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EMBRYO TRANSPLANTATION AND PRESERVATION

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Effective techniques for embryo transplantation in some laboratory and farm animals are now well established. Methods for collection and transfer of embryos in the pig were first developed in the early 1960s (Hancock and Hovell, 1962; Dziuk, Polge and Rowson, 1964; Vincent, Robison and Ulberg, 1964) and since then they have been applied mainly in research. Embryo transplantation has proved to be a valuable experimental tool in a number of studies concerned with early embryonic development, the survival of embryos *in vivo* or *in vitro*, migration and spacing of embryos within the uterus and factors affecting the maintenance of pregnancy. Future research is also likely to be concerned increasingly with cellular and genetic manipulation of eggs and embryos *in vitro* and the application of these techniques depends to a large extent on having reliable methods for the culture of embryos and their subsequent transfer.

Practical applications, particularly in farm animals, are also important and the best example is in cattle where methods developed for research have now been extended very successfully into the practice of animal breeding. Applications in pig husbandry have so far been on a relatively small scale. The high fecundity and reproductive rate in pigs compared with cattle will never provide the same economic incentive to apply such methods for the purpose of genetic improvement or to get more offspring from a few superior animals. On the other hand, strict control of disease, especially in large intensive units, is a most important aspect of modern pig husbandry and embryo transplantation should provide the safest method of introducting new genetic material into closed herds. It is mainly for this reason, and perhaps also for the possibility of transporting embryos between countries, that embryo transplantation in pigs is likely to be applied as a practical measure.

Methods of embryo collection and transfer

Techniques for collection and transfer of embryos used at different laboratories are basically very similar to those described by Hancock and Hovell (1962). The methods used routinely in experiments at the Animal Research Station are described here.

COLLECTION OF EMBRYOS

The tortuous nature of the cervix and uterus in the pig virtually precludes the collection of embryos from the uterus by non-surgical means. The surgical approach, however, is relatively simple and operations on donor animals can usually be completed in less than 30 minutes. Following mid-ventral laparotomy under general anaesthesia the reproductive tract is exposed and a region appropriate to the developmental stage of the embryos to be collected (time after ovulation) is then flushed. One-, 2- and early 4-cell embryos can be collected from the oviducts up to about 40 hours after ovulation. A fine glass cannula is inserted into the isthmus through a small hole made in the tip of the uterine horn. Flushing fluid passed down the oviduct from the fimbriated end is collected via the cannula into a glass cup or petri dish. Pig embryos normally enter the uterus much sooner and at an earlier stage of development than in many other species examined. This means that flushing the oviducts alone should be attempted only when the time of ovulation is known quite precisely. Estimates of ovulation time based on the assumption that the endogenous luteinizing hormone (LH) surge coincides with the onset of oestrus can sometimes be misleading. More precise timing of ovulation can be obtained by injection of human chorionic gonadotrophin (HCG) during late pro-oestrus (Dziuk and Baker, 1962; Hunter, 1972). When embryos enter the uterus at the 4-cell stage, it is then virtually impossible to flush them back through the oviducts due to the valve-like nature of the utero-tubal junction. The most efficient technique for obtaining uterine embryos, or indeed those that may be tubal or uterine, is to flush fluid down the oviduct and wash all the embryos away from the tip of the horn. The uterus is clamped with bowel forceps at an appropriate length from the tip of the horn and the flushing medium entering from the oviduct is massaged towards the clamp. A cannula with a larger bore than that used for insertion into the isthmus is introduced into the uterine lumen through a small incision made near the tip of the horn and the fluid is 'milked' back out. Up to 5–6 days after oestrus the embryos remain close to the tips of the horns and it is only necessary to flush a small section. Later, when the embryos have started to migrate throughout the uterus, the whole of each individual horn should be flushed. Several different media have been used successfully for embryo collection, but the medium now used routinely at the Animal Research Station is Dulbecco's phosphate buffered saline enriched with lactate, pyruvate and bovine serum albumin (Whittingham, 1971). An advantage of a medium buffered with phosphate over some others that may be buffered with bicarbonate is that pH is quite well maintained when the medium is exposed to air.

