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Research Article

# Biochemical Characterization of Follicular Fluid in Murrah Buffaloes (*Bubalus bubalis*)

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## Abstract

The aim of the present study was to characterize the composition of buffalo follicular fluid and to correlate the changes with follicular size. Ninety-two ovaries of healthy and non-pregnant adult Murrah buffaloes (n = 46) were collected immediately after slaughter and stored in cold normal saline. Follicular fluid was aspirated from small (3 - 5 mm; n = 124), medium (6 - 9 mm; n = 117) and large (10 - 12 mm, n = 121) ovarian follicles. Follicular fluid samples were analyzed for glucose, cholesterol, creatinine, proteins, triglycerides, bilirubin, urea, acid phosphatase, alkaline phosphatase, GGT (Gamma-glutamyl transferase), progesterone and estradiol. Results showed that follicular fluid concentrations of glucose and cholesterol increased ($P < 0.05$) from small to large follicles. The concentrations of proteins, triglycerides, bilirubin, urea, acid phosphatase, alkaline phosphatase and GGT decreased as the follicle became larger. The levels of progesterone in follicular fluid increased with the increase in follicular diameter. Lower levels of estradiol 17β were observed in small and large follicles as compared to medium follicles. It can be concluded that the composition of follicular fluid changes from small to large follicles and therefore, may affect the quality of oocyte and granulosa cells of buffalo.

***Keywords:*** *Biochemical; Characterization; Follicular Fluid; Buffalo*

## Introduction

Buffaloes are the primary dairy animals in many developing countries across Asia and form the backbone of India's dairy industry, accounting for over 60% of the nation's total milk production. India alone produces nearly two-thirds of the world's buffalo milk and around half of the global buffalo meat supply, underscoring its pivotal role in the global buffalo economy [1]. Despite their significance, buffalo productivity faces several reproductive challenges that hinder improvement through artificial breeding. These include silent heat, delayed sexual maturity, weak estrus expression, irregular estrous cycles, seasonal breeding patterns, anestrus, low conception rates, extended postpartum intervals, and repeat breeding [2-5]. Silent estrus, in particular, is a major obstacle in understanding reproductive physiology and implementing effective assisted reproductive technologies in buffaloes [6-9]. Follicular fluid (FF) molecules, and their

increase or decrease, can contribute to appropriate follicular growth and oocyte maturation, thus being related to female infertility conditions [10]. Follicular fluid is the avascular component of the ovary separated from blood follicle barrier. It constitutes not only the transudate of serum but also the metabolic substances produced locally from the follicular theca and granulosa cells [11,12]. Follicular fluid provides a suitable micro-environment for growth, development and maturation of oocytes [13]. It also protects the oocyte against proteolysis and having buffering tendency during adverse blood conditions [12]. The metabolic activity of the cells of the follicular wall and composition and quantity of the follicular fluid changes during growth of follicle [11,12] and hence a different biochemical composition of the follicular fluid in different size follicles could be expected. Follicular fluid contains specific constituents such as proteins, amino acids, enzymes, hormones, electrolytes and salts which play a crucial role in physiological, biochemical and metabolic aspects of the nuclear and cytoplasmic maturation of oocytes [14].

The characteristics of follicular fluid are affected by the physiological condition of donor cows and state of the follicles (size, growing phase and atretic phase). The components of the follicular fluid play vital, but as yet not fully defined, role in the complex mechanisms underlying follicular growth, development and maturation. The composition of follicular fluid serves as useful index for the requirement of oocyte and follicular growth. The biochemical composition of follicular fluid has been studied in cattle [15], goat [16], pig [17] and sheep [18]. Although protein, glucose and cholesterol concentrations have been studied in buffalo [19], detailed biochemical analysis of follicular fluid is completely lacking. To understand some of the basic biochemical changes that accompany follicular development, the present study was undertaken to delineate the changes in concentrations of metabolites (glucose, protein, triglyceride, cholesterol, creatinine, and urea), enzymes (acid phosphatase, alkaline phosphatase and GGT) and steroid hormones (progesterone and estradiol) of follicular fluid in relation to size of follicle in buffalo.

