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SOME ANTIOXIDANTS' ACTIVITIES, LIPID PEROXIDE AND NITRIC OXIDE LEVELS IN FOLLICULAR FLUID AND ITS RELATION TO OOCYTE QUALITY IN BUFFALO-COWS

(With 7 Tables)

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أنشطة بعض مضدات الاكسدة ومستوى اكسيد النيتريك (NO) وبيراكسيد الدهن في السائل الجريبي وعلاقته بنوعية البويضة في اناث الجاموس

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اجريت هذه الدراسة بغرض معرفة انشطة بعض مضدات الاكسدة وكذلك تقدير مستوى كل من اكسيد النيتريك (NO) وبيراكسيد الدهن في السائل الجريبي لجريبات المبيض في اناث الجاموس. تم اختيار عدد ٦٠ جريب الموجودة مع اجسام صفراء على مبايض اناث الجاموس في هذه الدراسة قسمت هذه الجريبات الى ثلاثة مجاميع طبقا لاقطارها على سطح المبيض الـــى جريبات صغيرة (٥ – ٨ ملم) ومتوسطة (٩ – ١٢ ملم) وكبيرة (اكبر من ١٢ ملم). تم تجميع السائل الجريبي من كل جريب على حده ثم تم فصل المحتويات الخلوية من هذا السائل مع فحص نوعية البويضة الموجودة في هذا المحتوى الخلوي. وبناءاً على مستوى كل من هرمون الاستروجين والبروجيستيرون في السائل الجريبي تم تقسيم هذه الجريبات في كل مجموعة الى جريبات نشطة (عالية مستوى الاستروجين) وجريبات غير نشطة (متدنية مستوى الاستروجين). وقـد اظهـرت هذه الدراسة ان معظم جريبات جـراف الصغيرة الحجـم كانت نشطـة (عالية مستوى الاستروجين) وان ٦٥ % من البويضات التي تم تجميعها من هذة جريبات الصغيرة كانت جيدة النوعية مقارنة بما تم تجميعه من جريبات جراف في المجاميع الاخرى. ووجد ان مستوى كل من السوبر أكسيد دسميوتيز واكسيد النيتريك (NO) وبيراكسيد الدهن يزداد معنوياً مع زيادة قطر جريب جراف. كما وجد ان مستوى كل من اكسيد النيتريك (NO) والقلوتاثيون أعلى في جريبات جراف الصغيرة مقارنة بالمتوسطة والكبيرة الحجم. كما وجد ان مستوى بيراكسيد الدهن له علاقة معنوية سالبة مع كل من اكسيد النيتريك (NO)، القلوتاثيون والسوبر أكسيد دسميوتيز والنسبة بين مستوى كل من الاستروجين والبروجيستيرون في السائل الجريبي. وبناءاً على

ذلك دلت هذه النتائج ان انشطة بعض مضادات الاكسدة والتغيرات في مستوى كل من اكسيد النيتريك (NO) وبيراكسيد الدهن من مكونات السائل الجريبي ذات التغيرات الملحوظة اثناء نمو جريبات جراف خلال فترة وجود الجسم الاصفر من دورة الشبق في اناث الجاموس. وان هذه التغيرات في البيئة الداخلية لجريبات جراف تعكس احتياجات هذه الجريبات خلال فترة النمو والنضج واستمرارية حيوية البويضات في جريبات جراف في اناث الجاموس.

