

Full Length Research Paper

Expression of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in normal and cystic follicles in sows

**Yanling Sun¹, Jing Zhang^{1#}, Zhiguang Ping², Lina Fan¹, Chunqiang Wang^{1,3}, Wanhong Li¹,
Chen Lu¹, Lianwen Zheng^{4*} and Xu Zhou^{1*}**

¹College of Animal Science and Veterinary Medicine, and Jilin Provincial Key Laboratory of Animal Embryo Engineering, Jilin University, 5333 Xi'an Avenue, Changchun, Jilin Province, 130062, P. R. China.

²College of Veterinary Medicine, China Agricultural University, Beijing, 100193, P. R. China.

³Liaoning Medical University, Jinzhou, Liaoning Province, 121001, P.R. China.

⁴Reproductive Medical Center, the Second Hospital of Jilin University, Changchun, Jilin Province, 130062, P.R. China.

Accepted 26 April, 2011

3 β -Hydroxysteroid dehydrogenase (3 β -HSD) performs essential roles in the regulation of follicular development and the level at which it is expressed may reflect the steroidogenic capacity of follicles and their physiological status. The aim of this study was to investigate the expression of 3 β -HSD protein and mRNA in normal follicles categorized by size into small (2 to 4 mm), medium (5 to 7 mm), large (8 to 10 mm) and cystic follicles (> 21 mm). The expression levels of 3 β -HSD protein and mRNA were evaluated by western blotting and quantitative real-time PCR, respectively; whereas, the immunolocalization of 3 β -HSD was examined in normal and cystic follicles. The results indicated that the expression of 3 β -HSD protein and mRNA increased as follicles grew. High levels of 3 β -HSD protein and mRNA ($P < 0.01$) were found in cystic follicles. Immunoreactions of 3 β -HSD were localized in the theca cells in the normal and cystic follicles, with reactions indicating 3 β -HSD also to be present in the granulosa cells of the cystic follicles, but to be less evident in or absent from the granulosa cells of normal follicles. Our results revealed that the expression and localization of 3 β -HSD differed in the cystic follicles and normal follicles and suggest that 3 β -HSD in cystic follicles plays an essential role in the formation and persistence of cysts. Our findings provide important additional insights into the pathogenesis of follicular cysts in sows.

Key words: 3 β -Hydroxysteroid dehydrogenase (3 β -HSD), expression, follicular cysts, sows.

INTRODUCTION

Ovarian follicular cysts (OFCs) are anovulatory follicular structures that have been identified in many mammalian species (Kaaijk et al., 2000). While they are known to lead to infertility, the pathogenesis of OFCs remains poorly understood. The most widely accepted hypothesis is the disorder of the hypothalamic- pituitary- gonadal axis (Bosu et al., 1987; Garverick et al., 1997; Ribadu et al., 2000), which suggests that hormones have important roles in the formation and persistence of cystic follicles. 3 β -HSD catalyzes the conversion of pregnenolone to

Co-first author.

progesterone, 17-alpha-hydroxypregnenolone to 17-alpha-hydroxyprogesterone and dehydroepiandrosterone (DHEA) to androstenedione, thus, is essential for the biosynthesis of mineralocorticoid, glucocorticoid and reproductive steroid hormones (Johnson et al., 1997). Alterations in the content of steroid hormones have been reported to be accompanied by changes in the expression and cellular distribution pattern of 3 β -HSD in the cystic ovaries of women (Takayama et al., 1996), rat (Cooke et al., 1993) and cow (Isobe et al., 2003). Changes of 3 β -HSD expression have also been reported to affect the content of progesterone, androgens and estrogens in ovarian structures (Jana et al., 2005). Clearly, 3 β -HSD not only has important roles in the synthesis of steroids and the maintenance of the ovarian

*Corresponding authors. E-mail: xuzhou65@vip.sina.com, davezheng@sohu.com. Tel: ++86-431-87835142. Fax: ++86-431-87836409.

Table 1. Sequence of primers and TaqMan probes used for quantitative real-time PCR.

