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Ovarian Expression of Inhibin-Subunits, 3 β -Hydroxysteroid Dehydrogenase, and Cytochrome P450 Aromatase during the Estrous Cycle and Pregnancy of Shiba Goats (*Capra hircus*)

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Abstract: The cellular localization of the inhibin subunits (α , β_A , and β_B), steroidogenic enzymes (3 β -hydroxysteroid dehydrogenase (3 β HSD) and cytochrome P450 aromatase (P450arom) were evaluated in the ovaries of cyclic (n=6) and pregnant (n=2) Shiba goats (*Capra Hircus*). The immunointensity of inhibin α and β_A subunits showed an increase in the granulosa cells (GC) of developing follicles. Inhibin β_B subunit and P450arom showed high expression in GC of antral follicles. 3 β HSD immunoreactivity was uniform in preantral and antral follicles. In follicular phase and late pregnancy, there was a strong expression of inhibin α subunit in GC of antral follicles. Although in mid pregnancy, antral follicles GC showed moderate immunostaining of inhibin β subunits, the immunoreactivity of inhibin β_A and β_B subunits was high during the follicular and luteal stages, respectively. While, immunoreactivity of GC to P450arom was moderate during all studied stages, and 3 β HSD immunoreactivity was plentiful in antral follicles during the luteal phase. The immunoreactivity to inhibin α subunit and P450arom was abundant during mid pregnancy in the luteal tissues. Immunoreaction to inhibin β subunits was faint-to-moderate in cyclic and pregnancy corpora lutea. Immunoexpression of 3 β HSD was maximal in late pregnancy corpora lutea. The present results suggest that, in goats, the GC of antral follicles are the main source of dimeric inhibins and that corpora lutea may partially participate in the secretion of inhibin. Changes in ovarian hormonal levels might depend on the synthesizing capacity of hormones in the follicles and corpora lutea to regulate the goat's reproductive stages.

Key words: goat, immunohistochemistry, inhibin, ovary, pregnancy

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Introduction

Laboratory animals are commonly used for evaluating the physiological properties of the mammalian ovarian follicle and the enclosed oocyte. The Shiba goat (*Capra Hircus var Shiba*), a non-seasonal Japanese native miniature goat with 20–21 days estrous cycle duration, is an excellent experimental animal [15] for studying the dynamic changes in the secretion of ovarian hormones occurring in conjunction with the morphological changes of ovarian follicular development [6]. Ovarian follicles secrete steroid and peptide hormones such as estradiol, progesterone and inhibin which play an important role in regulation of folliculogenesis through their feedback effects on pituitary gonadotropin secretion [5, 24].

Inhibin, a gonadal peptide hormone, is a disulphide-heterodimeric glycoprotein composed of α and β subunits. Combination of the α subunit with either of the β subunit (β_A or β_B) forms creates the bioactive inhibin molecule (α/β_A or α/β_B). Although the immunohistochemical expression of α , β_A and β_B subunits has been shown in granulosa cells of pre-antral and antral follicles in cow [20, 29] and sheep [2,19] ovaries in the absence of any signaling from the corpora lutea, the secretory source of inhibin in the ovary of the goat has not been investigated. In other species, the corpora lutea and placenta represent extra sources of inhibin [8, 30].

In the ovary, 3β hydroxysteroid dehydrogenase (3β HSD) and cytochrome P450 aromatase (P450arom) are steroidogenic enzymes which are crucial for catalyzing progesterone and estrogen from pregnenolone and androgens, respectively [9]. All luteal cells express 3β HSD and P450arom, but only weakly in late pregnancy in goats [31]. Immunohistochemical studies of the localization of the P450arom enzyme in the goat ovary during the estrous cycle have not yet been performed. Thus, the aim of the present study was to clarify the expression of inhibin subunits and steroidogenic enzymes in developing follicles and corpora lutea in relation to the reproductive stage to determine if they are **indicators of ovarian function(s) and to detect whether the ovarian structures and functions change during the estrous cycle and pregnancy**. We used Shiba goats which are an ideal animal model for ruminants and the immuno-peroxidase staining procedure.

