Influence of SLA haplotype on preimplantation embryonic cell number in miniature pigs

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Summary. Embryonic cell number in miniature pigs inbred for specific SLA haplotypes (*a*, *c*, and *d*) was determined on Day 6 by nuclear staining and, on Days 9 and 11, by DNA analyses (first day of oestrus = Day 0). Pigs exhibiting first behavioural oestrus at 08:00 h were hand-mated to an SLA homozygous boar 12 and 24 h later. Numbers of embryos flushed from uteri at 08:00–10:00 h on Days 6, 9 and 11 were greater ($P < 0.05$) for SLA^d females than for SLA^a or SLA^c females, which did not differ (8.2 vs 6.8 and 6.2, respectively). Recovery rates (embryos recovered/CL number) were similar, averaging 75.8% for all three SLA haplotypes. Embryos from SLA^d dams contained fewer blastomeres (23 cells) on Day 6 than did embryos from SLA^a (89 cells) or SLA^c (79 cells) females. The reduced cell numbers of SLA^d vs SLA^a or SLA^c embryos continued to Day 9 (28 vs 107 and 67 ng DNA/embryo) and Day 11 (167 vs 674 and 586 ng DNA/embryo). These results suggest an effect of the SLA complex on preimplantation embryonic development.

Keywords: pig; SLA complex; preimplantation; embryonic development

Introduction

In mature domestic pigs, embryonic mortality appears to be independent of fertilization rate, maternal oestrogen or progesterone concentrations, or uterine capacity before Day 30 of gestation (Pope & First, 1985). Instead, several lines of evidence suggest that, during early gestation in the pig, morphologically advanced blastocysts appear to have a greater survival potential than their less-developed litter-mates (Pope *et al.*, 1982; Pope & First, 1985; Wilmot *et al.*, 1985). This hypothesis is strengthened by the wide range of developmental stages among littermate embryos observed during the preimplantation period (Anderson, 1978; Ford *et al.*, 1982a; Geisert *et al.*, 1982). Further, Pope *et al.* (1982) and Morgan *et al.* (1987) have suggested that the biochemical synthetic activity of more developed embryos may stimulate a marked alteration in the uterine luminal environment which is detrimental to less developed embryos, incapable of adapting to the change.

A link has been established between the rate of embryonic development and the major histocompatibility complex (MHC) in mice (Verbanac & Warner, 1981; Goldbard & Warner, 1982; Goldbard *et al.*, 1982). These researchers demonstrated the existence of a gene controlling the rate of preimplantation embryonic development (Ped) which is located within the H-2 complex (mouse MHC). Vaiman *et al.* (1970) identified the MHC in pigs (SLA, swine leucocyte antigen complex) and Vaiman & Renard (1980) reported that SLA heterozygous offspring are more common than predicted for some matings, based on the known SLA haplotypes of the parents. Conley *et al.* (1988), using distinct genetic lines of miniature pigs with specific SLA haplotypes, observed significant effects of SLA genotypes on litter size and suggested an association of SLA genotype with embryo survival.

The present study utilized distinct genetic lines of miniature pigs with specific SLA haplotypes *a*, *c* and *d* (Sachs *et al.*, 1976) to investigate the effect of SLA genotype on preimplantation embryonic development. Embryos from homozygous matings were collected surgically and evaluated for developmental stage on Day 6 by nuclear staining and, on Days 9 and 11, by DNA quantitation (first day of oestrus = Day 0). These days after mating were chosen for the investigation because they represent the immediate post-hatching period (Day 6), a point before the start of elongation (Day 9) and the expected time of initiation of elongation (Day 11). The marked expansion of embryos after this time makes surgical collection of intact embryos extremely difficult. Embryo collection at slaughter is prohibitive due to the limited availability of animals with this genetic makeup.

Materials and Methods

Miniature sows and gilts homozygous for the SLA^a, SLA^c and SLA^d haplotypes were checked for oestrus twice daily, at 08:00 and 17:00 h, by exposing them to mature boars. Females exhibiting first behavioural oestrus at 08:00 h were given an injection of 500 i.u. hCG (i.m.) at that time and were subsequently hand-mated to a homozygous boar with the same genotype (SLA^a, SLA^c or SLA^d) 12 and 24 h later. Injection of hCG (500 i.u.) at the time of first detected oestrus lessened the possibility of a prolonged interval from oestrus to ovulation (Polge, 1967). Injection of hCG at this time is not expected to have any effect if the interval from the start of oestrus to ovulation is in the normal range, and did not alter the ovulation rates of these homozygous SLA haplotypes when compared to previously published results (Conley *et al.*, 1988).

