Comparison of follicular heterogeneity and ovarian characteristics in Meishan and Large-White hybrid pigs

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Comparisons were made between characteristics of pre-ovulatory follicles recovered from prolific Chinese Meishan gilts ($n = 12$) and from European Large-White hybrid gilts ($n = 13$) in the late follicular phase preceding their fifth oestrous cycle, to determine whether there is an ovarian basis for the enhanced prolificacy in the Meishan. A total of 177 follicles per breed was classified as pre-ovulatory, based on follicular fluid oestradiol concentrations. Results obtained demonstrated high variability in all follicular characteristics in both breeds and no decrease in heterogeneity was evident in the Meishan. The Meishan follicles tended to be smaller ($P < 0.06$) and had less follicular fluid ($P < 0.005$), but total oestradiol content per follicle was similar ($P > 0.1$) with the result that the concentration of oestradiol in follicular fluid tended to be higher ($P < 0.06$) in Meishan than Large-White hybrid pigs. There were no differences between breeds in terms of testosterone concentration in follicular fluid, hCG binding to granulosa cells or total DNA content of granulosa cells. Concentrations of inhibin in follicular fluid were similar in both breeds ($P > 0.1$) which resulted in a trend towards less total inhibin content in Meishan than Large-White hybrid follicles ($P = 0.065$). Corpora lutea were recovered from both breeds ($n = 12$ per breed) on days 27–31 of pregnancy after mating at first, second and third oestrus: corpora lutea were smaller ($P < 0.001$) and contained less progesterone per corpus luteum in the Meishan ($P < 0.05$) than in Large White hybrid pigs. However, since ovulation rate was higher in these particular Meishan pigs ($P < 0.005$), total ovarian progesterone content per animal was similar in both breeds. These results demonstrate that there was no decrease in the variability in follicular characteristics from Meishan pigs. However, both follicles and corpora lutea were smaller in Meishan than in Large-White hybrid pigs, and progesterone per corpora lutea was also lower in the Meishan. In spite of their smaller size, Meishan follicular oestradiol content was similar to that of Large-White hybrid pigs, such that the oestradiol concentration in follicular fluid tended to be higher in Meishan follicles.

Introduction

The Chinese Meishan breed of pig is extremely prolific compared with European breeds, and can produce up to 30–40% more live piglets per litter (Bolet *et al.*, 1986; Bazer *et al.*, 1988a, b; Haley and Lee, 1990). The precise physiological basis for this enhanced prolificacy is unknown as yet, but cross-breeding studies have indicated that it is a result of genes acting in the mother (Haley and Lee, 1990), which could be effective either through the oocyte or uterus. The greater number of piglets produced by the Meishans appears to be due mainly to increased prenatal survival (Bidanel and Legault, 1986; Bazer *et al.*, 1988a; Haley *et al.*, 1990). Conflicting reports have been presented about

whether the Meishans experience a higher ovulation rate (Bolet *et al.*, 1986; Bazer *et al.*, 1988a, b; Ashworth *et al.*, 1990; Biggs *et al.*, 1990) which could also contribute to increased litter size.

Several studies have suggested that variation in the rate of preimplantation embryo development within a litter is a likely cause of the high embryonic loss in European breeds of pig (Anderson, 1978; Pope *et al.*, 1986; Pope *et al.*, 1990), and indeed Bazer *et al.* (1988b) reported an increased uniformity of blastocyst development in the prolific Meishans. Furthermore, there is evidence that the variation in embryogenesis in European pigs is the result of heterogeneity in the pre-ovulatory follicle population (Foxcroft and Hunter, 1985; Grant *et al.*, 1989; Hunter *et al.*, 1989; Hunter and Wiesak, 1990; Pope *et al.*, 1990; Xie *et al.*, 1990a, b, c).

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The aims of the present study, therefore, were to compare the characteristics of pre-ovulatory follicles and corpora lutea in Meishan and European Large-White hybrid pigs and to determine whether differences in heterogeneity in follicular development could provide a basis for differences in embryonic survival.

