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Full Length Research Paper

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

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3β-Hydroxysteroid dehydrogenase (3β-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 3β-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 3β-HSD protein and mRNA were evaluated by western blotting and quantitative real-time PCR, respectively; whereas, the immunolocalization of 3β-HSD was examined in normal and cystic follicles. The results indicated that the expression of 3β-HSD protein and mRNA increased as follicles grew. High levels of 3β-HSD protein and mRNA ($P < 0.01$) were found in cystic follicles. Immunoreactions of 3β-HSD were localized in the theca cells in the normal and cystic follicles, with reactions indicating 3β-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 3β-HSD differed in the cystic follicles and normal follicles and suggest that 3β-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: 3β-Hydroxysteroid dehydrogenase (3β-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. 3β-HSD catalyzes the conversion of pregnenolone to 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 3β-HSD in the cystic ovaries of women (Takayama et al., 1996), rat (Cooke et al., 1993) and cow (Isobe et al., 2003). Changes of 3β-HSD expression have also been reported to affect the content of progesterone, androgens and estrogens in ovarian structures (Jana et al., 2005). Clearly, 3β-HSD not only has important roles in the synthesis of steroids and the maintenance of the ovarian

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

Gene		Sequence	Size (bp)
3β-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 3β-HSD has an essential role change during the normal process of follicular development. The expression of 3β-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 3β-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 3β-HSD in normal follicles of different size and in cystic follicles. The experiment will provide a theoretical foundation for studying the functional role of 3β-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 µm 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 3β-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 3β-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 µg) 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 3β-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 3β-HSD protein to β-actin protein.

RNA extraction and quantitative real-time RT-PCR

Quantitative assessment of the level at which 3β-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 (Invitrigen 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 µl contained 100 ng cDNA, 10 pmol primer and 0.15 µl 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×). Results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

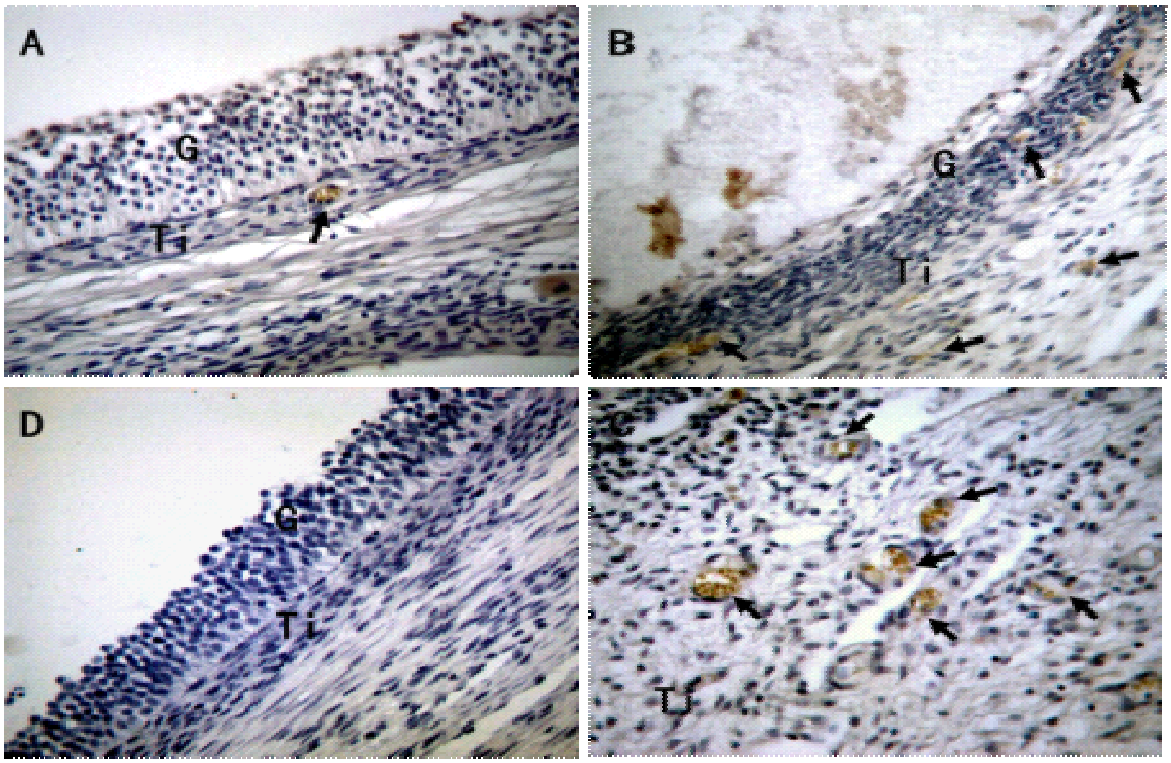

Figure 1. Light micrographs of normal (A), cystic; (B, C) porcine follicles, immunostained for anti- 3β-HSD antibody. Arrows show examples of 3β-HSD-positive cells; D, negative control; G, granulosa layer; Ti, theca interna. A, B and C magnification: ×100.

histochemistry experiments were each replicated at least three times. Data were expressed as the means ± 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 3β-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