Materials and Methods

Ovaries sampling, fixation, and slide preparation

The ovaries of eight adult Shiba goats (*Capra Hircus*), (cyclic; n=6 and pregnant; n=2) were excised immediately after euthanasia with an overdose of ketamine during the luteal (Day 10; n=2) and follicular (7, 5, 3, and 1 day before onset of estrus) phases, and pregnancy (90 and 120 days of gestation). These goats were originated from Animal Resource Science Center, the University of Tokyo (Ibaraki, Japan). The ovaries were dehydrated in a graded series of ethanol, embedded in paraffin wax, and sectioned at 6 μ m thickness. All procedures were carried out in accordance with guidelines established by Tokyo University of Agriculture and Technology.

Immunohistochemistry

Immunohistochemical staining for inhibin α , β_A and β_B subunits, 3β HSD and P450arom was carried out as described previously [23, 31]. Briefly, endogenous peroxidase was blocked by incubating the deparaffinized sections in 3% hydrogen peroxide in methanol for 30 min at room temperature and the epitopes were activated by autoclaving (at 121°C for 15 min, in 10 mM citrate buffer (pH 6.0). After rinsing with PBS/0.05 Tween20, non-specific staining was blocked with 10% normal goat serum at 37°C for 30 min. The sections were incubated with primary antibodies (Table 1) diluted in PBS overnight at 4°C, then reacted with biotinylated secondary antibody diluted in PBS containing 10% normal goat serum at 37°C for 45 min. Afterwards, the bound antibody was visualized with 3.3'-diaminobenzidine tetrachloride (DAB) 0.5% (Sigma Chemical Co., St. Louis, MO, USA) and 0.01% H_2O_2 until a brown precipitate formed or for a maximum of 20 min. Finally, the stained sections were counterstained in Mayer's haematoxylin (Merck, Tokyo, Japan). Controls were made by exposing to normal goat serum instead of primary antibodies. Some sections were also stained with haematoxylin and eosin for observation of the morphology of the follicular and luteal tissue.

Ovarian tissue analysis

Follicles were classified as previously described [22]: *primordial* (one layer of flattened granulosa cells around

Table 1. Identity, sources, and working dilutions of primary and secondary antibodies

Protein	Primary antibodies					Secondary antibodies	
	Antibody	Origin	Source	Dilution	Incubation time	Type	Source
Inhibin α subunit	Polyclonal antibody against [Tyr30] porcine inhibin α chain (1-30)NH2	Rabbit	Tokyo University of Agriculture and Technology	$\times 2,000$ – 4,000	Overnight at 4°C	Biotinylated anti-rabbit IgG	Vector Laboratories, Burlingame, CA, USA
Inhibin β_A subunit	Polyclonal antibody against synthetic inhibin β_A chain (C4)	Rabbit	Dr. W. Vale (The Salk Institute for Biological Studies, La Jolla, CA, USA).	$\times 2,000$			
Inhibin β_B subunit	Polyclonal antibody against synthetic inhibin β_B chain (C5)	Rabbit	Dr. W. Vale (The Salk Institute for Biological Studies, La Jolla, CA, USA).	$\times 2,000$			
P450arom	Polyclonal antibody against human placental aromatase cytochrome P450 (R-8-1)	Rabbit	Dr. Y. Osawa (Medical foundation of buffalo, buffalo, NY, USA)	$\times 4,000$			
3 β HSD	Polyclonal antibody against human placental 3 β HSD	Rabbit	Dr. J.I. Mason (Cecil H. and Ida Green center for reproductive science, University of Texas, Southern Medical center, Dallas, TX, USA)	$\times 1,000$			

the oocyte), *primary* (a single layer of cuboidal granulosa cells around the oocyte), *secondary* (oocyte surrounded with discrete layers of cuboidal granulosa cells), *tertiary*, or *antral follicle* (with a characteristic fluid-filled cavity, antrum). The parenchyma of the corpus luteum consisted of small (spindle-shaped cells with a low cytoplasmic/nuclear ratio) and large (polyhedral or irregular shape with a higher cytoplasmic/nuclear ratio with tapering cytoplasmic processes) luteal cells [11].

Immunohistochemical analysis

Eight-bit images of three random areas of the different ovarian structures were used for semi-quantitative densitometric analysis of immunostaining intensity in the cytoplasm using ImageJ software (National Institutes of Health, available at <http://rsb.info.nih.gov/ij/>) as previously described [3]. All images were converted to binary (black and white) using the calculated threshold of the currently displayed slice and the integrated intensity was determined using the particle analysis feature of ImageJ.

