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Changes in the Number of Preantral Follicles and Hormone Concentrations in the Bovine Fetus

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Abstract. The present study aimed to determine the relationship among changes in the number of primordial and primary follicles, ovarian weight and concentrations of androstenedione (A), estradiol-17 β (E₂), progesterone (P₄), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the bovine fetus between day-90 and -150 of gestation. Fetal ovaries were obtained from 25 pregnant cows. Histological sections of the ovaries were examined to determine the number of preantral follicles. The hormone concentrations were determined by enzyme immunoassay. Ovarian weight increased in parallel with fetal age. No clear changes in the number of primordial follicles were observed in this period. Primary follicles were observed in ovarian sections at day-100 and the number of primary follicles increased in parallel with fetal age between day-90 and -120, and afterwards it significantly increased ($p < 0.05$) at day-130. Around day-120, the number of primary follicles increased with the serum concentrations of A and E₂. However, no relationships among number of primary follicles and concentrations of FSH and LH were observed in this period. These results indicate that the number of primary follicles markedly increases around day-130 of gestation, the increase in the number of primary follicles is associated with an increase in the concentrations of A and E₂ in the fetal serum, and the growth and differentiation of primordial and primary follicles are independent of FSH and LH.

Key words: Primordial follicle, Primary follicle, Steroid hormone (A, E₂ and P₄), Gonadotropin (FSH and LH), Bovine fetal

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The mammalian ovary contains a large number of preantral follicles. About 2 million germ cells were found in the bovine fetal ovaries at day-90 of gestation [1, 2]. A drastic change in the number of preantral follicles as well as in the ovarian weight has been observed in the bovine fetus between day-90 and -150 of gestation [1, 3]. The number of follicles rapidly decreases after this period, but about 20 thousand preantral follicles were still found at birth in the calf [1]. From these follicles, only a few follicles grow and reach a final

ovulatory size throughout the reproductive life of the adult female. More than 99.9% of preantral and antral follicles undergo degenerative changes during growth and the maturation process [4]. This represents a tremendous loss of valuable genetic resources.

Follicle-stimulating hormone (FSH) induces follicular development in the ovaries of adult mammals [1]. FSH stimulates the activity of aromatase that converts androgens to estrogen in follicular cells [5]. Aromatase activity has been found in the bovine fetal ovaries at an early stage of pregnancy [6, 7]. FSH stimulates the activity of

aromatase and accelerates the production of estrogen. The proliferation of granulosa cells in the follicles is enhanced by an autocrine action of estrogen, which is stimulated by FSH. Estradiol production in fetal ovaries has also been shown in cattle [8, 9]. Recently, it has been demonstrated that bovine oocytes obtained from secondary follicles can achieve complete maturation *in vitro* [4]. However, a culture system for bovine primordial and primary follicles is still lacking [10–13]. Therefore, the present study aimed to determine the relationships among changes in the number of primordial and primary follicles, ovarian weight and concentrations of androstenedione(A), estradiol-17 β (E₂), progesterone (P₄), FSH and luteinizing hormone (LH) in bovine fetuses between day-90 and -150 of gestation.

Materials and Methods

Fetuses

Fetuses were collected from 25 pregnant Holstein cows at a local slaughterhouse and transported to the laboratory. Fetal age was estimated from the crown-rump length (CRL) according to the methods described by Salisbury and Van Demark [14]. The fetal ages ranged from day-90 to 150 of gestation and were divided into six groups (Group I: day-90 to 99, Group II: day-100 to 109, Group III: day-110 to 119, Group IV: day-120 to 129, Group V: day-130 to 139, Group VI: day-140 to 149 of gestation).

Fetal serum

Fetal blood was obtained from the heart using a 21-gauge needle connected to a plastic syringe, transferred to a 5-ml spits-tube, and centrifuged at $2,000 \times g$ for 20 min at 4 C. The supernatant was frozen and stored at -30 C until use.

Histology

Both ovaries were aseptically removed from the abdominal cavity, and separated from surrounding connective tissue. The weight of each ovary was measured separately and averaged for both ovaries. Each ovary was cut around the center along the long axis, fixed in 10% formalin solution and embedded in paraffin. Serial sections 4 μ m in thickness were cut and mounted on gelatin-coated slides (MUTO-GRASS, Japan). The slides were

stained with hematoxylin and eosin, and examined using a microscope (Nikon, Japan). Follicular stages were determined based on the follicular diameter and the morphology of the oocytes surrounding cells. The follicles were classified according to previously established methods [1, 15, 16] as follows: 1) primordial follicles; $\leq 40 \mu$ m of diameter, oocytes surrounded by germ epithelial cells (pre-granulosa cells), 2) primary follicles; $40\text{--}60 \mu$ m of diameter, oocytes surrounded by one layer of cubic or columnar granulosa cells without differentiated theca cells. Because no difference in the relative number of follicles was found in the serial sections of each ovary, the number of follicles counted in the section of both ovaries (left + right) was considered as the representative number of primordial and primary follicles for each fetus.

