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—Original Article—

Profiles of Circulating Steroid Hormones, Gonadotropins, Immunoreactive Inhibin and Prolactin During Pregnancy in Goats and Immunolocalization of Inhibin Subunits, Steroidogenic Enzymes and Prolactin in the Corpus Luteum and Placenta

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Abstract. The current study was performed to follow up the circulating hormonal changes and to correlate the findings with the physiological activity of the corpus luteum (CL) and placenta during pregnancy in goats. Blood samples were collected weekly from five goats during pregnancy for measuring steroid and protein hormones. A gradual increase was observed in immunoreactive (ir-) inhibin, with maximal levels at the 17th week. The plasma concentrations of estradiol and prolactin (PRL) showed nearly similar patterns during pregnancy, where they declined to basal levels during the first 4 weeks post-breeding and then increased significantly, with the maximal concentration during late pregnancy. The plasma FSH and LH concentrations were maintained at basal levels throughout the gestation period. The plasma progesterone concentration abruptly increased in the first week post-breeding and remained at high values throughout the pregnancy period. Immunohistochemical localization of inhibin alpha, $beta_A$, $beta_B$ and steroidogenic enzymes cytochrome P450 aromatase, 3alpha-hydroxysteroid dehydrogenase (3betaHSD), cytochrome 17alpha-hydroxylase P450 and cholesterol side-chain cleavage cytochrome P450 in the cyclic and pregnant goat CL revealed positive immunoreactivity without affinity differences between the luteal and pregnancy stages. The placental syncytiotrophoblasts also showed positive staining, except for inhibin $beta_A$ and 3betaHSD. The giant binucleate cells of the placenta showed positive immunoreactions to PRL. These results suggest that the high concentrations of ir-inhibin, estradiol and PRL during late pregnancy are of placental origin and that the placenta may have a vital role in the maintenance of pregnancy, regulation of mammary growth and preparation for kidding and lactation in goats.

Key words: Goat, Hormone, Inhibin, Placenta, Pregnancy

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Studying hormonal changes during the gestation period knocks down doors that block understanding of the regulatory mechanisms accompanying fetal development and maintenance of pregnancy; the most important stage of animal reproductive life. In goats, pregnancy is dependant on the corpus luteum (CL) with minimal contribution from placenta. However, day by day, scientists are working hard to discover the additional roles played by the placenta. The pattern of progesterone levels during pregnancy is changeable; it is low from mating until the 6th day [1], increases noticeably by the 3rd week and remains high until the 19th week [2–7]. Maintenance of progesterone secretion by the CL during pregnancy in the goat is luteinizing hormone (LH)-dependant; moreover, prolactin (PRL) is synergistic with LH in stimulating this function [8]. The highest concentration of estradiol in the blood is found 2 days before ovulation [1, 9]; the concentration decreases during the first 30 days after mating and then increases again between weeks 7 and 11, with the maximal output near parturition [7, 10].

Up till now, there have been no reports characterizing the changes in gonadotropic hormones and inhibin during pregnancy in the goat.

The aim of the present study was to investigate the changes of progesterone, estradiol, FSH, LH, inhibin and PRL during pregnancy in goats and to discover the relationship between these changes and the functional activity of the CL and placenta through the immunohistochemical localization of inhibin subunits, steroidogenic enzymes and PRL.

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Materials and Methods

Animals

Five cyclic Shiba goats (*Capra Hircus*) aged 2–5 years old and with average body weights of 27.67 ± 2.32 kg were monitored in

this study. An additional five goats were sacrificed on Day 10 (n= 2) and during pregnancy (2, 3, 4 months) for collection ovaries and placentae. The goats were kept in a sheltered paddock under natural daylight and temperature conditions and fed hay cubes (800 g/ head/day). Clean water and mineralized salt licks were available *ad libitum*. On Days 19–21 after mating, the animals were examined transrectally with an ultrasound for the signs of early pregnancy, such as presence of an anechoic fluid-filled graved uterine horn(s) and existence of clear hypoechoic corpora lutea in the ipsilateral ovary. Furthermore, fetal heart beats were detectable in all animals from Day 21 to 23 of pregnancy.