Materials and Methods

Animals

Twelve Meishan gilts and three Meishan boars were obtained from the Edinburgh Station of the Institute of Animal Physiology and Genetics Research at approximately 8 weeks of age. Thirteen prepubertal Landrace $\times$ (Landrace $\times$ Large White) gilts (Large-White hybrids) were obtained from the University of Nottingham herd. All gilts were group penned according to breed and checked daily for oestrous behaviour with mature vasectomized boars of the appropriate breed. Ovaries were recovered at slaughter or by ovariectomy on the day before the first day of their predicted fifth oestrus, based on the previous cycle lengths of each animal (equivalent to day 20 of the fourth oestrous cycle for an animal with a 21 day cycle).

Collection and treatment of follicular tissue

The handling and dissection of ovarian tissue, the collection of follicular fluid and the harvesting of granulosa cells were as described previously (Foxcroft *et al.*, 1987; Grant *et al.*, 1989; Hunter *et al.*, 1989), and all follicles ≥ 4 mm diameter were recovered. Briefly, this involved measurement of follicular diameter after dissection and recovery of follicular fluid using a Hamilton syringe. The fluid was immediately diluted into 1 ml Hanks Balanced Salt Solution containing Hepes buffer (Flow Laboratories, Irvine) followed by centrifugation at 160 *g* for 10 min to pellet any granulosa cells removed with the fluid. The supernatant was removed and stored at -20°C for subsequent radioimmunoassay. The collapsed follicle was bisected and the granulosa cells removed by scraping into phosphate-buffered saline (PBS). These cells were added to the previous pellet and the total complement collected by centrifugation. This pellet was then resuspended in PBS–glycerol and frozen at -196°C for subsequent analysis of human chorionic gonadotrophin (hCG) binding and deoxyribonucleic acid (DNA) content as described below.

Collection and treatment of luteal tissue

Ovaries were recovered from 12 pregnant Meishan gilts from the Edinburgh population which had been transferred to the Rowett Research Institute in Aberdeen, and from 12 contemporary pregnant Landrace $\times$ Large White (Large-White hybrid) gilts, on days 27–31 of pregnancy. Before pregnancy, Meishan gilts had experienced two or three oestrous cycles and the Large-White hybrid gilts had experienced one oestrous cycle. Six gilts of each breed were fed 20 mg allyltrenbolone (Regumate: Hoechst, Hounslow, Middlesex) daily for 18 days before mating, since these animals were used as part of a larger

study on embryo survival (not reported here). Dissected corpora lutea were frozen at -20°C and transported to Nottingham where they were thawed and weighed. Progesterone was extracted from luteal tissue by homogenization in double distilled ethanol as described previously by Hunter *et al.* (1988).

Hormone radioimmunoassays

Oestradiol and testosterone concentrations in follicular fluid were determined directly without extraction as described and validated by Grant *et al.* (1989). Limits of sensitivity of the assays were 4.2 and 14.5 pg per tube, respectively, and inter- and intra-assay coefficients of variation were 18.4 and 10.3% for oestradiol and 17.1 and 13.2% for testosterone, respectively.

Progesterone content of luteal tissue was determined from the ethanolic extract after evaporation to dryness and redissolving in buffer as described by Hunter *et al.* (1988). The limit of sensitivity of the assay was 10 pg per tube and inter- and intra-assay coefficients of variation were 14.2 and 12.7%, respectively.

Inhibin concentration in follicular fluid was determined as described by McNeilly *et al.* (1989). The sensitivity and inter- and intra-assay coefficients of variation were 2.5 pg per tube, 11.4 and 7.6%, respectively.

^{125}I -labelled hCG binding assay

Determination of hCG binding capacity by granulosa cells was carried out as described previously by Foxcroft *et al.* (1987), with the modification that after removal of the cells from liquid nitrogen and washing, cells were resuspended in 0.5 ml PBS with 0.1% bovine serum albumin (Sigma Chemical Company Ltd, Poole). Four aliquots of 50 μl were used for the binding assay and the remainder used for DNA estimation. Specific activity of the iodinated hCG was 99 c.p.m. pg^{-1} (59.5 $\mu\text{Ci } \mu\text{g}^{-1}$), and the active fraction was 35%. Inter- and intra-assay coefficients of variation were 2.9 and 5.5%, respectively.

