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# Comparative analysis of biochemical, hormonal, and mineral compositions of preovulatory and cystic ovarian follicles in buffalo during the non-breeding season

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## Research Article

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## Summary

This study is a comparative analysis of the biochemical, hormonal, and mineral compositions of follicular fluid in preovulatory and cystic follicles of water buffalo (*Bubalus bubalis*). In total, reproductive tracts from 215 buffalo along with intact ovaries were collected randomly from an abattoir. The incidence of cystic conditions found in this study was 3.72% (8/215), involving the right ovary in 62.5% of instances and the left ovary in 37.5% of instances during the non-breeding season. Follicular fluid was aspirated from preovulatory follicles (12–15 mm diameter, oestrogen-active, follicular phase or stage IV corpus luteum on one of the two ovaries, $n = 10$) and cystic follicles (at least 20 mm diameter, no corpus luteum on any one of the two ovaries, $n = 8$). The follicular fluid samples were assayed for biochemical components (uric acid, creatinine, blood urea nitrogen, cholesterol, total protein, glucose, ascorbic acid, and alkaline phosphatase), hormones (progesterone, estradiol, and insulin), and minerals (calcium, magnesium, phosphorus, copper, zinc, and cobalt). Cystic follicles had greater ($P < 0.05$) concentrations of creatinine, blood urea nitrogen, cholesterol, progesterone, copper, zinc, and cobalt, and lesser ($P < 0.05$) concentrations of uric acid, glucose, ascorbic acid, estradiol, insulin, calcium, magnesium, and phosphorus compared with preovulatory follicles. These results indicated the marked differences in follicular fluid composition between preovulatory and cystic follicles in buffalo. Some of the changes were indicative of oxidative stress and disturbed steroidogenesis, two important mechanisms shown to be associated with cystic ovarian disease in various species. Further studies are warranted to investigate whether these differences are directly or indirectly involved in the formation of cystic follicles or are mere manifestations of the condition.

## Introduction

Buffalo is an integral part of agriculture in Asian countries. In India, buffaloes contribute immensely in the form of milk, meat and draft power and are considered a reliable 'living bank' to serve the immediate needs of rural people, who constitute the vast majority of the total population in the country. However, buffalo reproduction has certain inherent challenges such as late sexual maturity, seasonal reproductive pattern, anoestrus, silent oestrus, and a long intercalving interval (Nanda *et al.*, 2003; Das and Khan, 2010; Phogat *et al.*, 2016). Ovarian cysts are an important cause of infertility in the species, leading to extended calving intervals and great economic losses (Khan *et al.*, 2011; Teshome *et al.*, 2016). There are several definitions used to describe ovarian follicular cysts, and the traditionally accepted definition is that they are 'follicular structures of 2.5 cm or larger that persist for a variable period in the absence of a corpus luteum' (Youngquist and Threlfall, 2007). A 2003 study by Hatler *et al.* (2003) pointed out that follicles typically ovulate at 17 mm in diameter, so follicles that persist at that diameter or greater may be considered to be 'cystic'. Similarly, Vanholder *et al.* (2006) suggested that cystic ovarian follicles (COF) should be defined as 'follicles with a diameter of at least 2 cm that are present on one or both ovaries in the absence of any luteal tissue and that clearly interfere with normal ovarian cyclicity'.

The incidence of ovarian cysts in dairy cattle may vary from 2.7% to 15.1% (Cattaneo *et al.*, 2014) or from 6% to 30% (Garverick, 1997) with peak incidences between the interval of 14 and 40 days postpartum (López-Gatius, 2003; Yimer *et al.*, 2018). The incidence of follicular cysts

