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Intrafollicular Content of Luteinizing Hormone Receptor, α-Inhibin, and Aromatase in Relation to Follicular Growth, Estrous Cycle Stage, and Oocyte Competence for In Vitro Maturation in the Mare[1]

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ABSTRACT

The intrafollicular content of LH receptor, α-inhibin, and aromatase are known good indicators of follicular status. We investigated the amounts of these proteins in granulosa and cumulus cells in relation to oocyte competence for in vitro maturation, follicular growth, and estrous cycle stage in the mare.

Follicular punctures were performed 34 h after an injection of crude equine gonadotropins, either during the follicular phase, at the end of the follicular phase, or during the luteal phase. The cumulus-oocyte complex, granulosa cells, and follicular fluid of follicles larger than 5 mm were collected. The nuclear stage of the oocytes after in vitro culture was determined microscopically. Granulosa and cumulus cell amounts of LH receptor, α-inhibin, and aromatase were assessed by the semiquantitative Western blot method and image analysis. Follicular fluids were assayed for progesterone (P_4) and estradiol-17β (E_2).

The three factors were expressed in mural granulosa and cumulus cells from all follicles from the gonadotropin-independent growth period until the preovulatory stage. Considering all the follicles punctured, the amounts of LH receptor and α-inhibin in granulosa cells were not different for the three physiological stages studied. The amounts of aromatase in granulosa cells, as well as the E_2:P_4 ratios, were higher for follicles punctured during the follicular phase than for the two other groups ($p < 0.05$). Considering the data from the three groups, the E_2:P_4 ratio and the LH receptor and aromatase contents, but not α-inhibin, in granulosa cells increased with an increase in follicular diameter ($p < 0.01$). The E_2:P_4 ratios and the amounts of LH receptor, α-inhibin, and aromatase in granulosa cells were lower in follicles 5–9 mm in diameter than in larger ones ($p < 0.05$). In cumulus cells, the amounts of the three factors were different neither between the three groups nor between the follicular diameters.

Although we could not establish any obvious relationship to oocyte competence for in vitro maturation, the influence of the follicle diameter on the content of LH receptors, α-inhibin, and aromatase in granulosa cells was similar to the influence of follicle diameter on oocyte competence. Therefore, one can hypothesize that, in the mare, there is a link between the acquisition of oocyte competence and the expression of these factors in the follicular cells.

INTRODUCTION

Typically in the mare, one follicle is selected and becomes dominant 8–10 days before ovulation, while the cohort follicles become atretic. The mare ovary also displays follicular growth during the luteal phase, and large antral follicles can be observed. These generally regress but occasionally reach the preovulatory size and ovulate [1]. Moreover, whereas the in vitro maturation rate of oocytes exceeds 90% in most domestic mammals (goat [2], sow [3], cow [4]), it remains low in the mare. Actually, whatever the culture conditions used, less than 70% of equine oocytes are in metaphase II at the end of the culture period [5] (for review, see [6, 7]). Moreover, the conditions for in vivo oocyte maturation in the mare are somewhat different from those in other domestic mammals: the ovulatory LH surge is a progressive increase lasting many days, with a maximum concentration occurring one day after ovulation [8, 9]. Thus, follicle and oocyte maturation in the mare could involve some species-specific mechanisms.

The reasons for the failure of equine oocytes to resume meiosis during in vitro culture are unknown. However, we showed recently that the oocyte competence for in vitro maturation increases with an increase in the follicular diameter and is lower in follicles 5–9 mm, which are thought to be gonadotropin-independent [6, 10–12]. Moreover, we showed that oocyte competence is influenced by the mare's reproductive status [6]. This allows us to assume that some local factors, in relation to follicular growth and maturation, whose expression varies during the estrous cycle, may play a crucial role in the acquisition of oocyte competence for in vitro maturation. Among them, steroid hormones, LH receptors, α-inhibin, and aromatase are good candidates.

The purpose of the present study was to investigate in the mare LH receptor, α-inhibin, and aromatase contents in mural granulosa and cumulus cells, as well as steroid concentrations in follicular fluid, in relation to 1) follicular size and estrous cycle stage, and 2) oocyte competence for in vitro maturation.

MATERIALS AND METHODS

Experimental Design

Ten adult cyclic mares (Selle Français) were used from April to June. They were in good body condition, from 8 to 19 yr old, kept indoors, and fed with concentrates. They received an initial synchronization treatment [13] with an intravaginal sponge containing 0.5 g altrenogest (Regumate; Roussel UCLAF, Romainville, France) plus 50 mg of estradiol benzoate (β-estradiol 3-benzoate; Sigma, La Verpillière, France) for 1 wk. On the day of sponge removal, mares received a prostaglandin $F_{2\alpha}$ analogue injection (cloprostenol, 250 μg/mare i.m.; Estrumate; Pitman-Moore, Meaux, France) to induce luteolysis, and all follicles larger than 5 mm were punctured to free the ovaries of atretic follicles. Healthy follicles developed afterwards. Ovarian activity was then assessed by routine rectal ultrasound scanning using an Aloka 210 (Tokyo, Japan) with a 5-mHz linear probe.

Follicular Punctures

To collect oocytes and follicular cells at various stages of the estrous cycle, 7 successive ultrasound-guided follic-

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ular punctures were performed for each of the 10 mares, either during the follicular phase (group DF; n = 3; see below), at the end of the follicular phase (group EF; n = 2; see below) or during the luteal phase (group DL; n = 2; see below). Follicles of five mares were punctured in the order EF, DL, EF, DL, DF, DF, DF; and those of five others in the order DF, DF, DF, EF, DL, EF, DL.

For punctures assigned to be performed at the end of the follicular phase (group EF), an i.v. injection of 25 mg of crude equine gonadotropin (CEG; [14]) was given when the largest follicle reached 35 mm, to induce ovulation; all follicles larger than 5 mm were punctured 34 h later, i.e., just before the expected ovulation. For punctures assigned to be done during the luteal phase (group DL), a CEG injection was performed when the largest follicle reached 16 mm, in order to increase the percentage of oocytes that completed meiosis after in vitro culture [6]; all follicles larger than 5 mm were punctured 34 h afterwards. On the day of the puncture in the luteal phase, luteolysis was induced by a prostaglandin $F_{2\alpha}$ analogue injection. During the follicular phase (group DF), the punctures were performed before the emergence of the dominant follicle: a CEG injection was given when the largest follicle reached 16 mm, and all follicles larger than 5 mm were punctured 34 h afterwards.