## Materials and Methods

### Collection of ovaries and processing of follicular fluid

Forty-six Murrah buffaloes in good health and with normal reproductive tracts upon macroscopical examination after slaughter were used for this study. Ovaries (n = 92) were collected immediately after slaughter and transported to the laboratory in 0.9% chilled (4°C) normal saline supplemented with gentamycin (50 mg/ml). Ovaries with normal functional corpus luteum and with more than one large follicle were selected. The ovarian follicles were graded into three groups: small (3 - 5 mm; n = 124), medium (6 - 9 mm; n = 117) and large (10 - 12 mm, n = 121). Antiproteolytic agents (PMSF: 25 mg/ml) and anti-clotting factor (Heparin: 25 IU/ml) were added to the follicular fluid. Cell debris was removed by centrifuging follicular fluid at 5000xg for 30 minutes. The supernatant after filtering through 0.2 mm filter were stored at -80°C until analysis except for estimation of enzymes which were estimated in fresh sample.

### Biochemical analysis

The determination of metabolites (glucose, total protein, cholesterol, triglycerides, creatinine, bilirubin and urea) and enzymes (acid phosphatase, alkaline phosphatase and GGT) was done by using clinical analyzer (Dade Bahering, Germany). Commercial kits used for estimation of glucose, total protein, cholesterol, triglycerides, creatinine, urea, bilirubin, acid phosphatase, alkaline phosphatase and GGT were procured from BIORAD (USA). All measurements were carried out according to the manufacturer's instructions. Normal saline (0.9%) was taken as control. Minimum detectable concentrations of triglycerides, glucose, cholesterol, urea, creatinine and bilirubin were 10.3, 1.5, 0.65, 0.68, 0.07 and 0.005 mg/dL, respectively. Minimum detectable concentration of protein was 0.26 g/dL. Minimum detectable concentrations of acid phosphatase, alkaline phosphatase and GGT were 18.6, 24.1 and 0.8 U/L, respectively. The intra and inter assay coefficients of variation for all analyses were below 7%. The concentrations of progesterone and estradiol 17β were quantified by the RIA kits procured from Immunetech, France. The sensitivity of the progesterone assay was 0.08 ng/ml and the inter-assay and intra-assay coefficients of variation were 9.0 and 5.8%, respectively. The sensitivity of the estradiol assay was 4.5 pg/ml and the inter-assay and intra-assay coefficients of variation were 11.2 and 12.1%, respectively.

### Statistical analysis

Results are expressed as mean ±SEM. Concentrations of each factor in three different classes of follicles were compared by using linear regression model. The data were analyzed by using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA).

## Results

The concentrations of metabolites (glucose, cholesterol, creatinine, triglyceride, protein and urea), enzymes (acid phosphatase, alkaline phosphatase and GGT) and hormones (progesterone and oestradiol 17β) in follicular fluid from small, medium and large follicles are presented in table 1. The concentrations of glucose were significantly ($P < 0.05$) higher in large follicles as compared to those in small and medium size follicles. Fluid from small follicles had lower cholesterol ($P < 0.05$) and creatinine concentrations than those from medium and large follicles. The concentrations of triglycerides were significantly ($P < 0.05$) higher in small follicles as compared to those in medium and large size follicles. Follicular fluid concentrations of protein and bilirubin were higher in small follicles than those of medium and large follicles. The concentrations of urea were significantly ($P < 0.05$) higher in small follicles as compared to those in medium and large follicles. Acid phosphatase and alkaline phosphatase activity were significantly ($P < 0.05$) higher in small follicles than in medium and large follicles. A significant difference ($P < 0.05$) in GGT activity was observed between the small and large follicles. Progesterone levels increased ($P < 0.05$) from small follicles to medium follicles and then to large follicles. However, oestradiol 17β concentrations were higher in medium sized follicles compared to small and large sized follicles.