Gene		Sequence	Size (bp)
3 β -HSD	FP	GGGATAATCAGTGCCAGATCTTG	118
	RP	GATGAGCGGGCGAACCT	
	TAMRA-FAM	FTGCAACAATCTTACAGGGCCACCCTCTP	
GAPDH	FP	TGGGCATGAACCATGAGAAG	117
	RP	CCACGATGCCGAAGTTGTC	
	TAMRA-FAM	FCCTCAAGATCATCAGCAATGCCTCCTGTP	

FP, forward primer; RP, reverse primer.

Steroidogenic activities in which 3 β -HSD has an essential role change during the normal process of follicular development. The expression of 3 β -HSD may also depend on the stage and physiological status of follicular development. However, it is still not clear whether any alteration in the expression and distribution pattern of 3 β -HSD occurs during follicular maturation in sows and is responsible for the formation of cystic follicles. The objective of this study was to investigate the expression and localization of 3 β -HSD in normal follicles of different size and in cystic follicles. The experiment will provide a theoretical foundation for studying the functional role of 3 β -HSD in the formation and persistence of cystic follicles and so make an important contribution to our understanding of the pathogenesis of follicular cysts.

MATERIALS AND METHODS

Collection of ovaries

Ovaries from sows with or without cystic follicles were collected from slaughterhouses and transported to the laboratory within 30 min of collection. Follicles from normal ovaries confirmed to be in the follicular phase of the ovarian cycle were manually dissected from the ovaries and sorted into three size categories: small (2 to 4 mm), medium (5 to 7 mm) and large (8 to 10 mm). Spontaneous follicular cysts were diagnosed if the follicle was greater than 21 mm and formed a fluid-filled structure with a smooth and thin wall in the absence of a corpus luteum (McGaughey, 1975). In total, five samples of each size class were collected and stored at -80°C, after aspirating the follicular fluid from the follicles with a 10 ml syringe. Some tissues from each group were fixed in 10% (v/v) formalin for histochemistry.

Immunohistochemistry

For immunohistochemistry assays, the formalin-fixed tissues were embedded in paraffin and sectioned into 3 to 4 μ m sections, which were de-waxed and rehydrated through descending grades of alcohol to distilled water, followed by incubation in 3% hydrogen peroxide to quench the endogenous peroxidase activity. After washing with PBS, sections were blocked for 2 h with blocking buffer and then incubated overnight at 4°C with a 3 β -HSD-specific antibody (1:50, sc-100466, Santa Cruz Biotechnology, Inc., CA, USA). Control sections were incubated with normal rabbit serum

(1:200, Santa Cruz Biotechnology, CA, USA). After washing with PBS, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1.5 h at room temperature. Immunoreactive sites were stained brown with diaminobenzidine (DAB). Multiple sections from each animal were examined in our experiment.

Protein extraction and western blotting analysis

The expression of 3 β -HSD protein in both normal and cystic follicles was estimated by western blotting. Total follicular proteins were extracted with the western lysis buffer (P0013, Beyotime Biotechnology, Jiangsu, PRC). After centrifugation at 4°C for 15 min, protein extracts were collected and determined by the method of Bradford (Bradford, 1976). Equal amounts (30 μ g) of proteins from the small, medium and large normal follicles and from the cystic follicles were analyzed by 12% SDS-PAGE and the separated proteins were then transferred onto PVDF membrane (Millipore Co., Billerica, MA, USA) at 80 v for 1.5 h. After being blocked with blocking solution (5% non-fat dried milk in PBST) for 1.5 h at room temperature, the membranes were incubated with polyclonal antibodies to 3 β -HSD (1:300, sc-100466, Santa Cruz Biotechnology, Inc., CA, USA), and β -actin (1:250, Beijing Biosynthesis Biotechnology Co., Ltd. Beijing, PRC) at 4°C overnight. After washing with PBST (3 x 15 min), the membrane was incubated with HRP-conjugated secondary antibody (1:3000; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, PRC) for 1.5 h at room temperature. Membranes were then washed several times and proteins were detected using SuperSignal substrate (Pierce Co., Rockford, IL, USA) and exposure to x-ray films. Data were expressed as the ratio of 3 β -HSD protein to β -actin protein.

