How does embryo manipulation fit into present and future pig reproduction?

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Summary. Available techniques for the collection and direct transplantation of pig embryos are simple and efficient and could be used for the expansion of new lines, for increasing selection pressure in nucleus herds and for extracting healthy stock from a diseased source. However, the reduced viability of pig embryos during culture *in vitro* and the inability as yet to preserve them by deep-freezing impose limits to the use of embryo transplantation for the export or import of potential breeding stock. The efficiency of breeding schemes could be improved by the sexing of embryos and the possibility of producing genetically identical twins or quadruplets by micromanipulation of embryos should improve the efficiency of animal experimentation. Chimaerism may be used to rescue embryos of a non-viable genotype such as parthenotes or those derived by hybridization, but the greatest revolution in pig breeding may be brought about by the introduction of foreign cloned genes into eggs and the production of transgenic animals. Eggs at an appropriate stage for microinjection may be provided in the future by techniques for the maturation and fertilization of oocytes *in vitro*. Animal breeders should be aware of the potential impact of techniques for the manipulation of eggs and embryos on future developments in animal production.

A simple answer to the question posed in the title of this paper is that as yet there has been very little application of even the relatively straightforward procedure of embryo transplantation, let alone any more sophisticated techniques, to the practice of pig breeding. However, there is now a marked growth of research into several aspects of cellular and genetic manipulation of embryos not only in laboratory animals, but also in the large domesticated species such as cattle, sheep and pigs. As this research develops it could provide the spur to new applications in pig reproduction, but how any of the techniques will fit into future breeding schemes will be determined by the practical opportunities that are provided and the economic or other advantages that might be gained.

The basis for application

Development and exploitation of techniques for embryo manipulation in any species, either for research or in practice, depend in the first instance upon the availability of effective methods for the collection and transplantation of eggs or embryos. The basic procedures for embryo transplantation in pigs were reviewed by Polge (1982a). These are now well established and, although surgical techniques are involved, they are simple and efficient. It has been shown that, if embryos are collected from inseminated donor animals 3–7 days after the onset of oestrus and transferred to unmated recipients which were in oestrus on the same day or 1 or 2 days later than the donors, a pregnancy rate of 85% can be achieved with 70% of the embryos surviving (Polge, 1982b). Pigs respond well to simple regimens for superovulation such as injection of PMSG on the 15th or 16th

day of the oestrous cycle (Hunter, 1964) and the natural ovulation rate can be increased by 2- or 3-fold without a reduction in fertility. If hCG is also used to induce ovulation, the timing of this event can be precisely determined so that embryos can be collected at any specific stage of development (Dziuk & Baker, 1962). Moreover, when care is taken during the surgical procedures, donor animals can be used repeatedly as, for example, in the case of one sow of special genetic merit from which embryos were collected on 14 occasions (J. E. James, personal communication). The time of oestrus in recipient animals can also be controlled quite effectively by administration of the orally active progestagen altrenogest (Regumate: Roussel-Uclaf, France) as described by Webel (1978) and Polge (1982a). All these factors make the pig an extremely useful animal for experiments on embryo manipulation and it is surprising that embryo transplantation has not also been used to a greater extent in practical breeding.

Superovulation and embryo transplantation could be used most effectively for the expansion of new lines or to increase selection pressure in nucleus herds. Embryo transplantation can also be used to extract healthy stock from a diseased source as described in a study in the U.S.A. in which embryos were recovered from sows that had been infected naturally with pseudorabies virus (PVR) some months previously. Although the donor animals still had positive titres for PVR antibodies in their serum, the embryos collected from the sows did not transmit PVR to the recipients or result in pigs with serum antibodies for PVR (James, James, Martin, Reed & Davis, 1983). In circumstances such as these, embryo transplantation may be a safer way of introducing new genetic material into closed herds than by introducing piglets that have been obtained by hysterectomy.