varies between 2.8% (buffalo heifers) to 4.2% (buffalo cows). This condition has been reported to be unilateral in most cases, involving mostly the right ovary (60.7%) compared with the left ovary (39.3%; Luktuke and Arora, 1972). Various studies have pointed out that the basic reason for cyst formation is a failure of the preovulatory luteinizing hormone (LH) surge to occur at the appropriate time in follicular maturation (Whitlock *et al.*, 2011; Yeo and Colledge, 2018). Follicular fluid plays a major role in autocrine and paracrine regulation and also in the physiological, biochemical and metabolic aspects of nuclear and cytoplasmic maturation of the oocyte and the process of ovulation (Hafez *et al.*, 2000). Because of its intimate contact with the oocyte and granulosa cells, follicular fluid can serve as a good index for the functional status of ovarian follicles (Da Broi *et al.*, 2018; BorŞ and BorŞ, 2020). Changes in the biochemical composition of follicular fluid may influence steroidogenesis, oocyte maturation and quality, ovulation, and transport of the oocyte to the oviduct, and preparation of the follicle for subsequent corpus luteum formation and function (Da Broi *et al.*, 2018). Therefore, the follicular fluid composition of follicular cysts may provide valuable insight into the pathogenesis of the cystic ovarian disease. The objective of the present study was to examine changes in the follicular fluid's biochemical, hormonal, and mineral profiles in cystic follicles of water buffalo.

## Materials and methods

In total, 215 buffalo reproductive tracts, along with intact ovaries, were collected randomly from a local abattoir during the non-breeding months (May to September) and transported to the laboratory on ice within 30 min of collection. Follicles exceeding a diameter of 20 mm, present in ovaries lacking a corpus luteum, were designated as follicular cysts (Khan *et al.*, 2011). Follicular fluid was aspirated from eight cystic follicles collected from eight buffaloes and stored at −20°C pending analysis. For comparison, follicular fluid samples from 10 preovulatory follicles (12–15 mm in diameter, oestrogen-active, collected during the follicular stage), each from a separate animal, were used.

### Biochemical, hormonal and mineral analysis

#### Ascorbic acid estimation

Ascorbic acid was estimated following the method of Zannoni *et al.* (1974). This method is based on the reduction of ferric iron by ascorbic acid followed by the formation of a complex of the ferrous iron product and α,α′-dipyridyl. Follicular fluid (60 µl) was mixed with 7.2 µl of 40% trichloroacetic acid (TCA) and allowed to stand on ice for 10 min, followed by centrifugation at 10,000 *g* for 15 min. The acidified supernatant was aspirated using a micropipette, and 20 µl was transferred to wells of a microtest plate. This was followed by sequential addition of 10 µl of 85% orthophosphoric acid, 80 µl of 1% α,α′-dipyridyl and 10 µl of 3% ferric chloride, with thorough mixing after the addition of each reagent. The plate was allowed to stand at room temperature for 15 min, and absorbance was measured using a microplate reader at 525 nm. The concentration of ascorbic acid in the samples was determined from a standard curve drawn using known standards and using GraphPad Prism software version 3.0.

#### Estimation of follicular fluid hormones

Progesterone ($P_4$) was assayed using an commercial diagnostic ELISA kit (Labserv Diagnostics Ltd, UK) following the manufacturer's instructions. The intra-assay and interassay coefficients of variance were 4% and 9.3%, respectively, and the minimum sensitivity was 0.05 ng/ml.

Estradiol and insulin were assayed in follicular fluid samples by radioimmunoassay (RIA) using a commercial RIA kit Beckman Coulter (Immunotech, France). The minimum sensitivity of the assay for $E_2$ was 6 pg/ml and the intra-assay and interassay coefficients of variation were 12.1 and 11.2%, respectively. The minimum analytical and functional sensitivity for insulin were 0.5 µIU/ml and 1.04 µIU/ml, respectively, and the intra-assay and interassay coefficients of variation were 4.3% and 3.4%, respectively.

#### Determination of other biochemical constituents

Follicular fluid samples were assayed for glucose, cholesterol, total protein, alkaline phosphatase, blood urea nitrogen (BUN), creatinine, and uric acid by spectrophotometric methods using commercial diagnostic kits (Span Diagnostics India Ltd) and adopting standard procedures, as per the manufacturer's instructions.

### Follicular mineral estimation

#### Estimation of macrominerals (calcium, phosphorus, and magnesium)

Follicular fluid calcium (Ca), phosphorus (P), and magnesium (Mg) were estimated using commercial diagnostic kits (Span Diagnostics, India Ltd, Surat, India) adopting the procedure recommended by the manufacturer.