During each follicular puncture attempt, mares were sedated with detomidine (0.6 mg/100 kg BW i.v. Domosedan; Smith Kline & French, Courbevoie, France), and the rectum was relaxed with prifinium bromide (45 mg/100 kg BW i.v. Prifinial; Vétoquinol, Lure, France). After puncture, the mares received an antibiotic injection (Mixtencilline: 1 600 000 IU penicillin/100 kg BW and 1.3 g dihydrostreptomycin/100 kg BW i.m.; Rhône Mérieux, Lyon, France).

Collection of Cumulus-Oocyte Complexes (COCs), Follicular Fluids, and Granulosa Cells

The follicles were punctured using a transvaginal ultrasound-guided follicular aspiration technique [15] with a 7.5-mHz sectorial probe (Kretz; Soframed, Truchtersheim, France). To improve the recovery rate of oocytes, two types of aspiration needles were used: a one-way needle (length, 600 mm; outer diameter, 1.8 mm; Thiebaud Frères, Jouvernex Margencel, France) was used for puncturing follicles $>$ 25 mm, and a two-way needle (length, 700 mm; outer diameter, 2.3 mm; internal diameter, 1.35 mm; CASMED, Cheam Surrey, England) was used for follicles $\leq$ 25 mm. After follicular fluid aspiration, the follicle was scraped with the needle and flushed five times with PBS (Dulbecco A, Unipath, Dardilly, France) containing heparin (50 IU/ml; LEO S.A., St-Quentin Yvelines, France) at 37°C. The one-way needle allows isolation of pure follicular fluid, while the two-way needle induces the recovery of follicular fluid diluted with PBS. All aspirated fluids were individually examined under a stereomicroscope for oocyte recovery and then centrifuged for 10 min at 1500 $\times$ *g* to obtain a pellet of granulosa cells. Pure and diluted follicular fluids were individually stored at −20°C.

Oocyte Culture and Examination, and Cumulus Cell Preparation

At recovery, oocytes were individually classified according to cumulus aspect, as expanded COCs or compact COCs [6]. Compact COCs were cultured individually in a humidified atmosphere (95% air:5% CO_2) at 38.5°C for 30 h in 500 μl of maturation medium, as previously described [6]. After culture, the COCs were stripped of their cumulus cells with small glass pipettes in 500 μl of PBS solution supplemented with 87.5 IU/ml hyaluronidase (type III, 875 IU/mg; Sigma) at 37°C. Totally denuded oocytes were rinsed in PBS with 1% inactivated fetal calf serum (Gibco, Eragny, France) at 37°C, stained with 1 μg/ml *bis*-benzamide (Hoechst 33342; Sigma) in PBS for 5 min at 37°C for DNA detection, and observed in a drop on a slide under a fluorescence microscope. They were classified according to chromatin configuration as germinal vesicle, dense chromatin, metaphase I, metaphase II, and degenerated [6]. Expanded COCs at recovery were immediately stripped of their cumulus cells, stained, and classified as described above.

The cumulus cell suspension was centrifuged (13 000 $\times$ *g*, 1 min) to obtain a pellet of cells. Pellets were suspended in 10 μl of a hypotonic buffer (KCl 10 mM, Tris 10 mM, EDTA 0.5 mM) containing 348 μg/ml PMSF (Sigma), 33.2 μg/ml *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma), and 34.1 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma). They were stored at −20°C until analysis.

Granulosa Cell Preparation

Follicular cell pellets were suspended in 2 ml of an NH_4Cl solution (8.3 g/L in Tris-HCl 10 mM, pH 7.5) and incubated for 5 min before the addition of an equal volume of PBS. After centrifugation (1500 $\times$ *g*, 10 min), granulosa cells were rinsed twice with PBS and were suspended in one volume of the hypotonic buffer containing PMSF, TLCK, and TPCK, as described above. They were stored at −20°C.

Gel Electrophoresis and Immunoblotting

One-dimensional (1D) SDS-PAGE was prepared according to the discontinuous buffer system of Laemmli [16] with a 12% separating gel and a 4% stacking gel. Acrylamide-*bis*acrylamide solution was purchased from Serva GmbH & Co. (Heidelberg, Germany), and other reagents were purchased from Sigma.

Pellets of cumulus cells and granulosa cells were sonicated for 2–15 min, diluted with an equal volume of electrophoresis buffer (Tris-HCl 160 mM pH 6.8, EDTA 10 mM, SDS 10%, β-mercaptoethanol 10%, glycerol 20%, and bromophenol blue), and boiled for 4 min before being loaded. In order to load enough proteins, 2–3 cumulus pellets were pooled in accordance with the nuclear stage of the oocyte. Electrophoresis was performed at a constant intensity of 25 mA per gel. At the end of migration, gels were electroblotted on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) overnight at 4°C.

Each membrane was used for 5 successive immunological detections (see below): LH receptor, actin, α-inhibin, aromatase, and plasma membrane proteins of erythrocytes. After each immunological detection, the membranes were stripped of bound antibodies by incubation in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) at 50°C for 30 min, and two rinses in TBS (10 mM Tris, 150 mM NaCl) containing 0.1% Tween 20, as recommended by the manufacturer (Amersham Life Science, Buckinghamshire, UK).

For LH receptor immunological detection, membranes were washed with TBS-Tween 20 and incubated for 1 h in blocking solution (5% dry milk, 0.2% Nonidet P40 in TBS) supplemented with 10% goat serum. The membranes were

then incubated for 3 h with a mouse monoclonal antibody raised against porcine LH receptors (775; provided by Dr. Salesse, INRA, Jouy-en-Josas, France; [17]). After two rinses in TBS-Tween 20 and a 30-min incubation in the blocking solution supplemented with 10% goat serum, membranes were incubated for 1 h with a peroxidase-conjugated rabbit anti-mouse IgG (Vector Laboratories, Burlingame, CA). Immunological detections of actin, α-inhibin, aromatase, and plasma membrane proteins of erythrocytes were performed using the protocol described above, except that the blocking solution was not supplemented with goat serum. The primary antibodies used were, respectively, a mouse monoclonal antibody raised against chicken actin (Amersham Life Science), a sheep antiserum raised against the 1–26 fragment of porcine α-inhibin (provided by Dr. Terqui, INRA, Nouzilly, France), a rabbit antiserum raised against equine aromatase (provided by Professor Séralini, IBBA, Caen, France [18]), and a rabbit anti-human erythrocyte membrane antibody (Dako, Glostrup, Denmark). The peroxidase-conjugated secondary antibodies used were either a rabbit anti-mouse IgG (Vector Laboratories), a donkey anti-sheep IgG (Sigma), or a goat anti-rabbit IgG (Institut Pasteur, Paris, France).