Embryo preservation

Perhaps the greatest potential use for embryo transplantation, however, would be to expedite the current international traffic in live animals for the purpose of moving stock of improved genetic merit around the world. Today quite large numbers of pigs are shipped by air freight from countries in which nucleus herds are located to others in which new breeding units are being established. The costs involved are very high, even for transportation of relatively light, young and untested animals. Moreover, expensive health tests are required on each individual. These costs could obviously be considerably reduced by shipping embryos rather than live animals and in some circumstances the savings achieved could outweigh the additional costs of preparing donor and recipient animals and carrying out the operations. From the economic point of view, the main advantage would be gained by the farmer or company exporting the embryos as the expense of keeping sows through pregnancy and rearing the piglets to maturity would be borne by the importer. A proportion of the piglets reared would probably also have to be culled as unsuitable for breeding. To the importer, however, these disadvantages would be more than acceptable if embryo transfer was the only method permitted by health authorities for the movement of potential breeding stock.

The feasibility of such intercontinental transfer of embryos has already been demonstrated (James *et al.*, 1980); the efficiency of such transfers has not been very high and the main limitation at the present time is the relatively short 'shelf-life' of the embryos. The survival rate of embryos kept in culture for up to 1 day during transportation has not been much greater than 25% (Davis, 1985) and even under more controlled experimental conditions embryonic survival rate was reduced to about 45% after 1 day of culture and to only about 20% after 2 days (Polge, 1982b). One of the first manipulative procedures that is required for pig embryos, therefore, is the development of more effective methods for their preservation *in vitro* and this would have an immediate application in the breeding industry.

It would be even more advantageous if it was possible to preserve pig embryos by deep freezing and to store them for prolonged periods of time in liquid nitrogen as has been achieved for cattle and sheep embryos (Willadsen & Polge, 1980). Conditions required to obtain a high survival of embryos of several mammalian species during freezing and thawing are now well established

(Whittingham, 1980; Lehn-Jensen, 1984). Important features of one of the best techniques include the use of glycerol or dimethylsulphoxide as a cryoprotective agent, slow cooling at a rate of about 0·5°C per min to a temperature of around −35°C before transfer to liquid nitrogen, and rapid rewarming at the time of thawing. There are variations between species in the stages of embryonic development that tolerate exposure to low temperatures. Cattle embryos, for example, have been found to be extremely sensitive to cooling during the first few days of their development, but by the time they have reached the blastocyst stage they become quite tolerant to cooling and can be frozen successfully (Willadsen, Polge & Rowson, 1978). In the commercial application of embryo transplantation in cattle, pregnancy rates of around 70% can now be achieved with frozen and thawed embryos (Lehn-Jensen, 1984). By contrast, pig embryos at any stage of development so far examined appear to be extremely intolerant to cooling (Polge, 1977) and there has been no authenticated or well-documented report of the establishment of pregnancy in pigs after transplantation of frozen embryos. In some experiments in which embryos were frozen and thawed at the expanded blastocyst stage, some of the cells survived and a few of the blastocysts hatched from the zona pellucida when they were cultured *in vitro*, but none survived to term after transplantation (C. Polge & S. M. Willadsen, unpublished). The reasons for the extreme sensitivity of pig embryos to cooling are still obscure, but may be associated with the composition of the cell membranes (Polge, 1977).

Further research on the deep freezing of pig embryos is urgently required and the development of a successful technique would greatly enhance the possibilities for international exchange of genetic material. Importing authorities would undoubtedly favour the use of frozen embryos since they could be maintained in quarantine pending double checking of the health status of donor herds. The necessity for close synchrony of oestrus in donor and recipient animals would be abolished and a single shipment could cope with the export of a whole herd since a suitable age spread in the recipient herd could be established by thawing and transplanting embryos when desired. Apart from these benefits, breeders could also establish 'banks' of frozen embryos in order to monitor the true rate of genetic improvement being obtained by any selection programme, because frozen embryos would not be subjected to the problem of genetic drift inherent in any conventional system.