SUMMARY

The objective of this study was to determine the intra-follicular levels of some antioxidant activities (SOD and GSH) and changes in LPO and NO levels and their association to oocyte quality were studied in buffalo-cows ovarian follicles. A total of 60 vesicular follicles (≥ 5 mm in diameter) on ovaries bearing fully developed corpora lutea (either ipsilateral or contralateral to CL) were selected and classified according to the follicular size into three groups [≥ 5-8 mm (small); ≥ 9-12 mm (medium) and > 12 mm (large)]. The follicular aspirate was centrifuged to separate the fluid from cell fractions, and then examined for oocyte quality. The levels of estrogen (E_2) and progesterone (P_4) were used to classify follicles in each diameter group into E_2 active and E_2 inactive follicles. Most of small follicles were E_2 active follicles and 65% of oocytes recovered from these follicles were of good quality in comparison to other follicular diameter groups. SOD activity and LPO level significantly increased as the diameter of the follicle increased. GSH and NO were significantly higher in small follicles. Moreover, LPO level was significantly negatively correlated with SOD, GSH, NO and E_2:P_4 ratio. In conclusion, the present study indicated that antioxidant activities and changes in LPO and NO levels were subjected to marked changes in the course of follicular growth during luteal phase of estrous cycle in buffalo-cows. These changes in the intra-follicular environment may reflect the follicular requirements for growth, maturation and maintenance of viability of oocytes in buffalo-cows.

Key words: *Antioxidants, Lipid peroxide, Nitric oxide, follicles, Corpus luteum, buffalo-cows.*

INTRODUCTION

In recent years, fertility preservation has been evolving rapidly as a result of clinical imperatives and advances in technology (Gosden and Nagano, 2002). *In vitro* production of buffalo embryos has received increasing interest due to poor adoption of artificial insemination and the low superovulatory response in buffaloes (Madan *et al.*, 1996). The

economical production of a large number of high quality embryos is a well defined goal of successful commercial embryo transfer programs (Nandi *et al.*, 2002). Essential attainment of this goal needs clear understanding of the factors that affect growth, maturation, and development of follicle and oocyte (Albertini *et al.*, 2001). The vesicular follicles (≥ 5mm in diameter) obtained from ovaries collected from slaughtered valuable buffalo-cows comprise an important genetic constitution. Unfortunately, these vesicular follicles sometimes were on the ovaries bearing corpora lutea (CL). It was suggested that the CL affected follicular maturation (Bellin *et al.*, 1984). The capacity of bovine oocytes to mature *in vitro* was found to be related to the presence or absence of cumulus cells (Shioya *et al.*, 1988), follicle size (Fukui and Sakuma, 1980), ovarian activity (Hagemann, 1999) and intra-follicular environment (Hazeleger *et al.*, 1995). In addition, Trounson *et al.* (2001) reported that follicular growth was predictive of the developmental competence of oocytes for *in vitro* fertilization (IVF).

The development of oocyte is a tightly regulated process, which is dependent on a balance between local intra-ovarian factors (steroidal and non-steroidal factors) and circulating hormones (Basini *et al.*, 1998; Yang *et al.*, 1998). In the ovarian follicle, the oocyte is bathed in a follicular fluid (FF) which is a reservoir for many substances produced by both the granulosa cells (GCs) and the theca cells (TCs) and a transudate of serum components, transported through the blood–follicle barrier (BFB) (Edwards, 1974). This metabolically active environment contains steroid hormones, growth factors, cytokines, free radicals, reactive oxygen species (ROS) and antioxidants (Aten *et al.*, 1992; Attaran *et al.*, 2000). Free radicals and associated agents play a number of significant and different roles in reproductive biology (Attaran *et al.*, 2000; El-Din Zain and Omar, 2001). It is well known that ROS [hydrogen peroxide (H_2O_2), Nitric oxide (NO), superoxide anion and hydroxyl radicals] and their product Lipid peroxide (LPO) are generally deleterious to tissue function (anti-gonadotrophic and anti-steroidogenic actions) when they overwhelm the antioxidant capacity (Shimamura *et al.*, 1995; Tarrin, 1996). These oxidants were suggested to be mostly originated from steroidogenesis (Rodgers *et al.*, 1995) triggering follicular atresia (Tilly and Tilly, 1995).