Results

Localization of inhibin α , β_A , β_B subunits, and P450arom and 3 β HSD proteins in goat ovarian follicles (Fig. 1)

Healthy ovarian follicles that had a well-organized, intact granulosa cell layer (s) showed positive immunostaining of inhibin α , β_A , β_B subunits and P450arom and 3 β HSD proteins. Meanwhile, the thin epitheloid theca cell layer(s) showed a weak affinity for these proteins (Fig. 1).

The primordial (Fig. 1A) and primary (Fig. 1B) follicles showed weak immunoreactivity to inhibin subunits β_A and β_B subunits, slight affinity for inhibin α and 3 β HSD and moderate immunostaining of P450arom proteins (Table 2).

Signals for inhibin α and β_A subunits gradually became greater in order of secondary, tertiary and large antral follicles. Furthermore, inhibin β_B and P450arom showed large expression on granulosa cells of tertiary and large antral follicles compared to secondary follicles. On the other hand, 3 β HSD immunoreactivity was uniform in secondary, tertiary and large antral follicles (Table 2).

Immunoreactivity of large antral follicles theca cells was exhibited a weak staining affinity for all inhibin

subunits and P450arom while faint staining was shown for 3 β HSD (data not shown).

Immunolocalization of inhibin α , β_A , β_B subunits, and P450arom and 3 β HSD proteins in cyclic and pregnant goat ovaries

Ovarian follicles (Fig. 2): The granulosa cells of large antral follicles showed clear positive immunostaining for inhibin α subunit that was strongest during the follicular phase and late pregnancy, moderate at mid pregnancy and low during the luteal phase. Antral follicles showed moderate affinity during the luteal phase and mid pregnancy and faint during the follicular phase and late pregnancy for inhibin β_B subunits (Table 3).

The immunoreactivity of granulosa cells of antral follicles to inhibin β_A subunit was moderate during the follicular phase and mid pregnancy and faint during the luteal phase and late pregnancy (Table 3).

The immunoreactivity of granulosa cells to P450arom was uniformly moderate at all the stages. Meanwhile, 3 β HSD immunoreactivity was highest in large antral follicles during the luteal phase and faint during the other stages (Table 3).

Corpora lutea (Fig. 3): The parenchyma of goat corpus luteum consists of two distinct cell types: small luteal and large luteal cells. The large luteal cells of the developing and mature corpus luteum expressed a moderate-to-strong immunoreactivity to inhibin α , β_A , β_B subunits, and P450arom and 3 β HSD. Meanwhile, small luteal cells showed no immunostaining reaction to these proteins (Table 4).

Immunoreactivity to inhibin α subunit and P450arom was strongest during mid pregnancy and comparatively faint during the follicular, luteal and late pregnancy stages, whereas immunoreaction to inhibin β_A and β_B subunits was faint and moderate in cyclic and pregnant corpora lutea, respectively (Table 4).

While immunoexpression of 3 β HSD in large luteal cells was strongest in late pregnancy, while it was moderate at mid pregnancy and faint in cyclic corpora lutea (Table 4).

tory mechanisms should facilitate the development of new approaches for monitoring and manipulating ovarian function and improving fertility in domesticated livestock, endangered species and humans. The present study demonstrated the immunoreactivity of the granulosa cells of developing follicles and corpora lutea to inhibin α , β_A , and β_B subunits and P450arom and 3 β HSD proteins in cyclic and pregnant goats. Likewise, weak staining of all inhibin subunits and P450arom and faint staining of 3 β HSD in theca cells implies their minor role in the synthesis of inhibin and steroid hormones in caprine species.

Immunostaining of inhibin subunits was only observed in granulosa cell layers of all classes of developed follicles, consistent with the cytological localization of mRNA for inhibin α in the granulosa cells of ovarian follicles in the bovine ovary [27], suggesting that granulosa cells are the major source of dimeric inhibin in the goat ovarian follicle and that theca cells have a negligible role in inhibin production. This conjecture is supported by evidence that *in vitro* removal of granulosa cells from the follicular wall [4] or follicular ablation [26, 28] resulted in reduction in inhibin production and lowered the serum inhibin concentration.

