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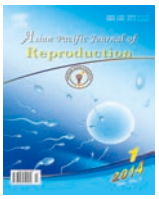

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Evaluation of follicular oxidant-antioxidant balance and oxidative damage during reproductive acyclicity in water buffalo (*Bubalus bubalis*)

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ABSTRACT

Objective: To investigate changes in follicular fluid concentrations of reactive oxygen species (ROS) and total antioxidant capacity (TAC) and degree of oxidative damage to follicular cells, using protein carbonyl (PC) as marker of oxidative stress, were investigated during reproductive acyclicity in buffalo. **Methods:** Follicular fluid was aspirated from follicles grouped into three classes depending upon their diameter [small (5.0-7.0 mm), medium (7.1-10.0 mm), and large (>10.0 mm)]. Progesterone and estradiol were estimated to determine functional status (P:E ratio) of the follicles. **Results:** Acyclic buffaloes had greater concentrations of ROS ($P<0.001$) and PC ($P=0.0412$) and lower concentrations of TAC ($P=0.0280$) than cyclic buffaloes. An interesting novel finding was the complete absence of low P:E functionally active follicles in acyclic buffaloes. Results indicated a pronounced follicular fluid oxidant-antioxidant imbalance and oxidative damage to follicular cells during acyclicity in buffalo. **Conclusion:** In conclusion, this study provided evidence about role of oxidative stress in pathogenesis of reproductive acyclicity.

1. Introduction

The water buffalo is an important livestock resource concentrated mostly in tropical and subtropical regions of the world. Although this species has a tremendous productive potential, its actual productivity is severely hampered by various reproductive disorders resulting in poor reproductive efficiency[1]. Reproductive acyclicity, characterized by failure of ovulation and lack of subsequent development of corpus luteum on the ovary, is one among those disorders with a major negative impact on the overall reproductive performance[2-4]. Recently, this condition has received a lot of attention with special emphasis on follicular development and follicular fluid microenvironment. Accumulating evidence suggests that underlying pathogenesis of acyclicity includes disturbances in follicular development associated with alterations in certain biochemical and hormonal components of follicular fluid[3,5,6]. Although a vast majority (87.5%) of acyclic buffalo population was observed to have small and medium sized follicles only, a very small proportion (12.5%) did show the presence of large sized, albeit estrogenically inactive, follicles[3]. Alterations in follicular fluid microenvironment during acyclicity in buffalo included reduced concentrations of estradiol, insulin, and ascorbic acid and elevated concentrations of nitric oxide and progesterone[3,6]. It was proposed that anestrus and anovulation during reproductive

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acyclicity are in part a result of reduced follicular estradiol, attributed to likely inhibition of aromatase activity by greater nitric oxide concentrations, and oxidative damage to follicles resulting from imbalance of nitric oxide and ascorbic acid concentrations[3].

Normal follicular growth and development represents a process accompanied by complex morphological, biochemical, and molecular transformations of various components of the follicle (oocyte, granulosa and theca cells, and follicular fluid). Composition of follicular fluid indicates the environment in which oocyte and granulosa cells are growing and maturing[7]. Follicular fluid holds considerable biological significance as it buffers internal environment of follicle against influence of external conditions presented by the blood stream[8]. Ovarian folliculogenesis not only involves gonadotropins and steroids, but it also involves local autocrine and paracrine factors. Reactive oxygen species (ROS) are some of the local factors influencing folliculogenesis, including the process of background follicular atresia[9-11]. Sufficient evidence has accumulated demonstrating that ROS are key signals in initiation of apoptosis in granulosa cells of antral follicles and that antioxidants protect against their potential damaging effects[12-14]. Oxidative stress occurs at the cellular level when reactive metabolites of oxygen are produced faster than they can be safely removed by antioxidant defense mechanisms[15]. Follicular fluid contains antioxidants that protect the ovarian follicle from ROS-induced damage[9,16,17]. Total antioxidant capacity is the cooperation of various preventive and chain breaking antioxidants, the estimation of which avoids cumbersome analysis of individual antioxidants[18]. It is well recognized that disruption of redox homeostasis mediates the accumulation of oxidized proteins that have a deleterious effect on cell function[19,20]. Protein carbonyls, generated as a result of protein modifications elicited either by direct oxidative attack on amino acid side chains or by modification of side chains with lipid peroxidation products, are relatively more stable and represent more general biomarkers for oxidative stress[20], while frequently used markers such as malondialdehyde serve as biomarkers for lipoxidation reactions only[21]. In buffalo, histological and apoptotic DNA fragmentation studies indicated greater degree of follicular atresia during reproductive acyclicity[22], which was also reflected in the predominantly inactive estrogenic status and higher alkaline phosphatase levels of the follicular fluid[3,5]. One of the reasons for greater follicular atresia in acyclic buffaloes could be oxidative stress due to imbalance in follicular fluid oxidant antioxidant equilibrium, as suggested by the associated alterations in nitric oxide and ascorbic acid concentrations[3]. The present study was designed to evaluate changes in total oxidant antioxidant balance in follicular fluid during acyclicity in buffalo. We hypothesized that acyclic buffaloes would have greater ROS and lesser TAC in follicular fluid than cyclic buffaloes. In addition, the degree of oxidative damage to the follicles was evaluated by measuring PC levels in follicular fluid.

