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Lipid and protein metabolism along with oxidative status of follicular fluid throughout the estrous cycle in Anatolian water buffalo

Oktay YILMAZ[1,*], Jerzy Jan JAROSZEWSKI[2], Aziz BÜLBÜL[3], Mehmet UÇAR[1]

[1]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey
[2]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Department of Physiology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

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Abstract: This study was performed to determine the concentrations of creatinine (CRE), high-density lipoprotein (HDL), low-density lipoprotein (LDL), blood urea nitrogen (BUN), nitric oxide (NO), and malondialdehyde (MDA) in follicular fluid (FF) in relation to follicular size and/or stage of the estrous cycle [early I luteal (n = 14), early II luteal (n = 16), late luteal (n = 16), and follicular (n = 16) phases] in Anatolian water buffaloes. The concentrations of CRE ($P < 0.001$), HDL ($P < 0.001$), LDL ($P < 0.05$) BUN ($P < 0.001$), and NO ($P < 0.01$) in FF of large follicles (LFs, ≥10 to <20 mm) during the early I luteal phase were lower than those of other stages. In FF of small follicles (SFs, ≥5 to <10 mm), LDL and BUN concentrations were lower during the early I luteal stage ($P < 0.05$), whereas MDA levels were the highest ($P < 0.001$). MDA concentrations in SFs were higher than those in LFs during the early I luteal stage ($P < 0.001$). In conclusion, higher CRE, HDL, LDL, BUN, and NO activity in FF of LFs during luteal stages (early II and late) revealed that these biochemicals may play some roles in follicle development prior to domination and/or ovulation.

Key words: Follicle, Anatolian water buffalo, nitric oxide, high-density lipoprotein, creatinine

1. Introduction

The Anatolian water buffalo (*Bubalus bubalis*) is a native breed and is commercially very important for the milk-cream industry in Turkey. It has been reported that calving and estrous activity occur in a seasonal pattern in Anatolian buffaloes (1,2). Poor reproductive management of dairy buffalo has resulted in various reproductive disorders causing infertility and thus economic losses in the milk industry (3). Therefore, studies on the follicular fluid (FF) microenvironment in buffalo may contribute to knowledge about the process of normal follicular development as well as the pathogenesis of some reproductive problems.

The FF consists of various nutrients, growth factors, hormones, electrolytes, and enzymes (4) and, therefore, plays a key role in the physiological, biochemical, and metabolic aspects of the maturation process of the oocyte (5). Follicular fluid can be an index of the functional status of the ovarian follicle and reproductive state of the animal (6). In this regard, the biochemical composition of FF possibly plays a role in the growth and development of follicles in buffaloes (6–8). FF is partly composed of exudates of serum and locally produced substances that are related to the metabolic activity of the follicular cells (9). It is well known that oxygen is vital for cell survival; however, the microenvironment of FF is a metabolically active system in which the metabolites of oxygen may alter the functions of the cells and, therefore, the development of follicles (10). Moreover, it has been suggested that the level of lipid peroxidation and antioxidant status gives supplementary information about the metabolic status of the animal (11). Nitric oxide (NO), with a short half-life, is a highly reactive free radical gas that has a variety of physiological actions in the reproductive system, such as ovarian function (12). However, NO can prevent the oxidation mediated by reactive oxygen species such as H_2O_2 and O_2^- that occurs at physiological levels of NO. In addition, NO protects against cell death mediated by H_2O_2, alkylhydroperoxides, and xanthine oxidase. It seems to be the major chemical mechanism by which NO may limit oxidative injury to mammalian cells. Moreover, cellular and physiological processes can be modulated by NO to limit oxidative injury via limiting processes such as leukocyte adhesion (13). Before focusing on effects of FF composition on oocyte quality, the investigation of physiological concentrations of some common metabolites obtained from different-sized follicles is

* Correspondence: oktayyilmaz@aku.edu.tr

needed. Moreover, despite the fact that biochemical constituents of FF have been implicated in follicular development, the evidence for the role of NO, the oxidant/antioxidant status, and some biochemical components of FF is limited throughout the estrous cycle in buffaloes. This study was, therefore, designed to examine the oxidant (NO and malondialdehyde [MDA]) and antioxidant (retinol, β-carotene and vitamin C) status, metabolites (creatinine [CRE] and blood urea nitrogen [BUN]), and concentrations of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) in relation to follicle size and stage of estrous cycle in Anatolian water buffalo.