The enhanced chemiluminescence detection system (ECL; Amersham Life Science) was used to detect immunoreactive polypeptides. The nitrocellulose membranes were exposed to Hyperfilm MP (Amersham Corp., Arlington Heights, IL) that was afterwards digitalized with an Eikonix 1412 scanner camera (Eastman Kodak, Rochester, NY). Patterns were quantified using the Kepler software (Large Scale Biology Corporation, Rockville, MD). The amounts of LH receptors, α-inhibin, and aromatase in cumulus and granulosa cells were analyzed as the ratio to the actin amount, after subtraction of the amount of actin due to the erythrocytes within each lane. The latter had been previously determined in a pre-experiment using blood cell pellets.

RIAs of Steroids in Follicular Fluid

Steroid concentrations in follicular fluid were determined by RIA without extraction, according to the methods of Saumande et al. [19] for progesterone (P_4) and Terqui et al. [20] for estradiol-17β (E_2), as previously described for the horse [21]. Intraassay coefficients of variation were 10.8% and 15.6%, respectively. For each steroid, all samples were analyzed in the same assay to avoid interassay variability. The results were expressed as E_2:P_4 ratio, considering that the dilution factor of the follicular fluid in PBS was unknown.

Statistical Analysis

The chi-square test was used to compare oocyte maturation rate in the three groups (DF, EF, and DL). Analysis of oocyte maturation after in vitro culture according to follicle diameter was performed by logistical regression analysis (Statistical Analysis System Software, Cary, NC). A nonparametric test (G-test = 2I-test) was used for comparison of nuclear maturation between the different classes of follicle diameter. Analysis of LH receptors, α-inhibin, and aromatase amounts was performed by unbalanced variance analysis (SAS Software; GLM procedure).

RESULTS

At the end of the follicular phase (group EF), follicles considered as preovulatory (larger than 35 mm in diameter), and nonpreovulatory subordinate follicles were punctured. During the follicular phase (group DF) and during the luteal phase (group DL), all follicles were nonpreovulatory.

Oocyte Recovery and Nuclear Stage

From the 20 puncture attempts performed at the end of the follicular phase, 27 preovulatory follicles were flushed, and 14 COCs were collected. All of them had an expanded cumulus; half of the enclosed oocytes were mature (7 of 14 in metaphase II).

From the 70 puncture attempts (20 EF, 20 DL, and 30 DF), 320 COCs were recovered from 647 nonpreovulatory follicles. The 299 oocytes with a compact cumulus were cultured in vitro and analyzed for nuclear stage after culture. The maturation rate increased with an increase in follicular diameter ($p < 0.05$) and was lower in follicles of 5–9 mm than in follicles of 10–34 mm ($p = 0.07$) (data not shown). Moreover, the maturation rate was higher for the oocytes recovered in group EF than for the oocytes recovered in group DL (51%, 52 of 101 vs. 34%, 30 of 88; $p < 0.05$); the maturation rate of oocytes collected in group DF was lower than rates of the other two (15%; 17 of 110; $p < 0.01$).

Intrafollicular E_2:P_4 Ratio

The E_2:P_4 ratio was significantly lower in follicular fluids aspirated during the luteal phase (group DL) than in fluids aspirated at the end of the follicular phase (group EF) ($p < 0.05$) when all follicles larger than 5 mm present on the ovaries were considered. Moreover, it was lower in the fluids of both group DL and group EF than in fluids collected during the follicular phase (group DF) ($p < 0.01$). Nevertheless, no significant difference between groups within each follicular size class studied was observed (Fig. 1).

Considering the data from the three groups, the E_2:P_4 ratio significantly increased with an increase in follicular diameter ($p < 0.01$); it was lower in 5- to 9-mm follicles than in 10- to 50-mm follicles ($p < 0.01$). Within each of the three groups, the E_2:P_4 ratio also increased with an increase in follicular diameter ($p < 0.01$ each; Fig. 1), and it was significantly lower in small follicles (5–9 mm in group DF, 5–14 mm in groups EF and DL) than in larger ones ($p < 0.05$ each).

No relationship was observed between the intrafollicular E_2:P_4 ratio and oocyte competence for in vitro maturation: this ratio was not significantly different between the nuclear stages of the corresponding oocytes after in vitro culture.

Cellular Content of Aromatase, α-Inhibin, and LH Receptor

A total of 61 samples of cumulus cells and 204 samples of granulosa cells were analyzed for actin, plasma membrane proteins of erythrocytes, aromatase, α-inhibin, and LH receptor amounts. The expected band for actin at 42 kDa was visualized in most samples (Fig. 2A); only 14 samples did not contain sufficient amounts of proteins. The antibody raised against plasma membrane proteins of erythrocytes revealed a specific band at 30 kDa in the samples contaminated with blood cells (Fig. 2B). The expected bands for aromatase and α-inhibin were visualized at 55 kDa (Fig. 2C) and 50 kDa (Fig. 2D), respectively. The LH

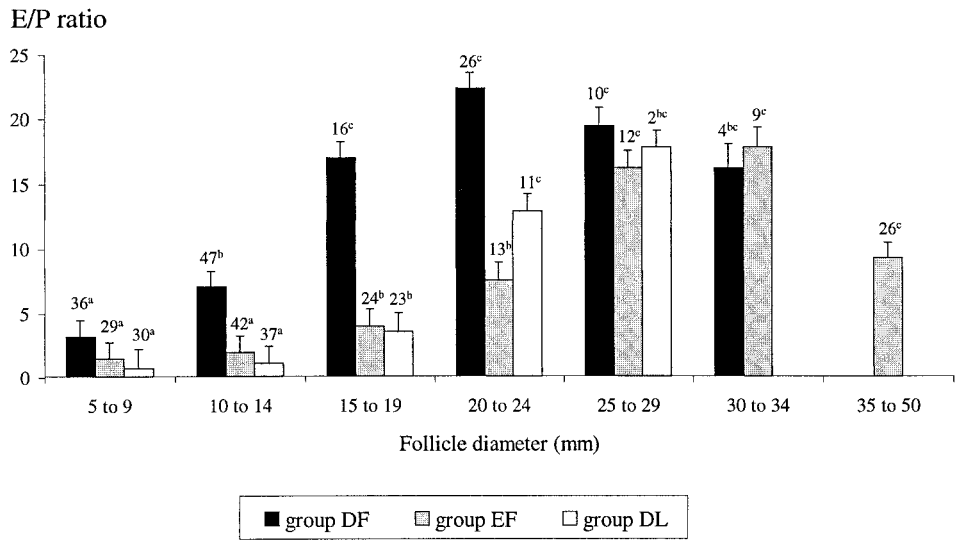

FIG. 1. Ratio of the concentrations of E_2:P_4 in relation to the group and the follicular diameter. Values with different superscripts within a group differ significantly ($p < 0.05$).

receptor antibody revealed two bands at 55 and 90 kDa and a doublet at 68–70 kDa (Fig. 2E).