DNA assay

The DNA content of an aliquot of granulosa cells from each follicle was determined according to the method of Labarca and Paigen (1980), and the range of the standard curve was 1.25 to 20 μg DNA. The inter- and intra-assay coefficients of variation were 8.3 and 10.5%, respectively.

Statistical analyses

Pre-ovulatory follicles were classified as those with a follicular fluid oestradiol concentration that fell above one standard deviation of the individual animal's overall mean follicular fluid oestradiol concentration: follicles which fell below were rejected as not destined to ovulate. It was decided that this was more appropriate than an arbitrary cut-off point, since follicular fluid concentrations varied not only within, but also between, animals. Predicted ovulation rates were compared between breeds by Student's *t* test.

The following analyses were performed using the GENSTAT statistical package (GENSTAT 5 Committee, 1989). An analysis of

Fig. 1. (a) Means and SED of follicular diameter, follicular fluid volume (FFV) and DNA content (μg per follicle) of granulosa cells (DNA) of preovulatory follicles ($n = 177$ per breed) from Meishan ($\square$; $n = 12$) and Large White ($\boxtimes$; $n = 13$) gilts. (b) Mean variances of follicular diameter, follicular fluid volume (FFV) and DNA content of granulosa cells (DNA) of preovulatory follicles ($n = 177$ per breed) from Meishan ($\square$; $n = 12$) and Large White ($\boxtimes$; $n = 13$) gilts.

variance was used to assess differences between mean values of follicular characteristics between breeds and the pooled variance estimate used to calculate the standard error of the difference (SED). Since there was much variation within and between animals, significance levels of $P < 0.07$ were considered to indicate a trend approaching significance, and thus have been included in the text. The heterogeneity of follicular characteristics was compared between breeds by analysis of deviance, and since independently estimated variances were being compared it was assumed that they followed a gamma distribution (McCullagh and Nelder, 1989). Linear correlation analysis was used to determine relationships between follicular characteristics in each of the breeds.

Results

Morphological characteristics

The total number of intact follicles ≥ 4 mm diameter recovered from Meishans ($n = 12$) was 223 and from Large-White hybrids ($n = 13$) was 256; a very small number (21) of follicles were burst during dissection, and rejected from any further analysis. A total of 177 follicles per breed was classified as pre-ovulatory, and thus the predicted mean ($\pm$ standard error of the mean (SEM)) ovulation rates per gilt were 14.8 ± 1.66 for Meishan and 13.6 ± 2.14 for Large-White hybrids ($P > 0.1$). These predicted ovulation rates are similar to those reported in both breeds during the fourth oestrous cycle (Biggs *et al.*, 1990) and confirm that the classification of pre-ovulatory follicles based on oestradiol concentrations of follicular fluid is appropriate at this stage of the oestrous cycle. Mean diameter, volume of follicular fluid and DNA content of the granulosa cells of pre-ovulatory follicles of each breed are shown (Fig. 1a). Follicles from Meishan pigs tended to be smaller ($P < 0.06$) and contained less follicular fluid ($P < 0.005$) than Large-White hybrid follicles. Granulosa cell number per follicle as assessed by DNA measurement was not different between breeds ($P > 0.1$). There were

no differences between breeds in variances in follicular diameter, follicular fluid volume or DNA content ($P > 0.1$) (Fig. 1b).

Follicular fluid hormones

The amount of hormone present in the follicular fluid was expressed as the concentration in ng ml^{-1} of fluid and also as a total follicular content in ng per follicle. Follicular oestradiol content was similar in both breeds ($P > 0.1$) (Fig. 2b); however, follicles from Meishan pigs tended to have higher concentrations of oestradiol in follicular fluid than did follicles from Large-White hybrid pigs ($P < 0.06$) (Fig. 2a). The concentrations of the other hormones were not significantly different ($P > 0.1$), although follicular inhibin content showed a trend towards being higher in Large-White hybrid than in Meishan follicles ($P = 0.065$), and testosterone content was similar in both breeds ($P > 0.1$). Variances in follicular fluid hormone concentrations and total follicular hormone content were similar between breeds except for testosterone concentration which was more variable ($P < 0.01$) in Meishan than Large-White hybrid follicles (Fig. 2c, d).