The methods of embryo recovery described are suitable for animals up to about 12 days after the onset of oestrus. If the uterus is flushed at later stages when the embryos have become extremely elongated, the embryonic membranes become entangled together. The efficiency of the technique is very high and the majority of the embryos within the reproductive tract are generally recovered. For example, in a recent experiment 205 gilts were used as embryo donors and the operations were performed 3–9 days after the onset of oestrus. Eggs or embryos were

recovered from all animals except one. In 57% of animals the recovery rate, estimated by counting corpora lutea and embryos, was 100% and in the remaining animals only a few embryos were missing. The average recovery rate for all animals (3234 corpora lutea) was 95%.

Repeated operations on donors inevitably tends to build up scar tissue and adhesions, and in our experience 3–4 operations are about the maximum that can be performed successfully on one individual.

Superovulation in donor animals can be achieved by administration of gonadotrophic hormones at an appropriate time in the cycle. A dose of 1000–1500 iu pregnant mare's serum gonadotrophin (PMSG) given early in the follicular phase (day 15 or 16 of the oestrous cycle) usually produces 25–30 ovulations in mature gilts (Hunter, 1964) although the response is quite variable. A mixture of 600 iu PMSG + 200 iu HCG given as a single dose will produce a similar result. Gonadotrophins can also be given after some treatments used for the synchronization of oestrus (Polge, Day and Groves, 1968). Heat normally occurs 3.5–4 days after PMSG treatment, but 500 iu HCG given during the third day after PMSG will synchronize the time of ovulation in a group of animals. Fertilization in superovulated animals is generally normal except when the ovulation rate is excessively high and a number of immature oocytes may be ovulated. Similar treatments can be used to induce ovulation and obtain embryos from prepubertal gilts (Dziuk and Gehlbach, 1966; Baker and Coggins, 1968).

EMBRYO TRANSFER

A similar surgical approach to that applied in donors is used for recipient animals. After exposure of the reproductive tract, embryos can be transferred either to the oviduct or uterus depending on the stage of embryonic development. When transferring to the oviduct the embryos are picked up in about 0.2 ml of fluid in a fine Pasteur pipette which is threaded down the lumen via the fimbria to a depth of about 5 cm. When embryos are transferred to the uterus, a small puncture is made in the isthmus region of the oviduct about 2 cm from the tip of the horn and the tip of the pipette is slid down into the uterine lumen through the utero-tubal junction. This method avoids making a puncture wound in the uterus itself and the consequent possibility of causing endometrial haemorrhage.

In early experiments (Polge, 1966) care was taken to transfer embryos at very early stages of development (2-cell and early 4-cell) only to the oviducts and all later stages to the uterus. In some species the site of transplantation in relation to the stage of embryonic development appears to be important. In cattle, for example, embryonic survival is reduced if embryos collected from the oviducts of donors are transplanted to the uterus of synchronized recipients one or two days earlier than the time they would normally enter the uterus (Newcomb and Rowson, 1975). In pigs, however, the length of time that embryos normally remain within the oviducts is quite short and in recent experiments it has been found that embryonic survival is not reduced when 2-cell embryos collected from the oviducts are transplanted to the uterus of recipients at the same stage of the reproductive cycle. An alternative method of transfer for all embryos can

therefore be used and this is simply to flush them down the oviduct and into the uterine lumen in a larger volume of fluid. It is generally only necessary to transfer the embryos to one side of the uterus since they will later migrate and become evenly spaced throughout both horns (Dziuk, Polge and Rowson, 1964).

Attempts at non-surgical transfer of embryos via the cervix in the pig have not been very successful (Polge and Day, 1968). It is possible, however, that embryo transfer could be achieved by means of laparoscopy (B.N. Day, personal communication). This approach could be most useful on farms where suitable facilities for surgery might be lacking.

Synchronization of oestrous cycles

An important factor affecting success in embryo transfer is the degree of synchrony between recipient and donor animals. In most species examined it has been found that pregnancy rate and embryonic survival are reduced if the stage of the reproductive cycle of the recipient is more than about one day out of phase with that of the donor.