RNA extraction and quantitative real-time RT-PCR

Quantitative assessment of the level at which 3 β -HSD mRNA was expressed in the normal and cystic follicles, was performed with real-time RT-PCR. Total RNA was extracted from the three size-classes of follicles with TRIZOL reagent (Invitrogen Life Technology Inc., USA). Extracted RNA was then measured by spectrometry at OD_{260/280} and equal amounts of RNA were reverse-transcribed into cDNA. Primers and probes were designed according to cDNA sequences from GeneBank (Table 1). The PCR was carried out in a mixture in which a final volume of 25 μ l contained 100 ng cDNA, 10 pmol primer and 0.15 μ l Ex Taq HS. Amplification was performed in an ABI 7000 Sequence Detection System (Applied Biosystems, USA) as follows: 95°C for 5 min; 94°C for 30 s; 60°C for 15 s; 72°C for 20 s (40 \times). Results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

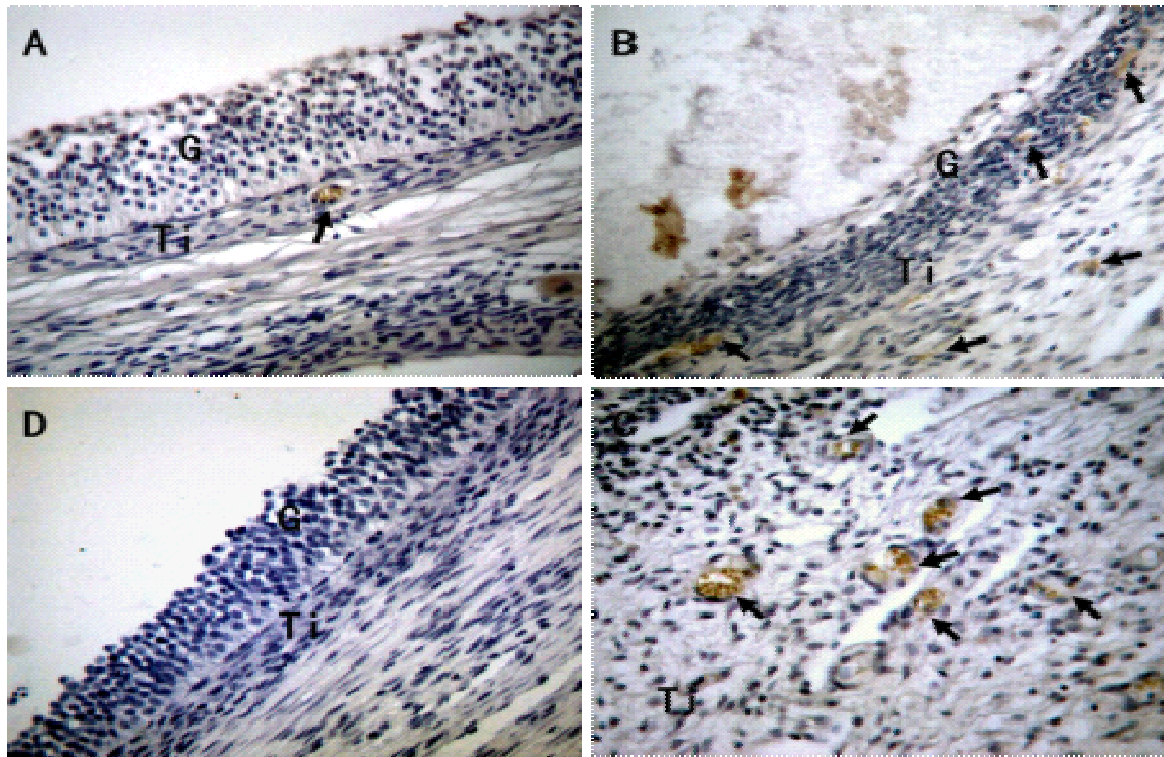


Figure 1. Light micrographs of normal (A), cystic; (B, C) porcine follicles, immunostained for anti- 3β -HSD antibody. Arrows show examples of 3β -HSD-positive cells; D, negative control; G, granulosa layer; Ti, theca interna. A, B and C magnification: $\times 100$.

