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RESEARCH ARTICLE

The Effect of Estrous Cycle on Oxidant and Antioxidant Parameters in Dairy Cows [1]

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Summary

Reactive oxygen species have fundamental roles in reproductive functions. To comprehensively evaluate the relation between reactive oxygen species and infertility, physiological variations across the estrous cycle in healthy cows have to be known. For this purpose 25 healthy multiparous Holstein dairy cows having regular estrous cycles were used. The estrous cycles were synchronized by ovsynch protocol. Oxidant [lipid hydroperoxide (LOOH), total oxidant status (TOS), oxidative stress index (OSI)], antioxidant parameters [total antioxidant status (TAS), total free sulfhydryl groups (SH), ceruloplasmin (CP), paraoxonase-1 (PON1), arylesterase (ARE), uric acid (UA)], lipid profile and progesterone levels were assayed at estrus, metestrus, diestrus and proestrus stages of the estrous cycle in the plasma samples. The plasma levels of oxidant (LOOH, TOS and OSI) and antioxidant (TAS, SH and UA) parameters were significantly decreased during the luteal phase compared to the follicular phase (at proestrus and at estrus) of the estrous cycle. There was also a significant positive correlation between TAS and TOS. The activity of PON1 and ARE significantly increased only at diestrus. Levels of high density lipoprotein, low density lipoprotein and total cholesterol elevated during the follicular phase (estrus) and declined during the luteal phase. In conclusion, oxidant/antioxidant status and lipid profile were affected by cyclic changes. Moreover, antioxidant defense system showed adaptive response to increased oxidative activities by occurring parallel increases and it may indicate that there is a dynamic balance between oxidant and antioxidant status during the estrous cycle in healthy cows.

Keywords: *Cow, Estrous cycle, Lipid peroxide, Lipid profile, Total antioxidant status, Total oxidant status*

Sütçü İneklerde Östrus Siklusunun Oksidan ve Antioksidan Parametreler Üzerine Etkisi

Özet

Reaktif oksijen türlerinin üreme fonksiyonları üzerinde önemli rolleri vardır. Sağlıklı süt sığırlarında reaktif oksijen türleri ve infertilite arasındaki ilişkiyi kapsamlı bir şekilde değerlendirmek için östrus siklusu boyunca reaktif oksijen türü seviyelerindeki değişimlerin bilinmesi gerekir. Bu çalışmada düzenli siklus gösteren 25 adet sağlıklı Holştayn ırkı sütçü inek kullanıldı. Östrus siklusları ovsynch protokolü ile senkronize edildi. Östrus siklusunun proöstrus, östrus, metaöstrus ve diöstrus dönemlerinde kan plazmasında oksidan parametreler [lipit hidroperoksit (LOOH), total oksidan seviye (TOS), oksidatif stres indeksi (OSI)], antioksidan parametreler [total antioksidan kapasite (TAS), total serbest sülfhidril gruplar (SH), seruloplazmin (CP), paraoksonaz-1 (PON1), arilesteraz (ARE), ürik asit (UA)], lipit profili ve progesteron seviyesi ölçüldü. Foliküler dönemle karşılaştırıldığında luteal dönemdeki plazma oksidan (LOOH, TOS, OSI) ve antioksidan (TAS, SH, UA) seviyelerinin önemli derecede düştüğü görüldü. Ayrıca TAS ve TOS değerleri arasında önemli bir pozitif korelasyon vardı. Paraoksonaz-1 ve ARE aktiviteleri sadece diöstrus döneminde anlamlı olarak arttı. Foliküler dönemde (östrus) artan yüksek yoğunluklu lipoprotein, düşük yoğunluklu lipoprotein ve total kolesterol seviyeleri luteal dönemde düştü. Sonuç olarak, oksidan/antioksidan denge ve lipit profili östrus siklusu sırasındaki değişikliklerden etkilenmiştir. Ayrıca, antioksidan savunma sistemi artan oksidatif strese karşı paralel artışlar göstererek uygun savunma cevapları oluşturmuştur. Bu da sağlıklı ineklerde östrus siklusu boyunca oksidan ve antioksidanlar arasında dinamik bir dengenin olabileceğini göstermektedir.