#### Estimation of microminerals (copper, cobalt, and zinc)

Copper (Cu), cobalt (Co) and zinc (Zn) were estimated in follicular fluid samples using an atomic absorption spectrophotometer (Model No. AAS 4141, Electronic Corporation of India) after acid digestion of the samples. Follicular fluid was digested following the procedure described by Kolmer *et al.* (1951). In brief, 0.5 ml of follicular fluid in a digestion test tube was mixed with 1.5 ml of concentrated $HNO_3$, kept at room temperature overnight, and followed by digestion on low heat (70–80°C) using a heat bench (digestion bench) until the volume of sample was reduced to 0.5 ml. To this, 3 ml of double acid mixture (3 parts concentrated $HNO_3$ and 1 part 70% perchloric acid) were added, and low heat digestion continued until the digested sample became clear and watery and emitted white fumes. If needed, the addition of a 3 ml double acid mixture followed by low heat digestion was repeated a couple of times. Further heating continued to reduce the volume to ~0.5 ml. The final volume of the filtrate was made up to 5 ml with triple distilled deionized water. The final concentration was calculated by multiplying the dilution factor by the value obtained from atomic absorption spectroscopy (AAS). The characteristic wavelengths were element specific and accurate to 0.01–0.1 nm. To provide the element specific wavelengths, a light beam from a lamp whose cathode was made of the element being determined was passed through a flame (Table 1).

### Statistical analysis

Differences in mean concentrations of the assayed intrafollicular components between the preovulatory and cystic groups were analyzed for statistical significance using the independent sample *t*-test. Data are reported as mean ± standard error of the mean (SEM) unless otherwise stated.

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**Table 1.** Operating conditions for AAS 400 for microelements

| Element | Lamp type | Lamp current (mA) | Wavelength (nm) | Slit width (nm) | Flame type (gases) | Linear working range (ppm) |
|---|---|---|---|---|---|---|
| Copper | C-HCL | 15 | 324.75 | 2.7/0.8 | Air–Acetylene | 0–1.50 |
| Zinc | C-HCL | 15 | 213.86 | 2.7/1.8 | Air–Acetylene | 0–0.30 |
| Cobalt | C-HCL | 15 | 240.73 | 2.7/1.8 | Air–Acetylene | 0–1.50 |

**Table 2.** Concentrations (mean ± standard error of the mean (SEM)) of hormones in follicular fluid of cystic and preovulatory follicles in water buffalo

| Parameter | Cyst ($n = 8$) | Preovulatory ($n = 10$) | Significance level ($P$-value) |
|---|---|---|---|
| Progesterone (ng/ml) | 46.34 ± 0.85 | 33.15 ± 2.25 | 0.001 |
| Estradiol (ng/ml) | 6.64 ± 1.22 | 12.60 ± 0.58 | 0.000 |
| Insulin (μIU/ml) | 0.80 ± 0.04 | 1.55 ± 0.23 | 0.010 |

## Results

The incidence of cystic disease in this study was 3.72% (8/215), involving the right ovary in 62.5% of instances and the left ovary in 37.5% of instances. As shown in Table 2, cystic follicles showed a significantly higher concentration of progesterone and a lower concentration of estradiol and insulin compared with normal preovulatory follicles.

The mean follicular fluid concentrations of different biochemical constituents and mineral profiles in follicular cysts and preovulatory follicles are presented in Table 3. Follicular cysts had a greater concentration of BUN, uric acid, creatinine, and cholesterol concentrations compared with preovulatory follicles. In contrast, ascorbic acid and glucose concentrations were less than in preovulatory follicles. Differences in total protein and alkaline phosphatase concentrations between the two groups were not significant. Follicular cysts had lower calcium and magnesium concentrations and higher copper and zinc concentrations compared with preovulatory follicles. However, the difference in phosphorus and cobalt concentrations between the two groups was not significant (Table 3).

