RESEARCH ARTICLE

Characterization of Growth and Atresia of Buffalo Ovarian Follicles by Follicular Fluid Hormonal Levels and Transcripts Expression Studies

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Abstract

The influence of CL on follicular development and the changes in the follicular fluid composition leading to attainment of follicular dominance and ovulation has not been intensively studied in the buffalo. In the present study, the buffalo ovarian follicular fluids were collected from live animals by transvaginal ultrasound guided follicular aspiration. Based on the size of the follicles and stage of the estrous cycle, follicles were classified into 3–5 mm (small), 5–8 mm (medium) and >8 mm (large ovulatory and non-ovulatory). The follicles were classified into healthy and atretic follicles based on nuclear quality as assessed by histological staining. The stage and health status of the follicles were confirmed by the serum progesterone and follicular fluid hormonal concentration. Hormone concentration (serum and follicular fluid) and HDL cholesterol were estimated in follicular fluids, and expression of LH and 3β hydroxysteroid dehydrogenase (3β-HSD) receptors was studied in granulosa cells of different sizes. The study revealed that the presence of CL influences the growth of the follicles of all stages. Significantly high follicular fluid progesterone in the ovulatory follicles indicated that the luteinization of granulosa cells starts even before ovulation of the dominant follicle. The medium-sized follicles with high estradiol concentration may attain dominance, whereas the follicle with high progesterone concentration may undergo atresia. A significantly low HDL cholesterol level in healthy follicles indicates effective steroidogenesis as evidenced by the higher expression of LH and 3β-HSD receptors in the granulosa cells.

Keywords: Atresia, Buffalo, Expression of genes, Follicle development, Follicular fluid hormones.

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Introduction

The riverine buffaloes are an economically important livestock species in many Asian, Mediterranean, and European countries. However, the reproductive efficiency in buffalo is comparatively lower than in cattle curtailing its economic advantage. Attempts to improve the fertility of buffalo require adequate knowledge of biomolecules in the follicular fluid adjunct to follicular development. One of the major constraints in buffalo reproduction is the limited number of follicles and oocyte reserves in the ovary. In buffaloes, the rate of follicular atresia is 70–90% (Palta *et al.,* 1998; Rajesha *et al.,* 2001) which is higher than cattle with 65–70% (Grimes *et al.,* 1987). The granulosa and theca cells from the follicle components synthesize steroid hormones (Hillier *et al*., 1994), and consequently, the follicular fluid steroid hormonal levels influence the fate of the follicles. The corpus luteum (CL) regulates reproduction in mammals (Niswender *et al*., 2000; Diaz *et al*., 2002), and regression of the CL in the absence of conception and the associated discontinuation of progesterone secretion is obligatory for the initiation of a new ovarian follicular wave and commencement of the next estrous cycle.

In bovine, although LH pulses are indispensable for follicle development beyond 9 mm in diameter, the role of LH receptors in follicular selection for dominance and ovulation is still not clear. It is observed that around the time of selection, LH receptor mRNA expression is initiated in granulosa cells (Luo *et al*., 2011). Further, the changes in 3β-HSD expression in granulosa cells have been reported (Zhang *et al.,* 2021), but the expression levels in different stages of follicle development have not been reported in detail. To understand the features of follicular growth, the biochemical and hormonal levels in follicular fluid and expression changes of steroidogenic receptors in granulosa

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cells have to be studied from the follicles of live animals. Hence the present study was conducted in the follicles aspirated using ultrasound-guided aspiration technique (i) to determine the changes in steroid hormones and HDL cholesterol levels and (ii) to assess the expression levels of the LH and 3β-HSD receptors in granulosa cells during the growth and atresia of buffalo ovarian follicles.

