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Original article

Assessment of lipid and protein peroxidation markers in non-pregnant and pregnant female dogs

M. Szczubiał¹, M. Kankofer², R. Dąbrowski¹, M. Bochniarz¹, R. Urban-Chmiel³

¹ Department and Clinic of Animal Reproduction, Faculty of Veterinary Medicine, University of Life Sciences, Głęboka 30, 20-612 Lublin, Poland

² Department of Animal Biochemistry, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland

³ Sub-department of Veterinary Prevention and Avian Diseases, Institute of the Biological Bases of Animal Diseases, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland

Abstract

The aim of the study was to investigate oxidative stress during normal pregnancy in female dogs based on an evaluation of plasma markers for lipid and protein peroxidation. Twenty clinically healthy female dogs (10 non-pregnant and 10 pregnant) were used in the study. Blood samples from the pregnant animals were collected at 19-21, 38-40, and 56-58 days of pregnancy. Blood samples from non-pregnant female dogs were obtained between 20 and 35 days after ineffective breeding. As indicators of oxidative stress, we measured the following using spectrophotometric and spectrofluorimetric methods: thiobarbituric acid reactive substances (TBARS), radical cations of N,N, diethylpara-phenylene diamine (RC-DEPPD), sulfhydryl groups (SH groups), bityrosine and formylkynurenine. The mean plasma TBARS concentration in the pregnant dogs ($0.486 \pm 0.071 - 0.581 \pm 0.191 \mu\text{mol/g protein}$) was significantly higher ($p < 0.05$) than that found in the non-pregnant animals ($0.274 \pm 0.111 \mu\text{mol/g protein}$). A marked, although not significant, decrease in SH group content, as well as an increase in bityrosine and formylkynurenine concentration were concurrently observed in the pregnant dogs. No significant differences were found in terms of the studied markers in the pregnant animals when comparing the values obtained during the investigated periods of pregnancy, although there was a progressive decrease in TBARS concentration and a progressive increase in RC-DEPPD, bityrosine and formylkynurenine contents. Our findings suggest that normal pregnancy in female dogs is associated with oxidative stress. Further studies are necessary to establish the physiological ranges of antioxidative/oxidative profiles in pregnant dogs and to explain if and how the intensity of oxidative stress might contribute to the risk of the complications of pregnancy.

Key words: oxidative stress, pregnancy, lipid peroxidation, protein oxidative damage, female dogs

Introduction

Oxidative stress is described as an imbalance in the production of reactive oxygen species (ROS) and the ability of antioxidant defenses to scavenge them (Myatt and Cui 2004). This may occur when ROS are produced faster than they can safely be neutralized by antioxidant mechanisms. In low concentrations ROS are important for several physiological processes, including the mammalian reproductive functions, such as ovarian follicular development, ovulation, fertilization, luteal steroidogenesis, endometrium receptivity, embryonic development, implantation and early growth and development (Agarwal et al. 2005, Al-Gubory et al. 2010). However, ROS excess, if not properly controlled by antioxidant mechanisms, leads to oxidative stress and induces cellular damage by acting on lipids and proteins, as well as other macromolecules (Halliwell 2006). Irreversible modification of cellular components by ROS leads to cell dysfunction. It is known that oxidative stress contributes significantly to the development of many diseases in humans and animals (Halliwell 2006, Lykkesfeldt and Svendsen 2007).

Lipid peroxidation is a non-enzymatic reaction based on the oxidation of mainly unsaturated fatty acids of cell membranes. This may influence the properties of cell membranes and their physiological functions (Gutteridge 1995). Once initiated, lipid peroxidation continues as a chain reaction to generate lipid hydroperoxides and aldehydes and a single oxidative event can thus affect many lipid molecules (Kruidenier and Verspaget 2002). Moreover, hydroperoxides and aldehydes are directly toxic to cells and organelles (Aw 1998). Lipid peroxidation processes may be detected by the determination of their intermediates and metabolites (Benzie 1996). Products of lipid peroxidation processes are frequently used as biomarkers of oxidative stress.

Proteins are the most abundant cell constituents, which make them important ROS targets. Although protein peroxidation is not as efficient as lipid peroxidation, it leads to the modification of amino acid residues, aggregation or fragmentation of protein molecules and the loss of the biological activity of proteins (Beal 2002). In consequence, the metabolic pathways might be impaired and disturbances may occur (Marnett et al. 2003). Protein peroxidative damage can be detected by the determination of end products such as nitrotyrosine and formylkynurenine or the content of the sulfhydryl groups (SH groups) (Goldstein et al. 1994, Halliwell and Whiteman 2004). Biomarkers of protein peroxidation are considered good indicators of oxidative stress.