Protection against ROS is provided by enzyme degradation [catalase, Superoxide dismutase (SOD), glutathione peroxidase], scavengers by antioxidants [(vitamins and glutathione (GSH)] and molecular repair (Aten *et al.*, 1992; Halliwell, 1994). These antioxidants

are present in the ovary and may be hormonally regulated (Aten *et al.*, 1992). In addition, Laloraya *et al.* (1988), and Doyle *et al.* (1990) reported that antioxidants activities in the ovarian follicles change in a similar manner to serum steroid concentrations and suggested an important role of these antioxidants in regulation of follicular growth and function. Although, most of recent studies were concerned with ovarian action of ROS and antioxidants activities in mechanical process of ovulation (Miyazaki *et al.*, 1991) and in CL during pregnancy (Shimamura *et al.*, 1995) and normal luteolysis (EL-Din Zain and Omar, 2001) yet, little information is available about these vital agents and their role on follicular growth and maturation of oocyte in bovine species (Carolan *et al.*, 1995). The present study was designed to clarify some antioxidant activities, changes in LPO and NO productions and its relations to oocyte quality recovered from ovarian follicles in buffalo-cows.

MATERIAL and METHODS

The ovaries were collected from a local slaughter house from forty-five water buffalo-cows, whose reproductive organs showed no macroscopic abnormalities. The animals ranged from 5 to 8 years of age. Immediately after slaughter, the paired ovaries from each animal were collected and transported within 1 to 2 h to the laboratory in a container containing ice bags (4°C). As the ovaries were obtained from buffalo-cows of unknown reproductive history, the stage of estrous cycle was identified using the criteria described by Okuda *et al.* (1988). The vesicular follicles on either ipsilateral and/or contralateral ovaries with fully developed corpora lutea (≥ 5 mm in diameter) at the middle of estrous cycle were selected for this study. These surface follicles were classified into three groups based on surface diameter: 5-8 mm (small); 9-12 mm (medium) and > 12 mm (large). Follicle-size selected on the basis of reported gonadotropin dependence and changes in the expression of steroidogenic enzymes and LH receptors mRNAs (Xu *et al.*, 1995).

The selected follicles were aspirated using 22-gauge needle attached to a syringe. The follicular aspirates were centrifuged (400 g; 5 min; 4 °C) to quickly separate the fluid from cell fractions. The cumulus–oocyte complex (COC), when retrieved, was picked up and examined under the stereo-microscope. The retrieved oocytes were analyzed on the basis of routine criteria described by de Loos *et al.* (1989). According to these criteria, they were classified as type A (good

quality) or type B (inferior quality) oocytes. The type A class included gametes of good quality, displaying compact multilayered corona radiata, homogeneous arrangement of cumulus oophorus, light and transparent aspect of total COC, adherent zona pellucida investment, and an ooplasm with homogeneously dispersed pigment. Type B oocytes displayed a heterogeneous arrangement of follicular cells (corona absent or with gaps, cumulus cells scattered in dark clumps or disconnected) and irregular ooplasm with pigment spots. The FFs from the aspirated follicles (n = 60) with recovered oocytes were used for further investigation (20 follicles in each size category) and were included in analysis.

Samples of FFs were further centrifuged (3000 g; 10 min.; 4°C). Aliquots of the processed follicular fluid (diluted 1:10, 1:100 and 1: 1000 with phosphate buffer solution, PBS, pH 7.0) were stored at –20°C until used for biochemical and hormonal assays. Protein concentration in FF was determined by the method of Lowery *et al.* (1951) using a commercial chemicals (Sigma Chemical St. Lious, USA). Total SOD activity in FF was measured by method described by Misra and Fridovich (1972), based on the ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2. Total SOD activity was expressed as ng/ mg protein. The GSH content of the FF was determined according to the method of Beutler *et al.* (1963) and the amount of GSH was expressed as a µg /mg protein. LPO in FF was assessed by the thiobarbituric reaction, which measures the secondary product of peroxidation, malondialdehyde (MDA) (Ohkawa *et al.*, 1979). The amount of LPO was expressed as nmol MDA/mg protein. Nitric oxide (NO) activity was measured using Gries reagent with sodium nitrite as a standard according to Ding *et al.* (1988) and activity was expressed as nmol/mg protein. The FF (diluted 1:100 and 1:1000 with PBS) was analyzed for P_4 and E_2 using a coat-A-count solid-phase RIA kit for P_4 and E_2 (Diagnostic Products Corp., Los Angeles, CA, USA). The levels of E_2 and P_4 in FF were used to classify these follicles as estrogen–active ($E_2 > P_4$; healthy, non-atretic) or estrogen inactive ($E_2 < P_4$, atretic) follicles (Sunderland *et al.*, 1994).