histochemistry experiments were each replicated at least three times. Data were expressed as the means \pm SE. The differences in mean values were statistically analyzed using the one-way ANOVA followed by Dunnet's multiple range test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Changes in the localization and expression levels of 3β -HSD protein in follicles

The expression of 3β -HSD proteins was clearly detectable levels in all the follicles tested, but was highest in the cystic follicles (Figure 2). In normal follicles, the levels of this enzyme were similar in the large and medium follicles and were significantly higher in these than in the small follicles ($P < 0.01$). In cystic follicles, the intensity of the immunoreactivity for 3β -HSD was detected at significant levels in the granulosa cells and the theca cells (Figure 1 b, c). However, in normal follicles only a weak or no 3β -HSD reaction was observed in the granulosa cells (Figure 1a).

Changes in the expression levels of 3β -HSD mRNA in follicles

The results of the quantitative analysis of the expression of 3β -HSD (118 bp) mRNA are presented in Figure 3. The levels of 3β -HSD mRNA differed among the normal follicles of different size, increasing with follicular size such that it was significantly higher in the large follicles ($P < 0.01$), than in the small and medium follicles. Moreover, we found that the expression of 3β -HSD mRNA was markedly higher in cystic follicles compared with the normal follicles ($P < 0.01$).

DISCUSSION

The mechanisms of follicular cysts formation are not completely understood, however, the results of this study in which we examined the localization and expression of 3β -HSD in normal and cystic follicles suggested that they are associated with the differential expression of 3β -HSD in cystic follicles. Expression of 3β -HSD in the cystic follicles was significantly greater than that in the normal follicles. The immunohistochemical study gave a strong reaction in the theca interna cells of pre-ovulatory follicles indicating localization of 3β -HSD in these cells, but only a weak or negative reaction in the granulosa cells was

observed. In the normal cells, since 3 β -HSD was expressed in pre-ovulatory follicles, theca interna cells

may have synthesized androgens for estradiol production in the granulosa cells during the pre-ovulation period. We