Pregnancy is a period when oxidative stress can be

expected due to the high energy demand and increased oxygen requirement, together with the increased metabolic activity in placental mitochondria (Gitto et al. 2002). Evidence for this concept can be found in studies demonstrating elevated levels of oxidative stress markers during normal pregnancy in women (Wickens et al. 1981, Iioka 1994, Toescu et al. 2002, Myatt and Cui 2004, Suhail et al. 2011). This increased oxidative stress has also been reported in several pathologic pregnancies in women (Bedaiwy et al. 2004, Myatt and Cui 2004, Wender-Ozegowska et al. 2004, Juaniaux et al. 2005, Harun et al. 2009, Al-Naemi et al. 2012). However, information is limited about the evaluation of the oxidative stress during pregnancy in animals, including female dogs (Vannucchi et al. 2007). Therefore, the aim of the study was to investigate oxidative stress during normal pregnancy in female dogs based on an evaluation of plasma markers of lipid peroxidation and protein oxidative damage.

Materials and Methods

Animals and sample collection

The study was approved by the Local Ethics Committee appointed by the University of Life Sciences in Lublin, Poland, and was performed in accordance with animal protection regulations.

Twenty clinically healthy female dogs of different breeds were used in the study. The animals were selected from among female dogs presented by their owners for routine pregnancy diagnosis and monitoring to the Clinic of Reproduction of the Faculty of Veterinary Medicine in Lublin. The dogs were aged 3-5 years and their weight ranged from 15 to 21 kg. A narrow range of age and weight for the female dogs was chosen to reduce the effect of these factors on oxidative stress markers. To rule out any underlying diseases, all the animals were clinically examined before blood sample collection, and routine hematological (Scil Vet ABC Plus+ Hematology Analyzer, Horiba ABX, Warsaw, Poland) and biochemical blood (Mindray BS-130 Chemistry Analyzer, Shenzhen Mindray Bio-medical Electronics Co., Ltd, Shenzhen, China) tests were performed as were urinalyses (for detection pH, leukocytes, protein, glucose, ketones, urobilinogen, bilirubin and occult blood (IDEXX VetLab&Urine Analyzer, IDEXX Laboratories, Inc., Westbrook, USA) and specific gravity by using refractometer). No signs of any diseases or abnormal clinical parameters were found on the physical examination. The results of haematological and biochemical blood analyses were within the normal range (Table 1).

Table 1. Results of hematological and biochemical blood analyses in the female dogs used in the study.

Parameter	Mean \pm SD (range)	Reference range*
RBC ($\times 10^{12}/l$)	6.8 \pm 0.8 (5.5 – 7.6)	5.5 – 8.0
Hemoglobin (g/dl)	16 \pm 1.7 (12.4 – 17.6)	12.0 – 18.0
Hematocrit (%)	41.8 \pm 2.4 (38 – 49)	37 – 55
WBC ($\times 10^9/l$)	9.2 \pm 0.6 (8.3 – 10.2)	9 – 10
Band neutrophils ($\times 10^9/l$)	0.2 \pm 0.07 (0 – 0.3)	0 – 0.3
ALT (U/l)	40.8 \pm 5.4 (29 – 43)	3 – 50
AST (U/l)	26.2 \pm 7.9 (10 – 33)	1 – 37
Glucose (mmol/l)	4.2 \pm 0.3 (3.8 – 4.8)	3.4 – 5.0
BUN (mmol/l)	6.5 \pm 1.3 (3.8 – 7.1)	3.3 – 7.5
Creatinine (μ mol/l)	108.0 \pm 18.7 (88.3 – 137.2)	88.4 – 150.3

* Reference range according to Basic Laboratory Tests in Veterinary Medicine, Winnicka A, SGGW Warsaw, Poland, 2008. RBC – Red blood cell count, WBC – Total white blood cell count, ALT – Alanine aminotransferase, AST – Aspartate aminotransferase, BUN – Blood urea nitrogen

No abnormal parameters were found on the urinalysis (specific gravity: 1.018 – 1.040; pH: 5.5 – 6.2; absence of detectable amounts of leukocytes, protein, glucose, ketones, urobilinogen, bilirubin and occult blood in urine). To minimize feed effect on the studied parameters, only animals receiving similar food were included in the study. According to information gained from the owners, all female dogs were fed commercial dog food for adult animals (product firms: Royal Canin, Orijen and Hill's Science Diet) and water was given *ad libitum*. The animals were divided into two groups – non-pregnant (control group) and pregnant (experimental group) – consisting of 10 females each. Pregnancy was confirmed *via* ultrasonography at the 3rd week after breeding (Honda HS 2000, Honda Electronics CO, LTD, Japan). The non-pregnant dogs were confirmed to be in diestrus by clinical history and vaginal cytology. All pregnant animals whelped spontaneously between 60 and 64 days of pregnancy. To exclude any influence of litter size or fetal death, only female dogs which had litters with 4-6 live puppies in total (no stillborns) were used in the study.