Cellular Content of Aromatase, α-Inhibin, and LH Receptor in Relation to Estrous Cycle Stage and Follicular Diameter

Within the whole follicular population studied, the amount of aromatase was significantly higher in granulosa cells recovered during the follicular phase (group DF) than in cells recovered at the end of the follicular phase (group EF; $p < 0.05$) or during the luteal phase (group DL; $p < 0.01$). The amounts of α-inhibin and LH receptors (calculated as the sum of all the bands) were not significantly different between the three physiological stages (groups DF, EF, and DL).

As illustrated in Figure 3, the amounts of aromatase increased with an increase in follicular diameter for granulosa cells collected at the end of the follicular phase (group EF, $p < 0.001$) and during the luteal phase (group DL, $p = 0.08$). The amounts of LH receptors increased with an increase in follicular diameter in group EF ($p < 0.01$). The amounts of aromatase and α-inhibin were lower in 5- to 9-mm follicles than in larger ones in group EF. The amounts of LH receptors were lower in 5- to 9-mm follicles than in larger ones in groups DF, EF, and DL (5–9 mm vs. > 10 mm).

Finally, considering the data from the 3 groups, the amounts of aromatase and LH receptor increased with an increase in follicular diameter ($p < 0.01$). Moreover, the amounts of aromatase, α-inhibin, and LH receptors were lower in 5- to 9-mm follicles than in larger ones ($p < 0.05$ each).

In cumulus cells, quantitative analysis of the amount of aromatase, α-inhibin, and LH receptor was limited by the low number of samples. The amounts of these factors in cumulus cells were not significantly different either between the three groups or between the classes of follicular diameter.

Cellular Content of Aromatase, α-Inhibin, and LH Receptor in Relation to Oocyte Competence for In Vitro Maturation

Quantitative analysis of the content of aromatase, α-inhibin, and LH receptor in granulosa and cumulus cells in

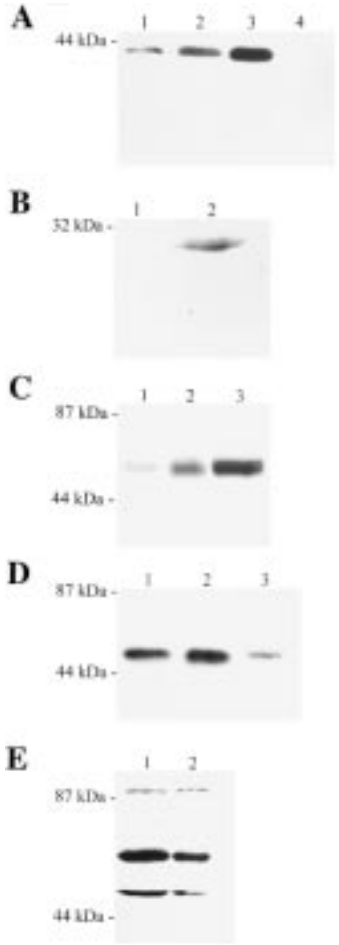

FIG. 2. **A**) Representative profiles of actin expression in equine follicular cells. Lanes 1–3, samples with increasing amounts of actin; lane 4, one sample with no detectable amount of actin. **B**) Representative profiles of expression of plasma membrane proteins of erythrocytes in equine follicular cells. Lane 1, one sample without any contamination with erythrocytes; lane 2, one sample contaminated with erythrocytes. **C**) Representative profiles of aromatase expression in equine follicular cells. Lanes 1–3, samples with increasing amounts of aromatase. **D**) Representative profiles of α-inhibin expression in equine follicular cells. Lanes 1–3, samples with various amounts of α-inhibin. **E**) Representative profiles of LH receptor expression in equine follicular cells. Lanes 1 and 2, samples with various amounts of LH receptors.