^{125}I -labelled hCG binding to granulosa cells

Mean binding to granulosa cells per follicle was not different between the breeds ($P > 0.1$) (323×10^3 versus 378×10^3 c.p.m. bound per follicle for Meishan and Large-White hybrids, respectively, $\text{SED} = 60.3 \times 10^3$). Variances were also similar between breeds ($P > 0.1$).

Characteristics of corpora lutea

There were no differences in any of the characteristics measured in either breed between animals that had been treated with allyltrenbolone and those that had not, so data were combined within breeds. A total of 238 and 186 corpora lutea were recovered from Meishan and Large-White hybrid gilts, respectively, and mean ovulation rate was significantly greater

Fig. 2. (a) Means and SED of oestradiol, inhibin and testosterone concentration (ng ml⁻¹) in the follicular fluid of preovulatory follicles. (b) Means and SED of total follicular oestradiol, inhibin and testosterone content (ng per follicle) in preovulatory follicles. (c) Mean variances of oestradiol, inhibin and testosterone concentration (ng ml⁻¹) in the follicular fluid of preovulatory follicles. (d) Mean variances of total follicular oestradiol, inhibin and testosterone content (ng per follicle) of preovulatory follicles. For all graphs, follicle number = 177 per breed from Meishan (□; *n* = 12) and Large White (▨; *n* = 13) gilts.

Fig. 3. (a) Mean and SED for weight of corpus luteum (mg) and progesterone content (ng mg⁻¹ tissue) from Meishan (□) and Large White (▨) gilts (*n* = 12 per breed). (b) Mean and SED for progesterone content (µg per corpus luteum) and total ovarian progesterone content (mg per animal) from Meishan (□) and Large White (▨) pigs (*n* = 12 per breed).

in Meishan than in Large-White hybrids pigs at 19.8 versus 15.5 SED = 1.3; $P < 0.005$. Corpora lutea recovered from Meishan pigs were significantly lighter than those from Large-White hybrids ($P < 0.001$), whereas progesterone content, per mg, was similar (Fig. 3a). As a consequence, progesterone content per corpora lutea was significantly greater in Large-White hybrid than in Meishan pigs ($P < 0.05$), but because of differences in ovulation rates, overall ovarian progesterone content per animal was not different between breeds ($P > 0.1$) (Fig. 3b).

Relationships between follicular characteristics and between breeds

Correlation analysis between follicular characteristics ($n = 177$ per breed) for each breed revealed highly significant positive correlations ($P < 0.001$) between follicular diameter and fluid volume ($r = 0.82, 0.90$ for Meishan and Large-White hybrids, respectively). These two characteristics also correlated with hCG binding to granulosa cells ($r = 0.44, 0.57$ and $0.44, 0.58$ for Meishan and Large-White hybrids and follicular diameter and fluid volume, respectively, $P < 0.001$), oestradiol concentration in follicular fluid ($r = 0.44, 0.36$ and $0.46, 0.32$ for Meishan and Large-White hybrids, respectively, $P < 0.001$), total follicular oestradiol ($r = 0.69, 0.68$ and $0.83, 0.71$ for Meishan and Large-White hybrids, respectively, $P < 0.001$) and total follicular inhibin ($r = 0.48, 0.64$ and $0.65, 0.70$ for Meishan and Large-White hybrids, respectively, $P < 0.001$). The testosterone concentration in the fluid was negatively correlated with follicular diameter and fluid volume, respectively ($r = -0.34, -0.21$ and $-0.43, -0.30$ for Meishan and Large-White hybrids, respectively, $P < 0.01$). Oestradiol, inhibin and testosterone fluid concentrations were positively correlated with total follicular oestradiol, inhibin and testosterone, respectively ($r = 0.84, 0.82$ and $0.81, 0.93$ and $0.47, 0.53$ for Meishan and Large-White hybrids, respectively, $P < 0.001$).