Analysis of variance-covariance was performed by the least squares method, employing the general linear model (GLM) of Statistical Analysis Systems (SAS, 2000), where the independent variables were follicular size and E_2:P_4 ratio in FF. The factors assigned as dependent variables were the concentrations of some antioxidants activities (SOD and GSH) and levels of LPO and NO in follicular fluids. The statistical significance of differences between groups of follicular

size for number of E_2 active follicles and good quality oocytes was tested by chi square analysis. Duncan's multiple range tests was used to test the difference of the antioxidants activities (SOD and GSH) and levels of LPO and NO in follicular fluids (mean ± s.e.m) in relation to quality of oocyte recovered. Pearson correlation coefficients were computed to study the relationships between biochemical constituents and the ratio of E_2: P_4 in FFs of different follicular sizes in buffalo-cows.

RESULTS

Overall-means, standard errors (S.E.), minimum and maximum values for steroid hormones (E_2 and P_4) and their ratio of selected follicles (different size categories) on ovaries bearing CL in buffalo-cows are shown in Table 1. Distribution of numbers of healthy follicles and quality of oocyte recovered from these follicles in relation to their size are presented in Table 2. The number and percentage E_2 active follicles as well as the good quality oocytes showed a decrease with increased follicular size. Most of small follicles were E_2 active follicles and their percentages were significantly ($P< 0.05$) higher compared with other follicular size categories (Table 2). Also, the number and percentage of quality of oocytes recovered were significantly ($P< 0.05$) different between the three follicular size categories. The percentage of inferior quality oocytes increased with increased follicular size with significantly ($P< 0.05$) highest percentage for group of large follicles compared with small follicles (Table 2).

Table 1: Steroid concentrations and E_2: P_4 ratio in follicles[1] selected for study.

Follicular size	Variable	Mean	S.E	Range Minimum	Maximum
Small	E_2 (ng/ml)	62.73	6.72	3.85	100.00
(5-8mm)	P_4 (ng/ml)	38.90	2.45	22.80	62.50
	E_2:P_4 ratio	1.74	0.23	0.11	3.66
Medium	E_2 (ng/ml)	62.50	7.77	8.51	125.18
(9-12 mm)	P_4 (ng/ml)	76.40	6.88	36.80	122.18
	E_2:P_4 ratio	1.11	0.20	0.10	2.75
Large	E_2 (ng/ml)	91.49	5.74	40.50	135.80
(>12 mm)	P_4 (ng/ml)	116.71	9.92	40.80	200.30
	E_2:P_4 ratio	0.92	0.13	0.41	3.20

[1]: 20 follicles in each diameter groups.

Table 2: Distribution of number of healthy follicles and quality of oocyte recovered in relation to size of follicles on ovaries bearing corpora lutea in buffalo-cows.

Follicular size (mm)	No. of follicles	Health state of follicles		Oocyte quality	
		E_2 active[1] (%)	E_2 inactive (%)	Good (%)	Inferior (%)
Small	20	16 (80)[a]	4 (20)[a]	13 (65)[a]	7 (35)[a]
Medium	20	10 (50)[b]	10 (50)[b]	7 (35)[ab]	13 (65)[ab]
Large	20	5 (25)[b]	15 (75)[b]	4 (20)[b]	16 (80)[b]

[1]: The ratio of E_2: P_4 = 1. 0 or more indicate estrogen active follicles.
[A,b]: Numbers with different superscripts in the same column differ significantly ($P < 0.05$).