Blood samples from animals of the experimental group were collected at 19-21, 38-40, and 56-58 days of pregnancy. Blood samples from the control group were obtained between 20 and 35 days after ineffective breeding. Nine milliliters of blood were collected from the saphenous vein into heparinized tubes and centrifuged immediately. The plasma was frozen at -70°C until further processing.

Biochemical investigations

As parameters of lipid peroxidation intensity, the concentration of thiobarbituric acid reactive substances (TBARS) and the content of radical cations of

N,N, diethylpara-phenylene diamine (RC-DEPPD) were used. As parameters of protein peroxidation, the content of sulfhydryl groups (SH groups), bi-tyrosine and formylkynurenine were determined.

Lipid peroxidation markers

The concentration of TBARS was determined according to the method described by Ledwozyw et al. (1986). The reaction products were measured spectrophotometrically at 532 nm (Ultrospec 2000, Pharmacia, Uppsala, Sweden). The calculation was based on a standard curve prepared with different dilutions of malondialdehyde (MDA). The results were expressed as $\mu\text{mol/g}$ protein. The intra-assay and inter-assay coefficients of variation were 6.9% ($n=10$) and 7.2% ($n=10$), respectively.

The concentration of RC-DEPPD was determined as described by Alberti et al. (2000). The estimation of radical cations (RC-DEPPD) formed in the reaction of alkoxy and peroxy radicals derived from hydroperoxides (present in the sample) was performed, by using N,N,diethyl-paraphenylene diamine (DEPPD) allowing the indirect estimation of the level of hydroperoxides. The incubation mixture contained 1 ml of acetate buffer (pH 4.8), 10 μl of an aqueous solution of DEPPD (Sigma, Poznan, Poland) (0.37 mol/l) and 20 μl of plasma. After 1.5 h incubation at 37°C absorbance was read at 505 nm against distilled water (Ultrospec 2000, Pharmacia, Uppsala, Sweden). In the control sample, 20 μl of distilled water replaced the plasma. Calculations were based on a standard curve prepared with six different dilutions of H_2O_2 . The results were expressed as nmol/g protein. The intra-assay and inter-assay coefficients of variation were 9.1% ($n=10$) and 8.9% ($n=10$), respectively.

Table 2. Mean \pm SD concentration of thiobarbituric acid reactive substances (TBARS) and radical cation of N,N-diethyl-para-phenylene diamine (RC-DEPPD) in blood plasma of the female dogs used in the study.

Parameter	Groups			
	control	experimental		
		19-21 days of pregnancy	38-40 days of pregnancy	56-58 days of pregnancy
TBARS ($\mu\text{mol/g}$ protein)	0.274 \pm 0.111 ^a	0.581 \pm 0.191 ^b	0.505 \pm 0.133 ^b	0.486 \pm 0.071 ^b
DEPPD (nmol/g protein)	0.019 \pm 0.008	0.017 \pm 0.007	0.020 \pm 0.004	0.023 \pm 0.006

^{a, b} different superscripts indicate statistically significant differences ($p < 0.05$)

Protein peroxidation markers

The content of SH groups was determined according to the procedure described by Rice-Evans et al. (1991). The reaction mixture absorbance was measured at 412 nm (Ultrospec 2000, Pharmacia, Uppsala, Sweden). The concentration of SH groups was calculated using a standard curve prepared with different dilutions of glutathione (GSH, Sigma, Poznan, Poland) ranging from 0 to 1 mmol/l in sodium phosphate buffer (10 mmol/l, pH 8.0) and expressed in mmol/g protein. The intra-assay coefficient of variation was 6.9% ($n=10$) and the inter-assay coefficient of variation was 7.1% ($n=10$).

Bityrosine was determined by the spectrofluorimetric method according to Rice-Evans et al. (1991). The plasma sample was excited by light at 325 nm and emission was measured at a wavelength of 420 nm. The standardization of the spectrofluorimeter (Jasco, Tokyo, Japan) to 100 deflections was performed with quinine sulfate (Sigma, Poznan, Poland) (0.1 $\mu\text{g/ml}$ in 0.1 mol/l H_2SO_4) at an excitation of 350 nm and emission of 445 nm. Samples were diluted with the sodium phosphate buffer (10 mmol/l, pH 8.0). The results were expressed as μg per mg protein. The coefficients of variation were: intra-assay 5.9% ($n=10$) and inter-assay 6.0% ($n=10$), respectively.

Formylkynurenine was determined by the spectrofluorimetric method according to Rice-Evans et al. (1991). The plasma sample was excited by light at 360 nm and emission was measured at a wavelength of 454 nm. The spectrofluorimeter (Jasco, Tokyo, Japan) was standardized as described above. The results were expressed as μg per mg protein. The coefficients of variation were: intra-assay 6.3% ($n=10$) and inter-assay 6.5% ($n=10$).