Experimental design

Estrus was synchronized by two doses of prostaglandin $F_{2\alpha}$ (Estrumate®, 125 μg, Schering Plough Animal Health, Kenilworth, NJ, USA) administered 11 days apart, with natural breeding following heat observation. The day of standing heat was designated as Day H. Blood samples were collected weekly from Day H until the 18th week of gestation through jugular vein puncture into heparinized vacutainer tubes (Venoject II, Terumo, Tokyo, Japan). The samples were centrifuged at 1700 × *g* for 30 min at 4 C for plasma separation, and the separated plasma samples were stored at –20 C until assayed for hormones.

Hormonal analysis

Plasma concentrations of progesterone and estradiol were determined by a double-antibody radioimmunoassay (RIA) system using ^{125}I-labeled hormones, as described by Taya *et al.* [11]. Antibodies against progesterone (GDN337) and estradiol-17β (GDN244) were kindly provided by Dr GD Niswender (Animal Production and Biotechnology, Colorado State University, Fort Collins, CO, USA). Plasma FSH concentrations were measured by RIA as described by Araki *et al.* [12] using anti-ovine FSH-1 (AFP-C5288113), NIDDK-FSH I-1 for iodination and NIDDK oFSH-RP1 as a reference standard. Plasma LH concentrations were measured by RIA as described by Mori and Kano [13] using anti-ovine LH (YM-18), NIDDK-LH I-3 for iodination and NIDDK-oLH-24 as a reference standard. The plasma concentrations of immunoreactive (ir-) inhibin were measured using antibody against bovine inhibin (TNDH-1) and ^{125}I-labeled 32-kDa bovine inhibin, as described by Hamada *et al.* [14]. For the PRL assay, anti-ovine PRL, NIDDK-anti-oPRL (AFP-C3581069II), and ovine purified PRL, NIDDK-oPRL-I-2 (AFP-7150B), for iodination and reference were used.

Immunohistochemistry

Tissue sampling, preparation and fixation: Goats were sacrificed by an overdose of ketamine, and ovaries and placentae were excised immediately. Ovaries were collected during the mid luteal stage (Day 10; n=2) and pregnancy (2, 3 and 4 months). Placentae were also collected at 3 months (n=1) and 4 months (n=1) of pregnancy. The obtained samples were immediately fixed for 24 h in 4% paraformaldehyde (Sigma Chemical, St. Louis, MO, USA) in 0.05 M PBS, pH 7.4, dehydrated in a graded series of ethanol and embedded in paraffin. The paraffin-embedded tissues were serially sectioned at 6 μm thickness and mounted on APS (3-aminopropyltriethoxysilane) coated glass slides (Matsunami Glass, Japan) and dried overnight at 37 C. All procedures were carried out in accordance with guidelines established by the Tokyo University of Agriculture and Technology.

Immunohistochemical technique: Immunohistochemical staining for the inhibin α, β_A and β_B subunits was carried out as described previously [15, 16]. Briefly, the sections were incubated with 10% normal goat serum to reduce non-specific binding and then incubated overnight with primary antibodies (× 1000–2000) diluted in 0.01 M PBS (pH 7.2–7.4) at 4 C. The primary antibodies used were as follows: 1) rabbit polyclonal antibody against [Tyr30] porcine inhibin α chain (1-30)-NH2, 2) rabbit anti-cyclic inhibin β_A(81-113)-NH2 (#334-274-ET), 3) rabbit anti-cyclic inhibin β_B (80-112)-NH2 (code#305-25-D), 4) anti-bovine adrenal cholesterol side-chain cleavage cytochrome P450 (P450scc), 5) anti human placental 3β-hydroxysteroid dehydrogenase (3βHSD), 6) anti-porcine testicular cytochrome 17α-hydroxylase P450 (P450c17), 7) anti-human placental aromatase cytochrome P450 (P450arom) and 8) rabbit polyclonal anti-ovine prolactin (oPRL; NIDDK346 anti-oPRL-2). The sections were then incubated with 0.25% (v/v) biotinylated secondary antibody (biotinylated anti-rabbit IgG from Vector Laboratories, Burlingame, CA, USA) diluted in PBS containing 10% normal goat serum and then with 2% avidin-biotin complex (Vector Laboratories). The reactions were visualized with 3,3'-diaminobenzidine (DAB) 0.025% (Sigma Chemical) in 0.05 M PBS. The stained sections were counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan). Control sections were exposed sections to normal goat serum instead of primary antibodies.