2. Materials and methods

2.1. Chemical reagents

All the reagents used in the present study were of analytical grade and obtained from Merck India Ltd., Indian Drug House and SISCO Research Laboratory, Mumbai, India, unless otherwise mentioned.

2.2. Experimental material

Apparently healthy female buffaloes were selected prior to slaughter and ovaries were collected post slaughter from reproductively acyclic (n=41) and cyclic (n=48) animals. Blood samples were collected from jugular vein in sterile vacutainer tubes prior to slaughter and serum was collected and stored at −20 ℃ till assay for progesterone. Ovaries were transported on ice to the laboratory within 30 min of collection. The classification into acyclic and cyclic ovaries was based upon the presence of cyclic corpus luteum/corpus albicans[3,6,23] and the retrospective analysis of serum progesterone, according to criteria reported elsewhere[24]. Acyclic buffaloes showed a serum progesterone concentration lesser than 0.5 ng/mL, whereas the concentrations were greater than 0.5 ng/mL during all the phases of estrous cycle[24]. Follicular fluid was aspirated from follicles grouped into three size classes depending upon their diameter [small (5.0−7.0 mm), medium (7.1−10.0 mm), and large (>10.0 mm)] and transferred into micro-centrifuge tubes kept in an ice bath. Ovaries bearing cystic follicles (>20 mm in diameter) and those from prepubertal animals (small sized and with no scars)[3] were excluded from the investigation. Follicular fluid samples were centrifuged at 3 000 rpm and 4 ℃ for 10 minutes in a refrigerated centrifuge. Aliquots of follicular fluid samples were prepared for different parameters and stored at −20 ℃ till further analysis.

2.3. Biochemical assays

2.3.1. Reactive oxygen species

Estimation of ROS was done using a high-throughput spectrophotometric assay as described by Hayashi and co-workers[25]. The reaction mixture contained 5 μL of follicular fluid samples, 140 μL of 0.1M sodium acetate buffer (pH 4.8) and 100 μL of the mixed solution prepared from R1 and R2 at the ratio of 1:25. The absorbance was measured at 505 nm

for 2 minutes at 15 s interval using a spectrophotometric plate reader. R1 solution contained 100 μg/mL of N,N-diethyl-para-phenylendiamine (DEPPD) sulphate in 0.1M sodium acetate buffer while R2 solution was prepared by dissolving ferrous sulphate 0.1M sodium acetate buffer to attain a final concentration of 4.37 μM. Ten different concentrations of hydrogen peroxide solution (50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mg/L) were used as standard. A calibration curve for the standard solutions was developed by calculating slopes (absorbance increase at 505 nm/min×1 000) and the level of ROS was expressed as unit of H_2O_2. One unit corresponded to 1 mg/L H_2O_2.

2.3.2. Total antioxidant capacity

Estimation of TAC was done according to the method of Koracevic and co-workers[18]. The reaction mixture contained 10 μL of follicular fluid samples, 0.49 mL of 100 mM sodium phosphate buffer, 0.5 mL of 10 mM sodium benzoate solution, 0.2 mL of Fe-EDTA mixture [prepared by mixing equal volumes of 2 mM EDTA solution and 2 mM $Fe(NH_4)_2(SO_4)_2$ solution] and 0.2 mL of 10 mM H_2O_2 solution. Each sample had its own control (blank) in which 1 mL of 20% acetic acid was added followed by the addition of 0.2 mL of Fe-EDTA mixture and 0.2 mL of 10 mM H_2O_2. Negative control was also prepared. After the reagents were added, the test tubes were vortexed and incubated at 37 ℃ for 60 min. This was followed by addition of 1 mL of 20% acetic acid (sample test tubes only) and thiobarbituric acid. The reaction tubes were incubated at 100 ℃ for 10 min. After cooling to room temperature, absorbance was measured spectrophotometrically at 532 nm against distilled water. Standards containing 1 mmol/L uric acid were used for calibration and TAC was expressed as mmol/l.

