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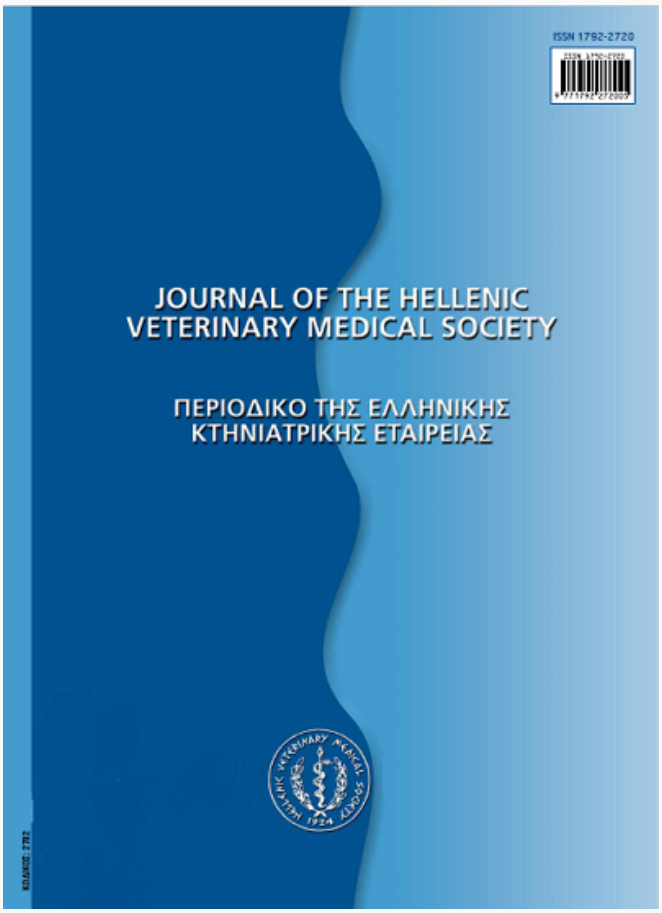

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The Impact of Endometritis on Specific Oxidative Stress Parameters in Cows

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ABSTRACT. This study was aimed to investigate the presence of uterine infection and the relationship between the severity of uterine infection and some oxidative stress parameters in cows in early postpartum period. Sixty six cows that were in the period between the 30th and 32rd days of postpartum were included in the study. Endometrial inflammation was scored according to the condition of the uterus at rectal and ultrasonographic examinations and the character and amount of uterine discharge from endometritis I through III, the latest being the most severe. Endometrial samples were taken from the clinically healthy animals by cytobrush method, and then stained with Giemsa for presence of polymorphonuclear leukocytes (PMN). Cows with a PMN percentage less than 18% were considered to be healthy (control, n=20). All cows were sampled for blood. Nitric oxide (NO) levels in Group I (Endometritis I; n=20), Group II (Endometritis II; n=16), Group III (Endometritis III; n=10) and the control group were determined to be 23.0±0.63 nmol/mL, 32.23±0.97 nmol/mL, 36.56±0.48 nmol/mL, and 11.10±0.29 nmol/mL, respectively. The differences among the groups were found to be statistically significant. Malondialdehyde (MDA) level was highest in Group III (6.38±0.13 μmol/L), and its level significantly decreased with the reduced severity of endometritis. The comparison of the groups for total antioxidant capacity (TAC) and total oxidant capacity (TOC) demonstrated that endometritis caused a decrease in TAC levels ($P<0.05$), but did not affect TOC levels. Endometritis increased serum NO and MDA levels, and decreased TAC levels, but had no effect on TOC levels. In conclusion, NO and MDA, as well as TAC can be used as a biochemical marker for estimation of the severity of endometritis in cows.