Analysis of variance-covariance showed that factors included in model had an effect on biochemical constituents of follicular fluid (Table 3). The highest accounts of variance were observed for NO level ($R^2 = 0.82$, $P< 0.001$) and LPO level ($R^2 = 0.77$, $P< 0.001$) followed by GSH content ($R^2 = 0.76$, $P< 0. 01$) and SOD activity ($R^2 = 0.75$, $P < 0. 01$). The greatest influences on biochemical constituents were follicular size followed by E_2:P_4 ratio (Table 3). While the follicular size and E_2:P_4 ratio had a significant effect on all the biochemical constituents examined in this study, the effect of E_2:P_4 ratio on SOD activity tended to be significant ($P < 0.06$) (Table 4). The GSH content and NO level in follicular fluid were significantly ($P< 0.01$ and 0.001, respectively) increased with decreased follicular size and increased E_2:P_4 ratio. The highest values were recorded in a group of small follicles and E_2:P_4 ratio more than 1.0 (Table 4).

Table 3: Analysis of variance-covariance for some antioxidants activities and changes in lipid peroxide and nitric oxide productions in follicular fluids in buffalo-cows.

Source of variation	Some constituents of follicular fluid								
	df	SOD		GSH		LPO		NO	
		MS	*P*	MS	*P*	MS	*P*	MS	*P*
Follicular size (mm)	2	28.09	0.01	81.79	0.01	32.97	0.001	112.21	0.001
E_2:P_4 ratio	1	9.04	0.06	243.15	0.001	34.78	0.01	66.01	0.01
Model	3	18.73	0.01	214.49	0.01	54.10	0.001	147.28	0.001
Error	56	0.34		3.56		0.82		1.72	
R^2			0.75		0.76		0.77		0.82
CV		23.02		24.67		17.93		23.70	

df: degree of freedom; MS: mean square; *p*: probability; cv: coefficient of variation.
SOD: Superoxide dismutase GSH: Glutathione LPO: Lipid peroxide NO: Nitric oxide.

Table 4: Effect of follicular size and E_2:P_4 ratio on some antioxidants activities and changes in lipid peroxide and nitric oxide productions in follicular fluid in buffalo-cows.

	Some constituents[1] of follicular fluid				
	No. follicles	SOD (ng/mg)[2]	GSH (ug/mg)[2]	LPO (nmol/mg)[2]	NO (nmol/mg)[2]
Follicular size					
Small(5-8mm)	20	1.07 ± 0.14^{a}	9.75 ± 0.45^{ad}	3.51 ± 0.21^{a}	8.05 ± 0.31^{a}
Medium(9-12mm)	20	3.03 ± 0.13^{fg}	7.79 ± 0.42^{ac}	5.48 ± 0.20^{fg}	5.58 ± 0.29^{b}
Large(> 12 mm)	20	3.50 ± 0.13^{gh}	5.41 ± 0.43^{b}	6.20 ± 0.21^{fgh}	2.96 ± 0.30^{c}
E_2:P_4 ratio					
≥ 1.0	31	2.44 ± 0.22^{i}	9.90 ± 0.65^{a}	3.93 ± 0.28^{a}	7.32 ± 0.52^{a}
< 1.0	29	2.63 ± 0.19^{i}	5.40 ± 0.39^{a}	6.27 ± 0.25^{b}	4.06 ± 0.37^{b}

[1]: least squares mean±SEM. [2]: mg protein.

[a,b,c]:Different superscripts within the same column indicate significance at ($p < 0.001$).

[d,e] :Different superscripts within the same column indicate significance at ($p < 0.01$).

[g,h] :Different superscripts within the same column indicate significance at ($p < 0.05$).

[fih] :Different superscripts within the same column indicate significance at ($p < 0.06$).

The NO levels showed only significant positive correlations with E_2:P_4 ratio ($P< 0.001$, r = 0.7633 and $P< 0.01$, r = 0.6783) in small and medium sized follicles, respectively (Table 5). SOD activity and LPO Level increased with increased follicular size with significant ($P < 0.001$) higher values were recorded in large sized follicles (Table 4). In addition, the LPO content significantly ($P< 0.001$) decreased with increased E_2:P_4 ratio. LPO levels also showed significant ($P< 0.01$) negative correlations with SOD activity and NO levels, irrespective of follicular size (Table 5). Moreover, LPO showed significant ($P< 0.001$, r = 0.6933 and r = 0.8115) negative correlation with E_2:P_4 ratio, only in first two sizes categories of follicles (small and medium), respectively (Table 5).