2. Materials and methods

2.1. Collection of ovaries and classification of follicles

Pluriparous, nonpregnant Anatolian water buffaloes (n = 62) aged between 5 and 8 years were used for this study. The study was approved by the local ethics committee of the Experimental Animal Research Center of Afyon Kocatepe University (Reference No: 49533702/116). Entire reproductive tracts of buffalo at different stages of the estrous cycle were collected from abattoirs for more than 1 year (from March 2014 to May 2015) in Afyonkarahisar Province, Turkey, and transported to the laboratory of the Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, on ice within 15 min to minimize any postmortem changes to the concentrations of the FF. Blood samples were taken into sterile blood collection tubes during exsanguinations and then centrifuged at 5000 rpm for 10 min. All sera were stored at –20 °C for later estimation of progesterone (P_4) and estradiol 17β (E_2) concentrations to confirm that the buffalo cows had normal ovarian function.

The estrous cycle was assessed on the basis of ovarian luteal and follicular morphology as being in the early I luteal (days 2 to 3), early II luteal (days 5 to 6), late luteal (days 15 to 17), or follicular (days 19 to 21) stage as described in Table 1. If the concentration of P_4 did not correspond to morphological findings, the data were excluded from analysis.

Immediately after slaughter, the follicular population was counted and measured using a water-bath ultrasonography technique. Individual follicles were classified into two size categories based on the diameter, i.e. small follicles (SFs, ≥5 to <10 mm) and large follicles (LFs, ≥10 to <20 mm). Ovaries having follicles greater than 20 mm in diameter were excluded from the study. Moreover, the large follicles that seemed morphologically atretic, identified macroscopically according to the criteria of Kruip and Dieleman (14), were not sampled. Briefly, follicles having a uniformly bright appearance, extensive and very fine vascularization, and no free-floating particles in the FF were classified as nonatretic, whereas follicles were accepted as atretic when they had a dull gray appearance, blood vessels either irregularly filled with clotted blood or empty, and many large free-floating globules in the antral cavity (14).

2.2. Follicular fluid aspiration and determination of biochemical parameters in FF

The FF in SFs and LFs was separately aspirated using a 5-mL syringe connected to an 18-G needle. For each buffalo, a different needle and syringe were used. The

Table 1. Classification of the estrous cycle [early I luteal (days 2–3), early II luteal (days 5–6), late luteal (days 15–17), and follicular (days 19–21) phases] based on the morphological appearance of the corpus luteum (CL), diameter of the follicle (F), and concentration of progesterone (P_4).[a]

Parameter	Criteria	Early I	Early II	Late	Follicular
CL	Surface	Bloody, visible rupture point	Bloody, visible vascularization	Covered by connective tissue	Covered by connective tissue
	Color	Red	Apex red/brown Rest grayish	Bright red/gray	Bright white/yellow
	Consistency	Soft	Soft	Compact	Hard
F[b]	<10 mm	(+)	(+)	(+)	(+)
	>10 mm	(–)	(±)	(+)	(+)
P_4[c]	<1	(±)	(–)	(–)	(+)
	>1	(±)	(+)	(+)	(–)

[a] Based on a previous report (29).
[b] (+) - presence of follicle; (–) - absence of follicle; (±) - variable presence of follicle.
[c] (+) - higher than 1 ng/mL; (–) - lower than 1 ng/mL; (±) - variable concentration of progesterone.