Statistical analysis

Hormonal data were expressed as means (± SEM) and analyzed for differences using one-way ANOVA. Dunnett's test was used for detection of significant differences during pregnancy as compared with before breeding. The correlations between hormonal (FSH, inhibin, progesterone-LH, estradiol-PRL) levels and between each hormone and stage of gestation were calculated using Pearson correlation and were interpreted using two-tailed P values. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) program. A value of $P<0.05$ was considered significant.

Results

Characterization of the prolactin assay

Displacement of tracer with highly purified ovine PRL, male, lactating, cyclic non-lactating and mid stage pregnant goat plasma, goat amniotic fluid and placental homogenates produced good dose-response curves. These curves were parallel to the ovine PRL standard curve, indicating that it was possible to measure the concentration of PRL in goats using the present RIA (Fig. 1).

FSH and ir-inhibin

The patterns of FSH and ir-inhibin changes in the goats during pregnancy are shown in Fig. 2A and 2D, respectively. The mean plasma FSH concentration was high around the onset of heat (preo-

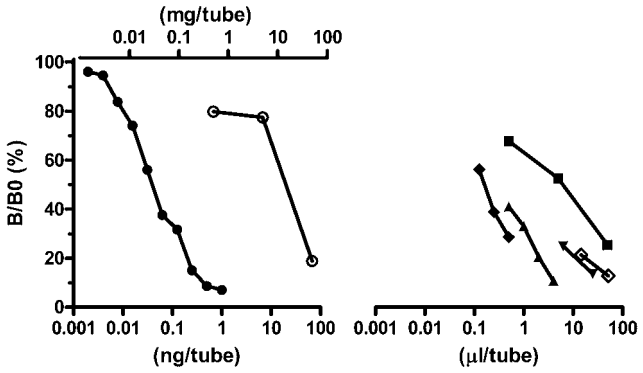

Fig. 1. Dose-response curve of ovine prolactin (●; ng/tube) and placenta homogenates (○; mg/tube) in the prolactin radioimmunoassay. Dose-response curves obtained by diluting plasma from lactating (◆), pregnant (▼), cyclic non-lactating female (◇) and male (▲) goats and amniotic fluid (■) are also shown. Each value represents the mean of triplicate values.

vulatory surge), decreased thereafter and remained low near the basal levels during the monitored period of gestation. There was a significant negative correlation between FSH and stage of pregnancy (r=–0.59, P<0.01). On the other hand, ir-inhibin showed a positive tendency to increase during pregnancy (r=0.42, P=0.06). The plasma ir-inhibin remained at the basal level during the first 12 weeks of gestation, then increased gradually from the 13th week and reached its highest levels in the 17th week of pregnancy.

Luteinizing hormone and progesterone

The patterns of LH and progesterone changes in the goats during pregnancy are shown in Fig. 2B and 2E, respectively. The preovulatory LH surge was consistent with the onset of heat, and the LH concentration subsequently decreased to its basal level. There was a significant negative correlation between LH and the stage of gestation (r=–0.45, P=0.048). The plasma progesterone concentration increased abruptly by the first week after breeding and remained at high levels throughout the monitored gestation period. There was a negative correlation between the plasma LH and progesterone concentrations throughout the studied period (r=–0.65, P=0.002). There was a non-significant negative correlation between progesterone and gestation stage (r=–0.33, P=0.18).

Prolactin and estradiol

Changes in the patterns of the plasma concentrations of PRL and estradiol are shown in Fig. 2C and 2F, respectively. There was a significant decrease in the plasma PRL concentration during the first 4 weeks of gestation and an abrupt increase from the 5th week till the end of the monitored period. The highest recorded values were from the 14th to 16th week. There was a significant positive correlation between PRL and gestation (r=0.65, P=0.0019). Similarly, the plasma concentrations of estradiol were high prior to mating, decreased to basal levels during the first 4 weeks of gestation, increased gradually from the 5th to the 8th week and after 10 weeks and finally reached the maximal values in the 17th week of gestation. There was a significant correlation between estradiol and stage of gestation (r=0.81, P<0.0001).

