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FSH binding capability of gonadotrophin responsive follicles and probable role of LH on their release

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ABSTRACT

Follicular fluid (FF) was collected from the isolated large (>5 mm diameter) and medium (3-5 mm diameter) size gonadotrophin dependent follicles of early and late luteal phase of goat ovaries of abattoir origin for FSH ELISA to know the pattern and efficiency of FSH binding, its release from follicles under the influence of LH, and to assess the probable role of LH in an *in vitro* culture model.

Nonatrectic, well vascularised follicles were cultured in TCM-199 having 5 µg FSH/ml for 30 min at 5%CO_2, 95% RH and 38°C. After FSH incubation they were made free of unbound FSH and cultured with 74 IU LH/ml in TCM-199 with 10% EGS for 4,8,12 and 16 hr in 1 ml culture fluid. Follicles of control group were cultured in a similar fashion but without LH. After culture follicles were subjected to immunohistochemistry (IHC) and FSH was estimated in the culture fluid.

Higher FSH levels in the FF of both types of follicles were observed during late luteal phase compared to the early luteal phase. Treatment of the follicles with FSH increased FSH binding in the FF and showed the presence of free receptors in FF. Medium sized follicles showed higher levels of FSH in culture fluid of LH treated group after 4hr of culture in comparison to large follicles. Maximum FSH concentration was observed at 8hr of culture in culture fluid with a decrease at 12 and 16hr. Control group had lower FSH levels compared to the LH treated group. At 0hr FSH was mainly localized in the FF and it decreased at 4hr. At 8hr it was mainly in the granulosa cells (GC) which further intensified in the GC at 12 and 16hr as compared to the FF. It may be concluded that the developing antral follicles synthesize FSH receptors that percolate in the FF and store FSH. On progesterone drop, FSH receptor complex of FF breakdown, releasing FSH, which goes out of the follicles and binds to the GC surface, probably due to more number of receptors on GC at progesterone fall. This enhances estradiol and progesterone synthesis from GC similar to that of proestrus. Hence proestrus rise of circulating FSH, if not total then partly contributed by follicular FSH.

Key words: Animal reproduction, FSH, FSH recepters, Follicles, Follicle stimulating hormone, Gonadotrophin

Follicle growth is a continuous process, is triggered and initiated from the pool of primordial follicles through integrated stages of follicular recruitment, selection and dominance. Central role of the classical hypothalamo-pituitary-ovarian axis in this process is well established (Webb *et al.* 1992, Campbell *et al.* 1995). Follicular development in cattle is thought to be regulated primarily by FSH and LH (Ireland and Roche 1987, Lucy *et al.* 1992). FSH and estrogens act synergistically to enhance follicular growth, follicular differentiation and steroid production (Wang and Greenwald 1993). Estradiol and FSH also stimulate 3', 5'-monophosphate production and cAMP dependent FSH and LH receptors formation by rat granulosa cells (Knecht *et al.* 1984). Dufour *et al.* (1979), Mcnatty *et al.* (1990) and Eckay *et al.* (1994) demonstrated that the early stages of folliculogenesis are not acutely dependent on gonadotrophin support. Antral follicular pool consists of gonadotrophin nonresponsive, responsive and dependent follicles (Gong and Webb 1996). Gonadotrophin dependent follicles of caprine origin were used in this study to know the pattern and efficiency of FSH binding and its release under the influence of LH, if any, to assess the probable role of LH using an *in vitro* follicle culture system.

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MATERIALS AND METHODS

Goat ovaries were collected from the local slaughterhouse in normal salt saline (NSS) immediately after slaughter, at 30-35°C for the transport to the laboratory. Ovaries were washed thoroughly in NSS followed by washings with sterile phosphate buffered saline (PBS). Finally the ovaries were exposed to D-PBS with antibiotics. Large (>5mm diameter) and medium (3 to 5 mm diameter) size follicles were isolated

and collected in D-PBS. Isolated follicles were examined under stereozoom microscope, nonatrectic well vascularised follicles were selected and washed in sterile tissue culture medium-199 (TCM-199) having HEPES and glutamine. Follicles (278) were isolated, out of which 108 were large and 170 were medium size follicles. All the follicles were first incubated with TCM-199 having 5 µg FSH/ml for 30 min at 38°C in 5% CO_2 and 95% RH. After FSH incubation, these follicles were washed with TCM-199 for 30 min with a change after every 5 min. After several washings follicles were randomly divided into 2 groups. In the first group an individual follicle was cultured in 1 ml of TCM-199 with 74 IU LH/ml and 10% estrus goat serum for 4,8,12,16 hr in 5% CO_2 and 95% RH at 38°C. The other group was also cultured in a very similar way except that there was no LH present in the culture medium. After each culture time follicles were carefully removed from the culture medium and fixed at –20°C for immunohistochemistry. Culture medium was collected and stored at –20°C until subjected for FSH estimation by ELISA. For immunohistochemistry (IHC) 10µ thin sections of the cultured follicles were made by cryomicrotome. They were fixed and processed for IHC of FSH and FSH receptors according to Sharma and Majumdar (1998) and Majumdar and Sharma (1999). In addition, all the above type follicles were also collected from the ovaries of early and late luteal phase. Follicular fluid was collected from them and subjected to ELISA for FSH estimation.