In the current study, the immunoreactivity of ovarian follicles to inhibin subunits showed differences in the various stages of follicular development. Immunostaining intensity was weak to moderate in pre-antral stages and strongest at the antral stage, confirming that the mature large follicles are the major source of inhibin in goats and that the pre-antral follicles contribute minimally to inhibin production. Further confirmation of this is provided by the **presence of mRNA encoding inhibin α and β_A** in the granulosa cells of most pre-antral and all non-atretic antral follicles, and its absence in primordial follicles or primary follicles with less than two layers of granulosa cells [1]. Furthermore, the amounts of mRNAs for inhibin α and β_A subunits increased coincident with follicle size, whereas inhibin β_A mRNAs remained unchanged [10]. The weak immunoreactivity of theca cells to inhibin α subunit in antral follicles in the present study was in accordance with an earlier demonstration of inhibin α subunit mRNA in bovine theca cells, even though, inhibin β_A subunit mRNA was absent [27], and indicates that inhibin β_A and

Discussion

Advances in our understanding of intraovarian regula-

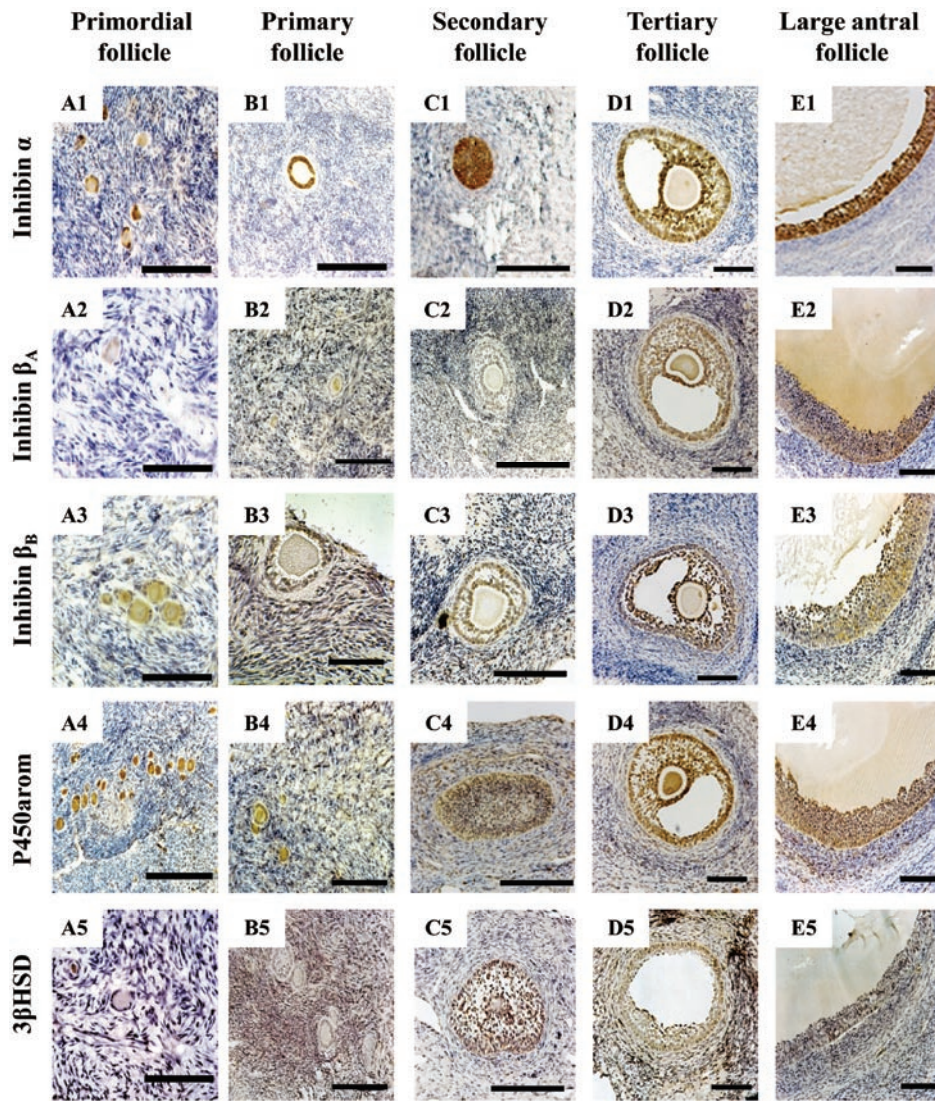


Fig. 1. Immunohistochemical localization of inhibin α , β_A , β_B subunits, and steroidogenic enzymes (P450arom and 3β HSD) in the primordial (A1–5), primary (B1–5), secondary (C1–5), tertiary (D1–5), and large antral (E1–5) follicles of goat ovaries. Note the increase in the intensity of staining to inhibin α and β_A subunits (large antral >tertiary >secondary follicles). Inhibin β_B and P450arom were strongly stained in granulosa cells of tertiary and large antral follicles compared to secondary follicles. 3β HSD immunoreactivity was uniform in all developing follicles. All photomicrographs were taken at the same magnification ($\times 20$). For demonstration of primordial and primary follicles, focused areas of the stained ovarian section(s) were selected. The scale bar represents 100 μ m.

Table 2. Immunohistochemical reactivity of inhibin α , β_A , and β_B subunits and steroidogenic enzymes in granulosa cells of goat ovarian follicles at different stages of development

	inhibin α	inhibin β_A	inhibin β_B	P450arom	3β HSD
Primordial follicle	w	w	w	++	+
Primary follicle	+	w	w	++	+
Secondary follicle	+	+	+	++	++
Tertiary follicle	++	++	++	+++	++
Large antral follicle	+++	+++	++	+++	++

w, +, ++, and +++: weak, low, moderate, and strong immunoreactivity of the ovarian tissue, respectively as judged by measuring the immunostained tissue integrated intensity using a public domain image processing and analysis program, ImageJ (<http://rsbweb.nih.gov/ij/>).