Immunolocalization of inhibin subunits and steroidogenic enzymes in goat CL and placental tissues

Immunoreactivity of inhibin α (Fig. 3–2, 3–6, 3–10 and 3–14), inhibin-β_A (Fig. 3–3, 3–7, 3–11 and 3–15) and inhibin-β_B (Fig. 3–4, 3–8, 3–12 and 3–16) was observed in all luteal cells of the cyclic and pregnant goats. Meanwhile, the placental trophoblastic epithelium and binucleated chorionic cells were positively stained against the inhibin α (Fig. 3–18 and 3–22) and inhibin-β_B (Fig. 3–20 and 3–24) subunits and devoid of positive staining for inhibin-β_A subunit (Fig. 3–19 and 3–23). Though the luteal cell showed a high intensity of staining for all inhibin subunits at 2 months of pregnancy, and for the inhibin α and inhibin-β_A subunits at 3 months of pregnancy, the immunoreactivity decreased for all subunits in the CL at 4 months. Nevertheless, the placental tissue showed increasing intensity of staining for the inhibin α and inhibin-β_B subunits from 3 to 4 months of pregnancy.

P450arom (Fig. 4–2, 4–7, 4–12 and 4–17), 3βHSD (Fig. 4–3, 4–8, 4–13 and 4–18), P450c17 (Fig. 4–4, 4–9, 4–14 and 4–19) and P450scc (Fig. 4–5, 4–10, 4–15 and 4–20) were detected in luteal cells in both during the luteal phase and pregnancy. Although 3βHSD was not detectable (Fig. 4–23 and 4–28), P450arom, P450c17 and P450scc immunoreactivity were observed in syncytiotrophoblasts of the goat placenta (Fig. 4–22, 4–24, 4–25, 4–27, 4–29 and 4–30). The immunoreactivity of luteal cells for P450scc and 3βHSD was highest in the cyclic CL and CL at 4 months of pregnancy, respectively. Although the P450arom intensity did not differ in the cyclic and pregnant luteal cells, placental immunoreactivity for P450arom, P450c17 and P450scc was higher in the 3rd month than in the 4th month pregnancy.

Immunolocalization of prolactin in the goat placenta

The goat placental tissue showed positive immunoreactivity for PRL, with the presence of variable size vesicles in the cytoplasm of binucleate cells (Fig. 5). The immnuoreactivity of the placental tissue was moderately higher at 3 months compared with 4 months of pregnancy.

Discussion

Understanding the hormonal changes during pregnancy furnishes a background for ovarian and placental function during this important reproductive stage. In the present study, we found that the plasma concentrations of ir-inhibin, estradiol and PRL increased from the mid- to late stages of gestation, while FSH and LH enormously decreased to basal levels. Furthermore, localization of the ir-inhibin subunits, steroidogenic enzymes and PRL in the goat CL and/or placenta implies their ability to secrete bioactive hormones and suggests their essential regulatory roles in maintaining pregnancy.

The ovarian follicular activity is FSH dependent; FSH increases prior to each wave emergence and decreases coincident with follicle selection [17–19]. The present study showed that the maximal FSH secretion was prior to estrus and that the secretion of FSH then