Anahtar sözcükler: *İnek, Östrus siklusu, Lipit peroksit, Lipit profili, Total antioksidan kapasite, Total oksidan seviye*

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INTRODUCTION

Many stress factors including heat stress and high milk yield are impairing the reproductive efficiency and performance of dairy cows. A common denominator of the responses to these stresses is the redox homeostasis. The redox homeostasis is maintained by the balance between the production of reactive oxygen species (ROS) and antioxidant defense system [1]. Oxidative stress occurs when the generation of ROS exceeds the scavenging capacity of antioxidants, either due to excessive ROS production or an inadequate availability of antioxidants. At physiological levels, ROS is required for reproductive functions including oocyte maturation, folliculogenesis, ovarian steroidogenesis, luteolysis, ovulation and cyclical endometrial changes [2]. However ROS, higher than physiological levels, contribute to several pathological conditions, such as retained placenta, udder oedema, mastitis, infertility which in turn may impair reproductive performances [3].

The estrous cycle is associated with several metabolic and hormonal variations. The mechanisms that relate ROS with female fertility are not completely understood. Investigation of the relationships between ROS and female fertility and reproductive outcomes may be masked by normal menstrual cycle variation [4]. In order to comprehensively evaluate the relation between ROS and infertility, physiological variations across the normal estrous cycle in healthy cows should be known. Up to date, few studies concerning to the effect of stages estrous cycle on the oxidative status on plasma in cows have been reported [5,6]. However, oxidant and antioxidant parameters were investigated as single parameters during the estrous cycle in these studies. Furthermore, effects of cyclic changes on plasma cumulative action of antioxidants (Total Antioxidant Status, TAS) and oxidants (Total Oxidant Status, TOS and Oxidative Stress Index, OSI) have not also been determined.

Lipids are one of the most susceptible substrates to ROS damage and biomarkers of lipid peroxidation are considered the best indicators of oxidative stress [7]. Especially, the compositional properties of low density lipoprotein cholesterol (LDL-C; e.g., lipid classes, fatty acids, antioxidants) relevant for its susceptibility to oxidation. In contrast, plasma high density lipoprotein cholesterol (HDL-C) particles exert potent antioxidant activity, which protects LDL-C against oxidative stress [8]. In addition, HDL-C particles transport enzymes exerting antioxidant activity, including paraoxonase (PON), an enzyme produced by the liver [9]. Paraoxonase has three known enzymatic molecules, including PON1, arylesterase (ARE), and dyazoxonase. Paraoxonase-1 hydrolyzes organophosphates such as paraoxon, hydrolyzes aromatic esters such as phenylacetate, and also decreases the accumulation of lipid peroxidation products. Serum PON1 acts in conjunction with ARE to function as a single enzyme having lipophilic antioxidant characteristics [10]. To our best knowledge, PON1 and ARE activities in cow plasma has not been evaluated during the estrous cycle until now.

In light of previous literatures, to be known physiological variations on oxidant/antioxidant parameters and lipids profile during the estrous cycles in healthy cows may help to understanding infertility problems. Therefore, this study was aimed to comprehensively evaluate oxidant/antioxidant status at different stages of the estrous cycle (at follicular phase: proestrus and estrus, and luteal phase: metestrus and diestrus) in cows by measuring cumulative indicator such as TAS, TOS, OSI and single parameters such as lipid hydroperoxide (LOOH), ceruloplasmin (CP), total free sulfhydryl groups (SH), uric acid (UA), PON1 and ARE in plasma. In addition, the relationship between oxidative stress and lipid profile throughout the estrous cycle was also examined.

MATERIAL and METHODS

Animal, Location and Experimental Protocol

All animal experimental procedures were approved by Harran University Animal Experimentation Local Ethics Committee (38/6, 13.01.2012). A total of 25 healthy multiparous Holstein cows having regular estrous cycles, aged 4-5 years, weighing 500±100 kg, in their 2nd to 4th lactation, at least 45 days postpartum were selected from a private dairy farm located in Sanliurfa province (southeastern Turkey) during the May and June. The animals were housed in a free stall barn, milked twice daily, and fed the same diets as total mixed ration.

