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Oxidative stress during cystic ovarian disease in water buffalo

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ABSTRACT

Follicular fluid and serum concentrations of antioxidants such as catalase, ascorbic acid (AA) and total antioxidant capacity (TAC) along with reactive oxygen species (ROS) and degree of oxidative damage to follicular cells, using protein carbonyl (PC) as marker of oxidative stress, were investigated during cystic ovarian disease in buffalo. Follicular fluid was aspirated from cystic follicles (>20 mm) and also from large size (10–17 mm) of cycling buffaloes. Estradiol and progesterone were estimated to determine functional status (E: P ratio) of the follicles. Cystic follicles had greater concentrations of ROS and PC and lesser concentrations of catalase, ascorbic acid and TAC than normal follicles. An interesting finding was a simultaneous decrease of TAC and increase of ROS in serum of cystic buffaloes. Results indicated a pronounced follicular fluid oxidant-antioxidant imbalance and oxidative damage to follicular proteins during cystogenesis. In conclusion, this study provided evidence about role of oxidative stress in pathogenesis of cystic ovarian disease.

Key words: Estrogenic status, Follicular cyst, Ovulation failure, Oxidative stress

Cystic ovarian disease (COD), a disorder of reproduction that affects bovine, ovine, caprine, porcine species, and an important cause of infertility, is characterized by anovulation, short inter-estrus interval, persistence of follicles with a diameter larger than the ovulatory follicle (Silvia *et al.* 2002). The development of ovarian follicular cysts is associated with an endocrine imbalance along the hypothalamo-hypophyseal-gonadal axis (Silvia *et al.* 2002). Although, insufficient preovulatory LH surge might lead to cyst formation, persistence of preovulatory follicle after ovulation failure may be the alternative cause of cysts. A combination of weak proliferative activity and low levels of apoptosis in the follicular wall of cyst could explain the slow growth of cystic follicles, then a static condition without degeneration leading to their persistence (Isobe and Yoshimura 2007). Cook *et al.* (1990) indicated that cysts possess morphological similarities to atretic follicles and have varying degree of thinning and degeneration of thecal and granulosa cells.

The buffalo forms the mainstay of dairy industry in Asia and contributes more than half of the total milk production in India (Das and Khan 2010). However, reproductive efficiency of buffalo is hampered by late sexual maturity, seasonal reproductive pattern, anestrus and long intercalving interval (Das and Khan 2010). Infertility may be attributed to follicular damage and failure of maturation and ovulation, especially in non functioning ovaries, whereas free radical production may play a role in the prolongation of postpartum anoestrus in dairy cows. El-Shahat and Kandil (2012) made a plea for greater research effort in identification of the specific roles of oxidative stress and antioxidants in etiopathogenesis of various reproductive problems in the species. Khan and Das (2012) demonstrated that a decrease in ascorbic acid predisposes follicle to free radical injury, such as due to higher nitric oxide, which under normal conditions is taken care of by the intra-ovarian antioxidant system. However, in COD, the antioxidant and oxidative stress parameters need to be analysed. The objective of the present study was to evaluate the antioxidant status and free radical generation in follicular fluid and serum of cystic buffaloes in order to get insights in the possible role of oxidative stress in the development of cystic ovarian disease in the species.

MATERIALS AND METHODS

Animals: Experimental females were derived from animals, culled mainly due to fertility disturbances. Every culled female was subjected to thorough clinical examination which comprised body condition scoring (BCS) and per-rectal examination. Animals having a body condition score >5 were selected. Animals, which were diagnosed as suffering from follicular cysts were selected and ovaries were obtained after slaughter. At the same time,

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animals with cycling ovaries having a large size follicle of 10–17 mm were also selected and kept as control. The selection of cyclic control was based upon the presence of corpus luteum of any stage present on one of the ovaries (Khan and Das 2011). Peripheral blood was collected by venipuncture in serum separation vials. The blood samples were centrifuged at 1,500 g for 10 min. The samples of follicular fluid from cystic and cyclic animals were collected by follicular aspiration. Serum and follicular fluid samples were stored at –20°C.