Twenty-five miniature gilts and sows (8 SLA^a, 9 SLA^c, 8 SLA^d) were mated as described, and then utilized for embryo collections between 08:00 and 10:00 h on Days 6, 9 or 11 after mating (first day of oestrus = Day 0) as described previously (Conley *et al.*, 1988). Anaesthesia was induced with sodium thiamylal (Surital: Parke-Davis Laboratories, Morris Plains, NJ: 666 mg/kg body wt) and maintained with a mixture of oxygen and fluothane (Halothane: Ayerst Laboratories, New York, NY). The reproductive tract was exposed through a midventral incision. Corpora lutea (CL) were counted, and embryos were flushed from each uterine horn with modified Krebs-Ringer bicarbonate (Davis & Day, 1978) without bovine serum albumin. The flushings were collected at the uterotubal junction through a funnel-tipped glass tube inserted through the uterine wall and firmly secured in the lumen with surgical silk. Two flushes of 20 ml each were conducted from the base of each uterine horn towards the uterotubal junction by inserting a sterile 18-gauge, 2.2-cm needle through the uterine wall to introduce medium. Once flushed from the uterus, Day-6 embryos were rinsed in phosphate-buffered saline (NaH₂PO₄·H₂O (4.5 g/l); Na₂HPO₄ (9.5 g/l); NaCl (74.0 g/l)) and stained for fluorescent counting of nuclei. Day-9 and -11 embryos were rinsed in saline and immediately frozen on solid CO₂, lyophilized, then stored in liquid nitrogen until assayed for DNA within 3 days by using a fluorometric DNA assay, as described below.

Fluorescent staining of nuclei. Day-6 embryos were utilized for fluorescent counting of nuclei by the method of Pursel *et al.* (1986). Embryos were transferred to a micro-culture slide (Thomas Scientific, Swedesboro, NJ) containing trypan blue (0.1%) in sodium citrate (2.3%). Staining was carried out at room temperature for 4 min. The embryos were then transferred to a slide containing a solution composed of 0.75 ml sodium citrate (2.3%), 0.25 ml absolute ethanol and 10 µl of a solution of Hoechst 33342 (1 mg/ml in distilled water) and incubated for 3–5 min at 37°C. Finally, the embryos were transferred to a fresh micro-culture slide containing saline to count nuclei (wet drop).

Embryos were examined for fluorescence under an Olympus Vanox-T Microscope using a 400-nm barrier filter and supplemental filter Y435. A ×10 planar objective was used to view fluorescence. Cell counts for each embryo were repeated four times, and the average was recorded. The overall coefficient of variation for counts on a single embryo was 4.65%.

Quantitation of embryonic DNA. The procedure for analysis of Day-9 and Day-11 pig blastocyst DNA was identical to that of Geisert *et al.* (1982), which was a modification of the fluorometric DNA assay for mouse blastocysts developed by Olds *et al.* (1973). Using a pool of pig splenocytes (10⁶ cells/sample), the intra-assay coefficient of variation was 10.5% (*n* = 4), and the inter-assay coefficient of variation was 15.9% (*n* = 5). In addition, the mean value obtained for a single pig splenocyte, 4.4 pg DNA, agrees well with literature values (Vendrey & Vendrey, 1949) for pig hepatocytes (5.0 pg DNA) and kidney cells (5.2 pg DNA). The sensitivity of this assay was ≈100 ng DNA. All embryos flushed from a single uterus on Days 9 or 11 were pooled for DNA quantitation. This value (DNA content per litter) was then divided by the number of litter-mate embryos to obtain a final estimate of DNA content per embryo. Embryonic cell number was estimated by dividing the average DNA content of embryos within a litter by 5, which was considered an estimate of the number of picograms of DNA per pig cell.

All variables were analysed by using least squares analysis of variance procedures (SAS, 1985). The model used to analyse the data included the effects of SLA genotype of the sow, day of pregnancy and the interaction of genotype and day. Comparison among least squares means and their standard errors are presented.

Results

The number of CL observed on the ovaries of SLA^d dams was significantly ($P < 0.05$) greater than those observed for SLA^a and SLA^c females, for which ovulation rates were similar. Numbers of ovulations were unaffected by age or parity (Table 1). Numbers of embryos recovered from uteri of SLA^d females from Day 6 to Day 11 were also significantly ($P < 0.05$) greater than numbers of embryos flushed from the uteri of SLA^a or SLA^c females. The number of embryos flushed from the uterus, as a percentage of the total number of CL on a female's ovaries, was constant across genotype, averaging $75.8 \pm 3.9\%$.

Table 1. Maternal differences in ovulation rate, number of embryos recovered and percentage recovery rate of pigs from Day 6 to Day 11 after mating

SLA genotype	No. of females	No. of CL/female	No. of embryos recovered/flush	% recovery rate*
a/a	8	8.9 ± 0.2^x	6.7 ± 0.6^x	77.0 ± 7.7^x
c/c	9	7.9 ± 0.6^x	6.2 ± 0.9^x	75.8 ± 7.8^x
d/d	8	11.8 ± 1.3^y	8.8 ± 1.0^y	74.6 ± 3.8^x

Values are mean $\pm$ s.e.m. for the no. of females indicated.

Values within a column with different superscripts differ ($P < 0.05$).

*Embryos recovered as a percentage of total CL number.

There was a significant ($P < 0.01$) increase in DNA content of embryos from Day 9 to Day 11 of gestation, regardless of genotype (Fig. 1). Moreover, SLA^d embryos contained less ($P < 0.05$) DNA than did SLA^a or SLA^c embryos, for which the DNA content was similar.

