 donors with the second method. This difference was found to be significant ($P<.025$) when the data were examined by χ^2 (Elsden, unpublished, in Table 5). Neither method affected fertility severely because the donors were subsequently bred and 11/14 (78%) subjected to collection from the oviduct were pregnant within 3 months, as were 35/39 donors (89%) from which collection was by the uterine flushing technique.

There are sufficient data on 480 of the donors listed in Table 5 to make the following generalizations about results obtained since Gordon's 1975 summary. The average ovulation rate for the 480 was 11.4 and 57% of the ova were recovered, a mean of 6.5 per flushed donor. Of these, 75% were fertilized, representing 43% of the ovulations or an average yield of 4.9 embryos (range 2.0-8.4) per flushed donor.

Recent developments in non-surgical ova collection techniques (see following pages), in which collection rates are equal to surgical uterine flushing methods, mean that the inherent risks of surgery are no longer warranted under most circumstances. The fact that older (uterine) embryos also tolerate cooling and freezing better (see pages 50 and 52) is also in favor of later collection. Surgical recoveries should be limited to situations where ova are to be collected within 4 days of estrus, for example in research applications and in certain infertility cases where the uterus is considered an unsuitable environment for embryonic development. It remains to be seen whether the pregnancy rates produced by the 'higher quality' fertilized eggs collected soon after ovulation (see page 25) at a higher rate of recovery (see above) can be improved sufficiently by culture before transfer to merit their further surgical collection from the oviduct.

EMBRYO COLLECTION BY NON-SURGICAL METHODS

A. Brand and M. Drost

Table 7 summarizes the advantages and disadvantages to be expected of non-surgical rather than surgical methods in embryo transfer.

Development of non-surgical recovery techniques before 1976 was exceedingly slow. Several workers over the years have described devices for the recovery of bovine embryos via the cervix from superovulated cows (Rowson and Dowling, 1949; Dowling, 1949; Dracy and Petersen, 1950; Dziuk et al, 1958). Success was variable and limited to an average of one ovum per attempt (range 0-20). Sugie et al (1972a,b) succeeded in recovering an average of 6.2 ova (range 1-18) from 45/60 superovulated cows using an apparatus comprising two or three cannulae, along with 2000-3000 ml of flushing medium.

TABLE 7. RELATIVE ADVANTAGES OF SURGICAL VS. NON-SURGICAL EMBRYO RECOVERY AND TRANSFER TECHNIQUES IN CATTLE

Surgical	Non-surgical
Advantages	
Patient immobilized	Repeatable
Sterile technique possible	Can be performed on the farm
Direct manipulation of uterus	No fasting required
Accurate evaluation of ovarian response	Timesaving (transfer)
20-50 ml recovery medium needed	No surgical risks
Disadvantages	
Formation of adhesions	Manipulative skills required
Surgical facilities needed	500 ml recovery medium required per cow
Risk of general anesthesia	Sterile procedure difficult to maintain
Surgical risk	Can only be performed in animals in which the cervical canal can be penetrated.
Difficult in lactating animals	
Fasting required	
Recovery period required	

More recently, Brand, Drost and Aarts (1976) obtained a recovery rate of 11.5% in flushing 26 non-superovulated cows using a modified Sugie apparatus. They also recovered 12/23 embryos from 4/5 superovulated cows. Using a transvaginal technique, Testart and Godard-Siour (1975) recovered eggs from 3/6 cows with a single ovulation and from 5/6 superovulated animals. The recovery rate was 43% of the number of CL (20/46). More extensive and intensive efforts on non-surgical methods have extended the later, more encouraging results and the experience of a number of workers (some of it unpublished) is summarized below. There is general agreement that dexterity, patience and practice are essential to achieve satisfactory results and that parous cows make better donors than heifers for non-surgical methods. Obviously, use of the technique is confined to donors after day 5, by which time most ova have reached the uterus in superovulated cattle as discussed on page 15.

A simple in-out flushing technique has been used by Testart et al in their transvaginal method and by Ayalon et al (1976) through the cervix. Testart reports recovery of ova from 21/23 donors with an average yield of eight per donor for one series of 14, and 4.3 per donor for another series of nine. Recovery was estimated to be 50% of ovulations as judged by palpation of CL. It seems that adhesions are not as important a problem as in

surgical recoveries. Ayalon et al use a simple polyvinyl chloride pipette fitted with an inflatable latex rubber cuff. Under epidural anesthesia the instrument is guided through the cervix and about 10 cm into a uterine horn by an arm in the rectum. The area posterior to the cuff is sealed off by inflating the cuff with air. A total volume of 300-400 ml of flushing medium (TCM-199) is infused in 50 ml aliquots using a plastic syringe attached to flexible polyethylene tubing and recovery is achieved by the siphon effect created when the syringe is removed after each infusion. Each collection is poured into flat-bottomed vials and allowed to stand 5 min before being searched for embryos. From 19 non-superovulated dairy cows flushed 12-17 days after estrus, Ayalon et al recovered 96% of the infused fluid and obtained ova or embryos from 11 (57%) of them. Fourteen of the 19 cows had had some pathological symptoms since calving. Twelve cows were inseminated at the first estrus following non-surgical flushing and then slaughtered 2-7 days later. Ten ova were recovered, eight of them fertilized, indicating that the technique did not impair fertility.

A continuous flow flushing technique has been used by Alexander in England, by Elsden and by J. M. Bowen in Colorado, by Bouters in Ghent, Schefels in Munich (personal communications), by Rowe et al in Wisconsin and by ourselves in Utrecht.

Alexander is undertaking all his collections non-surgically. The technique and instrument being used has been evolved over the past 3 years and a patent has now been applied for in several countries. Following an average collection of 6.8 eggs per donor in 15 donor operations last year, the technique is being applied commercially (Alexander et al, 1976). It has been found possible to pass the instrument through the cervix in 90% of heifers and in all cows tested on days 6-7. Over 90% of infused fluid is recovered and good post-flushing fertility has been demonstrated in donors treated with intra-uterine antibiotics after flushing.

At Utrecht, a non-surgical method requiring much simpler equipment has been developed (Drost et al, 1976). A Rusch two-way or three-way balloon catheter (3.6-6.0 mm diameter) is used, modified so that the tip of the catheter extends for 7-12 cm beyond the cuff. A metal stylet is inserted into the balloon catheter to give it rigidity and facilitate its manipulation through the cervix. To prevent straining, the donor is given an epidural injection (5-10 ml 2% xylocaine). The vulva is washed and disinfected with alcohol. To prevent contamination of the catheter inside the vagina, it is first introduced into the cervix via a 4 cm diameter vaginal speculum. After removal of the speculum, the catheter is manipulated through the cervix into one of the uterine horns until the balloon can be inflated with 7-20 ml of air and palpated inside the corpus uteri. The stylet is then removed and the catheter is connected by tubing to a bottle containing 500 ml of flushing medium (phosphate-buffered saline plus 4% bovine serum albumin) suspended 1 m above the level of the uterus. The second canal of the catheter is connected to a conical glass collecting vessel at ground level. With the three-way device, the medium is allowed to enter the uterus continuously under pressure (up to 150 mm Hg) or by gravity flow. With the two-way device, 75 ml medium is allowed to distend the uterine horn while the siliconized outlet tubing is blocked at a Y-junction outside the vulva. The inlet tubing is then clamped off and the outflow is released. This process is repeated six or seven times. In older cows, the uterus is retracted into the pelvic cavity with the help of a pair of forceps during irrigation. To maximize recovery of the medium from the tip of the uterine horn, which is normally situated below the level of the body of the uterus, the ipsilateral ovary is elevated and pulled cranio-dorsad. In superovulated animals, the procedure is repeated in the opposite horn using a second sterile speculum and device. After recovery, about 3 ml of medium is aspirated from the bottom of the conical collection vessels and examined under a stereomicroscope. Brand et al (1977) have reported on a series of 41 attempted flushes of superovulated dairy cows with this technique between days 7 and 12. In seven (17%) the cervix could not be penetrated (but no cervical dilators were used). The 34 flushed cows yielded an average of 2.7 normal embryos each, a recovery rate of 36% (as estimated from palpated CL), compared with 43% from a similar series of slaughtered animals. In the collected fluids, it was easier to locate embryos at days 12-13 than at earlier stages. Lugols iodine (30 ml, 20%) may be infused after collection to counter infection or pregnancy from residual embryos.

This method has been adopted, with very encouraging results, at Colorado (Elsden et al, 1976). Egg recoveries per attempt in non-superovulated cattle between days 5 and 8 were 36/51 (71%) in normal animals and 4/38 (11%) in animals with known fertility problems. In superovulated donors, eggs were collected in 24/26 attempts (92%), averaging 6.9 per recovery. Collections have been successfully repeated at up to four successive cycles. Considerable care is necessary in searching the flushing medium (up to 800 ml), which contains more mucus and cellular debris than medium collected surgically.

Rowe et al (1976) have used a technique very similar to the one described above, with additional agitation of flushing medium within the uterus by means of a syringe attached to a side arm. In a series of 19 non-treated, single ovulating and 15 superovulated heifers, recovery rates on days 6-9 ranged between 54 and 70% of estimated numbers of ovulations in various groups of animals. There were indications that these rates might be improved by increasing the number of flushes from five to eight for each uterine horn. In only two of the 34 heifers was it not possible to pass a Foley catheter through the cervix.

Bouters (personal communication) has used a modified two-way Sugie apparatus to flush the uteri of 10 superovulated cows. In 8/10 animals the technique was successful and 36 ova were collected, 46.8% of the number of corpora lutea counted after slaughter of the donors. As indicated in Fig. 7A, the flush is made principally from the base of the uterine horn to the tip. However, an additional 'reverse flush,' in through the ureteral catheter and collected from the same catheter, is felt to improve recoveries by creating extra turbulence near the uterotubal junction.

Schefels (personal communication) has flushed the uterus of nearly 100 cows with Sugie's instrument. In one third of the animals there were great difficulties in achieving a fluid-tight seal around the

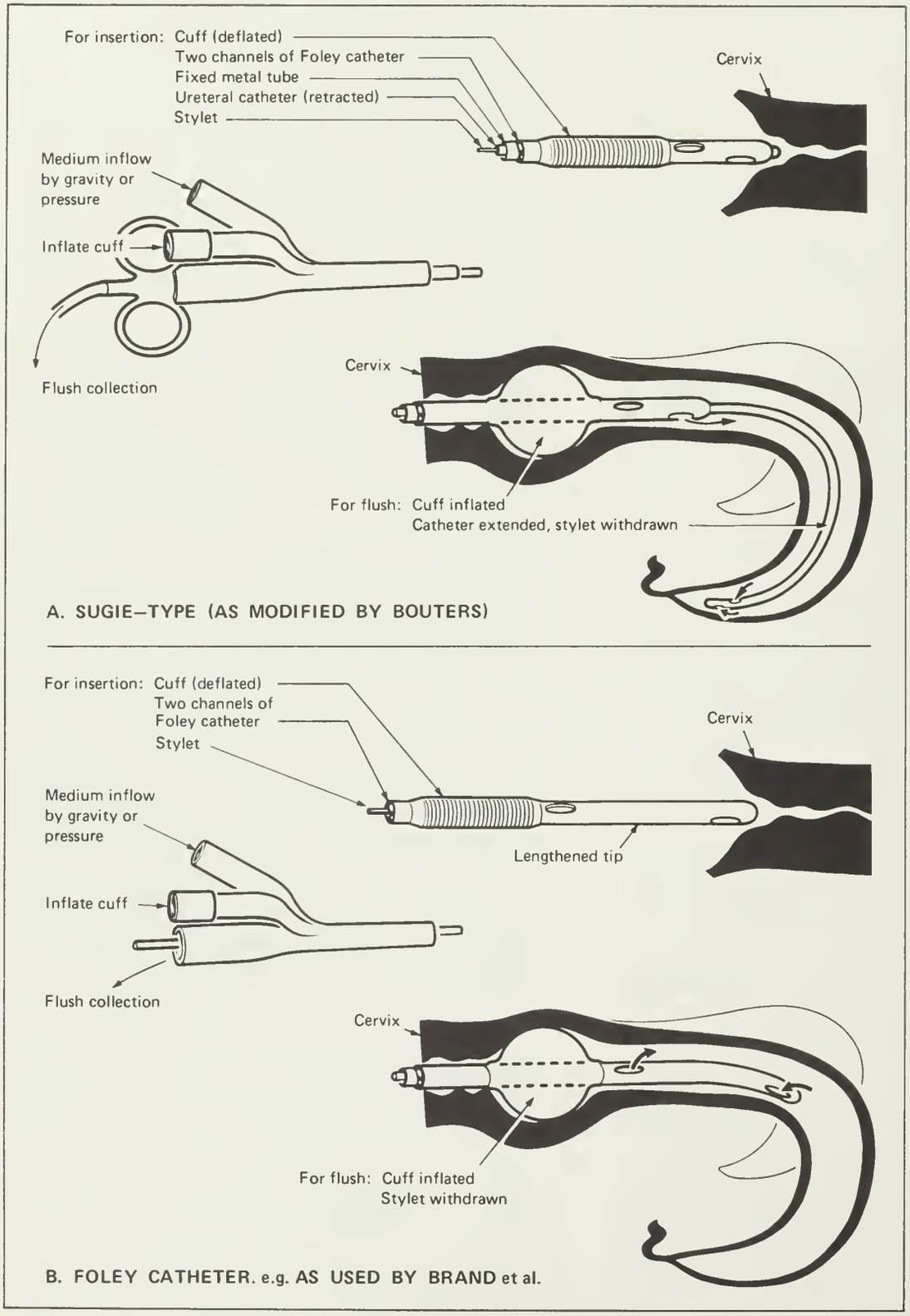
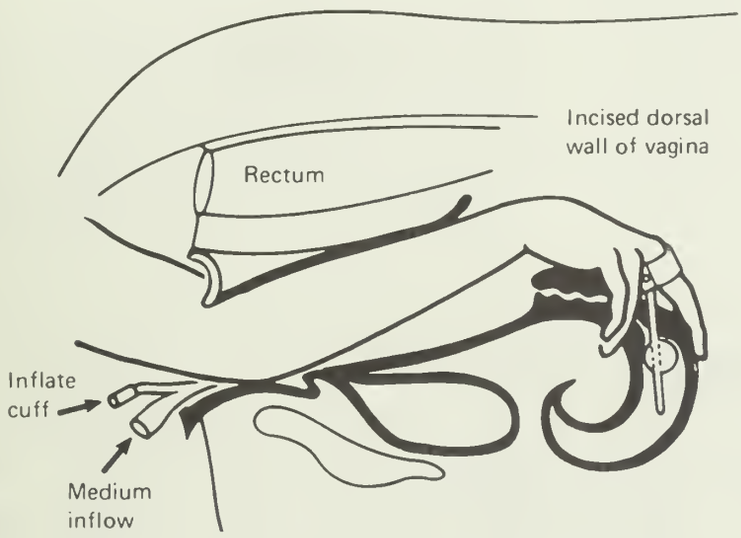
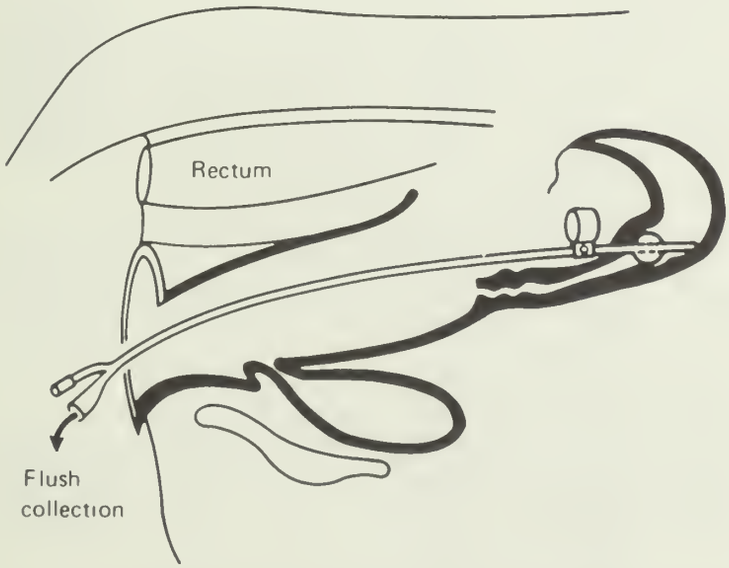


Figure 7. Three basic designs of apparatus for non-surgical collection of embryos from cattle.

instrument in the uterus and so losses of flushing medium were high and no ova were recovered. In another third the volume of flushing medium was reduced from 1000 to 100 ml per horn to reduce intrauterine pressure but, again, no ova were obtained. Some success was experienced in a few cases in the final third after enlarging the diameters of all tubes and using a combination of pressure on the inflow and vacuum on the outflow to increase flow rates and dislodge mucus. Schefels has also used a surgical vaginal approach on 30 cows, drawing the uterus into the vagina through an incision in its dorsal fornix. He gives no details of the flushing technique itself but was able to collect ova in only six animals. However, in those six, the recovery rate was 50-80% of the number of ovulations. Failures seemed due to technical difficulties only and Schefels considers that this approach could be useful in older cows.



Flushing medium introduced through cuffed catheter attached to a teat cannula on a finger ring.



Uterine horn elevated and utero–tubal junction occluded with fingers during collection of flush.

C. TRANSVAGINAL METHOD (TESTART AND GODARD–SIOUR, 1975)

Figure 7 (Cont'd). Three basic designs of apparatus for non-surgical collection of embryos from cattle.

SHORT-TERM MAINTENANCE AND CULTURE OF EMBRYOS

G. E. Seidel Jr.

The viability of bovine embryos must be maintained *in vitro* for successful embryo transfer and for studying basic aspects of embryonic development. Foote and Onuma (1970) have reviewed the literature to 1970. Until then, embryos had routinely been maintained in serum, saline or follicular fluid. Exceptions were Brinster (1968), who cultured two embryos in a chemically defined medium (BMOC-2); Onuma and Foote (1969), who used Ham's F-10 and Krebs-Ringer bicarbonate plus 10% serum; Sreenan, Scanlon and Gordon (1968), who used a synthetic medium; and Rowson et al (1969), who used TCM-199. In studies *in vitro*, development was usually limited to one cleavage division, although a few embryos underwent two or three divisions. Little success was reported in studies where embryos were transferred with the exception of Rowson et al (1969). After transferring one to three embryos to each recipient, they found pregnancy rates were high (13/20) if TCM-199 was used and zero (0/9) if bovine serum was used. Presumably the serum was not heat-treated to remove the embryotoxic factor (Chang, 1949). The low success rates of previous bovine embryo transfers were probably attributable in part to the use of serum as a culture medium.

Reports since 1970 of studies in which two or more culture media or systems were compared will be dealt with in chronological order and are summarized in Table 8. The storage of embryos below room temperature is reviewed elsewhere in this monograph (see pages 50-53). It must be emphasized that in many studies complete culture systems are compared rather than a single component of the system such as the medium. Some or all of the following may influence results: the stage of development of the embryos at the start of culture, age and superovulation treatment of the embryo donor, volume of culture medium, number of embryos per container, atmosphere, temperature, atmospheric pressure, use of paraffin oil or sealed containers to prevent evaporation, and individual variability among the donors. The latter is nearly impossible to deal

TABLE 8. EXPERIMENTAL TREATMENTS CONCERNING BOVINE EMBRYOS *IN VITRO*; A SUMMARY OF LITERATURE SINCE 1970

Author	Method	Atmosphere	Hours *in vitro*	Medium or treatment	Stage at beginning of study	No. embryos	Response	Response criteria	Comments
Seidel et al, 1971	Microdrops under paraffin oil	5% CO_2, 95% air	48	Modified Ham's F-10	2- to 3-cell	17	1.3	Mean no. cleavage divisions	Embryos recovered from calves
				TCM-199		15	0.7		
Tervit, Whittingham and Rowson, 1972	Stoppered test tubes	5% CO_2, 5% O_2, 90% N_2	144	SOF	1-cell	10	80	% developing to 16-cell	2/4 embryos transferred after culture developed into fetuses
			96		8-cell	5	60	% developing to blastocyst	
McKenzie and Kenney, 1973	Microdrops under paraffin oil	5% CO_2, 95% air	96	Recovered from oviducts	1- to 8-cell	78	36	% continued cleavage	Some donors were calves
				Recovered from uterus		30	20		Medium was BMOC-3
Seidel, 1974	Covered glass dishes	Air	1-10	TCM-199 with HEPES +5% serum	8-cell to morulae	88	53	% developing to fetuses after transfer	
	Medium covered with paraffin oil	5% CO_2, 95% air		Modified Ham's F-10		102	43		
Shea et al, 1974	Stoppered test tubes	5% CO_2, 5% O_2, 90% N_2	72-130	SOF with HEPES	8- to 12-cell	49	26	% developing to morulae	2 pregnant after transfer of morulae to 17 recipients
				BMOC-3	8- to 12-cell	54	57		
				SOF with HEPES	1-cell	54	6	% developing to 8-cell	
					2-cell	61	26		
					4-cell	18	78		
Boland et al, 1975	Covered glass dishes	Air	0-3	PBS + 15% FCS	Morulae or blastocysts	12	33	% developing to fetuses	Stored at 30°C, transferred non-surgically
				TCM-199		12	17		

TABLE 8 (cont'd). EXPERIMENTAL TREATMENTS CONCERNING BOVINE EMBRYOS *IN VITRO*; A SUMMARY OF LITERATURE SINCE 1970

Author	Method	Atmosphere	Hours *in vitro*	Medium or treatment	Stage at beginning of study	No. embryos	Response	Response criteria	Comments
Bowen et al, 1975	Medium covered with paraffin oil	5% CO_2, 5% O_2, 90% N_2 or 5% CO_2, 95% air	48	mOs Modified Ham's 270 F-10 270 300 300 SOF 270 270 300 300	2- to 8-cell Morulae 2- to 8-cell Morulae 2- to 8-cell Morulae 2- to 8-cell Morulae	16 9 12 8 31 20 22 15	75 89 33 62 48 80 23 53	% continued cleavage, 2- to 8-cell; % development to blastocyst for morulae	No significant differences between atmospheres or between SOF and Ham's F-10
Kanagawa et al, 1975	Stoppered test tubes	5% CO_2, 5% O_2, 90% N_2 or air	120	SOF BMOC-3 BMOC-3	8- to 32-cell	108	65 to 80	% development to blastocyst	No significant treatment differences
Sreenan et al, 1975	Covered glass dishes	Air	0-2 2-8	TCM-199	8-cell to blastocyst	62 48	73 42	% developing to fetuses after transfer	Hank's 199 with HEPES not different from Earle's 199 without HEPES
Gordon, 1976b	Stoppered test tubes with paraffin oil	Air	Various	TCM-199 +15% FCS PBS +15% FCS	2-cell to morulae 2-cell to morulae	20 45	5 60	% continued development	30°C
Trounson, Willadsen Rowson and Newcomb, 1976	Covered glass dishes	Air	1.5-7.5	Modified Dulbecco's PBS TCM-199	4- to 8-cell 8-cell to morulae 4- to 8-cell 8-cell to morulae	33 24 45 28	85 88 49 71	% normal development in the rabbit oviduct	Maintained at room temperature
Trounson, Willadsen and Rowson, 1976	Test tubes sealed with silicone bungs	Air	24 48 72 96 48	Modified PBS +20% FCS	Morulae	66 65 14 12 26	82 62 78 8 50	% normal development in culture % survival in 8/13 recipients	37°C; retarded embryos developed less well
Wright, Anderson, Cupps and Drost, 1976a	Microdrops under paraffin oil	5% CO_2, 5% O_2, 90% N_2 5% CO_2, 95% air	160	Ham's F-10 Others[1] Ham's F-10 Others[1]	2- to 8-cell	25 123 22 90	2.3 0.5 1.5 0.3	Mean number of cleavage divisions	Media were supplemented with FCS or BSA
Wright, Anderson, Cupps and Drost, 1976b	Microdrops under paraffin oil	5% CO_2, 5% O_2, 90% N_2	165	Ham's F-10 + BSA Ham's F-10 + FCS	1- to 2-cell 8-cell 1- to 2-cell 8-cell	11 18 14 20	0 0 21 55	% expanded blastocysts	
Renard, du Mesnil du Buisson, Wintenberger-Torres and Menezo, 1976	1 ml under paraffin oil in test tubes	5% CO_2, 5% O_2, 90% N_2	48-96	BMOC-3 + BSA Menezo's B2	Morulae day 6	16 20	18 65	% blastocysts hatching	Further development on transfer after 4d in B2

[1]Other media were MEM, TCM-199, BMOC-3, SOF, and Whitten's. There were no significant differences among these media

with statistically. It is not unusual to recover seemingly identical embryos from two donors and find no development *in vitro* (or *in vivo*) among those from one donor and excellent development among those from the other. Of the above complex of factors, the volume of medium and number of embryos per container are unlikely to affect development *in vitro* from all the evidence in mouse embryo culture.

In 1971, Seidel et al compared TCM-199 to modified Ham's F-10 (Seidel et al, 1976), both supplemented with 1.5% bovine serum albumin, for culture of embryos recovered from calves. The modified Ham's F-10 was slightly better than TCM-199, but neither medium supported embryonic development well. In these studies embryos were cultured under oil in an atmosphere of 5% CO_2 in air.

In 1972 Tervit, Whittingham and Rowson cultured bovine embryos in stoppered test tubes flushed with a mixture of 5% CO_2, 5% O_2 and 90% N_2 in the synthetic medium SOF (Synthetic Oviduct Fluid) based on the composition of oviduct fluid from sheep. They obtained considerable development after culturing 10 1-cell embryos for 4 days, and two pregnancies resulted from the transfer of four embryos that had been cultured from the 8-cell stage for 4 days. Although very small numbers of embryos were involved, this work was the most encouraging up to that time.

McKenzie and Kenny (1973) cultured bovine embryos in Brinster's medium under paraffin oil in a 5% CO_2-in-air atmosphere. No improvement over earlier work was noted. Some of their donors were inseminated with killed sperm. Embryos from these donors served as controls to rule out the occurrence of parthenogenetic cleavage or fragmentation resembling normal cleavage. Unfertilized, ovulated bovine oocytes rarely undergo seemingly normal cleavage if they are not subjected to temperature or osmotic shock or otherwise mishandled. However, the author has observed cleavage in unfertilized oocytes recovered from the ovaries of slaughtered heifers and cultured *in vitro*. Nuclei were visible in each cell after fixation and staining.

In 1974, Seidel compared modified Ham's F-10 to TCM-199 containing 35 mM $NaHCO_3$ buffered with 25 mM HEPES and supplemented with 5% heat-treated bovine serum for storing embryos for 1-10 h between collection and transfer. The TCM-199 was in covered petri dishes in air and the Ham's F-10 under paraffin oil in an atmosphere of 5% CO_2 in air. Following transfer, 47/88 embryos (53%) maintained in TCM-199 resulted in pregnancies, compared with 44/102 embryos (43%) in modified Ham's F-10. Although not different statistically ($P>.1$), the TCM-199 was recommended because paraffin oil and a 5% CO_2-in-air atmosphere were unnecessary.

Shea et al (1974 and unpublished) compared Brinster's medium (BMOC-3) and HEPES-buffered SOF with and without agitation during culture in stoppered tubes gassed with a mixture of 5% CO_2, 5% O_2 and 90% N_2. Agitation had no effect. When 8- to 12-cell embryos were cultured for 3 days, 26% (14/54) developed to morulae in BMOC-3, compared with 57% (28/49) in SOF. Two pregnancies resulted after transferring such morulae to 17 recipients. These workers also cultured embryos recovered in early stages of cleavage in SOF. Only 6% of fertilized 1-cell and 26% of 2-cell embryos developed beyond the 8- to 12-cell stage. However, 12/18 4-cell embryos developed to this stage and 2/18 became morulae. This and other published work illustrate that bovine embryos recovered in early stages of cleavage can rarely be cultured beyond the 8- to 12-cell stage. The author suspects that late 8-cell embryos are easier to culture to blastocysts than embryos that have just reached the 8-cell stage.

In 1975, Boland et al transferred embryos nonsurgically after storage in TCM-199 or phosphate-buffered saline (PBS) with 15% fetal calf serum. Four of 12 embryos stored in PBS resulted in pregnancy, compared with 2/12 in TCM-199.

Bowen et al (1975 and unpublished) compared factorial combinations of three media (SOF, modified Ham's F-10, and SOF supplemented with several amino acids), two osmolalities (270 and 300 mOs/kg), and two atmospheres (5% CO_2 in air and a mixture of 5% CO_2, 5% O_2, and 90% N_2). Embryos were recovered 3 or 6 days after estrus. No significant effects were observed due to atmosphere or medium. However, 270 mOs/kg proved superior to 300 mOs/kg for both 3-day and 6-day embryos ($P>.05$). At the lower osmolality 27/47 (57%) of the 2- to 8-cell embryos underwent at least one additional cleavage division, compared with 9/34 (26%) at the higher osmolality. Similarly, with 6-day embryos (morulae), 24/29 (83%) developed to the blastocyst stage at 270 mOs/kg, compared with 13/23 (57%) at 300 mOs/kg. Thirty-eight percent of blastocysts expanded. Thus, as in culturing embryos from several laboratory species, an osmolality slightly lower than blood serum is recommended.

Kanagawa et al (1975) cultured 8- to 32-cell embryos in SOF and BMOC-3 in test tubes gassed with a mixture of 5% CO_2, 5% O_2, and 90% N_2 and in BMOC-3 without gassing. They found no differences in embryonic development among these three treatments. From 65 to 80% of the embryos developed to the blastocyst stage. This occurred within 2-3 days for 32-cell embryos and 4-5 days for those cultured from the 8-cell stage.

Sreenan et al (1975) compared TCM-199 with Earle's salts to HEPES-buffered TCM-199 with Hank's salts for storing embryos between collection and transfer. Thirty-five of 58 (60%) and 30/52 (58%) embryos, respectively, developed into fetuses. They observed a decline in pregnancy rate with storage time. Of embryos transferred within 2 h, 73% developed into fetuses, compared with 42% of those transferred between 2 and 8 h. Rowson et al

(1969) also observed no decline in pregnancy rate for embryos stored up to 2 h in TCM-199.

Six papers of relevance have appeared so far in 1976. Crosby et al (cited by Gordon, 1976b) compared embryo storage at 30°C in either TCM-199 or Dulbecco's phosphate-buffered saline (PBS) supplemented as described by Whittingham (1971). Both media contained 15% fetal calf serum (FCS). Only 5% of the embryos continued development in TCM-199 but 60% did so in PBS.

Similar results were obtained by Trounson, Willadsen, Rowson and Newcomb (1976) who stored embryos in the modified PBS or TCM-199 for 1.5-2 h or 6.5-7.5 h and subsequently transferred them to rabbit oviducts to determine if normal development occurred. There was no effect due to storage time or medium for embryos recovered 5 days after estrus. An average of 79% developed normally after 2 days in the rabbit oviduct. For embryos recovered 3 days after estrus and stored 4 days in the rabbit oviduct, there was no difference between storage times of 1.5-2 and 6.5-7.5 h. However, the modified PBS proved superior to TCM-199 with 28/33 (85%) and 22/45 (49%), respectively, developing normally. Trounson, Willadsen and Rowson (1976) have also shown that days 6-7 morulae, cultured in the same enriched PBS supplemented with 20% FCS, not only develop normally in culture for up to 72 h but survive transfer to recipients. Of 26 embryos that had developed normally during 48 h in culture, 50% survived in 61% (8/13) recipients killed 3-18 weeks after transfer. FCS does not appear to be essential, as substitution of heat-inactivated sheep serum also consistently allows development of cow morulae. This work with phosphate instead of bicarbonate as a hydrogen-ion buffer is most encouraging, and is leading to widespread substitution of modified PBS for TCM-199 as a medium for routine transfers.

Wright, Anderson, Cupps and Drost (1976a) compared six tissue-culture media in drops under paraffin oil in atmospheres of either 5% CO_2 in air or a mixture of 5% CO_2, 5% O_2, and 90% N_2 using 260 embryos recovered in the 2- to 8-cell stage. Ham's F-10 supplemented with 10 or 50% heat-treated fetal calf serum proved superior ($P<.01$) to Minimal Essential Medium, TCM-199, BMOC-3, SOF, and Whitten's medium, among which there was no difference. All media were supplemented with bovine serum albumin (BSA) or FCS. The 5% oxygen atmosphere was superior to the 20% oxygen atmosphere ($P<.01$). However, with the best treatments, only two or three cleavage divisions occurred and with most treatments one cleavage or less.