**Table 3.** Concentrations (mean ± standard error of the mean (SEM)) of biochemical constituents and minerals in follicular fluid of cystic and preovulatory follicles in water buffalo

| Parameter | Cyst ($n = 8$) | Preovulatory ($n = 10$) | Significance level ($P$-value) |
|---|---|---|---|
| Uric acid (mg/dl) | 1.64 ± 0.13 | 2.03 ± 0.07 | 0.010 |
| Creatinine (mg/dl) | 2.85 ± 0.44 | 0.50 ± 0.14 | 0.000 |
| BUN (mg/dl) | 15.42 ± 0.63 | 13.49 ± 0.66 | 0.041 |
| Cholesterol (mg/dl) | 40.90 ± 2.78 | 30.11 ± 3.35 | 0.025 |
| Total protein (g/dl) | 2.26 ± 0.30 | 2.09 ± 0.09 | 0.573 |
| Glucose (mg/dl) | 25.07 ± 2.33 | 35.76 ± 3.07 | 0.019 |
| Ascorbic acid (μg/ml) | 8.23 ± 1.96 | 13.88 ± 1.06 | 0.009 |
| ALP (IU) | 156.78 ± 54.65 | 164.39 ± 18.93 | 0.883 |
| Calcium (mg/dl) | 8.03 ± 0.29 | 11.33 ± 0.65 | 0.001 |
| Magnesium (mg/dl) | 3.57 ± 0.36 | 4.61 ± 0.17 | 0.004 |
| Phosphorus (mg/dl) | 8.50 ± 0.65 | 12.95 ± 0.91 | 0.002 |
| Copper (μg/ml) | 27.97 ± 5.51 | 8.27 ± 1.42 | 0.002 |
| Zinc (μg/ml) | 17.27 ± 2.68 | 5.00 ± 0.11 | 0.000 |
| Cobalt (μg/ml) | 2.85 ± 0.44 | 2.00 ± 0.02 | 0.048 |

## Discussion

The incidence of cystic disease was almost similar to that of a previous report by Luktuke and Arora (1972). Follicular fluid originates mainly from the peripheral plasma by transudation and secretion from the follicular cells. Its composition reflects the changes in the secretory processes of the granulosa layer and theca interna, and changes in the components of plasma due to physiological or pathological processes (Bertevello *et al.*, 2020). Therefore, there is a vital balance between endocrine and biochemical constituents of follicular fluid and normal follicular development (Gerard *et al.*, 2002; Da Broi *et al.*, 2018). Changes in follicular fluid composition can be indicative of pathological conditions, including cystic follicles.

The availability of ascorbic acid in buffalo follicular fluid and its role in normal follicular development in the species has been studied previously (Meur *et al.*, 1999). Ascorbic acid plays an essential role in steroidogenesis, follicular membrane remodelling, collagen synthesis, and antioxidant systems (Luck *et al.*, 1995; Thomas *et al.*, 2001). In women there is sequestration of ascorbate in the follicular fluid to facilitate rapid follicular expansion during the approach to ovulation and/or post-ovulatory steroidogenesis (Aten *et al.*, 1992; Murray *et al.*, 2001). Similar findings have been observed across bovine follicular development (Pascu *et al.*, 1970). A lower level of ascorbic acid in the follicular cysts might be due to a rapid increase in fluid volume, and it is possible that this lower concentration predisposes the follicle to free radical injury and impaired steroidogenesis.

Brito and Palmers (2004) hypothesized various mechanisms that could potentially lead to cyst formation, although a commonly accepted hypothesis is that it results from failure of the hypothalamus to trigger the preovulatory surge of LH in response to estradiol (López-Gatius *et al.*, 2002; Whitlock *et al.*, 2011; Yeo and Colledge, 2018). It has also been postulated that greater concentrations of $P_4$ are possibly responsible for this hypothalamic defect (Silvia *et al.*, 2002; Robinson *et al.*, 2006). The presence of significantly higher progesterone and lower oestrogen concentrations in follicular cysts compared with preovulatory follicles in the present study supports

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the view that excess $P_4$ and, subsequently, low estradiol disturb the onset of the LH surge, resulting in the persistence of follicles as follicular cysts. However, the reason for this abnormal increase in $P_4$ and low estradiol is not yet clear and needs to be investigated in future studies.