Keywords: *endometritis, cow, NO, MDA, TAC*

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INTRODUCTION

Endometritis causes major economic losses as a result of uterine and ovarian dysfunction, reduced reproductive performance and culling of animals (Leblanc et al., 2002; Pleticha et al., 2009; Tsousis et al., 2010). While cases of clinical endometritis are scored according to the character and amount of uterine discharge (Sheldon et al., 2006; Williams et al., 2005), cases of subclinical endometritis are diagnosed by cytological examination (Barlund et al., 2008). In endometritis cases, the immune system is activated (Yaralioglu-Gurgoze et al., 2005) and an excessive amount of nitric oxide (NO) is secreted by the primary defence system (Gilbert, 2012; Pande et al., 2013). Therefore, NO a by-product of L-arginine (Ozcan and Ogun, 2015) increases in both the uterine discharge and the blood serum (Xue et al., 2015). NO, locally secreted from the ovaries, affects the size and functional phase of the follicle as well as ovulation and development and regression of the corpus luteum (Li et al., 2010; Pande et al., 2013). For rapid uterine involution during the postpartum period, uterine content is required to be expelled. NO shows direct effect on the smooth muscles of the uterus and causes muscle relaxation (Li et al., 2010; Xue et al., 2015). Accumulation of excessive amount of NO in uterine histiocytes causes it to react with superoxide anions to generate peroxynitrite radicals. Peroxynitrite increases lipid peroxidation and leads to the generation of free radicals. This, in return, aggravates inflammatory reaction resulting in tissue and organ damage. Uterine muscle relaxation causes for accumulation of inflammatory products in the uterine lumen, and this helps increasing the severity of uterine inflammation (Li et al., 2010; Xue et al., 2015). Oxidative stress, which results from an imbalance between the reactive oxygen radicals and antioxidants, has adverse effects on both animal health and production yields (Hanafi et al., 2008; Janowski et al., 2013; Xue et al., 2015). Free radicals at physiological levels are required for the capacitation of spermatozoids, the acrosome reaction, fertilization and embryonic development (Zhong and Zhou, 2013). However, oxidative stress, resulting from excessive production of free radicals in cases of endometritis, adversely affects sperm cells during the passage of spermatozoids through the uterus, and leads to the deformity of sperm and oocyte, and eventually, infertility (Hanafi et al., 2008; Janowski et al., 2013; Sanocka and Kurpisz, 2004; Zhong and Zhou, 2013). Apart from causing damage to the tissue structure, oxidative stress also causes apoptosis and necrosis, which lead to cell death (Lykkesfeldt and Svendsen, 2007). Oxidative stress causes greater damage during early embryogenesis, which is the period characterized by high mitochondrial activity (Zhong and Zhou, 2013), and reduces the percentage of ova developing into blastocysts (Gilbert, 2012). Free radicals readily react with unsaturated fatty acids found in the membrane structures, which results in the formation of lipid peroxidation products. Malondialdehyde (MDA) is one of the most important indicators of lipid peroxidation (Ercan et al., 2014; Oral et al., 2015). Free oxygen radicals can damage cells by disrupting the mechanism of the antioxidant system. The increase in free radical production reduces the total antioxidant capacity (TAC) and increases MDA levels. Antioxidants play a vital role in protection from infertility caused by endometritis (Mohamed, 2008; Pande et al., 2013). This study was aimed at investigating the relationship between the severity of uterine infection and specific oxidative stress parameters.

Material and Methods

This study was conducted pursuant to the approval of the Local Ethics Board for Animal Experiments of Kafkas University (KAÜ- HADYEK 2015/069).

Animals

The present study was carried out in 69 Brown Swiss cows (BCS=2.66±0.07, parity=2.05±0.18), raised at the Research and Practice Farm of Kafkas University, Faculty of Veterinary Medicine. Cows aged 3-6 years old were examined at 30-32 days in milking period postpartum.

Allocation Criteria

The vulvar, coccygeal and perineal regions were inspected for the presence of uterine discharge, and then internal examination was performed by vaginoscopy. The vulva was thoroughly cleaned with damp paper towels and then the vaginoscope was inserted into the vagina up to the level of the external os of the cervix.

Inspection of the cervix and vagina was performed with illumination from a penlight.The character of discharge (clear mucus, mucoprulent mucus or prulent mucus according to amount of prulent matter in exudate) were recorded. Following vaginoscopy the cervix and uterus were examined by transrectal ultrasonography (USG, 7.5 MHz, Titan®, Sonosite, USA). Rectal palpation findings (location of the uterus, symmetry of the cornua (yes/no), etc.) and ultrasonographic images of the uterus [diameter of the cornua (in cm), the presence of fluid in the uterine lumen (yes/no), texture of uterine wall (thick/thin) cervix diameter (<5 cm, 5-7.5 cm, >7.5 cm)] were recorded. Severity endometritis was were scored on the basis of uterus findings and the character and amount of uterine discharge, determined by rectal, ultrasonographic and vaginoscopic examinations. Animals with a hyperaemic vagina and uterine discharge containing mostly translucent mucus with a small amount of pus flakes were allocated to Endometritis I (Group I). Animals with an open cervix, ambiguous thickening associated with hyperaemia of the vaginal portion and a discharge containing yellow or white flakes of pus at a percentage less than 50% were allocated to Endometritis II (Group II). Animals displaying a thickening of the uterine wall and cervix and a uterine discharge containing more than 50% of yellow and white coloured flakes of pus, occasionally sanguineous, were assigned to Endometritis III (Group III) (LeBlanc et al., 2002). The clinically healthy animals were sampled with the cytobrush method for the collection of endometrial swabs. The cytobrush technique was performed using a brush approximately 3 cm long on a stainless steel rod (65 cm in length and 4 mm in diameter). Plastic sheaths were used to prevent contamination of the cytobrush in the vagina. Endometrial cytology samples were obtained by rotating cytobrushes a few times in the uterine endometrium. The samples were smeared onto slides and dried. After being fixed in methanol (Merck®, Turkey) for two minutes, the slides were stained with Giemsa solution (Merck®, Turkey) for 30 minutes. Then the neutrophil to leukocyte ratio (by counting a minimum of 100 cells at 400x magnification) was calculated using microscopy (Olympus CX23, Olympus corp, Japan). Subsequently, polymorphonuclear leukocytes (PMN) percentage was determined by microscopic examination, and cows with a PMN percentage greater than 18% were considered to suffer from subclinical endometritis (Kasimanickam et al., 2004). On the other hand, cows with a PMN percentage less than 18% were considered to be healthy.