Ovarian activities were checked by using transrectal ultrasonography (Pie Medical Scanner 100LC, The Netherlands) before estrous synchronization. The estrous cycle of cows were synchronized by Ovsynch protocol [11] consisting of 10 µg Buserelin acetate (Receptal®, Intervet, Turkey; 0. day), 500 µg cloprostenol sodium (Estrumate®, Intervet, Turkey; 7. day) and 10 µg Buserelin acetate (9. day) injections intramuscularly. The second 10 µg Buserelin acetate injection day were accepted as day 0 of the estrous cycle. Blood samples were collected from the jugular vein into evacuated 10 ml tube containing heparin on the 0 day (at estrus), 2 day (at metestrus), 16 day (at late diestrus) and 18 day (at proestrus). The ovarian ultrasonography was also used to confirm the stages of the estrous cycle. Plasma samples were immediately frozen and stored at -80°C, until analyzes.

Assay

Oxidative and antioxidative parameters were measured in the plasma samples by using the Aeroset automated analyzer (Abbott, IL, USA) and spectrophotometer (Cecil 3000, UK). Plasma lipid hydroperoxide level was evaluated

by the fluorimetric method based on the reaction between malondialdehyde and thiobarbituric acid [12]. Briefly, plasma was added to diethylthiobarbituric acid reagent in phosphate buffer and mixed at 95°C. Samples were placed in ice for 5 min and then added 5 ml of butanol. Fluorescence of the butanol extract was measured at excitation wavelength of 539 nm and emission wavelength of 553 nm.

Total oxidant status was measured using an automated colorimetric measurement method developed by Erel [13]. In this method, oxidants in the plasma sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The ferric ion builds a colored complex with xylenol orange in an acidic medium. The color intensity is measured spectrophotometrically.

Total antioxidant status was measured according to method of Erel [14]. In this method, the hydroxyl radical reacts with O-dianisidine to produce the colored dianisyl radical. Upon the addition of a plasma sample, the reactions initiated by hydroxyl radical are suppressed by the antioxidant components of the plasma, preventing the color change. Oxidative stress index, an indicator of the degree of oxidative stress, was calculated by using following formula; OSI = [(TOS, μmol/L)/(TAS, (mmol Trolox Eq./L) x 10]

Total free sulfhydryl groups of plasma was assayed according to the method of Hu et al.[15]. Briefly, plasma and buffer solution was added to a spectrophotometer cuvette followed by the addition of 5.5'-dithio-bis 2-nitrobenzoic acid (DTNB). Samples without DTNB were run for each sample as blanks. Following incubation for 15 min, absorbance was read at 412 nm. Ceruloplasmin activity was measured according to the method of Erel [16] based on the enzymatic oxidation of ferrous ion to ferric ion.

Paraoxonase activity was measured by using paraoxon substrate. The rate of paraoxon hydrolysis was measured by monitoring the increase of absorbance at 412 nm at 25°C [17]. Arylesterase activity was analyzed according to method of Haagen and Brock [18].

Plasma total cholesterol (TC), HDL-C and triglyceride (TG) levels were measured by an autoanalyzer (Aeroset, Abbott, IL, USA) using commercial kits (Abbott, IL, USA). Plasma concentration of LDL-C was calculated using the Friedewald equation [19]. Level of uric acid was measured by using commercial kits (Olympus, AU). Plasma progesterone concentration was determined with a validated electrochemiluminescence method using Roche commercially kits in autoanalyzer (Roche Elecsys E170, IN, USA).

Statistical Analysis

Statistical analysis was carried out with SPSS software 10.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using General Linear Model for repeated measures and Bonferroni test to determine the differences phases of the estrous cycle. Pearson correlation test was used for determination of correlations among oxidant, antioxidant and lipid parameters. Data were given as means and standard deviation (SD). The differences were considered to be significant when $P<0.05$.