| Composition | Small follicle | Medium follicle | Large follicle | Overall |
|---|---|---|---|---|
| **Metabolites** | | | | |
| Triglycerides (mg/dl) | $365.27 \pm 0.05^{a}$ | $307.39 \pm 2.38^{b}$ | $248.66 \pm 0.70^{c}$ | $15.54 \pm 4.66$ |
| Glucose (mg/dl) | $35.39 \pm 0.49^{a}$ | $57.59 \pm 0.68^{b}$ | $78.03 \pm 0.51^{b}$ | $56.81 \pm 0.98$ |
| Cholesterol (mg/dl) | $15.58 \pm 0.08^{a}$ | $24.83 \pm 0.10^{b}$ | $33.28 \pm 0.10^{c}$ | $24.48 \pm 0.39$ |
| Urea (mg/dl) | $20.68 \pm 0.04^{a}$ | $17.85 \pm 0.05^{b}$ | $16.40 \pm 0.04^{c}$ | $18.33 \pm 0.10$ |
| Total protein (g/l) | $7.38 \pm 0.03$ | $6.59 \pm 0.02$ | $6.30 \pm 0.02$ | $6.77 \pm 0.03$ |
| Creatinine (mg/dl) | $1.27 \pm 0.01$ | $1.54 \pm 0.01$ | $1.80 \pm 0.01$ | $1.53 \pm 0.01$ |
| Bilirubin (mg/dl) | $0.17 \pm 0.01$ | $0.15 \pm 0.03$ | $0.12 \pm 0.02$ | $0.15 \pm 0.01$ |
| **Enzymes** | | | | |
| Acid phosphatase (U/L) | $435.30 \pm 0.08^{c}$ | $322.42 \pm 0.05^{b}$ | $241.57 \pm 0.05^{a}$ | $334.06 \pm 4.21$ |
| Alkaline phosphatase (U/L) | $365.27 \pm 0.05^{c}$ | $307.39 \pm 2.38^{b}$ | $248.67 \pm 0.06^{a}$ | $307.59 \pm 2.63$ |
| Gamma-glutamyl Transpeptidase (U/L) | $25.98 \pm 0.65^{a}$ | $23.25 \pm 0.59^{ab}$ | $18.23 \pm 0.04^{b}$ | $22.50 \pm 3.27$ |
| **Hormones** | | | | |
| Progesterone (ng/ml) | $6.15 \pm 0.43^{a}$ | $13.07 \pm 1.83^{b}$ | $17.82 \pm 2.48^{ab}$ | $12.35 \pm 2.63$ |
| Oestradiol 17β (pg/ml) | $85.69 \pm 3.78^{a}$ | $274.33 \pm 7.95^{b}$ | $200.23 \pm 5.14^{ab}$ | $186.92 \pm 8.11$ |

***Table 1:*** *Concentrations (means ± SEM) of metabolites, enzymes and steroid hormones in buffalo follicular fluid in relation to follicular size.*

*Values with different superscripts in the same row differ significantly ($P<0.05$) (c >b>a).*

## Discussion

To the best of our knowledge this is the first study to report the detailed biochemical profiles of follicular fluid in buffalo. The result of the present study indicate that glucose concentration increased as the follicle diameter increased. These results are in agreement with earlier report in cattle [20-22], sheep [18], goats [23] and buffalo [24]. Glucose plays an important role in ovarian metabolism since it is

the major energy source for the bovine, mouse and human ovary, metabolized by the ovary through anaerobic pathways, leading to lactate formation [25-27]. It has been observed that glucose metabolism is less intensive in large follicles compared to small ones, resulting in a lower consumption of glucose from follicular fluid [21]. In large follicle a relatively smaller number of granulose cells consumed glucose from follicular fluid [28]. Probably the larger follicles can filter and reserve greater concentrations of glucose from circulation for utilization in their development to the graafian follicle [11,22]. Furthermore, increased permeability of the blood-follicle barrier during follicular growth might be another reason. The total protein content of the follicular fluid decreased as the follicle grew. Our results agree with earlier observation in sheep [29], goats [30], cattle [31] and buffalo [19]. It was reported that as the follicular fluid volume increased with an increase in follicle size the protein concentration declined [16,30].