Discussion

The number of pre-ovulatory follicles recovered in the present study from the Large-White hybrid females is consistent with those reported previously (Clark *et al.*, 1975; Grant *et al.*, 1989; Wiesak *et al.*, 1990). In addition, the predicted ovulation rates of 13.6 and 14.8 for Large-White hybrids and Meishan, respectively, are similar to the actual ovulation rates that have been reported for both breeds after an equivalent number of oestrous cycles (Bolet *et al.*, 1986; Bazer *et al.*, 1988a; Hunter *et al.*, 1991). In contrast, higher ovulation rates have been reported in other studies carried out on the same population of Meishans when compared with European breeds (Haley *et al.*, 1990; Ashworth *et al.*, 1990), which is consistent with the higher ovulation rate in the gilts from which luteal tissue was obtained in the present study (Ashworth *et al.*, 1992). We suggest that some of these discrepancies may be due to the number of oestrous cycles experienced by the animals of each breed at the time that comparisons are made, coupled with environmental influences.

Heterogeneity in the follicular population in pigs has been well documented (Foxcroft and Hunter, 1985; Hunter and Wiesak, 1990; Wiesak *et al.*, 1990; Xie *et al.*, 1990a, b, c), including

the implications this could have for oocyte maturation, luteal function and early embryonic development. Pope *et al.* (1990) have demonstrated a skewed distribution in both oocyte maturation and the pattern of ovulation. It has been reported that ovulation occurs over a shorter time span in Meishan than in Large-White hybrid pigs (Martinat-Botte *et al.*, 1989), and this, coupled with the increased uniformity of blastocyst development in Meishan pigs (Bazer *et al.*, 1988b) led to the hypothesis that increased embryo survival may be a direct consequence of a more synchronous follicular population. However, there was no evidence of decreased variability in the Meishan follicles at the time studied.

There is little information currently available on the process of folliculogenesis in Meishan pigs. Although ovulation rates were similar in the gilts used for the present follicular study, Meishan pre-ovulatory follicles were smaller than their Large-White hybrid counterparts and thus contained less follicular fluid. Follicles are smaller in prolific breeds of sheep (Driancourt *et al.*, 1985; Webb and Gauld, 1985; McNatty *et al.*, 1986) than in less prolific breeds. A reduced granulosa cell content was also noted in Booroola and Finn ewes, compared with Romanov and non-prolific ewes (Driancourt *et al.*, 1991). Previous studies in pigs have already highlighted the variability in granulosa cell number both within and between pigs (Foxcroft and Hunter, 1985; Grant *et al.*, 1989) and this was observed again in the present study. There was no difference overall in granulosa cell number as estimated by DNA content between breeds, in spite of the fact that the Meishan follicles were smaller, but this may be a reflection of the high variability within animals.

An important point is that, although they were smaller, Meishan follicles contained a similar amount of oestradiol as their Large-White hybrid counterparts, such that the higher concentration in the fluid in the Meishan approached significance. This suggests that the Meishan follicles may have been at a more advanced stage of development in terms of the intra-follicular steroid environment. The basis for this enhanced oestradiol concentration in Meishan follicles is not yet clear, particularly since granulosa DNA content and hCG binding were not different between breeds. Of several possibilities, the most likely are (i) earlier activation of aromatase activity in Meishan follicles, since it is known that aromatase activation is one of the earliest steps in follicular maturation in the pig (Foxcroft and Hunter, 1985), (ii) aromatase activity being higher on a per cell basis in Meishan than in Large-White hybrid follicles, (iii) a delay in the paracrine inhibitory effect of oestradiol on steroidogenesis (Hunter and Armstrong, 1987) or (iv) simply the same number of granulosa cells producing oestradiol into a smaller volume of follicular fluid in Meishan pigs. Such differences in aromatase activity have been reported in Booroola ewes (McNatty *et al.*, 1985), in which peak aromatase activity is achieved by smaller follicles than with non-prolific breeds. Furthermore, observations (C. Biggs, L. S. Faillace and M. G. Hunter, unpublished) have demonstrated an increase in aromatase activity of both the granulosa and theca cells in the Meishan follicles at day 20 of the cycle.

