Ovarian Activity and Oocyte Development during Follicular Development in Pigs at Different Reproductive Phases Estimated by the Repeated Endoscopic Method

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Abstract. The aim of the present study was to assess follicular and oocyte development in the same gilts during three phases of their reproductive life [prepuberal gilts (PP; 6.0 months of age), puberal gilts (P; 9.5 months of age) and primiparous sows (S)]. Follicular development was stimulated by the injection of 1,000 IU of equine chorionic gonadotropin (eCG) followed by 500 IU of human chorionic gonadotropin (hCG) 72 h later. Cumulus-oocyte-complexes (COCs) were recovered by endoscopic ovum pick up/aspiration from preovulatory follicles of the left ovary, and the follicular fluid (FF) from the right ovary was collected 34 h after the hCG treatment by endoscopy. Altogether, 19 pigs were used in the PP and P trials and 12 in the S trial. From the left ovaries, 168, 190 and 82 follicles were aspirated and 106, 125 and 42 COCs, respectively, were recovered (recovery rate 61 ± 27, 63 ± 21 and 53 ± 22%, respectively). The mean number of follicles was greater in the P phase than in the PP phase (19.7 ± 6.8 vs. 15.7 ± 6.8; p=0.06) and S phases (14.2 ± 4.0; p<0.05). More uniform oocytes with an expanded cumulus were aspirated in the P and PP phases than in the S phase (90 and 78 vs. 46%; p<0.05). Furthermore, the meiotic configuration in oocytes (T I/M II stage) differed between the three phases (56 and 62 vs. 0%; p<0.05). Progesterone (P4) levels in FF decreased from 590.0 ± 333.6 (PP) to 249.1 ± 72.6 (P) and 161.4 ± 75.2 ng/ml (S) (p<0.05). Estradiol-17β (E2) levels differed between PP and P gilts and S sows (9.3 ± 2.9, 21.9 ± 10.6 and 94.0 ± 15.9 pg/ml, respectively; p<0.05), and the P4/E2 ratio was 72, 15 and 5, respectively. These results indicate differences in follicular and oocyte development between the reproductive phases investigated. Puberal gilts should preferably be used in IVF and breeding programs. The lower reproductive potential of primiparous sows must be taken into consideration in breeding. Any prediction of lifetime performance based on individual ovarian reactions of prepuberal gilts is unreliable.

Key words: Endoscopy, Follicular fluid, Oocyte morphology, Sow, Steroid hormone (J. Reprod. Dev. 51: 109–115, 2005)

Nearly 50 years ago, it was recognized that oocytes were restricted in prophase I of meiosis till the ovulatory period [1]. Since the resumption of meiosis is triggered by the preovulatory luteinizing hormone (LH) peak which can be stimulated with exogenous gonadotropins, the most essential steps of oocyte maturation occur during the last couple of days before ovulation [2]. In the 1980-s, there were improvements to in vitro...
reproductive research in mammals. Although in vitro maturation (IVM) and in vitro fertilization (IVF) have been well studied in pigs, porcine studies are not as numerous as those for other domestic animals. The first IVF piglets were born after in vivo maturation of oocytes, however IVM prior to IVF was found to be less effective [3, 4]. Successful IVF programs following IVM were reported later, but the outcomes of such trials were much less reliable than that of IVF after in vivo maturation [5–7]. Approximately 90% of oocytes selected according to the cytoplasm and cumulus cells completed nuclear maturation [8], but artificial milieu affected this process [9, 10]. Different data have been published about the age and reproductive stage of oocyte donors. While some authors did not find a significant difference between oocyte maturation ability of prepuberal and puberal gilts [5, 7], other reports did not mention the age of slaughtered donors of oocytes [6]. Nevertheless, there is some evidence that the fertility of cumulus oocyte complexes (COCs) collected from puberal gilts is better than that of prepuberal animals [11–13]. Only limited data have been published on IVM/IVF capacity of subsequent estrus cycles [14]. Significant differences were found between steroid and electrolyte concentrations as well as the osmolarity of follicle fluid (FF) collected after slaughter or laparotomy [15, 16]. However, repeated laparotomy in the same gilts restricted the scope of these experiments. Smith et al. [17] analyzed steroid and plasminogen activator concentrations in FF in two estrus cycles. Endoscopic ovum pick up (OPU) and FF aspiration can overcome the disadvantages of laparotomic recovery [18, 19].

Since most researchers prefer to use ovaries and oocytes of slaughtered pigs, little information is available on the quality of oocytes from prepuberal and puberal gilts and primiparous sows. There is no information on these parameters from the same individuals during their reproductive life. The aim of this study was to analyze the morphology and maturation of oocytes, and the steroid hormone levels of FF collected from preovulatory follicles of the same animal in three typical reproductive phases (prepuberal and cycling ages, and following first parturition). The results may facilitate IVF studies as well as the pre-selection of gilts for early breeding.

