Effect of asynchronous transfer and oestrogen administration on survival and development of porcine embryos

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Summary. Results indicate that recovery of embryos on Days 11 and 13 of pregnancy was reduced for Day 5 embryos transferred to recipients on Day 6 of their oestrous cycle and was greatly reduced when embryos were transferred to recipients on Day 7 of the cycle ($P < 0.01$). Administration of oestradiol-17 β on Day 11 of the recipient's cycle did not appear to affect embryo development on Day 13.

Day 6 embryos transferred to recipients on Day 8 of the oestrous cycle deteriorated rapidly within 24 h of transfer; there was no recovery of embryos from the uterus after 36 h.

Treatment of pregnant gilts with 1 mg oestradiol-17 β (i.v.) on Day 10.5 resulted in total embryonic loss by Day 23, but pregnancy rates of gilts treated with oestradiol-17 β on Day 12 were similar to those of vehicle-treated gilts (60.6 vs. 71.4%).

Keywords: embryo; oestrogen; uterus; pig

Introduction

Successful establishment of pregnancy and survival of early conceptuses in gilts are partly dependent on conceptus synthesis of oestrogen and rapid trophoblastic elongation on Day 12 of pregnancy (Bazer *et al.*, 1986; Geisert *et al.*, 1990). Although conceptus oestrogen synthesis or exogenous oestrogen prolongs luteal function in gilts (Dhindsa & Dziuk, 1968; Frank *et al.*, 1978; Geisert *et al.*, 1987; van der Meulen *et al.*, 1988), maintenance of progesterone secretion does not ensure the survival of all embryos in this litter-bearing species. Approximately 20–50% of the early embryos represented by corpora lutea on the ovaries are lost prior to Day 25 of gestation in pigs (see review, Pope & First, 1985).

In some gilts, early embryonic loss may result from the diversity in morphological development of littermate embryos (Pope *et al.*, 1982; Wilmut *et al.*, 1985). Early embryonic loss could result from the ability of advanced embryos to synthesize oestrogen and expand rapidly within the uterine lumen before less developed littermates (Geisert *et al.*, 1982; Pope, 1988; Xie *et al.*, 1990).

Although data indicate that stage of embryo development within a litter may affect survival (Pope, 1988), the mechanism by which less developed embryos are lost during early pregnancy is unknown. Earlier production of oestrogen by more-developed littermate embryos could stimulate release of secretions into the uterine lumen which are detrimental to their less developed siblings (Morgan *et al.*, 1987a; Pope, 1988; Xie *et al.*, 1990). Stimulation of uterine secretion could affect less developed embryos by blocking their ability to undergo trophoblastic elongation (Morgan *et al.*, 1987a) or inhibiting developmental events required for conceptus attachment to the uterine epithelial surface (Morgan *et al.*, 1987b; Blair *et al.*, 1991). A delay in blastocyst elongation,

an intrinsic response regulated by individual embryo development (Geisert *et al.*, 1982; Morgan *et al.*, 1987a), may reduce endometrial surface area available for attachment, which could become restrictive at later stages of development (Wu *et al.*, 1989). The present experiments evaluated the role of the uterine environment on early development of porcine embryos and their survival, as affected by altering normal synchrony through embryo transfer and exogenous oestrogen administration.

Materials and Methods

Animals. Sexually mature crossbred gilts of similar age (7–8 months) and weight (100–130 kg) and having exhibited at least 2 oestrous cycles of normal duration (18–22 days) were used in all experiments. Gilts were observed for oestrous behaviour twice a day (08:00 and 18:00 h) in the presence of intact boars. Day of oestrus onset was designated Day 0. Gilts were mated to fertile boars when first detected in oestrus and 12 and 24 h later.