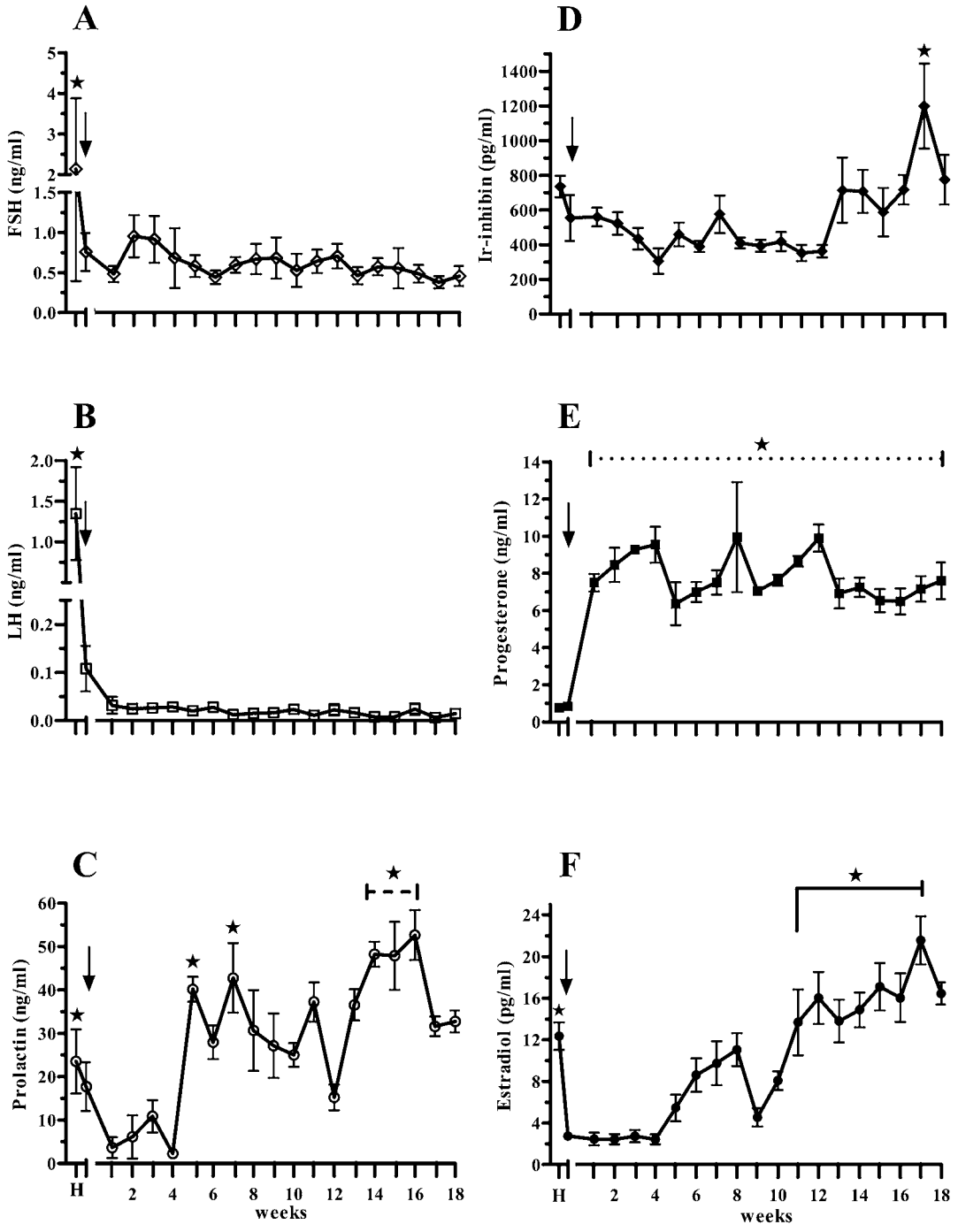

Fig. 2. Changes in the mean (± SEM) plasma concentrations of FSH (A; ◇), LH (B; □), prolactin (C; ○), ir-inhibin (D; ◆), progesterone (E; ■) and estradiol (F; ●) in goats (n=5) during pregnancy. Blood samples were collected weekly until week 18 of gestation. The day of estrus was designated as H. The arrows indicate the hormonal levels on the day of breeding. ★ $P<0.05$ compared with the values before breeding.

decreased and remained at basal levels throughout pregnancy. The circulating FSH levels in cows tend to be higher on the day of estrus (9-fold higher [20]) than in the follicular or luteal phases [21]. Moreover, in ewes, FSH is decreased twofold from days 60 to 90 and remains low for the remainder of gestation [22]. Moreover, the FSH levels are adversely correlated with the stage of preg-