In a second paper, Wright et al (1976b) compared BSA with FCS for supplementing Ham's F-10. With FCS, development to the expanded blastocyst stage occurred in 3/14 1- to 2-cell embryos and 11/20 8-cell embryos. BSA did not support similar development. Although small numbers of embryos were involved, this is the first report to show that bovine embryos can be cultured from the 1- to 2-cell stage to the expanded blastocyst *in vitro*.

Renard, du Mesnil du Buisson, Wintenberger-Torres and Menezo (1976) compared BMOC-3 with 5g/ℓ BSA and Menezo's medium B2 for culturing day-6 and day-7 embryos for up to 4 days. Medium B-2 proved superior in that 65% of embryos cultured in it for 4 days hatched from the zona pellucida, compared with 18% of those cultured in BMOC-3. Two embryos transferred after 4 days culture in B2 developed until the recipients were slaughtered at day 20 of gestation.

A number of researchers have used the rabbit oviduct for successful storage of embryos. Literature before 1971 has been reviewed by Lawson, Rowson and Adams (1972). These workers obtained pregnancies from 6/12 bovine recipients with embryos that had been in the rabbit oviduct for 4 days. Seidel (1974) also reported a pregnancy resulting from the transfer of an embryo that had been in the rabbit oviduct for 2 days. Although this method of storing embryos is quite successful, it involves several extra steps, which not only take time but sometimes result in the loss of embryos.

Great strides have been made in the last decade, but much remains to be done to develop media and methodology specifically for bovine embryos. For example, virtually nothing is known about the metabolism of bovine embryos or the incorporation of amino acids or nucleic acid precursors into macromolecules, and the effects of antibiotics, pH, osmolality, or various hydrogen-ion buffers on bovine embryos have not been studied adequately. Moreover, no studies have been published specifically comparing room temperature and 37°C for storage of embryos between recovery and transfer, although both holding temperatures have been used (Rowson, Lawson, Moor and Baker, 1972; Shea et al, 1976) as well as intermediate ones of 25-30°C (Sreenan, 1975; Boland et al, 1976a). While storage of embryos for more than 2 hours is detrimental sometimes, as mentioned above, this depends on the medium. The fact that 48 h culture can be followed by embryo survival rates in recipients only 8-15% lower than found after direct transfer (Trounson, Willadsen and Rowson, 1976) indicates that embryos can be held for longer periods, provided the culture system is suitable. In some cases, pregnancy rates may actually increase with increasing culture time. These problems remain to be examined systematically.

To date, *in vitro* fertilization has not been accomplished unequivocally in the bovine. Furthermore, though most media support one or two cleavage divisions of embryos recovered in the 2- to early 8-cell stages and the development of morulae to blastocysts, culture from the early cleavage stages through to the expanded blastocysts stage cannot yet be done reliably. Further, very few of the embryos that have been cultured for more than 24 hours have been transferred to determine if calves result.

Additional basic studies are needed so that improved methodology can be developed for maintaining bovine embryos *in vitro*.

METHODS OF ASSESSING THE VIABILITY OF EMBRYOS

K. J. Betteridge

The only methods that have been used to evaluate the quality of bovine embryos after collection or culture are morphological. Beyond the differentiation of unfertilized from fertilized ova, they therefore depend very much on experience and are subjective. The largest study of how direct assessment of embryos at the time of collection relates to subsequent pregnancy is that of Shea et al (1976). They scored embryos, usually collected at day 5, on a 1-5 scale on the basis of compactness, symmetry and density of blastomeres with a score of 1 representing a very poor-looking and 5 an excellent-looking specimen. Although highly scored embryos as a group generally produced higher success rates than lower-scored groups, this pattern was not invariable and, for any particular time, a more poorly rated group might result in high pregnancy rates. It appeared that scores were good predictors of results when pregnancy rates were higher than average but were less useful when pregnancy rates were low (October to December). The most consistently useful sign of the embryo's chances of establishing a pregnancy was its stage of development; the less advanced the embryo, the less its chances of surviving. Despite these broadly useful indications of whether an embryo is good or bad, the fact that some very poor-looking ones survive will always make it difficult to discard a potentially valuable embryo. This is no doubt part of the reason that pregnancy rates in commercial units tend to be lower than in more selective experimental use of embryo transfer. Comparable scoring techniques have been used by Renard and his colleagues (see following pages).

Stage of development has also been found to be a good indicator of the potential of day-5, -6 and -7 embryos to continue to develop in an *in vitro* culture system. Trounson, Willadsen and Rowson (1976) found that 94/131 (72%) embryos classified as normal at collection, but only 12/44 (27%) embryos classified as retarded (at a cleavage stage 24-48 h less advanced than expected), continued development *in vitro*. Cleavage stage at the beginning of a 48 h culture in the rabbit oviduct, however, did not appear to affect the ability of day-5 embryos to continue development (Trounson, Willadsen, Rowson and Newcomb, 1976). Culture systems such as these have been used to assess the viability of embryos after experimental manipulation, such as cooling and freezing, for obvious reasons of economy and convenience. They need to be validated by transfer work (as by Trounson, Willadsen and Rowson, 1976) to show that an embryo judged viable in this way can in fact produce a calf.

Morphological assessment of the effects of cooling has also been made on fixed and stained representatives of groups of embryos. The number and appearance of nuclei and the state of cell membranes have been used as criteria of normality, and it has been noted that degenerative changes not evident immediately after warming may appear after incubation in the rabbit oviduct (Trounson, Willadsen, Rowson and Newcomb, 1976).

With sheep embryos, a rough indication of viability is given by their ability to retain the vital dye neutral red for 2 h (Kardymowicz, 1972b), but there are no reports, yet, of comparable studies in cattle.

There is a real lack of knowledge of the possible use of biochemical or biophysical parameters that might reflect viability by measuring the metabolism of bovine embryos.

FACTORS INFLUENCING THE QUALITY OF OVA AND EMBRYOS

F. du Mesnil du Buisson, J. P. Renard and M. C. Levasseur

Work done with transfer and culture shows that many intrinsic and extrinsic factors are responsible for egg quality and we have drawn on evidence from a number of species in considering the possible effects of these in cattle.

Age of donor — Culturing eggs, Wright, Anderson, Cupps, Drost and Bradford (1976) have recently given an excellent demonstration of the effect of donor age on egg viability. In the same culture conditions, 39% of 8-cell eggs from adult ewes developed *in vitro* into blastocysts, but only 6% of 8-cell eggs from prepuberal ewes (5-7 months) developed to 64 cells. Intrafollicular oocyte size is perhaps a criterion of quality. In hamsters aged less than 23 days (Iwamatsu and Yanagimachi, 1975) the oocytes in the follicles never reached the size observed in the adult and were incapable of resuming nuclear maturation *in vitro*. Nuclear maturation of the oocyte is closely related to its size, as has been shown in the mouse (Sorensen and Wassarman, 1976). These specific factors related to the oocyte must be considered when attempting to explain the disappointing results obtained with ova collected from the superovulated calf (Onuma and Foote, 1969; Seidel et al, 1971; page 9 of this monograph). However, pregnancy induced in the 4-month-old pig (Ellicott et al, 1973) or the birth of seven lambs in a group of 75 prepuberal ewes (Wright, Anderson, Cupps, Drost and Bradford, 1976) indicates that immaturity in these species is not wholly incompatible with good quality egg production.

Lactation — The eggs of ewes suckling young have lower viability than those of dry ewes. Reciprocal transfer experiments have shown that the resultant infertility is definitely a donor effect (Cognie et al, 1975). We have no data on comparable effects in cattle but egg quality may be a problem in cows with high milk production which often have a particularly long lactational anestrus.

Hormonal environment before ovulation — In the adult cow, hormonal environment before treatment varies, depending on when the follicle stimulation leading to ovulation occurs. For Church and Shea (1976) 65% of fertilized eggs obtained after PMSG injection on day 16 of the cycle develop after transfer, as against only 40% if the injection is given on day 10. The yield of viable eggs that lead to development of embryos after transfer is the same in both cases because superovulation is more abundant on day 10. Progesterone level and the length of time that it is present during follicular stimulation are therefore probably important factors in determining egg quality. Enucleation of the cow's CL on day 10, followed by daily progesterone injection and a PMSG injection at day 16, contributed to improving the number of eggs developing in culture after recovery at day 2 or 3, compared with eggs from control cows in which the CL was not enucleated (McKenzie and Kenney, 1973).

Ovarian response and uterine environment — It is not necessarily true that egg quality after superovulation is inferior to quality of eggs ovulated spontaneously. However, several authors (Gordon, 1975; Hafez et al, 1963) observed fewer good quality eggs when there were more CL (aside from the lower number recovered). Church and Shea (1976) found 8-10 eggs per ovary optimum for egg quality. However, the pregnancy rate obtained from transferred eggs is the same (49-51%) whether eggs originate from donors producing 1-10, 10-20 or more than 21 ovulations (Table 3). The proportion of good quality eggs recovered between days 5 and 9 does not depend on the ovulation rate when it increases from 1 to 25 (examination of 600 cultivated or transferred eggs, Renard et al, unpublished). The number of good quality eggs varies greatly with the individual no matter how many eggs are ovulated, and a very high ovulation rate does not necessarily prevent most eggs from being of very high quality. Thus 30 calves were produced twice by the same cow in 1974 (Church and Shea, 1976).

Raising the PMSG dose from 1500 to 2500 IU was found to reduce egg quality independently of the number of CL produced (Sreenan and Beehan, 1976b). This perhaps occurred because of the large number of unovulated follicles that resulted and which would have caused high estrogen level during follicular stimulation and immediately after ovulation (Booth et al, 1975). However, in a study done on 70 Charolais heifers, we found no correlation between the number of good quality eggs, estimated after observation and culture, and the number of unovulated follicles.

Several experiments clearly show that the uterine environment of superovulated cows is harmful to egg development. First, recovery of eggs on days 3-4 after superovulation and storing them for 2-3 days in the tube of a rabbit improved transfer results on days 5-6, as compared with direct transfer. Egg survival rate increased after surgical transfer from 26 to 52% and after non-surgical transfer from 11 to 41% (Boland et al, 1976a). Second, when the uterotubal junction was ligated on day 3, normally developed eggs (82%) could be recovered in the tube on days 7-8. On the other hand, flushing tubal eggs towards the uterus on day 3 caused 69% of the eggs to degenerate (Newcomb, Rowson and Trounson, 1976). Third, these authors also found that the incidence of degenerate eggs almost doubled between days 7 and 8. Finally, using *in vitro* culture as a test of quality, the percentage of normal eggs recovered fell from 74% on day 5 to 42% on day 9, the drop in survival rate being particularly sharp between days 6 and 7 (Table 9).

In cattle, we do not know by what mechanism hormonal balance upset by ovarian stimulation, as described on pages 5-6, alters the uterine environment of the egg. However, it is noteworthy that estrogen levels are known to be high after ovulation in a large number of sterile cows (Domeki et al, 1975). Moreover, the high progesterone levels caused by superovulation in ewes dramatically accelerate blastocyst growth between days 8 and 11 of pregnancy (Wintenberger-Torres, 1967). We do not know if the same phenomenon occurs in cattle.

Indications that embryos might be adversely affected by early *post mortem* changes in the uterine environment before collection from slaughtered donors (Rowson et al, 1969; Tervit and Rowson, 1972) were not confirmed by Betteridge and Mitchell (1974). Using vital stain retention as a criterion of viability, Kardymowicz (1972b) found a rapid decline in the quality of sheep embryos after slaughter of the donors. Uteri removed within 8 min of slaughter yielded 75% living eggs, compared with 18% from uteri removed after 18 min.

[Some evidence that uterine environment in superovulated donors might not be quite as harmful to embryos, at least after day 7, as is suggested here is referred to on page 9. The evidence involving pre-transfer culture cited above could be interpreted

TABLE 9. QUALITY OF EGGS RECOVERED BETWEEN 5 AND 9 DAYS AFTER ESTRUS IN CATTLE

Recovery day	5	6	7	8	9
No. eggs examined	19	119	193	343	151
% eggs judged normal	73.6	67.2	46.6	41.6	41.7

From Renard et al (unpublished).

as being the result of some selection being effected in the rabbit oviduct (Boland et al, 1976a). The evidence that the uterus is hostile to embryos put there prematurely need not apply to embryos arriving there at the proper time. — *Editor.*]

Seasonal effects — The possible seasonal variations in responsiveness to PMSG are described on page 7. It may also be that egg quality is related to season. Many cattle transfer centers in Canada stop work during a part of the winter and pregnancy rates are reduced during the months of October-December (Shea et al, 1976). However, many other factors may contribute to this, as these authors point out. Seasonal variation in the frequency of abnormalities such as mongolism, Turner's syndrome and Klinefelter's syndrome are observed in women (Jongbloet, 1970).

Individual donor variation — Culture work has shown that there is a large variation in the viability of eggs of the same stage, put in the same conditions but coming from different cows (Trounson, Willadsen and Rowson, 1976; Seidel page 22 of this monograph). The general experience that pregnancy rates produced by transferred embryos also vary from donor to donor has been mentioned elsewhere (page 9). There is some direct evidence that the individual donor's effects on development *in vitro* and after transfer are related, for eight normal-looking embryos transferred and six cultured, all from the same two donors, failed to develop after a 0°C storage experiment (Trounson, Willadsen and Rowson, 1976).

Conclusions — We believe that the quality of eggs is a major problem in embryo transfer in cattle. The factors involved in the heterogeneous response at superovulation can only be solved by fundamental study of folliculogenesis in cows. Recent progress obtained in the culture of oocytes in their follicles (see page 10) constitutes a method of determining the metabolic and endocrine environment necessary to normal development of the bovine oocyte.

SEXING OF EMBRYOS

D. Mitchell

The ability to predetermine and preselect the sex of newborn farm animals would be of enormous practical importance (Rowson, 1971; Anon, 1973b). Since to date all efforts at separation of X- from Y-chromosome-bearing spermatozoa have resulted in no significant alteration in the sex ratio of live young (Beatty 1972; Anon, 1970, 1973a, 1975; Rhode et al, 1975), it is reasonable to predict that in the immediate future, sexing of early embryos is likely to prove a more practical method of manipulating sex ratios (Edwards and Gardner, 1968).

Although numerous studies involving micromanipulation and microsurgery have been conducted on laboratory animal embryos, particularly the mouse and rabbit, (Lin, 1971; Anon, 1973b; Gardner, 1975) it was not until 1968 that the first report of sexing and subsequent successful transfer of the embryo was published (Gardner and Edwards, 1968). This study was carried out using rabbit blastocysts 5-6 days old. Shortly before this it had been noted (Rowson and Moor, 1966b) that the removal of a portion of trophoblast from day-13 sheep embryos did not prevent their development, to full term, after transfer. This observation led to the development of successful sexing and transfer of 2-week-old bovine embryos using excised trophoblast cells for sex determination (as described below). There are two methodological approaches to sexing mammalian blastocysts.

By identification of sex chromatin — Sex chromatin makes its appearance in embryonic development as a feature of differentiation about the early morula stage. It is related to a change in the state of condensation of one of the X chromosomes and is only observed in cells containing more than one X chromosome. Its presence in embryos shows species, tissue and chronological differences and has been studied in the fowl, rat, rabbit, cat, dog, mink, rhesus monkey and man (Austin, 1966). Preliminary studies have been carried out in cattle and sheep (Bruere, 1968; Møller and Neimann-Sørenson, 1975); however, the distribution of the chromatin may make sex differentiation unreliable, at least in cattle. Using microsurgical techniques (Lin, 1971; Gardner, 1974), it has been found possible to remove 200-300 trophoblast cells and to fix, stain and examine these for the presence of sex chromatin before transfer in the rabbit (Gardner and Edwards, 1968). Although it was possible to sex 86% of the blastocysts examined, only 20% of those transferred survived to term. In other species in which sex chromatin is not reliably demonstrable in the early embryo, other means of sex determination are required.

By sex chromosome analysis — In the only study reported on successful sexing and transfer of farm animal embryos, a small portion of trophoblast was removed from 2-week-old bovine embryos (Hare et al, 1976; Mitchell et al, 1976). The cells were processed in a manner similar to that described in a study on the karyotype of 10-day-old pig embryos (McFeely, 1966). It was found possible to sex 68% of the biopsied bovine embryos with a 45% conception rate in recipients of sexed, biopsied embryos.

Chromosomal analysis is much the more accurate of the above two methods and allows not only determination of sex and sex chromosome aberrations, but also other chromosomal abnormalities when the complete karyotype is studied. The detection of a sex chromatin body within a cell nucleus

Figure 8. Eugena Carol, the first calf to be born after being sexed as an embryo, arrived at the ADRI with some panache – on Christmas morning 1975. Nine months previously she had looked like the 4 mm long embryo shown before and after biopsy on the right.

Photos from Agriculture Canada.

only indicates that two X chromosomes are present and provides no indication of chromosomal abnormalities. Differentiation of sex by chromosomal analysis is fairly simple in cattle, sheep, goats and pigs for experienced cytogeneticists. The use of differential staining techniques may improve the speed and accuracy of sex differentiation, but it is obvious that a great deal of further research is required to define the optimum stage of embryonic development at which sex determination should be performed. Methods of micromanipulation, microsurgery, processing and *in vitro* storage also need to be perfected for routine practical application with acceptable results.

EMBRYO TRANSFER BY SURGICAL METHODS

R. P. Elsden

Most workers have used a midline approach to the recipient's uterus, just as described for embryo collection (pages 10-13). Having brought the uterine horn up to the incision and after confirming the presence of a CL on the ipsilateral ovary, a probe (e.g., a blunted 16-gauge needle) is pushed through the myometrium into the lumen of the horn and then withdrawn. A Pasteur pipette containing the embryo in a minimal volume of medium is introduced through the passageway so made and the embryo is expelled by means of a syringe attached to the pipette. If the transfer is of a single embryo, it should be inserted into the horn on the same side as the CL, where the survival rate may be up to three times better than in the contralateral horn (see page 64). It is probably wise to place young uterine embryos near the tip of the horn rather than at its base (Boland et al, 1976a), though the evidence on whether or not this is critical is controversial, for Sreenan (1976b, c) and Sreenan and Beehan (1976c) obtained similar results whichever site was used (see Sreenan's review, pages 62-66 of this monograph).

A flank approach for embryo transfer is also in use but, in the author's opinion, the advantages of this method are dubious. Sometimes the operation is slightly faster, as only a local anesthetic is required. Facilities required are not as extensive as those for general anesthesia involving relatively large numbers of cattle. However, there is some evidence that resulting pregnancy rates are lower. During this procedure the recipient is restrained in a chute allowing access to either flank. The side of the current CL is determined by rectal palpation and a 30 cm square area in that flank is surgically prepared. Following a paravertebral block or a line infiltration with 2% procaine, a 15 cm skin incision

is made and muscle layers are separated in the usual manner.

The horn of the uterus is then pulled over into the incision by gentle traction and the embryo is introduced, either as described above or via a 0.5 ml inseminating straw attached to a flexible polyethylene tube with a 2 ml syringe fitted to one end. The incision is closed in only two layers: peritoneum and muscle together, with No. 3 or 4 chromic gut; the skin with a material such as Vetafil or Polydek using simple interrupted sutures. The disadvantages of the flank method include: increased risk of infecting the incision line from loss of asepsis due to movements of the cow during surgery; the necessity, in some cases, for extensive traction on the uterus resulting in trauma to the tissues, which are not relaxed as they are under general anesthesia; and the optimum site for depositing the egg is not always readily accessible. However, several commercial units have obtained satisfactory results by this approach (e.g., Hansen, 1976, in Table 11) which is probably more practicable in dairy than in range cattle.

Surgical factors that might affect success rates have been investigated by Nelson et al (1975). Any effect of recipients' pre-operative excitement on success rates was marginal: in 80 calm, 111 average and 74 excited recipients, 56.2, 64.0 and 52.7% of embryos developed into fetuses. The ease with which the reproductive tract could be exposed for transfer also had little bearing on results: for 241 easy, 90 moderate and 18 difficult exposures, success rates were 54.5, 56.7 and 61.1%, respectively, although no embryos developed in six obviously traumatized uteri.

OPTIMUM TIMING OF SURGICAL TRANSFER

Table 10 shows data indicating that surgical transfers within the first week after breeding give best results on days 5 and 6. With later transfers (days 10-16) there is no appreciable fall in pregnancy rates (13/17, 76.5% for twins; 36/75, 48.0% for singles, Betteridge et al, 1976) compared with day 4-7 transfers under the same conditions (7/9, 77.8% for twins; 36/68, 52.9% for singles, ADRI, unpublished, in Table 11). Transfers around days 8 and 9 are generally avoided because recently hatched blastocysts are small and more difficult to recognize without their distinctive zonae pellucidae.

INFLUENCE OF SYNCHRONY AND METHODS OF SYNCHRONIZATION ON RESULTS

R. Newcomb

In the farm species, it is necessary to ensure that the signs of estrus in donors and recipients occur either synchronously or within a limited time interval of one another if embryo transfer is to be successful. In cattle this can be achieved in two ways: either by having a large enough herd of recipients so that there are sufficient numbers in estrus naturally on any given day, or by using synchronizing agents to induce estrus in groups of animals as they are needed. Both methods, but especially the first, require close attention to herd management, estrus detection and accurate recording systems.

In cattle, Rowson et al (1969) showed that a degree of variation from exact synchronization to $\pm$ 2 days can be tolerated. However, Rowson, Lawson, Moor and Baker (1972) showed that although a high conception rate could be achieved with exact synchrony (91.1%), the conception rate was lower when the onset of estrus in the recipient differed from that of the donor by $+1$ or -1 day (56.5% and 52.2% respectively). Newcomb and Rowson (1975a) also achieved a higher conception rate with synchronized transfers than with transfers out of phase by $\pm$ 1 day. Sreenan and Beehan (1974) and Sreenan et al (1975) confirmed that the pregnancy rate of recipients out of synchrony by 1 day was lower, although not significantly so. Using embryos that had been cultured for 2 days, Trounson, Willadsen and Rowson (1976) found that they, too, produced more pregnancies in recipients that were synchronous or in estrus after the donor (7/8 became pregnant) than when recipients were in estrus before the donor (1/5 pregnant).

[Under large-scale commercial conditions, however, any such differences are much less pronounced. Thus, the data of Shea et al (1976) showed the transfer of 2016 morulae, rated as average or better, resulted in pregnancy in 62% of 1126 recipients in estrus on the same day as the donors, 60% of 334 recipients in estrus the day before the donors and 49% of 556 recipients in estrus the day after the donors. Other commercial users claim that recipients in estrus 1 day later than the donor give just as good results as synchronous ones. This, they claim, has the important practical advantage of allowing an unused '+1' asynchronous animal to be used the next

TABLE 10. PREGNANCY RATES FOLLOWING TRANSFERS OF BOVINE EMBRYOS OF VARIOUS AGES TO RECIPIENTS SYNCHRONIZED TO WITHIN 1 DAY OF THE DONOR

Day[1] of collection and transfer	3	4	5	6	7	Authors
No. recipients	67	51	40	23	10	Newcomb and Rowson, 1975a
% recipients pregnant	10.4	54.9	72.5	87.0	70.0	
No. recipients	54	194	141	43		Nelson et al, 1975
% recipients pregnant	29.6	53.6	63.1	62.8		

[1]Day 0 = donor's first day of estrus.

day as a synchronous '0' recipient for that day's donor. Shea et al point out that the apparent smoothing out of differences in pregnancy rates is as a result of a lower success rate in the exactly synchronous group. — *Editor.*]

There is little adequately controlled data to indicate whether the method used to synchronize donor and recipient is important. In earlier studies, both in cattle and sheep (Rowson et al, 1969; Rowson, Lawson, Moor and Baker, 1972; Moore and Shelton, 1964b; Rowson and Moor, 1966a) synchronization was achieved by simply selecting animals in which natural estrus in donor and recipient had been observed to be synchronous. There is no indication that the eggs recovered from donor cattle in which estrus is induced with prostaglandin are any less fertile than those recovered from donors after natural estrus (Rowson, Tervit and Brand, 1972; Sreenan and Beehan, 1974, 1976b; Sreenan et al, 1975; Betteridge, Sugden and Eaglesome, 1977). In most reports, transfers to recipients in which estrus had been induced with short-term (10-day) intravaginal progesterone or intramuscular prostaglandin treatment, were no less successful than in recipients after natural estrus (Rowson, Tervit and Brand, 1972; Sreenan et al, 1975; Sreenan and Beehan, 1976b; Betteridge et al, 1976), but Church and Shea (1976) obtained higher pregnancy rates in naturally cycling recipients than in two groups synchronized with PG. It would seem most unlikely that any method of estrus synchronization that results in normal fertility in inseminated cattle will in any way alter the synchronization requirements for egg transfer.

In sheep, the degree of asynchrony tolerated in advance of or after exact synchrony is not evenly distributed and varies with the age of egg transferred (Moor, 1965), but analogous data in cattle are not yet available. If it is the uterus that governs how much asynchrony is tolerable, one might expect young uterine eggs to fare better in 'older' uteri (more advanced in the estrous cycle) than in 'younger' (less advanced) uteri because the latter might expel real eggs as easily as they eject imitation ones (Tervit, 1973). At the other extreme it would be expected that embryos transferred near to the time of luteal regression (Moor and Rowson, 1966a; Betteridge et al, 1976) would survive better in a younger uterus than an older one. Data to test this hypothesis should be sought in cattle but Moor's (1965) comparison of transfer of day-5 and day-9 sheep embryos revealed an opposite trend that would argue against it.

It is also noteworthy that eggs may be removed from the bovine uterus and transferred to the oviduct of rabbits in various physiological states, with a perfectly normal conception rate on retransfer to recipient bovine uteri (Lawson, Rowson and Adams, 1972; Tervit, 1973). Eggs retained within the bovine oviduct after superovulation and after the time that they would normally be expected to have entered the uterus also develop quite normally, judged by their morphological appearance, at least up to day 7 or 8 (Newcomb, Rowson, Trounson, 1976). It appears from studies in the rabbit (Adams, 1971) that though the advanced progestational uterus is hostile to the cleaving morula, the adjacent oviduct allows it to develop further into an early blastocyst.

Progesterone secretion increases with time after estrus and, doubtlessly, plays an important role in the synchronization story; e.g., the injection of progesterone can completely alter the synchronization requirements between donor and recipients in sheep, as described on page 73 of this monograph. There is a need for similar studies in cattle and more work on the mechanisms whereby progesterone exerts its influence.

SUMMARY OF FACTORS AFFECTING SUCCESS RATES IN SURGICAL EMBRYO TRANSFER

K. J. Betteridge

The foregoing sections have reviewed how each of a large number of factors (quality of the egg and of the embryo, conditions under which it is held pending transfer, the timing of the transfer, the transfer procedure itself and the suitability of the recipient) can affect the outcome of embryo transfer. The beneficial effects of twinning on pregnancy rates (but not necessarily on embryo survival rates) are discussed on pages 63-70. Since all of these factors interact, each and every adverse influence along the chain of events reduces the chances of obtaining a calf. Another effect of this interaction is to make it extremely difficult to change just one factor in experimental comparisons of methods and equally difficult to compare and analyze data gathered in slightly different ways. This should be borne in mind in reviewing the results obtained since 1969, summarized in Table 11. Less fully documented results from commercial units are given on pages 60-61.

It is remarkable how few studies base their success rates on calving (Table 11). In experimental herds, the costs of keeping pregnant recipients to term is often prohibitive, but calving figures should be available to commercial units and it is obviously important to know whether significant losses are incurred later in pregnancies established by embryo transfer. Sreenan and Beehan (1976a) have presented good evidence that fetal losses following surgical transfer of twins occur no more frequently than normally. However, Hahn and Hahn (1976) noted a high rate of abortion after non-surgical transfers and G. B. Anderson et al (1976) have noted abortions of twins up to 7 months after surgical transfer. We have experienced considerable loss after day 45 (Table 12), but these data are difficult to interpret in the absence of any control (natural) pregnancies.

TABLE 11. RECENT RESULTS OF SURGICAL EMBRYO TRANSFERS IN CATTLE IN WHICH DONORS AND RECIPIENTS WERE SYNCHRONOUS ± 1 DAY

Day of transfer (donor)	Embryos per recipient	No. recipients	Pregnancies		Transferred embryos surviving		Criteria of pregnancy and comments	Reference
			No.	%	No.	%		
4-5	2	11	10	90.9	10	45.5	Calving	Rowson et al, 1969
4-7	2	32	22	68.8	36	56.2	Calving (8 cases) Slaughter d 60-90 (13 cases) Abortion (1 case)	Rowson et al, 1971
3-7	1-2	69	46	66.7	—	—	Calving and slaughter	Rowson, Lawson, Moor and Baker, 1972
4-8	1-3	31	27	87.1	33/43	76.7	In 23 slaughtered d 40	Sreenan and Beehan, 1974
5-10	1-3	12	9	75.0	14/23	60.9	Calving; from last 14 of a group of 24	Betteridge and Mitchell, 1974
3-7	2	72	55	76.4	65/110	59.1	In 55 slaughtered d 27-127; calving data on 17 not given	Sreenan et al, 1975
4-7	1-2	378	220	58.2	203/362 21/55	56.1 38.2	Singles } palpation; Twins } see also Table 10	Nelson et al, 1975
4-7	1-2	124	84	67.7	—	—	Palpation d 35-42; see also Table 10	Newcomb and Rowson, 1975a
3-8	2	135	97	72.0	—	—		Sreenan and Beehan, unpublished
5-6 direct	1 added	19	11	57.9	5	26.3	Slaughter d 30-40; all transfers to bred recipients	Boland et al, 1976a
5-6 after rabbit storage	1 added	21	14	66.6	11	52.4		
3-7	2	—	52	—	86	83.0	Slaughter d 27-117 (39 cases), calving (13 cases); unsuccessful transfers not enumerated	Sreenan and Beehan, 1976a
10-16	1	75	36	48.0	36	48.0	Palpation d 50	Betteridge et al, 1976
10-16	2	17	13	76.5	—	—		
4-7	1	68	36	52.9	36	52.9	Palpation d 50	ADRI, unpublished
4-7	2-3	9	7	77.8	—	—		
5 (usually)	1	2016	1162	57.6	1162	57.6	Palpation; selected morulae	Shea et al, 1976
5	1	239	131	54.7	131	54.7	Palpation; flank approach, paravertebral anesthesia	Hansen, 1976
5	2	48	36	75.0	55	58.3	Palpation d 45-60	G. B. Anderson et al, 1976

Several of the losses followed long-distance transportation of recipients soon after pregnancy was confirmed, and another possible contributory factor was frequent rectal palpation for experimental reasons. Losses following the late transfers may reflect the embryos' difficulties in fully overcoming the luteolytic function of the uterus. More knowledge of the reasons for these losses would be relevant to understanding the causes of fetal wastage early in normal pregnancy, which can also be considerable (Pope and Hodgson-Jones, 1975).

TABLE 12. PROPORTIONS OF PREGNANCIES LOST AFTER 45 DAYS FOLLOWING SURGICAL EMBRYO TRANSFER IN AN EXPERIMENTAL HERD OF CATTLE

Embryos transferred	Stage of transfer	
	Days 4-7	Days 10-16
Twins	2/7	4/9
Singles	7/33	3/13
Total	9/40	7/21

From ADRI (unpublished).

Pregnant recipients need be managed no differently to normally pregnant cattle unless they carry either twins and need to be fed accordingly (see pages 62-66), or calves of much larger breeds where Caesarian deliveries may be required (Hansen, 1976).

EMBRYO TRANSFER BY NON-SURGICAL METHODS

A. Brand and M. Drost

Early attempts at non-surgical transfer via the cervix failed to meet with success (Umbaugh, 1949; Rowson, 1951; Lamming and Rowson, 1952; Dracy, 1953; Avery et al, 1962). The first successful cervical transfer was reported by Mutter, Graden and Olds (1964). Workers in Cambridge, England, believed uterine infection (Lamming and Rowson, 1953; Rowson, Lamming and Fry, 1953a, b) and ejection of the embryos (Bennett and Rowson, 1961; Harper, Bennett and Rowson, 1961; Rowson, Bennett and Harper, 1964) to be major contributing factors to the lack of success after transfer via the cervix. It was further believed that inflation of the uterus with carbon dioxide gas just after deposition of the embryo reduced the loss of eggs from the uterus due to its relaxing effect or some unknown mechanism. Conception rates with this technique have been low (Sugie, 1965; Rowson and Moor, 1966c; Rowson et al, 1969; Vincent et al, 1969; Lawson, Rowson, Moor and Tervit, 1975). The last workers tentatively concluded that non-surgical transfer of fertilized eggs to heifers may best be done during midcycle, after day 6. Brand, Taverne, van der Weyden, Aarts, Dieleman, Fontijne, Drost and de Bois (1976) in a study of the electrical activity of the myometrium of the cow, demonstrated that myometrial activity started on day −3, culminated on days 0 (estrus) 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. Only non-specific electrical activity of the myometrium was noted during manipulation, both before and after day 5, which ceased immediately on removal of the instruments. They postulated that 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. Brand, Gunnink, Drost, Aarts and de Bois (1976) further demonstrated a significant reduction in the number of bacterial isolates obtained from the vulva to the external os of the cervix by using three concentric cannulae. They concluded that contamination of the uterus by introduction of pipettes through the cervix is not a major contributing factor to the low pregnancy rates after non-surgical transfer in the cow if adequate precautions have been taken. This conclusion is supported by the finding that the fertility of bred heifers was not adversely affected by sham transfer to the contralateral horn on days 6-16. This is in agreement with the work of Seidel et al (1975) who carried out sham transfers to the ipsilateral horn of bred heifers on day 6.