Protein content

The protein contents of plasma samples were measured by the biuret method using commercial assay kits (Cormay, Lublin, Poland) based on spec-

trophotometric measurement (Ultrospec 2000, Pharmacia, Uppsala, Sweden). The intra-assay coefficient of variation was 1.56% ($n=20$) and the inter-assay coefficient of variation was 8.20% ($n=60$). These values were only used for recalculations of the examined parameters for more objective comparisons between the different sample sources.

Statistical analysis

All data were presented as means \pm standard errors of the means (SEM). Statistical analysis was performed using STATISTICA 10.0 software (Statsoft, USA). Analysis the equality of the variances was tested with Leven's test. The Tukey's test was used for *post-hoc* tests of significant differences in the concentrations of the examined biomarkers between the study groups, as well as between the successive periods of pregnancy. Differences at $p < 0.05$ were considered statistically significant.

Results

Analysis of variance for samples in particular periods of pregnancy showed statistically significant differences ($p < 0.05$) in the concentrations of TBARS, RC-DEPPD, SH groups and formylkynurenine.

Lipid peroxidation markers

The mean values of parameters of the parameters of the lipid peroxidation intensity are presented in Table 2. The plasma concentration of TBARS was significantly higher ($p < 0.05$) in the experimental group of female dogs compared to the control group. The concentration of TBARS decreased at 38-40 and 56-58 days of pregnancy compared with that at 19-21 days, but no significant difference was found.

The concentration of RC-DEPPD did not differ significantly between the examined groups of female

Table 3. Mean \pm SD concentration of SH groups, bityrosine and formylkynurenine in blood plasma of the female dogs used in the study.

Parameter	Groups			
	control	19-21 days of pregnancy	38-40 days of pregnancy	56-58 days of pregnancy
SH groups (mmol/g protein)	0.00338 \pm 0.00064	0.00294 \pm 0.0007	0.00245 \pm 0.0007	0.00270 \pm 0.00027
Bityrosine (μ g/mg protein)	0.506 \pm 0.208	0.564 \pm 0.139	0.651 \pm 0.120	0.749 \pm 0.052
Formylkynurenine (μ g/mg protein)	0.250 \pm 0.139	0.236 \pm 0.055	0.283 \pm 0.070	0.313 \pm 0.024

dogs or between the studied periods of pregnancy, although at 38-40 days and 56-58 days of pregnancy this parameter was slightly higher than both at 19-21 days and in the control group.

Protein peroxidation markers

The mean values of parameters of the protein peroxidation intensity are presented in Table 3.

The concentration of SH groups in the experimental dogs remained constant throughout the sampling period and was insignificantly lower than that found in the control animals.

The plasma bityrosine concentration was markedly higher in the experimental group than in the control one; however, no significant differences were found between the groups. In the experimental group, the concentration of bityrosine gradually increased during pregnancy, but no significant differences between the successive time points were observed.

There were no significant differences between the studied groups in terms of formylkynurenine, although the level of this marker at 38-40 and 56-58 days of pregnancy was markedly higher than that determined in the control group. In the experimental group, the formylkynurenine concentration gradually increased, reaching the highest values at 56-58 days; however, no significant differences were found between the successive time points.

Discussion

In the present study, oxidative stress during normal pregnancy in female dogs was evaluated based on measurements of the products of lipid and protein peroxidation. Lipid peroxidation intensity was determined by the evaluation of the concentration of both intermediates which appear during the propagation phase of fatty acid peroxidation (RC-DEPPD) as well as end products of this process (TBARS). As par-

ameters of protein peroxidation, the content of sulfhydryl groups (SH groups), bityrosine and formylkynurenine – products of tyrosine and tryptophan peroxidative damage, respectively – were determined. Both a decrease in SH group concentration and an increase in the concentration of bityrosine and formylkynurenine may indicate peroxidative damage to proteins (Goldstain et al. 1994, Halliwell and Whiteman 2004, Halliwell 2006).

The present study revealed that the concentration of TBARS was significantly higher in the pregnant female dogs than in the non-pregnant animals. A marked, although not significant, decrease in SH group concentration, as well as an increase in bityrosine and formylkynurenine concentration were concurrently observed in the pregnant group. Moreover, in the pregnant group the concentration of RC-DEPPD, bityrosine and formylkynurenine gradually increased during pregnancy, reaching the highest values at 56-58 days. These findings proved the presence of the peroxidative damage of lipids and proteins which could be interpreted as symptoms of oxidative stress during pregnancy in female dogs, the intensity of which seems to increase towards the end of pregnancy.