Sex determination

The efficiency of pig breeding, in common with other livestock enterprises, could be considerably increased if it was possible to predetermine the sex of offspring to be produced. Unfortunately, none of the claims that have been made to alter the sex ratio significantly by the separation of spermatozoa with an X- or Y-chromosome from samples of semen used for insemination have been substantiated in practice. It is possible that some progress towards solving this problem may be made in the future if methods described for the separation of populations of spermatozoa on the basis of their DNA content by fluorescent labelling and cell sorting can be further developed (Keeler, Mackenzie & Dresser, 1983). However, in the absence of a satisfactory technique for the predetermination of sex, the opportunity to determine the sex of embryos to be transplanted would be an effective alternative.

The sexing of embryos by cytological methods has been reviewed by King (1984). These involve the analysis of chromosomes in cells obtained from early developing embryos or from trophoblast biopsies taken from slightly later stage embryos. However, the relatively small number of cells in metaphase at any one time limits the practicability of this approach for making an immediate diagnosis. The efficiency might be improved by culturing the cells in the presence of mitotic arresting agents, but this increases the time required to make a diagnosis and during this time the viability of the embryos, which would have to be maintained *in vitro*, would be diminished. Another problem to this approach in the pig is that in this species there are three pairs of metacentric

chromosomes in addition to the Y-chromosome which is also metacentric although somewhat smaller than others. Therefore, excellent chromosome spreads are required to make a good analysis.

More accurate methods for the determination of sex may become available with the development of DNA probes which are specific for the sex-determining sequences of the Y-chromosome (Singh & Jones, 1982). However, these methods would probably entail lengthy procedures involving in-situ hybridization and it would be necessary to be able to maintain the embryos from which the cells were derived in the frozen state.

Considerable interest has recently been evoked in the possibility of rapid methods for sex determination of mammalian embryos by immunological techniques. These are based on experiments to raise antibodies to the male-specific H-Y antigen. In mice, for example, it was reported that about 50% of 8-celled embryos were lysed when exposed to H-Y antiserum with complement and transplantation of surviving embryos resulted in a significant preponderance of females (White, Lindner, Anderson & Durant, 1982). If it is possible to produce effective monoclonal antibodies to H-Y antigen and to develop an enzyme-linked immunoassay, then rapid systems for sex determination may become available (Wachtel, 1984). However, more confirmation of the feasibility of this approach is required.

So far there have been no attempts to determine the sex of pig embryos before transplantation and it is impossible to assess the practicability of any of these methods. If cheap and effective techniques do become available, however, they could usefully be applied to reduce the cost of transplanting excessive numbers of male embryos and this might be an economic advantage in a relatively expensive export exercise. On the other hand, since embryo sexing does not result in the production of any more embryos of the desired sex, the only saving that can be achieved is in the use of fewer recipients and the methods would have to be extremely efficient to have a wider application.

Micromanipulation of embryos

Techniques for embryo splitting have been described by Robl & First (1985), but a more detailed assessment of their application is relevant. The possibility of artificially producing identical offspring in pigs by transplanting single blastomeres from early cleavage-stage embryos was first examined by Moore, Polge & Rowson (1969). Blastocysts were produced from single blastomeres from 4–6-celled embryos in which the zona pellucida had been only slightly damaged by the introduction of a very fine pipette and all except one of the blastomeres was destroyed. By contrast, when single blastomeres were removed from embryos at a similar stage of development and transferred to a foreign zona pellucida before transplantation to the reproductive tract, none survived. In this latter treatment the integrity of the foreign zona pellucida was quite severely disrupted. It was concluded that, although single blastomeres from 4- or 6-celled embryos were capable of further development, the practical exploitation of this potential to produce genetically identical animals would not be possible until suitable methods were devised for protecting the blastomeres during their early stages of development in the reproductive tract. The inefficiencies associated with culturing embryonic cells *in vitro* precluded any likelihood that blastomeres could be cultured successfully to a stage at which protection by the zona pellucida *in vivo* was no longer required.