Overall-means, standard errors (S.E.), minimum and maximum values for steroid hormones (E_2 and P_4) and its ratio in selected follicles in relation to quality of recovered oocyte are presented in Table 6. The mean concentrations E_2 and P_4 were 90.77± 5.02, 53.52 ± 7.16 and 59.88 ± 7.77, 93.22 ± 6.88 ng/ml in follicles with good and inferior quality oocytes, respectively (Table 6). The mean concentrations of some biochemical constituents of FFs in relation to oocyte quality recovered from follicles of different size categories are presented in Table 7. The GSH content and NO level were significantly ($P< 0.01$) higher in FFs from follicles contained good quality oocytes. On the contrary, the LPO level was significantly ($P< 0.01$) lower in follicles contained good

quality oocytes (Table 7). There was no change in SOD activities between follicles both containing good or inferior quality oocyte.

Table 5: Correlation coefficient between some antioxidants activities and changes in lipid peroxide and nitric oxide productions as well as the ratio of E_2:P_4 in follicular fluids of different size groups of follicles[1] on ovaries bearing corpora lutea in buffalo-cows.

	Follicular size groups		
	Small (5-8 mm)	Medium (9-12 mm)	Large (> 12 mm)
SOD / LPO	- 0.6137**	- 0.6253**	- 0.6032**
GSH / LPO	- 0.7767***	- 0.7402***	- 0.4999*
LPO / NO	- 0.8327***	- 0.8077***	- 0.6574**
SOD / E_2:P_4 ratio	0.5765**	0.6678**	0.5841**
LPO / E_2:P_4 ratio	- 0.6933***	- 0.8115***	- 0.3484
NO / E_2:P_4 ratio	0.7633***	0.6783**	0.3572

[1]: 20 follicles in each r group. *** P < 0.001 ** P < 0.01 * P < 0.05

Table 6: Steroid concentrations and E_2:P_4 ratio in relation to quality of recovered oocytes from ovarian follicles selected for study.

Oocyte quality	No. Follicles	Variable	Mean	S.E	Range	
					Minimum	Maximum
Good	24	E_2 (ng/ml)	90.77	5.02	20.11	135.80
		P_4 (ng/ml)	53.52	7.16	22.80	140.20
		E_2:P_4 ratio	2.07	0.17	0.88	3.66
Inferior	36	E_2 (ng/ml)	59.88	5.37	3.85	120.50
		P_4 (ng/ml)	93.22	7.30	34.50	200.30
		E_2:P_4 ratio	0.72	0.07	0.10	1.36

Table 7: Mean (± s.e.m) concentrations of some biochemical constituents of follicular fluids in relation to oocyte quality recovered from follicles on ovaries bearing corpora lutea in buffalo-cows.

	Oocyte quality	
	Good Oocyte (n = 24)	Inferior Oocyte (n = 36)
SOD (ng/mg)[1]	2.64 ± 0.26^{a}	2.46 ± 0.17^{a}
GSH (µg/mg)[1]	11.41 ± 0.56^{a}	5.14 ± 0.29^{b}
LPO (nmol /mg)[1]	3.24 ± 0.26^{a}	6.16 ± 0.22^{b}
NO (nmol/mg)[1]	8.49 ± 0.49^{a}	3.72 ± 0.30^{b}

[1]: mg protein. [a,b]: Different superscripts in the same row differ significantly (P < 0.01).