RESULTS

Significant cycle-dependent changes were observed on some parameters related with oxidant/antioxidant status and lipid profile during the estrous cycle in cows *(Fig. 1-4)*. The plasma levels of oxidant parameters such as LOOH and the cumulative indicator of oxidative status including TOS, OSI were significantly decreased ($P<0.05$) during the luteal phase of the estrous cycle (at metestrus and diestrus) *(Fig. 1,2)*. In addition, there were significantly positive correlations between TOS and OSI ($r=0.92$, $P<0.01$) or LOOH ($r=0.68$, $P<0.01$; *Table 1*). On the other hand, antioxidant parameters (SH, UA and TAS) were also decreased during the luteal phases of the estrous cycle *(Fig. 1,2)*. Interestingly, similar fluctuations were observed both oxidant and antioxidant parameters as mentioned above. There was also a significant positive correlation between TAS and TOS ($r=0.55$, $P<0.01$). Activity of CP did not change during the estrous cycle *(Fig. 3)*. The activity of PON1 and ARE significantly increased only at diestrus ($P<0.05$) *(Fig. 3)*. There was also a significant correlation between PON1 and ARE ($r=0.96$, $P<0.01$). On the contrary to our expectation, no significant correlations were observed between HDL-C and PON1-ARE activities ($P>0.05$).

Lipoprotein cholesterol levels were observed to change over the estrous cycle. Levels of TC, HDL-C and LDL-C elevated the highest during the follicular phase of estrous cycle and declined during the luteal phase *(Fig. 4)*. The changes in TC levels were accompanied by a change in the levels of the HDL-C ($r=0.79$, $P<0.01$) and LDL-C ($r=0.96$, $P<0.01$) respectively). TG levels did not vary throughout the estrous cycle. Moreover, the concentration of plasma progesterone showed a normal pattern according to the stage of the estrous cycle increasing until day 16, and then decreasing rapidly during luteal regression.

DISCUSSION

The present study was conducted to evaluate the possible variations in oxidant/antioxidant parameters and the related factors such as lipid profile which affect these parameters during the estrous cycle in lactating dairy cow. To minimize individual animals effects on those parameters, each animal were used for all studied stages of the estrous cycle. Furthermore, the stages of estrous cycle in cow were defined by both their ovarian ultrasonography and plasma progesterone level.

Oxidative stress were evaluated by measuring the levels

Table 1. *The correlation between some oxidant and antioxidant parameters and progesterone levels during the estrous cycle in dairy cows*
Tablo 1. *Sütçü ineklerde östrus siklusu boyunca bazı oksidan ve antioksidan parametreler ile progesterone seviyesi arasındaki korelasyonlar*

Parameters	TAS	TOS	OSI	LOOH	SH	CP	ARE	PON	UA	TG	TC	HDL	LDL
TOS	0.55**												
OSI	0.18	0.92**											
LOOH	0.58**	0.68**	0.53**										
SH	0.30**	0.48**	0.44**	0.33**									
CP	0.14	0.14	0.10	0.09	0.02								
ARE	-0.30**	-0.08	0.04	-0.17	0.37**	0.08							
PON	-0.35**	-0.08	0.07	-0.17	0.40**	0.02	0.96**						
UA	0.79**	0.56**	0.30**	0.59**	0.23*	0.11	-0.21*	-0.23*					
TG	-0.19	0.04	0.15	0.02	0.31*	0.05	0.26*	0.33**	-014				
TC	0.48**	0.07	-0.14	0.23*	-0.15	-0.09	-0.36**	-0.43**	0.42**	-0.46**			
HDL-C	0.57**	0.14	-0.09	0.28*	-0.07	0.05	-0.30**	-0.39**	0.51**	-0.48**	0.79**		
LDL-C	0.38**	0.02	-0.15	0.17	-0.19	-0.15	-0.35**	-0.41**	0.32**	-0.44**	0.96**	0.62**	
P	-0.41**	-0.38**	-0.27**	-0.38**	-0.22*	-0.1	0.11	0.08	-0.48**	0.02	-0.09	-0.18	-0.04