Blood Sampling

Between days 30-32 postpartum, after being examined, all of the animals were sampled only once for blood, and the samples were taken from the coccygeal vein into gel-coated serum vacutainers (8.5 ml). The samples were centrifuged at 1200 g for 10 minutes. The sera seperated from the blood samples were transferred into Eppendorf tubes, and stored at -20 C° until being used for analysis.

Analysis of Specific Oxidative Stress Parameters

Serum nitric oxide concentrations were measured as described by Miranda et al. (2001) in that nitrate is reduced to nitrite by vanadium (III) chloride and then in acidic environment nitrite was reacted with sulphanilamide to produce colored complex diazonium compound, which was read spectrophotometrically (Epoch®, Biotek, USA) at 540 nm (Karapehlivan et al. 2014). The levels of nitrate and nitrite were measured separately, and the sum of both was considered as the NO level. NO levels were given in nmol/ml. MDA levels were measured as described by Yoshioka et al. (1979). MDA forms a pink coloured complex with thiobarbituric acid and by means of the spectrophotometric measurement (Epoch®, Biotek, USA) of the absorbance of the solution containing this complex at 535 nm, the severity of lipid peroxidation can be ascertained. MDA levels were measured in μmol/l. The TAC and TOC (Rel Assay Diagnostic®, Turkey) measurements of the serum samples were performed colorimetrically using a commercial kit (PowerWave XS, BioTek, Instruments, USA).

Statistical Analysis

Statistical analyses were performed using the SPSS® (SPSS 20, IL, USA) statistical software package. NO, MDA, TAC and TOC levels observed in each group were tested for normality using the Shapiro-Wilk test. The differences among the groups were compared

with the one-way ANOVA (Tukey-HSD) test. Results were given as X±SE. A value of P <0.05 was considered statistically significant.

RESULTS

Postpartum Examinations Results

The number of cows with endometritis at postpartal examination was as follows; 20 endometritis I, 16 endometritis II, 10 endometritis III. The cows sampled with the cytobrush method (n=23), only 3 were diagnosed to have subclinical endometritis. As these animals were very few in number and their data could not be statistically analysed, they were not included in the study. The remaining animals were considered to be healthy and were allocated to the control group (n=20).

Levels of Specific Oxidative Stress Parameters in Groups

The NO, MDA, TAC and TOC levels in groups were given in Table 1. NO and MDA levels were higher in the animals suffering from endometritis as compared to the control group,these increases were in accordance with the severity of the endometritis recorded. TAC levels were highest in the control group, and the levels decreased as the score of endometritis increased. TOC levels did not show any differences between the control (0.67±0.02 μmolH_2O_2 Eqv/L) and the other groups (I, II and III) with endometritis (0.65± 0.02 μmolH_2O_2 Eqv/L, 0.68±0.02 μmolH_2O_2 Eqv/L, 0.68±0.03 μmolH_2O_2 Eqv/L, respectively).

DISCUSSION

Presence of free oxygen radicals is responsible for development of many diseases (Lobo et al., 2010). It is well known that free radicals play an important role in the pathogenesis of endometritis as well (Yaralioglu-Gurgoze et al., 2005). Free radicals lead to changes in NOS levels in endometrium (Alghamdi et al., 2005). In a study conducted by Li et al. (2010), it was observed that NO levels were higher in cows suffering from endometritis, in comparison to healthy animals. It was demonstrated that NO increased to higher levels with increased severity of endometritis. In another study carried out by Oral et al. (2015), it was determined that NO levels increased as a result of stress induced by intravaginal applications. Similarly, in the present study, it was shown that NO levels had increased in parallel with the severity of endometritis. The increased NO concentration in blood is speculated as an inflammatory response of uterine tissue. High levels of serum nitric oxide that cause the relaxation of smooth muscle and also the accumulation of inflammatory products in the uterus are considered to play an important role in the increased severity of infection of the uterus.