RESULTS AND DISCUSSION

Significantly higher levels of FSH (1.824 and 0.172 µ/ml) were observed in the follicular fluid of the medium sized than the large sized follicles (0.167 and 0.062µ/ml) during both early and late luteal phase as revealed by ELISA. Treatment of follicles with FSH increased binding of FSH in the follicular fluid showing presence of free receptors in it (0 hr, Table 1).

In vitro follicle culture in presence of LH, revealed that FSH level in FF of medium size follicle was 0.144 milliunits/ml in the medium follicular group compared to 0.166 milliunits/ml for the large follicular group. Maximum FSH concentration in culture fluid was observed at 8 hr of culture with the values of 0.345 and 0.406 for the medium and large size follicles respectively (Table 2). FSH level decreased after 12 and 16 hr though it was more than the 4-hr group (Table 2).

Immunohistochemical study of the follicular sections revealed that FSH was localized in the follicular fluid only (Fig. 1, Table 1) at 0 hr of culture. After 4 hr of culture FSH decreased in the follicular fluid (Fig. 2). With an increase in the culture time i.e at 8 hr of culture with LH treatment the follicular sections showed an increased FSH localization in the granulosa cells (Fig. 3) and after 12hr of culture, a gradual fall in the FSH levels of the culture fluid was noted (Table 2) with further intensified FSH binding on the granulosa cells (Fig. 4).

In goats very few gonadotrophin receptors were present in the follicular cells during the early luteal phase and hence less steroidogenesis occurred during the early luteal phase compared to the late luteal phase (Sharma and Majumdar 1998). In the present study also, FSH concentration was less in the follicular fluid collected during the early luteal phase as compared to the follicular fluid collected during late luteal phase. Comparatively less amount of FSH in the follicular fluid of large size follicles in comparison to medium size follicle may be due to increased volume of follicular fluid in those follicles or that some of the follicle is proceeding to atresia as on initiation of atresia or preovulatory follicle the follicular fluid increases. Kumari and Channing (1979) observed that granulosa cells from small follicles contain more

Table 1. Localization of FSH and FSH receptors in the treated and control group of follicles

Follicle size	Culture time									
	0 hr		4 hr		8 hr		12 hr		16 hr	
	FF	GC	FF	GC	FF	GC	FF	GC	FF	GC
Medium 3–5 mm										
Treated										
FSH	8+	–	6+	–	2+	2+	–	3+	–	4+
FSH-R	10+	–	2+	2+	8+	3+	–	5+	–	5+
Control										
FSH	8+	–	6+	–	5+	1+	4+	1+	3+	2+
FSH-R	10+	–	8+	–	7+	2+	6+	2+	6+	2+
Large >5mm										
Treated										
FSH	6+	–	4+	1+	2+	2+	–	3+	–	4+
FSH-R	8+	–	6+	1+	5+	3+	–	4	–	5+
Control										
FSH	6+	–	5+	–	4+	1+	3+	1+	3+	2+
FSH-R	8+	–	7+	–	6+	2+	5+	2+	5+	2+

Table 2. FSH levels (milliunits/ml) in the culture fluid after LH incubation

Follicular size	Incubation time							
	4 hr		8 hr		12 hr		16 hr	
	C	T	C	T	C	T	C	T
Medium 3-5 mm	1.318	0.144	0.085	0.345	0.083	0.2.1	.540	0.183
Large >5 mm	0.0122	0.166	0.026	0.406	0.020	0.369	0.076	0.382

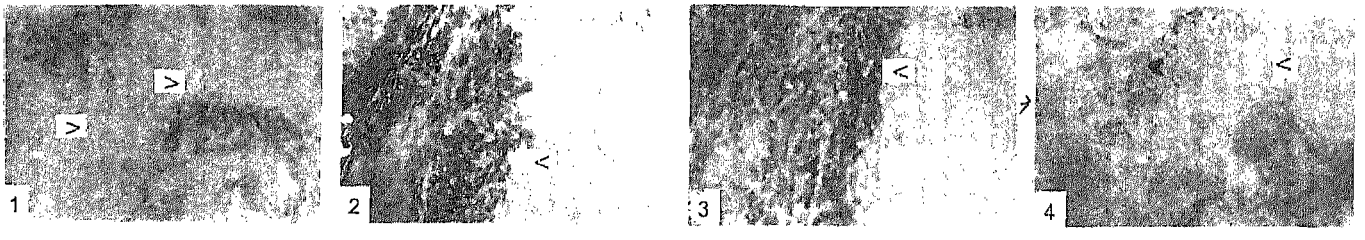
number of FSH receptors, which percolate into the follicular fluid and bind FSH; hence higher concentration of FSH was available in the follicular fluid of medium size follicle. Slight level of FSH increase at 8 hr which further decreased at 16 hr of culture could be due to the presence of LH in the estrous goat serum as 10% EGS was used in the culture fluid.