Oxidative stress: A panel of biomarkers was used to evaluate the relation between oxidative stress and development of follicular cysts. FF and serum activities were analysed for catalase (Aebi 1983), ascorbic acid (Zannoni *et al.* 1974), total antioxidant capacity (Koracevic *et al.* 2001), reactive oxygen species (Hayashi *et al.* 2007) and protein carbonylation (Levine *et al.* 1990).

Hormone assays: Estradiol (E2) and progesterone (P4) concentration in follicular fluid were using solid-phase radioimmunoassay kits using manufacturer's protocol. Follicles were divided into cyclic and cystic follicle groups, which were further divided into 2 groups based on estrogenic status**.** 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.

Statistical analysis: Ovaries were classified as cyclic estrogenic, cyclic non-estrogenic, cystic estrogenic and cystic non-estrogenic (n= 12 each). Data were tested for normality by Shapiro-Wilk test and transformed to square roots, natural logarithms or ranks when indicated. Data were analysed for the main effect of group (cyclic vs cystic), main effect of status (estrogenic vs non-estrogenic) and the interaction of group-by-status using the Mixed Procedure of SAS (Version 9.2) using following model

$$Y_{ijk} = \mu + G_i + S_j + (GS)_k + \varepsilon_{ijk}$$

where, Y_{ijk}, k^{th} data value of j^{th} level of factor S (status) and i^{th} level of factor G (group); μ, grand mean; G_i, i^{th} level of G; S_j, j^{th} level of S; $(GS)_k$, k^{th} interaction; ε_{ijk}, residual error. A significant interaction between rows and columns is when the difference between rows is not the same at each column, equivalent to variations between columns that is not the same at each row. Serum concentration of various parameters were analysed by DMRT followed by LSD test to find difference between individual groups. A probability value of $P \leq 0.05$ indicated that the difference was statistically significant while a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as mean±SEM.

RESULTS AND DISCUSSION

Cystic follicles had significantly higher (P<0.01) P4 and E2 concentrations as compared to cyclic follicles. Similarly, significantly greater P4 and E2 concentrations (P<0.05) in non-estrogenic follicles derived from cystic ovaries than those of cyclic counterparts. In both cyclic as well as cystic ovaries, the concentration of P4 was significantly higher (P<0.01) in non-estrogenic follicles as compared to estrogenic follicles. In contrast, the E2 concentration was significantly higher in estrogenic as compared to non-estrogenic follicles (Table 1).

Table 1. Hormonal concentrations in the follicular fluid of cyclic and cystic follicle according to estrogenic status

Parameters	Follicular status	Group (G)	
		Cyclic	Cystic
Estradiol (ng/ml)	Estrogenic	162.11±13.87D	176.34±12.68D
	Non- estrogenic	24.29±3.03a,C	55.11±3.42b,C
Progesterone (ng/ml)	Estrogenic	23.27±3.59C	29.28±2.01C
	Non-estrogenic	61.94± 6.96c,D	162.18± 10.30d,D
E2/P4 ratio	Estrogenic	9.03±2.34D	6.29±0.60D
	Non-estrogenic	0.42±0.05C	0.35±0.03C

Uppercase [A: B (P<0.05); C: D (P<0.01)] within group (cyclic or cystic) and lowercase [a: b (P<0.05); c: d (P<0.01)] within follicular status (estrogenic, E or non-estrogenic, NE) indicate significant difference. *NS, nonsignificant.

Cystic follicles, whether estrogenic or non-estrogenic, had significantly (P<0.05) lower concentrations of catalase, AA and TAC as compared to cyclic follicles whereas the ROS and PC levels were significantly (P<0.05) higher as compared to cyclic follicles. Furthermore, estrogenic follicles from cyclic ovaries had significantly (P<0.05) higher concentration of catalase, AA and TAC and, a lower concentration of ROS and PC as compared to non-estrogenic counterparts (Table 2). The results obtained for antioxidants, ROS levels and protein carbonylation in serum are presented in Table 3.