Materials and Methods

The experiment was conducted in seven buffaloes maintained at Experimental Livestock Unit, ICAR-National Institute of Animal Nutrition and Physiology (ICAR-NIANP), Adugodi, Bangalore, after approval of the Institutional Animal Ethics Committee. These animals were maintained at standard feeding practices with a body condition score of 3 (Score1-5, 1-lean, 5-fatty). Blood samples were collected daily for two consecutive cycles starting from the day of the experiment up to the end of the experiment. Serum was separated by centrifugation at 1210 g for 15 minutes and stored at -20°C until used for progesterone estimation.

Assessment of Follicle Development Using Transrectal Ultrasonography

A real-time B-mode ultrasound diagnostic instrument (Aloka SSD-500, Japan) equipped with a linear array 5 MHz transducer was used. The probe was adjusted to enable scanning of the ovary from various angles to visualize all the follicles ≥3 mm in diameter. The diameters of all the follicles were measured with the built-in calipers after freezing the ultrasound image. The diameter of non-spherical follicles was calculated by taking the average of the follicle's longest and widest measured points. The number and diameter of all follicles with an antral diameter of ≥3 mm were recorded at each examination. The follicles were then classified based on their diameter as small (3–5 mm), medium (5–8 mm), and large (≥8 mm). The animals were scanned for two estrous cycles. Though the ovary contained many 3–8 mm diameter follicles, only one follicle was observed at above 8 mm diameter. Hence the follicle above 8 mm diameter was considered as a dominant follicle. The dominant follicle was considered non-ovulatory if the animal had a CL in any one of the ovaries. A follicle was considered the ovulatory follicle when the animals showed estrous symptoms with >8 mm follicles in the absence of a CL in both ovaries. The disappearance of the largest follicle seen at previous examination was considered to mark the occurrence of ovulation. The daily growth (in mm) of the follicle was measured and averaged for each size category, small (3–5 mm), medium (5.1–8 mm), and large (>8 mm).

Collection of Follicular Fluid from Live Animals

Ultrasound-Guided Follicular Fluid Aspiration Technique (Leory *et al.,* 2004) was used to collect follicular fluids from live animals with a modification to avoid excessive follicular fluid dilution for more accurate biochemical estimation. A 2 mL sterile syringe was filled with 1 mL normal saline (0.9% NaCl) and connected to an aspiration needle through a capillary tube of around 1 mm diameter. The normal saline was introduced into the lumen of the needle until it appeared at the needle's tip. Then the aspiration set-up was assembled with the probe, and the follicular fluid was aspirated through negative pressure using the syringe.

The follicular fluid was immediately transferred to separate 1.5 mL sterile microcentrifuge tubes at 4°C. The exact volume of the follicular fluid was noted.

The aspirated medium and follicular fluid were transported to the laboratory in a portable cooler (4°C) within 30–45 minutes. The samples were thoroughly mixed, and 5 µL of fluid was taken to clean slide processed for Feulgen's stain to detect apoptotic nuclei. The remaining follicular fluid was subjected to centrifugation at 600 g (Remi cooling centrifuge, Mumbai) at 4°C for 10 min, and the follicular cells and fluid were separated. After centrifugation, the supernatant was aspirated, and the dilution ratio of follicular fluid, i.e., follicular fluid volume (mL) to media volume (1 mL), was calculated. The follicular fluid was stored at -20°C for further process. The granulosa cell pellet was snap-frozen in liquid nitrogen and stored at –80°C.

Classification of the Follicles

Based on the size of the follicle and stage of the estrous cycle, follicles were classified into 3–5 mm (small), 5–8 mm (medium), and >8 mm (large ovulatory and non-ovulatory). The follicles were classified into atretic and nonatretic based on granulosa cells pyknosis (>5%) and nuclear fragmentation (Fig. 1) employing histological studies, hematoxylin, and Feulgen's staining (Sreejalekshmi *et al.,* 2011). In addition to granulosa cell pyknosis and fragmentation, serum hormonal profiles were considered to classify the dominant follicles into ovulatory and non-ovulatory.