FF was centrifuged at 2500 rpm and 4 °C for 10 min in a refrigerated centrifuge and the supernatant was stored at –20 °C until further analysis of CRE, HDL, LDL, and BUN levels using an autoanalyzer (Tokyo Boeki Prestige 24i, Japan) following the protocols according to the manufacturer's instructions. NO level was quantified indirectly by measuring nitrites (NO_2) and nitrates (NO_3), using the Griess method. Briefly, 100 µL of sample was added to a 96-well plate and incubated for 30 min in the presence of 50 µL of NEDD (0.1%, w/v), 50 µL of SULF (2%, w/v), and 100 µL of vanadium(III) chloride (50 mM) at 37 °C. After incubation, the absorbance of each sample was measured with a plate microplate reader (MWGt Lambda Scan 200, Bio-Tek Instruments, USA), with an emission filter set at 545 nm. NO_2/NO_3 concentration (0.25–200 mM) was calculated using the NO_2 standard curve. Plasma lipid peroxidation was determined using a procedure in which MDA, an end product of fatty acid peroxidation, reacts with TBA to form a colored complex with a maximum absorbance at 532 nm.

Serum P_4 and E_2 concentrations were estimated by electrochemiluminescence immunoassay using a commercial test kit (Elecsys Progesterone II, Roche Diagnostics GmbH, Germany) in an immunological test analyzer (COBAS e601, Roche Diagnostics GmbH, Germany). The sensitivities of assay for P_4 and E_2 were 0.15 ng/mL and 12.00 pg/mL, respectively. The average intraassay and interassay coefficients of variation were kept under 20% for both investigated hormones.

Retinol and β-carotene concentrations in serum were estimated using a spectrophotometer (Shimadzu UV-1601, Japan). One milliliter of each serum sample was mixed with 1.0 mL of 96.5% ethanol in a test tube, followed by 3.5 mL of 98.5% hexane. Tubes were shaken for 10 min and centrifuged at 800 × *g* for 10 min. Spectrophotometric absorbance of supernatants was measured at 453 nm and 325 nm for β-carotene and retinol, respectively. The β-carotene and retinol concentrations (µg/dL) were calculated by related equations. The serum vitamin C concentrations were measured using an enzyme-linked immunosorbent assay/spectrophotometric reader (MWGt Lambda Scan 200, Bio-Tek Instruments, USA). Briefly, after precipitation of proteins by phosphotungstic acid, spectrophotometric measurement was employed to determine vitamin C level on the basis of the color formation in response to reaction between protein precipitate and ascorbic acid.

2.3. Statistical analysis

Differences between the metabolites in SFs and LFs and the metabolite, P_4, and E_2 levels in blood during the different stages of the estrous cycle were compared using analysis of variance (ANOVA) followed by the Tukey test (SPSS 13.0). The differences between metabolites in SFs and LFs in each stage of the estrous cycle were compared with the Student t-test. Data were considered to be significantly different at $P < 0.05$.

3. Results

The mean blood P_4 concentration was higher ($P < 0.001$) in the late luteal stage as compared with the early I luteal, early II luteal, and follicular phases, whereas mean E_2 concentrations did not differ throughout the estrous cycle (Table 2).

Tables 3 and 4 summarize concentrations of biochemical metabolites in LFs and SFs, respectively. In LFs, significantly lower concentrations of CRE ($P < 0.001$), HDL ($P < 0.001$), LDL ($P < 0.05$), and BUN ($P < 0.001$) were observed during the early I luteal phase as compared with other stages of the estrous cycle. The concentration of NO was higher in the early I luteal stage ($P < 0.01$), whereas MDA was higher during the late luteal and follicular stages of the estrous cycle ($P < 0.01$).

In SFs, concentrations of CRE during the early I luteal, early II, and late luteal stages did not show any significant differences, whereas in the follicular stage higher concentrations than those seen in the early I and II luteal stages were observed ($P < 0.05$). Concentrations of HDL did not significantly differ during the early II luteal, late

Table 2. Mean (±SEM) serum progesterone (P_4) and estradiol 17β (E_2) concentrations during different stages of the estrous cycle in Anatolian water buffalo.

	Concentrations	
Stages of estrous cycle	P_4 (ng/mL)	E_2 (pg/mL)
Early I luteal (n = 14)	0.77 ± 0.22^a	15.58 ± 1.30^a
Early II luteal (n = 16)	3.97 ± 1.10^b	18.74 ± 3.46^a
Late luteal (n = 16)	6.73 ± 1.46^c	15.67 ± 1.20^a
Follicular (n = 16)	0.37 ± 0.13^a	17.09 ± 1.01^a

^{a,b} - Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 3. Mean (±SEM) concentrations of creatinine (CRE), high-density lipoprotein (HDL), low-density lipoprotein (LDL), blood urea nitrogen (BUN), nitric oxide (NO), and malondialdehyde (MDA) in large follicle fluid during different stages of the estrous cycle.