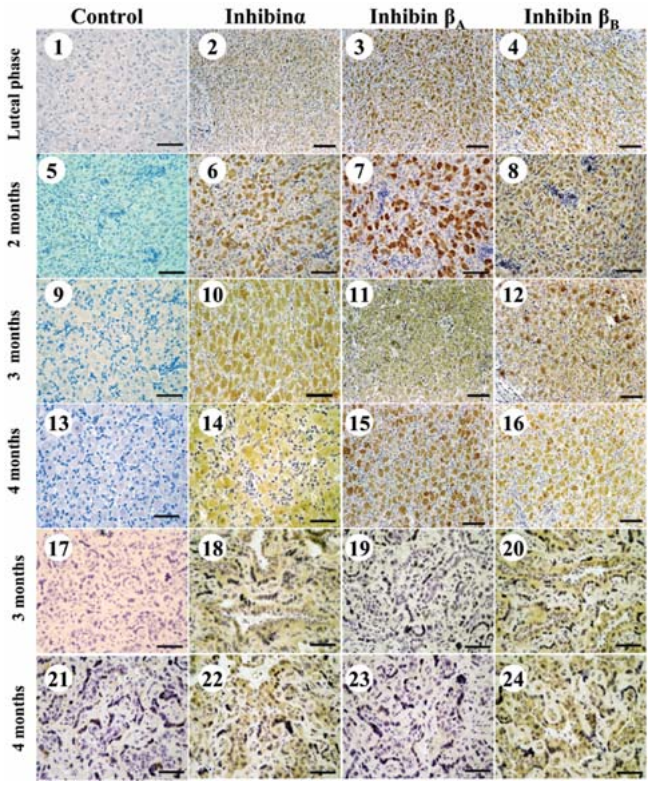

Fig. 3. Immunohistochemical localization of inhibin α, inhibin-β_A and inhibin-β_B subunits in the corpus luteum representing the luteal phase (2–4); two (6–8); three (10–12) and four (14–16) months of pregnancy; and placental tissue from three (18–20) and four (22–24) months of gestation in Shiba goats. As a negative control (1, 5, 9, 13, 17 and 21), normal goat serum was substituted for the first antibodies. Scale bar=100 μm.

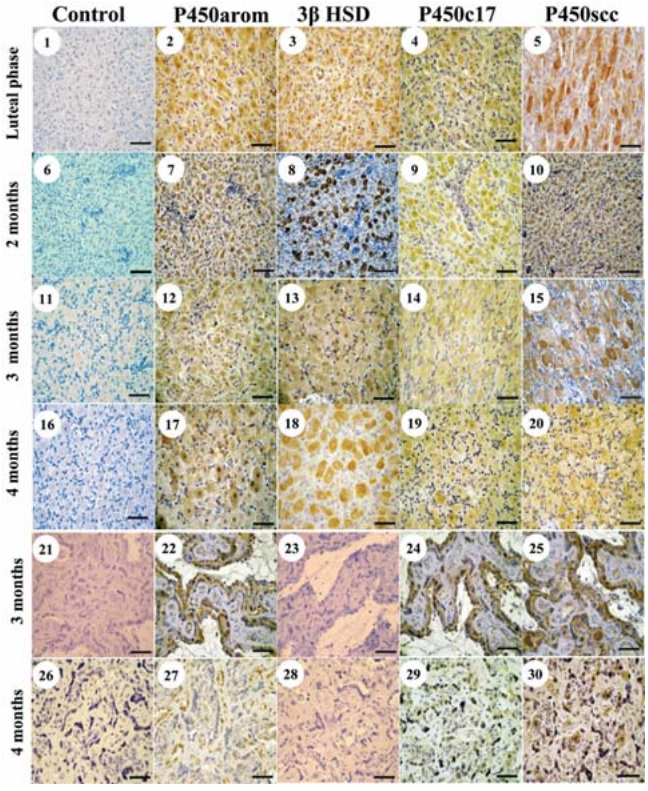

Fig. 4. Immunohistochemical localization of cytochrome P450 aromatase (P450arom), 3β hydroxysteroid dehydrogenase (3βHSD), cytochrome 17α-hydroxylase P450 (P450c17) and cholesterol side-chain cleavage cytochrome P450 (P450scc) in the corpus luteum obtained at the luteal phase (2–5) and two (7–10), three (12–15) and four (17–20) months of pregnancy in addition to placental tissue from three (22–25) and four (27–30) months of gestation in Shiba goats. As a negative control (1, 6, 11, 16, 21 and 26), normal goat serum was substituted for the first antibodies. Scale bar=100 μm.

nancy. This may explain the marked reduction in the number of medium size follicles (3–5 mm) starting at day 80 of pregnancy [23] and the absence of large size follicles at day 125 of gestation [24].