Follicular Fluid HDL Cholesterol Estimation

The follicular fluid HDL Cholesterol was estimated as per the procedure given in the kit (Span Diagnostic, Surat, India)

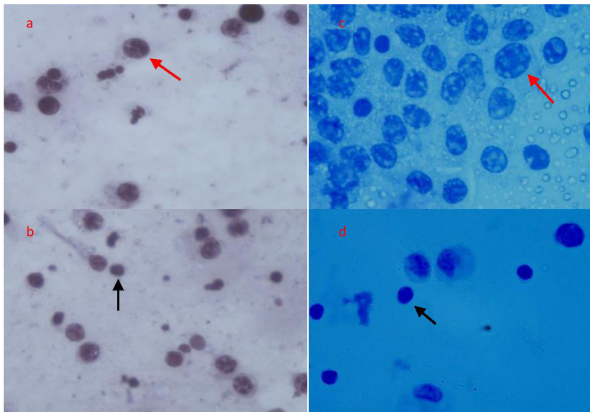

Fig. 1: Histological classification of follicle by Hematoxylin (Fig.: a, b) and Feulgen's staining (Fig.: c, d) of granulosa cells (Red arrow showing mitotic nuclei; Black arrow showing pyknotic nuclei)

Hormones Estimation

Estimation of estradiol-17β and androstenedione in follicular fluid and progesterone (17-a-OH progesterone) in follicular fluid and serum was carried out using RIA kits (Immunotech SAS®, France) as per the procedure given by the manufacturer. The inter-assay and intra-assay variation was less than 15 and 10%, respectively, for all the hormones.

Expression Studies

According to the manufacturer's instructions, the total RNA was isolated from granulosa cells by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The total RNA was eluted in 30 µL RNase-free water. For cDNA synthesis, an aliquot of 1 µg of total RNA from each pool of granulosa cells was independently reverse-transcribed using the superscript-II reverse transcriptase (Invitrogen, USA). The cDNA was stored in -20°C for further use.

Polymerase Chain Reaction

PCR reactions were carried out using 2 µL cDNA as template in 25 µL reaction mixture containing final concentrations of 50 mM $MgCl_2$, 10 mM of dNTP mix, 2.5 µM of forward and reverse primers of 3β-HSD (F: TGTTGGTGGAGGAGAAGG and R: GGCCGTCTTGGATGATCT, NM_174343.3 Product length 360 bp) and LH-R (F: CATCACGGGAAATGTGACTG and R: TAGACCGGGAGGGCTTATTT; XM_010809903.3 Product length 911 bp) (Chromus Biotech, Bangalore) and 5 units Taq DNA polymerase (Fermentas, USA). PCR's thermal cycling profile consisted of initial denaturation at 95°C for 5 minutes, and then 40 cycles of amplification included heating at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 sec. The same was repeated for 40 cycles, and final elongation of the sample was done at 72°C for 5 min. All the reactions were performed in Real-time PCR (RT-PCR) machine (myiQ, Bio-Rad, USA). β-Actin was used as the housekeeping gene, and the expression levels were calculated using DDct method.

Statistical Analysis

The data were analyzed using SPSS.15 software. The values were presented as mean ± SEM. The statistical significance between two groups (atretic rate between the ovary with CL and without CL, hormonal difference between dominant follicle of 5–8 and >8 mm, and expression of genes in atretic and nonatretic follicles) were analyzed using paired "t" test. The statistical difference in follicular fluid hormonal and biochemical parameters among different sizes of the follicles were analyzed using one-way ANOVA. The P-value <0.05 was considered statistically significant.

Results and Discussion

Rate of Growth and Development of the Follicles

The mean growth rate (mm/day) in small (3–5 mm), medium (5–8 mm) and large (>8 mm) size follicles was 0.60 ± 0.1 (range: 0.25 to 1.4), 1.13 ± 0.21 (0.4 to 1.7) and 1.15 ± 0.24 (0.34 to 2.3), respectively. It was found to be significantly ($p < 0.05$) lower in small size follicles than medium and large size follicles. The maximum follicular diameter observed in the dominant follicle was 13.2 mm. The dominant follicle size observed in the present study is in concurrence with the earlier reports in water buffalo (Gaur and Purohit, 2019). The maximum average diameter of ovulatory follicles in buffalo has been reported up to 22.60 ± 3.06 mm in Murrah buffalo (Baruselli *et al.,* 1997).