The technique developed by the authors in Utrecht, and since used in Colorado by J. M. Bowen and Seidel and by Elsden (personal communications), for introducing the embryos involves the use of three concentric stainless steel tubes and an inner No. 5 or 6 French gauge 70 cm Rusch ureteral catheter. The outer metal tube acts as a vaginal speculum and is 30 cm long, outside diameter (OD) 8 mm, inside diameter (ID) 6 mm; the second metal tube is 35 cm long, OD 4 mm, ID 3 mm; the third tube is 51 cm long, OD 2.5 mm, ID 2 mm. The distal end of the second tube is covered by non-absorbent paper held in place by sensitivity tape. Equipment is autoclaved except for the Rusch catheter which is in the manufacturer's sterile pack. The recipient is given an epidural anesthetic and the vulva and surrounding area are thoroughly cleansed and disinfected. With the left arm in the emptied rectum of the heifer, the largest tube is placed in the vagina by the right hand and held against the external os of the cervix. The second tube is then inserted into the first tube and gently manipulated approximately 1-2 cm into the cervical lumen until resistance is felt. The third tube, which is inside the second tube, is then carefully extended, breaking through the paper covering and manipulated through the anterior portion of the cervix and body of the uterus until it reaches approximately 2-3 cm into the horn ipsilateral to the CL. An assistant then breaks the end of the sterile pack containing the Rusch catheter, exposing only the tip, which is introduced into the inner metal tube and carefully pushed through. As the Rusch catheter emerges from the end of the metal tube, the uterine horn is extended by rectal manipulation so that the assistant is able to continue pushing the flexible catheter freely toward the uterotubal junction. A modified, fire-polished Pasteur pipette with an ID of approximately 0.25 mm at the tip is connected to a 2 ml syringe and used to pick up the ovum. First 0.5 ml of air is drawn up, then 0.2 ml of medium followed by the ovum in a further 0.8 ml of medium. The tip of the pipette is then placed into the exposed end of the Rusch catheter and the ovum is slowly expelled into the horn of the uterus. After removal from the cow the catheter is flushed with medium into a watch glass, which is checked to see if the ovum remained within the lumen of the tube. Bowen and Seidel found the egg intended for transfer still within the catheter in two out of 44 cases and, on two other occasions, the recipient's own unfertilized ovum was picked up in the transfer tube on its withdrawal.

Results obtained with this technique are shown in Table 13. The apparent superiority of results obtained after day 6.5 by Bowen and Seidel was not confirmed by Elsden. Bowen and Seidel found that a pre-operative vaginal douche with penicillin and

TABLE 13. RESULTS OF NON-SURGICAL EMBRYO TRANSFER IN CATTLE

Technique	Day of transfer	Embryos per recipient	No. recip-ients	Pregnancies		Transferred embryos surviving		Criteria of pregnancy	Comments	References
				No.	%	No.	%			
Simple pipette, rigid or flexible	2-6	2-3	8	3	38			Calvings? and 1 abortion	CO_2 insufflation	Rowson and Moor (1966c)
	3-5	1-3	20	4	25	4	11	Calvings	CO_2 insufflation	Rowson et al, 1969
	6-9	1	20	7	35	7	35	Slaughter after 3 months	Control	Lawson, Rowson, Moor and Tervit, 1975
	3-5	1	10	1	10	1	10		Control	
	6-9	1	10	1	10	1	10		CO_2 inflation	
	3-5	1	10	0	0	0	0		CO_2 inflation	
	6-9	1	20	8	40	8	40		Fluothane anesthesia	
	3-5	1	10	1	10	1	10		Fluothane anesthesia	
Bypassing cervix	4-6		2	2	100				CO_2 insufflation	Sugie, 1965
	3-7	1-3 or 1 added	56[1]	17	30	19	?	12 calvings and 5 abortions	Collection also non-surgical; CO_2 insufflation; 2 bred recipients	Sugie et al, 1972
	4-7	1-4	14	6	43	9	27	1 slaughter d 45; 5 calvings		Testart, 1975
	5-10	1 added	17	(11)	(65)	3	18	Calvings and 1 abortion	Bred recipients	Testart, Godard-Siour and du Mesnil du Buisson, 1975
	6-8	1 added	15	(10)	(67)	7	47	Calvings and 1 abortion	Bred recipients	Testart, Godard-Siour and du Mesnil du Buisson, 1976
Concentric cannula(e) and pipette	6-7	1	15	6	40	6	40			Brand, unpublished
	5-6	1	13	1	8	1	8			Bowen and Seidel, unpublished
	6.5-8	1	27	8	29	8	29			
	5-6	1	20	6	30	6	30			Elsden, unpublished
	7-8	1	20	3	15	3	15			
	4-7	1	42	10	24	10	24			Hahn et al, 1975
	4	1	13	1	8	1	8			J. Hahn, unpublished
	5-6	1	47	20	43	20	43			
Cassou gun	6	2	8	4	50	4	25	Slaughter d 40-42		Sreenan, 1975
	6-8	1 added	24	(14)	(58)	6	25	Slaughter d 40-56	Bred recipients	Sreenan et al, unpublished
	5-7	1 added	24	(15)	(63)	6	25	Slaughter d 30	Bred recipients	Boland et al, 1975
	5-6	1 added	26	(14)	(54)	3	12	Slaughter d 30-40	Bred recipients, direct transfer	Boland et al, 1976a
	5-6	1 added	22	(15)	(68)	9	41		Bred recipients, after culture in rabbit	
	9 & 10	?	9	4	44	?	?	Palpation d 42	Direct transfer	Renard and du Mesnil du Buisson, 1976
	9 & 10	?	11	3	27	?	?		After 16 h culture	
	7-10	2	20	12	60	18	45	Palpation		du Mesnil du Buisson et al, unpublished

[1]Recipients in estrus from 1 day before to 2 days after donor.

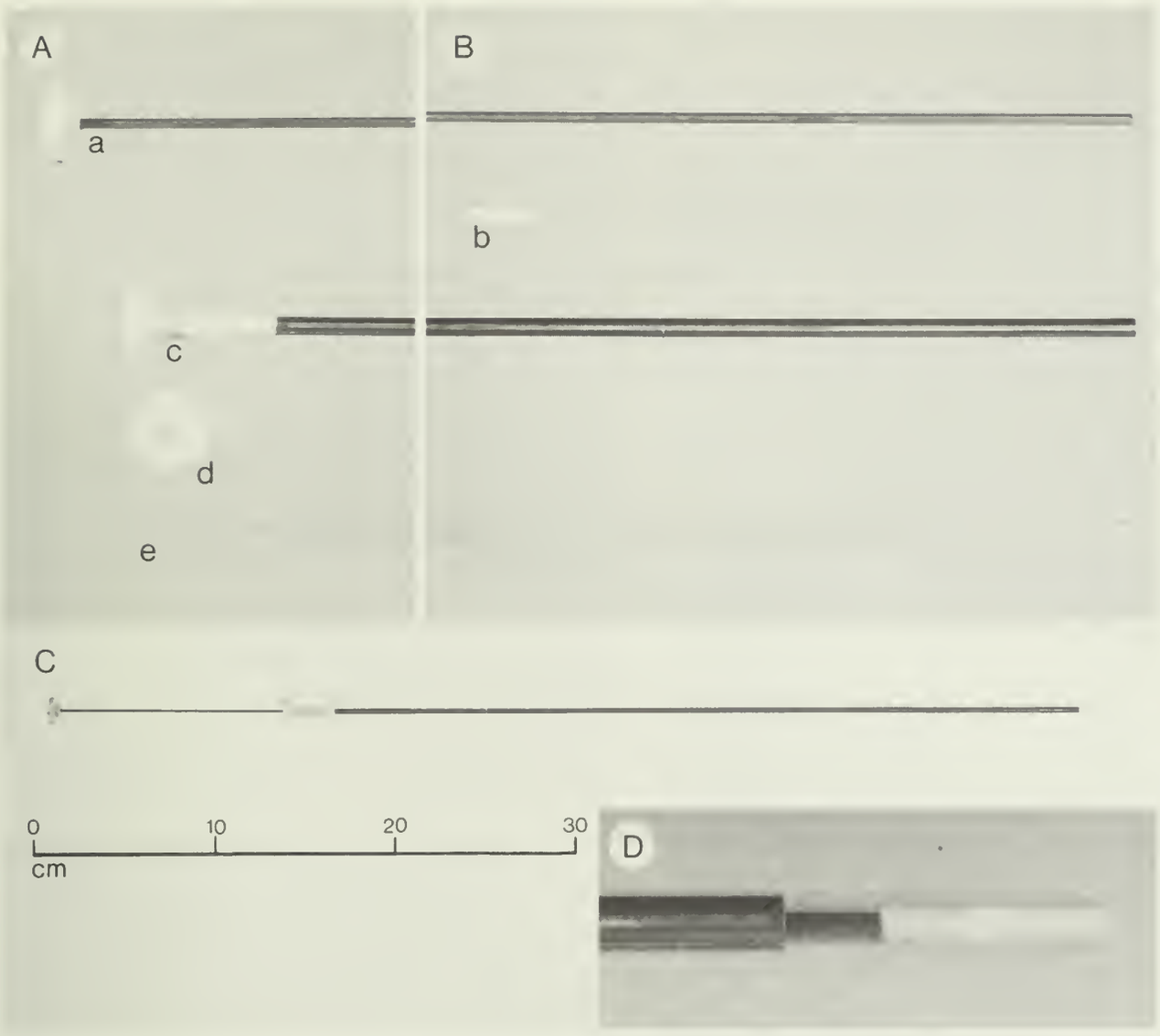


Figure 9. A Cassou AI gun used for non-surgical embryo transfer in cattle.

A and B. The proximal and distal ends, respectively, of its component parts.
These are: (a) a stainless steel plunger with a plastic hub; (b) a 0.5 ml plastic straw with a white plug of polyvinyl alcohol between two lengths of wick; (c) a stainless steel barrel with a plastic hub; (d) a plastic locking ring and (e) a clear plastic pipette with a tapered tip.
The straw, after being filled with medium containing the embryo, fits into the distal end of the barrel. The clear plastic pipette holds it there by fitting over the barrel and being locked to the hub of the barrel by the ring. The plunger expels the contents of the straw by pushing the plug through to its distal end.

C. The assembled gun with the plunger inserted as far as the proximal end of the straw, ready to expel its contents.

D. The distal end of the gun after discharge. The plug can be seen pushed to the end of the straw that protrudes from the barrel inside the plastic pipette. In practice, the straw and pipette are usually shortened to achieve better rigidity and would extend only just beyond the metal barrel.

Photos from ADRI.

streptomycin and the use of new, rather than used, ureteral catheters seemed to improve results in their series.

J. Hahn (personal communication) uses a simplified version of the above method, with one metal catheter to pass through the cervix and a flexible one within it, which is passed further into the uterine horn. As shown in Table 13, he has obtained much better results after day 5 than before. Pregnancy rates have been similar in heifers (5/11) and cows (15/36) after day 5.

The transvaginal method of Testart and his colleagues initially produced pregnancy rates of 18-27%, but was tested in part under adverse field conditions. More recently, this method has yielded embryo survival rates of 47% when used on cows bred 5-10 days previously, establishing a twinning rate of 60% (Table 13; see also page 63).

Good results are also being achieved by non-surgical means using 0.25 ml or 0.5 ml straws in the Cassou gun developed for AI (Fig. 9). This approach was first described by Sreenan (1975) and has since been used by Boland et al (1975, 1976a, b), Renard and du Mesnil du Buisson (1976) and Rowson et al (personal communication). The straw is first shortened by approximately 1.5 cm and used to aspirate a small volume of medium, an air bubble, the embryo in a minimal volume of medium, another air bubble and, finally, another small volume of medium before being loaded into the gun. Introduction through the cervix is just as for AI except that Rowson covers the pipette with a sterile cellophane tube which is not punctured until the cervix is reached. Boland et al insert the instrument as far as possible into the horn, which is never further than the mid-horn position. Sreenan et al, on the other hand, are of the opinion that it is better to minimize interference and trauma and they make no attempt to place the egg too far forward. There is general agreement that transfers should be performed after day 7 (Rowson reports a best pregnancy rate of 59% on day 9 for an unspecified number of transfers) and that cervical plug material creates difficulties. Boland et al (1976a) found that heifers in which entry through the cervix was easy had a higher pregnancy rate (72% vs. 48%) and embryo survival (32% vs. 17%) than when entry was difficult. This, like most data on the use of the Cassou gun, relates to heifers that were themselves mated before having an extra embryo added (see also pages 63-65), but pregnancy rates of 40-60% are obtainable in unmated recipients (Sreenan, 1975; Renard and du Mesnil du Buisson, 1976; Rowson, personal communication; Table 13). In mated recipients, the method produced results almost as good as those obtained surgically in a direct comparative study, pregnancy rates being 62.5 and 60.4% and twinning rates 48 and 38% by surgical and non-surgical means, respectively (Boland et al, 1976a). Normal twin calves have been born following the procedure (Boland et al, 1976b).

Of the authors listed in the summary of results by non-surgical transfer (Table 13), only Sugie et al (1972a, b) transferred embryos that were collected non-surgically. Elsden et al (unpublished) have obtained 10 pregnancies after non-surgical collection and transfer. As has been pointed out, early failures experienced in non-surgical transfers may have been due in part to the use of bovine serum as a medium for holding the ova. This was subsequently shown to contain an embryotoxic factor (see page 20). Many of the early experiments also used early (day-3) embryos, which Newcomb and Rowson (1975a) have shown will not readily survive in the uterine environment.

As discussed for surgical transfers (see page 29), few reports give calving rates and Gordon has pointed out that Onuma and Foote (1970) show some incidence of abortion in seven of the 16 reports that they reviewed in detail. Table 13 can add to that list and indicates the need for a better understanding of whether this is a problem exaggerated by non-surgical transfer and, if it is, the underlying causes.

TECHNIQUES AND RESULTS IN SHEEP AND GOATS

INTRODUCTION

R. A. S. Lawson

Early attempts to transfer embryos recovered from the reproductive tracts of ewes after slaughter were encouragingly successful (Warwick, Berry and Horlacher, 1934; Warwick and Berry 1949). Lopyrin et al (1950a) reported the birth of nine lambs after the transfer of fertilized ova and also follicular oocytes into the oviducts of unmated and mated ewes, respectively. Later, Lopyrin et al (1951) had nine lambs born after the transfer of 46 day-1 zygotes.

The potential of egg transfer in domestic animals became reality with the demonstration of a procedure that gave an acceptable and repeatable conception rate. Hunter et al (1955) transferred 19 2- to 16-cell embryos to 18 recipient ewes; eight lambs were born. Averill (1958) reported the survival of 24/30 transferred embryos. The procedures used by these workers have remained the basic methods used for surgical embryo transfer in domestic animals.

Results of experiments on embryo transfer in goats have been published by Nishikawa, Horie, Sugie, Onuma and Niwa (1963a, b); Niwa, Sugie, Onuma, Soma and Nishikawa (1960); and Moore (1974) (see also review by Foote and Onuma, 1970).

In using embryo transfer as a research tool, McDonald (1969) reported that embryo transfer between ewes was more successful than the re-transfer of embryos within the same ewe. However, Killeen (1974) found a conception rate of 65% after homologous embryo transfers.

The steps in the procedure will be considered in the same order as for cattle.

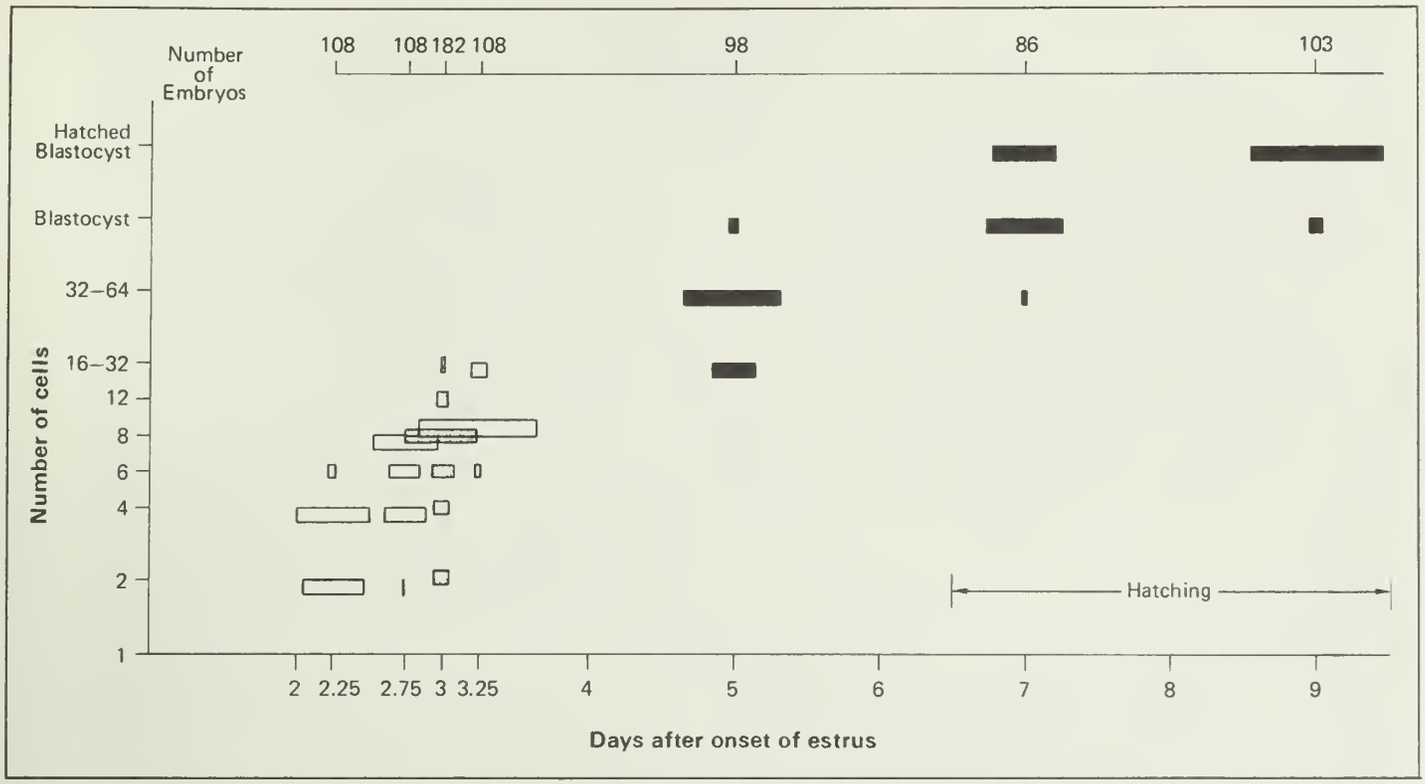


Figure 10. The cleavage and hatching of embryos in superovulated sheep. For each day, the proportion of recovered embryos in each developmental stage is represented by the length of the horizontal bar.

Data from: Averill, 1958 (stippled bars); Moore and Shelton, 1964b (open bars); and Rowson and Moor, 1966b (solid bars).

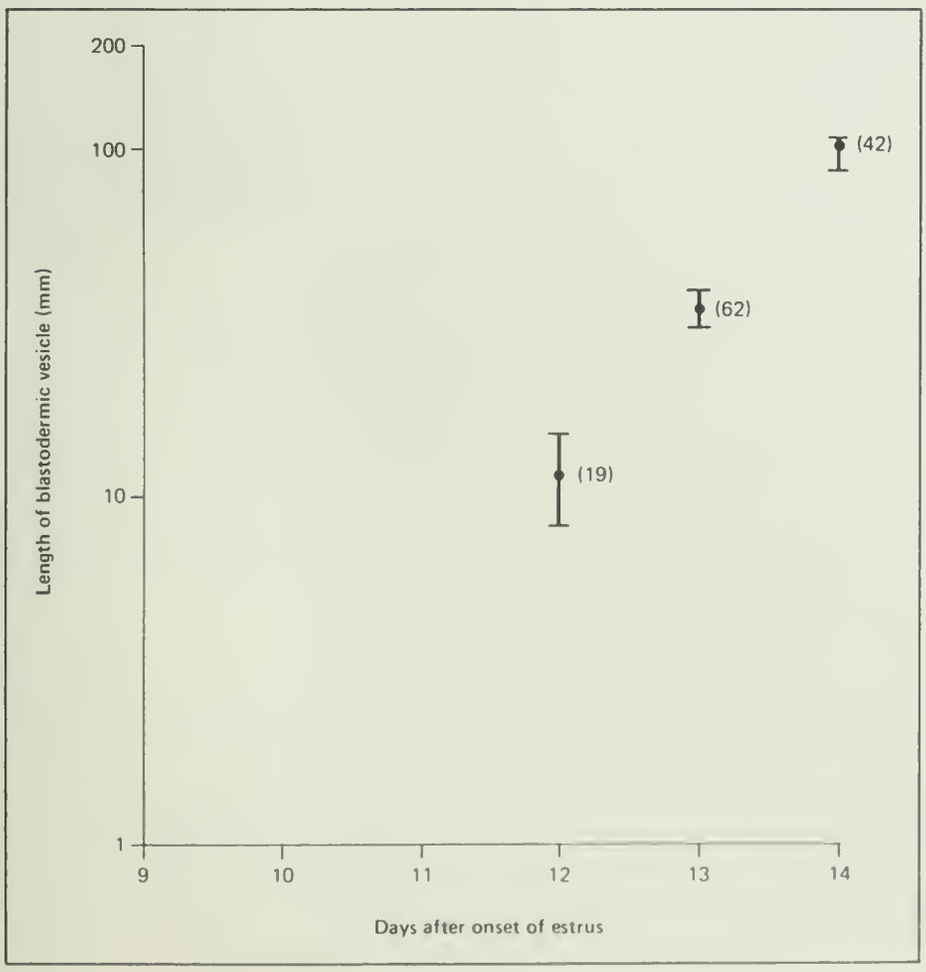
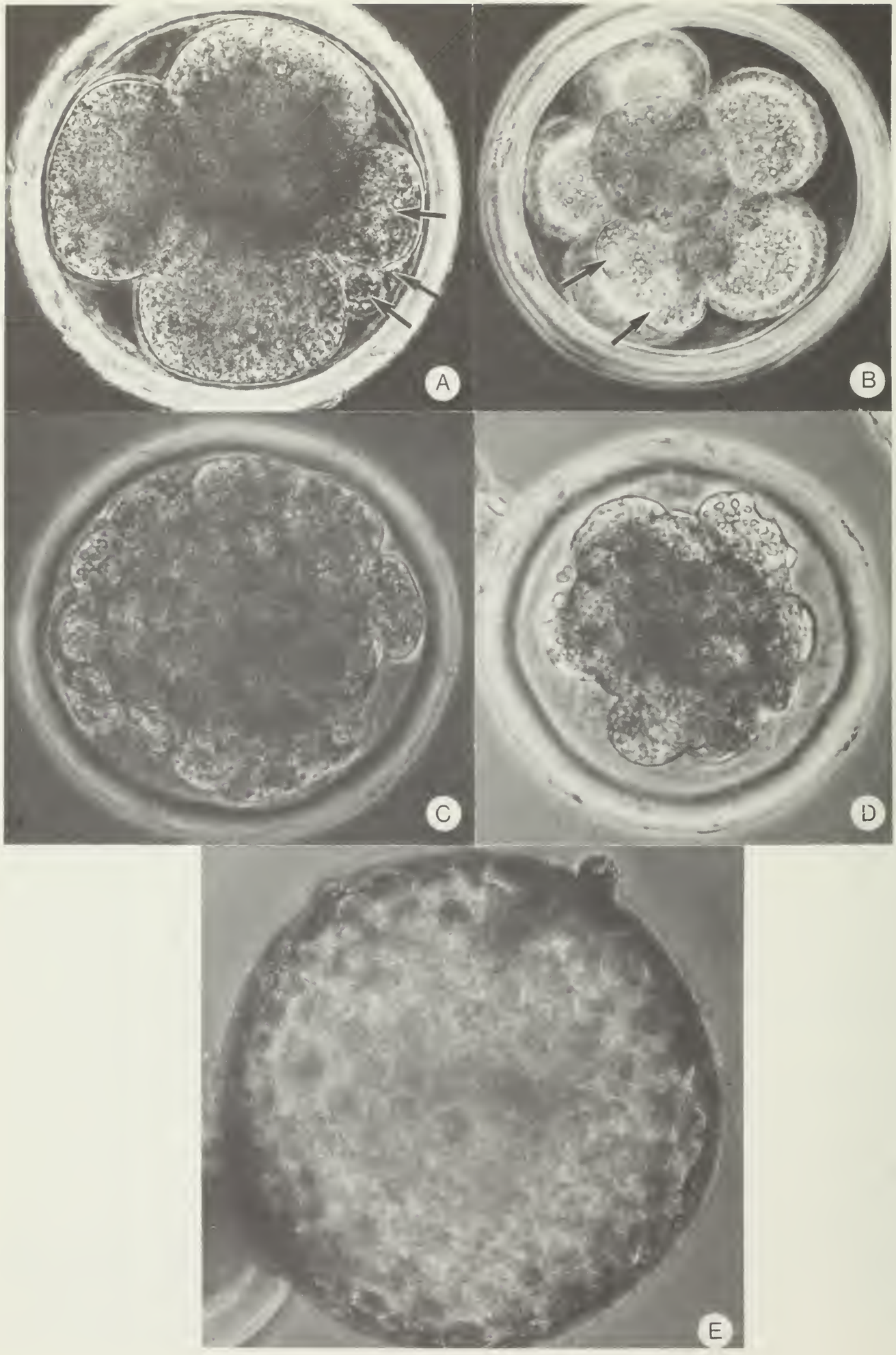


Figure 11. The growth of hatched embryos in superovulated sheep. Mean lengths are indicated on a logarithmic scale with the number of embryos for each point in parentheses.

Data replotted from Rowson and Moor, 1966b.


A
B
C
D
E

SUPEROVULATION

K. J. Betteridge and N. W. Moore

Sheep — The principles of inducing superovulation in sheep are the same as in cattle: a follicle-stimulating gonadotrophin is administered either near the end of the luteal phase of the cycle (days 11-13) or around the conclusion of treatments with progestogens designed to control the time of ovulation. The use of prostaglandins to induce luteolysis after follicle stimulation in sheep has been described by Trounson, Willadsen and Moor (1976). The PG analog Cloprostenol (ICI), given as a single IM injection of 100 μg between days 4 and 13 proved very effective for synchronizing estrus in 84 ewes that had been treated with PMSG 24-72 h previously. All but two (which probably ovulated silently) came into estrus, 71 of them within 36 h of the PG, and a large proportion of ovulations occurred between 30 and 36 h after PG. Embryos recovered from such animals between days 3 and 8 were transferred to eight recipients (also synchronized with Cloprostenol) which all became pregnant. Two out of 10 donor ewes had regressing CL at the time of embryo recovery, but this did not seem to be related to PG treatment since a similar proportion of animals showed premature CL regression after receiving PMSG alone. Lawson (personal communication) has also encountered premature CL regression in ewes treated with PMSG and PG; non-functional CL followed superovulation in 4/6 animals receiving 1000 IU PMSG and 7/10 treated with 1600 IU PMSG. A similar phenomenon occurs in cattle, as described on page 6 of this monograph.

In naturally cycling sheep, PMSG induces a dose-related response, the average number of ovulations increasing from 2.8 to 9.1 as the dose rises from 700 to 1300 IU given on day 12 or 13 of the cycle (Averill, 1958). Doses are often given on a body-weight basis at the rate of about 20 to 45 IU/kg, or up to 2000 IU/animal, and this induces estrus 2-4 days later with multiple ovulations in 74-100% treated ewes with average ovulation rates being in excess of four (Hancock and Hovell, 1961; Lynch, 1968, cited by Gordon, 1975; Gordon, 1969, and Quirke and Hanrahan, 1973, cited by Quirke and Hanrahan, 1975). Of the anterior pituitary preparations containing FSH, the one from the horse (HAP) has been used with particular success when given as three equal daily doses beginning on day 12. It increases the average number of ovulations from four to 11 as the total dose is increased from 60 to 135 mg (Moore and Shelton, 1964a; Boland, 1973, cited by Gordon, 1975). Optimum responses to HAP were obtained in ewes coming into estrus 24-48 h after the end of treatment when over nine fertilized eggs per ewe were obtained with less variability than found with PMSG (Moore and Shelton, 1964a).

Superovulation at predetermined times can be induced by PMSG or pituitary extracts (e.g., HAP) given in conjunction with progestogens administered by injection or as intravaginal pessaries (Robinson, 1959; Gordon, 1969, cited by Quirke and Hanrahan, 1975; Moore, 1970; Quirke and Hanrahan, 1975). PMSG is given as a single injection 24 h before, or at the time of, the last injection or removal of pessaries. HAP needs to be given as three equal consecutive daily injections commencing on the day before the last injection or removal of pessaries. Estrus normally commences 36-48 h after the end of progestogen treatment.

Irrespective of type of superovulation treatment, fertilization frequently fails, particularly in ewes showing a high ovulatory response. Fertilization failure is equally evident in ewes inseminated naturally or artificially and appears to be due to faulty transport of spermatozoa through the cervix. The problem can be overcome by the direct deposition, under local anesthesia, of semen into the uterus (Trounson and Moore, 1974a). Fertilization rates in excess of 90% of ova recovered were achieved by injecting 0.02 ml semen into the tip of each uterine horn around the time of onset of estrus. It is not necessary to use HCG after PMSG or FSH to bring about superovulation. In sheep given a moderately stimulating dose of PMSG (750 IU), HCG increased the proportion of follicles ovulating if given within 3 h after the onset of estrus (Killeen and Moore 1970a), but was without effect on ovulation rate when given before estrus in PMSG-treated synchronized ewes (Quirke and Hanrahan, 1975). Gonadotrophin-releasing hormone (GnRH) was also ineffective in the latter study and has, by itself, been unable to induce superovulation in anestrous ewes (Kinder et al, 1976), although GnRH treatment on day 12 in cycling sheep can increase average ovulation rates from 1.09 to 1.36 (Findlay and Cumming, 1976). Prepuberal lambs can be superovulated (Mansour, 1959), but do not appear to provide embryos suitable

Figure 12. Sheep embryos at various transferable stages of development. (X 435 approx.)

A. 4-cell egg, day 2. Note the small particles (arrowed) making this an 'atypical' rather than an abnormal embryo.
B. 8-cell egg, day 3. This embryo is also atypical although at the expected stage of development.
C. Early morula, day 5, containing more than 20 cells.
D. Late morula, day 6, showing condensed inner cell mass.
E. Expanded, hatched blastocyst, day 8. Note the loss of the zona pellucida.

Photos A and B from Killeen, 1969, by kind permission; and photos C, D, E by courtesy of N. W. Moore.

for transfer. Surgical insemination may be required to obtain fertilization.

Goats — According to Japanese workers (Nishikawa and Onuma, 1963; Nishikawa, Horie and Onuma, 1963), goats differ from cattle and sheep in their response to PMSG in that they require a supplementary injection of an ovulating hormone such as HCG if multiple follicles are to rupture. The regimen evolved by the Japanese workers in Saanen goats was to give 1500 IU PMSG between days 16 and 20, either as a single injection or as two equal doses 1 day apart. Estrus occurred 2-5 days (usually on the second day) after PMSG and would last 1-5 (average 2.5) days unless HCG was given. The essential HCG (1000 IU), given at the onset of estrus, shortened its duration to 1-2 (average 1.3) days. In 11 treated animals, 10-48 (average 25.5) follicles developed, of which 27-80% ruptured to produce 6-26 (average 13.3) ovulations per animal. Eight of the 11 goats had more than 10 ovulations.