Embryo transfer. Embryo transfer procedures were performed following induction of anaesthesia with a 5% solution of thiopentone sodium (Abbott Laboratories, North Chicago, IL, USA) and 1 litre oxygen/min. The reproductive tract of the donor gilt was exposed via midventral laparotomy. After isolation of the oviduct, a small incision was made into the lumen of the isthmus with a cutting needle, 1.0 cm from the utero-tubal junction. A modified polyvinyl catheter with 2 side ports cut 1.5 cm from the tip was inserted into the oviduct and gently threaded 3–4 cm into the lumen of the uterine horn. The uterine horn was clamped between finger and thumb 20 cm from the uterine body while 20 ml of modified Dulbecco's phosphate-buffered saline (mDPBS; Sigma Chemical Co., St Louis, MO, USA) containing 1% (v/v) fetal calf serum was infused into the lumen. The fluid was gently massaged down the horn and then back through the catheter into a sterile dish. Embryos were immediately identified under a binocular microscope and transferred to a dish containing short-term culture medium (mDPBS containing 10% (v/v) fetal calf serum). Embryos were washed twice in short-term culture medium at room temperature before transfer to recipient gilts.

Recipient gilts were prepared for surgery as described previously. The tip of each uterine horn was exposed and an incision was made in the isthmus. Embryos were drawn into the tip of a sterile Pasteur pipette (siliconized and fire-polished) in 0.5 ml of medium. The tip was gently passed through the puncture wound and into the lumen of the uterine horn for at least 4.0 cm, where the embryos were deposited. The laparotomy incision was closed routinely. Gilts undergoing surgical procedures were injected (i.m.) with 20 000 iu procaine penicillin G/kg body wt.

Experiment 1. The experiment was designed to determine whether advancement of uterine secretion by exogenous oestrogen on Day 11 affected the ability of embryos to elongate on Day 12; and the effect of 1 or 2 days of asynchrony on embryo survival to Day 11 and their subsequent elongation. Embryos were collected from donor gilts ($n = 33$) on Day 5 of pregnancy. An average of 12 (range 9–16) morphologically normal embryos (morulae and blastocysts) was transferred into recipient females. Recipient gilts ($n = 33$) were randomized and assigned to one of the following treatment groups: (1) synchronous ($n = 6$), Day-5 embryos transferred to recipients on Day 5 of the oestrous cycle; (2) asynchronous +24 h ($n = 13$), Day-5 embryos transferred to recipients on Day 6 of the oestrous cycle; (3) asynchronous +48 h ($n = 9$) Day-5 embryos transferred to recipients on Day 7 of the oestrous cycle; and (4) asynchronous +24 h oestradiol-17 β , same as treatment 2, but recipients received an i.m. injection of 5 mg oestradiol valerate (R. J. Legere & Co., Scottsdale, AZ, USA) on Day 11 of the oestrous cycle ($n = 5$). Recipient gilts were unilaterally ovariio-hysterectomized on Day 11, via midventral laparotomy as described by Morgan *et al.* (1987b). After removal, the excised uterine horn was flushed with 20 ml of sterile saline (9 g NaCl/l) and examined for the presence of embryos. The diameter of each embryo was measured to estimate the approximate time of trophoblastic elongation in the remaining uterine horn. If embryos were present in the uterine flushing, the remaining horn was removed and flushed 12–48 h later according to the estimated time of trophoblastic elongation predicted from stage of embryo development in the first uterine horn, as described by Geisert *et al.* (1982). If no embryos were present after flushing on Day 11, the intact uterine horn was removed 48 h later. Only recipients which contained embryos on Day 11 received oestrogen in treatment 4.

Experiment 2. Failure to recover the Day-5 embryos transferred to recipient gilts on Day 7 of the oestrous cycle in Expt 1 suggested that embryo mortality occurred shortly after transfer. To determine more clearly when embryo loss was taking place, Day-6 embryos were surgically transferred into the uterine horns of either synchronous (Day 6 of oestrous cycle, $n = 5$) or asynchronous +48 h (Day 8 of oestrous cycle, $n = 4$) recipient gilts as described for Expt 1. Gilts were ovariio-hysterectomized 24–36 h after embryo transfer. Each uterine horn was flushed with 20–40 ml of sterile saline and embryos were evaluated with a binocular microscope.