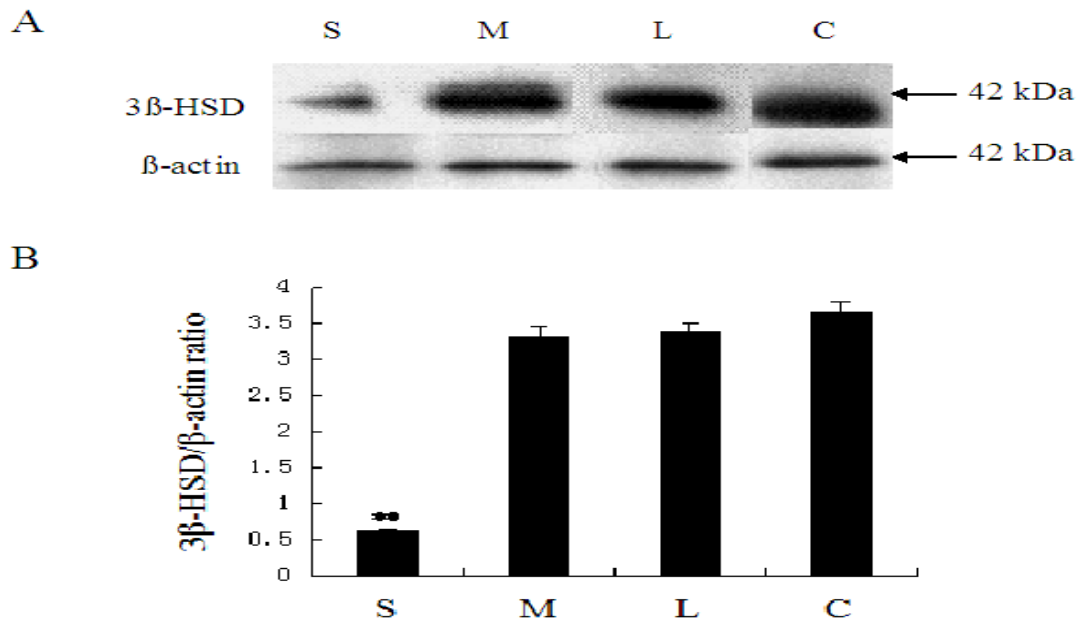


Figure 2. Western blotting of 3 β -HSD in the cystic follicles and normal follicles of different sizes. A, Representative photographs of western blotting for 3 β -HSD and β -actin (as an internal control); B, proteins levels were expressed as a ratio of the 3 β -HSD protein to the β -actin protein in the small, medium, large normal follicles and cystic follicles. All data are shown as means \pm SE. **indicates a statistically significant difference (P < 0.01). S, small follicle; M, medium follicle; L, large follicle; C, cystic follicle.

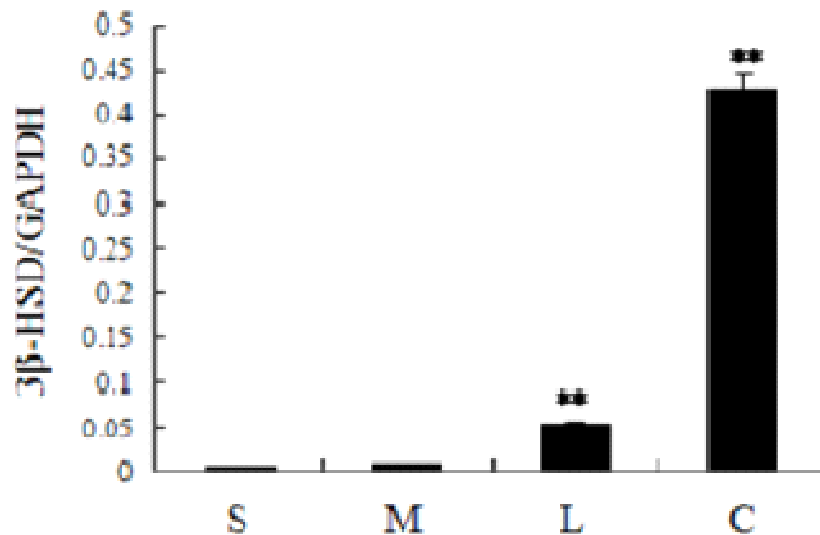


Figure 3. Quantitative real-time PCR of 3 β -HSD mRNA expression in small (S), medium (M), large (L) normal follicles and cystic (C) follicles. The relative mRNA levels represent the amount in femtograms of the mRNA expression corrected to the ratio of GAPDH gene mRNA. **indicates a statistically significant difference (p < 0.01).

therefore suggest that granulosa cells cannot metabolize steroid hormones, but derive them mainly from theca

interna cells in pre-ovulatory follicles. However, in the cystic follicles, we observed a high intensity of the 3 β -

HSD immunoreaction in the granulosa cells and the theca interna cells, whereas the immunoreaction in the granulosa cells of the normal follicles was weak or absent. These results are consistent with previous studies which
6188 Afr. J. Biotechnol.

have reported 3 β -HSD expression in granulosa and theca cells in bovine cysts (Calder et al., 2001). However, other studies have reported the presence of 3 β -HSD only in theca cells of polycystic ovaries of women (Kaaijk et al., 2000). The presence of the 3 β -HSD protein in granulosa cells of cystic follicles may provide additional precursors for conversion to androgens in theca cells and so cause a greater production of androgens; whereas in cystic follicles, the presence of the 3 β -HSD protein might be associated with the luteinization of granulosa cells, which might then lead to persistent follicles gradually becoming luteinic granulosa cells. Moreover, in the study we found that the expression of the 3 β -HSD protein increased as the follicles grew, suggesting that the steroidogenic activity of follicles increases in pre-ovulatory follicles. High levels of the 3 β -HSD protein in the cystic follicles may therefore imply a steroidogenic activity of the cystic structure, as well as being associated with the formation of cystic follicles.