Pant *et al.* (1977) observed that during mid luteal phase there is an increase in the FSH pulse but at the same time FSH levels are low in blood. Padmanabhan and Mc Neilly (2001) further reported that slower frequency of GnRH, which occurred during midluteal phase favours FSH release but faster frequency of GnRH favour LH release from pituitary gonadotrophin. Normally faster frequency is observed during proestrus to estrus. It can be explained based on the present study that this FSH goes and binds to the follicular receptors of gonadotrophin responsive follicles during follicular phase, as follicular fluid contain large number of FSH receptors that bind FSH (Majumdar and Sharma 1999). The increase in FSH level in blood at follicular phase though less FSH pulse as was observed during this time may be explained from these results that the follicular FSH releases with increase in low level of LH, that helps in leaching out of FSH receptor complex located in the follicular fluid releasing FSH which percolates from the follicle to blood. Thus it is presumed that this follicular FSH also contributes to the blood FSH level at proestrus besides FSH released from pituitary.

Immunohistochemical (IHC) results also support the observation that on culture of follicles with LH in a constant volume of culture fluid, the FSH level increased at 4 to 8 hr of culture in cultured fluid but decreased than that of 8, 12 and 16 hr with an increase in binding on granulosa cells. It seems that FSH receptors grow on the surface of granulosa cells on LH release/progesterone drop and then the FSH binds over the granulosa cells, as was observed earlier (Majumdar and Sharma 1999). At 0 hr of culture, the presence of FSH in the follicular fluid is because of the presence of the receptors in the fluid only. No FSH was observed in the granulosa cell surface at 0 hr because these follicles were isolated from the ovaries having corpus luteum and therefore higher levels of progesterone which does not allow FSH to bind with the granulosa cells and FSH receptors were also lacking on the surface of these cells at this hour. After 12 and 16 hr of culture revealed that FSH concentration started decreasing in the culture fluid. This raised a question that where is the FSH going as the follicles were cultured in a close system? The answer came through the IHC where it was noticed that the FSH was localized in the granulosa cells only in much more intensified manner, because of the increase in the FSH receptors with the low levels of progesterone and elevated levels of estradiol.

Camp *et al.* (1991) have indicated that the gonadotrophin receptor genes are regulated in a complex fashion during the recruitment, maturation and ovulation of the ovarian follicle. In this experiment we did not observe any FSH receptor on granulosa cell surface till proestrous even in large follicles. It seems that FSH receptors that are synthesized in granulosa cells percolate in follicular fluid in presence of high level of progesterone (Majumdar and Sharma 1999). Thus at 0 hr FSH could bind only in follicular fluid but not on granulosa cell surface.

Abdennebi *et al.* (1999) reported that in healthy follicles, LH receptors levels in granulosa cells increased with



Figs 1-4. 1. Section of an isolated follicle at 0 hr of culture showing FSH localization exclusively in the follicular fluid but not at all in the granulosa cells (20×). 2. Isolated follicle section after 4 hr of *in vitro* culture in presence of LH showing FSH decline in follicular fluid. (20×). 3. Isolated follicle section at 8 hr of culture in the presence of LH showing an increased binding of FSH in the granulosa cells with a further decrease of FSH in the follicular fluid. (20×). 4. Isolated follicle section at 16 hr of culture showing localization of FSH. It is mainly localized in the granulosa cells but not in the follicular fluid. (20×).

increasing follicular size ($P<0.001$), while FSH receptor levels decreased ($P<0.05$). In granulosa cells of large follicles, LH receptor mRNA levels were greater in the late follicular phase than in the early follicular phase.

It may be concluded from this study that developing antral follicles synthesize FSH receptors but percolate in the follicular fluid that binds and store FSH. On drop of progesterone with slight rise in GnRH and LH this FSH receptor complex breakdown releasing FSH, which comes out of follicles and probably due to fall of progesterone the FSH receptors grow on granulosa cell surface that bind FSH and enhance estradiol and progesterone synthesis from granulosa cells to some extent similar to that of proestrus where FSH level increases in circulation but decreases on the onset of estrus. Hence proestrus rise of circulating FSH, if not total but partially contributed by follicular FSH.

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