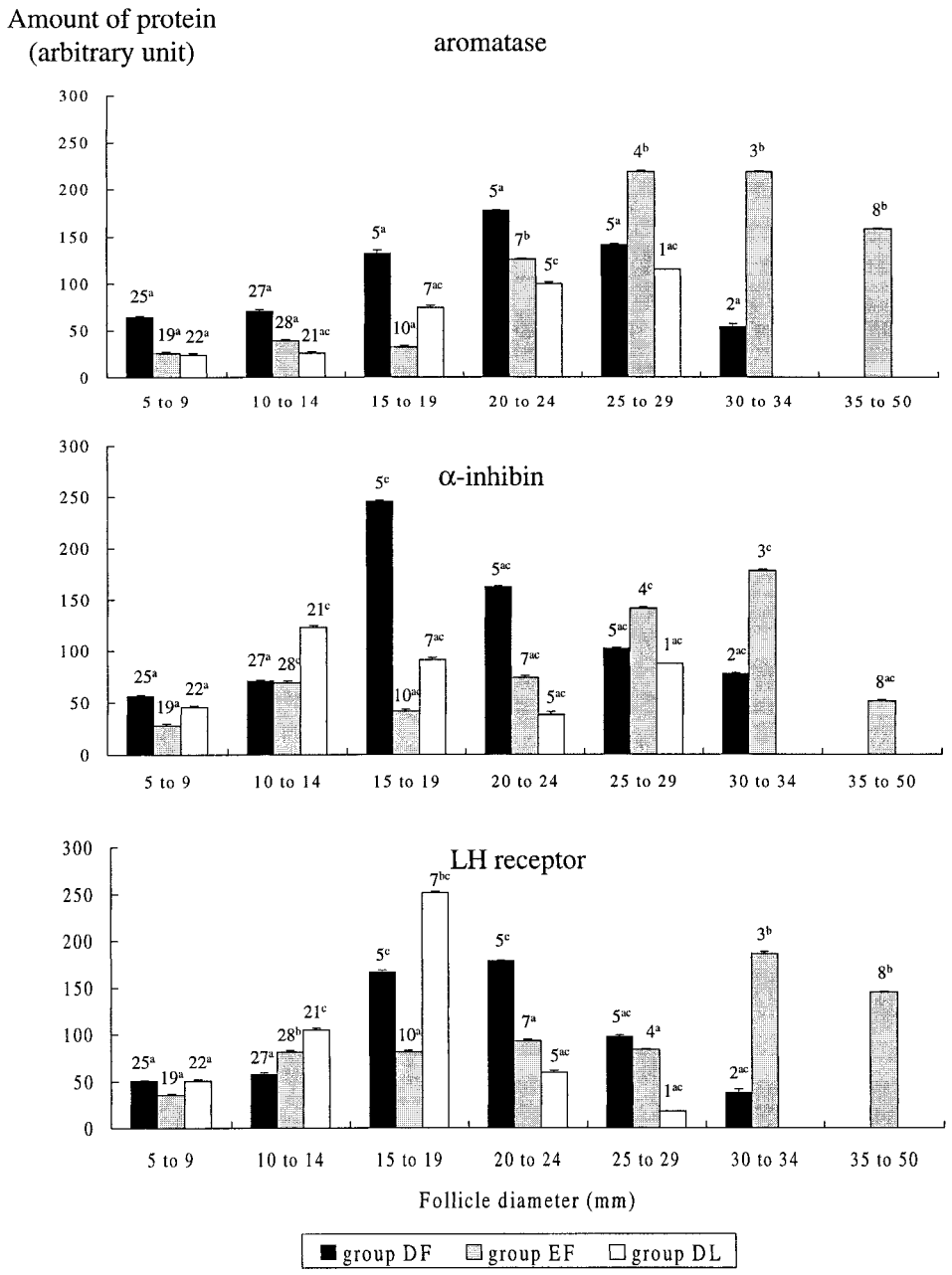

FIG. 3. Quantitative analysis of the expression of aromatase, α-inhibin, and LH receptors (arbitrary unit) in granulosa cells in relation to the group and the follicular diameter. [a,b] Values with different superscripts within a group differ significantly ($p < 0.05$). [a,c] Values with different superscripts within a group tend to differ ($p < 0.10$).

relation to oocyte competence for in vitro maturation is illustrated in Figure 4. The amounts of these three proteins in granulosa cells and in cumulus cells were not significantly different between the nuclear stages of the corresponding oocytes after in vitro culture. However, the amounts of these three proteins in cumulus cells tended to decrease as the oocyte progressed in nuclear maturation.

Moreover, as shown in Table 1, the amounts of aromatase, α-inhibin, and LH receptor in cumulus cells tended to be lower for oocytes that reached metaphase II after in vitro maturation than for preovulatory oocytes that reached metaphase II in vivo.

DISCUSSION

The aim of this study was to analyze the amounts of LH receptor, α-inhibin, and aromatase in equine mural granulosa and cumulus cells, as well as the E_2:P_4 ratio in follicular fluid, in relation to 1) follicular size and estrous cycle stage and 2) oocyte competence for in vitro maturation. In order to normalize the various experimental groups, all mares received 35 mg i.v. crude equine gonadotropins 34 h before ovarian punctures. We observed that LH receptor, α-inhibin, and aromatase were expressed in mural granulosa and cumulus cells from all follicles from the gonadotropin-independent growth period to the preovulatory stage. Their amounts in granulosa cells were influenced by the follicle diameter and the estrous cycle stage. No obvious relationship with the oocyte competence for in vitro maturation was observed.

The LH receptor antibody used in the present study revealed two bands at 55 and 90 kDa, respectively, and a doublet at 68–70 kDa. Similar molecular sizes were observed in the pig [17, 22]. In the pig [22] and the rat [23], the sequence of the receptor cDNA allows calculation of a molecular mass of 75 kDa. The 90-kDa band may thus correspond to the whole glycosylated receptor, whereas the

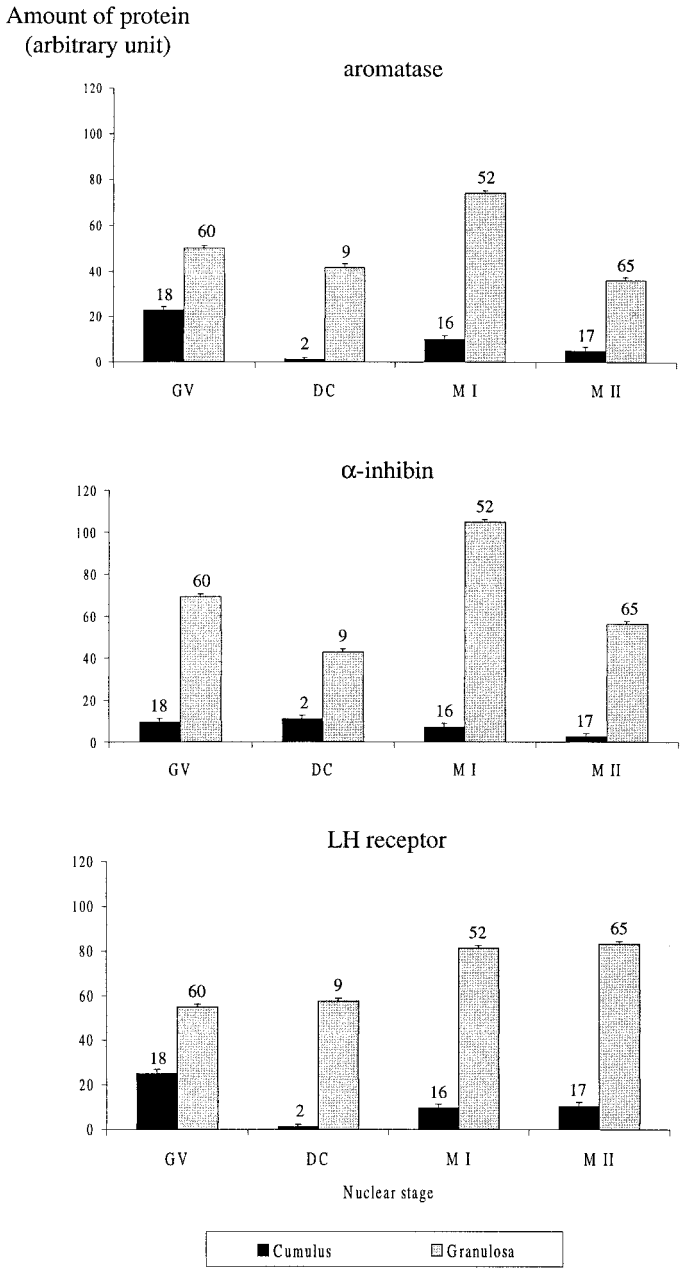

FIG. 4. Quantitative analysis of the expression of aromatase, α-inhibin, and LH receptors (arbitrary unit) in granulosa and cumulus cells in relation to the nuclear stage after in vitro culture of the corresponding oocytes. GV, Oocytes in germinal vesicle stage; DC, oocytes with dense chromatin; MI, oocytes in metaphase I; MII, oocytes in metaphase II. Within granulosa cells and cumulus cells, the amounts of aromatase, α-inhibin, and LH receptors were not significantly different between the nuclear stages of the corresponding oocytes after in vitro maturation.