Moore (1974), however, did not find that HCG was essential. He used horse anterior pituitary extract (HAP) injected SC at 12 or 15 mg/day for 3 successive days beginning on the day before the final injection of a 16- to 18-day course of progesterone injections (12 mg/day). Of 43 treated does, 39 came into estrus, 31 of them within 2 days of the final HAP injection. The 39 does had 386 ovulations (range 0-21) when examined on days 4-5.5.

IN VITRO FERTILIZATION AND USE OF FOLLICULAR OOCYTES

These topics have been reviewed in a general context on pages 9-10, where it will be seen that the sheep has served as a model in developing systems for maturing oocytes in cultured follicles and that such oocytes have been successfully transferred, 50% of them giving rise to lambs. Earlier Russian work resulting in the birth of lambs after oocyte transfer is mentioned on page 74.

EGG AND EMBRYO COLLECTION METHODS

N. W. Moore

Collection and transfer procedures for the ewe and goat doe are essentially similar and have changed little from those described and illustrated by Hunter et al (1955). Collections are usually carried out under general anesthesia with the ovaries, oviducts and uterus exposed by a mid-ventral incision. The oviducts are cannulated (glass or polythene cannulae) via the fimbria and a portion, or all, of the uterine horns and oviducts are flushed by the gentle expression of flushing medium along the horns and through the oviducts. In both the ewe and goat, embryos enter the uterus around the 3rd to 4th day after estrus, but irrespective of the time after estrus at which collections are attempted, flushing through the oviducts results in high rates of recovery of embryos, around 80% in both the ewe (Trounson and Moore, 1974a) and the doe (Otsuki et al, 1960; Nishikawa, Horie, Sugie, Onuma and Niwa, 1963a, b; Moore, 1974). In both species there appears to be little effect of collection time after estrus on rate of recovery. Repeated collections from individual animals appear to have been rarely attempted. The results of one study in the ewe indicated no effect of a first collection on the rate of recovery, or fertilization, of embryos at a second collection 1 year later (Moore and Shelton, 1962b). No data were provided on the nature or extent of any adhesions resulting from the first collection. In the ewe and the doe, nonsurgical collection as described for the cow and the mare is not possible because of the tortuous nature of the cervix.

MEDIA, STORAGE AND CULTURE CONDITIONS

N. W. Moore

A variety of media ranging from complex tissue culture media (e.g., TCM 199) to simple balanced salt solutions enriched with serum, or serum albumins, have been used for the collection and holding of sheep and goat embryos before transfer. Some of the earliest workers even tried aqueous humor (Warwick and Berry, 1949). In early studies in the ewe, Hunter et al (1955), Averill and Rowson (1958), Moore et al (1960) and Hancock and Hovell (1961) achieved successful transfers using sheep serum containing penicillin (500-1000 IU/ml). Following the development of effective methods of *in vitro* culture, serum has been fully or partly replaced by bicarbonate- or phosphate-buffered solutions of osmolarity around 300 mOs and pH 7.2-7.6, enriched with either 2-3% bovine (or ovine) serum albumin or 10-20% sheep serum. For collection and transfer purposes phosphate buffers (e.g., Dulbecco's phosphate buffer) have an advantage over bicarbonate buffers in that they do not have to be maintained under a gas phase to maintain pH. Antibiotics (penicillin around 100-200 IU/ml and streptomycin sulphate around 50-100 IU/ml) are usually added and media are generally sterilized by passage through cellulose acetate filters (e.g., Millipore) of about 0.5 μ pore size. As serum rapidly clogs cellulose filters, it is advisable to filter it separately through asbestos (e.g., Seitz) filters. However, it may then be necessary to discard the first 200-300 ml of filtered serum as it has been reported from tissue culture studies that asbestos filters contain growth-inhibiting substances (House, 1964; Brown, Cartwright and Newman, 1965). The growth-inhibiting substances appear to be removed from the filters by the first 200-300 ml of filtrate. Filtered serum can be stored frozen and added to the buffered solutions as required.

Successful methods of *in vitro* culture of sheep and goat embryos at 37-38°C have been achieved and they provide a method of rapidly assessing the capacity of embryos for further development. Development of 1-, 2- and 4-cell embryos to eight cells, and development of embryos of 20 cells or more to expanded and hatched blastocysts, can be readily achieved in a variety of media including TCM 199 enriched with fetal calf serum or sheep serum, bicarbonate- and phosphate-buffered solutions enriched with serum or serum albumins (Moore, 1970; Moor and Cragle, 1971; Moore and Spry, 1972). Development has been achieved in a wide range of osmolarities (307-320 mOs) and pH (6.8 to 7.8). However, development through the 8-cell stage seems to require specific conditions, which can be satisfied by incubating under low oxygen tension (Tervit, Whittingham and Rowson, 1972; Trounson and Moore, 1974b). A similar situation appears to exist for goat embryos (R. J. Bilton and N. W. Moore, unpublished data).

In vitro culture offers a method of short-term storage, but the duration of storage is limited to 4-5 days. Alternative methods of short-term storage at low temperatures have been developed and are discussed on pages 50-52 of this monograph. A further method of short-term storage lies in the use of the rabbit. Sheep embryos show continued development in the oviduct of the rabbit and, when retransferred to recipient ewes, normal pregnancies have resulted (Averill et al, 1955). The technique has provided a method for the transport of sheep embryos (Hunter et al, 1962). Recent reports on the development of methods of effective frozen storage of sheep and goat embryos (see pages 52-53) seem likely to limit further exploitation of short-term storage.

METHODS OF ASSESSING THE VIABILITY OF EMBRYOS

N. W. Moore

The ultimate test of viability of embryos must be in their potential for development *in vitro* to normal young. However, for transfer it is important that their potential be rapidly assessed by visual appraisal shortly after collection. As for cattle (page 24), stage of development in relation to time of mating and the general appearance of embryos are the major means of appraisal.

Extensive data are available on the rate of development of sheep embryos (Averill, 1958; Moore et al, 1960; Moore and Shelton, 1964b) and when embryos are collected at known times after estrus the stage of embryos can be predicted (Fig. 10). Not infrequently, one or more relatively underdeveloped embryos are collected with others that are at the expected stage of development, e.g., 2-cell embryos with those of eight or more cells. In the ewe and doe, as in other species, little direct evidence is available on the viability of the underdeveloped embryos. However, they may well be suspect, as they show little or no development in culture *in vitro*, but nearly all of those at the expected stage of development show continued development in culture (N. W. Moore, unpublished data).

In transfer, donors will in all likelihood have been treated with gonadotrophins to induce superovulation. As stated in the introduction (page xvii), there appears to be no evidence to suggest any substantial effect of superovulation treatments on the subsequent incidence of abnormalities. Abnormalities in sheep embryos have been reported (Averill, 1958; Tervit and McDonald, 1969; Hancock and Hovell, 1961) but their incidence and effects on viability require examination. In one study, Killeen and Moore (1971) classified over 50% of embryos collected from donor ewes 2-3 days after estrus as abnormal. Most contained one or more anucleate cells of variable size together with apparently normal nucleated blastomeres. However, the presence of this type of 'abnormality' did not have any marked effect upon subsequent development in recipient ewes and they concluded that most of the 'abnormal' embryos would have been better classed as atypical rather than abnormal. Gross abnormalities involving the nuclear elements of sheep embryos have been described (Braden, 1964; Killeen and Moore, 1970b, 1971) but their incidence is low (less than 1-2% of embryos examined). Most are the result of the involvement of more than one spermatozoon in the fertilization process. In the ewe, it is doubtful if polyspermic embryos advance beyond the pronuclear stage and under normal transfer procedures they would not be distinguished from unfertilized ova and would be rejected for transfer.

Irregularly shaped embryos (D-shaped, involuted, etc.) have been described, and embryos with one or more fractured and dispersed blastomeres are frequently encountered. Irregularities and damage to blastomeres may well result from collection procedures but little is known of their viability. However, in the ewe (N. W. Moore, unpublished data) as in the rabbit (Seidel, 1952; Moore et al, 1968) and pig (Moore et al, 1969), single blastomeres of 2-, 4- and 8-cell embryos have the potential for full development to normal young, and damage to one or more blastomeres of early embryos may not markedly affect their subsequent viability.

Kardymowicz (1972b) has used vital stain retention as a rough indicator of sheep embryo viability.

The age of embryos at the time of transfer can affect their subsequent viability. In the ewe, the survival of embryos collected within the first 2-4 days after estrus increases with age and this occurs whether they are transferred to the oviducts or to the uterine horns (Moore and Shelton, 1964b; Table 14), whereas with 5-9 day embryos there appears to be no effect of age (Rowson and Moor, 1966a). Transfer of sheep embryos older than 9 days is rarely attempted because of problems associated with identification and manipulation but they, too, can survive

TABLE 14. NUMBERS OF EWES THAT LAMBED AND LAMBS BORN IN RELATION TO AGE OF TRANSFERRED EGGS

Site of transfer	Age of eggs (h)	Cell stage	Ewes that lambed (n = 27)		Lambs born (n = 54)	
			Number	%	Number	%
Tubes	48-60	2-6	13	48.1	19	35.2
	60-72	4-8	15	55.6	23	42.6
	72-84	6->8	20	74.1	30	55.6
	Total		48/81	59.3	72/161	44.7
Uterus	48-60	2-6	10	37.0	12	22.2
	60-72	4-8	10	37.0	12	22.2
	72-84	6->8	15	55.6	21	38.9
	Total		35/81	43.2	45/161	30.0

From Moore and Shelton (1964b)

considerable damage without loss of viability (Rowson and Moor, 1966b). Other general aspects of factors that may affect embryo viability are those discussed for cattle on pages 24-26.

In goats, 80-86% rates of cleavage (fertilization) in recovered eggs have been recorded (Otsuki et al, 1960; Nishikawa et al, 1963a, b; Moore 1974).

SEXING OF EMBRYOS

The general principles of sexing procedures and the observations in sheep that led to sexing techniques in cattle are described on pages 26-27. Polge and Rowson (1973) reported an experiment by Dain and Rowson in which the sex of six embryos was determined before transfer by Y-chromosome identification in metaphase spreads found in squash preparations of tissue samples from day-12 embryos.

EMBRYO TRANSFER METHODS

N. W. Moore

Surgical transfer may be carried out under general anesthesia, or under local anesthesia with the animals restrained in a laparotomy cradle, as described by Lamond and Urquhart (1961). Transfer using local anesthesia has the advantages of speed and economy. Approach is invariably by mid-ventral incision, and there would seem to be no advantage in employing other approaches. Although there are no reports on its use, transfer using laparoscopic techniques might provide an attractive alternative to laparotomy.

The site to which embryos should be transferred has been assumed to be determined by their age and stage of development. Certainly, with sheep embryos collected up to 3½ days after estrus the oviducts provide a more favorable site than the uterus does (Moore and Shelton, 1964b; Table 14). With older embryos, high survival rates (70-75%) result from uterine transfer (Rowson and Moor, 1966a) and transfer to the oviducts of these older embryos would seem inappropriate. Similar, critical studies have not been reported for the goat but, as with sheep, the adopted practice has been to transfer embryos of less than 3-4 days old to the oviducts, and older embryos to the uterine horns. In goats, Sugie et al (1969) obtained a pregnancy rate of 17/22 (77%) and an embryo survival rate of 35/56 (63%). Moore (1974) obtained a pregnancy rate of 46/158 (29%) and an embryo survival rate of 68/275 (25%) and ascribed these poorer results to maternal failure rather than to lack of embryo viability.

Non-surgical transfer via the cervix has not been attempted in the ewe. In the doe, the cervix is a little less tortuous and can sometimes be penetrated with small-bore catheters. Otsuki and Soma (1964) transferred day-3 embryos via the cervix to the uterus in 23 goats but only one of the 23 subsequently produced a kid.

INFLUENCE OF SYNCHRONY AND METHODS OF SYNCHRONIZATION ON RESULTS

R. Newcomb

In sheep, there is a requirement for synchrony of estrus between egg donor and recipient, similar to that in cattle (pages 28-29), but apparently with a wider tolerance of variation from synchrony. Rowson and Moor (1966a) found that, when synchronization was exact, 75% of all recipients became pregnant and a high proportion of pregnancies were also obtained when the onset of estrus in the recipient differed from that of the donor by ± 2 days. However, a difference of 3 days between the onset of estrus in the donor and recipient appears to be incompatible with pregnancy (Moor, 1965) or to result in extremely low conception rates (8%) (Rowson and Moor, 1966a). The degree of synchronization necessary for embryonic survival remains relatively constant from day 2 to day 12 (Moore and

Shelton, 1964b; Moor 1965), although there is some indication that the most favorable asynchronous combination of donor and recipient alters as the age of the transferred embryo increases (Moor 1965).

The use of progesterone to synchronize estrus in donor or recipients (Hunter et al, 1955; Shelton and Moor, 1966) does not adversely affect fertility in sheep. In goats, the use of progestogen sponges and PMSG to synchronize estrus in 354 recipient does gave good synchronization but was followed by poor conception rates (Moore 1974); however, it is not clear whether the disappointing results were due to the synchronization method. Encouraging results following PG synchronization of donors and recipients are described on page 37.

Experimentally, the injection of progesterone can completely alter the synchronization requirements between donors and recipients, as described on pages 72-74 of this monograph.

OTHER FACTORS INFLUENCING RESULTS

The possible influences of a number of factors, mostly associated with recipients, on results are reviewed by Lawson on pages 72-78. These include: number of embryos transferred to each recipient; progesterone levels or numbers of CL in recipients; inter-strain and intra-strain transfers; experimental heat stress; recipient breed, liveweight, face cover, age, nutrition and lactational status.

TECHNIQUES AND RESULTS IN PIGS

SUPEROVULATION

K. J. Betteridge

References to earlier work on the use of PMSG in normally cycling pigs are cited by Guthrie et al (1974), in a study of the hormonal as well as the ovulatory responses to such treatment. Mean ovulation rates in response to treatment on day 15 with 0, 600 or 1200 IU PMSG were approximately 14, 19 and 27, respectively, and 1500 IU resulted in averages of 33 and 38 ovulations per gilt in other studies (Hunter, 1964, 1966). Estrus and the time of the LH peak is advanced by about 1 day, occurring 4 days after PMSG, in treated animals and the pre-ovulatory estrogen (but not LH) and post-ovulatory progesterone (but not estrogen) levels are directly related to the dose of the gonadotrophin. Previous suggestions that the level of food intake on the 1st day of estrus could influence ovulation rates were not confirmed.

Pigs synchronized with methallibure (Aimax, ICI) fed to them at 100 mg per gilt daily for 20 days can be superovulated very reliably with a subcutaneous injection of 1500 IU PMSG 24 h after the last feeding with methallibure (Christenson et al, 1973). Estrus occurred 3-4 days later in 93-95% of 80 PMSG-treated animals. Ovulation rates averaged 25

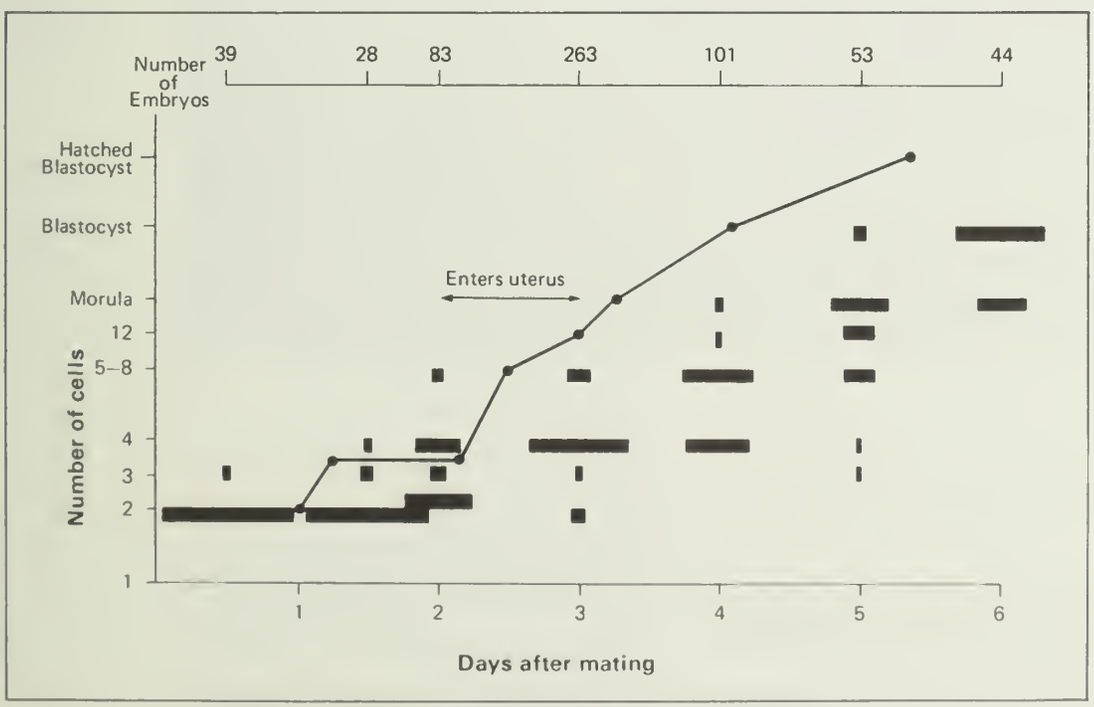


Figure 13. The cleavage of embryos in pigs. For each day, the proportion of recovered embryos in each developmental stage is represented by the length of the horizontal bar for the data of Hancock, 1961. The graphed points (●——●) represent the earliest times at which given stages are reached (from the data of Hunter, 1974, allowing 6-8h between mating and activation).

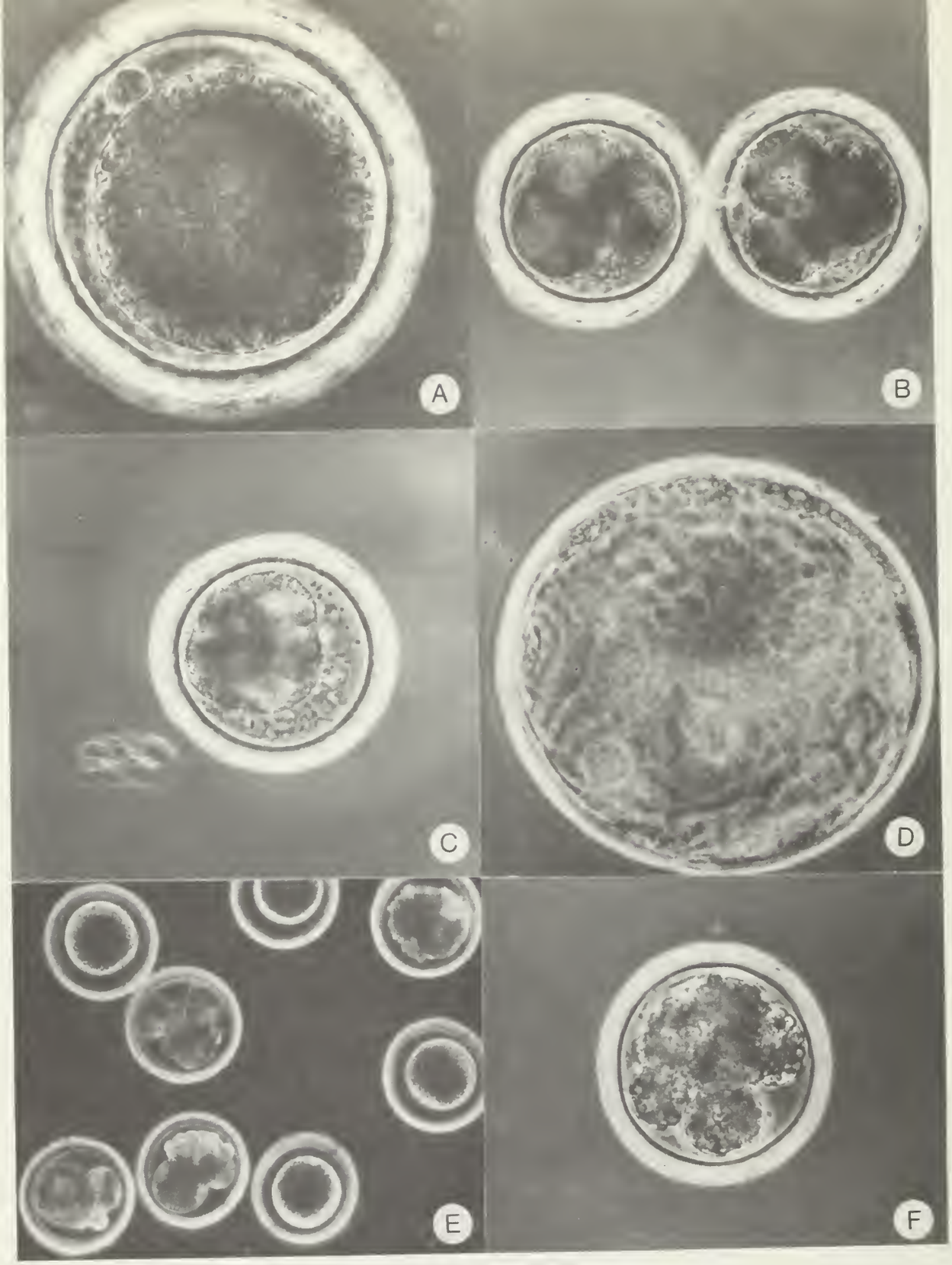


Figure 14. Pig embryos at transferable stages of development and degenerating pig eggs.

A. An uncleaved pig egg recovered from the oviduct and probably fertilized. Note the prominent polar body and numerous spermatozoa in the zona pellucida. (X 400 approx.)
B. Two 4-celled pig eggs. Blastomeres are of equal size, dense, and well defined in comparison with the fragmenting egg shown in F. Spermatozoa are evident in the zona pellucida. (X 200 approx.)
C. A morula recovered from the uterus. Individual blastomeres are not obvious in the compact group of cells and normal morulae may appear similar to unfertilized and fragmenting eggs. Spermatozoa are evident in the zona pellucida. (X 200 approx.)
D. An expanded blastocyst recovered from the uterus. The zona pellucida is still intact but thinner. The blastocoele is well developed. (X 400 approx.)
E. Uncleaved and fragmenting pig eggs at lower magnification. Note the absence of spermatozoa in zonae pellucidae and the lack of cellular organization in fragmenting eggs. (X 100 approx.)
F. A fragmenting pig egg. Compare the appearance and density of spermatozoa in its zona pellucida with B and C. The absence of well defined blastomeres indicates atypical cleavage. (X 200 approx.)

Photos by courtesy of B. N. Day.

in animals receiving no further treatment and 28 in those receiving 500 IU HCG IM 80 h after PMSG, compared with a rate of 13 in synchronized but unstimulated gilts. The main advantage of HCG was in synchronizing ovulation so that artificial inseminations could be at set intervals of 12 and 24 h after HCG, rather than 12 and 24 h after the onset of estrus. Unfortunately, methallibure has been withdrawn from the market because of its teratogenic effect when fed to pregnant sows.

Alternative methods of synchronization using gonadotrophins and prostaglandin are under development (Guthrie and Polge, 1976). PG alone is impractical in the pig because it is only luteolytic after day 10. The new method therefore uses PMSG and HCG to prolong the luteal phase by inducing accessory CL, which are made to regress with PG about 10 days after formation. Estrus follows in 4-7 days.

Prepuberal (5-6-month-old) gilts can be superovulated as a satisfactory source of embryos either by giving between 250 and 2000 IU PMSG followed by 500 IU HCG 48 h later (Baker and Coggins, 1968) or by a combination of the two hormones as a single injection of 300 or 400 IU PMS and 200 IU HCG (Baker et al, 1974). Gonadotrophin-releasing hormone did not prove a satisfactory substitute for HCG in the combination treatment. A marked seasonal variation in response to PMSG has been recorded, responses being twice as high in March and April as in other months (Webel et al, 1970b). Fertilization rates remain normal after any of the above superovulation treatments in pigs.

IN VITRO FERTILIZATION AND USE OF FOLLICULAR OOCYTES

C. Polge

The general principles involved in this topic were introduced on pages 9-10.

Primary pig oocytes collected from follicles of about 2 mm in diameter or greater will resume meiosis when incubated *in vitro* in a variety of culture media (McGaughey and Polge, 1971). The rate of nuclear maturation *in vitro* is generally a little slower than that observed *in vivo* following LH stimulation (Hunter and Polge, 1966), but after 40-50 h of culture 50-70% of the oocytes reach the second meiotic metaphase. Cytogenetic analysis of *in vitro* matured oocytes has revealed a fairly high incidence of chromosomal abnormalities (McGaughey and Polge, 1971). In addition, nuclear maturation *per se*, even if apparently normal, is probably a poor criterion of the potential for normal fertilization and embryonic development. Cytoplasmic factors as well as nuclear maturation are likely also to be involved. At the ARC Unit of Reproductive Physiology and Biochemistry, Cambridge, pig oocytes matured *in vitro* have been transferred to the oviducts of inseminated gilts. Sperm penetration occurred in a proportion of the oocytes, but none developed normally to the blastocyst stage. There was a high incidence of polyspermy suggesting that the mechanism for the block of polyspermy, which *in vivo* develops concomitantly with the maturation of the nucleus (Polge and Dziuk, 1965), was abnormal in some oocytes. Failures in embryonic development might be expected if fertilization occurs before maturation has been achieved because, even following maturation *in vivo*, oocytes removed from follicles before the attainment of anaphase I and transferred to recipient gilts failed to develop normally if penetrated by sperm at this stage (Baker and Polge, 1976). Nevertheless, some normal embryos have been obtained from gilts following transfer of oocytes collected from gilts as early as 24-28 h after injection of HCG, which is about 12-16 h before the expected time of ovulation (Leman and Dziuk, 1971). Thus, the transfer of oocytes matured *in vivo* for fertilization in a recipient animal might have some application in certain circumstances. Further work on maturation *in vitro* should always be backed up by observations on the potential for normal fertilization and development, and more information is required on the follicular contribution to oocyte maturation.

In vitro fertilization of pig oocytes following maturation *in vivo* has been claimed in a few unconfirmed reports (Harms and Smidt, 1970), but the evidence presented was far from convincing and the experiments have not been repeated. On morphological grounds, clear evidence of sperm penetration, pronuclear development and syngamy is required; the ability to develop normally following transfer should also be tested. None of these criteria have been met. In our experience, in experiments involving over 500 oocytes collected from follicles shortly before ovulation or from the oviducts shortly after ovulation, sperm penetration was never achieved *in vitro* (Baker and Polge, 1976). The oocytes were incubated in droplets of various culture media with or without the addition of fluids from the oviduct or pre-ovulatory follicles. The sperm added to the cultures were washed ejaculated sperm, epididymal sperm or sperm recovered from the oviduct, uterus or vagina of inseminated estrous gilts. Although none of the oocytes was penetrated by sperm *in vitro*, sperm penetration did occur if the mixtures of oocytes and sperm after 4 h incubation were transferred to the oviducts of unmated estrous recipients. Thus failure of sperm penetration *in vitro* appeared to be associated with an inability to achieve capacitation of the sperm in these circumstances.

EMBRYO COLLECTION METHODS

C. Polge

Collection and transfer procedures normally adopted today are similar to those described in the early 1960's (Hancock and Hovell, 1962; Dziuk et

al, 1964; Vincent et al, 1964; Polge, 1966). Following general anesthesia, usually maintained by a closed circuit system, the reproductive tract is exposed via a mid-ventral incision. Eggs from the oviducts (up to 40 h after ovulation) can be collected by means of a cannula inserted in the fimbria or through the uterotubal junction. Uterine eggs cannot be collected by flushing them back through the oviducts due to the valve-like nature of the uterotubal junction and they are therefore collected by means of a larger cannula inserted through the uterine wall. Different flushing media — Tyrodes solution TCM-199, Dulbecco's PBS — have all been used successfully. Very high recovery rates (over 90%) are usually achieved up to 6 days following ovulation. Recovery rates of hatched blastocysts after this time are often slightly lower. There is a greater loss of embryos in superovulated animals than in normal animals by the 10th day after ovulation. Up to three repeated recoveries have been carried out successfully in individual donors before the build-up of adhesions seriously interferes with these procedures.

The length and tortuous nature of the cervix and also of the uterine horns in the pig virtually rule out the possibility of non-surgical collection of embryos.

SHORT-TERM MAINTENANCE AND CULTURE OF EMBRYOS

C. Polge

There is little published information on the culture or storage of pig embryos, but considerable experience in these techniques has been obtained at the ARC laboratories. Observations have been based on cleavage *in vitro* and on the viability of embryos transferred to recipients. Culture media examined have included Brinster's medium, TCM 199, Tyrode's solution, Ham's F-10 and Dulbecco's PBS enriched with pyruvate and lactate. To these media have been added homologous heat-treated serum or bovine albumin. In cultures with media containing a bicarbonate buffer system, the gas phase has been 5% CO_2 in air or 5% CO_2, 5% oxygen and 90% nitrogen. In media with a phosphate buffer system the gas phase has been air. Optimal pH is 6.9-7.2.

A very high proportion (over 90%) of 1- or 2-cell embryos collected from the oviducts divided to four cells. A similar proportion of 6- to 8-cell embryos collected from the uterus developed to morulae or blastocysts. At one time it was considered extremely difficult to culture 1- or 2-cell embryos through to the blastocyst stage, but this was before the natural 'lag' at the 4-cell stage was appreciated. Eight-cell embryos were cultured for 48 h to the blastocyst stage and transferred to 19 recipients. Ten recipients were pregnant at autopsy 21 days later and, of the 229 embryos transferred, 51 were represented by living fetuses. The survival of cultured embryos was therefore considerably lower than normal.

Four-cell embryos have been stored at 20°C for 24 h and transferred to five unmated recipients. All were pregnant at autopsy and the embryo survival rate was 43%. However, storage temperature is critical, and no embryos have survived following storage at temperatures lower than 15°C.

A high proportion of pig embryos continue development when transferred to the oviducts of estrous or pseudopregnant rabbits ligated at the uterotubal junction. Ninety percent of embryos recovered from donor gilts at 8 h after fertilization and kept for 2 days in the rabbit continued development when re-transferred to recipient gilts. Longer periods of storage in the rabbit and the use of older embryos resulted in decreased survival.

These methods provide a means for short-term storage of pig embryos, but long-term storage at very low temperatures has not been achieved.

PREDICTION OF VIABILITY OF EMBRYOS

C. Polge

There is usually little difficulty in classifying completely degenerate embryos in the unstained state, but mistakes can be made. Fragmentation of unfertilized eggs does not normally occur in the oviduct during the first 40 h following ovulation, but fragmentation does occur very commonly after this time when the eggs have entered the uterus (Dziuk, 1960). Sometimes this fragmentation takes the form of a very even cleavage to two cells, but it is more generally an uneven fragmentation of the cytoplasm (Fig. 14 E, F). Polyspermically fertilized oocytes frequently fail to divide in the oviducts. In normally developing fertilized eggs the blastomeres become fused together at the 8-cell stage and from then on it is impossible to count accurately the number of cells present within an embryo in unfixed preparations (Fig. 14 C).

Accurate information is available on the normal rate of development of pig embryos following fertilization (Hunter, 1974). Cleavage from one to four cells takes place in the oviducts during the first 36 h of development. They enter the uterus at this stage and, therefore, the recovery of 2-cell embryos from the uterus usually represents an abnormality. At these ARC laboratories, observations on several thousand embryos have revealed that development is arrested at the 4-cell stage for up to 48 h and 4-cell embryos are commonly still recovered as late at 70-80 h after ovulation. When cleavage is resumed, there is roughly a 12 h cell cycle. However, not all embryos resume cleavage at the same time and it is not uncommon to recover 4-cell embryos in the presence of embryos of 8-16 cells. There is no evidence that the potential for normal development of the 'delayed' embryos is in any way diminished. Likewise, following hatching of blastocysts from the zona pellucida, which occurs between the 6th and 7th day following ovulation, a great variation in size

of blastocysts is seen, but any relationship between size and viability that might exist has not been established.

EMBRYO TRANSFER METHODS

C. Polge

Usually 1- or 2-cell eggs are transferred to the oviduct via the fimbria and embryos at later stages of development are transferred to the tip of the uterine horn by means of a fine pipette inserted into the lumen. Perhaps because the length of time the eggs normally remain in the oviducts is quite short, normal embryonic development has been achieved following transfer of 2-cell eggs, collected 24 h after ovulation, to the uterus. Normally, migration of the embryos down the uterine horns does not take place for 5 or 6 days, but 4-cell embryos have been transferred successfully to the cervical end of the uterus. Another method of transferring eggs to the uterus has been to flush them into the lumen via the oviduct. This avoids puncturing the uterine wall and the possibility of inducing bleeding; it also ensures entry into the lumen. Egg transfers need only be carried out to one side of the uterus, because later migration of the embryos ensures even spacing through both uterine horns and maintenance of pregnancy (Polge and Dziuk, 1970). However, pregnancy is not often maintained if less than four embryos are transferred (Polge et al, 1966).