During pregnancy in women circulating markers of oxidative stress have been shown. Numerous studies have demonstrated higher concentrations of lipid peroxidation products in healthy pregnant women than in non-pregnant women (Uotila et al. 1991, Qanungo et al. 2000, Toescu et al. 2002, Djordjevic et al. 2004, Hung et al. 2010, Idonije et al. 2011). Some authors have found that plasma concentrations of TBARS and other markers of lipid peroxidation gradually increase during pregnancy, reaching the highest values in the third trimester (Carine et al. 1993, Djordjevic et al. 2004, Hung et al. 2010), but other authors have reported that levels of the products of lipid peroxidation reach their maximum concentrations in the second trimester and then decline until term (Uotila et al. 1991, Qanungo et al. 2000). Also, a comparison of the levels of the markers of protein peroxidation

processes in women indicates the occurrence of oxidative stress during pregnancy (Wisdom et al. 1991).

Relatively few studies have investigated oxidative stress in pregnant animals. Previously, only Vannucchi et al. (2007) examined oxidative profiles in blood plasma of pregnant and non-pregnant female dogs. They determined TBARS as a marker of lipid peroxidation and carbonyl proteins as a marker of the occurrence of protein oxidation. They found that concentrations of TBARS and carbonyl proteins were slightly higher in the pregnant group compared to the non-pregnant group, but they found no significant differences between the studied groups or between the subsequent periods of pregnancy regarding these markers of oxidative stress.

Contradictory information is available on oxidative status in normal pregnancy in other animals. Erisir et al. (2009), similar to Oztabak et al. (2005), did not find any significant differences between plasma concentrations of lipid peroxidation markers (TBARS, MDA) in pregnant and non-pregnant ewes. In contrast, Joshi et al. (2013) found that serum biomarkers of oxidative stress were significantly higher in pregnant buffaloes than in non-pregnant animals. Burchieri-Ronchi et al. (2011) investigated oxidative DNA damage as a marker of oxidative stress in pregnant sows. They showed an increased oxidative stress in middle and late pregnancy in comparison with early pregnancy. In turn, Staicu et al. (2011) determined the oxidative stress markers (MDA, carbonyl proteins and SH groups) in blood of pregnant and non-pregnant female rats. They observed that levels of these biomarkers were lower in pregnant than in non-pregnant rats.

Oxidative stress can arise from increased production of ROS and/or a decrease in antioxidant capacity. It is thought that the high metabolic demand during pregnancy may induce the production of ROS. Several organs during pregnancy have been found to reveal an increased basal oxygen consumption and changes in substrate energy use resulting in the production of ROS (Toescu et al. 2002, Mutinati et al. 2013). The placenta is another local source of ROS (Myatt and Cui 2004). As pregnancy and fetal growth progress, an overproduction of ROS in placental and fetal mitochondria is observed (Kim et al. 2005). It is thought that the placenta is a major source of oxidative stress in pregnant females (Gitto et al. 2002, Myatt and Cui 2004, Hung et al. 2010). A significant part of the total indicators of pregnancy-related oxidative stress may derive from the placenta's mitochondrial mass, which increases with gestational age (Wang and Walsh 1998). Mitochondria are considered primary generators of superoxide anions

(O₂⁻), the major ROS molecule (Cadenas et al. 2010). Oxidative stress has been shown in placental tissues and its intensity increases significantly as pregnancy proceeds (Quanungo and Mukherjea 2000, Garrel et al. 2010). These findings indicate that lipid and protein peroxidation processes may be restricted to specific tissues such as placenta tissue and in this site the oxidative stress intensity might be higher than that in blood plasma.

Another cause of oxidative stress during pregnancy may be a decrease in antioxidant capacity. It is known that in response to ROS overproduction there is often up-regulation of antioxidant defenses, but persistent overwhelming oxidative stress leads to the consumption and depression of antioxidants (Myatt and Cui 2004). Several studies in humans and animals have shown a decrease in the concentration of various non-enzymatic antioxidants, such as vitamin A, C, and E, during pregnancy (Vannucchi et al. 2007, Burchieri-Ronchi et al. 2011, Mohebbi-Fani et al. 2012). Vannucchi et al. (2007) found that profiles of vitamins A, E and magnesium were significantly lower in pregnant female dogs compared to those determined in non-pregnant animals. They speculated that the increased consumption of these antioxidant elements might have neutralized the oxidative stress during pregnancy in female dogs. The lowest levels of vitamins A and E in the cited study were observed during the 3rd and 5th weeks of pregnancy. This suggests that the oxidative stress generated during the mentioned period of pregnancy leads to a greater consumption of these antioxidants. Another cause of deficiency of these antioxidants during pregnancy could be placental transfer. Significant changes in the antioxidant enzyme activities have also been observed in pregnant females (Watson et al. 1997, Djordjevic et al. 2004, Jauniaux et al. 2005, Oztabak et al. 2005, Erisir et al. 2009, Hung et al. 2010).