P<0.05, ** P<0.01,* ***TAS:* *Total antioxidant status;* ***TOS:*** *Total oxidant status;* ***OSI:*** *Oxidative stress index;* ***LOOH:*** *Lipid hydroperoxide;* ***SH:*** *Total free sulfhydryl groups;* ***CP:*** *Ceruloplasmin;* ***ARE:*** *Arylesterase activity;* ***PON:*** *Paraoxonase activity;* ***UA:*** *Uric acid;* ***TG:*** *Triglyceride;* ***TC:*** *Total cholesterol;* ***HDL-C:*** *High density lipoprotein cholesterol;* ***LDL-C:*** *Low density lipoprotein cholesterol;* ***P:*** *Progesterone*

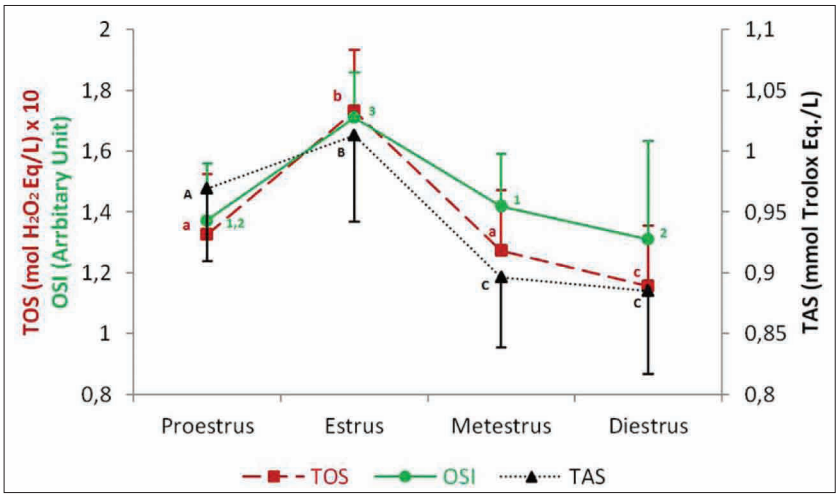
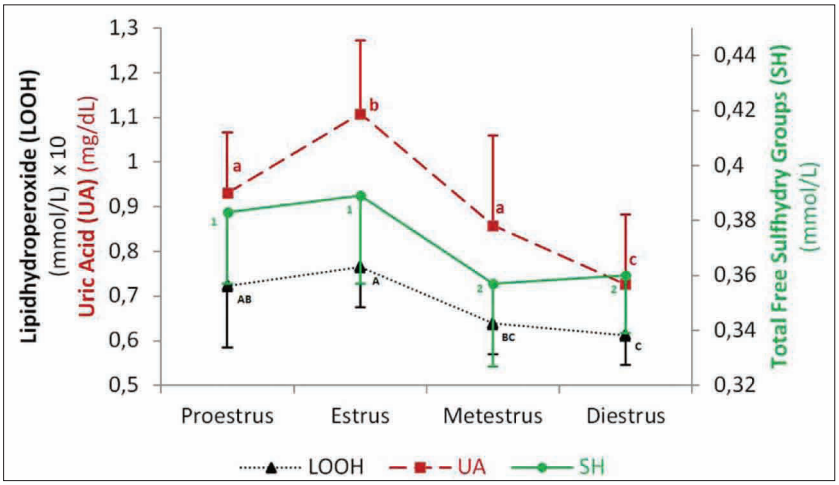


Fig 1. Total Oxidative status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) parameters during the estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 1. Sütçü ineklerde östrus siklusu boyunca total oksidan stress (TOS), total antioksidan kapasite (TAS) ve oksidatif stres indeksi (OSI) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır

Fig 2. Lipid hydroperoxide (LOOH), uric acid (UA) and total free sulfhydryl groups (SH) parameters during the estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 2. Sütçü ineklerde östrus siklusu boyunca lipit hidroperoksit (LOOH), ürik asit (UA) ve total serbest sülfhidril grup (SH) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır



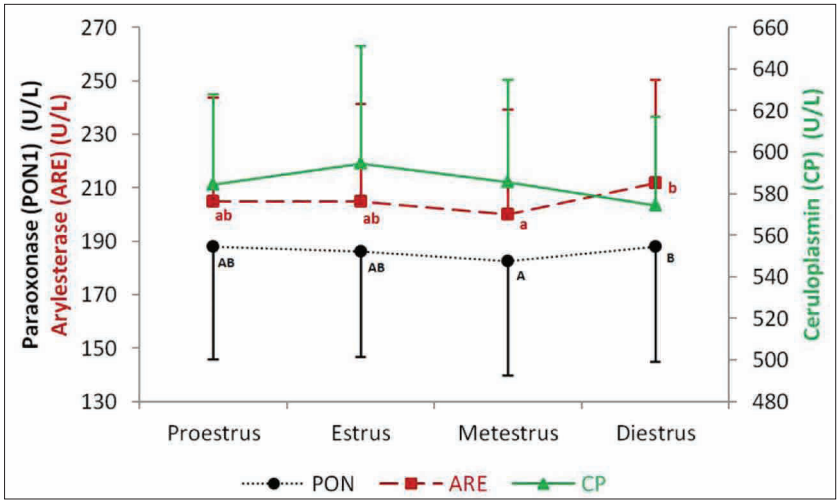
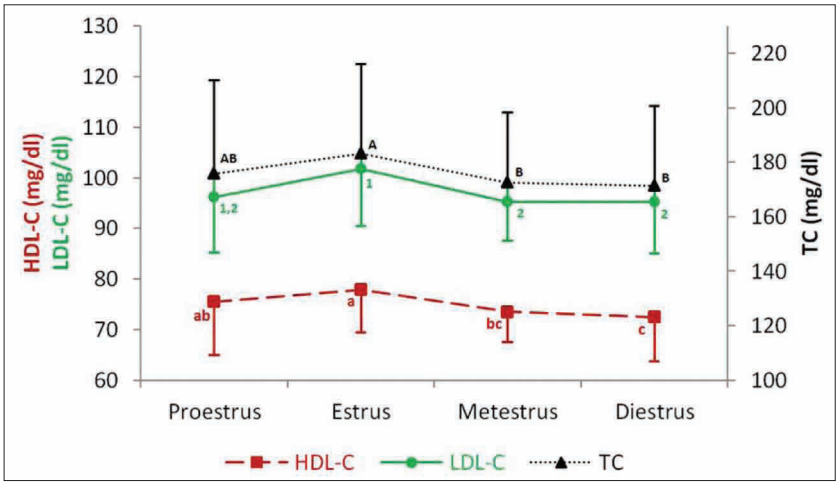


Fig 3. Paraoxonase (PON), arylesterase (TAS) and ceruloplasmin (CP) activities during the estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 3. Sütçü ineklerde östrus siklusu boyunca paraoksonaz (PON), arilesteraz (ARE) ve serulo-plazmin (CP) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır

Fig 4. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) levels during estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 4. Sütçü ineklerde östrus siklusu boyunca total kolesterol (TC), yüksek yoğunluklu lipoprotein kolesterol (HDL-C) ve düşük yoğunluklu lipoprotein kolesterol (LDL-C) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır



of lipid peroxidation as a single, TOS and OSI as cumulative indicator in the plasma. The measuring of TOS considers the cumulative action of all the oxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable oxidant [3]. It was observed that both LOOH and TOS levels increased the luteal phase of the estrous cycle (P<0.05). Some studies suggest that dynamic changes in metabolism and energy consumption in many organs can generate byproducts on an extraordinary scale in the estrus phase of the estrous cycle [20]. Among these byproducts, ROS are generated during the physiological process of oxygen consumption [21]. In our study, an increase on oxidative stress in plasma during the follicular phase of the estrous cycle may be closely related to the oxygen demand as a result of cell growth and synthesis activity [20]. In addition, lipids, especially due to compositional properties of LDL-C, are one of the most susceptible substrates to lipid peroxidation [22]. Because of LDL-C susceptibility to oxidation, its high level may be a reason of increased oxidative stress at the follicular phase of the estrous cycle. As for total antioxidant status, it considers the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable antioxidant [23]. We observed that TAS levels in the luteal phase were significantly lower (P<0.05) than the follicular phase *(Fig. 1)*. Among the other antioxidants examined the concentrations of UA and SH showed similar pattern to those of TAS, peaked during the follicular phase and decreased during the luteal phase of the estrous cycle *(Fig. 1)*. Specially, there was a high significant correlation between TAS and UA (r=0.79, P<0.01). Uric acid is a well-known antioxidant that contributes significantly to the plasma antioxidant capacity. These findings are in accordance with previous studies reporting that UA is contributing to approximately 70% of plasma total antioxidant capacity, when measured by FRAP method [24]. In addition, a negative correlation was observed between UA and progesterone level (r= -0.48, P<0.01) in the present study. The lower UA level in the luteal phase of the estrous cycle may be result from that progesterone increases glomerular filtration rate and urinary UA clearance [25]. Total free sulfhydryl groups are mainly responsible for antioxidant response to oxidative stress [26]. In our study, the lower SH level in the follicular phase of the estrous cycle due to elevated oxidative stress compared to the luteal phase of the estrous cycle (P<0.05) seems reasonable and predictable. The activity

of ceruloplasmin, acute phase protein, also shown at tend to be lower profile (P>0.05) during the luteal phase of the estrous cycle and to elevate during the follicular phase of the estrous cycle like other antioxidant parameters *(Fig. 3)*. Similarly, Fox et al.[27] also observed the elevation in plasma CP ferroxidase activity during the follicular phase and they indicated that it may be induced by oxidative stress.

The activity of PON1 and ARE significantly increased only at late diestrus (P<0.05) *(Fig. 3)*. There was also a significant correlation between PON1 and ARE (r=0.96, P<0.01) like the report of Miyamoto et al.[28]. On the other hand, there were no correlations between activities of PON1 or ARE and TAS or HDL-C levels (P>0.05). This result is somewhat surprising because it would be expected that the antioxidant property of PON1 is responsible for the antioxidant action of HDL-C [9] which correlated with TAS (r=0.57, P<0.01) in present study.

As for lipid profile, it undergoes cyclic changes during the estrous cycle in this study *(Fig. 4)*. This pattern is characterized by a marked reduction TC level during the luteal phase of the estrous cycle in plasma as reported earlier by Mumford et al.[29]. The changes in TC levels were accompanied by a change in the levels of the HDL-C and LDL-C (r=0.79, r=0.96; P<0.01 respectively; *Table 1*), whereas TG levels varied no significantly throughout the estrous cycle. Since cholesterol is the precursor of ovarian steroids, lower TC level during luteal phase may be a result of enhanced utilization by steroidogenesis [30].

Interestingly, both oxidant and antioxidant parameters shown similar fluctuations as mentioned above. Moreover, there was a significant positive correlation between TAS and TOS values (r=0.55, P<0.01). The correlation of some antioxidants with oxidative status indicates that anti-oxidative mechanisms are activated to cope with oxidative stress in physiologic conditions. On the other word, an increased antioxidant capacity in plasma may not necessarily be a desirable condition if it reflects a response to increased oxidative stress, or vice versa in physiologic condition such as the estrous cycle [24].

As a result, cyclic changes in oxidant/antioxidant status and lipid profile during the estrous cycle were shown by the present study in lactating cows. Especially antioxidant defense system (TAS, SH and UA) showed adaptive response to increased oxidative activities (TOS and LOOH) during the follicular phase of the estrous cycle and it may indicate that there is a dynamic balance between oxidant and antioxidant status in healthy cows during the estrous cycle. Taken together, these findings suggest the stage of the estrous cycle should always be taken into account when evaluating oxidative status and lipoprotein cholesterol levels in cyclic cows in order to improve interpretation in clinical settings and in future research. It would also indicate a need for studying the nutritional, metabolic and endocrine features that regulate this relationship.

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