Hormone determinations

The serum concentrations of the hormones in each fetus were determined in duplicate by double-antibody enzyme immunoassay (EIA) using 96-well ELISA plates (Corning Glass Works, Corning, NY). Serum concentrations of steroids (E₂, A and P₄) were determined after diethylether extraction. The EIA for E₂ was carried out as described previously [17]. The standard curve ranged from 2 to 2,000 pg/ml, and the ED₅₀ of the assay was 105 pg/ml. The intra- and inter-assay CVs were 6.5 and 7.3%, respectively. The EIA for A has been described previously [18]. The A standard curve ranged from 2 to 1,000 pg/ml, and the ED₅₀ of the assay was 130 pg/ml. The intra- and inter-assay CVs were 6.8 and 8.1%, respectively. The EIA for P₄ was also performed as previously described [19]. The standard curve ranged from 20 to 20,000 pg/ml, and the ED₅₀ of the assay was 850 pg/ml. The intra- and inter-assay CVs were 4.8 and 6.6%, respectively. The recovery rates of A, E₂ and P₄ were 81, 75 and 92%, respectively.

The serum levels of LH and FSH were also determined by EIA. The EIA for LH determination was based on the streptavidin-biotin technique as previously described by Mutayoba *et al.* [20]. The standard curve for LH ranged from 0.2 to 200 ng/ml and the ED₅₀ of the assay was 10.5 ng/ml. The intra- and inter-assay CVs were 8.5 and 13.5%, respectively. FSH was measured by a modification of the method previously reported by Watanabe *et al.* [21]. The standard curve ranged from 0.02 to 40 ng/ml and the ED₅₀ of the assay was 1.1 ng/ml.

The intra- and inter-assay CVs were 13.8 and 16.7%, respectively.

Statistical analysis

All values are presented as the mean $\pm$ SEM. The quantitative data obtained from EIA (A , E_2 , P_4 , FSH and LH) were compared among the different groups using ANOVA (Excel) followed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

Ovarian weight

The mean weight ($\pm$ SEM) of the ovaries from the bovine fetuses at day-90 was 51.2 ± 4.0 mg ($n=4$). This value increased significantly ($P < 0.05$; $R^2 = 0.17$) with gestational age at day-140 (63.3 ± 10.1 mg; $n=5$) (Fig. 1).

Primordial and primary follicles

Primordial follicles were observed only in the cortex layer of the ovary (Fig. 2-A). Changes in the number of primordial follicles in fetal ovaries between day-90 and -150 of gestation are shown in Fig. 3-A. During this period, significant changes in the number of primordial follicles were not found. Primary follicles were observed in the deep cortex

layer (Fig. 2-B). The mean number of primary follicles in each ovarian section is shown in Fig. 3-B. Primary follicles were observed in ovarian sections at day-100 and the number of primary follicles increased in parallel with fetal age, and significantly increased ($p < 0.05$) at day-130. No secondary follicles ($\geq 60 \mu\text{m}$ of diameter, an oocyte surrounded by one layer of cubic or columnar granulosa cells) or early antral follicles were observed in the fetal ovaries examined in this study.

Serum levels of hormones

Fig. 4 shows the change in serum levels of A , E_2 , P_4 , LH and FSH in the fetus. The mean concentration of E_2 in Group I was 4.4 ± 1.9 pg/ml. Afterwards, the E_2 level gradually increased up to day-120 (26.0 ± 6.9 pg/ml), when the number of primary follicles began to increase. The serum A concentration in the Groups III, IV, V (mean value for three groups: 274.8 ± 23.4 pg/ml) was significantly higher ($p < 0.05$) than those of Group I (70.2 ± 20.6 pg/ml). The serum levels of A and E_2 increased up to day-120. The mean P_4 concentration from day-90 to 99 was 39.0 ± 14.2 pg/ml. The P_4 concentration gradually increased up to day-120 (101.5 ± 25.0 pg/ml), but afterwards the P_4 values decreased to the same levels observed at day-90 to 99. The mean LH concentration from

Fig. 1. Changes in ovarian weight with fetal age in the bovine fetus ($n=25$).

Fig. 2. Representative primordial (A) and primary (B) follicles in bovine fetal ovaries. Scale bars: 20 μ m.

day-90 to 99 was 3.4 ± 0.9 ng/ml, and did not change during the observation period. The mean FSH concentration from day-90 to 99 was 1.0 ± 0.5 ng/ml. At day-110, the FSH concentration tended to be higher than the values of earlier periods, but decreased at around day-120 of gestation.