Table 2. Follicular fluid concentrations (mean±SEM) various oxidative stress parameters in cystic and cyclic buffaloes

Parameters	Follicular status (S)	Group (G)	
		Cyclic	Cystic
Catalase (mIU/ mg protein)	E	1.82±0.20a	1.25±0.13b
	NE	1.54±0.12	1.12±0.13
Ascorbic acid (μg/ml)	E	10.65±0.77c,C	7.31±0.62d,A
	NE	7.59±0.61a,D	5.25±0.41b,B
Total antioxidant capacity (mmol/l)	E	1.43±0.20c,C	0.78±0.08d
	NE	0.89±0.12a,D	0.49±0.11b
Reactive oxygen species (unit of H_2O_2)	E	180.28±16.73a,A	263.80± 29.70b,A
	NE	271.79 ±29.46a,B	362.52 ±32.10b,B
Protein carbonylation (nmol/mg protein)	E	8.00±0.61c,A	13.43±0.89d,C
	NE	10.92±0.57c,B	16.63±1.04d,D

Uppercase [A: B (P<0.05); C: D (P<0.01)] within group (cyclic or cystic) and lowercase [a: b (P<0.05); c: d (P<0.01)] within follicular status (estrogenic, E or non-estrogenic, NE) indicate significant difference.

Table 3. Serum concentrations (mean±SEM) of various oxidant-antioxidant parameters in cystic and cyclic buffaloes

Parameters	Cyclic		Cystic	
	Estrogenic (E)	Non-estrogenic (NE)	Estrogenic (E)	Non-estrogenic (NE)
Catalase (mIU/mg protein)	2.60±0.27	2.55±0.25	2.30±0.18	2.17±0.29
Ascorbic acid (µg/ml)	2.46±0.33	2.18±0.21	2.08±0.16	2.29±0.24
TAC (mmol/l)	2.30±0.27^{b}	2.17±0.22^{b}	1.55±0.15^{a}	1.41±0.19^{a}
ROS (unit of H_2O_2)	196.01±22.81^{a}	225.24±28.67^{a}	250.47±23.72^{ab}	300.09±31.93^{b}
PC (nmol/mg protein)	8.89±0.82	9.59±0.88	10.1±0.67	10.99±0.81

Different superscripts indicate significant difference (P<0.05) between groups based on DMRT.

The finding of a disturbed redox status and oxidative modification of follicular proteins in dominant follicles of cystic buffaloes supported the hypothesis that altered pro-oxidant/ antioxidant status is involved in the development of COD in buffalo. The presence of both progesterone dominant as well as estrogen dominant cysts indicated that the cysts are endocrinologically dynamic. Progesterone dominant cysts are associated with significantly higher serum progesterone indicating that progesterone dominant cysts do not represent atretic state but instead are progesterone secreting cysts due to varying degree of luteinization. It was found that the protein carbonylation in the follicular fluid of cystic buffaloes increased drastically. This increase could be due to simultaneous disturbance in follicular redox status, characterized by elevation of ROS and reduction of TAC in follicular fluid of cystic buffaloes, which indicated that the antioxidant mechanism operating in the follicular micro-environment is less efficient in cystic animals.

Normal reproductive rhythm in any livestock species involves 3 intricate processes, viz. folliculogenesis, ovulation, and the subsequent formation of the corpus luteum. Once follicular growth gets under way, it is a continuous process without a cutoff phase, ending at ovulation (Vasconcelos *et al.* 2001). Ovulation is characterized by undergoing morphological and functional adjustments of granulosa cells, leading to transformation of highly proliferative, oestrogen secreting granulosa cells of preovulatory follicles to the non-proliferative progesterone secreting luteal cells of corpus luteum (Salvetti *et al.* 2009). The follicular unit is subjected to a fine balance between survival factors and insults, interaction of which may either allow a follicle to grow or enter into atresia (Gupta *et al.* 2011), through a cooperative regulation of different endocrine, paracrine and autocrine factors. A Graafian follicle contains its own potential sources of reactive oxygen species, including large numbers of macrophages, neutrophils and metabolically active granulosa cells (Gupta *et al.* 2011). At controlled levels, reactive oxygen species are capable of exerting physiological effects and mediating processes such as tissue remodelling, hormone signalling, oocyte maturation, folliculogenesis, tubal function, ovarian steroidogenesis, cyclic endometrial changes, and germ cell function. However, increase of ROS to pathological levels is capable of inflicting significant damage to cell structures. ROS is controlled and kept within physiological limits within the ovary by various antioxidant systems. In the present study, there were significant alterations in antioxidant profile together with free radical generation and oxidative damage of proteins in buffaloes with ovarian cysts. These findings support the notion that oxidative stress may lead to failure of ovulation and failure of responsiveness to apoptotic mechanisms and hence persistence of follicles. The expression of various markers of oxidative stress was demonstrated in normally cycling ovaries (Gupta *et al.* 2011, El-Shahat and Kandil 2012) and implicated in various reproductive pathologies including polycystic ovarian syndrome (PCOS).