In many of the early experiments on embryo transfer in the pig, methallibure was used to control the time of onset of oestrus in donor and recipient gilts. This drug was a most effective synchronizing agent and, in addition, gonadotrophins could be used following treatment either to stimulate superovulation or induce ovulation at a predetermined time (Polge, Day and Groves, 1968). Since the use of methallibure was withheld in many countries in the early 1970s on the grounds that it was a teratogenetic agent, alternative methods for oestrus synchronization have been sought. In pigs, analogues of prostaglandin $F_{2\alpha}$ do not induce luteolysis effectively when given earlier than day 12 or 13 of the oestrous cycle (Guthrie and Polge, 1976a) and there is no way therefore that these compounds alone can be used to synchronize oestrus in randomly cycling animals. Various approaches have been attempted in order to create groups of gilts in which the ovaries of all animals contained corpora lutea which were old enough to respond to exogenous prostaglandin. The methods included the induction of accessory corpora lutea (Guthrie and Polge, 1976b) and the extension of luteal function by oestrogen. Perhaps the most successful method was simply to prolong luteal function by means of pregnancy. In animals injected with cloprostenol (ESTRU-MATE, ICI Ltd) 12–40 days after mating, abortion was induced and a very high proportion returned to oestrus 4–7 days later. Insemination at the synchronized oestrus resulted in normal levels of fertilization and embryonic survival (Guthrie and Polge, 1978).

A simpler and more effective method of oestrus synchronization is now available however. In the past, several orally active progestational agents had been examined in pigs, but there were always major drawbacks to their uses, including reduced fertility after treatment and the induction of follicular cysts. Encouraging preliminary results with a new orally active progestin, allyl trenbolone or altrenogest (REGU-MATE, Roussel Ltd) were first described at a recent Nottingham Easter School (Webel, 1978) and since then trials have been carried out at the Animal Research Station.

In these trials two hundred and twenty eight mature, crossbred gilts that had had at least one oestrus were divided into groups of six and fed a normal diet containing altrenogest. Treatment was started on any day of the oestrous cycle and a dose of 15 or 20 mg altrenogest/pig/day was given for a period of 18 days. Twice daily testing for oestrus with a boar was carried out during and after treatment. Oestrus was effectively suppressed in all animals during treatment, but following withdrawal of the compound there was a spontaneous rebound to follicular activity. Oestrus was detected in 98% of animals within eight days after treatment and about 80% of the heats occurred on the fifth or sixth day (*Table 14.1*). The maximum oestrus

Table 14.1 OESTRUS SYNCHRONIZATION IN GILTS

		Altrenogest treatment for 18 days	
		15 (mg/day)	*20* (mg/day)
Number treated		144	84
Number showing oestrus after treatment		141	83
Day of onset of oestrus after treatment (% of animals)	4	8.5	–
	5	58.9	25.3
	6	27.0	53.0
	7	5.7	16.9
	8	–	4.8
Average number of corpora lutea/animal		15.5	15.6
% eggs fertilized		89.8	92.3

response was on the fifth day after treatment in animals that had received 15 mg/day and on the sixth day after treatment in animals that had received 20 mg/day. Most of the gilts in this trial were used in embryo transfer experiments. About half of them, which were used as donors, were therefore artificially inseminated (two inseminations with 50 ml fresh undiluted semen on the second day of oestrus). The fertilization rate of eggs collected 3–9 days later was over 90%. At laparotomy in donor and recipient animals it was also possible to examine the state of the ovaries and the incidence of follicular cysts was found to be negligible. Altrenogest, therefore, appears to be an exceedingly effective drug for oestrus synchronization in pigs. The only problem relating to its use in embryo transfer experiments, however, may be that the induction of superovulation in donor animals by means of PMSG and HCG has so far produced rather variable results (Polge, 1981).

Early embryonic development

Early embryonic mortality is a common feature in most animals and in the pig it has been estimated that about 30% of the potential embryos are lost by the 25th day of gestation (Hanly, 1961). In a study on early pregnancy in the pig, Perry and Rowlands (1962) made observations on gilts and sows killed between 2 and 40 days after mating and determined the incidence of embryonic loss and the time and stage of development when the loss

occurred. An important observation was that 22% of the embryos recovered from the uterus between the sixth and ninth days after mating appeared to be degenerating.