Embryo survival rates of about 60% have been achieved following transfer up to 5 days following ovulation. These results compare favorably with normal survival rates. However, the survival of embryos falls dramatically when transfers are carried out more than 6 days after ovulation (Hunter et al, 1967; Webel et al, 1970a), by which time the blastocysts have hatched from the zona pellucida and no pregnancies have been established following transfers at 9 days after ovulation.

The features of the cervix referred to above also impose difficulties on non-surgical transfer. Nevertheless, 1/32 gilts became pregnant following attempted non-surgical transfer of embryos via the cervix on day 4 of the cycle (Polge and Day, 1968).

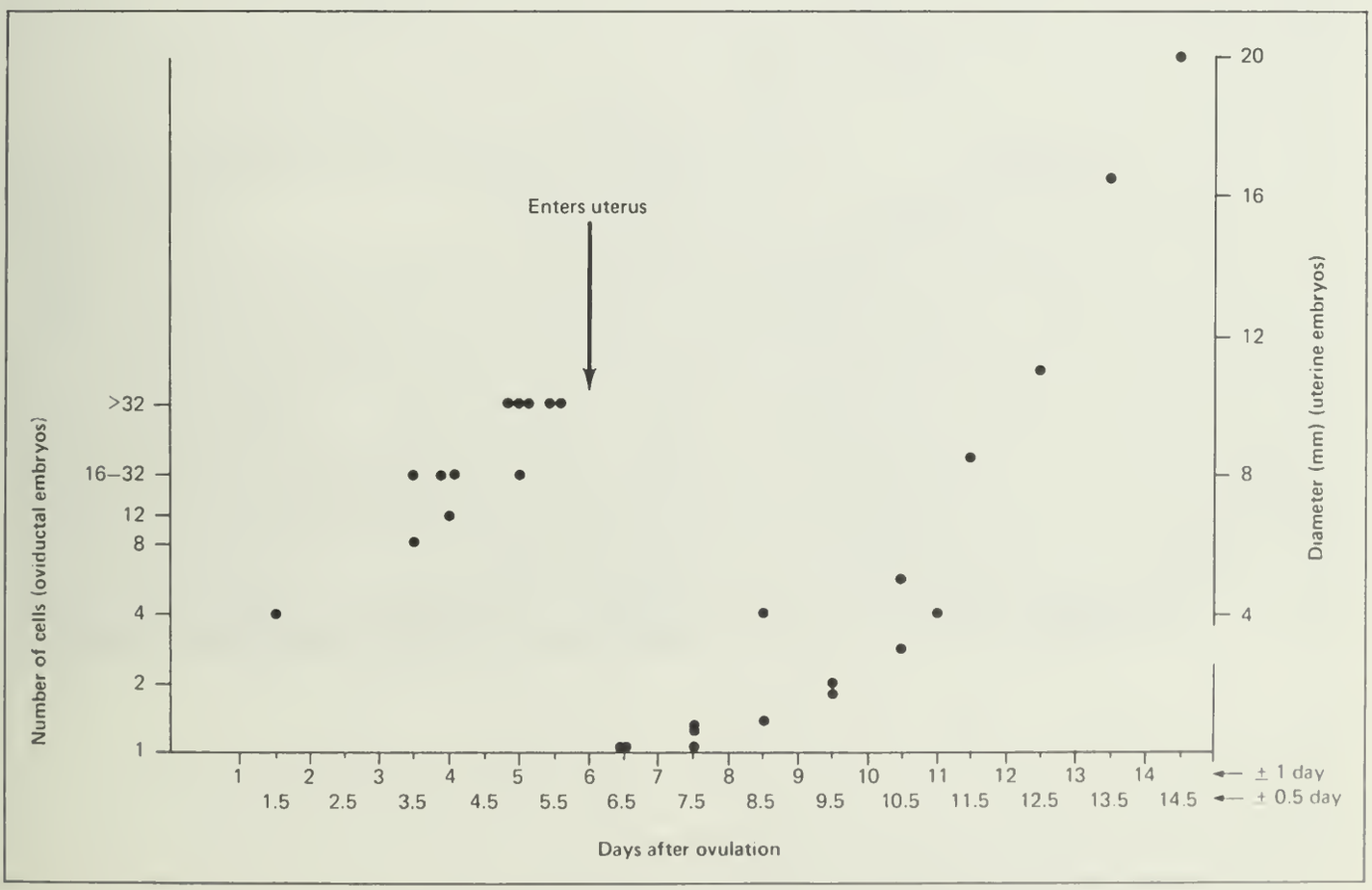


Figure 15. The cleavage and growth of embryos in pony mares. Each point represents a single embryo.

Data from Flood, Betteridge, Mitchell and Eaglesome (in preparation) and ADRI, unpublished.

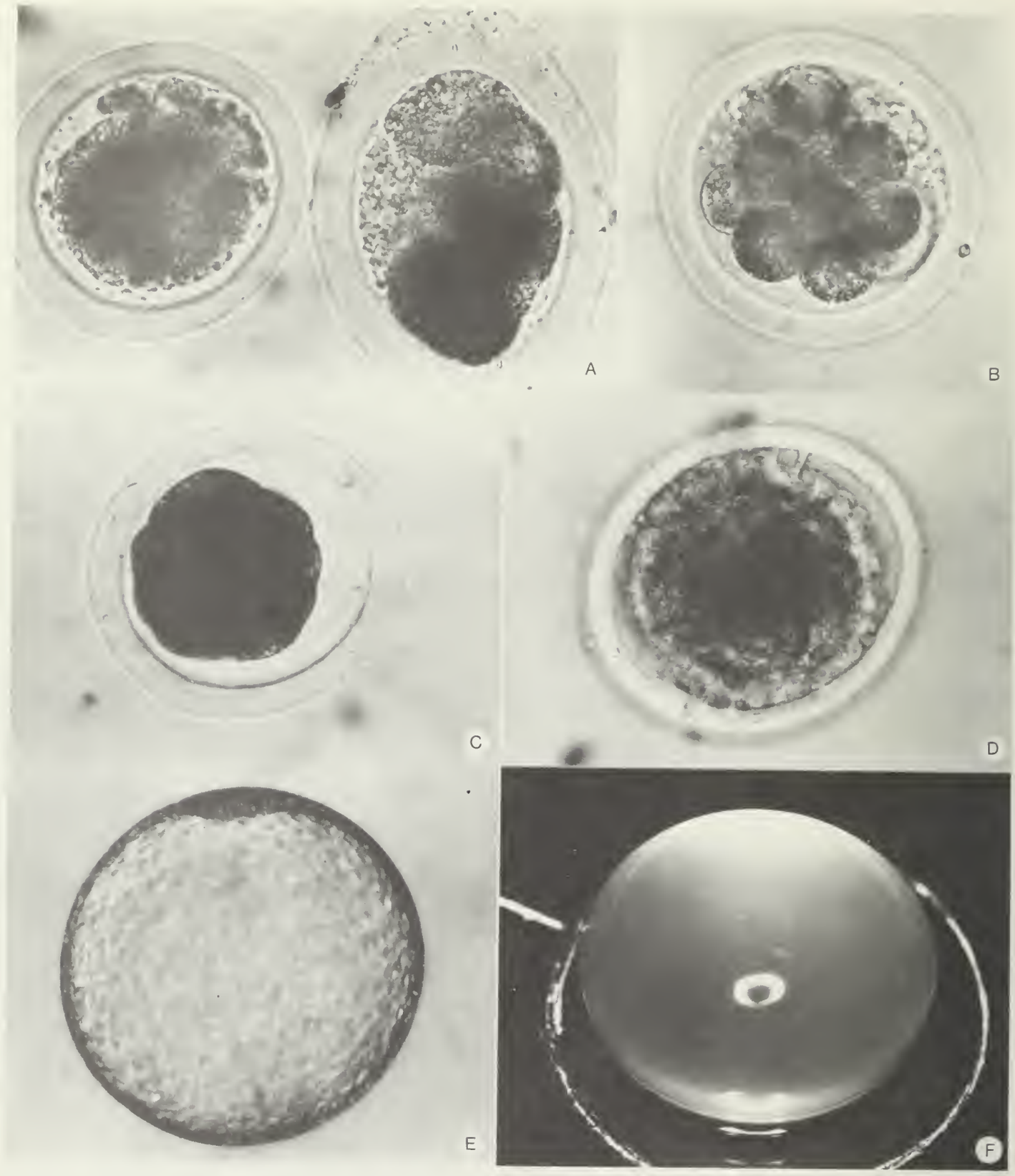


Figure 16. Horse embryos at various transferable (?) stages of development.

A. 4-cell egg (right) next to an old, unfertilized egg flushed from the oviduct of the same mare 1½ ± ½ days after ovulation. (X 330)
B. 12-cell egg from the oviduct 4 ± 1 days after ovulation. (X 335)
C. Morula from the oviduct 5 ± 1 days after ovulation. (X 335)
D. Early blastocyst from the uterus 6½ ± ½ days after ovulation. (X 320)
E. Expanded blastocyst from the uterus 7½ ± ½ days after ovulation. (X 100)
F. Expanded blastocyst from the uterus 12½ ± ½ days after ovulation. (X 4)

Photos from ADRI

INFLUENCE OF SYNCHRONY AND METHODS OF SYNCHRONIZATION ON RESULTS

In a study of the effects of asynchrony between donor and recipient animals (Webel et al, 1970a) it was concluded that transfers in which the donor was 1 or 2 days earlier or 1 day later than the recipient were as successful as synchronous transfers. The method of synchronization involved the use of methallibure followed by the induction of ovulation with PMS and HCG.

TECHNIQUES AND RESULTS IN HORSES

W. R. Allen

The horse has remained the 'Cinderella' among the farm animals in all aspects of embryo recovery, culture, storage, transport and transfer, and there have been very few published reports on this species to date. What little work has been done has shown clearly that both surgical and non-surgical methods of embryo recovery and transfer can be performed with relative ease and considerable success in the mare. The present block to further progress results from the continuing refusal of major breed registration authorities to accept for registration progeny that have been obtained by either artificial insemination or embryo transfer. This seems a pity since the very high values of individual horses, and the large financial loss that occurs as a result of pregnancy wastage in the Thoroughbred and other breeds, make the whole process of embryo transfer and transport highly desirable from both genetic and commercial points of view.

Techniques for embryo transfer in horses will be considered in the same order as for the other species.

SUPEROVULATION

A major drawback to the development of experimental studies of embryo transfer in horses is the great difficulty of inducing superovulation in the mare by administering exogenous gonadotrophins. Day (1940) originally reported that single and multiple injections of PMSG and large quantities of a crude equine pituitary extract given to anestrous and cycling Pony and Thoroughbred mares stimulated the development of one follicle only. Similarly, the author and his colleagues, in many unpublished studies, have completely failed to induce superovulation in Welsh Mountain Pony mares given, at various stages of the estrous cycle, single and multiple injections of either highly or partly purified gonadotrophin preparations from various species and of both pituitary and chorionic origin. The mechanism of this marked resistance of the mare's ovary to exogenous gonadotrophic stimulation remains unknown and therefore a major hurdle. However, Lapin et al (1975, 1976) recently reported up to four ovulations occurring in mares treated daily for 14 days during anestrus or for 6 days during the estrous cycle with either a crude or partly purified extract of equine pituitary gland. The cost and difficulty of obtaining sufficient material for this purpose, together with the very long nature of the treatment, seem to make it impractical from the commercial point of view. It is, however, the only glimmer of light to date on an otherwise dark horizon so far as attempts to recover large numbers of horse embryos for experimental or other purposes are concerned.

EGG AND EMBRYO COLLECTION BY SURGICAL METHODS

The technique used for the surgical recovery of unfertilized eggs and embryos from the Fallopian tubes and uterine horns of mares is virtually identical to that developed originally for cattle and sheep by Lamming and Rowson (1952). The uterus and ovaries are exteriorized through a ventral midline incision with the animal in dorsal recumbency, a short length of bent glass or polythene tubing with beveled ends is ligated into the anterior one third of the uterine horn on the side of the recent ovulation, and 30-50 ml of medium is flushed through the Fallopian tube and into the ligated uterine horn via a blunted 18-gauge needle held in the fimbria (Allen and Rowson, 1975). Satisfactory embryo recovery rates of 77% have been achieved between days 1 and 6 after ovulation by this method. The main problem is the difficulty of exteriorizing the ovary and fimbria in some 2-year-old and maiden mares. This is caused by the 'shortness' and immaturity of the ovarian and broad ligaments in these animals and is more pronounced with the right ovary than the left. In recent studies, Allen, Rowson and Pashen (unpublished) have achieved good embryo recovery rates in small, 2-year-old Pony mares between days 7 and 10 after ovulation, by injecting 60 ml medium into the tip of the uterine horn and collecting the flushings via a cannula inserted at the base of the same horn.

The presence in the mare of a pronounced and very effective one-way-valve-like papilla at the uterotubal junction makes it physically impossible to 'milk' flushing medium back up the Fallopian tube from the ligated uterine horn as has been done in cattle and sheep. However, Betteridge and Mitchell (1975) have overcome this barrier by inserting a cannula through the uterotubal junction via a surgical incision in the tip of the uterine horn. These authors have reported a high recovery rate of fertilized and unfertilized eggs by this method.

There have been no reports to date of surgical egg recovery in the mare via a laparotomy incision in the flank. The depth and extent of the lateral abdominal wall musculature in the horse make this

route of exposure difficult. Also, post-surgical problems, including severe pain and wound breakdown occur more frequently after flank than ventral midline surgery in the horse.

EMBRYO COLLECTION BY NON-SURGICAL METHODS

The two reports to date of non-surgical recovery and transfer of horse embryos have given very encouraging results. Using adaptations of the instrument originally designed for use in cattle by Sugie (1965), and employing a continuous flow of medium, Oguri and Tsutsumi (1972) and Allen and Rowson (1975) have reported embryo recovery rates of 47 and 40%, respectively, when flushing a single uterine horn of mares between days 6 and 8 after ovulation. Using an instrument designed to occlude the cervix and flush the whole uterus with a continuous flow of 1500 ml of physiological saline, Oguri and Tsutsumi (1974) obtained the very high embryo recovery rate of 90% in 20 mares. Mitchell et al (ADRI, unpublished) have used two non-surgical systems to flush 15 mares on 27 occasions 6-14 days after ovulation. The embryo was recovered in 5/17 attempts with a simple in-out apparatus using 350-1000 ml of medium per flush and in 6/10 attempts with an arrangement using 100-700 ml of medium by continuous flow. One mare yielded an embryo on five successive flushes. The large size and ease of dilation of the mare's cervix, and the straight, tubular nature of her uterine horns allow the use of much bigger and more easily manipulated flushing instruments than is possible in cattle.

MEDIA, SHORT-TERM MAINTENANCE, CULTURE, AND PREDICTION OF VIABILITY OF EMBRYOS

None of these have been critically investigated in horses. Oguri and Tsutsumi (1972) used physiological saline supplemented with either 2% gelatine or mare's serum to recover embryos. Their successful transfers (Oguri and Tsutsumi, 1974) were with a medium comprising mare's serum and Ringer's solution in equal volumes and containing penicillin. Allen and his colleagues used TCM-199 in earlier transfers and Dulbecco's PBS, modified as described by Whittingham (1971), for more recent ones. The morphology of early embryos has been described and illustrated by Oguri and Tsutsumi (1972) and in Fig. 16 of this monograph. Tubal flushes may contain old unfertilized eggs in addition to the most recently ovulated one (Betteridge and Mitchell, 1975).

EMBRYO TRANSFER BY SURGICAL METHODS

The surgical method used for embryo transfer in the mare is identical with that used routinely in cattle. The embryo in a minimal volume of medium is inserted into the oviduct (days 1 and 2 after ovulation) or anterior end of the uterine horn (days 3-10 after ovulation), via a glass Pasteur pipette pushed gently through a small hole made initially in the myometrium and endometrium with the blunt end of a surgical needle.

As reported for cattle (Newcomb and Rowson, 1975a) and sheep (Moore and Shelton, 1964b), the success rate of surgical embryo transfer in the mare seems to increase as the interval between ovulation and transfer increases; although only 1/8 embryos transferred between days 1 and 3 after ovulation continued to develop in Welsh Pony mares, 6/8 embryos transferred between days 4 and 6 after ovulation resulted in pregnancies (Allen and Rowson, 1975).

Any effect that the site of transfer might have on results has not been examined but since a very high rate of transuterine migration of embryos is known to occur spontaneously in the mare (Day, 1940; Bain and Howey, 1975) it seems likely that the side of embryo transfer in relation to the corpus luteum may be less important than in other species. This contention is supported by experience with non-surgical transfers (see below).

EMBRYO TRANSFER BY NON-SURGICAL METHODS

Even more encouraging than embryo recovery figures, has been the pregnancy rate following non-surgical transfer to synchronized recipient mares between days 6 and 8 after ovulation. Allen and Rowson (1975) reported 5/7 (71%) successful transfers and, since that report, a further 3/4 successful non-surgical transfers have been carried out. In most of these transfers, the embryo was simply deposited in the body of the uterus of the recipient mare and no special attempt was made to place it in the uterine horn ipsilateral to the corpus luteum. Thus, from the results of the few studies undertaken, it appears that both the technique and the results of non-surgical embryo recovery and transfer may prove better in the horse than the cow. The results so far also indicate a pregnancy rate for non-surgical transfer that is equal to or better than what can be achieved by surgical transfer in mares.

INFLUENCE OF SYNCHRONY AND METHODS OF SYNCHRONIZATION ON RESULTS

Although the precise degree of synchrony of ovulation between donor and recipient for successful embryo transfer has yet to be determined in the mare, reported studies in cattle and other species suggest that this interval should not be any greater than 48 h. The long period of estrus exhibited by the mare, and the marked individual variation between mares in both length of estrus and time of ovulation in

Figure 17. Coney, this Welsh Mountain Pony foal, was conceived and collected in England, transported to Poland in a rabbit, transferred and born there to her new 'mother' Morfina in time to be displayed to delegates at the 8th International Congress on Animal Reproduction and Artificial Insemination at Krakow in July 1976.

Photo by courtesy of W. R. Allen.

relation to the onset of estrus, have made accurate synchronization of ovulation in donor and recipient mares difficult. This problem has largely been overcome by the use of prostaglandins, and a host of studies recently have demonstrated the luteolytic action in the mare of $PGF_{2\alpha}$ and various synthetic PG analogs. As in the cow, it is not possible to synchronize estrus with a single injection of PG in all the individuals of a group of mares selected at random and therefore at various stages of the estrous cycle. To overcome this difficulty, Palmer and Jousset (1975) adapted for use in the mare the double PG injection technique originally developed for cattle by Cooper and Rowson (1975). All the mares of the group are given a luteolytic dose of Fluprostenol (Equimate, ICI) on day 0, designed to induce luteolysis followed by a return to estrus in 3 days in those animals that happen to be between days 4 and 13 of diestrus. All the mares are then given an ovulation-inducing dose of HCG (2000-3000 IU) on day 6 to hasten ovulation in those in estrus at the time. On day 14, when all the mares should be between days 4 and 15 of diestrus, they are again treated with Fluprostenol to induce luteolysis and a return to estrus in 3 days. Finally, on day 20 or 21, the donor mares are covered or inseminated and all the mares again injected with HCG to induce ovulation. Preliminary results using this technique for synchronization are most promising and in a recent experiment involving the successful transport of horse embryos between England and Poland in the ligated oviducts of rabbits (Allen et al, 1976) ovulation occurred during a 24 h period in 5/7 English donor mares and 5/6 Polish recipient mares, treated simultaneously with Fluprostenol and HCG as described above.

CONCLUSIONS

The now standardized techniques of embryo recovery, transport and transfer have been tried and shown to work well in the mare. Many further experiments are required to validate and fully define the optimum techniques and results obtainable. However, until such time as the dual problems of ovarian resistance to gonadotrophic stimulation and breed-society resistance to scientific advancement are overcome, there seems little commercial future for the techniques in this species.

[A service is now being offered by one commercial company in Ontario. — *Editor.*]

LOW TEMPERATURE PRESERVATION OF EMBRYOS

D. G. Whittingham

INTRODUCTION

Embryo storage is potentially a valuable adjunct to the embryo transfer technique. Apart from relieving the demand for having all recipients at the correct stage of synchronization for embryo transfer on the day of embryo collection from the donor, other major advantages of embryo storage accrue; e.g., transport of embryos between countries, conservation of valuable genetic material, control of genetic stability within breeds, possibility of shortening the generation time for pregnancy testing and the preservation of rare breeds.

Until 1972, when mouse embryos were shown to survive storage at −196°C (Whittingham et al, 1972; Wilmut, 1972a), the few attempts to preserve mammalian embryos at low temperatures had met with limited success (Whittingham, 1973, 1974). These earlier investigations indicated that short-term storage at temperatures above freezing point was more successful than storage at lower temperatures, but observations were made on small numbers of embryos and no assessment was made of the most suitable preimplantation stage(s) for preservation. The following account reviews progress that has been made over the past 4 years on low-temperature storage of embryos from farm animals at 0°C and above (short-term storage) and at sub-zero temperatures (long-term storage).

SHORT-TERM STORAGE

Logically, before invoking procedures for the deep-freeze preservation of embryos, it is necessary to determine the potential viability of embryos cooled to around 0°C. Clearly, storage of embryos at temperatures near 0°C can be of limited duration only, because of the instability of cellular components, especially enzymes, at such temperatures. Nevertheless, storage of embryos for 1 or more days around 0°C would enable embryo transfer to be spread over several days and even allow transport of embryos between countries. The most important advantage of this method of storage is that the embryos do not have to be subjected to the procedures of freezing and thawing.

Table 15 summarizes some of the main observations that have been made on short-term storage *in vitro* at 0°C and above. Viability of stored embryos varies at different developmental stages and also between species. Attempts to cool 8-cell pig embryos below 15°C have failed (Wilmut, 1972b) although a few cells of the early blastocyst may survive cooling (Polge, personal communications).

In cattle, sensitivity to cooling and storage appears to decrease during pre-attachment development. Survival at the blastocyst stage is high, 92, 67 and 48% surviving storage for 30 min, 24 h and 48 h, respectively (Trounson, Willadsen and Rowson, 1976). Reports on the survival of morulae are variable and may reflect differences in the actual developmental stage of the embryos at the time of collection. Only limited development of earlier cleavage stages was observed, but both Sreenan et al (1970) and Trounson, Willadsen, Rowson and Newcomb (1976) indicated that slow cooling increased the survival rate although the differences were not significant.

In sheep, only one report so far indicates that early embryos (2- to 16-cell) are more sensitive to cooling to 0°C than morulae stages, but the number of embryos used in the study was small (Willadsen, Polge, Rowson and Moor, 1976). Other workers have shown that early sheep embryos, especially the 8- to 16-cell stage, store quite successfully at temperatures ranging between 0 and 13°C for periods of up to 10 days (Table 15). Varying the cooling rate to 5°C does not appear to affect subsequent survival of sheep embryos (Moore and Bilton, 1973).

In goats, late morulae and early blastocysts stored at 5°C for 1-2 days, continued development *in vitro* and kids were born following transfer. Cooling of earlier stages was not examined (Bilton and Moore, 1976b).

Media for collection and storage have varied greatly between studies, e.g., from 100% homologous serum and mixtures of serum and physiological salt solutions to physiological salt solutions plus bovine serum albumin (Table 15). Very little work has been done on evaluating media for storage at low temperatures. Trounson, Willadsen, Rowson and Newcomb (1976) found no beneficial effect of adding fetal calf serum to PBS for cooling cattle embryos. Obviously, media without serum are preferable since the risk of disease transfer is reduced. Differences in the types of collection media do have marked effects on subsequent survival of uncooled embryos following transfer (see pages 20-24 of this monograph). It is interesting to note in this connection that early hamster embryos (1- and 2-cell) fail to develop when collected and transferred in a bicarbonate-buffered medium (Tyrodes), but continue development when the procedures are performed in PB1 (Whittingham and Bavister, 1974). Also, early mouse embryos survive storage at 0°C for longer periods (up to 5 days) in PB1 (Whittingham, 1974). Early embryos are probably more sensitive to variations in pH, an aspect warranting further examination.

In conclusion, it is difficult to draw valid comparisons between the different reports since conditions and procedures for short-term storage vary greatly. Differences in sensitivity to cooling between species and embryonic stages are apparent

TABLE 15. TECHNIQUES USED IN AND RESULTS OBTAINED WITH EMBRYO STORAGE AT TEMPERATURES BETWEEN 0 AND 10°C

Minimum temp, °C	Storage time	Medium	Developmental stage	Subsequent development	Reference
Cattle					
10	24 h	Bovine serum	2-cell to morula	Few cleavage divisions *in vitro*	Pincus (1949)
10	20-24 h	Bovine follicular fluid	4- to 10-cell	Limited number of cleavage divisions in rabbit oviduct	Sreenan et al (1970)
0-7.5	2 min-24 h	PBS and PBS + 20% FCS	8- to 24-cell	Limited development to blastocyst in rabbit oviduct	Wilmut et al (1975) Trounson, Willadsen, Rowson and Newcomb (1976)
0-7.5	2 min-24 h	PBS and PBS + 20% FCS	Morula (~ 32-cell)	Variable development to blastocyst stage (7-78%) in rabbit oviduct; birth of calves following transfer	
10	24 h	Calf serum: Locke's solution (1:1)	Morula (~ 32-cell)	Less than 20% normal development *in vitro*	Renard, Wintenberger-Torres and du Mesnil du Buisson (1976)
0	30 min-48 h	PBS + 20% FCS	Blastocyst	Good survival *in vitro* (up to 92%); normal fetuses 12-26 weeks after transfer	Trounson, Willadsen and Rowson (1976)
6	1-4 days	PBS + 25% bovine serum	Late morula and blastocyst	Blastocyst expansion *in vitro*; fetuses 8 weeks after transfer	Bilton and Moore (1976a)
Sheep					
0-8	6-72 h	Sheep serum	2- to 12-cell	Birth of lambs	Averill and Rowson (1959)
	6-9 h	Ringers	2- to 12-cell	None	
0	24 h	Sheep serum	~ 8-cell	Birth of lambs	Loginova (1961)
4-13	1-10 days	Sheep serum: Locke's solution (1:1)	8- to 16-cell	Birth of lambs	Kardymowicz et al (1963, 1964, 1966, 1971) Kardymowicz (1972) Renard, Wintenburger-Torres and du Mesnil du Buisson (1976)
5	10 min-2 days	PBS + 20% sheep serum	1-cell to morula	Cleavage *in vitro*; birth of lambs	Moore and Bilton (1973)
0	15 min	PBS	2-cell to morula	Cleavage in rabbit oviduct	Willadsen, Polge, Rowson and Moor (1976)
Goats					
5	24-48 h	PBS + 25% goat serum	Morula and early blastocyst	Blastocyst expansion *in vitro*; birth of kids	Bilton and Moore (1976b)
Pigs					
0-20	> 2 h (?)	PBS	8-cell	No survival after storage at 0 and 10°C but good survival at 15 and 20°C	Wilmut (1972b)

and this is an important area for further investigation. Closer examination of changes occurring in the embryos during cooling to 0°C is necessary. Are there configurational changes occurring in cell and mitochondrial membranes that might be observable at the ultrastructural level or indicated by measuring ionic fluxes across the cell membranes of the blastomeres during cooling?

So far, the results on the storage of embryos at low temperatures above freezing point are encouraging; there is reasonable survival of cow embryos after 48 hrs at 0°C (Trounson, Willadsen and Rowson, 1976) and of sheep embryos after 10 days at about 10°C (Kardymowicz, 1972). This aspect of embryo storage has distinct advantages over deep freezing for short-term preservation, as mentioned earlier, and should be examined in much more detail for it has an immediate practical application.

LONG-TERM STORAGE

The demonstration that mouse embryos survive storage at −196°C (Whittingham et al, 1972; Wilmut, 1972a) was an exciting finding for cryobiologists, reproductive physiologists and mammalian geneticists. The ability to store mammalian embryos at temperatures where no observable deterioration of mammalian cells with time has been observed provides a unique opportunity for the preservation and conservation of valuable genetic material.

The development and application of the technique to farm animals has been carried out almost solely at the Animal Research Station in Cambridge. Table 16 summarizes the results obtained for cattle, sheep and goat embryos. In general, the technical procedures of freezing and thawing are similar to those used for mouse embryos and are based on the initial finding that embryos are especially sensitive to high rates of cooling and warming.

Dimethylsulfoxide (DMSO) has been used as the cryoprotective agent in all studies except for goats where glycerol appeared to afford better protection (Bilton and Moore, 1976b). However, the numbers of embryos were small and the two additives were compared at only one concentration level. Whittingham et al (1972) showed that glycerol was not as effective as DMSO in protecting mouse embryos during freezing and thawing but, in subsequent work, high rates of survival of 8-cell mouse embryos were achieved with concentrations of glycerol ranging between 0.5 to 4.0 M (Leibo and Mazur, 1974). Optimal survival of cattle and sheep embryos at the stages examined occurs with cooling rates ranging between 0.13 and 0.30°C/min and warming rates for morulae and early blastocysts of 1.2 to 12°C/min. Late cow blastocysts (days 10-13) were warmed most effectively at 360°C/min (Wilmut and Rowson, 1973b) but only a few early sheep and cattle blastocysts survived re-warming at this rate (Willadsen, Polge, Rowson and Moor, 1974, 1976; Willadsen, Trounson, Polge, Rowson and Newcomb, 1976)

TABLE 16. TECHNIQUES USED IN AND RESULTS OBTAINED WITH EMBRYO STORAGE BELOW 0°C

Minimum temp, °C	Storage time	Medium	Development stage	Subsequent development	Reference
Cattle					
− 196	2 h-several months	PBS + 1-2 M DMSO	8-cell and morula	Poor survival in rabbit oviduct	Wilmut and Rowson (1973a) Willadsen, Trounson, Polge, Rowson and Newcomb (1976)
− 196	2 h-several months	PBS + 1-2 M DMSO	Late morula and blastocyst	Re-expansion *in vitro*; birth of calves	Wilmut and Rowson (1973b) Willadsen, Trounson, Polge, Rowson and Newcomb (1976) Bilton and Moore (1976a)
Sheep					
−15 to −60	0	PBS + 1.5 M DMSO	Morula and blastocyst	Majority continued in rabbit oviduct	Willadsen, Polge, Rowson and Moor (1976)
− 196	12 h-1 month	PBS + 1.5 M DMSO	Morula and blastocyst	Some formed expanded blastocysts in rabbit oviducts; birth of lambs	
Goats					
− 196	2-4 weeks	PBS + 25% goat serum + 2 M DMSO; PBS + 25% goat serum + 1 M glycerol	Morula and early blastocyst	Blastocyst expansion *in vitro*; birth of kids	Bilton and Moore (1976b)

Figure 18. Frosty II at 1 day old. A healthy bull calf born in Cambridge from an embryo that had been kept for a week of suspended animation in a deepfreeze. This was the first calf born after freezing as an embryo.

Photo from British Information Service.

as in the initial report with mouse embryos (Whittingham et al, 1972). It appears that these very low cooling rates are necessary to effect dehydration of the embryos (by osmotic loss of water) before the whole embryo itself becomes frozen. This prevents or minimizes the amount of intracellular ice formation which is one of the main factors causing damage during thawing. Any factors that cause a sudden increase in the rate of cooling, e.g., supercooling or transfer of embryos directly to liquid nitrogen before the embryos have become adequately dehydrated, are deleterious to the embryos. For optimal survival of mouse embryos, slow cooling to approximately $-60°C$ is necessary before transfer to liquid nitrogen (Leibo, Mazur and Jackowski, 1974) and, as a safeguard, embryos are usually slowly cooled to $-70°C$ or $-80°C$ before direct transfer to liquid nitrogen.

At present, the ability to preserve large-animal embryos is determined, in part, by their survival after cooling to 0°C. Without effective procedures for cooling early stages of cow embryos to 0°C, their preservation by freezing is not possible.

To date, the viability of later-stage cattle, sheep and goat embryos after freezing and thawing has been demonstrated. Considering the limited numbers of embryos that have been frozen and stored at $-196°C$ (about 54 sheep and 250 cattle embryos) the results are very encouraging and great credit is due to the technical expertise of the workers concerned.