It is well known that the changing hormonal activity of ovaries has an important role in oxidative stress (Pajovic et al. 2003). Some investigators assume that both estrogens and progesterone have antioxidant properties modulating antioxidant enzyme activities and reduce oxidative stress (Roof and Hall 2000, Pajovic et al. 2003), while the others, on the contrary, state that progesterone abrogates the antioxidant effects of estrogens (Wassman et al. 2005). In the present study, both pregnant and non-pregnant female dogs were under the influence of progesterone dominance. It is known that the duration of the luteal phase in pregnant and non-pregnant dogs in diestrus is similar and there is no significant difference in mean serum progesterone concentration when comparing pregnant with non-pregnant female dogs in diestrus (Concannon et al. 2009). Therefore, we con-

cluded that the differences in the oxidative status demonstrated in our study between pregnant and non-pregnant female dogs were not dependent on sex steroid hormones.

This study has one limitation. We could not completely exclude any effect of nutrition on the studied parameters. All the animals included in the study were selected from among female dogs brought by their owners to the Clinic of Reproduction, Faculty of Veterinary Medicine in Lublin with the aim of diagnosing and managing their pregnancy; therefore, we were unable to feed them the same food. It is known that most commercial foods for dogs are supplemented with antioxidants, such as vitamin E and selenium (Se) at variable concentrations (Simcock et al. 2005). Moreover, commercial dog foods may be supplemented with inorganic or organic Se. These forms of Se differ in bioavailability in dogs and as a result in antioxidant activity (Todd et al. 2012). In turn, the concentration of vitamin E in commercial dog foods may decrease during long storage. We chose the animals which received commercial dog food for adult animals supplemented with similar concentrations of Se. Since, in our study, female dogs were fed similar but not the same commercial dog foods, we cannot exclude the possibility of the effect of nutrition on the parameters studied.

Although our findings point to the fact that oxidative stress may occur during pregnancy in female dogs, the question as to whether this might increase the risk connected with the outcome of pregnancy and health of mother and fetuses, similar to that observed in women, is still unanswered. Numerous human studies have shown that oxidative stress is associated with pregnancy complications, such as fetal growth retardation, embryo resorption, embryo and fetus anomalies, abortions, pre-eclampsia, preterm labor and gestational diabetes (Bedaiwy et al. 2004, Myatt and Cui 2004, Wender-Ozegowska et al. 2004, Min et al. 2009, Al-Gubory et al. 2010). However, limited information and research studies are available about the possible role of oxidative stress on pregnancy outcomes in animals. Al-Naemi et al. (2012) showed a negative correlation between oxidative stress during pregnancy and pup count in rats. In this study, histopathological examination of placental tissues demonstrated that oxidative stress induced macroscopically visible degeneration of deciduae with the microscopic appearance of vasculitis and hemorrhage. Some studies have suggested that oxidative stress is implied in the development of gravidic toxemia in ewes (Kulcsar et al. 2006, Al-Qudah 2011).

In conclusion, our findings suggest that normal pregnancy in female dogs is associated with oxidative stress. Further studies are necessary to establish the

physiological ranges of antioxidative/oxidative profiles in pregnant dogs and to explain if and how the intensity of oxidative stress might contribute to the risk of the complications of pregnancy.