During the course of experiments on embryo transplantation at the Animal Research Station an opportunity has been afforded to examine a large number of embryos collected during the first 10 days after oestrus. These studies are interesting from the point of view of the efficiency of fertilization and the extent of early embryonic degeneration in pigs. In the experiment referred to in an earlier section, the embryos collected from 205 donors 3–9 days after the onset of oestrus were examined. Since the average recovery rate was 95%, very few embryos were lost through technical reasons and in the majority of cases the whole 'litter' representing all the eggs that had been ovulated was available. A total of 3085 embryos was examined. Animals had been treated with altrenogest and inseminated on the second day of oestrus as described above. Out of the 204 animals from which eggs or embryos were recovered, none of the eggs was fertilized in 11 (5%) pigs. In 82% of the remaining animals all the eggs were fertilized whereas in 18% there was a mixture of fertilized and unfertilized eggs. Eggs were classified as unfertilized if they were single cell or fragmenting and contained no sperm in the zona pellucida. The proportion of unfertilized eggs in individual animals with partial fertilization varied quite considerably; in some it was just one or two eggs out of the total, but in others the number was much higher. The main cause of fertilization failure related to unilateral sperm transport within the uterus and fertilized eggs were recovered from one uterine horn only. Overall, 34% of the eggs were unfertilized in the animals in which fertilization was not complete. Apart from fertilization failure there was very little evidence of embryonic loss up to nine days after the onset of oestrus. In all embryos classified as fertilized, less than 1% showed any obvious signs of degeneration. These observations are clearly not in agreement with those of Perry and Rowlands (1962). It should be noted, however, that these authors treated their data with some caution and stated that it was possible that some of the ova collected 6–9 days after mating and classified as degenerating were, in fact, viable. They agree that if this were the case, then mortality among fertilized eggs up to the ninth day must be very small.

Our data certainly suggest that embryonic mortality up to the ninth day is very small, but these observations should also be treated cautiously. When embryos are used in transfer experiments it is possible only to examine them in a relatively superficial manner under a stereomicroscope. It may be that a more detailed examination of fixed and stained embryos could reveal abnormalities which are not immediately obvious. For example it was often noted that some of these embryos at the late morula and early blastocyst stage appeared to have some cells developing outside the main body of the embryos themselves. These were not classified as degenerate. Also, after the blastocysts had hatched, there were frequently considerable discrepancies in the size of embryos within a 'litter', although all appeared to be viable. Whether such features reflect the ability of embryos to survive at a later stage is not known. Genetic abnormalities resulting from errors arising around the time of fertilization have been suggested as a cause for early embryonic mortality. However, in a detailed

cytogenetic study of pig embryos collected from animals up to 12 days after ovulation, very few chromosomal abnormalities could be detected (Lupse, 1973).

Other observations, also recorded in the study of Perry and Rowlands (1962), suggest that a large amount of embryonic loss, at least during the first 2–2.5 weeks of pregnancy, is not characteristic of most animals. Embryonic loss in thirteen pigs killed between the 13th and 18th days was 28.4%, but the greater part of this loss occurred in two of the animals. The average loss in the remaining 11 animals was only 12%. Undoubtedly, however, embryonic losses are higher in the majority of animals by the 30th day of pregnancy.

Results achieved in embryo transfer

When embryos have been collected from donors 2–5 days after the onset of oestrus and transferred to synchronous unmated recipients, the pregnancy rate achieved has been 60–70% with embryonic survival in pregnant animals (usually determined about the 30th day of pregnancy) also around 60–70% (Dziuk, Polge and Rowson, 1964; Vincent, Robison and Ulberg, 1964). Transfer of embryos at later stages, seven or eight days after the onset of oestrus, has resulted either in a complete failure to establish pregnancy (Webel, Peters and Anderson, 1970) or in much reduced pregnancy rates (Hunter, Polge and Rowson, 1967). Relatively little is known about the importance of synchrony between donors and recipients in the pig, although in one experiment there was evidence that transfers in which oestrus in the donor was one or two days earlier or one day later than the recipient were as successful as synchronous transfers (Webel, Peters and Anderson, 1970).