Experiment 3. The experiment was designed to determine whether advancement in uterine secretion by oestrogen treatment on Day 10.5 affected embryo survival to Day 23 of pregnancy compared with gilts receiving oestrogen on Day 12 (after embryo elongation). Seventeen mated gilts were assigned randomly to receive either vehicle (0.8 ml ethanol and 0.5 ml saline) on Day 10.5 of pregnancy, oestradiol-17 β (1 mg in 0.5 ml ethanol and 0.5 ml saline) on Day 10.5 or oestradiol-17 β in vehicle on Day 12 of pregnancy. Injection was into an ear vein. Gilts treated with vehicle or

oestradiol-17 β on Day 10.5 were hemi-ovariohysterectomized 12 h after injection. Gilts treated with oestradiol-17 β on Day 12 of pregnancy had been hemi-ovariohysterectomized on Day 11 and the uterine horn flushed with 20 ml of sterile saline (9 g NaCl/l). Diameters and developmental stages of embryos were recorded, the uterine flushings centrifuged at 10 000 *g* for 15 min at 4°C and the supernatants stored at -70°C until analysed for calcium, protein and acid phosphatase activity (for methods, see Geisert *et al.*, 1982). The remaining uterine horn was removed on Day 23 of pregnancy and the number of embryos was recorded.

Statistical analysis. Data were analysed by least-squares analysis of variance using the general linear models procedure of the Statistical Analysis System (Barr *et al.*, 1979). Differences in embryonic survival in Expts 1, 2 and 3 were analysed by χ^2 analysis. Effects of oestrogen on recoverable calcium, protein and acid phosphatase activity in uterine flushings on Day 11 in Expt 3 were analysed for treatment effects. Orthogonal contrasts of means were made for vehicle and oestradiol-17 β administered on Day 12 vs. oestradiol-17 β administered on Day 10.5, and vehicle vs. oestradiol-17 β administered on Day 12.

Results

Experiment 1: effect of oestrogen and asynchrony on embryo loss and development on Days 11 and 13

Asynchronous transfer of embryos to a uterine environment 24 h before their development resulted in a nonsignificant reduction in embryo survival at Day 11, but transfer of embryos to recipients 48 h more advanced than the donor female resulted in failure to recover any embryos on Day 11. Oestradiol-17 β treatment of gravid recipient gilts on Day 11 did affect conceptus development to Day 13: 11 of 18 gilts (61%) were pregnant on Day 11 after transfer of Day-5 embryos to Day-6 recipients (treatments 2 and 4) compared with 6 of 6 pregnant gilts when donor and recipient gilts were synchronous (treatment 1). Only the recipient gilts with embryos on Day 11 were used to evaluate effects of oestradiol-17 β for treatment 4. Oestradiol administration on Day 11 did not affect embryonic development and survival: all 5 of recipient gilts +24 h and treated with oestradiol-17 β and 5 of 6 vehicle-treated +24 h recipient gilts had filamentous conceptuses on Day 13 of pregnancy. Conceptuses from one +24 h vehicle-treated recipient were 2–6 mm in diameter when recovered on Day 13. None of the nine +48 h recipients ($P < 0.01$) contained conceptuses in uterine flushings on Day 11 or Day 13.

Experiment 2: effect of 48 h of asynchrony on early embryo loss

Transfer of embryos to an advanced (+48 h) uterine environment resulted in embryo degeneration within 36 h of transfer. Embryo recovery rates 24–36 h after transfer between synchronous Day-6 donor and recipient gilts were 10/12, 11/12, 8/9, 4/6 and 9/11 (42/50, 84%). In contrast, only 36.6% (15/41) of the embryos transferred to recipients in which the uterine environment was 48 h in advance of the donors were recovered. Individual embryo recovery from +48 h recipients was 8/9 and 7/12 (24 h after transfer) and 0/9 and 0/11 (36 h after transfer). Although 15 embryos were recovered from two +48 h recipient gilts 24 h after transfer, the embryos appeared to be undergoing degenerative changes (Fig. 1a) as indicated by cell disorganization and cytoplasmic darkening. Embryos were very adhesive and tended to stick to cell debris in the uterine flushings. Synchronous Day-6 embryos (Fig. 1b) had developed to expanded blastocysts by 24 h after transfer; some were still within the zona pellucida with clear evidence of the embryoblast, while others had completed hatching. Unfertilized ova were evident in uterine flushings from 2 synchronous recipient gilts (Fig. 1b). The total number of viable embryos collected 24–36 h after transfer was greater ($P < 0.01$) for synchronous (38/50) than for +48 h (0/41) recipient gilts.