55-kDa band may correspond to the extracellular domain. The bands at 68–70 kDa may correspond to a partly degraded or nonglycosylated receptor; in the present study, the relative abundance of these bands may reflect proteolysis of the receptor during the collection and preparation of the cells. We detected LH receptors in granulosa cells of all follicles from 5 mm onwards. In other domestic mammals, LH receptors, as revealed by mRNA content or I^{125}-hCG binding, do not appear in granulosa cells before the large antral follicle stage (rat; see [24–26] for review; porcine [27, 28]; ovine [29]; bovine [30, 31]), which corresponds to equine follicles 20–25 mm in diameter. The intracellular LH receptor level in all equine follicles studied could explain, at least in part, the improvement in oocyte competence for in vitro maturation after exogenous LH injection in the mare [6]. We showed that LH receptor content in the granulosa cells increased with an increase in follicular diameter, as previously observed in the mare by using I^{125}-hCG binding [32]. This is in agreement with findings in bovine [33, 34], porcine [27, 35], and ovine [29, 36, 37] species. In cumulus cells, as observed in mural granulosa cells, we detected LH receptors from all equine follicles from 5 mm onwards. LH receptors were detected in porcine cumulus cells from preovulatory follicles [27], but not in corresponding bovine cells [38], whereas conflicting results have been reported in the rat [25, 39, 40]. The presence of LH receptors in equine cumulus cells could be involved in the positive effect of gonadotropins during equine oocyte in vitro culture (see [5] for review).

TABLE 1. Expression of aromatase, α-inhibin, and LH receptor (arbitrary unit; mean ± SEM) in cumulus cells of oocytes that reached metaphase II after in vitro vs. in vivo maturation.

	Maturation	
Protein	In vitro (17 lanes)	In vivo (4 lanes)
Aromatase	5.1 ± 1.7	147.9 ± 1.1
α-Inhibin	2.6 ± 1.5	89.1 ± 1.2
LH receptor	10.2 ± 1.8	165.9 ± 1.1

Equine α-inhibin subunit was observed in granulosa cells at 50 kDa, as previously shown in equine granulosa-theca cell tumors [41], whereas an 18-kDa band was observed in equine follicular fluid [42]. Until this study, no data were available in the mare on the expression of inhibin in relation to the follicle diameter or the estrous cycle stage. We observed that, at the end of the follicular phase (group EF), the granulosa cells contained lower amounts of α-inhibin in 5- to 9-mm follicles than in larger ones. Despite the fact that few studies are available on small follicles in other species, our results are consistent with findings in the pig [43, 44] and rat [45]. Moreover, equine cumulus cells showed an immunostaining for α-inhibin in agreement with findings in the rat [46], the sheep [47, 48], and the human [49].

Aromatase has previously been immunohistochemically localized in the granulosa cells of various mammalian species, including the mare [18, 50]. In the present study, aromatase was observed in granulosa cell lysates at 55 kDa, at the three physiological stages and across the range of diameters studied. During the luteal phase (group DL), the low amount of aromatase in granulosa cells and the low E_2:P_4 ratio in fluids highlight the fact that granulosa cells are primarily P_4 producers during the luteal phase. During the follicular phase (group DF), we observed a higher amount of aromatase in granulosa cells as well as a higher E_2:P_4 ratio than at the end of the follicular phase (group EF). This is in agreement with estradiol concentrations in equine follicular fluid, which decrease at the preovulatory stage after LH stimulation and are very low in subordinate follicles [51, 52]. On the other hand, we showed that the amount of aromatase in granulosa cells, and consequently the E_2:P_4 ratio, increased with an increase in follicular diameter, as previously observed by using immunohistochemistry [18]. This result is in agreement with aromatase expression in the rat [53], the pig [28], and the sheep [54]; with estradiol and P_4 levels in equine follicular fluids [52]; and with mRNA amounts in the pig and the cow [28, 31].

Such an increase in aromatase mRNA in the equine species has yet to be tested. In equine cumulus cells, aromatase was observed at the three physiological stages and across the range of diameters studied, whereas in humans and in superovulated cattle, pigs, and sheep, the cumulus cells lack aromatase [55–57]. To our knowledge, aromatase has been localized in cumulus cells only from rat preovulatory follicles [58].

The second purpose of the present study was to investigate the amounts of LH receptors, α-inhibin, and aromatase in mural granulosa and cumulus cells in relation to oocyte competence for in vitro maturation. Actually, these factors play a role in oocyte maturation in several species: the addition of inhibin to the culture medium stimulates primate and bovine oocyte maturation in vitro [59, 60], inhibin levels in porcine follicles were double when the oocytes resumed meiosis in comparison to immature oocytes [61], and an improvement in in vitro maturation of equine oocytes is obtained with the addition of estradiol or gonadotropins in the culture medium ([62]; see [5] for review). Nevertheless, we did not observe any obvious relationship between the amounts of α-inhibin, LH receptors, and aromatase and the nuclear stages of the corresponding oocytes after in vitro culture. Moreover, our results show that the influence of the follicle diameter on the intracellular levels of LH receptors, α-inhibin, and aromatase in granulosa cells is similar to the influence of follicle diameter on oocyte competence for in vitro maturation. On the one hand, we observed that the amounts of aromatase and LH receptors increased with an increase in follicular diameter, and that the follicles 5–9 mm in diameter contained lower amounts of α-inhibin, LH receptors, and aromatase than the larger ones. On the other hand, we showed, in the present study as previously [6, 10], that the oocyte maturation rate increased with the follicle diameter and was lower in follicles 5–9 mm in diameter than in larger ones. This reinforces our hypothesis that the follicles of 5–9 mm represent the basal gonadotropin-independent follicular growth [6, 10] and allows us to imply a relationship between oocyte incompetence and the expression of α-inhibin, LH receptors, and aromatase in granulosa cells.

Finally, the amounts of aromatase, α-inhibin, and LH receptors in cumulus cells tended to be lower for oocytes that reached metaphase II after in vitro maturation than for preovulatory oocytes that reached metaphase II in vivo. This could be linked to the higher amounts of these factors in cells from the preovulatory follicle in comparison with the small follicles, or to unsuitable culture conditions.

In conclusion, our results demonstrate that aromatase, α-inhibin, and LH receptors in the equine species are expressed in mural granulosa and cumulus cells from all follicles from the gonadotropin-independent growth until the preovulatory stage. No obvious relationship between the amounts of these three factors and oocyte competence for in vitro maturation could be established. However, the expression of the three proteins studied, as well as oocyte competence, was influenced by the follicle diameter, showing a link between the acquisition of oocyte competence and the expression of aromatase, α-inhibin, and LH receptors in follicular cells.