Overall survival of frozen-thawed mouse embryos to fetuses and live births in a recent fairly extensive study was approximately 36% (479/1330, Whittingham and Lyon, 1976). Survival rates of frozen-thawed cattle and sheep embryos have yet to be determined on a similar scale but will need to be higher than this if long-term embryo storage is to become a practical procedure.

SECTION TWO

APPLICATIONS OF EMBRYO TRANSFER IN ANIMAL PRODUCTION

Contributors to this section review the present and potential uses to which embryo transfer may be put in the agricultural industry. Commercial uses are direct and their advantages and shortcomings can be more readily appreciated than the indirect benefits accumulating through the use of embryo transfer in reproductive research.

THE GENETICS OF BREED IMPROVEMENT

R. B. Land

Genetic improvement necessitates the substitution of genetically superior animals for those of little genetic merit. The early stages of an improvement program may involve the change from one breed to another, either directly or by crossing, and it is here that embryo transfer may be used to increase the reproductive rate of valuable animals, and so facilitate their distribution. Where it is appropriate to improve performance by crossing, embryo transfer may also aid the introduction of new breeds if veterinary controls restrict the movement of livestock. In general, however, and particularly in countries with a developing livestock industry, a logical sequence may be first to import semen and evaluate the performance of crosses and possibly backcrosses with native breeds. Only then would it be appropriate to consider the introduction of purebred animals, by embryo transfer if necessary, to give a local source of semen. Once the most suitable breed has been chosen for a particular purpose, further improvement depends on increases in the frequencies of favorable genes already segregating in the population. It is pertinent to consider the possible contribution of embryo transfer to the rate of such improvement.

The rate of genetic improvement within a breed or population depends on: 1. the accuracy with which genetically superior animals can be identified and selected as parents of the next generation; 2. the superiority of selected animals above the mean performance of the group from which they were selected; and 3. the time interval between generations. To explain these further:

1. The genetic merit of an individual can be estimated from its own performance and/or indirectly from the performance of its relatives. Either way, the accuracy of the assessment depends on the heritability of the trait selected for, which is measured statistically as the proportion of the overall variation in the trait that arises from genetic sources. The higher the heritability, the more accurately will an individual's performance, or the performances of its relatives, reflect its genetic merit.
2. The superiority of an individual, or a group of individuals, above the overall population mean depends on both the intensity of selection (i.e., the proportion of individuals chosen) and the variance of the trait in question. The more intense the selection and the higher the variance, the greater the superiority of selected animals.
3. The generation interval is the mean of the ages of males and females when their offspring are born.

Embryo transfer may help in genetic improvement through increasing the reproductive rate, especially in conjunction with superovulation, in several ways. The higher the reproductive rate, the smaller the proportion of animals that need to be selected for breeding and, hence, the greater their superiority. The more offspring an individual produces the more accurate is its progeny test. Further, if maternal effects are important, they may be eliminated by embryo transfer, and the accuracy of selection increased. As with all genetic selection the number selected and the population size will be influenced by the rate of inbreeding tolerable. These contributions of embryo transfer to genetic improvement can be considered under several headings.

INCREASE IN THE ACCURACY OF PROGENY TESTS

The reproduction rate of males is sufficient for them to be progeny-tested as accurately as testing facilities allow, i.e., male reproductive rate is not limiting, and embryo transfer would not be of any advantage. With females this is not so, and increasing the number of offspring per female by embryo transfer would facilitate the progeny testing of females and the accuracy of female selection. The relevant question, however, is how much extra information the progeny test would provide. With dairy cattle, for example, Miller (1975) shows that the response per generation would be improved by 20% if four progeny records were available in addition to a single cow record, or 12.5% if four progeny records were added to five records of the cow's own yield. However, the collection of progeny records would extend the time interval between generations, especially if, as would be reasonable, only females with good first lactation records were subsequently progeny-tested. The increased accuracy would therefore be counterbalanced by the longer generation interval. Miller (1975) and Hill and Land (1976) conclude that the progeny testing of females would add little to the progeny testing of males in national or large-scale breeding programs.

INCREASE IN SELECTION INTENSITY

The reproductive rate of males is high enough that, not only can they be progeny tested efficiently, but the intensity of selection among them can be independent of their reproductive rate. Among female cattle and sheep, selection is usually at the expense of lengthening the interval between generations. Improvements in the reproductive rate, therefore, would enable the intensity of selection among females to be increased, potentially to a level comparable with that already achieved for males. The selection intensity per year is the arithmetic mean of that among males and that among females divided by the mean generation interval of the two sexes. As a result, no matter how great the number of offspring per female, the greatest increase in the rate of response through changes in the selection

intensity would be two-fold. Detailed considerations showed that most of this increase could be achieved with about six offspring per donor (Land and Hill, 1975). Increases in egg yield above this level had only a small further effect on the selection intensity and, hence, on the rate of response to genetic selection. Such a two-fold improvement could be well worthwhile, especially in large-scale (possibly national) breeding programs where the improvement made in an elite herd can be spread over a large population, for example by AI.

The possible doubling of the rate of improvement applies where the selected trait can be measured in both sexes. However, many traits of economic importance are sex-limited to females, e.g., reproductive and lactational traits. Superovulation might have a role to play here, for selection is restricted to females, and males can only be chosen on the basis of the performance of their female relatives. With dairy cattle, the use of AI in most developed improvement schemes gives a ready-made structure for progeny testing. Nevertheless, Skjervold (1974) Hansen and Neimann-Sørensen (1974) and Cunningham (1976) all conclude that the addition of superovulation and embryo transfer to existing schemes could only lead to improvements of around 2.5% in the overall rate of genetic progress by increased selection among bull-dams. Further improvement may also be made by improving selection among cow-dams, but ultimately this would mean that all reproduction was by embryo transfer, so that the expense would be far greater. Miller (1975) concludes that this would not be worthwhile. The use of superovulation and embryo transfer in selection for milk yield would therefore have a marginal effect and certainly less than could be achieved by optimizing schemes using conventional reproduction.

The existence of very large AI schemes complicates the establishment of small elite herds amenable to embryo transfer, and in which the costs can be carried by the distribution of the improvement to a much larger population. In a closed dairy herd, for example, the improved genetic progress that would accrue from an increased ability to select among females would be counterbalanced by the difficulty of assessing the breeding value of bulls if the herd were too small for progeny testing. To be useful, therefore, such a herd would require the availability of additional facilities for the progeny testing of its males, as proposed under conventional reproduction by Hinks (1977).

Krausslich (1976) suggests that in a dual-purpose breed it may be possible to increase selection applied to growth through performance testing while maintaining that applied to milk. For example, it would be possible to select among several sons of a chosen bull dam according to their growth characteristics. Such a system would not, unfortunately, enable selection to be made on dairy conformation without reducing potential selection for yield as both traits are sex-limited to females.

By contrast to lactation, where there is a specific progeny-testing system, suitable records are not available in several situations where it may be desirable to improve production. Land and Hill (1975) conclude that superovulation and transfer may make selection for twinning a possibility in a population of beef cattle. Even then, however, the rates of response may only be of the order of 0.5% per year and very dependent upon the incidence of twinning in the base population. With Bowman (1976), they conclude that selection in dairy cattle should be concentrated on milk production rather than twinning. Twinning is more fully discussed on page 62 of this monograph.

In sheep, and particularly in pigs, higher female reproductive rates reduce the potential benefits of embryo transfer for genetic improvement within populations. The advantages of embryo transfer have been considered low, and have received little attention in the literature. As with cattle, however, the economic merit partly depends on the size of the population to be 'served' by an elite nucleus breeding stock.

GENETIC CONTROLS AND GENE BANKS

The assessment of genetic improvement schemes through comparison with individuals of the original population, conserved as embryos (see pages 52-53), is now a possibility. The relative merits of the use of conserved embryos or conserved semen from the base population as a source of comparison partly depends on the objects of the experiment. If the improvement scheme is designed to produce better sires for crossing, the more useful comparison would be between crossbred offspring sired by potentially improved versus original males. If, however, the object were to be to produce animals whose productivity as purebreds was important, the more useful comparison would be with individuals conserved as embryos. In the crossbreeding situation, the additional conservation of crossbred embryos would assist the separation of possible improvement in the male from changes in the female.

The usefulness of gene banks would similarly depend on the objectives. If it were to be regarded strictly as a reserve of alleles that might be useful in the future, the storage and transfer of embryos would offer no advantage over the conservation of semen. If, however, the productive performance of breed were thought to be potentially useful, and if this performance were considered to be dependent on gene interactions within that breed and hence difficult to recreate by crossing, the conservation of embryos would have definite advantages.

FUTURE DEVELOPMENTS

Simplified transfer may aid the movement of animals and, hence, breed improvement by crossing.

However, the effects of future developments on the rate of genetic gain within populations are unlikely to be large. Existing techniques are sufficient to facilitate selection among females and, if properly utilized, the benefits are already sufficient to carry the cost when spread over a large 'service' population. Non-surgical recovery and insertion, more predictable embryo yields, and the sexing of embryos might, for example, reduce the cost, but would not significantly increase the potential rate of improvement. Larger embryo yields would have a small effect and, similarly, the availability of embryos from very young females would only make a significant contribution if criteria for the selection of young animals could also be developed.

As opposed to the *rate of improvement,* the availability of large numbers of embryos from elite cows would increase the *average genetic merit* for the population over which it could be spread.

Theoretically, the maximum improvement could be about two-fold; in practice it will almost certainly be less. In relation to AI, very large numbers of embryos will have to be available and inserted at low cost before embryos displace semen.

CONCLUSIONS

The potential benefits from the use of superovulation and embryo transfer are most likely to be realized in situations where the cost can be carried by an ability to spread the superiority of elite selected herds to a relatively large population, e.g., with the use of AI. This advantage would apply directly to traits that can be measured on growing animals of both sexes, e.g., beef traits. Where extensive progeny testing is used to facilitate the improvement of sex-limited traits, such as milk yield, selection within elite herds may be advantageous only with the use of outside testing facilities. As with all genetic selection, the financial benefits will only be as great as the economic importance of the trait in question.

COMMERCIAL EMBRYO TRANSFER IN NORTH AMERICA AND AUSTRALASIA

R. P. Elsden

CATTLE

The application of embryo transfer to the cattle industry has been an interesting but controversial subject. There are now (1976) 20 listed commercial embryo transfer units in North America, three in Australia and six in New Zealand. These numbers fluctuate as new units start and as some of the older ones become defunct, having experienced the unpredictable and uncontrollable problems of obtaining viable embryos from donors. The unreliability of and risks involved in the procedure have led to a tenuous economic existence for the professional embryo transfer units. Capital investment in many cases has been considerable and naturally has led to many units continuing in business with the hope that something new will turn up, or that their own practical, but of necessity limited, research would solve the problems. Methodology was secretive and surrounded by an aura of mystique which, with the aid of misleading advertising in some cases, carried the embryo transfer procedure on the crest of a wave that had to run out when the exotic breed boom predictably crashed, or when no new information came forward from the research organizations.

The reasons given for the establishment of commercial embryo transfer units are:

1. To multiply exotic breeds of cattle.
2. To obtain progeny from genetically superior cows.
3. To obtain calves from proven infertile cows and prepuberal heifers.
4. To provide a mechanism to progeny-test females.
5. To build a herd of dairy or beef cows from a few superior base cows.
6. To provide embryos so that commercial beef cows can be twinned.
7. To transport embryos instead of animals.

The multiplication of exotic breeds has been the incentive for the establishment of embryo transfer units, but it is a short-term venture. The following discussion covers all the above points plus the possibilities of long-term projects, based on sound objectives, utilizing embryo transfer techniques.

With the publication of the Cambridge work (Rowson et al, 1969), in which 91% pregnancy rates were obtained, there was a rekindled interest in this method of reproduction. It was fortunate that at that time the exotic breed boom came along, providing both a stimulus and funding for embryo transfer. From 1968, first Charolais and then other European breeds showed superior weight gains over the traditional British breeds in progeny testing of beef bulls in Britain (Milk Marketing Board, 1973-4). This stimulated interest in the United States and Australasia, with the result that the 'new' breeds quickly gained popularity. Their supply was limited by stringent quarantine regulations and could not meet the rapidly increasing demand. American imports had to come via Canada; and Australian farmers were at the end of a line commencing in France, then to England and from there to New Zealand before finally reaching Australia at a highly inflated, unrealistic price. This situation provided an ideal environment for embryo transfer with the chance to obtain many calves from one female in the same year.

Similarly, multiplication of animals or certain families popular today in the market place, remains a good reason for utilizing embryo transfer and is a short-term profitable venture for a few owners of these animals.

The possibilities and shortcomings of embryo transfer as a tool in genetic improvement have been discussed in the preceding pages.

Embryo transfer has proved a useful method by which offspring can be obtained from infertile cows (Bowen et al, 1977). The infertility of potential donors should not be of genetic origin but as a result of disease, injury or aging. Success rates are of course lower in this group than those obtained from healthy donors. Thirty-seven pregnant recipients have resulted from superovulating and transferring embryos from 34 operations on 25 cows which had had extended periods of infertility before being admitted to our program at Colorado State University. In 11 of the cows no embryos were transferred. Pregnancies per donor ranged from none to seven and were obtained from 13 cows. Many infertile cows have to be treated for their problems on admittance and it usually takes longer to establish estrous cycle lengths in these animals. When considering the extended preparatory period plus the lower pregnancy rates, costs are naturally greater. For an infertile cow to be selected by the owner for embryo transfer, she must be superior to her contemporaries, either genetically or as a popular cow whose progeny will realize a quick profit.

Superovulation of calves or prepuberal heifers is still in the research stage as described on pages 6-9.

For twinning to be economically feasible, embryos must be transferred non-surgically. Developments in this area are discussed on pages 31-34.

Success rates in commercial practice are difficult to assess. In some reports all donors tried are included in the results, but some units only take into their calculations the successful donors, i.e., those cows yielding embryos resulting in pregnancies. After consulting members of the industry, it appears that an efficient organization using fertile donors averages between two and three pregnant recipients per embryo recovery attempt. In 1974, Graham reported the results from seven embryo transfer units. Of the donors treated, 73.5% responded to the gonadotrophin treatment, yielding an average of 8.2 ova. Of the collected ova, 65.5% were fertilized, giving an average of 5.3 per donor that were considered transferable. Of these, only 2.2 per donor resulted in pregnancies. However, the number of recipient pregnancies from one recovery attempt ranged from 0 to 32.

It is said that most donors superovulated on repeated occasions give decreasing responses and that animals in which response is improved on the second attempt are usually limited to those responding poorly on the first occasion. Firm evidence for this is lacking (see page 1), but a very real limitation to repeated successful surgical recoveries of embryos is the increased difficulty in exteriorizing the uterus and cannulating the oviduct due to deposition of scar tissue. Therefore, most donors undergo two surgical recoveries and are then bred to carry their own pregnancy. It has been estimated that even with the best techniques and experience 1-2% will be rendered infertile and 10% subfertile after the first surgery. With two surgeries risks are presumably doubled.

Conclusions — It has become apparent that commercial embryo transfer in cattle is a tenuous economic proposition when this activity is the only source of income. This is due to unreliable superovulatory responses, risks of causing damage to the donor using surgical methods of recovering ova and the decreasing monetary value of individuals in the exotic breeds. However, the technique can be an advantage under special circumstances, and, with recent progress in non-surgical recoveries, at least one risk has been largely eliminated.

SHEEP AND GOATS

There are no reported embryo transfer units dealing with sheep or goats in North America or Australasia. There is, however, a suitable environment in Australia for the establishment of such a unit. Three million dollars worth of Mohair is imported into Australia annually, as there are very few Angora goats in the country. Imports are severely restricted because of stringent quarantine regulations and so the resident Angora is at an unrealistically high price, with the demand exceeding the supply (see also page 77).

PIGS

The first reported practical application of the technique in pigs was described by Curnock et al in 1975. There are now six commercial pig embryo transfer units in North America, but as yet none listed in Australasia. Reasons for establishment of commercial embryo transfer units are:

1. To utilize the technique for health programs by introducing new blood lines into closed herds; and by enabling owners of herds with known disease problems to sell progeny.
2. To recover embryos from infertile sows.
3. To multiply the progeny from proven sows.

The efficacy of control and elimination of swine diseases through re-population with specific-pathogen-free (SPF) pigs, utilizing hysterectomy techniques, is well established. Curnock et al (1975) have demonstrated that superovulation and embryo transfer is an effective alternative method for introducing disease-free pigs into an SPF herd. They reported that the donor can be re-bred sooner than she

could have been had she undergone a normal pregnancy and, obviously, more offspring can be obtained from each donor than is possible by hysterectomy.

Jason (personal communication) reports good success rates with infertile sows, sometimes recovering 100-150 embryos from one donor. The average number of corpora lutea produced as a result of PMSG stimulation is 25-30. Although fertilization rates vary from 0 to 100%, 90% is not unusual. Numbers of embryos collected vary from 0 to 30, but average 11.8; and, of 37 sows receiving an average of 11.5 embryos, 28 (78%) gave birth to 7.8 pigs each. Surgical recovery can be repeated every 3 weeks and apparently good results are obtained without the use of gonadotrophins as superovulatory agents.

It would appear that success rates in the pig are greater than those obtained in cattle. An additional advantage in the sow is that much less scar tissue follows surgery, so that surgical recoveries can be repeated several times.

[It should be noted that Polge (page 44), from long experience, estimates that surgical recoveries are usually limited to three operations. — *Editor*].

COMMERCIAL EMBRYO TRANSFER IN EUROPE

R. Newcomb

The involvement of commercial services in the field of embryo transfer has to date been restricted to work in cattle. The main interest has been centered in Britain and Ireland which are free of foot-and-mouth disease, and the stimulus for activity was almost totally generated by the inflated prices paid for some exotic (European) breeds of cattle in North America. The first group was operating in Britain in 1972, but others did not follow with the same enthusiasm as in North America until later. At the peak of activity in 1974-75 some eight groups of assorted sizes were active in Britain and two in Ireland.

The bursting of the exotic bubble associated with the economic recession in the West (1975-76) led to an equally rapid reduction of activity and in liquidation of some commercial groups. Only one group remains active on any scale in Britain (1976) but there is no loss of interest in the technique, just the absence of commercial justification. There are, in fact, signs of a renewal of activity at a level less dependent on the high-priced exotic cattle. A new group in Britain is offering a non-surgical egg recovery service and interest is increasing in the prospect of deep-freezing bovine eggs, both as a means of rationalizing the use of excess numbers of eggs and for export purposes.

In France, there is interest and activity in minor surgical recovery and transfer of the eggs of outstanding cows. This interest is channeled through SERSIA (Société d'Etudes et de Recherches Scientifiques sur l'Insemination Artificielle).

In Denmark, the technique has been used surgically by one group for multiplication of a non-native breed of cattle (the Belgian Blue); and Rasbech reports that a non-surgical service is being offered on the farm through a cooperative AI center and the veterinary college in Copenhagen.

In Germany, the technique is being used for genetic research and a commercial, non-surgical service will be offered in the latter part of 1976.

In Poland, egg transfer is used for breed improvement through a university clinic of obstetrics.

Many organizations and, in particular, the existing AI networks are standing on the sidelines waiting for a time when there is justification for their involvement. Some AI organizations such as SERSIA and the Milk Marketing Board of England and Wales are actively sponsoring research.

The scale on which units have operated has been small compared with North American organizations, with 145 donor operations during the year 1974 being the most achieved by any of the 11 groups replying to a questionnaire. The methods used and success rates of five groups providing satisfactory information are shown in Table 17.

Of all donor cattle entering the egg transfer program, about one third were unproductive. More than half of this failure was attributable to inadequate or total absence of superovulation in response to treatment. The reasons stated by groups replying to this questionnaire for having used egg transfer and anticipated uses in cattle were the same as those advanced in North America (page 59), plus the use of outstanding Friesian cows as potential bull mothers. In general, commercial units have been centered on farms with exotic donors and veterinarians are employed by the unit owners. Custom services centered on veterinary practices (as in North America) are rare in Europe.

It would appear that one of the most probable ways in which the technique will be applied non-surgically in future will be through veterinary practices unless the value of some breeds increases to inflated levels again, irrespective of their individual genetic value.

The use of the egg transfer technique by AI organizations may be limited unless incorporated into schemes of genetic improvement and for the collection of eggs from potential bull mothers. The more speculative aspects of non-surgical recovery from the better cows belonging to participating members could then be a quite natural extension of these programs. The cost of egg transfer will always be higher than AI, and its application to general farm practice on a wide scale must ultimately depend on the efficiency of the techniques and the market value of the calf produced.

TABLE 17. RESULTS ACHIEVED FROM SURGICAL EMBRYO TRANSFER IN CATTLE BY FIVE COMMERCIAL GROUPS IN EUROPE

Group	No. cattle						Donors[1]							
	Jan 74/75		Jan 75/76		Projected Jan 76/77		Minimum ovulations acceptable	Estrus	Failing %	Succeeding				
										%	Eggs/donor		No. recipients	
	Donor	Recip-ient	Donor	Recip-ient	Donor	Recip-ient					Total	Normal	Per donor	Pregnant per donor
A	0	0	35	178	50+	—	8	PG or progestin synchronized	36	64	—	3.6	—	2.3
B	55	650	70	750	0	0	4-5	PG synchronized or natural	35	65	9.1	5.9	5.7	2.4
C	10	65	4	29	?	—	3	PG synchronized	25	75	9	7	7	3.5
D	0	—	75	—	0	—	1	PG synchronized or natural	25	75	5.7	3	5.1	2.6
E	—	—	12	59	0	—	—	—	25	75	8	5	6	2.1

[1]All superovulated with PMSG (1800-2500 IU) and flushed surgically.

EMBRYO TRANSFER FOR THE INDUCTION OF TWINNING IN CATTLE

J. M. Sreenan

INTRODUCTION

The main cost of production in the beef cow herd is that associated with cow maintenance. Because of this, cows producing twin calves would be more efficient than cows producing single calves. Total calf crop from the beef cow herd is the main factor affecting production and increased production therefore means increase in output per cow. In the dairy herd, twin calvings also have relevance, particularly to that portion of the herd not required for replacement breeding, and in this way could increase the proportion of beef calves produced by the dairy herd. In both of these situations, a practical method of twinning would allow an increase in beef calf numbers without a consequent increase in cow numbers. Some management and economic aspects of twinning (Bowman, 1976), as well as some genetic implications (Cunningham, 1976), have been considered elsewhere.

FREQUENCY OF NATURAL TWIN BIRTHS IN CATTLE

Estimates of the incidence of naturally occurring twin births in cattle vary between location, breed and parity of dam but generally fall within the range of 1-4% (Gordon et al, 1962; Hendy and Bowman, 1970; Scanlon et al, 1974). The relationship between parity of dam and frequency of multiple births is well established. Scanlon et al (1974) report a 2.8% twin-birth rate in 2323 pregnancies with the highest frequency of multiple births (8%) occurring at the fifth pregnancy. Other reports confirm this in a variety of breeds (Hereen, 1957; Brodauf, 1963). Breed differences in the frequency of multiple births also exist, with dairy breeds showing a higher incidence of twin births than beef breeds (Scanlon et al, 1974).

METHODS OF TWIN-PREGNANCY INDUCTION IN CATTLE

There are three main approaches towards increasing the frequency of twin births in cattle, viz., genetic selection, gonadotrophin administration and ovum transfer. This brief review relates mainly to ovum transfer but the other methods are discussed for comparison. Another recent review of the topic is that of Lamond (1974).

Genetic selection — Few estimates are available of the heritability for twinning in cattle. Bowman, Frood and Wood (1970) analyzed the calving records of 2862 cows and the resulting estimate of the heritability for twinning was 0.043 $\pm$0.012. These authors quote an estimate of 0.031 having been reported by Maijala (1964). Because of the low

heritability value, breeding schemes designed to select for twinning have met with little success so far (Mechling and Carter, 1964; Donald and Gibson, 1974). However, following the work of Turner (1969) in achieving rapid progress in selection for multiple births in sheep, by selecting animals with extremely high reproductive performance, it is possible that similar breeding techniques could take advantage of genetic variation in cattle and result in more rapid progress. Bowman (1975) estimates that using such procedures, an increase of about 7% could be achieved over a 5-year period but after that the rate of increase would decline and become less predictable. However, selection for twin ovulations, even if highly successful, would be unlikely to result in a high rate of twin calvings in the cow. Unlike the sheep, transuterine migration of ova rarely occurs in the cow. Estimates of the frequency of migration in the cow generally fall between 0.1 and 1.5% (Perkins et al, 1954; Gordon et al, 1962; Scanlon, 1972). This means that even if selection for twin ovulations were successful, both ova shed would be confined to one uterine horn in 50% of cases. Rowson et al (1969; 1971) have shown that the ability of a single uterine horn to sustain two conceptuses is quite limited, but a high rate of twinning resulted from bilateral transfers. Following the induction of multiple ovulation in cows with PMSG, Gordon et al (1962) reported similar data. Thus, to successfully induce twin pregnancy in the cow, it is preferable that the two ova be located in different uterine horns.

Hormonal induction of twinning — Many reports have appeared on the use of FSH and compounds with similar properties (mainly PMSG) for the induction of mild superovulation in cattle. The common aim in all studies has been to induce a limited number of ovulations (up to three), but so far the results have not been consistent. Wide variation in response to standard treatments has been reported (Hammond and Bhattacharyra, 1944; Gordon et al, 1962; Turman et al, 1971; Mauleon et al, 1970; Wildt et al, 1975). Recent work indicates that the use of partly purified FSH products results in a more consistent response than the use of PMSG (Bellows et al, 1969; Wildt et al, 1975), but only a small proportion of animals have yielded two to four ovulations. Moreover, as in selection for twinning, no control is possible over ovulation site and, because of the problems of transuterine migration and unilateral twin ovulations, a high rate of twin calving would be unlikely.

TWIN PREGNANCY FOLLOWING SURGICAL EMBRYO TRANSFER

Pregnancy rates ranging from 66.6 to 91% have been obtained following transfer of one or two fertilized ova to recipients in estrus on the same day or within 1 day of the donor, as has been discussed in Section One of this monograph. Some factors of particular relevance to the induction of twinning by such techniques need to be emphasized.

Twin-pregnancy rates — There are only a limited number of reports dealing with twin-pregnancy induction following surgical ovum transfer and the main reports are summarized in Table 18. Some of these are bilateral transfers to non-mated recipients and some are single egg transfer to mated recipients. Successful transfers of the latter type suggest that any immunological effect that might prevent a dam carrying twins of two different origins

TABLE 18. TWIN-PREGNANCY RATES FOLLOWING SURGICAL EMBRYO TRANSFER IN CATTLE

No. recipients	No. ova and site of transfer		Pregnancy rate, %	Twinning rate of pregnant recipients, %	Reference
	+CL	−CL			
11	2	–	90.9	0.0	Rowson et al, 1969
15	2	–	66.6	50.0	Rowson et al, 1971
17	1	1	70.5	75.0	
9	1	1	66.6	50.0	Tervit, Whittingham and Rowson, 1972
31	1	1	87.0	68.4	Sreenan and Beehan, 1974
55	1	1	70.9	66.6	Sreenan et al, 1975
19	M[1]	1	57.9	27.3	Gordon, 1976a and
21	M[2]	1	66.7	64.3	Boland et al, 1976a
135	1	1	72.0	71.0	Sreenan and Beehan, unpublished
17	1	1	76.5	—	Betteridge et al, 1976
9	1	1	77.8	60.0	ADRI, unpublished (see Table 11)
48	1	1	75.0	52.8	G. B. Anderson et al, 1976

[1]Recipients mated before transfer.
[2]Ova transferred to rabbits and then to mated recipient cows.

(see page 70) is not important enough to preclude twin induction in this way. From Table 18 it can be seen that high pregnancy rates (57-91%) and high twinning rates (27-75%) are feasible following ovum transfer. Other reports such as Betteridge and Mitchell (1974), where up to three ova were transferred to some recipients, also record high pregnancy rates (71%) and the birth of multiples. Possible reasons for pregnancy rates following twin transfers being superior to those obtained after single transfer are advanced on page 70.

Site of transfer — To induce twin pregnancy in a high proportion of recipients, it would seem necessary to locate one embryo in each uterine horn. Rowson et al (1969, 1971) reported either no twin pregnancies or a relatively low level when they transferred two fertilized ova to one uterine horn. The range of twinning rates reported following bilateral transfers or contralateral transfers to bred recipients (Table 18) is 27-75%, but they generally fall in the region of 60%. The failure of one uterine horn to support twin pregnancy in a high proportion of animals agrees with other reports (Gordon et al, 1962).

Where single ovum transfers have been carried out, most authors state transfers were to the horn ipsilateral to the CL. Sreenan et al (1975), Sreenan and Beehan (1976c) and Sreenan (1976b, c) reported that of 20 single pregnancies following bilateral transfers in two separate trials, 13 (65%) survived in the horn adjacent to the CL. Further data collected on the site of transfer in relation to site of CL indicate that there may be a local uterine-embryo interaction. Embryo survival rates (single ovum transfer) of 61.3 and 18.1% have been recorded ipsilateral and contralateral to the CL, respectively (Sreenan 1976b, c). Newcomb et al (unpublished) obtained 6/13 pregnancies following transfer of one or two eggs to the ipsilateral horn but 0/13 following transfer to the contralateral horn. However, Tervit et al (1977) found that pregnancy

Figure 19. The first twin calves born as a result of surgical embryo transfer in Ireland, where embryo transfer work has been largely directed to increasing beef production through twinning. More recently, twins have been born following non-surgical transfers.

Photo by J. Walshe, Galway, courtesy J. M. Sreenan.

rates following single transfers to the ipsilateral horn (15/28, 54%) did not differ significantly from those obtained by transfer to the contralateral horn (11/28, 39%).

Sreenan and Beehan (1976c) and Sreenan (1976b, c) examined the effect of surgically depositing embryos either at the tip (ovarian end) or near the common body of the uterus on days 4-8. At 30 days of gestation they did not find any difference in pregnancy rates (8/10 and 7/10 for the tip and body groups, respectively) or embryo survival rates (13/20 and 12/20 for the respective groups). On the other hand, Boland et al (1976a) compared tip and mid-horn surgical transfer sites for insertion of an additional embryo into inseminated recipients using both direct transfer and transfer after 2-3 days culture in rabbit oviducts. With both groups of embryos a higher pregnancy rate was obtained following transfer to the tip of the horn, but the difference was not statistically significant (direct transfers resulted in 5/9 pregnant after mid-horn insertion, 6/10 after tip insertion; transfers after culture produced 5/10 and 9/11 pregnancies in the two respective positions). The tip of the horn also favored the rate of survival of transferred embryos, 12/21 (57.1%) surviving in that position as opposed to 4/19 (21%) surviving after transfer to the middle of the horn. This difference was statistically significant ($P<0.025$) and was consistent whether transfers were direct (4/10 vs. 1/9) or after culture in the rabbit (8/12 vs. 3/10).

Embryonic survival in twin pregnancy — Little information is available on the incidence of multiple ovulation in the cow under natural conditions and, therefore, the extent of prenatal loss in multiple pregnancies is unknown. Following a series of bilateral transfers, Rowson et al (1971) reported an embryo survival rate of 82% at day 90. Sreenan and Beehan (1976a) have examined embryo survival rates at various stages of gestation through to calving following bilateral transfers; they found no difference in survival rate at any stage between day 27 through to calving. It would seem that most loss occurs before day 27 and that once pregnancy is established uterine capacity does not limit the ability of the cow to carry twins located in separate uterine horns (but see also pages 29-30).

TWIN PREGNANCY FOLLOWING NON-SURGICAL TRANSFER

To be of value, egg transfer techniques must be capable of operation at farm level. The disappointing early results and the recent development of more effective non-surgical techniques which are bringing this closer to reality have been described on pages 31-34 of this monograph. Recent results are summarized in Table 13.

TWIN-CALVINGS FOLLOWING EMBRYO TRANSFER

Many of the data in the literature dealing with twin-pregnancy induction are based on pregnancy rates obtained either by palpation in early pregnancy or following slaughter of the recipients. Little information has yet become available on the outcome of twin calvings following ovum transfers. Twin calving under natural conditions is associated with an increased incidence of retained placenta, high rate of calf mortality at or near birth and reduced performance (productive and reproductive) on the part of the dam (Bowman and Hendy, 1970; Hendy and Bowman, 1970). However, because twin-pregnancy diagnosis at farm level has not been possible, most of these data refer to the outcome of twin calvings in dams managed as single-bearing dams.