Experiment 3: effect of uterine advancement by oestrogen on embryo survival to Day 23

Embryos were obtained from all gilts when the uterine horn was flushed on Day 11 of pregnancy. The number of corpora lutea (CL) and embryos was similar in the treatment groups (Table 1). Diameter of spherical blastocysts ranged from 2–10 mm, most gilts containing blastocysts of

Fig. 1. Representative embryos recovered in uterine flushings from recipient gilts: (a) degenerative embryos collected 24 h after transfer to a recipient 48 h before donor female's cycle; (b) embryos collected from a synchronous recipient gilt 36 h after transfer. Note the presence of the recipients' unfertilized ova (arrow) and the thin zona pellucida surrounding the transferred blastocysts.

Table 1. Number of corpora lutea and embryos and total calcium, protein and acid phosphatase activity in uterine flushings from gilts on Day 11 of pregnancy

Treatment	<i>n</i>	Number of corpora lutea	Number of embryos	Calcium (mg)	Protein (mg)	Acid phosphatase activity (μmol Pi/min)
Vehicle	7	5.3	5.0	0.06 ^a	15.2	11.75 ^c
Oestradiol-17β						
Day 10.5	5	7.0	7.0	1.13 ^b	18.5	45.91 ^d
Day 12.0	4	7.6	5.4	0.13 ^a	15.1	21.2 ^c
Overall s.e.m.		±0.96	±0.93	±0.178	±1.87	±9.94

Values with different superscripts are different at a, b $P < 0.01$ and c, d $P < 0.05$.
Data are means.

3–6 mm in diameter. Content of protein in uterine flushings was similar in treatment groups; but administration of oestrogen on Day 10.5 increased total recoverable calcium ($P < 0.01$) and acid phosphatase activity ($P < 0.05$) in uterine flushings compared with vehicle-treated gilts and those treated with oestrogen on Day 12 (Table 1).

Treatment of gilts with oestrogen on Day 10.5 of pregnancy reduced the number of gilts containing embryos in the uterine horn removed on Day 23 of pregnancy ($P < 0.02$). None of the 5 gilts treated with oestrogen on Day 10.5 contained embryos on Day 23 compared with 5 of 7 (71.4%) vehicle-treated gilts and 3 of 5 (60.0%) gilts treated with oestrogen on Day 12. All gilts administered oestrogen on Day 10.5 and 2 gilts injected on Day 12 of pregnancy appeared to be returning to oestrus on Day 23, as large corpora albicantia and developing follicles were present. The 2 nonpregnant control gilts returned to oestrus by Day 21. Numbers of CL and embryos on Day 23 were similar in vehicle-treated gilts (6.8 ± 1.0 and 3.8 ± 0.9) and those treated with oestrogen on Day 12 (7.0 ± 1.6 and 6.5 ± 1.5).

Discussion

Using our model of asynchronous embryo transfer and oestrogen treatment, the results of the present study suggest that there are 2 periods of uterine selectivity during early conceptus development in pigs. The first period of embryonic loss is not caused by competition among littermate embryos, but is related to asynchrony between development of the embryo and its uterine environment. Polge (1982) demonstrated that the success of establishing pregnancy with the transfer of advanced (+24–48 h) embryos was comparable to that obtained using embryos synchronous with the recipient's oestrous cycle. However, in contrast to embryo transfer results reported in sheep and cattle (see Wilmut *et al.*, 1985), pregnancy rates decreased for embryos transferred 24 h behind and were negligible when transferred embryos were ≥ -48 h from synchrony with the recipient. Failure to establish pregnancy with embryos transferred 48 h behind the recipient's oestrous cycle is not related to trophoblastic elongation or synthesis of oestrogen as these embryos did not survive to Day 11 of pregnancy in Expt 1. Early loss was caused by rapid deterioration of the embryos within 24 h following transfer of Day-6 embryos to Day-8 recipients in Expt 2. Rapid deterioration of Day-4 embryos transferred into an advanced (+48 h) uterine environment has also been reported by Jarrell *et al.* (1990). Retransfer of Day-4 embryos into a synchronous recipient after a short-term (24 h) exposure to an advanced (Day 6) uterine environment also resulted in a significant increase in degeneration and loss of embryos by Day 11 of pregnancy. Collectively, these data indicate that early embryonic survival is not inhibited when embryos are advanced compared with the recipient's uterine environment, but younger embryos are intolerant of an advanced uterine environment.