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REFERENCES

1. Ginther OJ. Characteristics of the ovulatory season. In: Ginther OJ (ed.), Reproductive Biology of the Mare, Basic and Applied Aspects. 2nd Edition. Cross Plains, WI: Equiservices; 1992: 173–232.
2. De Smedt V, Crozet N, Gall L. Morphological and functional changes accompanying the acquisition of meiotic competence in ovarian goat oocyte. J Exp Zool 1994; 269:128–139.
3. Singh B, Meng L, Rutledge JM, Armstrong DT. Effects of epidermal growth factor and follicle-stimulating hormone during in vitro maturation on cytoplasmic maturation of porcine oocytes. Mol Reprod Dev 1997; 46:401–407.
4. Sirard MA. Practical aspects of in vitro fertilization in cattle. J Reprod Fertil Suppl 1989; 38:127–134.
5. Squires EL. Maturation and fertilization of equine oocytes. Vet Clin N Am Equine Pract 1996; 12:31–45.
6. Goudet G, Bézard J, Duchamp G, Gérard N, Palmer E. Equine oocyte competence for nuclear and cytoplasmic in vitro maturation: effect of follicle size and hormonal environment. Biol Reprod 1997; 57:232–245.
7. Hinrichs K, Williams KA. Relationships among oocyte-cumulus morphology, follicular atresia, initial chromatin configuration, and oocyte meiotic competence in the horse. Biol Reprod 1997; 57:377–384.
8. Whitmore HL, Wentworth BC, Ginther OJ. Circulating concentrations of luteinizing hormone during estrous cycle of mares as determined by radioimmunoassay. Am J Vet Res 1973; 34:631–636.
9. Irvine CHG, Alexander SL. The dynamics of gonadotrophin-releasing hormone, LH and FSH secretion during the spontaneous ovulatory surge of the mare as revealed by intensive sampling of pituitary venous blood. J Endocrinol 1994; 140:283–295.
10. Goudet G, Bézard J, Belin F, Duchamp G, Palmer E, Gérard N. Oocyte competence for in vitro maturation is associated with histone H1 kinase activity and is influenced by estrous cycle stage in the mare. Biol Reprod 1998; 59:456–462.
11. Wesson JA, Ginther OJ. Influence of season and age on reproductive activity in pony mares on the basis of a slaughterhouse survey. J Anim Sci 1981; 52:119–129.
12. Driancourt M-A, Prunier A, Palmer E, Mariana J-C. Seasonal effects on ovarian follicular development in pony mares. Reprod Nutr Dev 1983; 23:207–215.
13. Palmer E. Recent attempts to improve synchronisation of ovulation and to induce superovulation in the mare. Equine Vet J Suppl 1984; 3:11–18.
14. Duchamp G, Bour B, Combarnous Y, Palmer E. Alternative solutions to hCG induction of ovulation in the mare. J Reprod Fertil Suppl 1987; 35:221–228.
15. Duchamp G, Bézard J, Palmer E. Oocyte yield and the consequences of puncture of all follicles larger than 8 millimeters in mares. In: Sharp DC, Bazer FW (eds.), Equine Reproduction VI. Madison, WI: Society for the Study of Reproduction; 1995: 233–241.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
17. Vuhai-Luuthi MT, Jolivet A, Jallal B, Salesse R, Bidart J-M, Houllier A, Guiochon-Mantel A, Garnier J, Milgrom E. Monoclonal antibodies against luteinizing hormone receptor. Immunochemical characterization of the receptor. Endocrinology 1990; 127:2090–2098.
18. Almadhidi J, Séralini G-E, Fresnel J, Silberzahn P, Gaillard J-L. Immunohistochemical localization of cytochrome P450 aromatase in equine gonads. J Histochem Cytochem 1995; 43:571–577.
19. Saumande J, Tamboura D, Chupin D. Changes in milk and plasma concentrations of progesterone in cows after treatment to induce superovulation and their relationships with number of ovulations and embryos collected. Theriogenology 1985; 23:719–731.
20. Terqui M, Dray F, Cotta J. Variations de la concentration en oestradiol-17β dans le sang périphérique de la brebis au cours du cycle oestral. C R Acad Sci 1973; 277:1795–1798.
21. Bézard J, Goudet G, Duchamp G, Palmer E. Preovulatory maturation of ovarian follicles and oocytes in unstimulated and superovulated mares. In: Sharp DC, Bazer FW (eds.), Equine Reproduction VI. Madison, WI: Society for the Study of Reproduction; 1995: 261–271.