This problem was solved when Willadsen (1979) developed a method for embedding sheep embryos in agar following radical microsurgery. The agar effectively sealed any holes or defects in the zona pellucida and allowed normal development to continue when the manipulated embryos were returned to the female genital tract. By the use of this technique, monozygotic twins have been produced from sheep 'half' embryos made by separating blastomeres from 2-, 4-, or 8-celled embryos into two equal groups (Willadsen, 1980). In a similar manner monozygotic triplets and

quadruplets have been produced from 'quarter' embryos derived from single cells from 4-celled embryos or pairs of cells from 8-celled embryos (Willadsen, 1981). The techniques of microsurgery and agar embedding have also been applied to cattle embryos to produce genetically identical twins and triplets (Willadsen & Polge, 1981).

Although there has been less experience of the application of these techniques to early developing pig embryos, at least two sets of genetically identical twins have been produced (S. M. Willadsen & C. Polge, unpublished). Groups of two blastomeres derived from 4-celled pig embryos were transferred to foreign zonae pellucidae and embedded in agar. After culture for 4 days *in vivo* in the oviduct of a sheep, 8 pairs of embryos that had developed to the early blastocyst stage were released from the agar and transferred to the uterus of a recipient pig. Nine piglets were born of which 7 survived and blood typing confirmed that amongst these there were two sets of identical twins.

Enough experience has now been gained with sheep embryos to indicate that the viability of half embryos is probably equivalent to that of whole embryos and the viability of quarter embryos is only slightly reduced. Although a further reduction of cell number in early embryos is not generally compatible with normal development and differentiation, chimaerism can sometimes be used to produce a larger number of identical animals derived from a single embryo (Willadsen & Fehilly, 1983). These techniques, therefore, provide an excellent practical opportunity for the production of genetically identical animals on demand. The main use for such animals will probably be to enhance the efficiency of experimentation and to reduce the numbers required for particular purposes. It is not difficult to envisage many examples of experiments on nutrition, behaviour and disease in which the availability of genetically identical animals of pre-selected parentage would be an enormous advantage.

It seems unlikely that the rather intricate procedures involved in the manipulation of early cleavage stage embryos could be considered an economic proposition simply for the purpose of increasing reproductive efficiency. However, methods of splitting sheep and cattle embryos into two equal halves at the late morula or blastocyst stage have more recently been developed (Willadsen & Godke, 1984; Williams, Elsden & Seidel, 1984). This has the advantage that the divided embryos can be returned directly to the reproductive tracts of recipients since protection from the zona pellucida *in vivo* is no longer required. The simplicity of the technique makes it far more attractive in a practical breeding programme and since it doubles the number of embryos available for transplantation it provides an opportunity for the more rapid multiplication of offspring from particular animals. Pig embryos split at the blastocyst stage appear to be less robust than sheep or cattle embryos (S. M. Willadsen, personal communication) but some have been shown to be capable of surviving to term (Rorie, Voelkel, McFarland, Southern & Godke, 1985). The cloning of embryos on a more extensive scale by means of nuclear transplantation may indeed become possible in the future, although much basic research is still required (Robl & First, 1985).

The ability to produce chimaeric embryos by the aggregation of embryonic cells from two or more embryos or by the injection of cells from one embryo into the blastocyst cavity of another could have some interesting applications in animal breeding. Apart from the opportunity that these techniques provide to increase the number of genetically identical offspring that can be produced from a single embryo, perhaps the most important feature of chimaerism is the way in which embryonic cells of different origin are able to collaborate in order to overcome incompatibilities in development. The most striking example of this phenomenon is the demonstration that chimaerism can be used to 'rescue' cells of parthenogenetic embryos which by themselves have only a restricted potential for development. In mice, for example, cells from parthenogenetic embryos have been mixed with those derived from normally fertilized eggs and the chimaeric embryos so formed have been shown to be capable of normal development and resulting in offspring which contained cells of parthenogenetic and normal origin (Surani, Barton & Kaufman, 1977). If the parthenogenetic cells enter the germ line, they can give rise to eggs (Stevens, 1978).