Monitoring of the ir-inhibin profile in the present study showed a substantial decline of ir-inhibin to basal levels during the first 12 weeks and then a gradual increase thereafter, with the maximal recorded values at the 17th week of gestation. Additionally, there was a positive correlation between the plasma ir-inhibin concentrations and pregnancy. Previous studies have shown that the ovary is the main source of circulating inhibin and that inhibin subunits are mainly expressed in the granulosa cells of pre-antral and antral follicles [25]; moreover, inhibin secretion increases during follicular maturation and decreases abruptly after ovulation [26]. Accordingly, the unchanged basal plasma ir-inhibin concentration until the 12th week of gestation mirrors to the suppressed follicular growth during this time and is in accordance with previous observations in the pregnant ewe [22, 27]. However, the unexpected increase of ir-inhibin from the 11th week may be of placental or fetal origin, not follicular origin. In the present study, we demonstrated immunoreactions for the inhibin subunits in luteal cells in addition to the

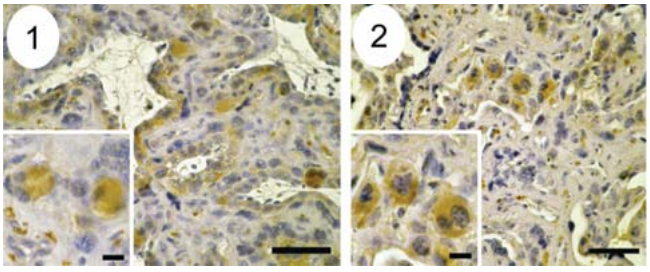

Fig. 5. Immunohistochemical localization of prolactin in the Shiba goat placenta obtained at three (1) and four (2) months of gestation. Scale bar=100 μm. The small, lower left image shows a higher magnification of positive giant binucleate cells. Scale bar=10 μm.

placenta at 3 and 4 months of pregnancy, which suggests that they have the capability to secrete inhibin and that they may be responsible for the late term increase of plasma ir-inhibin in goats. The earlier observation of high concentrations of inhibin in the amniotic fluid suggests that the fetal membranes and the placenta may be possible sources in ruminant species [28]. Additionally, during mid- and late gestation, high concentrations of inhibin are found in the gonads and plasma of male and female fetuses of horses [29, 30], sheep and cattle [28], suggesting a possible role of the fetus in such increase.

The data of the current study showed the maximal LH levels around the onset of heat (LH surge), and this was followed by sustained low basal levels throughout gestation. In cows [31, 32] and sheep [33], except for the highest levels on the day of estrus, LH does not vary significantly throughout pregnancy. Additionally, we recorded a concurrent increase in the plasma concentration of progesterone, and this is in agreement with previous reports in pregnant goats [1–7]. These findings confirm the selective suppressing effect of progesterone on the pituitary secretion of LH through a negative feedback mechanism. Supporting this concept, the total content of pituitary LH shows a significant drop by day 40–50 and reaches its lowest value by 120–135 days of pregnancy in the ewe [34].