Metabolites	Early I luteal (n = 14)	Early II luteal (n = 16)	Late luteal (n = 16)	Follicular (n = 16)
CRE (mg/dL)	0.53 ± 0.19[a]	1.64 ± 0.15[b]	1.95 ± 0.12[b]	1.70 ± 0.12[b]
HDL (mg/dL)	11.62 ± 3.70[a]	42.12 ± 2.01[b]	33.50 ± 2.88[b]	40.25 ± 4.62[b]
LDL (mg/dL)	4.12 ± 2.09[a]	12.25 ± 1.12[b]	14.25 ± 1.62[b]	12.37 ± 3.71[b]
BUN (mg/dL)	16.14 ± 5.45[a]	50.57 ± 6.32[b]	58.00 ± 5.22[b]	49.75 ± 3.20[b]
NO (µmol/L)	22.81 ± 0.89[a]	17.93 ± 0.84[b]	19.25 ± 0.77[b]	18.60 ± 0.85[b]
MDA (nmol/mL)	3.01 ± 0.30[a]	3.50 ± 0.11[ba]	3.86 ± 0.17[cb]	4.40 ± 0.28[c]

[a,b,c] - Values within rows with different superscripts differ significantly ($P < 0.05$).

Table 4. Mean (±SEM) concentrations of creatinine (CRE), high-density lipoprotein (HDL), low-density lipoprotein (LDL), blood urea nitrogen (BUN), nitric oxide (NO), and malondialdehyde (MDA) in small follicle fluid during different stages of the estrous cycle.

Metabolites	Early I luteal (n = 14)	Early II luteal (n = 16)	Late luteal (n = 16)	Follicular (n = 16)
CRE (mg/dL)	0.09 ± 0.02[a]	0.13 ± 0.04[a]	0.75 ± 0.26[ab]	1.18 ± 0.39[b]
HDL (mg/dL)	2.62 ± 0.46[a]	8.85 ± 5.27[ab]	20.50 ± 7.11[b]	23.37 ± 6.51[b]
LDL (mg/dL)	1.98 ± 0.18[a]	6.69 ± 1.77[b]	7.25 ± 1.62[b]	7.37 ± 1.67[b]
BUN (mg/dL)	3.66 ± 0.36[a]	12.30 ± 1.86[b]	16.37 ± 4.96[b]	22.92 ± 7.02[b]
NO (µmol/L)	12.25 ± 0.90[a]	13.00 ± 0.98[a]	11.87 ± 0.62[a]	16.68 ± 0.84[b]
MDA (nmol/mL)	4.59 ± 0.22[a]	2.14 ± 0.17[b]	2.40 ± 0.14[b]	2.65 ± 0.17[b]

[a,b] - Values within rows with different superscripts differ significantly ($P < 0.05$).

luteal, and follicular stages, whereas in the early I luteal stage lower concentrations ($P < 0.05$) were measured as compared with the late luteal and follicular stages. Significantly lower ($P < 0.05$) LDL and BUN concentrations were observed during the early I luteal stage as compared with the other stages of the estrous cycle. The highest ($P < 0.001$) NO concentrations were observed during the follicular stage, whereas MDA concentrations were the highest ($P < 0.001$) during the early I luteal stage.

The serum concentrations of NO did not differ during the early I luteal, early II luteal, and follicular stages of the estrous cycle, whereas higher concentrations were observed in the late luteal phase as compared with the early luteal II ($P < 0.01$) and follicular ($P < 0.01$) stages. Serum MDA and vitamin C concentrations did not significantly differ among the stages of the estrous cycle. The highest ($P < 0.05$) serum retinol and β-carotene concentrations were observed during the follicular stage as compared with the other stages of the estrous cycle (Table 5).