2.3.3. Protein carbonylation

Carbonylation of proteins was measured by the method described by Levine and co-workers[26]. Briefly, 100 μL of follicular fluid was taken in a test tube and derivatization of protein was done with 400 μL 10 mmol/L 2,4-dinitrophenylhydrazine (DNPH) in 2.5 mol/L HCl. Protein in the samples was extracted with 500 μL 20% trichloroacetic acid (TCA), and pellet of protein was separated by centrifugation. This was followed by further addition of TCA and pelleting of the protein and three successive washings of the pellet with ethanol-ethylacetate (1:1) to remove the excess DNPH. The derivatized protein was dissolved in 2 mL of 6 mol/L guanidine hydrochloride solution. The carbonyl contents were calculated from the peak absorbance (370 nm) using an absorption coefficient of 22 000 mol/L-cm against appropriate blank and expressed as nmol/mg protein. The protein concentration of samples was estimated using a microplate Lowry protein assay method as described by Fryer and co-workers[27].

2.4. Hormone assays

2.4.1. Estrogen and progesterone in follicular fluid

Follicular functional status (Progesterone:Estradiol, P:E) was determined by estimating concentration of estradiol (E2) and progesterone (P4) in follicular fluid using solid-phase radioimmunoassay kits (Immunotech, France) using manufacturer's protocol. Serum progesterone was estimated using radioimmunoassay kits from the same manufacturer. The minimum sensitivities of the assay for E2 and P4 were 6 pg/mL and 0.05 ng/mL and the corresponding intra-assay and inter-assay coefficients of variation were <12.1% and <11.2%, and <6.5% and <7.2%, respectively.

2.5. Statistical analyses

Data were analysed for normality distribution by Shapiro-Wilk test and transformed to natural logarithms, square roots or ranks when indicated. The data were analysed for the main effects of group (G; acyclic and cyclic), follicle size (S; small, medium, and large), and follicular functional status (St; low, medium and high P:E) and their interaction using the Mixed Procedure of SAS (Version 9.2; SAS Institute, Inc., Cary, NC, USA). Differences between means were further examined by Least Significant Difference test or Student's *t*-test as required. A probability value of $P<0.05$ indicated that the difference was statistically significant. Associations between different parameters were analysed for statistical significance using Pearson's correlation co-efficient. Data are presented as mean±SEM, unless otherwise stated.

3. Results

In total, 31 small, 30 medium, and 28 large follicles were used in this study.

3.1. Progesterone – estradiol ratio

To evaluate the relationship between P:E ratio in FF and follicular oxidant/antioxidant status, follicles were assigned to one of three groups based on ratio of P:E in FF [high, P:E >10 (n=27); medium, 1<P:E <10 (n=45); low, P:E <1 (n=17)]. Cyclic ovaries displayed all the three groups of follicles on the surface, but in acyclic ovaries only high P:E and medium P:E follicles were observed. Small sized follicles of cyclic ovaries were either high or medium P:E but the medium and large sized follicles were either medium or low P:E type. However, different sized follicles of acyclic ovaries were having either high or medium P:E. Owing to the absence of data in some categories (low P:E follicles in acyclic animals) and large differences in distribution of follicles with different functional status in different follicle size categories of acyclic and cyclic buffaloes, the effect of three way interaction *viz*. group-by-size-by-status on

follicular oxidant and antioxidant status was not considered.

3.2. Reactive oxygen species

Follicular fluid ROS levels revealed a significant ($P< 0.001$) group effect indicating greater overall concentrations in acyclic (174.30±4.61 units) than in cyclic (153.00±4.03 units) buffaloes. Although there was no group-by-size interaction, differences in ROS concentrations were observed in medium and large sized follicles between cyclic and acyclic buffaloes and between different follicle size categories within each of the two groups as shown (Table 1). A significant ($P<0.001$) group-by-status interaction indicated greater ROS concentrations in high P:E follicles of acyclic buffaloes than those of cyclic buffaloes. Within cyclic buffaloes, the concentrations were lower in high P:E compared to medium and low P:E categories. However, within cyclic animals high P:E follicles had greater ROS concentrations than medium P:E follicles (Table 2).

Table 1
Follicular concentrations (Mean±SEM) and group-by-size interaction of ROS, TAC and PC.