References

- Agarwal A, Gupta S, Sharma RK (2005) Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 3: 28-48.
- Alberti A, Bolognini L, Macciantelli D, Caratelli M (2000) The radical cation of N,N-diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. *Res Chem Intermed* 26: 253-267.
- Al-Gubory KH, Fowler PA, Garrel C (2010) The roles of cellular reactive oxygen species, oxidative stress and antioxidants pregnancy outcomes. *Int J Biochem Cell Biol* 42: 1634-1650.
- Al-Naemi RS, Abdullah QH, Ibrahim SA (2012) Impact of oxidative stress on pregnancy outcome in albino rats. *Iraqi J Vet Sci* 26: 93-99.
- Al-Qudah KM (2011) Oxidant and antioxidant profile of hyperketonemic ewes affected by pregnancy toxemia. *Vet Clin Pathol* 40: 60-65.
- Aw TY (1998) Determinants of intestinal detoxication of lipid hydroperoxides. *Free Radic Res* 28: 637-646.
- Beal MF (2002) Oxidatively modified proteins in aging and disease. *Free Radic Biol Med* 32: 797-803.
- Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J, Agarwal A (2004) Differential growth of human embryos in vitro: role of reactive oxygen species. *Fertil Steril* 82: 593-600.
- Benzie IF (1996) Lipid peroxidation: a review of causes, consequences, measurement and dietary influences. *Int J Food Sci Nutr* 47: 233-261.
- Berchieri-Ronchi CB, Kim SW, Zhao Y, Correa CR, Yeum KJ, Ferreira AL (2011) Oxidative stress status of highly prolific sows during gestation and lactation. *Animal* 5: 1774-1779.
- Cadenas S, Aragonés J, Landazuri MO (2010) Mitochondrial reprogramming through cardiac oxygen sensors in ischaemic heart disease. *Cardiovasc Res* 88: 219-228.
- Carine D, Loverco G, Greko P, Capuno F, Selvaggi L (1993) Lipid peroxidation products and antioxidant enzymes in red blood cells during normal and diabetic pregnancy. *Eur J Obstet Gynecol Reprod Biol* 51: 103-109.
- Concannon PW, Castracane VD, Temple M, Montanez A (2009) Endocrine control of ovarian function in dogs and other carnivores. *Anim Reprod* 6: 172-193.
- Djordjevic A, Spasic S, Jovanovic-Galovic A, Djordjevic R, Grubor-Lajsic G (2004) Oxidative stress in diabetic pregnancy: SOD, CAT, and GSH-Px activity and lipid peroxidation products. *J Matern Fetal Neonatal Med* 16: 367-372.
- Erisir M, Benzer F, Kandemir FM (2009) Changes in the rate of lipid peroxidation in plasma and selected blood antioxidants before and during pregnancy in ewes. *Acta Vet Brno* 78: 237-242.
- Garrel C, Fowler PA, Al-Gubory KH (2010) Developmental changes in antioxidant enzymatic defences against oxidative stress in sheep placentomes. *J Endocrinol* 205: 107-116.