DISCUSSION

Understanding the dialogue between the follicle and its oocyte may certainly generate information useful for selection of follicle or its oocyte to improve the present IVM/IVF/IVP technology, especially in buffalo-cows. Since the FF is in intimate contact with the oocyte and granulosa cells, changes in concentrations of different intra-follicular factors, steroidal and non-estradiol factors may reflect the follicular requirements for growth, maturation and maintenance of viability of oocyte. The present study showed that the antioxidant activities and changes in LPO and NO are subjects to marked changes during the course of follicular growth during the estrous cycle in buffalo-cows. Moreover, this study indicated different physiological states as well as different quality of oocytes recovered from these vesicular follicles of different sizes. These data are consistent with previous findings about the possible effects of follicular size and intra-follicular environment on variability in developmental capacity and quality of bovine oocytes (Fukui and Sakuma, 1980; Shioya *et al.*, 1988; Hazeleger *et al.*, 1995; Smith *et al.*, 1996 and Yang *et al.*, 1998).

P_4 and E_2 are major regulators of follicular development and atresia. The results of the present study showed that concentrations of E_2 was higher in large follicles as compared with small ones, which is in agreement with previous study by Mekkawy *et al.* (1988) in buffalo-cows. This may be related to the ability of granulosa cells in large follicles to convert androgens and P_4 to E_2 (Henderson *et al.*, 1982). Expression of LH receptor mRNA increased in granulosa cells of healthy follicles and was related to follicular diameter (Xu *et al.*, 1995). Moreover, E_2 was found to be associated with growth divergence, growth termination and eventual regression of follicles (Bodensteiner *et al.*, 1996). Sequential growth and atresia of ovarian follicles occurred at any stage of estrous cycle (Hirshfield, 1991). Data of the present study indicated that during middle of the estrous cycle, most of large follicles on ovaries bearing corpora lutea were E_2 inactive follicles. This is in agreement with previous study by Takagi *et al.*, (1993) who reported that approximately 70% of follicles (> 10 mm in diameter) were E_2 inactive follicles and that more than 95 % of them are morphologically degenerated in bovine ovaries. Moreover, the data presented in this study showed that most of ovarian follicles (≥ 5 up to < 12mm in diameter) were E_2 active follicles, which seem to be in agreement with data previously reported by Henderson *et al.* (1982). The later authors

recorded that 83% of the ovarian follicles (> 3-8 mm in diameter) were E_2 predominate. Recently, divergent intra-follicular transcriptional/ mitogenic and antioxidant roles of estradiol that influence granulosa cell function and oocyte maturation have been defined (Lund *et al.*, 1999). The results of present study indicated that most of E_2 active follicles irrespective of follicular size contained good quality oocytes. This could be explained through protection effect of E_2 on granulosa cells against an ROS insult either by a non-genomic receptors-independent mechanism (Murodoch, 1998) and/or serve directly (at high concentration) as an oxidant scavenger (Mooradian, 1993). Moreover, the present data indicated that almost all oocytes recovered from E_2 inactive follicles were of bad quality. These data are consistent with previous findings that showed P_4 predominant follicles or large vesicular follicles (> 10 mm in diameter) on ovaries bearing corpora lutea mainly contained degenerated oocytes in dairy cows (Takagi *et al.*, 1993) or in buffalo-cows (EL-Din Zain *et al.*, 1997). In general, the present result and previous work by EL-Din Zain *et al.* (1997) suggested that CL had only adverse effect on large vesicular follicles (> 10mm in diameter). This is in agreement with previous findings of Boediono *et al.* (1995) that high P_4 concentration caused regression of dominant follicles and turnover of follicular waves in bovine species. Moreover, Boediono *et al.* (1995) showed that higher blastocyst production in vitro could be achieved from oocytes obtained from luteal phase cows and from ovaries bearing an active CL. Thus, changes in the relationship of concentrations of estradiol to other steroids within the follicle during the estrous cycle reflect either alteration in capacity to synthesize estradiol or essential intra-follicular environment for oocyte maturation.