Quite a large scale experiment has recently been undertaken at the Animal Research Station in order to determine more precisely the effects relating to degree of synchrony between donors and recipients and stage of embryonic development at which transfers are made. Although this experiment has not yet been completed, enough has been achieved to provide some interesting results. Embryos have been collected from donors on days 3, 4, 5, 6, 7, 8 and 9 (onset of oestrus = day 0). Transfers have been made to recipients in which the onset of oestrus was either synchronous with that of the donors or one or two days earlier or later. Five recipients have been allocated to each group and thus the experiment involves 175 transfers of which results are now available for 140. The time of onset of oestrus in groups of animals was controlled by feeding altrenogest and the methods for collection and transfer of embryos were those described earlier. The average number of embryos transferred to recipients was 14 (range 11–18) and embryonic survival in pregnant animals was determined at slaughter on the 30th day of pregnancy.

The results are shown in *Figures 14.1* and *14.2*. Pregnancy rate was over 70% when transfers were made to recipients in which the onset of oestrus was either synchronous with that of the donors or one or two days later. In fact, the highest pregnancy rate (86%) was achieved in recipients which came on heat two days later than the donors *Figure 14.1(a)*. By contrast,

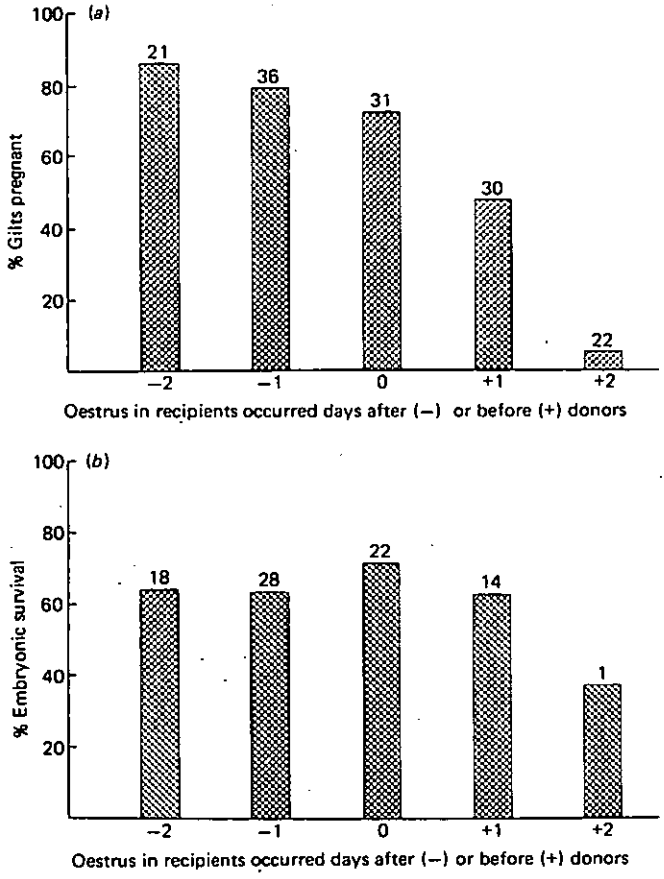

Figure 14.1 Embryo transplantation in gilts: differences in synchrony between recipients and donors. (a) Pregnancy rate following transfer of embryos collected from donors 3–9 days after the onset of oestrus. (b) Embryonic survival rate in pregnant gilts on the 30th day of pregnancy. Numbers above columns refer to the numbers of recipients in (a) and the numbers of pregnant animals in (b)

pregnancy rate fell dramatically in recipients which were ahead of the donors and only one animal out of 22 became pregnant in the groups that came on heat two days before the donors. Results were similar when embryos were collected from donors at any stage of the cycle from days 3–9. The average embryonic survival rate in pregnant animals was 65% and was lowest in the one animal that was two days ahead of the donor (*Figure 14.1(b)*. In *Figure 14.2*, the results have been presented according to the day of the cycle of the donor on which embryos were collected. The figures include results from all recipients except those which were two days ahead of the donors since virtually no pregnancies were achieved in these groups. The main point of interest is that, although the results were somewhat lower in groups receiving embryos collected from donors on days 8 and 9, there was by no means a dramatic fall in pregnancy rate or embryonic survival following transfer of these older embryos.