Follicle size	ROS (Units of H_2O_2)		TAC (mmol/L)		PC ((nmol/mg protein)	
	Cyclic	Acyclic	Cyclic	Acyclic	Cyclic	Acyclic
Small	143.72±7.95^{a}	151.95±4.98^{a}	0.93±0.08^{a}	0.95±0.07^{a}	5.52±0.47^{a}	6.54±0.61
Medium	149.99±4.57^{ab}	172.88±7.00^{*b}	1.11±0.09^{a}	1.05±0.09^{a}	6.07±0.59^{a}	6.20±0.79
Large	166.83±6.88^{b}	200.04±6.09^{*b}	1.69±0.16^{b}	0.49±0.07^{*b}	2.56±0.51^{b}	7.97±1.18*

Asterisk (*) indicate significant differences between groups within a size category, whereas different lowercase letters (a,b) indicate significant differences between follicle sizes within a group.

Table 2
Follicular concentrations (Mean±SEM) and group-by-status interaction of ROS, TAC and PC.

Status (P:E)	ROS (Units of H_2O_2)		TAC (mmol/L)		PC ((nmol/mg protein)	
	Cyclic	Acyclic	Cyclic	Acyclic	Cyclic	Acyclic
High	116.74±4.43^{a}	186.00±7.01^{*a}	1.25±0.07^{a}	0.74±0.08*	7.46±0.61^{a}	9.53±0.47^{*a}
Medium	156.82±4.95^{b}	162.11±4.70^{b}	0.85±0.05^{b}	0.94±0.08	5.51±0.43^{b}	4.09±0.27^{*b}
Low	160.26±6.70^{b}	–	1.77±0.12^{c}	–	2.76±0.47^{c}	–

Asterisk (*) indicate significant differences between groups within a status category, whereas different lowercase letters (a,b) indicate significant differences between status categories within a group; –: absence.

3.3. Total antioxidant capacity

A significant ($P=0.0280$) main effect of group on total antioxidant capacity was observed with lower concentration in acyclic (0.84±0.06 mmol/L) than in cyclic (1.22±0.08 mmol/L) buffaloes. A significant ($P<0.001$) group-by-size interaction indicated less concentrations in large follicles of acyclic buffaloes than in those of cyclic buffaloes. Within cyclic buffaloes, large follicles had greater TAC concentrations than small and medium sized follicles, whereas in acyclic buffaloes the trend was exactly opposite (Table 1). The group-by-status interaction was significant ($P=0.009$) indicating lesser TAC concentrations in high P:E follicles of acyclic buffaloes than those of cyclic buffaloes and differences in concentrations between each of the three P:E categories within the cyclic group (Table 2).

3.4. Protein carboylation

Follicular fluid PC concentrations revealed a significant ($P=0.0412$) group effect indicating greater overall concentrations in acyclic (6.88±0.51 nmol/mg protein) than in cyclic (4.78±0.37 nmol/mg protein) buffaloes. A significant ($P<0.001$) group-by-size interaction indicated greater PC concentrations in large sized follicles of acyclic buffaloes than in those of cyclic buffaloes and less PC concentrations in large sized follicles than in small and medium sized follicles within the cyclic group (Table 1). Further, a significant ($P<0.001$) group-by-status interaction was observed, owing to differences between cyclic and acyclic buffaloes within different P:E categories and between different P:E categories within each of the two groups (Table 2).

3.5. Correlation analyses

There was a significant positive correlation between PC and ROS in acyclic but not in cyclic buffaloes. In contrast, the correlations between PC and TAC and between ROS and TAC were negative in both cyclic and acylic buffaloes (Table 3).

Table 3
Association between PC, ROS and TAC concentrations in cyclic and acyclic buffaloes.

Buffaloes		PC *vs.* ROS	PC *vs.* TAC	ROS *vs.* TAC
Cyclic	*r*	0.213	–0.596**	–0.532**
		$P > 0.1$	$P < 0.001$	$P < 0.001$
Acyclic	*r*	0.562**	–0.376*	–0.602**
		$P < 0.001$	$P = 0.015$	$P < 0.001$