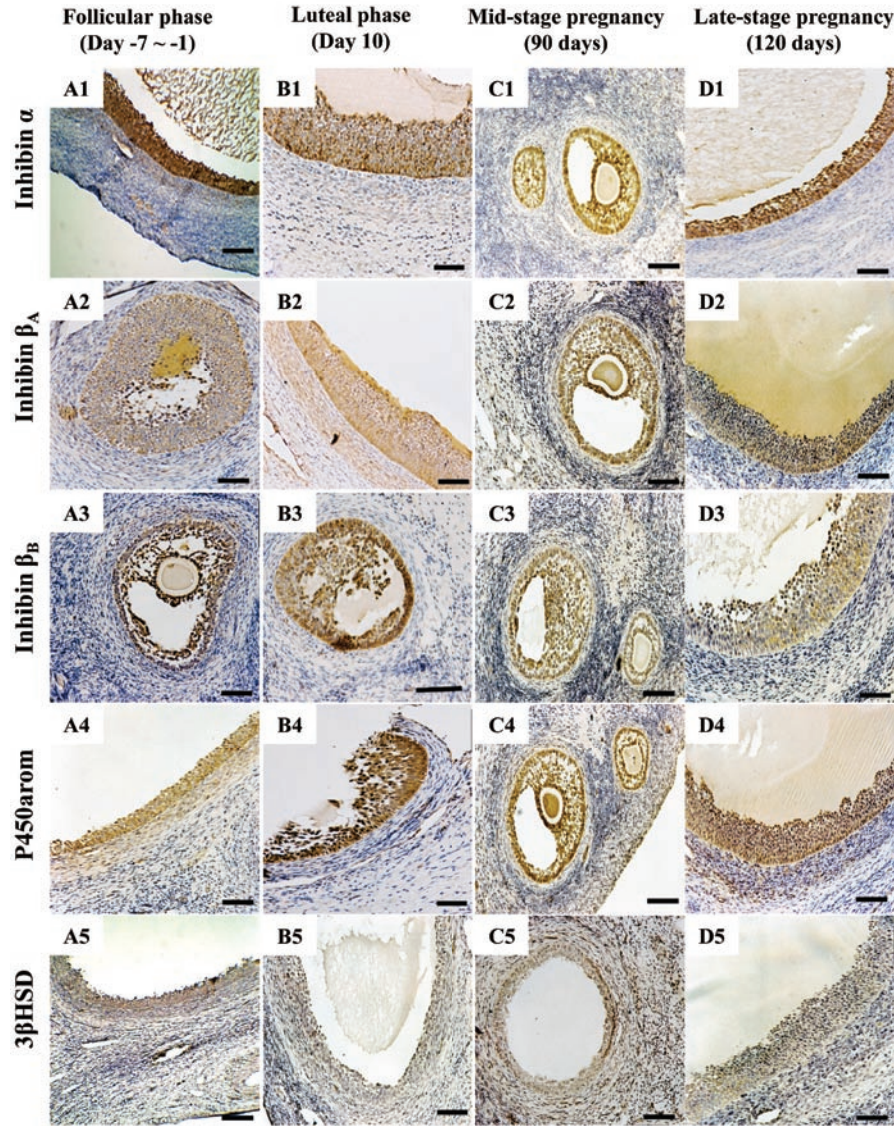


Fig. 2. Immunohistochemical localization of inhibin α , β_A , β_B subunits, and steroidogenic enzymes (P450arom and 3 β HSD) in the wall of antral follicles during the follicular phase (A1–5), luteal phase (B1–5), mid-stage (C1–5) and late-stage (D1–5) pregnancy in goats. The follicular wall showed strong immunostaining of inhibin α during the follicular phase and late-stage pregnancy. Moderate staining of inhibin β_A and β_B was observed in granulosa cells during follicular and luteal phases, respectively. The immunoreactivity of granulosa cells to P450arom was uniform across all stages. 3 β HSD immunoreactivity was highest in late pregnancy antral follicles. All photomicrographs were taken at the same magnification ($\times 20$) and the scale bar represents 100 μ m.

Table 3. Immunohistochemical reactivity of inhibin α , β_A , and β_B subunits and steroidogenic enzymes in granulosa cells of large antral follicles of cyclic and pregnant goat ovaries

	inhibin α	inhibin β_A	inhibin β_B	P450arom	3 β HSD
Follicular phase	+++	++	+	++	+
Luteal phase	+	+	++	++	++
Mid-stage pregnancy (90 days)	++	++	++	++	+
Late-stage pregnancy (120 days)	+++	+	+	++	+

+, ++, and +++: low, moderate, and strong immunoreactivity of the ovarian tissue, respectively as judged by measuring the immunostained tissue integrated intensity using a public domain image processing and analysis program, ImageJ (<http://rsbweb.nih.gov/ij/>).