The measurement of lipid peroxidation level (MDA levels) enables a better understanding of the level of oxidative damage caused by free oxygen radicals. Increased MDA levels in animals with clinical endometritis indicate a high level of production of reactive oxygen species (ROS) in leukocytes during inflammation. ROS lead to increased levels of MDA and limit the immune system response by damaging immune cells (Heidarpour et al., 2012). Heidarpour et al. (2012) reported that serum MDA levels were

Table 1. A comparison of the serum NO, MDA, TAC and TOC levels in study groups

Variables	Group I (n=20)	Group II (n=16)	Group III (n=10)	Control (n=20)
NO (nmol/mL)	23.00±0.63^{a}	32.23±0.97^{b}	36.56±0.48^{c}	11.10±0.29^{d}
MDA (μmol/L)	3.71±0.13^{e}	5.29±0.34^{f}	6.38±0.13^{g}	2.32±0.57^{h}
TAC (mmolTrolox Eqv/L)	1.38±0.24^{x}	1.14±0.09^{y}	0.84±0.35^{z}	1.57±0.31^{w}
TOC (μmolH_2O_2 Eqv/L)	0.65± 0.02*	0.68±0.02*	0.68±0.03*	0.67±0.02*

n = cows number; *=statisticlally not significant, NO= a:c:<0.001; b:c:=0.001; a:b:d:<0.001; MDA= e:g:h:<0.001; f:g:=0.005; e:f:h:<0.001; TAC= x:y:<0.001; x:z:<0.001; x:w:=0.03; y:z:<0.013; y:w:<0.001; z:w:<0.001; TOC= >0.05

significantly higher in cows with clinical endometritis than in healthy cows. Similarly, in a study conducted by Mohamed (2008), it was demonstrated that serum MDA levels were higher in camels diagnosed with clinical endometritis, when compared to healthy camels. In agreement with these researches, in the present study, it was ascertained that the MDA levels of the cows with endometritis were significantly higher than those of the healthy animals, and the increase in MDA levels were higher as the severity of endometritis increased. This increase is possibly, due to the excessive production of the hydroxyl (OH) radicals, which cause damage to the DNA and cell membrane, during infection in mares (Yaralioglu-Gurgoze et al., 2005).

Antioxidant systems are of particular importance in the establishment and persistence of endometritis, and in protection from the adverse effects of free radicals to the organism (Heidarpour et al., 2012). It is well known that inflammatory diseases, including endometritis, disrupt the antioxidant defence mechanism. Accordingly, in a previous research it was shown that TAC levels of camels diagnosed with clinical endometritis (0.156±0.004 mmol/L) were significantly lower than those of healthy camels (0.201±0.006 mmol/L) (Mohamed, 2008). Similarly, in a study conducted by Heidarpour et al. (2012), it was ascertained that the TAC levels of cows suffering from endometritis (3.67±1.22 mmol/L) were lower than the TAC levels measured in healthy cows (4.65±0.97 mmol/L). Hanafi et al. (2008) also reported that the TAC levels of animals with endometritis (0.45±0.05 mmol/L) were lower than those of healthy animals (1.43±0.08 mmol/L). In agreement with the previous investigations, the TAC level in the present study was highest in the healthy animals, and it decreased as the severity of endometritis increased.

TOC is one of the major components of the antioxidant system, which provides protection against oxidative agents, and it has been demonstrated that TOC levels in the uterine fluid increase in parallel with increased NO levels (Xue et al., 2015). Atakisi et al. (2010) reported that TOC levels were higher in diseased animals, in comparison to healthy animals. Similarly, Xue et al. (2015) determined that the TOC levels of cows with clinical endometritis (9.04±0.48 U/mL) were higher than the TOC levels of healthy animals (7.89±0.4 U/mL). However, contrary to the results of the previous studies, no statistically significant difference was determined in TOC levels between the control group and the groups with endometritis, in the present study.

In conclusion, it was determined that endometritis in cows resulted in decrease of TAC levels in blood serum, but had no effect in TOC levels therein. This study also demonstrated that concentration of MDA and NO in blood serum was related to the degree of endometritis. These results support the notion that free radicals play a role in the pathogenesis of endometritis. Serum NO and MDA, as well as TAC levels can be used as a biochemical marker for estimation of the severity of endometritis in cows.

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