Our results indicated a clear decrease in catalase and ascorbic activity and also the total antioxidant capacity in follicular fluid of estrogen dominant as well as progesterone dominant cysts, although the later were more severely affected. This was followed by a parallel increase in generation of reactive oxygen species in both types of cysts as compared to normal preovulatory follicles. The serum of affected animals also had higher levels of ROS than normal cyclic animals. The results are in agreement with similar findings, higher levels of melondialdehyde (MDA) and reduced glutathione in cystic buffaloes (Ahmed *et al.* 2010). However, given the current uncertainity on the exact significance of lipid peroxidation and apoptosis in granulosa (Combelles *et al.* 2011), carbonylation of proteins was taken as an index of oxidative stress in the present study. Protein carbonylation was significantly lower in the follicular fluid of preovulatory follicles of cycling buffaloes, possibly due to a gonadotropin induced expression of antioxidant genes; an effect that accounts for the protective effects of gonadotropins on the developing follicle. Exposure of rat ovaries to chorionic gonadotropin (with FSH and LH activity) *in vivo* increases the expression of some antioxidants (Tilly and Tilly 1995). However, in cystic buffaloes, protein carbonylation of large follicles was pathologically high, suggesting a disturbance in the pro-oxidant/antioxidant status of acyclic buffaloes. The higher levels of PC in ovaries of cystic buffaloes are suggestive of the fact that oxidative stress has already ensued resulting in damage to granulosa cells and thereby rendering them unresponsive to gonadotrpin surge. Collagenolysis was considered a prelude to stigma formation and ovarian rupture (Imai *et al.* 2003). Tumor necrosis factor α (TNFα), plasmin, matrix metalloproteinases are upregulated in mature Graffian follciles approaching ovulation (Murdoch and Gottsch 2003). It seems logical to speculate that in cystic ovaries, high levels of oxidative modification of ovarian

proteins do not spare the above mentioned proteins. The irreversible inhibition of these enzymes by carbonylation may be responsible for anovulation together with a yet to be percieved mechanism resulting in persistence of follicles in cystic buffaloes.

Mature follicles of buffalo ovaries accumulate ascorbate acid (Khan and Das 2011), promote steroidogensis (Luck *et al.* 1995) and prevent enzymatic machinery of estradiol synthesis, especially lecithin-cholesterol acyltransferase (LCAT) from oxidant induced insult (Cigliano *et al.* 2002). Reactive oxygen species cause LH receptor to uncouple from adenylate cyclase and also prevents conversion of cholesterol to pregnenolone most likely through impaired production of steroidogenic acute regulatory protein (STAR), an intracellular cholesterol transport protein (Ruder *et al.* 2008). Ovulation is generally compared to an acute inflammatory process. In an *in-vitro* perfusion model, reactive oxygen species have been shown to play a role in the mechanical process of ovulation and their ablation hinders ovulation as well as a whole repertoire of essential preovulatory responses (Shkolnik *et al.* 2011). Ovarian surface epithelim has been shown to undergo apoptosis and the subsequent weakening of the apical follicular wall leads to ovarian stigma formation and ovulatory rupture (Murdoch and Gottsch 2003). The increased levels of follicular ROS in cystic buffaloes as comapred to cyclic buffaloes goes against the above notion of involvement of ROS in ovulation. This may be due to a more pathological increase in follicular ROS, which seems to be sufficient to illicit an ROS induced damage to granulosa cells, thereby making them irresponsive to preovulatory surge. Increased expression of TAC is neccesary to limit the negative influences on granulosa cells and to preserve the physiological role of ROS in promoting ovulation. However, such an increase in total antioxidant capacity was not noticed in cystic buffaloes. The present study provides evidence for the role of disturbed oxidant/antioxidant balance in the etiopathogenesis of cystic ovarian disease. Future studies should focus on the oxidative modification of specific proteins and enzymes involved in the ovulatory process.

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