Follow up of the changes in estradiol and PRL during pregnancy in the current study showed that after a temporary period of hormonal decline during the first 4 weeks, the levels of estradiol and PRL increased gradually with the progression of pregnancy and reached their highest values at the 16th and 17th week of gestation for PRL and estradiol, respectively. On the other hand, the decrease of goat placental weight [35] and prolactin-related protein gene expression from Day 90 of gestation [36] could result in an increase in type I pituitary PRL secreting cells during pregnancy. However, the low PRL content [37] in these pituitary cells might be responsible for the second PRL drop in the circulation at the 12th week of pregnancy in the present study because after day 120, the PRL concentration is significantly higher than before and continues to increase up to 800 ng/ml around parturition [38]. The positive immunoreactivity of the goat placenta for P450arom and PRL in the present study suggested that the high levels of estradiol and PRL during late pregnancy might be of placental origin. These findings may indicate a shift in the origin of PRL and estradiol secretion from pituitary or ovarian to placental sources. Estradiol and PRL act synergistically in the luteotrophic process through PRL-mediated estrogen receptor stimulation in the CL graviditatis [39]. In addition, PRL prevents progesterone catabolism by silencing the 20α-hydroxysteroid dehydrogenase (20α-HSD) enzyme throughout pregnancy [40]. Moreover, the presence of increased quantities of PRL in the blood is essential successful induction of mammary growth and lactation with estradiol and progesterone in virgin [41] and pregnant [42] goats.

In the current study, we verified positive immunoreactivity of goat luteal cells for the inhibin α, inhibin-β_A and inhibin-β_B subunits during the luteal and pregnancy stages, which suggest that the CL has the ability to secrete inhibin. Further studies are needed to investigate the nature and biological activity of the inhibin subunits secreted by luteal cells. A previous study showed that the goat CL expresses inhibin/activin A protein and mRNA [43]. Additionally, mRNA for inhibin α subunit has been found in the cow CL, and the mRNA concentration in ovarian tissue decreases slightly after $PGF_{2\alpha}$ injection [40]. Furthermore, there is a great deal of evidence suggesting that the sheep CL might be able to secrete inhibin [45–47]. In contrast, earlier studies revealed that cow and sheep CLs are devoid of inhibin α and β_A subunit proteins and mRNA [48–51] and fail to produce detectable amounts of inhibin in cell cultures *in vitro* [52, 53].

In the current study, positive immunolocalization of the inhibin α and β_B subunits in the goat placenta at 3 and 4 months of pregnancy was coincident with high circulating concentrations of ir-inhibin, suggesting that the placenta might be responsible for inhibin B secretion during pregnancy in goats.

Demonstration of P450arom, 3βHSD, P450c17 and P450scc proteins in the goat CL during the luteal and pregnancy stages indicated the ability of luteal cells to synthesize androgen and estradiol in addition to progesterone. However, the lack of expression of 3βHSD in the placenta is strong proof that the CL is the unique source of progesterone during gestation in goats. Our previous study reported this in goats [16]. Moreover, we found that P450scc was positively expressed in the goat placenta, with a decrease in intensity and distribution from the 3rd to 4th months. The present results do not agree with the lack expression of P450scc found in the placenta at 120 days of gestation [16] but do agree with earlier findings in cows that showed the presence of P450scc protein and mRNA in the placenta [54, 55]. In goats, the placenta cannot synthesize estradiol from progesterone due to the lack of 3βHSD. The placenta must rely on precursor androgens formed by the maternal and fetal adrenal glands [56]. Placental P450scc is essential for conversion of cholesterol to pregnenolone, which in the fetal adrenal is converted to dehydroepiandrosterone sulfate (DES) [56]. DES enters the placenta, where it can be converted to estrone and estradiol [56].

The present immunohistochemical results showed PRL expression in the giant binucleate cells of the goat placenta concomitant with an increased plasma concentration of PRL. This implies that the placenta has a crucial role in regulation of mammary gland growth during late pregnancy. Supporting our hypothesis about the placental origin of circulating PRL, we detected ir-PRL by RIA in goat term placenta homogenate and amniotic fluid. Moreover, previous studies have shown the presence of mRNAs of several PRL-related proteins in goat trophoblast binucleate cells [57].

In summary, the endocrine milieu is dramatically changed during pregnancy in goats. The substantial decrease of FSH and LH reflected the decrease in the ovarian follicular activity. Alternatively, the increases of the plasma levels of estradiol, ir-inhibin and PRL in association with the placental expression of steroidogenic enzymes, inhibin subunits and PRL proteins indicate an increase in the placental secretory functions during the late stage of pregnancy in goats.

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