**Materials and Methods**

**Animals and treatments**

Altogether 19 crossbred gilts (Landrace x Large White) were involved in the present experiment: prepuberal gilts at 6.0 months of age, cycling gilts at 9.5 months of age and after the first parturition (primiparous sows). The first estrus was induced in prepuberal gilts at 6.0 months of age by intramuscular injection of 1,000 IU of eCG (Folligon®; Intervet, Amsterdam, The Netherlands) and 72 h later by 500 IU of hCG (Choriogonin®; Richter, Budapest, Hungary). In puberal gilts at the age of 9.5 months, estrus was synchronized by daily oral administration of altrenogest (16 mg Regumate®/day/animal, Janssen Animal Health, Neuss, Germany) for a 15-day period. Thus, 25 h after the last Regumate® application, animals were injected with 1,000 IU of eCG and then 500 IU of hCG 72 h thereafter. In the following estrus cycle, all gilts were inseminated and 12 gilts became pregnant. These primiparous sows were weaned after a 28-day suckling period. Their estrus was synchronized by administering 1,000 IU of eCG 24 h after weaning and 72 h later, 500 IU of hCG.

**Oocyte retrieval and evaluation**

Cumulus-oocyte-complexes (COCs) and FF were recovered 34 h after the injection of hCG by endoscopic OPU [18]. Laparoscopic surgery was carried out under general anesthesia [15 ml ketamine (Ursotamin®), Serumwerk Bernburg, Germany, and 5 ml xylazine (Xylavet®), Lavet Pharmaceuticals, Budapest, Hungary]. The COCs were recovered using a two-way cannula and an electric aspiration pump (model 3014; Labotect, Göttingen, Germany) with a vacuum pressure of 100 mm Hg [20]. The aspiration cannula was inserted into a follicle, the content was aspirated, and the follicle was refilled and aspirated twice with heparinized phosphate-buffered saline (PBS). The COCs were recovered in each case from follicles of the left ovary, and the FF was collected from follicles of the right ovary by using a one-way cannula without flushing the follicles. Only macroscopically healthy follicles (translucent follicles with good vascularization and a diameter of more than 5 mm) were punctured.

The morphology of recovered COCs was determined using an inverted microscope at × 60 magnification. COCs were classified as compact,
expanded or denuded [21]. Thereafter, COCs were liberated from cumulus cells in PBS containing 100 IU/ml of hyaluronidase (Hylase®; Impfstoffwerke Dessau, Germany). Oocytes were mounted on slides and fixed for > 24 h in a mixture of acetic acid/alcohol/chloroform (3:6:1) before staining with 2% (w/v) orcein in 60% (v/v) acetic acid. The nuclear configuration of oocytes was checked under a phase-contrast microscope at a magnification of 250 to 630. According to their nuclear development oocytes were classified as follows: (1) immature, germinal vesicle (GV) with diplotene chromatin; (2) meiosis resumed, GV breakdown and diakinesis (M-I to A-I); and (3) mature, T-I and M-II.

**Immunoassay for steroid hormones**

The progesterone (P4) levels in the FF were determined using a direct, single-antibody ³H-radioimmunoassay [22]. The [1,2,6,7-³H]progesterone (³H-P4; used as a tracer) was purchased from Amersham-Buchler (Berlin, Germany). The antibody was raised in rabbits by immunization with 11α-hydroxyprogesterone conjugate (Steraloids Newport, MA, USA). The antiserum was purified by affinity chromatography on protein A Superose (Pharmacia, Uppsala, Sweden), and used at a final titer of 1:200,000. The range of the standard curve was between 12.5 and 800.0 pg/ml. Fluid (5 µl) from each follicle was diluted with 200 µl of phosphate buffer (pH 7.0), and the P4 analysis was performed in 25 µl of this diluted fluid. The B/F separation of this assay was performed by the dextran-charcoal method. Radioactivity was measured using a LSC with an integrated RIA program (Rackbeta 1219; Wallace, Uppsala, Sweden). The intra-assay coefficient of variation (Cv) was between 7 and 10%, and the inter-assay Cv between 9 and 12%.

Estradiol-17β (E2) was measured in 50-µl duplicates of diluted follicular fluid with a ³H-radioimmunoassay [22]. The antibody against E2 raised in rabbits was purified and used at a titer of 1: 55,000. Incubation, B/F separation and counting were performed as described for P4. The sensitivity of the assay was about 2 pg/ml, and intra-assay and interassay Cvs were 8.5 and 12.0%, respectively.