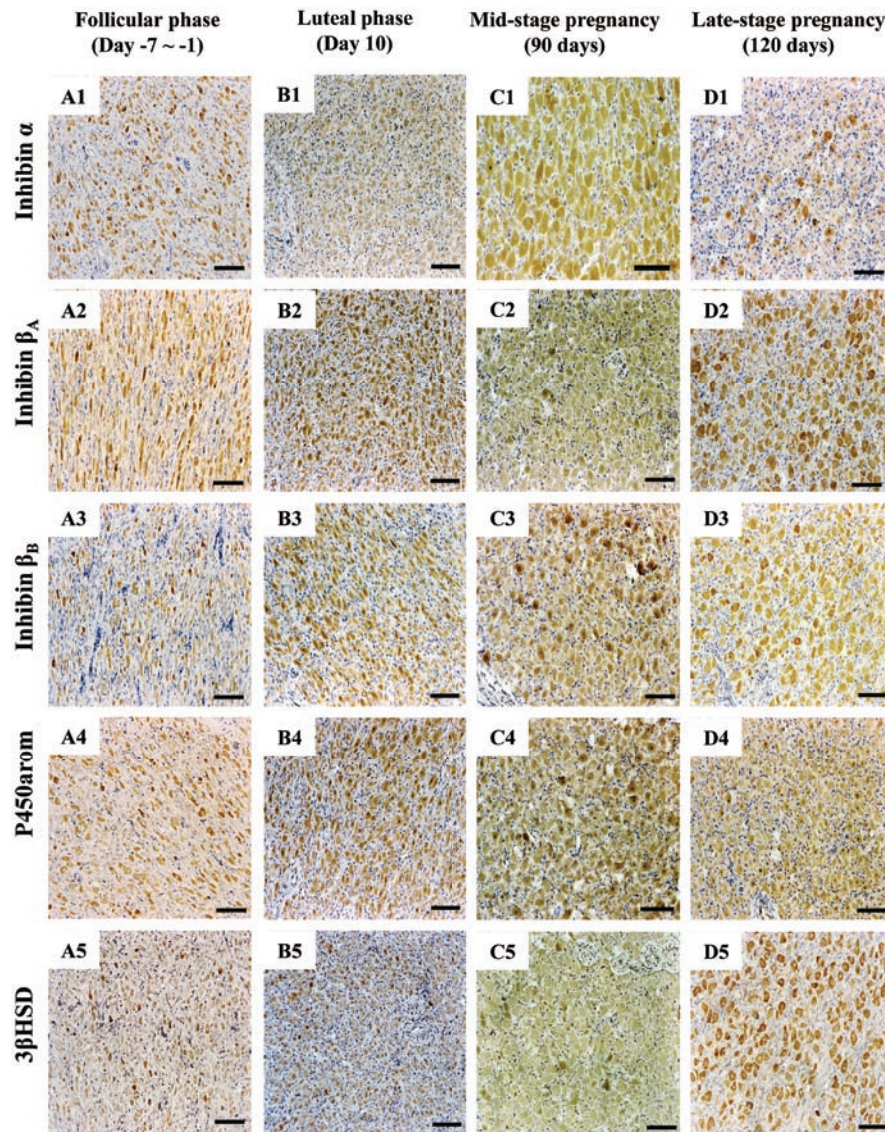


Fig. 3. Immunohistochemical localization of inhibin α , β_A , β_B subunits, and steroidogenic enzymes (P450arom and 3β HSD) in the luteal cells during the follicular (A1–5) and luteal (B1–5) phases, and mid-stage (C1–5) and late-stage (D1–5) pregnancy in goat corpora lutea. Note the presence of immunoreactive cells to inhibin subunits, P450arom and 3β HSD in goat corpora lutea. Inhibin α and P450arom immunostaining was strong in mid-stage pregnancy luteal cells. Immunoreaction to inhibin β subunits was faint to moderate. 3β HSD was strongly stained in late-stage pregnancy luteal cells. All photomicrographs were taken at the same magnification ($\times 20$) and the scale bar represents $100\ \mu\text{m}$.

Table 4. Immunohistochemical reactivity of inhibin α , β_A , and β_B subunits and steroidogenic enzymes in corpus luteum cells of cyclic and pregnant goat ovaries

	inhibin α	inhibin β_A	inhibin β_B	P450arom	3β HSD
Follicular phase	+	+	+	+	+
Luteal phase	+	+	+	+	+
Mid-stage pregnancy (90 days)	+++	++	++	+++	++
Late-stage pregnancy (120 days)	+	++	++	+	+++

+, ++, and +++: low, moderate, and strong immunoreactivity of the ovarian tissue, respectively as judged by measuring the immunostained tissue integrated intensity using a public domain image processing and analysis program, ImageJ (<http://rsbweb.nih.gov/ij/>).

β_B proteins may have spread from the nearby granulosa cells.

Follicular development and differentiation are closely associated with increasing steroidogenesis. P450arom is a crucial regulatory enzyme that catalyzes the conversion of testosterone to estradiol and 3β HSD plays a pivotal role in the synthesis of progesterone; it converts androgen precursor, pregnenolone, to progesterone via the $\Delta 4$ steroidogenic pathway. The present study showed that all ovarian follicles showed immunoreactivity to P450arom and 3β HSD enzymes, suggesting that granulosa cells are able to synthesize not only estradiol but also progesterone. Biochemical studies in several species have identified granulosa cells as the major site of follicular estrogen biosynthesis [9]. The increase of immunostaining intensity in the tertiary and antral stages to P450arom over those of the earlier development stages in the present study indicates that estrogen production maximizes with follicle maturation. Concurrently, the granulosa cells of large pre-ovulatory follicles have the highest levels of aromatase (estrogen synthetase) activity [9]. Similarly, P450arom mRNAs were found only in granulosa cells of early antral follicles granulosa cells and increased with follicle size [32]. On the other hand, 3β HSD expression increased in secondary follicles but then remained constant even in large antral follicles, demonstrating the significance of progesterone for follicle growth and oocyte competence as it inhibits coordinated oocyte apoptosis [16].