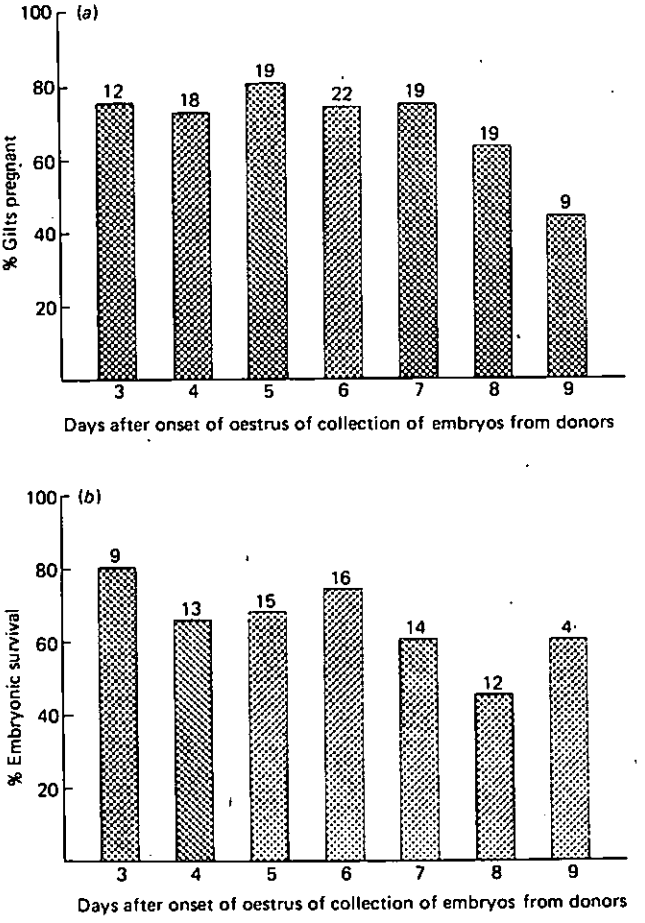

Figure 14.2 Embryo transplantation in gilts: transfer of embryos collected from donors 3–9 days after the onset of oestrus. (a) Pregnancy rate in recipients in which onset of oestrus varied from one day before to two days after that of donors. (b) Embryonic survival in pregnant gilts on the 30th day of pregnancy. Numbers above columns refer to the numbers of recipients in (a) and the numbers of pregnant animals in (b)

Embryonic survival following asynchronous transfer in pigs now confirms observations made in some other species which show that deviation on the side of transferring 'older' eggs to 'younger' uteri is often better tolerated than the reverse situation. How much 'younger' than two days the uteri could be without jeopardizing embryonic survival remains to be determined. In the rabbit it has been shown that exposure of the early embryo to a more advanced progestational uterus for less than 24 hours is incompatible with survival (Adams, 1971). Pig embryos also fail to survive in uteri which are 48 hours in advance even when transfers are done relatively early in the cycle. It would be interesting to know whether the embryos die soon after transfer or whether, being so much 'younger' than the uterus, they are unable to prevent luteolysis.

Earlier experiments (Webel, Peters and Anderson, 1970) in which no embryos survived when transferred to recipients on days 7 and 8, led to the suggestion that perhaps embryos need to be present in the uterus of the pig before day 7 for pregnancy to be established and maintained. Our recent results do not confirm this suggestion. A possible explanation of the poor results achieved following transfer of older embryos in some earlier experiments could relate to the type of medium used for collection. We have noted that when embryos have hatched from the zona pellucida they are very easily damaged by adverse environmental conditions. Media that will support development of early embryos *in vitro* may be unsuitable for the storage of embryos at a later stage (Robl and Davis, 1981).

Embryo transplantation in research

Embryo transplantation has been used as an experimental tool in a number of studies on relationships between the uterus and embryos during early stages of gestation. In most pregnancies some embryos probably migrate from one uterine horn to the other before implantation in order to establish even spacing throughout the uterus. The incidence of transuterine migration and the mixing of embryos within the uterus has been studied experimentally by transferring genetically marked embryos to each uterine horn (Dziuk, Polge and Rowson, 1964). When embryos from black donors were transferred to the tip of one uterine horn and embryos from white donors were transferred to the tip of the opposite horn, it was found that not only did they migrate from the horn of origin, but in most cases they became interspersed throughout the uterus.