**Statistical analyses**

Statistical calculations were performed with Statistical Analysis System (SAS®) software. Numbers of follicles and aspirated follicles were analyzed using the GENMOD procedure with an identity link function and Poisson distribution. All possible differences of LS-means and standard errors were computed and tested with Wald’s chisquare test. The recovery rate, serum steroid concentrations and the P4/E2 ratio were subjected to GLM repeated measurement analysis of variance (repeated factor: Group) using the GLM procedure. The differences between groups due to cumulus expansion and oocyte maturation (immature, resumption of meiosis and mature) were analyzed with a contingency table. Each value represents the mean ± SE. Differences of p<0.05 were considered significant.

**Results**

Differences in the number of follicles between prepuberal and puberal gilts, and primiparous sows are shown in Fig. 1. In most cases, the number of follicles increased from prepuberal gilts (15.7 ± 6.8) to puberal gilts (19.7 ± 6.8; p=0.06) but decreased in primiparous sows (14.2 ± 4.0; p<0.05) (Fig. 1A). However, in some pigs, the number of follicles increased from prepuberal gilts to primiparous gilts, continuously decreased from prepuberal gilts to primiparous sows, or increased from prepuberal gilts to puberal gilts but decreased from puberal gilts to primiparous sows (Fig. 1B).

The average size of punctured follicles was independent of the animal’s age (6.3 ± 0.3; 7.2 ± 0.9 and 6.5 ± 0.7 mm for prepuberal gilts, puberal gilts and primiparous sows, respectively). However, the shape differed, i.e. in prepuberal and puberal gilts, follicles were definitely vaulted over the ovarian surface and the follicular wall was softer, whereas in sows, they were flatter lying deeper in the stroma and the follicular wall was markedly stronger.

Altogether 440 follicles were punctured in the left ovaries for the recovery of COCs. Results regarding collection, morphology and oocyte maturation are summarized in Table 1. The collection rate was comparable in all groups and ranged from 53 to 63%. Cumulus morphology was more consistent in prepuberal gilts. The number of oocytes with an expanded cumulus investment decreased from the prepuberal to primiparous
phases (p<0.05). Oocyte maturation varied between age groups. In prepuberal and puberal gilts, fewer oocytes with an immature chromatin configuration and more mature oocytes were found than in primiparous sows (p<0.05).

Follicular fluid was collected from 245 follicles of the right side ovaries. Mean FF steroid concentrations are presented in Table 2. Higher P4 and lower E2 levels were noted in follicles of prepuberal gilts compared to puberal gilts and primiparous sows. Similarly, the P4/E2 ratio varied with age (72–43 vs. 15–11 vs. 5–3 in prepuberal and puberal gilts and primiparous sows, respectively). Due to the methodical approach used in this study, the relationship between steroid concentrations and oocyte maturation in the same follicle could not be analyzed. Since the number and morphology of follicles was similar between the right and left ovaries, a link between oocyte maturation and follicular steroid content appears reasonable. Consequently, higher P4 and lower E2 concentrations in FF were associated with a higher incidence of oocyte maturation (meiosis resumed/mature; Fig. 2).

**Discussion**

Little information is available on oocyte maturation and hormone concentrations in FF of the same female pigs. Only two reports have provided data on the development of embryos in vitro, and on steroid and protein hormone concentrations from the same gilts during the first and third estrus cycle [14, 17]. Endoscopic ovum

![Fig. 1. Tendencies in the follicular development of prepuberal gilts, puberal gilts and primiparous sows (n=19). In most cases, the number of follicles increased from prepuberal gilt to puberal gilt, and decreased in primiparous sow (A). In some pigs, the number of follicles increased from prepuberal gilts to primiparous sow, decreased from prepuberal gilt to primiparous sow, or decreased from prepuberal gilt to puberal gilt and decreased from puberal gilt to primiparous sow (B).](image)

| Table 1. Oocyte recovery, cumulus-oocyte complex (COC) morphology and chromatin configuration of oocytes, an indicator for oocyte maturation, in prepuberal gilts, puberal gilts and primiparous sows |
|---|---|---|
| | Prepuberal gilts | Puberal gilts | Primiparous sows |
| Gilts/sows (n) | 19 | 19 | 11 |
| Aspirated follicles (n) | 168 | 190 | 82 |
| Recovered COCs (n) | 106 | 125 | 42 |
| Recovery rate (%) | 61 ± 27 | 63 ± 21 | 53 ± 22 |
| Rate of oocytes with expanded cumulus (%) | 90<sup>a</sup> | 78<sup>b</sup> | 46<sup>c</sup> |
| Evaluated oocytes (n) | 92 | 112 | 37 |
| Oocyte maturation (%) | | | |
| Immature stage | 4<sup>a</sup> | 6<sup>a</sup> | 49<sup>b</sup> |
| Resumption of meiosis stage | 40<sup>a</sup> | 32<sup>a</sup> | 51<sup>a</sup> |
| Mature stage | 56<sup>a</sup> | 62<sup>a</sup> | 0<sup>a</sup> |

<sup>a-c</sup>: p<0.05 between different letters; χ²-test.
pick up in pigs makes the repeated recovery of oocytes and FF possible without affecting the forthcoming ovarian processes [18]. In the present study, follicular and oocyte development were analyzed in the same animals at three typical reproductive phases.