The present study indicates that the CL favored follicular development in buffalo (Fig. 2) This suggests that the CL prevents the percentage of follicles undergoing atresia in all classes of follicles. However, the influence of CL on follicular development decreased as the follicle grew in size. A positive relationship exists between the CL and follicular development. Approximately 63% of dominant follicles have been reported to be developed in the ovary ipsilateral to the CL (Savio *et al.,* 1988). The ovary not bearing a CL had lower follicular activity than the CL bearing one (Driancourt *et al.,* 1991).

In contrast, Vassena *et al.* (2003) reported no significant intraovarian effects of the CL on characteristics of the dominant follicle, such as growth rate and maximum diameter. Further, Pierson and Ginther (1987) found an inhibitory effect of the CL, whereby the size and number of medium to large follicles were greater in the ovary contralateral to the CL. Thus, the interplay between the CL and the follicle appears to be complex, as reported earlier (Ginther, 2019), and the present study in buffalo suggests positive influence of CL on follicular development.

Serum and Follicular Fluid Hormonal Levels

The serum progesterone concentration (ng/ml) was significantly ($p < 0.05$) lower during estrus (0.27 ± 0.1), in which the follicles aspirated were considered as ovulatory follicles. In addition, the serum progesterone levels were higher during diestrus (3.62 ± 0.4), in which the follicles aspirated were considered atretic dominant follicles. These findings were confirmed using the histological findings from the granulosa cells.

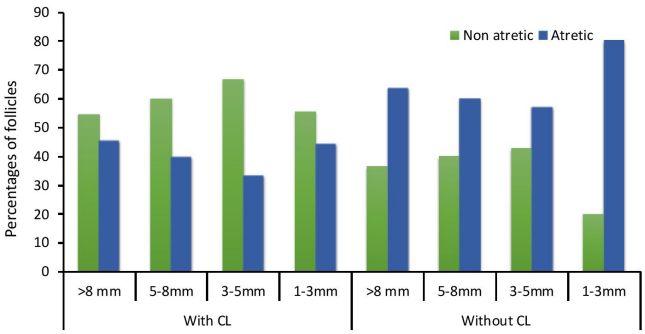

Fig. 2: Influence of CL on follicular development characteristics at different stages of growth in buffalo

The dominant non-atretic follicle in an ovary containing CL had significantly ($p < 0.05$) higher progesterone levels as compared to atretic follicles with and without CL (Table 1). Among the dominant atretic follicles, the progesterone and androstenedione concentrations were non-significantly higher in the follicles associated with CL than without CL.

Medium (5-8 mm) and Dominant (>8 mm) Follicles

In the follicles of 5-8 mm size, the follicular fluid progesterone and androstenedione concentrations (ng/mL) were non-significantly lower in non-atretic follicles (Table 2). The follicular fluid estradiol 17β concentration (ng/mL) was significantly higher in non-atretic follicles than atretic follicles. In the follicles of >8 mm size, the follicular fluid progesterone concentration ng/mL was significantly higher in non-atretic follicles as compared to atretic follicles. The follicular fluid estradiol-17β and androstenedione concentrations ng/mL did not differ significantly between the non-atretic and atretic groups of follicles.

Intrafollicular concentrations of estradiol promote granulosa cells proliferation. Follicular estradiol also enhances the acquisition of luteinizing hormone receptors. In non-atretic antral follicles, the estradiol level in follicular fluid increases as the follicle grows until reaching the preovulatory follicle. After the preovulatory LH surge, which occurs within first 6 hours after standing estrus (Bloch *et al.,* 2006), the estradiol levels start to fall, accompanied by an increase in the progesterone level. In the preovulatory follicle, estradiol ceases to be synthesized by granulosa cells and is replaced by progesterone. The physiological role of progesterone in follicle function is not fully understood. However, this event is indirectly attributable to the LH surge and meiotic maturational events in the follicles. Hence, the maturation of the follicle is presumably influenced by the changes in estradiol/progesterone balance within the follicle.