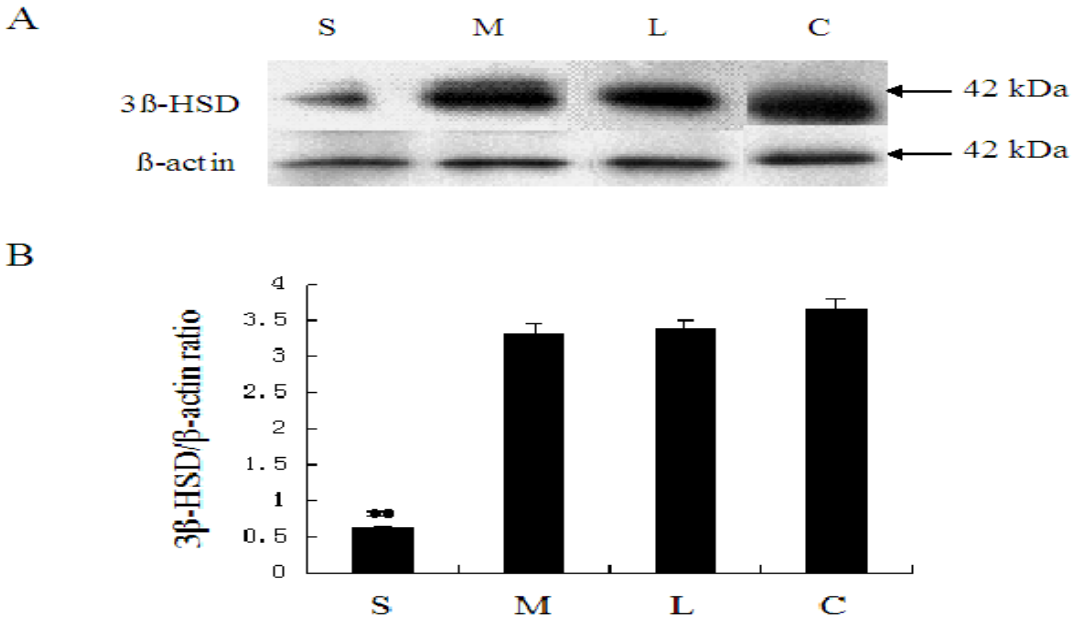

Figure 2. Western blotting of 3β-HSD in the cystic follicles and normal follicles of different sizes. A, Representative photographs of western blotting for 3β-HSD and β-actin (as an internal control); B, proteins levels were expressed as a ratio of the 3β-HSD protein to the β-actin protein in the small,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

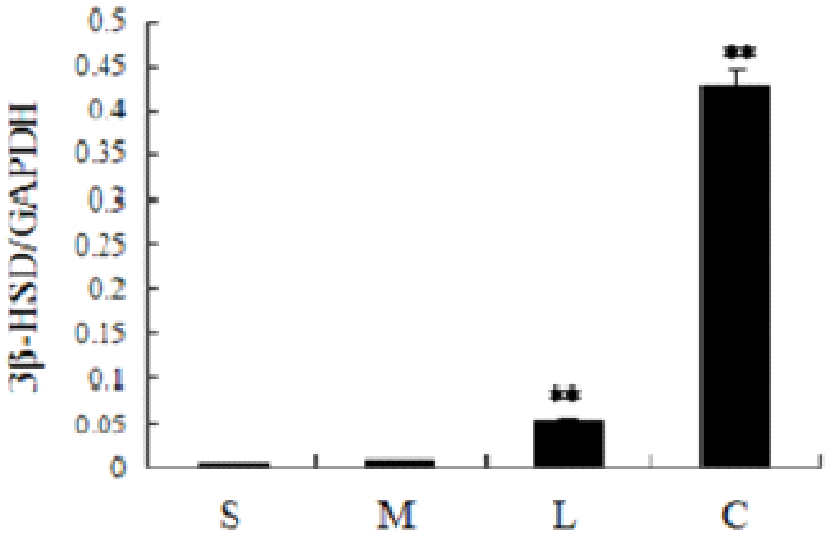

Figure 3. Quantitative real-time PCR of 3β-HSD mRNA expression in small (S), medium (M), large (L) normal follicles and cystic (C) follicles. The relative mRNA levels represent the amount in femtograms of the mRNA expression corrected to the ratio of GAPDH gene mRNA. **indicates a statistically significant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

have reported 3β-HSD expression in granulosa and theca cells in bovine cysts (Calder et al., 2001). However, other studies have reported the presence of 3β-HSD only in theca cells of polycystic ovaries of women (Kaaijk et al., 2000). The presence of the 3β-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 3β-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 3β-HSD protein increased as the follicles grew, suggesting that the steroidogenic activity of follicles increases in pre-ovulatory follicles. High levels of the 3β-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 3β-HSD mRNA in both normal and cystic follicles. In normal follicles, the expression of 3β-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 3β-HSD mRNA is raised in the pre-ovulatory follicles of cattle (Tian et al., 1995). Elsewhere, it has been reported that 3β-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 3β-HSD mRNA expression in large follicles. In our experiment, we also found the expression of 3β-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 3β-HSD expression in sows is not currently well-understood. LH is the major stimulator of 3β-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 3β-HSD activity in humans. These studies imply that gonadotropin and steroid hormones might regulate the expression of 3β-HSD in the sow as well. The expression of 3β-HSD is correlated with follicular fluid concentrations of the steroid hormones, production which is catalyzed by the enzyme 3β-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 steroidogenases (Lucki et al., 2008). 3β-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 3β-HSD, leading to androstenedione synthesis via Δ_4 metabolism. DHEA can be metabolized to androstenedione (Δ_5 pathway to estrogen synthesis). 3β-HSD activity is ultimately required for estrogen production, but a predominance of 3β-HSD over P450c17 activity in the follicles might inhibit estrogen by promoting progesterone formation (Lucki et al., 2008). Expression levels of 3β-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 3β-HSD expression in cystic follicles.

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

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