Evidence suggests that granulosa cell aromatase activity, certainly in the initial stages, is controlled by follicle-stimulating hormone (FSH) acting via the adenylate cyclase system (Erickson and Hsueh, 1978; Ainsworth *et al.*, 1990), but currently there is no information available on FSH secretion

during the early follicular phase in Meishan pigs. During the late follicular phase at about the time of the luteinizing hormone (LH) surge, FSH concentrations were not significantly different between Meishan and Large-White hybrid pigs (Hunter *et al.*, 1991). It has previously been shown that androgen substrate availability is a major limiting factor for oestradiol production by pig and sheep follicles (McNatty *et al.*, 1984; Foxcroft and Hunter, 1985; Grant *et al.*, 1989), and the theca tissue in Meishan follicles may have increased capacity to produce androgen substrate. Although there was no significant difference in follicular fluid testosterone concentrations between the two breeds, this could have resulted from enhanced conversion to oestradiol in the Meishan follicles.

The trend towards a lower total inhibin content of the Meishan follicles is of interest particularly since follicle number was not significantly different between breeds. A similar reduction in inhibin secretion by individual pre-ovulatory follicles has been reported for prolific Booroola and Romanov ewes (Driancourt *et al.*, 1991), but these animals also secreted less oestradiol and in the case of the Booroola there were fewer granulosa cells. McNatty *et al.* (1985) also reported fewer granulosa cells; however, in this case, the preovulatory follicles contained more oestradiol at a smaller diameter.

The significant positive correlations between follicular diameter and follicular fluid volume in both breeds are consistent with previous observations (Grant *et al.*, 1989). Hormone concentrations of follicular fluid correlated with their respective total follicular hormone content, in both Meishan and Large-White hybrid pigs. The morphological characteristics correlated significantly with hormone content indicating that for both breeds the larger follicles exhibited a higher oestradiol concentration and total inhibin and oestradiol content. Testosterone concentration however was higher in smaller follicles in both breeds.

Although the present study found no evidence of decreased variability of follicular characteristics in Meishan pigs, the trend towards a higher oestradiol concentration in follicular fluid may indicate advanced maturity of these follicles.

Corpora lutea were smaller in Meishan than in Large-White hybrid pigs although there was no difference in follicular granulosa cell DNA content, but follicles were indeed smaller; however, the corpora lutea were recovered from pregnant Meishan pigs that had experienced more oestrous cycles before mating. Again, smaller corpora lutea have been reported in the prolific Booroola ewe, and this has been thought to be due to the lower granulosa cell numbers in these ewes (Scaramuzzi and Radford, 1983). The other point is that the corpora lutea recovered for this study were obtained from Meishan with a higher ovulation rate, the possible reasons for which are discussed above. Progesterone concentration per mg of tissue was similar between the breeds indicating comparable steroidogenic capacity on a cellular basis. Owing to their smaller size, Meishan corpora lutea contained less total progesterone than Large-White hybrid corpora lutea, but the overall ovarian progesterone content was the same for both breeds owing to the higher ovulation rate in Meishan pigs. These observations on luteal function need to be repeated in animals with similar ovulation rates, as well as an assessment of circulating progesterone concentrations.

In conclusion, although the present study found no differences in follicular heterogeneity between the breeds, it has demonstrated some differences between Meishan and Large-White

hybrid pigs in both follicular and luteal characteristics. In particular, follicles from Meishan pigs, although smaller, had a similar follicular oestradiol content to their larger Large-White hybrid counterparts and tended to have higher oestradiol concentration in the follicular fluid. This suggests that these follicles may have been at a more advanced stage of maturation than their Large-White hybrid counterparts and whether this is subsequently reflected in oocyte maturation is currently under investigation.

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