Migration of embryos from the tips of the horns usually occurs soon after the sixth day of gestation. In experiments in which embryos were permitted to enter the uterus from one side only it was found that they started to enter the opposite horn around day 8 or 9 and the uterus was occupied completely by day 15 (Dhindsa, Dziuk and Norton, 1967). The time of cessation of intrauterine migration was studied by restricting the embryos to anterior sections of the uterine horns by means of ligatures applied soon after mating. Removal of the ligatures between days 8 and 13 showed that embryos could still migrate beyond the ligature sites up to day 11 and pregnancy was then maintained. Gilts in which the ligatures were removed on day 13 did not remain pregnant (Polge and Dziuk, 1970).

There is now much evidence to show that the presence of embryos throughout a large part of the uterus by days 12 or 13 is essential for the maintenance of pregnancy. Non-gravid sections of the uterus have been established experimentally, in some cases by transferring embryos to isolated segments (du Mesnil du Buisson, 1966; Anderson, Rathmacher and Melampy, 1966; Day *et al.*, 1967; Dhindsa and Dziuk, 1968). It has been shown that a relationship exists between the length of the non-pregnant uterine segment and maintenance of pregnancy. Pregnancy is usually interrupted when over one half of a uterine horn does not contain embryos. However, pregnancy continues when a smaller sterile uterine segment is isolated adjacent to one ovary even though corpora lutea in the ipsilateral ovary may regress. Effects of non-pregnant uterine segments are similar whether they are located at the tip or base of the uterine horns.

Restriction of embryos into isolated segments of the uterus has shown that pregnancy fails regardless of the nubmer of embryos present within the isolated segment. Maintenance of pregnancy does not therefore depend upon having a sufficient number of embryos to provide a luteotrophic stimulus. It is more important that the embryos are in contact with the greater part of the endometrium in order to prevent luteolysis (see F.W. Bazer, Chapter 12). In pregnancies with a small number of embryos, the embryonic membranes of individual embryos are longer than those of embryos which are more crowded within the uterus. However, when the number of viable embryos is very small around days 12–15, there is still likely to be a considerable area of the endometrium which is not in contact with the embryonic membranes and the chances of maintaining pregnancy are reduced. When the number of embryos present within the uterus has been reduced experimentally by means of embryo transfer, it has been shown that more than four embryos are necessary in gilts for the consistent maintenance of luteal function during early pregnancy (Polge, Rowson and Chang, 1966; Polge *et al.*, 1967).

Embryo transplantation has also been used to increase above normal the number of embryos present within the uterus and to determine whether 'uterine capacity' is an important factor affecting embryonic survival and litter size (Dziuk, 1968; Bazer *et al.*, 1969; Pope *et al.*, 1972). The best evidence supports a conclusion that uterine crowding is not a major factor limiting litter size in pigs during the first 25–30 days of pregnancy. Embryonic survival rates were similar at about day 25 in gilts to which either 12 or 24 embryos had been transferred (Pope *et al.*, 1972). A similar result was obtained in an experiment at the Animal Research Station (*Table 14.2*). Thus, early embryonic mortality appears to be more closely

Table 14.2 EMBRYONIC SURVIVAL IN RECIPIENT GILTS TO WHICH 12 OR 24 EMBRYOS WERE TRANSPLANTED (SYNCHRONOUS TRANSFER ON DAY 5)

Number of embryos/ recipient	*Number of recipients*	*Number pregnant on day 30*	*% pregnant*	*Embryo survival*			
				Total transferred	*Total live embryos on day 30*	*% survived*	*% survived in pregnant gilts*
12	21	15	71.4	244	102	41.8	58.6
24	20	18	90.0	408	247	60.5	61.9
Total	41	33	80.5	652	349	53.5	60.9

associated with intrinsic embryonic factors interacting with the uterine environment than with the ability of the uterus to support only a limited number of embryos. Nevertheless, uterine capacity is an important factor affecting foetal survival during later stages of gestation since litter size at parturition has not generally been increased above normal either by transferring additional embryos or by superovulation (Fenton *et al.*, 1970; Longenecker and Day, 1968).