Insulin stimulates the proliferation of follicular cells (Spicer and Stewart, 1996) and oestradiol-17β production in the granulosa cells (Butler *et al.*, 2004; Da Broi *et al.*, 2018; BorŞ and BorŞ, 2020) and is also involved in the selection of the dominant follicle towards ovulation (Fortune *et al.*, 2004; Walters *et al.*, 2006). Insulin, together with increasing oestradiol levels, stimulates the dominant follicle to reach final maturation, which in turn leads to LH surge and ovulation (Kawashima *et al.*, 2007). Therefore, low insulin levels cause insufficient oestradiol-17β production. The reduced oestradiol-17β production disrupts the hypothalamic–pituitary–gonadal axis. This finally results in an aberrant LH surge and the subsequent development of a cystic follicle (Braw-Tal *et al.*, 2009). The findings of the current study support this notion, as low levels of insulin and oestradiol were noted in cystic follicles.

Glucose plays an important role in ovarian metabolism as it is the major energy source. A lesser concentration of glucose in the cystic follicles compared with the preovulatory follicles can be attributed to the lesser insulin concentration (Spicer and Echternkamp, 1995) and also the active influx of the molecule in the preovulatory follicle (Landau *et al.*, 2000) and/or the dilution due to excessive increase in follicular fluid volume. Cholesterol in follicular fluid is derived from two sources, cellular synthesis from acetate and uptake from plasma lipoprotein (Alkalby *et al.*, 2012). Cholesterol, present in follicular fluid, is bound to the high-density lipoprotein fraction (HDL); the low-density lipoprotein (LDL) fraction is too large to pass through the blood–follicle barrier (Bauchart, 1993; Kim *et al.*, 2017). The significantly higher total cholesterol concentration in cystic follicles might be attributed to the reduced conversion of cholesterol to steroid hormones, oestrogen and progesterone during steroidogenesis, and/or as size increases there may be chances of increased permeability of the cystic follicular wall because of free radical injuries and prolonged persistency of cystic follicles, as it has been reported that larger sized follicles have greater permeability compared with small-sized follicles, permitting the entrance of the larger HDL fraction (Wehrman *et al.*, 1991; Bloom *et al.*, 2014).

It is well established that high-yielding cows generally suffer from NEB and are more prone to the development of cystic conditions (López-Gatius *et al.*, 2002; Hooijer *et al.*, 2003). In NEB conditions, the cow's body is conditioned for a low energy supply. Therefore, both fatty acids and amino acids are consumed and urea increases. High levels of BUN in cystic cows have also been reported by various workers (Lak, 2007; Yousefdoost *et al.*, 2012). Lak (2007) suggested that high amounts of BUN in cystic cows may be related to interrupted protein metabolism. A very high correlation for urea between follicular fluid and blood serum was reported by Leroy *et al.* (2004). Therefore, the increased concentration of urea nitrogen in cystic follicular fluid compared with normal preovulatory follicles possibly reflects elevated serum urea levels in the affected animals.

Creatinine is a waste product of creatine and phosphocreatine, a supplier of energy to the muscle, and is found almost exclusively (90%) in skeletal muscle tissues and formed during normal muscle contraction, and level in the blood remains unchanged from day to day. Cows with cystic ovarian disease frequently showed nymphomaniac behaviour and also, in chronic cases, the development of masculine characteristics. All these increased activities and muscle mass may lead to an increased level of creatinine in the serum of cystic cows, which might be reflected in the follicular fluid, as many serum biochemical metabolites have a very strong correlation with their follicular fluid concentration.

The observations on total protein concentration were similar to those reported earlier by various workers. The total protein content of the follicular fluid did not differ between follicle classes in dairy cows, described by Leroy *et al.* (2004), in buffaloes by Arshad *et al.* (2005) and Abd Ellah *et al.* (2010) and in cystic buffaloes by Khan *et al.* (2011). In the present study, concentrations of uric acid were significantly low in cystic follicular fluid compared with the preovulatory follicle. There was no significant difference in the concentration of alkaline phosphatase in the cystic follicle compared with the preovulatory follicle, which is similar to that reported previously by Khan *et al.* (2011).