The number of preovulatory follicles was smaller in prepuberal gilts than cycling ones but similar to that in primiparous sows after estrus was stimulated. However, such a uniform trend was not identified in all animals. Thus, the ovulation rate in later life cannot be predicted on the basis of the prepuberal ovarian response. Data in the literature vary as well. While some authors found that the number of ovulated follicles increased from first to third estrus, i.e. 9.5 vs. 11.1 vs. 13.1 [23], others reported similar rates at the first and third ovulation: 10.3 ± 0.4 vs. 9.0 ± 0.9 [17]. In previous studies, where prepuberal and puberal gilts were treated with 1,000 IU of eCG, the average number of follicles ranged from 19.6 to 25.5 and 18.6 to 24.3, respectively [24 – 27].

The diameter of follicles did not differ much in gilts and sows, but the shape did. Follicles of primiparous sows were less vaulted, embedded deeper under the ovarian surface, flat and less vascularized. Such features can be characteristic of less developed follicles [28].

Since the expansion of the cumulus layer precedes nuclear maturation, it is an indicator of oocyte maturation and developmental capacity [21, 29, 30]. In our study, the proportion of oocytes with an expanded cumulus was higher in the prepuberal than puberal or primiparous phase. Consequently, the size of the population of gonadotropin-stimulated follicles and their maturation competence was more consistent in prepuberal gilts.

Although an earlier study reported a lower percentage of mature ova at first estrus compared to third estrus [12], we found more mature oocytes or oocytes resuming meiosis in younger animals than in primiparous sows (96, 94 vs. 51 %). Surprisingly, in our study, no mature oocytes were found in primiparous sows. However, several studies have shown that follicular and oocyte development as well as ovulation in sows are strongly affected by the previous lactation and nutrition [31–33]. Apparently in the present experiment, the punctured follicle population in primiparous sows was less mature at the time of recovery, because as well as steroid concentration, the shape and vascularization of the follicles in sows evidently differed from those in gilts.

Consistent with previous studies [34, 35], the highest P4 and lowest E2 levels were measured in FF collected at the prepuberal age. Due to the

<table>
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<tr>
<th>Group</th>
<th>Prepuberal gilts</th>
<th>Puberal gilts</th>
<th>Primiparous sows</th>
</tr>
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<tbody>
<tr>
<td>Gilts/sows (n)</td>
<td>19</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>Aspirated follicles (n)</td>
<td>86</td>
<td>114</td>
<td>45</td>
</tr>
<tr>
<td>Concentration of estradiol (pg/ml; mean ± SD)</td>
<td>9.3 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.9 ± 10.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94.0 ± 15.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Concentration of progesterone (ng/ml; mean ± SD)</td>
<td>590.0 ± 333.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>249.1 ± 72.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>161.4 ± 75.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a-b</sup>: p < 0.05 between different letters; χ²-test.
significant reduction in follicular capacity to produce E2 and the increase in P4 production, both gonadotropin-stimulated and spontaneously growing late preovulatory follicles have a higher P4/E2 ratio [34–38]. Along with heterogenous follicular development, we could identify a marked dissimilarity in follicular E2 and P4 levels of gilts and sows. Such an asynchrony of Graafian follicles according to morphology and biochemistry may continue in the immediate periovulatory period, and follicles may have a different response to the LH (or hCG) surge, reflecting their actual maturational stage [34, 39–41]. The higher E2 and lower P4 levels in accordance with oocyte maturation and follicular morphology in primiparous sows indicate less maturation compared to prepuberal and puberal gilts. Since the number of mature oocytes increases with the reduction in the follicular production of E2 and increase in the synthesis of P4 [34], follicles of primiparous sows evidently need a longer time to mature. This may explain the longer estrus cycle of sows than of gilts.

It can be concluded that in female pigs, follicular and oocyte development changed during the periods investigated. Morphological and biochemical heterogeneity of follicles was always identified. Puberal gilts appear to be more suitable for breeding as well as for in vitro fertilization because they have a more balanced intrafollicular environment, oocyte maturation and presumably a higher ovulation rate. On the basis of ovarian reactivity at the prepuberal age we cannot predict the later reproductive performance. The lower reproductive potential of primiparous sows should be considered in breeding programs with special reference to the longer follicle/oocyte maturation.

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References