- Gitto E, Reiter RJ, Karbownik M, Tan DX, Gitto P, Barberi S, Barberi I (2002) Causes of oxidative stress in the pre- and perinatal period. *Biol Neonate* 81: 146-157.
- Goldstein S, Czapski G, Cohen H, Meyerstein D (1994) Free radicals induced peptide damage in the presence of transition metal ions: a plausible pathway for biological deleterious processes. *Free Radic Biol Med* 17: 11-18.
- Gutteridge JM (1995) Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem* 41: 1819-1828.
- Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol* 141: 312-322.
- Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* 142: 231-255.
- Hung TH, Lo LM, Chiu TH, Li MJ, Yeh YL, Chen SF, Hsieh TT (2010) A longitudinal study of oxidative stress and antioxidant status in woman with uncomplicated pregnancies throughout gestation. *Reprod Sci* 17: 401-409.
- Idonije OB, Festus O, Okhiai O, Akpamu U (2011) A comparative study of the status of oxidative stress in pregnant Nigerian women. *Res J Obstet Gynecol* 4: 28-36.
- Iioka H (1994) Changes in blood level of lipid peroxide and vitamin E during pregnancy: clinical significance and relation to the pathogenesis of EPH gestosis. *Gynecol Obstet Invest* 38: 173-176.
- Jauniaux E, Burton GJ (2005) Pathophysiology of histological changes in early pregnancy loss. *Placenta* 26: 114-123.
- Joshi A, Kataria N, Sharma A, Arora S, Abhimanu, Maan R, Asopa S, Arya N, Khan S, Kataria AK (2013) Detection of oxidative stress in pregnant and non-pregnant Murrah Buffaloes during extreme ambiances. *Vet Res* 6: 27-31.
- Kim A, Murphy MP, Oberley TD (2005) Mitochondrial redox state regulates transcription of the nuclear-encoded mitochondrial protein manganese superoxide dismutase: a proposed adaptive response to mitochondrial redox imbalance. *Free Radic Biol Med* 38: 644-654.
- Kruidenier L, Verspaget HW (2002) Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease – radicals or ridiculous? *Aliment Pharmacol Ther* 16: 1997-2015.
- Kulcsar M, Danko G, Delavaud C, Mircu C, Nikolic AJ, Gaspard A, Cernescu H, Chilliard Y, Cseh S, Rudas P, Huszenicza G (2006) Endocrine characteristics of late pregnant hyperketonaemic ewes and their reproductive performance following the induction of ovarian cyclicity out of the breeding season. *Acta Vet Hung* 54: 235-249.
- Ledwozyw A, Michalak J, Stepien A, Kadziolka A (1986) The relationship between plasma triglycerides, cholesterol, total lipids and lipid peroxidation products during human arteriosclerosis. *Clin Chim Acta* 155: 275-283.
- Lykkesfeldt J, Svendsen O (2007) Oxidants and antioxidants in disease: oxidative stress in farm animals. *Vet Journal* 173: 502-511.
- Marnett LJ, Riggins JN, West JD (2003) Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. *J Clin Invest* 111: 583-593.
- Min J, Park B, Kim YJ, Lee H, Ha E, Park H (2009) Effect of oxidative stress on birth sizes: consideration of window from mid pregnancy to delivery. *Placenta* 30: 418-423.
- Mohebbi-Fani M, Mirzaei A, Nazifi S, Shabbooe Z (2012) Changes of vitamins A, E, and C and lipid peroxidation status of breeding and pregnant sheep during dry seasons on medium-to-low quality forages. *Trop Anim Health Prod* 44: 259-265.
- Mutinati M, Piccinno M, Roncetti M, Campanile D, Rizzo A, Sciorsci R (2013) Oxidative stress during pregnancy in the sheep. *Reprod Domest Anim* 48: 353-357.
- Myatt L, Cui X (2004) Oxidative stress in the placenta. *Histochem Cell Biol* 122: 369-382.
- Oztabak K, Civelek S, Ozpinar A, Burcak G, Esen F (2005) The effects of energy restricted diet on the activities of plasma Cu-Zn SOD, GSH-Px, CAT and TBARS concentrations in late pregnant ewes. *Turk J Vet Anim Sci* 29: 1067-1071.
- Pajović SB, Sačić ZS, Spasić MB, Petrović VM (2003) The effect of ovarian hormones on antioxidant enzyme activities in the brain of male rats. *Physiol Res* 52: 189-194.
- Qanungo S, Mukherjea M (2000) Ontogenic profile of some antioxidants and lipid peroxidation in human placental and fetal tissues. *Mol Cell Biochem* 215: 11-19.
- Rice-Evans CA, Diplock AT, Symons MC (1991) Techniques in free radical research. In: Burdon RH, van Knippenberg PH (eds) *Laboratory techniques in biochemistry and molecular biology*. Elsevier Science Publishers BV, Amsterdam.
- Roof RL, Hall ED (2000) Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. *J Neurotrauma* 17: 367-388.
- Simcock SE, Rutherford SM, Wester TJ, Hendriks WH (2005) Total selenium concentrations in canine and feline foods commercially available in New Zealand. *New Zeal Vet J* 53: 1-5.
- Staicu ML, Muresan A, Tache S, Moldovan R (2011) Effects of exogenous antioxidants on oxidative stress in pregnancy. *J Med Life* 4: 163-167.
- Suhail M, Patil S, Khan S, Siddiqui S (2011) Antioxidant vitamins and lipoperoxidation in non-pregnant, pregnant, and gestational diabetic women: erythrocytes osmotic fragility profiles. *J Clin Med Res* 2: 266-273.
- Todd SE, Thomas DG, Bosh G, Hendriks WH (2012) Selenium status in adult cats and dogs fed high levels of dietary inorganic and organic selenium. *J Anim Sci* 90: 2549-2555.
- Toescu V, Nuttall SL, Martin U, Kendall MJ, Dunne F (2002) Oxidative stress and normal pregnancy. *Clin Endocrinol (Oxf)* 57: 609-613.
- Toy H, Camuzcuoglu H, Celik H, Erel O, Aksoy N (2009) Assessment of serum paraoxonase and arylesterase activities in early pregnancy failure. *Swiss Med Wkly* 139: 76-81.
- Uotila J, Tuimala R, Aarnio T, Pyykko K, Ahotupa M (1991) Lipid peroxidation products, selenium-dependent glutathione peroxidase and vitamin E in normal pregnancy. *Eur J Obstet Gynecol Reprod Biol* 42: 95-100.
- Vannucchi CI, Jordao AA, Vannucchi H (2007) Antioxidant compounds and oxidative stress in female dogs during pregnancy. *Res Vet Sci* 83: 188-193.
- Wang Y, Walsh SW (1998) Placental mitochondria as a source of oxidative stress in pre-eclampsia. *Placenta* 19: 581-586.
- Wassmann K, Wassmann S, Nickenig G (2005) Progesterone antagonizes the vasoprotective effect of estrogen

- on antioxidant enzyme expression and function. *Circ Res* 97: 1046-1054.
- Watson AL, Palmer ME, Jauniaux E, Burton GJ (1997) Variations in expression of copper/zinc superoxide dismutase in villous trophoblast of the human placenta with gestational age. *Placenta* 18: 295-299.
- Wender-Ozegowska E, Kozlik J, Biczysko R, Ozegowski S (2004) Changes of oxidative stress parameters in diabetic pregnancy. *Free Radic Res* 38: 795-803.
- Wickens D, Wilkins MH, Lunec J, Ball G, Dormandy TL (1981) Free radical oxidation (peroxidation) products in plasma in normal and abnormal pregnancy. *Ann Clin Biochem* 18: 158-162.
- Wisdom SJ, Wilson R, McKillop JH, Walker JJ (1991) Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. *Am J Obstet Gynecol* 165: 1701-1704.