We also detected the expression of 3 β -HSD mRNA in both normal and cystic follicles. In normal follicles, the expression of 3 β -HSD mRNA was significantly greater in the large normal follicles ($P < 0.01$), compared with the smaller sizes. This observation is consistent with previous studies, which have reported that the expression of 3 β -HSD mRNA is raised in the pre-ovulatory follicles of cattle (Tian et al., 1995). Elsewhere, it has been reported that 3 β -HSD mRNA is localized in the theca interna of all growing follicles and in the granulosa cells of follicles greater than 8 mm in diameter (Bao et al., 1997); the latter might contribute to the high levels of 3 β -HSD mRNA expression in large follicles. In our experiment, we also found the expression of 3 β -HSD mRNA to be significantly higher in the cystic follicles ($P < 0.01$) than in the normal follicles and that protein levels were also greater, suggesting greater steroidogenic activity in these structures.

The hormonal regulation of 3 β -HSD expression in sows is not currently well-understood. LH is the major stimulator of 3 β -HSD mRNA expression in granulosa cells in rats and humans (Martel et al., 1990; Dupont et al., 1992) and both FSH (Jone et al., 1982) and estradiol (Spiegel et al., 1978) are known to increase 3 β -HSD activity in humans. These studies imply that gonadotropin and steroid hormones might regulate the expression of 3 β -HSD in the sow as well. The expression of 3 β -HSD is correlated with follicular fluid concentrations of the steroid hormones, production which is catalyzed by the enzyme 3 β -HSD. The biosynthesis of these chemical messages occurs in specialized steroidogenic tissue which is catalyzed by cytochrome P450 superfamily monooxygenases and HSD, though numerous signaling mediators control steroidogenesis (Lucki et al., 2008). 3 β -HSD has important roles in estrogen synthesis. The study has reported that pregnenolone can be converted to DHEA by

P450_{c17} (Δ_5 metabolism) or to progesterone by 3 β -HSD, leading to androstenedione synthesis via Δ_4 metabolism. DHEA can be metabolized to androstenedione (Δ_5 pathway to estrogen synthesis). 3 β -HSD activity is

ultimately required for estrogen production, but a predominance of 3 β -HSD over P450c17 activity in the follicles might inhibit estrogen by promoting progesterone formation (Lucki et al., 2008). Expression levels of 3 β -HSD could reflect the steroidogenic capacity and physiological status of follicles. In our laboratory, the concentrations of estradiol and progesterone in follicular fluid have been examined in previous studies (Supplementary file; Sun et al., 2011), which showed the ratio of estradiol to progesterone to be significantly low. The ratio of estrogen to progesterone in follicular fluid is used to estimate follicular estrogenic activity and health status of follicles (Ireland, 1987). Therefore, the change of steroid hormone concentrations might be associated with high levels of 3 β -HSD expression in cystic follicles.

In conclusion, the present study has shown that the expression and localization patterns of 3 β -HSD differed between normal follicles and cystic follicles. Our results suggest that 3 β -HSD affects follicle growth by controlling ovarian steroidogenesis and that it might play an essential role in the formation and persistence of cystic follicles in sows.