** Correlation is significant at the 0.01 level (2-tailed),*Correlation is significant at the 0.05 level (1-tailed).

4. Discussion

Reproductive acyclcity in buffalo has been associated with alterations in biochemical and hormonal composition of follicular fluid microenvironment[3,5,6]. Although the reduction of follicular ascorbic acid and elevation of nitric oxide in acyclic buffaloes[3] gave the initial clue, results of the present study provided a more conclusive evidence about an altered follicular oxidant-antioxidant balance and the resulting oxidative damage to follicles during acyclicity. An interesting novel finding was the absence of low P:E follicles in acyclic buffaloes. It is already known that the presence of high and medium P:E in follicular fluid indicates follicles in which atresia has already ensued and follicles in a transitional state from non-atretic to an atretic condition, respectively[28,29], whereas low P:E indicates estrogenically active follicles in which granulosal cells have a greater capacity to bind gonadotropins[28]. Histological, biochemical, and endocrinological studies have demonstrated that a vast majority of follicles in acyclic buffaloes are atretic and estrogenically inactive[3,5,22]. Taken together, these observations suggest that follicular development during reproductive acyclicity is impaired such that only functionally inactive antral follicles with reduced potential for estradiol production and elicitation of gonadotropin surge and/or reduced affinity for gonadotropin binding and response are generated, consequently, resulting in anestrus and anovulation.

In the ovary, ROS are generated through leakage of electrons from the inner mitochondrial membrane during oxidative phosphorylation and ATP generation as well as activity of cytochrome P450 enzymes[13]. Although ROS at low concentrations play important physiological roles such as oocyte maturation and acquisition of developmental competence [30, 31], ROS overproduction can have deleterious effects on cellular function by inducing oxidative damage of intracellular components and inducing apoptosis[32,33], especially when the levels are too high to be neutralized by the antioxidant system. The overall greater concentrations of ROS coupled with the reduced concentrations of TAC in acyclic buffaloes suggest involvement of oxidative stress in development of reproductive acyclicity. Although there was a progressive increase in ROS production during follicular development in cyclic buffaloes resulting in greatest concentrations in large follicles, a concurrent increase in TAC levels could ensure maintenance of oxidant-antioxidant balance, thereby, preventing the onset of oxidative stress. Antioxidant defense in the follicular fluid of buffalo has been shown to be composed of both enzymatic and non-enzymatic components[17,34] and variation in concentration of enzymatic and non enzymatic antioxidants with follicle size and stage of estrous cycle have been reported[17,35]. In contrast to cyclic buffaloes, the presence of greater ROS and lesser TAC levels in acyclic buffaloes indicates oxidant-antioxidant imbalance, which could lead to oxidative damage to follicles. The above observations coupled with the significant negative correlation between ROS and TAC underscore the importance of a precise oxidant-antioxidant balance in the follicular fluid for normal follicular development and function.

Protein carbonyls are increasingly being used as biomarkers of oxidative stress, owing to their greater stability and availability of experimental evidence that proteins are the major initial targets of ROS[20]. The significant associations of PC with ROS (positive) and TAC (negative) observed in the present study suggest that PC concentrations in follicular fluid can be a good marker of oxidative stress in the buffalo ovary. The presence of greater overall PC concentrations in acyclic buffaloes indicates greater oxidative damage to follicles during acyclicity. More specifically, large follicles in acyclic buffaloes were subjected to extensive oxidative damage, as indicated by greatly increased PC concentrations in the follicular fluid. There is evidence that acyclic buffaloes have lesser concentrations of follicular fluid estradiol[6] and majority of the follicles are estrogenically inactive[3], which could be attributed in part to granulosa cell oxidative damage resulting in a lower steroidogenic capacity and destruction of steroids by free radicals. Evidence for this also comes from the significant differences in PC concentrations between high, medium, and low P:E follicles in this study. Besides the apparent effects of oxidative stress on steroidogenic potential and gonadotropin binding, a direct effect on proteins involved in ovulation is a possible consequence. It is known that gonadotropic stimulation of urokinase-type plasminogen activator secretion by ovarian surface epithelial cells bordering the preovulatory follicle elicits a localized increase in tissue plasmin, which activates latent collagenases and secretion of tumor necrosis factor-α (TNF-α) from thecal endothelium. TNF-α potentiates collagenolysis (via matrix metalloproteinase gene expression) and (at elevated concentrations) mediates epithelial/vascular dissolution[36]. Future studies are warranted on the carbonylation of specific proteins involved in ovulation and the potential involvement of the protein modification in the development of reproductive acyclicity.

In conclusion, the present study provided evidence that reproductive acyclcity in buffalo is associated with changes in the follicular fluid concentrations of ROS and TAC. The resulting imbalance in follicular oxidant-antioxidant system leads to severe oxidative damage to follicular cells, as reflected in highly increased PC concentrations and absence of estrogenically active follicles in acyclic buffaloes. Taken together, these findings indicate the role of oxidative stress in the pathogenesis of reproductive acyclicity.

Conflict of interest statement

The authors have no conflict of interest to declare.

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