The differences between the concentrations of metabolites in SFs and LFs during the estrous cycle are shown in Figures 1A–1D. In the early I luteal stage the concentrations of CRE, HDL, LDL, BUN, and NO were higher ($P < 0.05$ to $P < 0.001$), whereas MDA was lower in LFs as compared to SFs ($P < 0.05$). In the early II and late luteal stages the concentrations of all parameters examined were higher ($P < 0.05$ to $P < 0.001$) in LFs. In the follicular stage, only concentrations of HDL ($P < 0.01$), BUN ($P < 0.05$), and MDA ($P < 0.001$) in LFs were higher than those in SFs.

4. Discussion

There have been well-documented reports investigating the FF profile in cattle (15–17), camels (18), and buffaloes (6,8,19). However, the data obtained throughout the estrous cycle are limited. In the current study, we have found that during the early I luteal stage there were lower concentrations of HDL in the FF of both SFs and LFs.

Table 5. Mean (±SEM) serum concentrations of nitric oxide (NO), malondialdehyde (MDA), vitamin C (Vit C), retinol, and β-carotene during different stages of the estrous cycle.

Metabolites	Early I luteal (n = 14)	Early II luteal (n = 16)	Late luteal (n = 16)	Follicular (n = 16)
NO (μmol/L)	18.00 ± 1.81^{ab}	17.22 ± 2.25^{b}	23.52 ± 1.96^{a}	12.12 ± 1.53^{b}
MDA (nmol/mL)	3.71 ± 0.13^{a}	3.70 ± 0.21^{a}	3.29 ± 0.70^{a}	3.73 ± 0.14^{a}
Vit C (mg/dL)	1.54 ± 0.12^{a}	1.50 ± 0.10^{a}	1.52 ± 0.12^{a}	1.40 ± 0.22^{a}
Retinol (μg/L)	12.76 ± 1.09^{a}	9.32 ± 1.86^{a}	12.24 ± 1.50^{a}	22.13 ± 5.78^{b}
B-carotene (μg/L)	25.58 ± 2.62^{ab}	19.37 ± 2.83^{a}	25.19 ± 2.78^{ab}	37.30 ± 6.79^{c}

^{a,b,c} - Values within rows with different superscripts differ significantly ($P < 0.05$).

However, other luteal stages and follicular phases did not show any significant difference in SFs, in accordance with Acar et al. (19), who detected no difference in FFs obtained from follicles 3–8 mm in diameter. LFs showed higher HDL concentrations as compared with SFs throughout the estrous cycle. Moreover, it was seen that as the follicle became larger, the concentration of HDL increased, which was in agreement with findings in cows (20). However, our results differed from other studies that reported that follicular size had no effect on HDL concentrations in Graafian follicles of dromedary camels (21) and that SFs had higher HDL concentrations than LFs in the ovulatory follicles of one-humped camels (18). It has been reported that HDLs are transuded from the blood into the follicles and provide cholesterol to the granulosa cells for steroidogenesis (22). In healthy growing follicles, HDLs maintain progesterone production at a low rate (23). Thus, HDLs might support steroidogenesis during follicular growth and prevent cholesterol accumulation in the largest follicles (18). It has been reported that the blood–follicle barrier permits the passage of LDLs into preovulatory follicles only during the final phase of follicle development (24). Similar to HDL, lower concentrations of LDL were found in SFs during the early I, early II, and late luteal stages as compared with large follicles in our study. This might be due to the dominance of the large follicles throughout the estrous cycle. Moreover, there was no significant difference in the concentration of LDL during the follicular stage, in accordance with findings in camel (18).

The CRE concentrations found during the follicular stage in the current study were in accordance with the results reported in camels (18), buffaloes (8), and cattle (15). Moreover, CRE levels in both SFs and LFs did not change in the luteal stage of the estrous cycle and this was in accordance with the results obtained from buffalo (7).

In the current study, the higher BUN concentrations seen at the end of protein metabolism in LFs indicated that there was local urea production by the follicular cells (24) and the protein catabolism was higher in the FFs of LFs than SFs. However, the BUN concentrations did not differ in SFs during the early II luteal, late luteal, and follicular phases in accordance with Acar et al. (19). Our finding suggests that when the follicle becomes larger, the protein metabolism increases.