It is also to be noted that the progesterone concentration ng/mL in the dominant ovulatory follicle was significantly higher than in the atretic follicles of the same class. Similar findings have been reported in buffalo, wherein the progesterone concentration in the follicular fluid of the dominant follicle before hCG injection was 38 ± 7.6 ng/mL and increased dramatically to reach 195 ± 24.6 ng/mL at 24 hours post-hCG injection (Jyotsna and Medhamurthy, 2009). Together these findings suggest that the luteinization of the follicle of >10 mm diameter might be due to preovulatory LH surge.

In the subordinate atretic follicles, higher progesterone concentration was observed. Similar results have been reported in cattle (Echternkamp, 2000). Small antral follicles are androgenic, regardless of whether they are destined to ovulate or become atretic. The concentration of androstenedione and testosterone in follicular fluid decreased gradually, increasing follicle size. In the atretic follicles, the androstenedione levels were higher than healthy follicles probably due to failure in conversion of androgens to estrogen as the androgens synthesized by thecal cells are obligatory substrates for estrogen biosynthesis.

Follicular Fluid HDL Cholesterol Levels

The follicular fluid HDL cholesterol concentration in >8 mm diameter atretic follicle was significantly ($p < 0.05$) higher than non-atretic follicle (Fig. 3). Similar trend was observed in the cholesterol concentration in 5-8 mm follicles, but was non-significantly higher in atretic than non-atretic follicles. In the present study, high-density lipoprotein (HDL) cholesterol was estimated as this is the sole lipoprotein pass through the blood follicular barrier (Brantmeier *et al.,* 1987). It was observed that in buffalo, a significant reduction in HDL cholesterol concentration occurs as the follicles grow from small to medium and large sizes. In contrast, in the bovine follicular fluid, the HDL concentrations increased with follicular maturation (Brantmeier *et al.,* 1987). This discrepancy could be due to species differences or confounding causes.

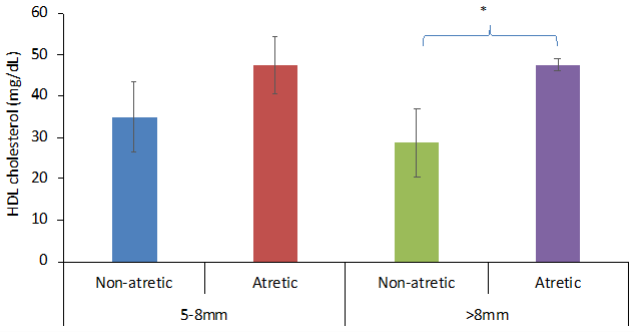

Fig. 3: HDL Cholesterol (mg/dl) in the follicular fluid of different sizes and health status of the follicles.

Table 1: Influence of CL on follicular fluid hormonal profile in dominant (>8 mm diameter) follicles

Hormone concentration (ng/mL)	*Follicles type*		
	Non atretic (without CL)	*Atretic (without CL)*	*Atretic (with CL)*
Progesterone	138.58 ± 47.1[a]	33.1 ± 19.7[b]	78.6 ± 23.9[b]
Estradiol	5.4 ± 2.4	5.4 ± 2.3	3.8 ± 1.3
Androstenedione	3.2 ± 0.3	16.2 ± 5.5	23.9 ± 14.5

Values with different superscript [a,b] differ significantly ($p<0.05$)

Table 2: Follicular fluid hormonal concentration in follicles classified based on size (5-8 mm and >8 mm) and histological features (non-atretic and atretic).