Culture and preservation of embryos

In experiments on embryo transplantation, the time elapsing between collection of embryos from donors and their transfer to recipients is

generally not more than about one hour. Since the survival rate of embryos has been normal, at least with embryos collected from donors up to seven days after the onset of oestrus, the phosphate-buffered saline medium (Whittingham, 1971) which is used routinely, appears to be quite satisfactory for temporary storage. However, this medium may be less suitable for the storage of hatched blastocysts collected at later stages.

For longer-term preservation of embryos, media and conditions are required which will support normal development *in vitro*. Various media have been examined for the culture of pig embryos; for a review, see Wright and Bondioli (1981). A characteristic finding in the majority of experiments has been that development *in vitro* of 1- and 2-cell embryos is generally very limited and few progress beyond the 4-cell stage (Polge and Frederick, 1968; Rundell and Vincent, 1969). By contrast, a variety of media has been found to support development *in vitro* of embryos collected at later stages. Late 4-cell embryos and morulae have been cultured to hatched blastocysts (Wright, 1977; Lindner and Wright, 1978). Indeed, very simple media appear to support cleavage and development. Using a modified Krebs Ringer bicarbonate medium with various additives, Davis and Day (1978) found that more eggs cultured in medium without lactate and pyruvate formed blastocysts than when lactate and pyruvate were included. Pyruvate alone inhibited development and 4-cell eggs formed blastocysts when only bovine serum albumin was added to the inorganic salt solution.

The ability of embryos to continue to cleave *in vitro*, however, may be a poor indication of their ability to survive when transplanted back to recipients. Transplantation of embryos cultured *in vitro* for 24 hours has resulted in normal embryonic survival. By contrast, embryo survival has been negligible in gilts to which embryos have been transferred after 48 or 72 hours culture (Pope and Day, 1977; Davis and Day, 1978).

The lack of development beyond the 4-cell stage of 1- and 2-cell embryos cultured *in vitro* is an interesting phenomenon. We have noted that cleavage *in vivo* is also arrested at the 4-cell stage. Following fertilization, the first cleavage *in vivo* normally occurs at 16–18 hours and by 30 hours after fertilization the majority of embryos have cleaved to 4-cells. In many pigs, however, embryos recovered at 60–70 hours after ovulation are still at the 4-cell stage. Soon after this, mixtures of 4- to 8-cell embryos are recovered (Lupse, 1973). Studies on protein synthesis of pig embryos during early stages of development *in vivo* show that a number of new proteins are synthesized during the 'lag' phase at 4-cells. In some 1- and 2-cell embryos cultured *in vitro*, however, the new pattern of protein synthesis is not observed (Osborn and Polge, unpublished observations). If this new pattern of protein synthesis is essential for further normal development of the embryos, it would provide an explanation for the lack of development *in vitro* beyond the 4-cell stage. Similar problems could be involved in longer-term culture of embryos *in vitro* which could account for their poor survival following transplantation. Changing patterns of metabolism and protein synthesis during early development might also be related to early embryonic mortality. Some embryos, for example, may not 'switch on' as soon as others. It has already been shown that embryonic survival is reduced when they are transplanted to a uterine environment

which is more advanced than that from which they have been recovered. It seems unlikely, however, that there is any very specific uterine factor in the pig which regulates early embryonic development since pig embryos survive and develop normally when transferred to the oviduct of a rabbit (Polge, Adams and Baker, 1972).

Preservation of embryos by freezing and storage in liquid nitrogen has now been achieved in a number of species. So far, however, pig embryos have not been frozen and thawed successfully. A major problem is their sensitivity to cooling *per se* and few have survived after exposure to temperatures below +15 °C (Polge, Wilmut and Rowson, 1974). During cooling and rewarming it has been observed that there is a loss of intracellular lipids, which are very abundant in the pig embryo. It would be interesting to speculate that it is the lipid composition of the embryonic membranes which affects their sensitivity to cooling (Polge, 1977).

Despite the inability to freeze pig embryos, some successful long-distance shipments of embryos have been achieved (Baker and Dziuk, 1969; James *et al.*, 1980). In the latter experiment, embryos were transported in culture medium from the USA to England and transplanted to recipients within 20–27 hours after collection from donors. Seven of the 12 recipients farrowed producing 58 piglets from 227 transferred embryos.

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