Discussion

In the present study, bovine fetal ovarian weight increased with gestational age from day-90 to 150 of gestation. Other studies [1, 3] have reported that fetal ovarian weight remains relatively constant between day-120 and -177, but markedly increases after day-180 of gestation. The period between day-90 and -150 of gestation is a time of drastic increase in the number of germ cells and preantral follicular development in the fetal ovary [1, 2]. The growth of the follicles including the increase in the diameter of the oocyte, proliferation of the granulosa cells and differentiation of the surrounding stromal cells into theca cells are followed by the formation of new blood vessels in the adjacent interstitial tissue. These changes in the follicular structure may result in an increase in the ovarian weight and mass during this period.

The results of the present study are consistent with those of Rüsse [2], who reported that the number of primordial follicles did not change between day-90 and -150 of gestation. However, in the study of Rüsse [2], primary follicles were first observed in fetal ovaries at day-140 of gestation. On the other hand, Tanaka *et al.* [1] reported that

the primary follicles appeared at day-100, as was also observed in the present study. The mechanism leading to spontaneous growth, development, and differentiation of some resting primordial follicles to primary follicles remains unknown at present. The first sign that primordial follicles and their corresponding oocytes have entered into the growth phase is the morphological change of the surrounding granulosa cells. The granulosa cells proliferate and change from flattened-shape in primordial follicles to cuboidal-shape cells in primary follicles. Following the growth process, follicles increase in size through a series of mitotic divisions of the granulosa cells [4], and unilaminar primary follicles are converted into multilaminar secondary follicles. In the present study, the secondary follicle was not observed up to day-150 of pregnancy.

In the present study, primary follicles were observed in ovarian sections at day-100 of gestation. The number of primary follicles increased markedly around day-130 of gestation. This increase in the number of primary follicles was associated with an increase in the concentrations of A and E₂ in the fetal serum. The main route for steroid production in the bovine fetal ovary appears to be the androstenedione-estrone-estradiol pathway [7], and it was postulated that this shift in steroid biosynthetic pathways might be related to changes in cellular events from mitosis to meiosis in fetal ovaries. In the present study, the serum levels of A, E₂ and P₄ increased in parallel up to day-120. The concentration of P₄ after day-120 decreased to the same levels observed between

Fig. 3. Changes in the number of primordial (A) and primary (B) follicles in bovine fetal ovaries (n=25).

day-90 and -99, while A and E_2 concentrations remained relatively high. Androstenedione is mainly synthesized from P_4 through the $\Delta 4$ pathway after LH stimulation in fetal ovaries [8]. As primordial follicles do not have differentiated theca cells, LH receptors may be absent in primordial follicles. Therefore, it seems that the

changing pattern of P_4 and other steroids (A and E_2) may be independent. Shemesh *et al.* [8] reported that P_4 and E_2 concentrations started to increase in the fetal ovary on day-30 and -42, respectively. In other studies [8, 9], E_2 was undetectable in fetal ovaries at day-70 and -105 of pregnancy. Acosta *et al.* [18] showed that P_4 concentrations in the fetal

Fig. 4. Changes in serum concentrations of steroid hormones (A, E₂, P₄) and gonadotropins (FSH, LH) in the bovine fetus. a, b, c: Values with different superscripts are significantly different ($P < 0.05$).

sera were lower than that in the maternal sera, but the fact that the concentrations of A and E₂ in fetal sera were higher than those in maternal sera suggests that these two steroids (A and E₂) are of fetal origin. From the above result, since the

increase in two steroids (A and E₂) was coincident with the appearance of primary follicles, we consider that fetal originated steroid hormones may support the growth of primordial follicles into primary follicles.

The concentrations of gonadotropins (FSH and LH) showed a large variation and did not show clear changes during the observation period. It was reported that LH was not able to cross the placental barrier in the sheep [22]. Therefore, it is likely that the LH detected in the present study was of fetal origin. Secondary follicles may develop LH receptors in their differentiated theca cells. As theca cells were not observed in primordial and primary follicles, it appears that LH may not affect follicular growth during the observation period of the present study. FSH has been reported to promote follicular development in cultured bovine preantral follicles [1], and FSH may play a role in the early follicular development that occurs in the bovine fetal ovary [23]. Bao *et al.* [5] reported that FSH receptors are present in the granulosa cells of bovine primary follicles which may respond to a low concentration of FSH. However, our results

and those of a previous study [24] suggest that the development of primordial and primary follicles is not associated with either gonadotropin.

In conclusion, the number of primary follicles markedly increased around day-130 of gestation in the bovine fetal ovary, the increase in the number of primary follicles was associated with increases in the concentrations of A and E₂ in the fetal serum, and the growth and differentiation of primordial and primary follicles appeared to be independent of FSH and LH.

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