Gestation length — Under natural conditions, gestation length is longer for singles than twins (Gordon et al, 1962; Scanlon et al, 1974). The estimates of difference in gestation length vary and are probably related to level of nutrition. Data on gestation length at first calving following ovum transfer are given in Table 19 for first pregnancies. Five of the heifers carrying twins were fed on a low plane of nutrition (5.1 kg DM daily) and had a mean gestation length of 277.6 days; the other 18 were fed on a medium to high plane (6.8-8.0 kg DM daily) and had a mean gestation length of 276.1 days. The data of Gordon et al (1962), however, showed an effect of plane of nutrition on gestation length with cows on an 'inadequate' level of nutrition having a mean pregnancy duration of 17.3 days less than cows on an 'adequate' level of nutrition.

Retained placenta — Twin calving has been associated with a high incidence of retained placenta (Bowman and Hendy, 1970; Kay et al, 1976). Retained placenta has been associated with premature twin births (Gordon et al, 1962). Of 23 twin calvings following ovum transfer, Sreenan, Sheehan and Beehan (unpublished data) have recorded retained placenta in four animals, which were all on a low plane of nutrition (5.1 kg DM daily). Of 18 animals receiving 6.8-8.0 kg DM daily, none had retained placenta. The incidence in single pregnancies reported by Gordon et al (1962) is 6%.

TABLE 19. EFFECT OF TWIN PREGNANCY ON GESTATION LENGTH FOLLOWING EMBRYO TRANSFER IN CATTLE

No. pregnancies	Gestation length, days	
	Mean	Range
14 (single)	281.9	269-289
23 (twin)	276.4	262-285

From Sreenan et al (unpublished).

Subsequent dam reproductive performance — There is little accurate data on the effect of twin calvings on subsequent dam reproductive performance. Preliminary data recorded by Sreenan et al (unpublished) suggest that twin births do not affect dam reproductive performance if nutrition is adequate both pre- and post-partum. Of eight twin-calving heifers, five were again used as recipients and subsequently calved five sets of twins within an average calving interval of 365 days; the other three were inseminated once and one subsequently calved within a calving interval of 365 days (Sreenan et al, unpublished).

Twin-calf performance — Twin-calf birth weights have been estimated to be 67-80% of single-born calves' weights (Gilmore, 1952; Meyer, 1964; Sreenan et al, unpublished). Estimates of the mortality rate of twin-born calves vary widely, but are generally put 2-12% higher than for single calves (Berge 1942; Richter, 1955; Gordon et al, 1962). It must be realized that data are mainly collected from twin-calving cows that had been managed as single-bearing cows. Data on the outcome of twin calvings following ovum transfer are given in Table 20. The indications are that plane of nutrition of the dam over the last third of pregnancy has a definite effect on the mortality rate of twin calves at or near calving. Preliminary observations suggest that the high calf mortality from dams on the low plane of nutrition may be due to the inability of the dams to produce adequate levels of colostrum. All twin calves from dams on the medium-high plane were live-born and mortality rate at 21 days was 8.3%, compared with 8.4% in single-born calves.

Data on comparative growth performance of twin and single calves under controlled conditions are limited. Kay et al (1976) point out that contradictory reports on growth performance appear in the literature. In their own study, they found that growth rate and feed conversion ratio from weaning to 3 months were similar for 14 twin and 14 single calves; and, although the twin calves weighed significantly less at birth, weaning and 3 months of age, the difference at 1 year was not significant. Likewise, Russel (1976), in a study involving 350 single and 250 twin calves, reported that at 18 months of age single calves had only a 1% superiority over twin calves for unadjusted body weight. The difference in birth weight between single and twin calves could be reduced by adjusting the level of nutrition of the dam pre-partum (last third of gestation). Recent data from Ortavant (personal communication, 1976) suggest that total estrogen level in peripheral plasma at about day 220 of gestation can be used to diagnose single or twin pregnancy. Thus, it would be possible to anticipate twin calvings for nutritional and management adjustments.

TABLE 20. CALF MORTALITY FOLLOWING TWIN BIRTHS RESULTING FROM EMBRYO TRANSFER IN CATTLE

	Total		Pre-partum nutrition level of dam			
			Low		Medium to high	
	S[1]	T[2]	S[1]	T[2]	S[1]	T[2]
No. calves	14	46	1	10	13	36
No. live-born	13	44	1	8	12	36
No. alive at 3 weeks	12	37	1	4	11	33

[1]Single calves.
[2]Twin calves.
From Sheehan and Sreenan (unpublished).

CONCLUSIONS

Under the right conditions, high pregnancy and twinning rates have been obtained following surgical embryo transfers and recent reports indicate that non-surgical transfer techniques *per vaginam* are showing improved results, as discussed in Section One of this monograph.

Data are now beginning to accumulate on production aspects of twin calvings and their post-partum effect on the dam.

Beyond the many aspects that require further research to improve the technology of embryo transfer as a means of inducing twinning, it is essential to develop further a method of twin-pregnancy diagnosis (based perhaps on circulating levels of estrogen) and to investigate the nutrient requirements of the twin-bearing cow under controlled conditions, together with the effects of nutrition on subsequent calf and dam performance.

EMBRYO TRANSFER FOR IMPORT AND EXPORT AND POSSIBLE DANGERS OF TRANSMITTING INFECTIOUS DISEASE

D. Mitchell and K. J. Betteridge

INTRODUCTION

With the development and perfection of a variety of methods for storing mammalian embryos, increasing interest in the practical application of this means of international movement of livestock can be expected.

Successful long distance transport of embryos has been recorded in rabbits (Marden and Chang, 1952), sheep (Adams et al, 1961; Hunter et al, 1962; Welch, 1969; Baker et al, 1971; Kardymowicz and Kramer, 1971; Kardymowicz et al, 1976), pigs (Baker and Dziuk, 1970; Wrathall et al, 1970), mice (Whittingham and Whitten, 1974) and horses (Allen et al, 1976). These embryos have been stored during transport either *in vivo* in the Fallopian tube of the

rabbit, or *in vitro* in a variety of media and at various temperatures and, for mice, frozen.

Embryo transfer has also been used to import 'exotic' breeds of cattle into New Zealand (Morcan, 1972). Friesians and Jerseys were used as recipients of the required embryos in England and were therefore eligible to pass the importing country's health regulations.

Although the technology now exists to utilize this technique for transportation of animals, the problem of ensuring that the transported embryos are free from infectious disease has been a major factor in preventing its wider implementation for commercial purposes.

DISEASE TRANSMISSION

Only one of the above reports (Wrathall et al, 1970) addressed itself seriously to the problem of monitoring the health status of donor and recipient animals. In this study, strict quarantine precautions were instituted and tests for brucellosis, leptospirosis, porcine enteroviruses, parvovirus, pseudorabies, hog cholera, swine influenza, transmissible gastroenteritis, vesicular stomatitis and hemagglutinating encephalomyelitis virus were undertaken on the recipient sow and the resulting progeny. All results were negative and the study clearly demonstrated that, provided strict quarantine and test procedures are performed, the risk of disease transmission can be minimized. Indeed, embryo transfer is being used as an alternative to hysterectomy for introducing disease-free pigs into closed, minimal disease herds as described on pages 60 and 61. It is interesting to note that early mouse embryos, once removed from a cytomegalovirus-infected uterus that could retard their development and block their implantation, developed normally in an *in vitro* culture system (Neighbour, 1976) and the cytomegalic virus involved was not believed to infect the embryos themselves.

Conversely, however, some viruses have been shown to pass through the zona pellucida in early mouse embryos (Gwatkin, 1967), others can be introduced via the spermatozoa in rabbits (Brackett et al, 1971) and mice (Ericsson et al, 1971) and bacterium-like particles can be found in rat blastocysts after the zona has been shed (Wu and Meyer, 1972). It is probable that a large proportion of such early infections are self-limiting through embryonic death. Glass et al (1974) found this to be true after experimental injection of mouse blastocysts with an avian virus, but they did not study survivors beyond birth. They point out the possibility that such early exposure could render the animal tolerant to later infection or could provide a basis for long-term colonization of the animal with the virus.

What is more, it has been shown that certain virus infections can be vertically transmitted via the transferred embryo in mice (Mims, 1966; Bentvelzen et al, 1970; Calarco and Szollosi, 1973; Jaenisch et al, 1975; Whittingham, 1974). C-type virus particles have also been demonstrated in preimplantation baboon embryos (Kalter et al, 1975). These reports and others (see reviews by Gwatkin, 1971; Ferm, 1971; Fenner, et al, 1974; Whittingham, 1974) indicate the risks of disease transmission and related problems of certification of freedom from infection that confront regulatory authorities responsible for authorizing international trade in mammalian embryos. Obviously, there is no room for complacency and a need for further research.

This problem has been considered by animal health officials in several countries but as yet no uniform international guidelines have been formulated. Individual approaches have varied from the detailed scientific requirements laid down by Denmark, to the outright prohibition (except in special cases) imposed by the Netherlands. It is obvious that certification requirements, as for importation of live animals, will largely depend on existing disease situations in the exporting and importing countries.

CONCLUSIONS

In the light of present knowledge it seems reasonable to predict that any import-export program with mammalian embryos for commercial purposes will require health monitoring of donor males and females and of recipient females both before and after embryo collection and transfer. The extent and nature of testing demanded will depend on the countries involved but should be similar to that presently required for international trade in live animals and semen. Breed societies will probably also request blood typing information before permitting registration of the resulting progeny.

In addition to tests for specific diseases it can be anticipated that there will be a need to construct a facility or facilities designed specifically for this purpose. These would be licensed in a similar manner to cattle artificial insemination centers. A limited quarantine period, with concurrent disease monitoring, would be mandatory for recipient females.

The advantages and disadvantages of engaging in an embryo import program under these conditions may be summarized as follows:

Advantages

1. Reduction in risk of introducing exotic disease, backed by quarantined recipients doubling as test animals.
2. Reduction in transportation costs.
3. Minimal depletion of numbers of superior genetic strains from the exporting country.
4. Greater control over genetic selection of embryos imported in relation to benefits to be gained, particularly in terms of increased production.

5. Possibly more rapid adaptation of progeny to environmental and disease stresses when exotic embryos complete gestation in an indigenous female.

Disadvantages

1. Increased costs related to collection and transfer procedures, which will decrease as non-surgical and freezing techniques become more available.
2. Maintenance of a suitable herd of recipient females essential.
3. Necessity for specialized facilities and trained personnel at selected locations.
4. Unpredictable percentage of successful transfers and sex of resulting progeny (unless sex is pre-determined).

In summary, it can be stated that future use of embryo transfer as a means of long distance transportation of animals, particularly at the international level, will depend on improvement in storage, sexing, collection and transfer techniques; economic benefits to be derived; agreement on regulatory requirements to minimize the risk of disease transmission; and establishment of units licensed for this purpose.

There can be few who have examined embryos through the microscope without being excited. Today's ascetic editors leave little room for the expression of feelings but in any case it is doubful whether a day of excitement in the laboratory could be any better described than was December 8th, 1932:

"Five days after we had seen our first living ovine egg, we operated on a goat which had been bred two days previously. We examined both ovaries for corpora hemorrhagica and found one typical corpus. We then removed the corresponding oviduct and proceeded to attempt to find the egg. We were successful; and soon were gazing in awe at a beautiful four-celled goat in saline under the binocular. We wondered why we had been so skeptical of our abilities, and regretted that we did not have a recipient animal of either species ready to become the uterine foster parent of this handsome animal. This animal seemed so beautiful to us that we could not bear to let it die of neglect, so we hastily decided to put it back in its original home for five months. When, 147 days later, we again looked at this same animal after normal parturition, a good many changes had taken place". (Warwick and Berry, 1949).

RESEARCH APPLICATIONS OF EMBRYO TRANSFER IN CATTLE

I. Gordon

Despite the demonstration by Heape of embryo transfer in the rabbit as early as 1890 at Cambridge, it was not until midway through the 20th century that the technique began to be considered in earnest as a method for use in cattle breeding improvement and as a research tool in farm animal reproduction. Much of the pioneering work in cattle embryo transfer has been carried out by the Cambridge group and several excellent reviews by Rowson (1971, 1973a, b, 1974) include thoughts on ways in which the transfer technique might be employed in research. This review considers some of the ways in which embryo transfer might be used in investigating the physiology and endocrinology of pregnancy in cattle, in separating maternal effects from those arising from the embryo itself (especially as these may relate to the causes of prenatal mortality), in looking at the problem of freemartinism and in testing the developmental potential of bovine eggs after they have been subjected to experimental treatments or manipulations. It should be mentioned that such application as yet has been extremely limited, largely because a technique capable of producing acceptable results was not available until a few years ago.

PHYSIOLOGY AND ENDOCRINOLOGY OF PREGNANCY

Those concerned in physiological studies may well be expected to exploit the embryo transfer technique in examining many questions relating to embryonic and placental growth and on pre-natal mortality, not only in single pregnancies but also in multiples.

Anti-luteolytic action of the early embryo — Considerable interest exists currently in the endocrine activity of the early embryo in many mammalian species. It appears that the embryo transmits a signal, apparently hormonal in nature, which indicates to the mother that she is pregnant. Certainly, as reviewed by Rowson (1971), egg transfer work has shown that in the cow there is a unilateral luteolytic action of the uterus and that the embryo acts in an anti-luteolytic manner. One consideration is the time at which the hormonal signal is first emitted by the embryo. In the ewe, which has an estrous cycle of 16-17 days, the embryo must be present within the uterus by day 12½, otherwise regression of the corpus luteum cannot be prevented (Moor and Rowson, 1966a). Corresponding information is now available for the cow, showing that embryo transfer can prevent luteolysis at least up until day 16 (Betteridge et al, 1976). The fact that the bovine embryo can progress such a considerable way in its development means that certain examinations (e.g., cytogenetic analysis) and manipulations can be attempted in a way that would be impossible or much less feasible with earlier embryos. In examining the origin of the anti-luteotrophic activity in the bovine embryo, for example, it might be possible to separate the trophoblast and inner cell mass to determine in which of these the anti-luteolytic activity resides.

Fetal hormones — As well as yielding information on the way in which the early embryo maintains the corpus luteum, embryo transfer may help in studying the question of hormone production by the embryo and fetus at later stages of pregnancy. Robertson and King (1975) and Eley et al (1975) have presented preliminary data showing estrogen synthesis by the pre-attachment embryo of the cow in the 6th week of pregnancy. The detection of fetal hormones could eventually open the way to a method for the diagnosis of multiple pregnancy in cattle, the importance of which has been indicated (pages 65-66). In the ewe, the progesterone level in peripheral blood can be used to predict the number of fetuses carried (Gadsby et al, 1972) but the same approach cannot be applied in the cow, in which progesterone is apparently derived almost exclusively from the CL in pregnancy with little placental production until shortly before parturition. For that reason, it is unlikely that peripheral plasma levels would indicate twin pregnancy in cows that possess a single CL (after egg transfer to a single-ovulating recipient cow). However, a test for twin pregnancy based on the measurement of fetal estrogen, or perhaps even of a gonadotrophin, might be feasible especially since it would not be required until the final trimester of pregnancy (see also page 66).

Multiple pregnancy — Information on many aspects of multiple pregnancy in cattle is limited, especially on those pregnancies initiated by embryo transfer and maintained by a single CL. The nutritional requirements of the twin-bearing cow are very much an untouched area of animal husbandry research and deserve much greater attention now that the means of producing twins at will is available. For sheep, it is well established that the nutritional level maintained through the final 6 weeks of the gestation period has a major effect on the birth weight of the fetus, especially in ewes possessing twins (Wallace, 1948). Not only is it a question of fetal growth and size at parturition, but also a matter of looking at the implications of induced multiple pregnancy as it affects production (in milking cattle) and subsequent reproductive ability. Some promising beginnings in studies of this kind are described on pages 65-66. The development of the fetus in multiple pregnancy may be influenced by placental area, which in turn can be influenced by the number of placentomes. French

workers (Testart and du Mesnil du Buisson, 1970) who induced twin pregnancies by gonadotrophin treatment, and those at Cambridge (Rowson et al, 1971) who used the egg transfer approach, have reported that the number of placentomes that developed was much greater in heifers with bilateral twins than in those with unilateral twins. This is one argument that would favor employing egg transfer rather than selective breeding (Rutledge, 1975) or the hormonal stimulation of multiple ovulations as the approach to the induction of cattle twinning as described elsewhere (pages 62-66).

Immunological aspects — Although Rowson et al (1971), in reporting their first successful production of twin calves by bilateral two-egg transfer, allude to the possibility of twinning by introducing a second egg into a previously mated cow, the Cambridge group did not subsequently report on this approach. Rowson (1971), however, mentions attempts to produce twins of differing breeds by transferring an egg to the uterus of the bred animal and states that results for both sheep and cattle suggested that a physiological or an immunological effect was operating in the recipient's uterus, leading to the loss of either the native or transferred egg. Although it is probably true that some of the immunological consequences of embryo transfer need more precise definition, work in France (Testart, Godard-Siour and du Mesnil du Buisson, 1975) and Ireland (Boland et al, 1975, 1976a, b), in which one-egg transfers have been successfully made to the bred recipient, would not appear to support the possibility of an immunological effect. As noted by Dawes (1976), evidence that antigenic dissimilarity between mother and fetus might influence the magnitude of placental and fetal growth is at present conflicting. Any effect on pregnancy arising from antigenic differences, if it does exist, appears to be small.

DISTINGUISHING BETWEEN MATERNAL AND EMBRYO EFECTS

The transfer technique can be applied in the cow for many studies in pre-natal mortality, on the effect of the maternal (uterine) environment on embryo survival, ability of the uterus to carry several young and in various utero-ovario-embryo relationship studies. The relevance of these to understanding the causes of fetal wastage in normal pregnancy has been discussed (page 30).

Conception rate — Bovine fertility, as measured in terms of the pregnancy rate to a single service, can vary markedly among cattle in different areas of the world. Laing (1970), after reviewing data from various sources, quoted a pregnancy rate of 60-65% for dairy cattle bred by artificial insemination in northern Europe. This is despite the very high fertilization rate (95-100% in heifers) that can be expected after normal breeding (Laing, 1949; Bearden et al, 1956; Wishart and Young, 1974). Much of the evidence suggests that responsibility for embryo mortality lies with the cow, but the precise causes remain ill defined. Embryo transfer might be employed in achieving a substantial improvement in the conception rate in cattle. In this connection, it is of interest to note that pregnancy rates (90% +) in some of the Cambridge surgical transfer reports (Rowson et al, 1971; Rowson, Lawson, Moor and Baker, 1972) were substantially above those considered normal in cattle. Although the suggestion is made that these were a result of eliminating abnormal and retarded eggs and maintaining exact estrous cycle synchrony between donors and recipients, the explanation may lie rather in the fact that two eggs and not one were used in transfers. One means of achieving a higher conception rate may be in providing the cow with two eggs, giving her two chances rather than one of staying pregnant. Data in Table 11 would appear to support this suggestion. Newcomb et al (unpublished), in another experimental series, obtained two pregnancies from six single egg transfers but four pregnancies after seven transfers of two eggs. Certainly, it remains for those transferring single eggs to record pregnancy rates (not to be confused with embryo survival rate, discussed below) approaching those that obtain with twin-egg transfer. This would seem to contrast with the situation in sheep (see page 75), where pregnancy rates are not improved by increasing the number of embryos transferred.

Synchrony — The importance of synchrony has been clearly demonstrated, as discussed on pages 28-29. Although, in general, the greatest success has been with donors and recipients at identical stages, there may be certain occasions (since manipulation and storage may be expected to slightly retard embryo development) when better results can be obtained if the donor is more advanced in the estrous cycle than the recipient (Newcomb and Rowson, 1975a). The importance of data about synchrony is in explaining some of the ways in which embryo loss may occur in the normal cow. If certain conditions of feeding and management induce a state of asynchrony between the physiological development of uterus and embryo, then despite the presence of a perfectly normal embryo, the pregnancy may not continue.

Embryo survival — The egg transfer technique can be employed in investigating embryo survival in the cow, especially factors involved in the capacity of the uterus to support embryos. Early attempts to establish twin pregnancy by two-egg transfers to the one uterine horn were largely unsuccessful because of the loss of one of the embryos. Nelson et al (1975) have also reported that significantly more embryos developed from single-transferred eggs than from those that they transferred in

pairs (but whether to one horn or both is not stated). When bilateral two-egg transfers are made, however, it is now clear that a high percentage of normal twin pregnancies can be established and the same appears to be true with one-egg transfers to the bred recipient (see pages 34, 63 and Table 18). The cause of the pre-natal loss when both embryos are confined to the one uterine horn is obscure, as indeed is the apparent failure of the bovine embryo to migrate from one horn to the other as normally occurs in sheep and pigs. Embryo survival rate in sheep is similarly depressed as the number transferred to a single recipient increases (see page 75).

Large and small breeds — The fact that the uterus of the recipient largely determines the size of the offspring has already been well established by embryo transfer studies in sheep. It could be of interest to develop the embryos of some of the small breeds (Jerseys) in the uteri of large breeds (Friesians) to determine how development and production characteristics are affected (Rowson, 1971).

Pathogenesis of reproductive diseases — Extension of studies analogous to those of Neighbour (1976, see page 67) could cast light on whether embryos or maternal environment are chiefly affected by certain infectious agents. Embryo transfer can also help show whether vertical disease transmission occurs before attachment of the embryo or by crossing the placenta later in pregnancy.

FREEMARTINISM IN CATTLE

In some ways, the explanation of the bovine freemartin remains as elusive today as it was a half-century ago. Quite apart from the intrinsic biological interest in the phenomenon itself, the growing possibility of employing embryo transfer as a means of getting twin calves means that much more needs to be known about the freemartin condition. Where the objective on the farm is the provision of twin calves for meat production, the occurrence of the freemartin may be of minor importance, but the same would not be true when using cattle twinning in any breeding improvement program. One way of overcoming the problem would be in sexing twin embryos before transfer, using an approach such as that described by Hare et al (1976). Conversely, the same approach allows freemartinism to be produced by design, a powerful research tool.

Incidence and nature of freemartinism — A recent review by Marcum (1974) gives the incidence of the freemartin in twins of unlike sex as 92% from the literature. Embryo transfer could be employed to examine the incidence of the condition more precisely in relation to whether twins are bicornual or unicornual.

Lillie (1917) posed the question as to whether transformation of the gonads might proceed to such a degree that production of spermatocytes and spermatozoa could occur in freemartins. Certainly, there have been reports of remarkable transformations in the male direction (Buyse, 1936; Hay, 1950), but conclusive evidence of spermatogenesis in the freemartin has not been reported and would not be expected in view of the abdominal temperature at which the transformed gonal would be held.

Whether in twins or larger litters, one male fetus can result in the other female littermates becoming freemartins. It is not clear what factors may influence the degree to which the freemartin is transformed in the male direction. Embryo transfer may be expected to help in resolving some of these such as the effects of the embryo's position in the uterus, the number of males and females in the uterus, the age of embryos at the time of vascular anastomosis and the extent of the anastomoses.

Germ-cell chimerism — The possibility that blood-borne elements other than hemopoetic tissue can circulate between bovine twins, as a result of anastomosis of fetal blood vessels, has been demonstrated by several workers (review by Marcum, 1974). Evidence suggesting germ-cell chimerism was reported by Ohno et al (1962) in two bulls born co-twins to freemartins, but not in the freemartins themselves. This was supported in the reports of Ohno and Gropp (1965) and Jost and Prepin (1966) who showed that germ cells entered the circulation of the 25- to 34-day-old bovine embryo, at a time when anastomosis has occurred between partners. Teplitz et al (1967) also detected germ cell chimerism in three bulls that were co-twins to freemartins.

Evidence on whether germ cells that migrate from the female into the bull testes are capable of later giving rise to spermatozoa appears to be conflicting. Dunn et al (1968) have presented data suggesting a deviation from the normal sex ratio in favor of females in the progeny of a bull that was co-twin to a freemartin, but other reports have not confirmed this. Certainly, evidence from chimeric mice of XX/XY constitution show that the sex ratio in their offspring is normal. It appears that when gonads are testes in the chimeric mouse of XX/XY constitution, then the XY cells provide the germ-cell line; when the gonads are ovaries, the XX germ cells are functional (Mintz, 1965).

Besides its fundamental interest, a better understanding of germ-cell chimerism may have considerable practical significance because there is some evidence that bulls born twin to freemartins may be subfertile (Dunn et al, 1968; Stafford, 1972). Bovine freemartinism is thus an important model system in studies of germ-cell chimerism and is one that can be produced at will by embryo transfer.

TESTING BOVINE EMBRYOS AFTER EXPERIMENTAL TREATMENTS

There are many studies involving the production, manipulation or storage of the early bovine embryo that would depend on the transfer technique to establish the developmental potential of the experimental embryo, whether after storage, *in vitro* fertilization, injection for chimera production or, more distantly perhaps, after nuclear transplantation to achieve fertilization.

Bovine chimeras — As mentioned above, there is some evidence that in multiple births in cattle, germ-cells may migrate from one partner to the other. If this occurs with twin bull calves, then the possibility exists that such interchanged germ cells could develop through to spermatozoa and allow one bull to sire some proportion of his brother's offspring. Cambridge workers have transferred two eggs from different color-marking breeds (one egg to each uterine horn) with a view to producing bulls that can be used for the insemination of a large number of cattle (Rowson, 1973a; Rowson and Newcomb, 1976). It should be obvious from the color markings of the calves whether either of the bulls possesses germ-cells of the other.

Advantage can also be taken of the natural production of chimeras in most bovine twin pregnancies, even when the twins concerned are both heifers; for such calves, which can be of differing breeds, would show tissue tolerance to each other. As noted by Rowson (1971), it would be possible to investigate questions bearing on various milk production characteristics, not only by examining what happens as a result of erythrocyte chimerism but also at the cellular level after reciprocal interchange of half the udder by grafting operations.

Apart from the chimeras produced naturally, attempts have been made in various mammalian species in recent years to produce chimeras artificially by the injection of embryonic, fetal and adult cells into developing eggs and blastocysts, while they are still incapable of an immune response. The feasibility of this technique has been well demonstrated in mice (Gardner, 1968), rabbits (Gardner and Munro, 1974) and sheep (Tucker et al, 1974). It is perhaps possible that adult cells from a particularly valuable cow could be injected into embryos to establish tissue tolerance between the cell donor and the resulting calves. This might allow the subsequent grafting of ovarian tissue from the cell donor into the sterilized ovaries of the chimeric cattle, thus opening the way to proliferation of a particularly valuable strain by grafting operations.

Identical calves — Embryo transfer could be employed to produce identical twins, triplets or possibly larger litters. If necessary, more than one recipient could be used to carry the identicals. Such production could follow the separation of individual blastomeres at, say, the 4-cell stage and transferring these individually into suitable recipients. Several studies along these lines have already been reported for the rabbit (Moore et al, 1968) and the pig (Moore et al, 1969), but it is in cattle that the greatest interest in the production of identicals would lie. For many years, bovine monozygotic twins have been used in several areas of research in animal production. One problem in getting isolated blastomeres to develop is to provide them with a suitably sealed zona pellucida (using the rabbit oviduct to provide a mucin plug has been attempted) to enable them to survive transfer at an early cleavage stage. An alternative might lie in developing them in the laboratory to the blastocyst stage, at which time they should survive transfer in the absence of the zona.

Storage of embryos — The importance of using transfer in assessing the normality, or otherwise, of bovine embryos after their having been stored in various ways or after *in vitro* fertilization of oocytes has already been stressed (see pages 9 and 24).

Nuclear transplantation — The years ahead are likely to bring exciting developments in ovum physiology. Nuclear transplantation is one technique that could have immense impact in cattle breeding by allowing genotype selection. Although the technique of nuclear transplantation was initially developed in the large amphibian egg (reviewed by King, 1966), recent work by Bromhall (1975) has shown limited success in the introduction of the nuclei of embryonic rabbit cells into unfertilized rabbit eggs by both micro-injection and virus-induced fusion. Although the difficulties in working with the minute mammalian egg are formidable, it is perhaps not impossible to think forward into a future in which calves can be obtained from eggs provided with transplanted nuclei that would also determine sex according to their gender of origin.

RESEARCH APPLICATIONS OF EMBRYO TRANSFER IN SHEEP AND GOATS

R. A. S. Lawson

Most experimental uses of embryo transfer in sheep are analogous to those described for cattle, and sheep have often provided the model for work in the larger species.

PHYSIOLOGY OF PREGNANCY

Synchronous relationship of embryo and uterus — In establishing procedures for embryo transfer in sheep, early investigators recognized the requirement for synchrony between the estrous cycles of

donor and recipient which had previously been established in laboratory species. Averill (1956) and Moore and Shelton (1964b) found that, although 2- to 16-cell embryos could be successfully transferred between ewes in estrus within 48 h of each other, conception rates were maximized when estrus was closely synchronized. This aspect was examined systematically and confirmed by Rowson and Moor (1966a) who transferred 5-, 7- and 9-day embryos to recipients in estrus within 3, 2 or 0 days of the donor.

The necessity for this synchronous relationship has been assumed to be related to the requirement that the embryo exert a precisely timed anti-luteolytic influence on the maternal system on day 12 (see below). Recent investigations by Lawson and Cahill (1975, 1976a) have shown that the synchronous relationship is probably controlled by progesterone. Exogenous progesterone given on days 0-3 of the ewe's estrous cycle shortened it by approximately 4 days. However, embryos from donors in estrus 4 days earlier could be transferred into the uteri of such ewes and they survived equally as well as embryos transferred synchronously in untreated control recipients.

This experiment indicated a further use of embryo transfer; it could be used to assay the state of the uterine environment. However, dependence on conception is an impractical measure in many situations. We have, therefore, further investigated the development of embryos following asynchronous transfer (Lawson and Cahill, 1976b). These findings, which are summarized in Table 21, showed that 16-cell embryos entering the uterine environment of a recipient ewe that had proceeded to a more-advanced stage of diestrus than the donor made more rapid growth at all stages up to day 12 in the recipient than did synchronously transferred embryos. Embryos entering a less-advanced recipient were retarded. Despite their rapid growth, the asynchronously transferred embryos were unable to prevent luteolysis.

Accelerated growth by the sheep embryo during days 7-11, when progesterone levels in the ewe are elevated, has also been reported by Wintenberger-Torres (1968) and Wintenberger-Torres and Rombauts (1968). Their findings, and our own described above, suggest that the effect of elevated levels of progesterone may be to induce a degree of asynchrony between the embryo and uterus.

The use of embryo transfer and growth to day 11 or 12 to determine the suitability of the uterine environment may prove useful as a sensitive measure in investigations of factors affecting embryo survival.

Embryo-maternal interactions and luteolysis — The site to which embryos are transferred in the reproductive tract was suggested by Averill and Rowson (1958) to have a bearing on embryo survival. Moore et al (1960) and Moore and Shelton (1962a) found that the survival rate of embryos recovered 48 h after the onset of estrus was depressed if they were transferred into the oviduct rather than the uterus. However, Moore and Shelton (1964b) found that with embryos collected 48-84 h after the onset of estrus, oviduct transfers were significantly more successful. The uterine environment may be unsuitable to early embryos, whereas older embryos survive best when placed in the uterus (Shelton and Moor, 1966). Wintenberger-Torres (1956) showed embryos could continue to develop normally up to day 7 in the oviduct, but they became retarded if they were withheld from entering the uterus after this time.

TABLE 21. EFFECT OF ASYNCHRONOUS TRANSFER ON THE SIZE OF BLASTODERMIC VESICLES RECOVERED AT VARIOUS INTERVALS AFTER TRANSFER IN SHEEP

Treatment of embryos	Days in recipient	Chronological age at recovery, days		No. embryos	Size of embryos, mm ± SE
		Recipient	Embryo		
Control	—	9	9	48	0.23±0.07
Synchronous transfer, day-4 embryo to day-4 ewe	6	10	10	21	0.29±0.03
Asynchronous transfer, day-4 embryo to day-6 ewe	6	12	10	22	0.45±0.01 **
Synchronous transfer, day-4 embryo to day-4 ewe	4	8	8	19	0.19±0.02
Asynchronous transfer, day-4 embryo to day-7 ewe	4	11	8	15	0.75±0.10 **
Synchronous transfer, day-4 embryo to day-4 ewe	8	12	12	12	2.23±0.36
Asynchronous transfer, day-4 embryo to day-7 ewe	5	12	9	9	2.12±0.58 NS
Synchronous transfer, day-4 embryo to day-4 ewe	10	14	14	10	51.71±6.44
Asynchronous transfer, day-4 embryo to day-7 ewe	7	14	11	12	8.03±2.50 **

From Lawson and Cahill (1976).