The process of follicular growth and maturation requires additional fine-tuning by non-steroidal intra-ovarian factors acting as autocrine or paracrine regulators of hormonal signaling (Basini *et al.*, 1998). Among these, SOD and NO as labile messenger molecules have emerged to be important intra-and intercellular messengers controlling many biological processes (Aten *et al.*, 1992 and Rodgers *et al.*, 1995). Reactive oxygen species such as hydroxyl radicals and superoxide anions generated during normal metabolic reactions, if not sufficiently neutralized, pose a serious threat to cellular viability (Tarrin, 1996). In the present study, the presence of SOD in follicular fluid in buffalo-cows is demonstrated. Previous work by Fridovisch (1986) isolated SOD, the specific inhibitor of superoxide anion, in several forms which differ in transition at the active center in their cell location. The result of this

study demonstrated the existence of a dependent relationship between SOD activity, LPO production and E_2:P_4 ratio in different size categories of examined follicles. Superoxide anion created during mitochondrial electron-transport system and cytochrome P_{450} lead to decrease steroidogenesis and increase in production of LPO due to damage of follicular cells, if not detoxified by SOD and other scavenges (Weiss, 1986). Moreover, data of this study demonstrated increased SOD activity with increased follicular diameter. This may be related to increase in steroids synthesis by granulosa cells with development and growth of ovarian follicles as observed with increase of E_2 and P_4 concentrations in follicular fluids. Sugino, *et al.* (1993) reported that SOD, in the presence of catalase, apparently blocks the antisteriodogenic effect of superoxide anions and other ROS.

Glutathione is free thiols that function in many biological processes including DNA and protein synthesis and cellular protection during oxidative stress (Zuelke *et al*., 1997). The presence of GSH in higher levels in small and medium sized follicles than larger ones coincide with previous finding by Zuelke and Perreault (1994) who demonstrated the rapid increase in GSH concentrations that associated with *in vivo* meiotic maturation of oocyte. In bovine species, Fair *et al.* (1996) reported that the ability of oocyte to complete meiosis to reach metaphase II increased if recovered from follicles above 4 mm in diameter. Moreover our study demonstrated a high level of GSH in follicles contained good oocyte. This could be attributed to coupling between these cells and oocyte via heterologous gap junctions which is critical for oocyte maturation (de-Matos *et al.*, 1997). In addition, present study found a negative correlation with LPO production. This is in agreement with recent work by de-Matos *et al.* (2002) who reported a decrease in LPO production with increased of GSH content.

The present study revealed that LPO production increased with increase in follicular size in buffalo-cows. This marked increase in LPO production coincided with increase in steroidogenic activity of large follicles (> 12 mm in diameter) as observed by increased mean levels of steroids in follicular fluid. Increases in ROS (superoxide radicals) with decreased antioxidant activities as well as NO, as observed in the present study, may initiate apoptosis of follicular cells. Therefore, most follicles in class > 12 mm in diameter are atretic follicles containing inferior quality oocytes. Nitric oxide has emerged as a novel regulator of several ovarian events, such steroidogenesis, oocyte meiosis and apoptosis (Basini *et al.*, 1998 and Nakamura *et al.*, 2002). The data of the present

study demonstrated the presence of NO in follicular fluid in buffalo-cows and its levels decreased with increased follicular size. This agrees with previous finding by Basini *et al.* (1998) who reported a higher NO content in small size (< 5 mm in diameter) than large (> 5 mm in diameter) sized follicles. Also results of this study found that large follicles, mostly E_2 inactive or atretic follicles and contained inferior quality oocytes, contained lower level of NO compared with E_2 active follicles. This may be due to decrease in NO-synthase (NOS) that regulate production of NO (Adams *et al.*, 1992). Recent investigation suggested that NO/NOS system exerts a regulatory role on steroidogenesis (Boiti *et al.*, 2000), as well as, plays an important role in oocyte maturation (Basini *et al.*, 1998). Moreover, the lower concentration (as observed in follicles more than 12 mm in diameter) stimulated cellular apoptosis of large follicles (Basini *et al.*, 1998). This supports the findings of present study that most of large follicles on ovaries bearing corpora lutea were atretic follicles.

In conclusion, the antioxidant activities and changes in LPO and NO levels are subjects of marked changes in the course of follicular growth during the luteal phase of the estrous cycle in buffalo-cows. These changes in the intra-follicular environment may reflect the follicular requirements for growth, maturation and maintenance of viability of oocytes in buffalo-cows.

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