22. Loosfelt H, Misrahi M, Atger M, Salesse R, Vu Hai-Luu Thi MT, Jolivet A, Guiochon-Mantel A, Sar S, Jallal B, Garnier J, Milgrom E. Cloning and sequencing of porcine LH-hCG receptor cDNA: variants lacking transmembrane domain. Science 1989; 245:525–528.
23. McFarland KC, Sprengel R, Phillips HS, Köhler M, Rosemblit N, Nikolics K, Segaloff DL, Seeburg PH. Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science 1989; 245:494–499.
24. Camp TA, Rahal JO, Mayo KE. Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. Mol Endocrinol 1991; 5: 1405–1417.
25. Peng X-R, Hsueh AJW, Lapolt PS, Bjersing L, Ny T. Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. Endocrinology 1991; 129:3200–3207.
26. Richards JS. Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol Rev 1980; 60:51–89.
27. Meduri G, Vuhai-Luuthi MT, Jolivet A, Milgrom E. New functional zonation in the ovary as shown by immunohistochemistry of luteinizing hormone receptor. Endocrinology 1992; 131:366–373.
28. Yuan W, Lucy MC, Smith MF. Messenger ribonucleic acid for insulin-like growth factors-I and -II, insulin-like growth factor-binding protein-2, gonadotropin receptors, and steroidogenic enzymes in porcine follicles. Biol Reprod 1996; 55:1045–1054.
29. England BG, Webb R, Dahmer MK. Follicular steroidogenesis and gonadotropin binding to ovine follicles during the estrous cycle. Endocrinology 1981; 109:881–887.
30. Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod 1995; 53:951–957.
31. Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS. Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. Biol Reprod 1997; 56:1158–1168.
32. Fay JE, Douglas RH. Changes in thecal and granulosa cell LH and FSH receptor content associated with follicular fluid and peripheral plasma gonadotrophin and steroid hormone concentrations in preovulatory follicles of mares. J Reprod Fertil Suppl 1987; 35:169–181.
33. Stewart RE, Spicer LJ, Hamilton TD, Keefer BE, Dawson LJ, Morgan GL, Echternkamp SE. Levels of insulin-like growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 1996; 137:2842–2850.
34. Bodensteiner KJ, Wiltbank MC, Bergfelt DR, Ginther OJ. Alterations in follicular estradiol and gonadotropin receptors during development of bovine antral follicles. Theriogenology 1996; 45:499–512.
35. Tilly JL, Kowalski KI, Schomberg DW, Hsueh AJW. Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 aromatase. Endocrinology 1992; 131:1670–1676.
36. Carson RS, Findlay JK, Burger HG, Trounson AO. Gonadotropin receptors of the ovine ovarian follicle during follicular growth and atresia. Biol Reprod 1979; 21:75–87.
37. Webb R, England BG. Identification of the ovulatory follicle in the ewe: associated changes in follicular size, thecal and granulosa cell luteinizing hormone receptors, antral fluid steroids, and circulating hormones during the preovulatory period. Endocrinology 1982; 110: 873–881.
38. Van Tol HTA, Van Eijk MJT, Mummery CL, Van Den Hurk R, Bevers MM. Influence of FSH and hCG on the resumption of meiosis of bovine oocytes surrounded by cumulus cells connected to membrana granulosa. Mol Reprod Dev 1996; 45:218–224.
39. Bortolussi M, Marini G, Reolon ML. A histochemical study of the binding of ^{125}I-HCG to the rat ovary throughout the estrous cycle. Cell Tissue Res 1979; 197:213–226.
40. Lawrence TS, Dekel N, Beers WH. Binding of human chorionic gonadotropin by rat cumuli oophori and granulosa cells: a comparative study. Endocrinology 1980; 106:1114–1118.
41. Piquette GN, Kenney RM, Sertich PL, Yamoto M, Hsueh AJW. Equine granulosa-theca cell tumors express inhibin α- and βA-subunit messenger ribonucleic acids and proteins. Biol Reprod 1990; 43: 1050–1057.
42. Moore KH, Dunbar BS, Bousfield GR, Ward DN. Initial characterization of equine inhibin. Biol Reprod 1994; 51:63–71.
43. Guthrie HD, Rohan RM, Rexroad CE Jr, Cooper BS. Changes in concentrations of follicular inhibin α and βA subunit messenger ribonucleic acids and inhibin immunoactivity during preovulatory maturation in the pig. Biol Reprod 1992; 47:1018–1025.
44. Westhof G, Westhof KF, Hasegawa Y, Miyamoto K, diZerega GS. Inhibin secretion by individual porcine follicles perifused in vitro. Fertil Steril 1990; 54:718–724.
45. Van Cappellen WA, van Leeuwen ECM, Meijs-Roelofs HMA, Kramer P, Sander HJ, de Jong FH. Inhibin and oestradiol-17β in antral follicles of various size classes of cyclic rats. J Endocrinol 1995; 146: 323–330.
46. Kobayashi M, Yamoto M, Minami S, Imai M, Nakano R. Immunohistochemical localization of inhibin α- and βA-subunits in the ovary of immature female rats. Eur J Endocrinol 1995; 132:97–102.
47. Engelhardt H, Smith KB, McNeilly AS, Baird DT. Expression of messenger ribonucleic acid for inhibin subunits and ovarian secretion of inhibin and estradiol at various stages of the sheep estrous cycle. Biol Reprod 1993; 49:281–294.
48. Braw-Tal R. Expression of mRNA for follistatin and inhibin/activin subunits during follicular growth and atresia. J Mol Endocrinol 1994; 13:253–264.
49. Roberts VJ, Barth S, El-Roeiy A, Yen SSC. Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle. J Clin Endocrinol Metab 1993; 77:1402–1410.
50. Sirois J, Kimmich TL, Fortune JE. Steroidogenesis by equine preovulatory follicles: relative roles of theca interna and granulosa cells. Endocrinology 1991; 128:1159–1166.
51. Gérard N, Monget P. Intrafollicular insulin-like growth factor-binding protein levels in equine ovarian follicles during preovulatory maturation and regression. Biol Reprod 1998; 58:1508–1514.
52. Gérard N, Duchamp G, Goudet G, Bézard J, Magistrini M, Palmer E. A high molecular weight preovulatory stage-related protein in equine follicular fluid and granulosa cells. Biol Reprod 1998; 58:551–557.
53. Hickey GJ, Chen S, Besman MJ, Shively JE, Hall PF, Gaddy-Kurten D, Richards JS. Hormonal regulation, tissue distribution, and content of aromatase cytochrome P450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: relationship to estradiol biosynthesis. Endocrinology 1988; 122:1426–1436.
54. Tsonis CG, Carson RS, Findlay JK. Relationships between aromatase activity, follicular fluid oestradiol-17β and testosterone concentrations, and diameter and atresia of individual ovine follicles. J Reprod Fertil 1984; 72:153–163.
55. Inkster SE, Brodie AMH. Expression of aromatase cytochrome P-450 in premenopausal and postmenopausal human ovaries: an immunocytochemical study. J Clin Endocrinol Metab 1991; 73:717–726.
56. Laurincik J, Kolodzieyski L, Elias V, Hyttel P, Osawa Y, Sirotkin A. Immunocytochemical localization of aromatase in the ovary of superovulated cattle, pigs and sheep. Acta Vet Scand 1994; 35:185–191.
57. Laurincik J, Kolodzieyski L, Hyttel P, Osawa Y, Niemann H, Schmoll F, Brem G, Schellander K. Granulosa-cumulus-corona expansion and aromatase localization in preovulatory follicles in superovulated heifers. Acta Vet Scand 1996; 37:99–107.
58. El-Maasarany S, Brandt ME, Majercik MH, Zimniski SJ, Puett D. Immunocytochemical localization and enzymatic distribution of rat ovarian aromatase. Biol Reprod 1991; 44:550–560.
59. Alak BM, Smith GD, Woodruff TK, Stouffer RL, Wolf DP. Enhancement of primate oocyte maturation and fertilization in vitro by inhibin A and activin A. Fertil Steril 1996; 66:646–653.
60. Stock AE, Woodruff TK, Smith LC. Effects of inhibin A and activin A during in vitro maturation of bovine oocytes in hormone- and serum-free medium. Biol Reprod 1997; 56:1559–1564.
61. Miller KF, Xie S, Pope WF. Immunoreactive inhibin in follicular fluid is related to meiotic stage of the oocyte during final maturation of the porcine follicle. Mol Reprod Dev 1991; 28:35–39.
62. Bézard J, Palmer E. In vitro maturation of horse oocytes from slaughtered ovaries. In: Proceedings of the 12th International Congress of Animal Reproduction; 1992; The Hague, The Netherlands. Abstract 315.