The concentration of cholesterol in the present study increased with increase in follicular size. These reports agree with earlier report in goat [16,23] and cattle [31]. Cholesterol present in follicular fluid is bound to the high density-lipoprotein fraction (HDL) because other cholesterol containing lipoprotein fraction is so large that they could not pass the blood-follicle barrier [32,33]. The higher cholesterol content in large follicles might be due to increased permeability of the follicular wall allowing the entrance of the larger HDL fraction [34,35]. Cholesterol was the precursor for synthesis of steroids and the follicular fluid contained only high-density lipoprotein. The avascular granulosa cells of the follicles depended on the cholesterol from high density lipoprotein derived from plasma by crossing the basement membrane of granulose cells [16]. However, our results differed from that of Kor., *et al*. [22] wherein follicular fluid cholesterol gradually decreased with increase in follicular size in dairy cows. Our results also differed from those of in pigs [17] and buffalo [19,24]. The decreased cholesterol level in large follicles could be attributed to the conversion of cholesterol to steroid hormones during steroidogenesis. Follicular fluid concentration of triglyceride in small follicles was significantly higher as compared to that of medium and large follicles. Our results are supported by the similar findings in cattle [21,22] and buffalo [24]. The continuous and rapid use of triglycerides might have caused the lower concentrations in larger follicles as compared with smaller [17,22]. The triglyceride concentrations were higher in small follicles and might be an alternate source of energy for the cells [22,36]. The high concentrations of triglyceride might be due to inability of triglyceride to pass through the follicular membrane [37]. The concentrations of urea decreased as the follicle grew and lower urea concentration favour oocyte development [21]. Kor., *et al.* [22] reported that there were significant differences between follicular fluid concentrations of glucose and cholesterol while non significant difference was observed for total proteins, urea and creatinine among the follicles of different size in dairy cattle. Similar results for the follicular fluid urea and creatinine concentration were also documented in case of buffaloes [24].

Phosphatases and lysosomal enzymes catalyze various reactions in the body and associated with the active transport of phosphatase across the cell membrane, synthesis of protein and DNA turnover in nucleus [38]. The concentration of acid and alkaline phosphatases was greatest in the small follicle and decreased with increase in follicular size. These results are in agreement with earlier report in cattle [39] and goats [16,40]. However, there were no significant differences in acid and alkaline phosphatase activity among different follicles [41]. High concentration of acid phosphatase in follicular fluid of small antral follicles might limit their ability to respond to gonadotropin stimulation [39]. The higher alkaline phosphatase activity in small follicles might be due to a progesterone and androgen dominant environment that exists in small follicles. The decrease in alkaline phosphatase activity in the large size follicle could be due to the shift in the follicular hormonal milieu from androgen to estrogen dominant with the development of the follicle. The progesterone levels in follicular fluid tended to increase significantly with the advancement in development of follicles. Our results are in agreement with earlier report in sheep and goat [30,38]. This could be attributed to higher estradiol level in medium follicles which initiates LH receptor for the action of LH to cause luteinization of the granulosa cells to change the 4-delta pathway of steroidogenesis to 5-delta pathway. The estradiol levels were found to higher in medium follicles in comparison to small and large size follicles. The lower concentrations of estradiol in large follicles could be due to greater secretion of this steroid into blood circulation or due to lower rate of its synthesis in

theca cells. The peak level of estradiol in circulation on the day of estrus when ovarian follicles are in preovulatory or ovulatory stage also substantiated greater secretion of this hormone into blood circulation [42,43].

## Conclusion

We found that the concentrations of glucose, cholesterol and progesterone increased while those of urea, triglyceride, protein, bilirubin, acid phosphatase, alkaline phosphatase and GGT decreased as the follicle size increased. Characterization of follicular fluid components has the potential for optimization of the culture conditions of oocytes and granulosa cells in buffalo.

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