REFERENCES

- Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS (1997). Expression of messenger RNA encoding 3 β -hydroxysteroid dehydrogenase Δ^4 , Δ^5 isomerase during recruitment and selection of bovine ovarian follicles: identification of dominant follicles by expression of 3 β -HSD mRNA within the granulosa cell layer. *Biol. Reprod.* 56: 1466-1473.
- Bosu WTK, Peter AT (1987). Evidence for a role of intrauterine infections in the pathogenesis of cystic ovaries in postpartum dairy cows. *Theriogenology*, 28: 725-736.
- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Calder MD, Manikkam M, Salfen BE, Youngquist RS, Lubahn DB, Lamberson WR, Garverick HA (2001). Dominant bovine ovarian follicular cysts express increased levels of messenger RNAs for luteinizing hormone receptor and 3 β beta-hydroxysteroid dehydrogenase delta (4), delta (5) isomerase compared to normal dominant follicles. *Biol. Reprod.* 65: 471-476.
- Cooke GM, Brawer JR, Farookhi R (1993). Steroidogenic enzyme activities in rat polycystic ovaries. *Can. J. Physiol. Pharmacol.* 71: 247-253.
- Dupont E, Labrie F, Luu-The V, Pelletier G (1992). Immunocytochemical localization of 3 β - hydroxysteroid dehydrogenase/delta 4-isomerase in human ovary. *J. Clin. Endocrinol. Metab.* 74: 994-998.
- Garverick HA (1997). Ovarian follicular cysts in dairy cows. *J. Dairy. Sci.* 80: 995-1004.
- Ireland JJ (1987). Control of follicular growth and development. *J. Reprod. Fertil.* 34: 39-54.
- Isobe N, Nakao T, Yoshimura Y (2003). Distribution of cytochrome P450-side chain cleavage in the theca interna layers of bovine small antral and cystic follicles. *Reprod. Domest. Anim.* 38: 405-409.
- Jana B, Dzienis A, Rogozińska A, Piskula M, Jedlińska-Krakowska M, Wojtkiewicz J, Majewski M (2005). Dexamethasone induced changes in sympathetic innervation of porcine. *J. Reprod. Dev.* 51: 715-725.

Johnson M, Everitt B (1997). *Essential Reproduction*. 4th Edn. Blackwell Scientific Publications.

Jone BB, Hsueh AJ (1982). Regulation of ovarian 3 beta-hydroxysteroid dehydrogenase activity by gonadotropin-releasing hormone and folliclestimulating hormone in cultured rat granulosa cells. *Endocrinology*, 110: 1663-1667.

Sun et al. 6189

Kaaijk EM, Sasano H, Suzuki T, Beek JF, Van Der Veen F (2000). Distribution of steroidogenic enzymes involved in androgen synthesis in polycystic ovaries: an immunohistochemical study. *Mol. Hum. Reprod.* 6: 443-447.

Lucki NC, Sewer MB (2008). Multiple roles for sphingolipids in steroid hormone biosynthesis. *Subcell. Biochem.* 49: 387-412.

Martel C, Labrie C, Dupont E, Couet J, Trudel C, Rheume E, Simard J, Luu-The V, Pelletier G, Labrie F (1990). Regulation of 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase expression and activity in the hypophysectomized rat ovary: interactions between the stimulatory effect of human chorionic gonadotropin and the luteolytic effect of prolactin. *Endocrinology*, 127: 2726-2737.

McGaughey RW (1975). A comparison of the fluids from small and large ovarian follicles of the pig. *Biol. Reprod.* 13: 147-153.

Ribadu AY, Nakada K, Moriyoshi M, Zhang WC, Tanaka Y, Nakao T (2000). The role of LH pulse frequency in ACTH induced ovarian follicular cysts in heifers. *Anim. Reprod. Sci.* 64: 21-31.

Spiegel J, Jones E, Synder BW (1978). Estradiol-17 beta interference with meiotic maturation of rana pipiens ovarian follicles: evidence for inhibition of 3 beta-hydroxysteroid dehydrogenase. *J. Exp. Zool.* 204: 187-191.

Sun YL, Ping ZG, Li CJ, Sun YF, Yi KL, Chen L, Li XY, Wang XL, Zhou X (2011). Comparative proteomic analysis of follicular fluid from normal and cystic follicles. *Reprod. Dom. Anim.* doi: 10.1111/j.1439-0531.2011.01760.x.

Takayama K, Fukaya T, Sasano H, Funayama Y, Suzuki T, Takaya R, Wada Y, Yajima A (1996). Immunohistochemical study of steroidogenesis and cell proliferation in polycystic ovarian syndrome. *Hum. Reprod.* 11: 1387-1392.

Tian XC, Berndtson AK, Fortune JE (1995). Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome P450 side-chain cleavage, 3-hydroxysteroid dehydrogenase, and P450 17a-hydroxylase, but not P450 aromatase. *Endocrinology*, 136: 5102-5110.