Some studies have reported the biochemical constituents of FF in buffaloes, but few have examined the oxidant status of the FF throughout the estrous cycle, which may play a possible role in the process of follicular development (6). Therefore, the present study also focused on the oxidant status of FF and the oxidant–antioxidant status of blood serum. It was observed that buffalo cows showed disturbed oxidant and antioxidant status in the blood, with increased NO levels during the late luteal stage, stable MDA levels throughout the estrous cycle, and increased retinol and β-carotene concentrations during the follicular stage. It has been reported that at the early stage of corpus luteum (CL) development, NO promotes P_4 production (25), but at the late phase of the luteal stage, NO plays a direct luteolytic role in regression of the CL (26). In the current study, increasing serum NO levels during the luteal period may have been due to the development and regression processes of the CL, as previously reported in cows (25) and buffaloes (27). The NO composition of FF showed that the highest NO concentration in LF fluids was evident during the early I luteal stage, whereas the highest NO concentration in SF fluids was seen during the follicular stage. It has been reported that the blood flow of follicles is associated with the first follicular wave in cows (28) and follicles with follicular blood flow have higher NO concentrations in FF (17). In buffaloes, the first follicular wave appears approximately on the day of ovulation (between days 1 and 4) (29). The highest NO activity in LFs during the early luteal stages of the estrous cycle might be due to development of a new follicle wave at the beginning of the estrous cycle and, therefore, the