Fig. 1. Content of DNA from SLA^{a/a} (▼), SLA^{c/c} (■) and SLA^{d/d} (●) matings on Days 9 and 11 of gestation. Values are mean $\pm$ s.e.m. for 8, 9 and 8 females.

The overall effects of day of embryo collection and SLA genotype on the average number of cells an embryo contained within a litter are depicted in Fig. 2. There was a significant effect of genotype on the number of cells that an embryo contained on Day 6 through Day 11 of gestation ($P < 0.01$), with SLA^d embryos containing fewer cells than SLA^a or SLA^c embryos. In addition,

Fig. 2. Average number of cells in an embryo in litters of SLA^a, SLA^c and SLA^d pigs from Day 6 to Day 11 of gestation. Values are mean $\pm$ s.e.m. for the no. of litters indicated. Values with different superscripts differ ($P < 0.05$).

the variation in average embryonic cell numbers between litters was less for SLA^d embryos compared to SLA^a or SLA^c embryos on Day 6 and Day 11.

Discussion

As previously reported for the same strains of miniature pigs (Conley *et al.*, 1988), SLA^d dams had a significantly higher ovulation rate than did SLA^a or SLA^c females (11.8 vs 8.9 and 7.9, respectively). These results support those of Rothschild *et al.* (1984) who reported that commercial pigs selected exclusively for ovulation rate also indirectly selected for some SLA haplotypes. Together, these observations confirm an association between the genes controlling ovulation rate and the MHC of pigs.

The numbers of embryos flushed from uteri of SLA^d dams were also greater than numbers flushed from the uteri of SLA^a or SLA^c females (8.8 vs 6.7 and 6.2, respectively). Using these three strains of NIH miniature pigs, we have previously reported (Conley *et al.*, 1988) that litters from matings involving either sires or dams with SLA^{-d} or SLA^{d/d} genotypes were significantly larger than those of other matings. The equivalent effect of both sire and dam on litter size in the study of Conley *et al.* (1988) argues against only a major maternal SLA haplotype effect, such as ovulation rate or uterine environment, on the number of embryos surviving to term.

One reason for the increased litter size from matings involving an SLA^{-d} or SLA^{d/d} sire or dam may be alterations in the rate and uniformity of preimplantation development. Conley *et al.* (1988) observed no obvious difference in blastomere number among SLA^a, SLA^c or SLA^d embryos on Days 2–4, during which embryos ranged from a single cell on Day 2 to 8 cells on Day 4. Conley *et al.* (1988) also observed no differences in oestrone, oestradiol-17 β or progesterone concentrations in systemic blood or uterine flushings from Day 2 to 6 of gestation, suggesting no marked differences

in ovarian function among the three strains of NIH miniature pigs. In contrast, litter-mate embryos collected from SLA^d dams on Days 6, 9 and 11 after mating in the present study contained fewer embryonic cells than did embryos collected from SLA^a or SLA^c females. Log transformation of the data (Fig. 2) indicated that the variation in embryonic cell numbers among genotypes was proportional to the means, suggesting that the apparent reduction in the variation of SLA^d litters was due to differences among the stages of development within a day rather than the variation inherent within a genotype. These results lend support to the existence of an MHC-linked gene in the pig, similar to the Ped gene in the mouse (Warner, 1986), which may be expressed between Days 4 and 6 after mating. From Day 6 to Day 11, however, the rate of increase in embryonic cell numbers was similar across all three SLA genotypes, suggesting no further differences in embryonic growth rate.

Bazer *et al.* (1987) observed differences in preimplantation embryonic growth rate and uniformity of development between Large White and prolific Chinese Meishan pigs. As previously stated, considerable variation exists in the morphological stage of development among litter-mate embryos from Day 7 or 8 of gestation and is exaggerated by Day 10–12 (Anderson & Parker, 1976). Associated with this morphological variability is variation in the ability of each embryo to initiate new synthetic pathways. Specifically, the morphologically more developed Day-11 litter-mate embryos synthesize oestrogen sooner than do the less-developed embryos (Ford *et al.*, 1982a). Embryonic oestrogen production in pigs has been linked to alterations in secretion of uterine proteins (Geisert *et al.*, 1982), sequestration of intrauterine prostaglandin F_{2a} (Frank *et al.*, 1978) and increased uterine blood flow (Ford *et al.*, 1982b). It is possible that the more developed embryos, by synthesizing oestrogen earlier, advance the biochemical environment within the uterus, resulting in the death of the less-developed embryos. Evidence for this hypothesis is the failure of exogenously administered oestradiol to affect embryo survival in the pig when administered on Days 12 and 13 (after initiation of embryonic oestrogen production) and its marked embryotoxic effect when administered on Days 9 and 10 of gestation (Pope & First, 1985).

The results of the present study suggest an effect of SLA genotype on preimplantation embryonic development. More specifically, they suggest an effect of the *d* haplotype on delaying preimplantation embryonic development and increasing uniformity of litter-mate embryos.

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