Our results suggest that a second period of uterine selectivity to embryonic development and survival occurs after the period of conceptus elongation and oestrogen synthesis between Days 11 and 12 of pregnancy. Results of Expt 2 indicated that, if asynchronous (transfer 24 h behind the recipient's oestrous cycle) embryos are present in the recipient uterus on Day 11, administration of oestrogen does not interfere with the ability of the conceptuses to undergo rapid trophoblast elongation, which is consistent with the results of Morgan *et al.* (1987b). Although the administration of oestrogen results in a systemic effect, treatment of gilts with oestradiol-17 β on Day 11 stimulates a physiological response in uterine release of calcium, protein and uteroferrin as occurs during early pregnancy (Geisert *et al.*, 1982).

Although results of previous studies indicated that oestrogen administration can inhibit conceptus attachment after Day 12 in pigs, stimulation of the uterus with oestrogen on Days 9 and 10, as in the study of Morgan *et al.* (1987b), is not physiological. Induced release of uterine secretions by oestrogens synthesized by more-developed littermate embryos would only occur between Days 11 and 12 of pregnancy. The abnormal endometrial protein synthesis (Gries *et al.*, 1989) and loss of the uterine epithelial glycocalyx (Blair *et al.*, 1991) indicates that oestrogen administration on

Days 9 and 10 is an inappropriate model for studies of embryonic mortality caused by the diversity in development of littermate embryos as proposed by Pope *et al.* (1990). Administration of oestrogen to pregnant gilts via i.v. injection (Expt 3) allowed us to stimulate a physiological release of intraluminal calcium and acid phosphatase activity within 8–12 h, as reported for cyclic gilts by Young *et al.* (1987). Uterine environmental changes induced by oestrogen in Expt 3 occurred when the majority of gilts contained spherical blastocysts 4–5 mm in diameter. Therefore, uterine secretions were stimulated at least 20–30 h before conceptus trophoblastic elongation. Given the diversity in embryonic development that can occur during pregnancy (Wilmut *et al.*, 1985; Pope *et al.*, 1988) and the association of oestrogen synthesis with development within a litter (Pusateri *et al.*, 1990), the advanced uterine secretions induced by oestrogen would mimic the timing of oestrogen secretion by embryos advanced in development and would not result in the abnormal development of the uterine epithelium observed when treated on Days 9 and 10. Results of Expt 3 indicate that advanced stimulation of uterine secretions on Day 11 results in total embryonic loss by Day 23. Although some animals treated with vehicle or with oestrogen on Day 12 did not maintain pregnancy after the initial surgery, the majority contained embryos on Day 23. Maintenance of pregnancy in gilts treated with oestrogen after conceptus elongation (Pope *et al.*, 1986; present study) demonstrates that embryonic loss is a result not of exogenous oestrogen, but of the time at which it is administered. Previous results indicate that conceptus elongation is not affected by oestrogen treatment; therefore, early embryonic death could result from either inappropriate timing of uterine secretion (Morgan *et al.*, 1987a) or the inability of the conceptus to attach to the uterine epithelial glycocalyx, which is stimulated by embryo oestrogen on Days 11–12 of pregnancy (Blair *et al.*, 1991) or after administration of oestrogen (Keys & King, 1988).

Results of the present study indicate that the uterus of pigs selects against embryos which are too asynchronous in development with the uterine environment after Day 8 of pregnancy. After Day 10, synthesis and release of oestrogen by more-advanced embryos may result in loss of less developed littermates after Day 12 of pregnancy. The effects of oestrogen stimulation on embryonic survival on Day 12 of pregnancy emphasize the importance of embryo uniformity in increasing litter size and embryonic survival rate, as occurs in Chinese Meishan pigs (Bazer *et al.*, 1988).

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