The circulatory inhibin levels vary according to the stage of the reproductive cycle or presence of pregnancy. Increased output of inhibin by the selected dominant follicle(s), in addition to its endocrine role to suppress FSH secretion, may upregulate LH-induced androgen secretion that is required to sustain a high level of estradiol secretion during the pre-ovulatory phase [17].

The present immunohistochemical study showed that the granulosa cells of large antral follicles were strongly immunostained for inhibin α subunit during the follicular phase and late pregnancy, while, there were moderate affinities to inhibin β_A and β_B subunits during the follicular and the luteal phases, respectively. Furthermore, mid pregnancy ovarian follicles moderately expressed both inhibin β_A and β_B subunits. These results suggest that inhibin A and B are the main forms of in-

hibin expressed during the follicular and luteal phases, respectively, while in mid pregnancy, follicles secreted both forms of inhibin, with free inhibin α subunit secreted during the follicular phase and late pregnancy. The highest levels of inhibin A were measured during the growth phase of the estrous cycle dominant follicles [14]. In golden hamsters, the ovarian follicles secrete large amounts of inhibin A and inhibin B during the second half of pregnancy [18], and the late stage of pregnancy in goats is associated with an increase in plasma ir-inhibin levels [13]. The present results show that both P450arom and 3β HSD expression in follicles is nearly stable in cyclic and pregnant goats ovarian follicles. Despite this, 3β HSD showed increased immunoreactivity during the luteal phase. Moreover, follicular steroid production changes from predominantly estradiol and androgen before the LH surge to an increase in progesterone production after the LH surge [7].

The formation of corpus luteum involves a series of biochemical and morphological changes in the pre-ovulatory follicle following the LH surge, including the luteinization of theca and granulosa cells into small and large luteal cells, respectively [21]. Although immunohistochemical staining is not quantitative, when luteal tissues from various stages of the estrous cycle and pregnancy were analyzed, changes in the inhibin intensity in corpora lutea were observed, indicating dynamic changes in inhibin accumulation in the corpus luteum of cyclic and pregnant animals.

Our present results show strong immunoreactivity to inhibin α subunit in mid pregnancy corpus luteum with immunoreactivity being at its lowest in cyclic animals and late pregnancy, while affinity for inhibin β_A and β_B subunits was stronger in pregnant animals than in that cyclic corpora lutea, indicating that corpora lutea have distinct secretion patterns of inhibin isoforms during the estrous cycle and pregnancy in goats. Earlier studies [18, 23] demonstrated all inhibin subunits and inhibin/activin A mRNA in goat luteal tissue. Furthermore, inhibin immunization during goat pregnancy is associated with an increase in plasma FSH levels [12], confirming the endocrine regulatory role of inhibin on pituitary secretion of FSH in pregnant animals.

Previous studies have shown that P450arom immunoreactivity in luteal tissue is close to that of inhibin α

subunit and is characterized by its greater abundance in mid pregnancy than in cyclic corpora lutea samples, indicating that the goat luteal estrogen activity might parallel that of inhibin α secretion. Earlier studies showed that all luteal cells express P450arom without difference in affinity between the luteal phase and pregnancy, however, weak positive staining was observed in late pregnancy [31] and this was associated with an increase in plasma estradiol levels during mid pregnancy in goats [13]. On the other hand, 3 β HSD showed a unique expression in luteal cells characterized by a gradual increase from the luteal phase to late pregnancy, with the maximum intensity being observed during late pregnancy. This indicates an increase in luteal steroidogenic function, particularly that of progesterone, during late pregnancy in goats, which is important for relaxing of the uterine wall and the maintenance of pregnancy. This was confirmed by the reduction of progesterone after treatment of goats in late pregnancy with 3 β HSD inhibitor and the initiation of parturition [25].

From the results of the present study, we can conclude that the granulosa cells of the tertiary and large antral follicles are the main source of dimeric inhibin with little/or no contribution from theca cells, and that corpora lutea may also secrete inhibin in goats. The ability of goat luteal cells to synthesize estrogen as well as progesterone during the luteal phase suggests the role of estrogen in the maintenance of corpus luteum function. Inhibin secreted from the corpora lutea during the gestation period might play a role in modulation of the ovarian function and folliculogenesis during pregnancy in goats.

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