In animal breeding, some of the characteristics on which selection is based may be expressed in

one sex only. Prolificacy in sows may be one such characteristic and natural breeding may result in dilution of the desired genotype. Could chimaerism be used in such circumstances to proliferate the number of animals of special merit by the production and rescue of parthenogenetically derived embryos? Other opportunities may also be provided for the creation of new genotypes by hybridization. These in themselves could be developmentally incompetent, but also capable of surviving in collaboration with normal embryonic cells.

Gene injection

Although some of the concepts already discussed are aspects of genetic engineering, there is no doubt that the introduction of foreign cloned genes into mammalian eggs by direct microinjection and the production of transgenic animals represent the most revolutionary prospects in animal breeding. The techniques of gene injection and factors affecting their integration, expression and regulation have been discussed in detail by Robl & First (1985). There will obviously be an enormous potential for the use of these techniques in fundamental research, but to the animal breeder they may provide a means of accelerating the progress usually made by selection through the introduction of particularly desirable characteristics into animals without dilution of an existing genome. Such opportunities, however, will depend upon the identification of genes for characteristics of special economic importance such as the efficiency of growth, milk production or disease resistance and the ability to obtain controlled expression and regulation of these genes.

Of the various genes that have been successfully introduced into mice, special interest from the economic point of view has been generated in growth hormone gene constructs as some of the transgenic animals have expressed considerably enhanced growth (Palmiter *et al.*, 1982). Some success has also been achieved in introducing copies of similar gene constructs into pig eggs and several transgenic pigs have been produced (Hammer *et al.*, 1985). In mice it had been shown that the integration of foreign DNA into the genome was best obtained by direct injection into the nucleus of an egg shortly after fertilization, but with pig eggs this proved difficult because of the opacity of the cytoplasm. This problem was overcome, however, by centrifuging the eggs so that the opaque cytoplasm was concentrated to one pole and the pronuclei or nuclei then became readily visible under the microscope.

Evidence of the expression of the foreign genes in some of the pigs was obtained in that mRNA was detected in tissues of the ears and tails and they had elevated concentrations of plasma growth hormone. However, body weight was not increased dramatically. These experiments represent an important step towards the feasibility of genetic manipulation in pigs, although much remains to be learned about the type of gene constructs which will produce a desired physiological response.

At the present time the efficiency of gene transfer is rather low and a very large number of eggs must be injected to obtain a positive result. This type of experimentation is therefore very expensive, especially when applied to the large domestic species. A more readily available and cheaper source of pronucleate eggs for injection could perhaps be provided by harvesting immature oocytes from the ovaries of animals that have been slaughtered. These oocytes could then be matured and fertilized *in vitro* before injection and transplantation to recipient animals. Effective culture conditions have been described for sheep oocytes which enable them to be matured outside the follicles in such a way that they acquire full developmental competence (Staigmiller & Moor, 1984), and similar techniques could probably be developed for pig oocytes. Moreover, reliable methods have also been developed for the fertilization of pig oocytes *in vitro* (W. T. K. Cheng & C. Polge, unpublished) using a medium based on that described by Pavlok (1981) for the capacitation of spermatozoa and a fertilization system in which the spermatozoa and oocytes are incubated at the normal body temperature of pigs.

Conclusion

The manipulation of eggs and embryos of the large domestic species could have a major impact on

the efficiency of animal production in the future. Although much of the research is in its infancy, it is growing fast and it is essential that all those concerned with animal breeding are aware of the possibilities and ensure that new developments are applied in the most beneficial way.

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