Blood calcium concentration varies across the oestrous cycle, being maximum at oestrus in cattle (Burle *et al.*, 1995), indicating the critical role of this ion during the follicular phase, especially in and around oestrus. Calcium content of the follicular fluid increases with the advancement of the follicle from the early follicle to the ovulatory stage, providing an optimum osmotic gradient across the follicular wall necessary for the movement of water from the blood to the antrum (Kalmath and Ravindra, 2007). Furthermore, it was suggested that calcium is involved in the disruption of cumulus cell cohesiveness by regulating the gap junctions between the cells (Peracchia, 1978). Moreover, the calcium-induced increase in the plasmin activity is believed to be a factor in weakening the follicular wall (Espey, 1994) therefore initiating the process of ovulation. The lesser calcium concentration in cystic follicles could be one of the factors causing failure of ovulation, therefore leading to their persistence.

Release of LH–RH occurs in a Mg-dependent manner (Burrows and Barnea, 1982). In an *in vitro* assembly, the same authors reported that Mg and ATP acted jointly to facilitate the release of LH–RH in hypothalamic granules, although Mg alone can also release LH–RH to a lower magnitude. In humans, magnesium deficiency has been reported to increase the risk of polycystic ovarian syndrome (PCOS), which was 19 times greater in Mg-deficient patients compared with those who had normal serum Mg concentrations (Sharifi *et al.*, 2012; Hamilton *et al.*, 2019). Low Mg levels have been associated with anovulation. In our study a significantly low level of magnesium in cystic follicles supported the view that low levels of magnesium predispose to cyst formation in buffalo.

Classical manifestations of phosphorus deficiency on reproductive processes involve changes in oestrus and decreased ovarian activity (Ahmed, 2007; Safari *et al.*, 2022) characterized by anoestrus, delayed maturity (Ahmed *et al.*, 2010), subestrus, and irregular cycles (Hurley and Doane, 1989). Follicular fluid is more concentrated compared with blood in terms of phosphorus content (Tabatabaei and Mamoei, 2011; Dastorani *et al.*, 2018). In the present study, lower levels of phosphorus on cystic follicles strengthened the view that phosphorus deficiency may lead to anovulation and subsequent cyst formation.

Copper concentration in cystic follicular fluid was significantly higher compared with preovulatory follicles, similarly much higher serum copper concentrations were reported in cystic cows compared with the healthy group (Yousefdoost *et al.*, 2012; Sun *et al.*, 2019) leading us to speculate that this higher content in cystic follicular fluid might be influxed from higher serum concentrations or locally produced by cystic follicle metabolism; further study is warranted to confirm this.

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Lower zinc levels have been reported to be associated with suboptimal steroid hormone concentrations, i.e. oestrogen and progesterone (Akhtar *et al.*, 2009; BorŞ and BorŞ, 2020) that were attributed to the involvement of this ion in steroidogenesis (Hurley and Doane, 1989). Ziaee (2009) found that cystic primiparous cows had higher concentrations of zinc in serum compared with healthy multiparous cows. A higher concentration of zinc in cystic follicles could be due to the increased permeability of the blood–follicle barrier during excess follicular growth.

The concentration of cobalt was significantly high in cystic follicles compared with preovulatory follicles. This could be due to the excess permeability of the cystic follicles due to free radical injury, as it is well established that cobalt has the capacity to produce reactive oxygen species (ROS), which are highly reactive against DNA and other biomolecules (Valko *et al.*, 2005).

In conclusion, from this study, significant changes in biochemical, hormonal, and mineral compositions of the follicles are associated with cystic ovarian disease in buffalo. Cystic ovarian follicles had higher follicular $P_4$, cholesterol, urea nitrogen, creatinine, copper, zinc, and cobalt and lower $E_2$, insulin, ascorbic acid, glucose, uric acid, calcium, magnesium and phosphorus concentrations. These changes are consistent with some of the previously proposed mechanisms underlying the development of cystic ovarian disease such as oxidative stress, aberrant endocrine function, and nutritional imbalance.

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**Conflict of interest.** The authors declare that there is no conflict of interest in publishing this article.

**Ethical approval.** The authors assert that all experimental procedures were approved by the Institute Ethical Committee of ICAR-IVRI, U.P., India.

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