The period during which embryos can be transferred between ewes has been shown to range from follicular oocytes (to mated recipients) and 1-cell zygotes on day 0 (Lopyrin et all, 1951; Woody and Ulberg, 1963) to blastodermic vesicles on day 12 (Moor and Rowson, 1966a). Interest has centered on the factors involved in fixing the upper limits. In a now classical series of experiments using embryo transfer it was shown that the presence of an embryo in the uterus was essential on or before day 12 to prevent luteolysis (Moor and Rowson, 1966a, b). By confinement of transferred embryos to isolated portions of the uterus this was shown to be a local relationship between the embryo, the uterus and the adjacent ovary (Moor and Rowson, 1966c, Niswender and Dziuk, 1966).

Moor et al (1969) used embryo transfer to produce unilaterally pregnant sheep in which the lifespan of CL induced during pregnancy was studied. They found that the unilateral relationship between embryo and CL was preserved up to day 31, but CL induced at day 51 or 71 were maintained even when on the ovary adjacent to the non-pregnant uterine horn.

The mechanism by which the embryo prevents luteolysis remains to be elucidated. Since homogenates of early embryos can prevent luteolysis if they are infused into the uterus from before day 12 (Rowson and Moor, 1967), it appears that the embryo produces an anti-luteolytic factor.

Although the period about day 12 appears to be critical in the establishment of pregnancy, the development of the embryo before this is not wholly passive. In the previously mentioned series of experiments of Lawson and Cahill (1976b), it was shown by embryo transfer and recovery that the uterus could stimulate or retard embryo growth before day 12. One tenable conclusion from this work was that a change in the uterine environment that stimulated blastocyst growth occurred normally about the time of loss of the zona pellucida. A further change that directly affected embryo growth occurred after day 12. From this time synchronously transferred embryos entered the phase of rapid blastodermic vesicle elongation. Asynchronously transferred embryos were unable to respond to this stimulus despite their equivalent size when the recipient had reached day 12. Thus, at recipient day 12, embryos transferred synchronously at the 16-cell stage had reached 2.23 $\pm$ 0.36 mm in diameter, and embryos transferred 3 days asynchronously and now 9 days old were 2.12 $\pm$ 0.58 mm. On recipient day 14 these embryos measured 51.71 $\pm$ 6.44 and 8.07 $\pm$ 2.37 mm, respectively. Retarded growth rate after day 11 by embryos in ewes with elevated progesterone levels has also been recorded (Wintenberger-Torres, 1968, Wintenberger-Torres and Rombauts, 1968).

The relationships between these postulated stimuli from the maternal system about days 7 and 12 to affect embryo growth, and the proven stimulus from the embryo to the maternal system to prevent luteolysis on day 12, remain unknown. Embryo transfer will be an essential tool in the exploration of these interactions.

Endocrinology of gestation — Embryo transfer has enabled attempts to be made to partition the roles of the main ovarian hormones, estradiol-17β and progesterone, in the establishment of gestation. Exogenous hormones can be administered to a spayed animal and conception after embryo transfer is used to assess the normality of the treatment.

Moore and Rowson (1959) showed that transferred embryo could survive when ewes spayed at the time of transfer were treated with progesterone alone. Trounson and Moore (1974c) found that in ewes ovariectomized on days 3 or 6 of their estrous cycles, graded doses of progesterone rising to 12 mg/day on day 7 were sufficient to enable embryos transferred on days 3 or 6 to survive. The addition of estradiol to mimic the surge in blood estrogen levels, which has been reported to occur in the ewe on days 3-4, did not increase the rate of success of embryo transfers.

Subsequently, Moore and Miller (1976) have investigated the roles of estrogen and progesterone in the long-term spayed ewe. They found that to produce a uterine environment in which transferred embryos could survive to day 21, a regime of estrogen (E) — progesterone (P) — estrogen (E) — progesterone (P) was most suitable. Embryos failed to survive after E, P, E, —; E, —, E, P; or E, —, —, P. They concluded that an entire cycle of progesterone secreted before estrus and estradiol at estrus was necessary to suitably prime the uterus.

Further studies are needed in this area. We have found (Lawson, unpublished observations) that apparently normal embryos were almost invariably recovered on day 15 after embryo transfer 10 days previously to long-term (3 months) spayed ewes treated with progesterone only.

Another factor that needs to be controlled in the above types of experiment is the possibility that small quantities of estrogen of adrenal origin could be playing a role. Cumming et al (1974) showed that progesterone alone could maintain gestation after ovariectomy and adrelectomy on day 3.

Uterine capacity — By enabling breed, CL number and the numbers of embryos in the uterus to be varied independently, embryo transfer has provided the opportunity for their effects on litter size to be systematically investigated.

An early experiment was that of Moore et al (1960), who transferred either two or five embryos to superovulated and untreated ewes. Superovulation did not affect embryo survival and conception rates were similar in groups of ewes with two or five embryos. Though embryo survival was depressed when five rather than two embryos were transferred, litter size was still significantly greater after the transfer of five embryos.

In investigations of this type, two factors must be considered; conception rate and litter size in the ewes that conceive. The extent to which they are independent is not clear. The best published evidence that conception after embryo transfer is maternally controlled comes from the data of Cumming and McDonald (1970) who transferred one, two or four embryos to groups each of 36 recipient ewes. The percentages of ewes that failed to conceive were 47, 50 and 50%, respectively. Other experiments in which conception rate has remained constant when varying numbers of embryos were transferred have been those of Moore (1968) and Bradford et al (1974). These findings confirm the general observation of many workers that conception may be strictly a maternally (confounded with operator) regulated phenomenon that cannot be improved upon by increasing the number of embryos transferred. This appears to contrast with the situation in cattle where twin transfers seem to improve pregnancy rates (see page 71).

Where the number of embryos transferred to ewes of similar breeding has been varied in all of the experiments cited above, it has been consistently observed that the chances of a transferred embryo surviving have been depressed as the number of embryos transferred was increased. This loss appeared to occur before days 17-18 (Moore et al, 1960; Moore, 1968). The mechanism by which embryos are lost following multiple transfers awaits systematic investigation. This is consistent with the situation in cattle (see page 71).

Analyses of embryo survival in relation to the number of CL in the recipient ewes within the same breed have failed to show any significant effect of CL numbers on survival (Moore et al, 1960; Cumming and McDonald, 1970; Bradford et al, 1974). Kardymowicz and Stepinski (1957) suggested that superovulation of recipients aided conception after embryo transfer, but Schmidt (1961) was unable to detect such an effect. Where interbreed comparisons have been made (see below), breed and CL numbers have been confounded. In this type of investigation, the number of CL is probably too crude an estimation of progesterone production to be of value. Investigation of conception and embryo survival in relation to the pattern of progesterone production during the first weeks of gestation could prove to be more significant.

Besides the intrabreed studies mentioned earlier, several interbreed comparisons have been made to find out if the capacity of the uterus to support large litters significantly limits fertility (Moore, 1968; Lawson and Rowson, 1972; Bradford et al, 1974). Limited numbers of animals have been a problem in these experiments. The general conclusion of all workers has been that ovulation rate is the main factor limiting fertility in breeds of lower fertility. All of the breeds studied have had the uterine capacity to carry larger numbers of offspring than occur naturally. However, there were indications that Finnish Landrace ewes (Lawson and Rowson, 1972; Bradford et al, 1974) and Border Leicester ewes (Moore, 1968) tended to maintain larger litters more regularly than the less fecund breeds with which they were compared. In each case breed and natural ovulation rate in the recipient were confounded.

An interesting result not previously observed when interbreed transfers have been made was reported by Trounson and Moore (1972) following reciprocal transfers of embryos between strains of Merinos selected for and against multiple births. More of the unselected group of ewes conceived and lambed after embryo transfer and inter-strain rather than intra-strain transfers were associated with improved conception rate; the latter effect was significant in unselected recipients. Relatively large numbers (119) of recipients were available in this experiment. This 'heterosis' effect requires further investigation.

Gestation length — The role of the embryo in determining gestation length has been confirmed in interbreed embryo transfer experiments. Moore (1968) found the gestation length of single or multiple Border Leicester lambs was around 145-146 days and of Merino lambs 150-152 days in both Border Leicester and Merino recipients. The gestation length of Finnish Landrace lambs was 142-143 days in Finnish Landrace, Border Leicester and Welsh Mountain ewes (Bradford et al, 1972). In Romney Marsh, Suffolk and Finnish Landrace ewes, Lawson (1970) found that the gestation length of Clun Forest lambs was 144-145 days, but Romney Marsh ewes produced Romney Marsh lambs after 148.4 $\pm$ 0.6 days and Finnish Landrace lambs after 143.9 $\pm$ 0.5 days. When embryos of two breeds of differing gestation lengths are transferred, gestations are of intermediate lengths but closer to that of the breed with the shorter gestation (Bradford et al, 1976).

PATHOLOGY OF PREGNANCY

Embryo survival — Investigations of environmental and management factors affecting embryo survival in sheep have been notoriously difficult because of the problem of obtaining repeatable treatment effects. A few attempts to use embryo transfer in these investigations have been reported.

Alliston and Ulberg (1961) attempted to demonstrate the mechanism by which embryo survival was depressed by high environment temperatures. Reciprocal transfers of embryos between ewes subjected to ambient temperatures of 21°C and 32°C indicated that embryos from heat-stressed ewes had suffered damage that was not morphologically apparent within 3 days of conception. However, transfers of embryos after day 3 from unstressed to

stressed ewes were also less successful than transfers between unstressed ewes. Subsequently Woody and Ulberg (1964) showed, by transferring unfertilized ova between ewes at 21°C and 32°C, that the reproductive tract of ewes subjected to the higher temperature appeared to be deleteriously affected by the end of estrus.

The effects on embryo survival of liveweights and face cover, which have been associated with barrenness in ewes, were studied by Cumming and McDonald (1970). After transfer of one, two or four embryos, they were unable to discern any relationship between liveweight and embryo survival. A significant association between face cover and embryo survival was found after the transfer of two embryos, but this relationship was not apparent in other groups of ewes; and it should be noted that it has since been shown that there is no relationship between face cover and fertility in some flocks (Martin and Watson, 1976).

To investigate the effects of age and nutrition on embryo survival, Cahill et al (1976) transferred two embryos to 1½- and 4½-year-old ewes and two or four embryos to mature ewes, which were either fully fed or had a restricted feed intake from the estrus preceding embryo transfer. Embryo survival to day 30 was significantly depressed in young ewes (44% vs. 63%). Despite a loss of 20% of their liveweight to day 30, no effect of nutritional restriction on embryo survival was evident.

In studies of embryo survival in sheep it has been well established that most losses occur within the first 3 weeks of gestation. Experiments that rely for their result on failure of conception, though this is the significant practical consideration, really see only the extreme effect of the treatment applied. More precise investigations must await elucidation of the mechanism of the establishment of pregnancy in the hope that a more easily measurable parameter may be found. Possibly assessment of embryonic development to a stage between days 11 and 15 could give a more accurate measure of the quality of the environment to which the embryo has been subjected.

Embryo transfer was employed by McDonald and Rowson (1962) to investigate the reasons for low fertility in ewes following estrus induced during lactation. They found that the conception rate after transfer of embryos increased from 7 to 37 and 60% when ovulation was induced at 20, 36 and 65-90 days after parturition, respectively. A similar use of this technique was made by Schmidt (1961), Shelton and Moore (1966) and O'Reilly and O'Byrne (1973) in investigating the infertility of ewes mated after treatment with progestogens. Progestogen treatment did not affect the survival of transferred embryos.

Intergeneric embryo transfers between sheep and goats has enabled investigations of the physiological mechanisms underlying the failure of these related species to interbreed. Studies by Warwick et al (1934), Warwick and Berry (1949), Bowerman and Hancock (1963), Hancock (1964) and Hancock and McGovern (1968) have shown that, though fertilization occurs in does inseminated with rams' semen, hybrid offspring failed to survive beyond the 2nd month of gestation. In all cases where reciprocal embryo transfers have been made between sheep and goats, and transfers of hybrid embryos to does and ewes with or without a native embryo present, the alien embryo has failed to survive the 2nd month of gestation. An immunological interaction, possibly of the maternal system against the alien embryo, has been suggested as the cause of embryonic death (Hancock, McGovern and Stamp 1968; Dent et al, 1971).

McGovern (1974), after studying freshly fertilized sheep oocytes transferred to mated does as well as fertilization in ewes after uterine inseminations with goat semen, concluded that the failure of fertilization following the mating of ewes to goats was due to factors affecting the survival, transport or capacitation of goat sperm.

GENOTYPE PROLIFERATION

Two obvious applications of embryo transfer in sheep and goats are for the introduction of new genotypes and the expansion of valued genotype by increasing the reproductive rate of females.

The former application has been demonstrated to be feasible, though in practice a problem limiting its use has been quarantine considerations. Hunter et al (1962) reported the birth of four lambs in South Africa following the aerial transport from Cambridge, England, of 21 embryos in the oviducts of pseudopregnant rabbits. The embryos from Border Leicester and Welsh Mountain ewes were transferred from the rabbit to Dorper and German Merino ewes.

The use of the rabbit oviduct for the short-term incubation of sheep embryos was pioneered by Averill et al (1955) and also experimented with by Loginova et al (1968). Although techniques for freezing sheep embryos (Willadsen, Polge, Rowson and Moor, 1976) are likely to supersede this method of storing embryos for long periods, the rabbit has certain advantages as a short-term, fully automatic incubator. Lawson, Adams and Rowson (1972) showed that 87% of sheep embryos could be recovered from the ligated oviducts of pseudopregnant or estrous rabbits; 93% of these had developed normally. On re-transfer to ewes after 3 days in the pseudopregnant rabbit, 69% of the embryos developed to term. This survival rate after ewe-rabbit-ewe transfers was comparable to that in ewe-ewe transfers. The viability of embryos kept in the rabbit oviduct declined when the period in the rabbit was extended to 5 or 7 days. Baker et al (1971) reported the birth of two lambs from 14 embryos transferred after incubation during transit between Chicago and Montreal in silicone rubber tubes.

Moore and Shelton (1962a) used embryo transfer to multiply a flock of polled Merino ewes. From

74/86 treated ewes, 441 developing embryos were transferred to produce 236 lambs. i.e., a final lambing rate of 2.7 lambs per ewe in the selected flock.

In the remnants of an Australian Wiltshire Horn flock that had been reduced to 10 ewes and a ram, all of indeterminate ages, Lawson (unpublished data) treated eight ewes. Fifty-two ova were recovered but only eight showed normal development. From these, eight lambs were born. Five progeny of the above ewes were again treated and 24 ova recovered; only five normal embryos were transferred. Possibly the breeding failure in this case was associated with a high level of inbreeding in the flock.

Goat breeders in Australia have shown considerable interest in the use of feral does as recipients of embryos from Angora flocks. Procedures for estrous cycle synchronization, superovulation and embryo transfer have been described by Moore (1974). Moore (personal communication) has had 177 Angora kids born after the transfer of 308 embryos from 35 Angora does to 280 feral recipients, 165 of which kidded.

MATERNAL AND GENETIC EFFECTS ON FETAL DEVELOPMENT

Birth weight — In several studies, interbreed embryo transfers have been used to investigate the factors controlling the birth weight of lambs. Maternal effects on birth weight were first investigated using embryo transfer by Lopyrin, Loginova and Karpov (1950 a, b; 1951) and Lopyrin and Loginova (1960). Reciprocal transfers between Welsh Mountain and Border Leicester ewes were made by Hunter et al (1954, 1955) and Hunter (1956), and between Welsh Mountain and Lincoln ewes by Dickinson et al (1962). Both series of experiments showed a significant maternal effect on birth weight, but in the latter, larger experiment it was shown that the genotype of the lamb was the more important factor in determining birth weight. They concluded that the ewe was able to respond to the genetically determined demands of the lamb. Maternal ability was improved with increasing parity.

A study of interbreed transfers involving Oxford Down, Finnish Landrace, Border Leicester, Southdown, Tasmanian Merino, Welsh Mountain and Soay sheep, and litter sizes ranging from one to five was made by Bradford et al (1974). Transfers between Lincoln and Southdown ewes were studied by Karihaloo and Combs (1971). Litter size, breed of ewe and genotype of lamb were all significant in determining lamb weight at birth.

Both Bradford et al (1974) and Dickinson et al (1962) commented that the limit upon maternal capacity appeared to be reached at a total birth weight approaching three times that of singly born lambs.

In the above experiments, litter size affected post-natal survival through its effect upon birth weight. However, the particular viability of Finnish Landrace lambs, despite their small birth weight, has been noted by Lawson (1970) and Bradford et al (1974). In comparing the birth weights of Romney Marsh and Finnish Landrace lambs in litters of one to four from Romney Marsh ewes, Lawson (1970) noted that Finnish Landrace lambs were able to maintain their birth weight as litter size increased to four, but that of Romney Marsh lambs was severely depressed.

Fleece characteristics — Lopyrin, Loginova and Karpov (1950b, 1951) noted maternal effects on the birth coats of lambs following reciprocal transfers of embryos between Merino, Chuntuk and Karakul ewes. Studies of the effects of maternal environment upon a variety of fleece characteristics have been made by Wiener and Slee (1965), Burns (1972) and Burns and Ryder (1974). All three experiments showed that transfer to another maternal breed affected follicle density, sulfur:phosphorus ratio, and fiber medullation, diameter and length. These changes were in the direction of the recipient breed. Burns and Ryder (1974) concluded that these effects following embryo transfer occurred as a direct result of the maternal environment, rather than as a consequence of change in fetal size.

Mother-lamb transfer of immunoglobulins — The mechanism whereby the vigorous Finnish Landrace lamb attained exceptionally high concentrations of maternal immunoglobulins was investigated by Halliday (1973). Finnish Landrace lambs were found to have high levels of immunoglobulins at 2 days of age both when born to Finnish ewes or ewes of other breeds. Halliday concluded that this was not a maternal effect, but most likely a consequence of their vigorous sucking behavior. Superior intestinal absorption could also have been a factor.

MANIPULATION OF EMBRYOS

Identical twins — The potential of single blastomeres from early embryos to develop normally to term has been demonstrated in several species. An attempt to produce identical twins by mechanically dividing 4-, 6- and 7-day sheep embryos has been reported by Trounson and Moore (1974b). Although some 25% of the halved 6- and 7-day embryos developed during culture into blastocysts classified as normal, only two lambs were born from 19 blastocysts that were re-transferred.

In the process of examining embryos before transfer in sheep, Rowson and Moor (1964) discovered twin embryonic discs on two of 200 blastocysts aged 7-9 days and on two of 250 blastodermic vesicles aged 12-14 days. This study suggests that identical twinning could be relatively common in sheep, though the survival rate to birth is not known.

Chimera production — Two experiments have been described in which the production of chimeras

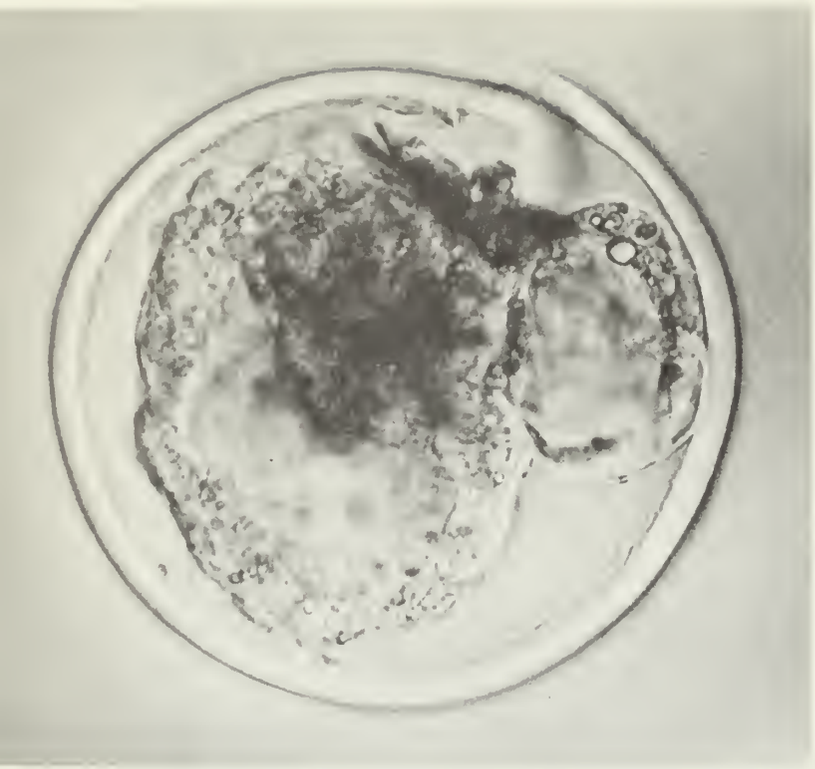


Figure 20. Monovular twin bovine blastocysts before hatching? Do identical twins sometimes separate this early rather than developing from two embryonic discs on the same blastocyst or blastodermic vesicle as described on page 77.

From ADRI, unpublished.

in sheep has been attempted. Pighills et al (1968) transferred 22 composite embryos that had been fused *in vitro*. Two lambs and one stillborn at 136 days were produced. From serum transferrin patterns of the animals involved it was demonstrated that the stillborn lamb was chimeric.

Tucker et al (1974) injected disaggregated cells from 3-7 day embryos into a further 92 intact embryos. From these, 33 lambs were produced after transfer. On the basis of red-cell antigens, serum transferrin and albumin types it was shown that three of the offspring were of mixed (tetraparental) parentage and one had donor type blood only. All four lambs were phenotypically male, though two had mixed male and female karyotypes.

Freemartinism — Freemartins in sheep have been identified by Ewen and Hammason (1947), Stormont et al (1953), who also cited one other case, and Moore and Rowson (1958). Each of these three cases was identified by chance from the occurrence of male traits in a phenotypic female, by erythrocyte mosaicism and by tolerance to skin grafting, respectively. Stormont et al calculated that freemartinism could occur in up to 0.8% of births in sheep. A systematic survey of sex chimerism in sheep was made by Dain (1971) who identified two chimeric sets in 161 mixed sex pairs of twins.

No attempts to study freemartinism in sheep using embryo transfer appear in the literature.

The techniques of sexing, and of producing identical twins and chimeras in sheep offer remarkable possibilities for the study of the physiological control of productive traits in sheep. Identical twinning, if it could be induced with some degree of ease, would aid experimentation in many fields of animal research. Further experimentation on these techniques should receive a high priority as research objectives.

RESEARCH APPLICATIONS OF EMBRYO TRANSFER IN PIGS

P. J. Dziuk and B. N. Day

Embryo transfer allows one to control the number, age and genetic background of embryos and the site of their deposition in the uterus of the recipient sow. This capability is useful in studies requiring such information for analysis of observations on uterine migration, spacing and survival of embryos.

An answer to the question on the extent of migration of embryos from one uterine horn to the other was provided by a study using embryos of two donors whose fetuses were distinguishable by color. Embryos from a black sow were transferred to the top of one horn of the recipient and embryos from a white sow were transferred to the tip of the opposite horn. After day 85 of gestation, when the fetuses had developed skin color, the recipient mothers were killed and the fetuses examined for color and their position in the uterus (Dziuk et al 1964). Embryos had migrated from one uterine horn to the other in every case and in most cases embryos had mixed with embryos from the opposite side. Thus embryos can pass the uterine body and each other quite readily.

To obtain information on the effect of differences between the age of the embryo and the stage of gestation, Webel et al (1970a) transferred embryos from donors at various stages to recipients of the same and differing stages. They concluded that a 24-hour asynchrony was about as much as could be tolerated and still have many of the embryos survive. They also found that embryos at about day 5 were more likely to develop than older embryos. This effect of age of embryos was also noted by Hunter et al (1967).

To study the possible local effect of the ovaries on maintenance of gestation and initiation of parturition, Martin (1976) transplanted ovaries either to the uterine horn or to the body wall. The ovaries were transplanted in two stages; first, they were sutured either to the surface of the uterine horn or to the external surface of the muscle of the body wall through a small incision; second, about 3 weeks later, the stalk of the ovary and the usual vascular

connections were cut and tied leaving the only connection to the ovary from either the surface of the uterus or the external body wall. The gilts with transplanted ovaries had normal estrous cycles. Embryos were transferred to the uteri of these gilts. Five pregnancies went to term after 26 embryo transfers in gilts with ovaries on the uterus and three pregnancies went to term after 22 transfers to gilts with ovaries on the body wall under the skin. The length of gestation was normal, the progesterone and estrogen levels were also normal. The gilts farrowed normally, nursed their litters and resumed normal estrous cycles after the piglets were weaned.

Varying the number of embryos and fetuses in the uterus to determine the effect of numbers on maintenance of pregnancy and embryo survival can be done systematically by embryo transfer. Dhindsa (1967) transferred one, two or four embryos to one horn only in each of five gilts and also transferred this same number of embryos to each horn in five gilts each. Pregnancy was maintained in none of the 10 gilts receiving one embryo per horn per gilt; in 2/10 receiving two embryos and in 2/10 in gilts receiving four embryos. This indicates that whereas a pregnancy can be maintained with two embryos, it is unlikely to be maintained with one embryo, and is more likely to be maintained with four than with two. At the other extreme, addition of embryos to already existing litters (superinduction) or the creation of large litters of known numbers has been done by embryo transfer. Dziuk (1968) compared the average number of embryos at 25 days of gestation of superinducted and unoperated control gilts. Gilts receiving approximately 13 additional embryos 4 days following insemination had an average of 4.1 additional embryos at 25 days of pregnancy. Several studies at North Carolina State University have employed the use of superinduction in gilts to investigate uterine capacity during early and late stages of gestation as limiting factors on litter size (Bazer, Clawson, Robison and Ulberg, 1969; Bazer, Robison, Clawson and Ulberg, 1969; Fenton, Bazer, Robison and Ulberg, 1969; Fenton, Robison and Ulberg, 1969).

Pope et al (1972) transferred 12 or 24 fertilized ova to unmated recipient gilts to determine the effects of embryo number on embryo survival rate to 25 days of gestation. Gilts receiving 24 fertilized eggs had significantly more embryos at 25 days (16.3 vs. 6.8 embryos). Embryo survival rates were 67.9% for gilts receiving 24 fertilized eggs and 56.7% in the 12-embryo transfer group. All of the gilts receiving 24 eggs were pregnant at 25 days and the number of viable embryos in 9 of the 10 pregnant recipients ranged from 14 to 23. The CL count of gilts receiving 24 eggs varied from 11 to 15 and averaged 12.9. Embryo transfer, therefore, provided a means to evaluate the effect of an increased number of embryos on embryo survival during early pregnancy in gilts that had not been superovulated. The average of 16.3 viable embryos present at 25 days following transfer of 24 fertilized eggs demonstrates that additional CL are not a requirement for development and growth of an increased number of embryos in superovulated gilts.

EMBRYO TRANSFER IN PERSPECTIVE

K. J. Betteridge

The preparation of this monograph during the last quarter of 1976 coincides with a trough in the cyclic emphasis given to embryo transfer as a tool in animal production that has been a feature of the subject since the late 1940's. The trough has followed an unprecedented wave of commercial use of the technique in cattle as described on pages 59 to 62. The 'artificial' nature of the commercial values of exotic cattle that occasioned the wave has been properly emphasized but should not obscure the fact that the use of the technique was nonetheless 'real' and that new artificial markets are likely to occur. A current one is in exporting cattle to the Middle East. Furthermore, the commercial boom has taught us much about both the potential and shortcomings of embryo transfer in the field and has directly and indirectly brought about considerable technical advances. These deserve summary, saying at the outset that it is disappointing that a wealth of useful results obtained commercially has been lost because relatively few units have published their experiences.

The wave began in cattle with entirely surgical techniques stimulated by the success achieved by L. E. A. Rowson and his colleagues at Cambridge. It is clear that, when two embryos are transferred to each recipient, these methods can lead to pregnancy rates of 70-90% and embryo survival rates of 40-60%. Transfers of single embryos however, at least under commercial circumstances when even 'doubtful-looking' embryos must be used, can only be expected to result in 50-60% of recipients becoming pregnant. Verification of figures like these has been an important result of large-scale studies during the boom and it is to be hoped that techniques such as freezing and sexing will be subject to similar extensive testing.

It seems quite possible that surgical rates of success may be matched, but not exceeded, by the non-surgical methods that have improved so much during the past 2 years. Calculations of embryo transfer costs can therefore be built around those figures for the recipients. The requirements of commercial users of embryo transfer played no small part in stimulating the advances that have been made in non-surgical techniques.

The donor still constitutes the less predictable half of the transfer system. The proportion of gonadotrophin-treated cattle that respond with more

than three or four ovulations ranges between 60 and 90% in heifers, is lower in mature cows and is one of the most poorly documented of embryo transfer's vital statistics. Animals that do respond can be expected to yield an average of five transferable embryos, but averages can be virtually meaningless when it is the yield from one particular donor that is of paramount concern. We are still unable to tailor treatments to the individual to guarantee superovulation. As a consequence, predicting the outcome of committing a single donor to embryo transfer is still a gamble. This, again, is a serious shortcoming when individual donors are important but much less serious when a population of donors can be used to provide embryos, as in twinning for beef production, for example. In either circumstance, the long-term yield of embryos from donors should improve markedly as non-surgical methods supervene, provided refractoriness to repeatedly used gonadotrophins does not prove to be too big a problem when really put to the test.

The prospect of obtaining potential embryos indiscriminately from slaughtered females' oocytes seems a little more real as a result of follicle culture work but is not yet a practical alternative to the superovulation of donors. Regardless of the source of embryos, successful transfer depends on close attention to detail in everything from herd management to glassware washing and, during the commercial boom, many learned the hard way that running a transfer unit cannot be a part-time occupation.

The commercial boom also revealed just how expensive a procedure surgical embryo transfer can be. This aspect of the subject has not been dealt with in this review, but J. M. Bowen, in a paper presented to the International Embryo Transfer Society in 1975 (and in personal communications), has shown that the real cost of obtaining a pregnant recipient in a commercial unit using natural synchrony and surgical methods is about $1900 in North America or £1600 in the United Kingdom. Adding donor costs and allowing a profit of 33.3% means that pregnant recipients need to have a market value of about $3000 or £2100 to make the procedure pay.

By far the largest part ($1660 or £1530) of the cost per pregnancy results from the maintenance of a large recipient herd. Costs can be reduced, therefore, either by using prostaglandins to reduce the numbers required or by cooperating with a feedlot from which recipients can be drawn as needed. Bowen calculates that prostaglandins at $5 per treatment can reduce the cost per pregnancy by $240 and cooperation with a feedlot effects a reduction of $650 per pregnancy.

Using non-surgical collection but surgical transfer methods together with prostaglandins makes little impact on costs, each pregnancy costing about $1200.

Although it is early yet to quote success rates with frozen embryos, current studies suggest that about 33% of embryos submitted to the freezing procedure may subsequently produce calves. Working with this figure, Bowen calculates the cost per pregnancy with frozen embryos to be about $1000.

Wilmut and Hume (cited by Cox, 1976) estimate that costs, after allowing for losses in production by donors and recipients, could be reduced to £68 per calf using non-surgical methods for both recovery and transfer. Even at this price, they show that the procedure would be far too expensive for most commercial uses, with the notable exceptions of producing beef sires from elite herds and moving livestock internationally. Bowen (personal communication) considers that their costings as cited are unrealistically low, being based on optimistic success rates and making no allowance for the cost of maintaining recipients. This would reinforce, rather than detract from, the conclusion of Wilmut and Hume ". . . that embryo transfer will have only limited commercial application (and) is unlikely to be affected by changes in milk and meat prices or technological advances in the near future." It has been experience gained during the commercial boom that has allowed the role of embryo transfer in agriculture to be appraised realistically. Such appraisals should counter the more outlandish claims made by some promoters of embryo transfer during the boom.

It is important now that the trough following the most recent wave of emphasis given to embryo transfer should not be too deep. Even the most pessimistic assessors of the future of the procedure in agricultural production agree that it is an invaluable research tool. It may well be through research that money and effort expended on embryo transfer pays biggest dividends. Our knowledge of uteroovarian interrelationships, for example, owes much to embryo transfer and has been the key to the development of methods of estrus synchronization with prostaglandins. Since recent technical advances have made it an easier tool to use, embryo transfer can be expected to be applied to a wide range of investigations in the future. It is impossible to predict whether these in turn will result in sufficient improvement in technology to disprove Wilmut and Hume's conclusion, but it can be safely assumed that unusual production demands, best met by embryo transfer, will continue to occur in all the farm species. Fortunately, the achievement of results that would allow more widespread direct commercial application of embryo transfer need not be considered the only criterion of usefulness if the research to which the procedure is applied is itself worthwhile. Thus, whatever the immediate future of commercial embryo transfer, one can feel justified in proposing, like Walter Heape, ". . . to continue . . . experiments and to extend them . . ."

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