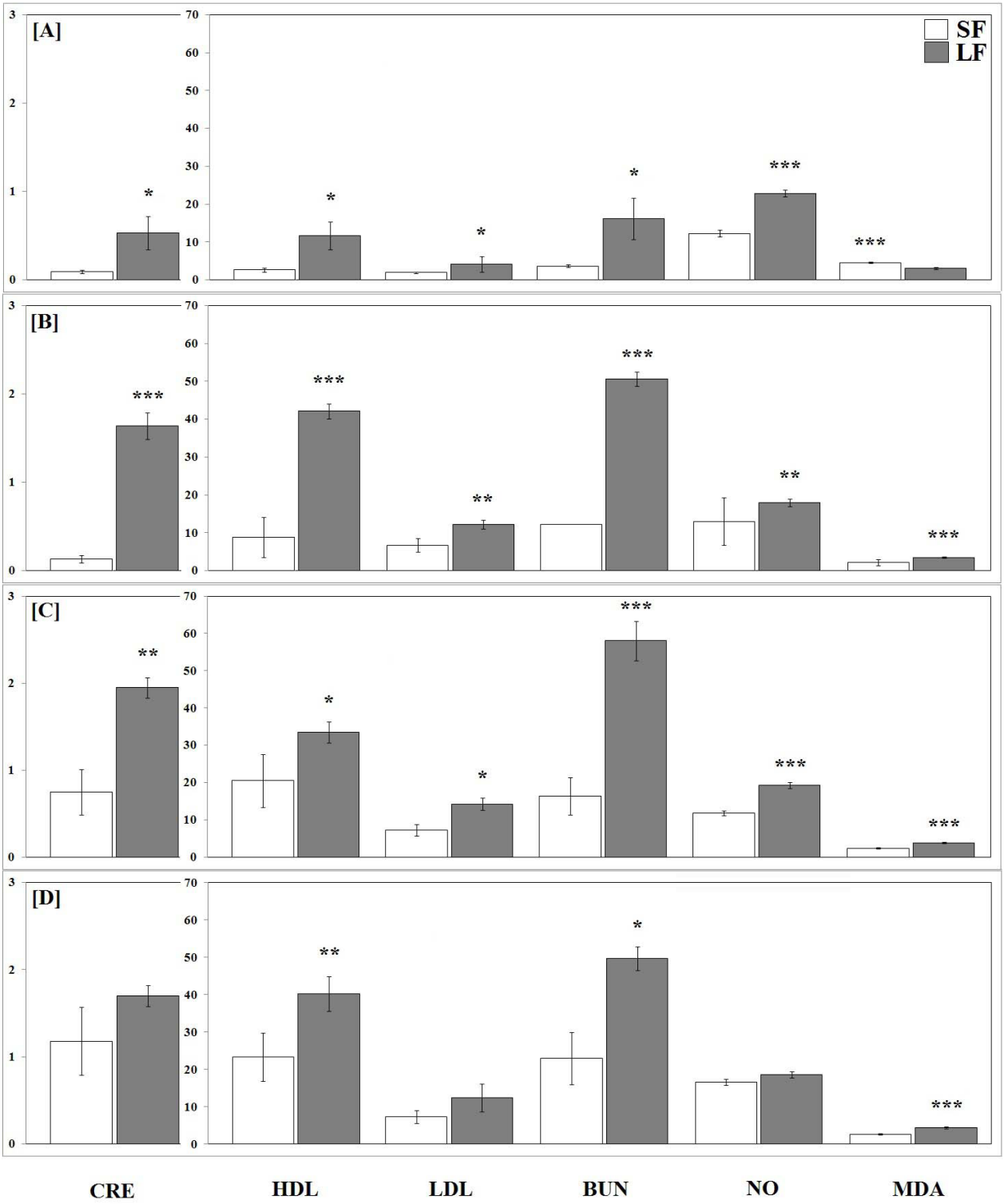

Figure 1. Mean (±SEM) concentrations of creatinine (CRE, mg/dL), high-density lipoprotein (HDL, mg/dL), low-density lipoprotein (LDL, mg/dL), blood urea nitrogen (BUN, mg/dL), nitric oxide (NO, µmol/L), and malondialdehyde (MDA, nmol/mL) in both small and large follicle fluids during (A) early I luteal phase (n = 14), (B) early II luteal phase (n = 16), (C) late luteal phase (n = 16), and (D) follicular phase of the estrous cycle. SF - small follicle; LF - large follicle. *P < 0.05, **P < 0.01, ***P < 0.001.

high oxidant activity. It has been indicated that NO may play a role in selecting one of the follicles that will ovulate and NO induces ovulation in part by stimulating the production of prostaglandins (12). The observation of no significant difference in the concentrations of NO in LF fluids during the early II and late luteal and follicular stages might be due to the dominance process of the follicle. In the present study, the concentrations of NO in SFs were significantly higher during the follicular phase. It has been indicated that after the deviation process of growing follicles have higher NO concentrations (16) and among bovine granulosa cells, those from SFs produce more NO than do those from LFs (30) and estradiol 17β modulates NO synthase activity (26). Since the presence of a positive correlation between NO concentrations and estradiol 17β has been demonstrated in cattle (17), the presence of estradiol 17β and a low level of progesterone might be related to increased NO levels in this stage.

MDA concentration in LFs did not differ during the late luteal and follicular stages. Similarly, MDA concentrations in SFs did not differ during the early II luteal, late luteal, and follicular stages and these findings were in accordance with those of El-Shahat and Kandil (31). It was expected that LF fluids would have more oxidant activity due to the high metabolic activation. In our study, MDA concentrations in LFs were higher than those in SFs, except during the early I luteal stage. This might be due to the high metabolic activity of SF fluids during the initiation of the new follicle wave and the dominance of the follicle may be related to high oxidant activity. Importantly, the serum oxidant/antioxidant status showed a different nature than that determined in FF. This observation may support the suggestion that the metabolic activity of follicles directly affects the follicle microenvironment itself.

In conclusion, our results showed that a different biochemical composition existed in the FF of buffaloes throughout the estrous cycle. It seems that low concentrations of CRE, HDL, LDL, and BUN as well as a high concentration of NO in LFs may be used as an indicator at the beginning of the luteal stage (early I luteal stage) of the estrous cycle in buffaloes, since other stages do not show any significant differences. Similarly, lower LDL and BUN concentrations and higher MDA contents in SFs may also be related to onset of the luteal stage (early I luteal). Furthermore, the luteal stage (early II and late luteal) has higher CRE, HDL, LDL, BUN, and NO activity in LFs, suggesting their possible role in the dominance of the follicle. Moreover, it is also suggested that higher HDL, BUN, and MDA contents in LFs may indicate that these LFs could potentially be preovulatory follicles.

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