Hormone concentration (ng/mL)	*5-8 mm*		*>8 mm*	
	Non-atretic (n = 4)	*Atretic (n = 6)*	*Non-atretic (n = 4)*	*Atretic (n = 6)*
Progesterone	30.68 ± 3.35	114.68 ± 32.8	302 ± 28.3[a]	127 ± 10.2[b]
Estradiol-17β	20.60 ± 1.5[a]	12.21 ± 2.63[b]	10.37 ± 4.41	16.02 ± 5.7
Androstene-dione	3.09 ± 0.82	4.66 ± 3.03	37.2 ± 4.3	28.8 ± 14.6

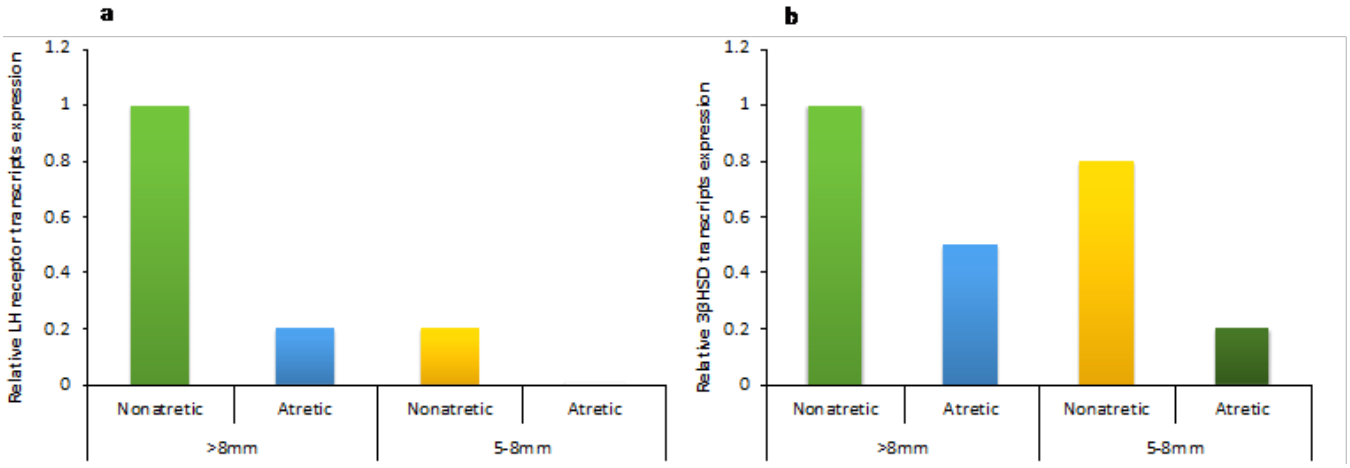

Fig. 4: The relative expression of LH (Fig.: a) and 3β-HSD (Fig.: b) receptors in the granulosa cells of different sizes (>8 and 5-8 mm) follicles

Greater than 95% of the cholesterol in follicular fluid was HDL cholesterol, with a more significant proportion of HDL cholesterol sequestered by estrogen active than estrogen inactive follicles (Wehrman *et al.*, 1991). Perhaps increased exposure to HDL cholesterol *in vivo* enhances granulosa cell maturational rate. The possible reason for significantly low cholesterol concentration in healthy compared to atretic follicles could be due to the metabolism of cholesterol for steroidogenesis.

Expression of LH and 3β-HSD Receptors

The expression of LH and 3β-HSD receptors were higher in the granulosa cells of non-atretic follicles of different sizes (>8 and 5–8 mm) (Fig. 4). The present study revealed that the follicular atresia was associated with reduced expression of gonadotropin receptor mRNAs in the buffalo, which agrees with earlier reports in bovine (Bao and Garverick, 1998). In bovine granulosa cells, the LH receptor mRNA was detected only in healthy follicles of > 9 mm in diameter, but the expression of LH receptor mRNA was not detected in the regressing dominant follicles (Xu *et al.,* 1995). The findings also suggest that LH and 3β-HSD receptors play a significant role in ovarian follicular maturation in buffalo.

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