Effect of Pregnancy and Foetal Number on Diameter of Corpus Luteum, Maternal Progesterone Concentration and Oxidant/Antioxidant Balance in Ewes

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Contents

The aim of this study was to determine the changes in diameter of corpus luteum (CL), maternal progesterone (P) concentration, lipid peroxidation and non-enzymatic antioxidant levels along with enzymatic antioxidant activities in pregnant ewes bearing single and twin foetuses. The ewes were selected from healthy animals that were brought to the abattoir for slaughtering. The ewes were divided into three groups: Group 1 (non-pregnant, non-oestrous, n = 30), Group 2 (pregnant bearing a single foetus, n = 30) and Group 3 (pregnant bearing twin foetuses, n = 12) after they were slaughtered. Pregnant ewes were in the first half of the pregnancy. The diameter of CL and P concentration of pregnant ewes bearing a single foetus or twin foetuses were found higher than that found in non-pregnant ewes. Similarly, the P concentration of pregnant ewes bearing twin foetuses was higher than that found in pregnant ewes bearing a single foetus. Malondialdehyde (MDA) level in pregnant ewes bearing twin foetuses was higher than that found in both non-pregnant and pregnant ewes bearing a single foetus. The serum glutathione (GSH) level and glutathione-peroxidase (GSH-Px) activity of pregnant ewes bearing twin foetuses were found lower than that found in non-pregnant ewes. Additionally, the GSH-Px activity of pregnant ewes bearing twin foetuses was found lower than that found in pregnant ewes bearing a single foetus. No significant difference was found between pregnant ewes bearing female and male foetus with respect to diameter of CL, P concentration and oxidative stress parameters. There were significant positive correlations between foetal number (0, 1, 2) and diameter of CL, P concentration, MDA level, and between P concentration and diameter of CL, MDA level. However, significant negative correlations were found between foetal number (0, 1, 2) and GSH level, GSH-Px activity, and between P concentration and GSH-Px activity. In conclusion, the diameter of CL enlarges, P production increases and oxidant/antioxidant balance impairs because of the gestation stress in ewes during pregnancy.

Introduction

The *corpus luteum* (CL) develops after the collapse of the follicle at ovulation in cyclic and pregnant mammals and CL of pregnancy is known as the *corpus luteum vernum*. Sheep CL is necessary to maintain pregnancy until at least day 60 (Al-Gubory et al. 1999). The major secretory product of CL is the progesterone (P). It is the hormone of pregnancy and unequivocally required in all mammals for maternal support of the conceptus (embryo/foetus and associated membranes) survival and development (Spencer and Bazer 2002). P increases throughout the whole length of gestation and reaches its highest level at weeks 19–20 and then declines 2 weeks before parturition in sheep (Ranilla et al. 1994). In cyclic and pregnant sheep, there is a positive correlation between the total volume of luteal tissue and the P concentration (Kaulfuss et al. 2003). It has been reported by many authors (Kalkan et al. 1996; Manalu and Sumaryadi 1998; Kaskous et al. 2003; Müller et al. 2003; Kulcsar et al. 2006) that increased foetal number results in increased maternal circulating P concentration in different sheep breeds. However, it has been reported that sex of foetuses does not affect the P concentration during gestation (Mukasa-Mugerwa and Viviani 1992; Kalkan et al. 1996).

Free radicals are highly reactive molecules that include reactive oxygen species (ROS) and reactive nitrogen species (RNS). The most prominent ROS are the superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$) and the hydrogen peroxide (H_2O_2). Nitric oxide (NO), is one of the most significant RNS, is synthesized during the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS, Agarwal et al. 2005). Free radical reactions are essential for host defence mechanisms involving neutrophils, macrophages, and other cells of immune system; however, excessive production of free radicals can lead to tissue injury and cell death and result in antioxidant depletion. Antioxidants are substances or enzymes present in tissues with the capacity to balance or neutralize these free radicals (Chen and Scholl 2005). Placenta is rich in mitochondria and highly vascularised with high metabolic rate. Thus, with increased leakage of electrons from the mitochondrial respiratory chain, there is increased generation of free radicals in normal pregnancy. This physiological response, in the presence of transition metals, potentially contributes to damage of DNA, lipids and proteins by ROS (Casanueva and Viteri 2003; Poston and Raijmakers 2004). Many studies have compared biomarkers of oxidative stress in normal pregnant women and non-pregnant control subjects (Zachara et al. 1993; Morris et al. 1998; Mihailovic et al. 2000; Arıkan et al. 2001). Results of such studies suggest that erythrocyte or plasma malondialdehyde (MDA), a by-product of lipid peroxidation, and lipid peroxides are generally higher during the second trimester (Mihailovic et al. 2000) as well as late in gestation (Morris et al. 1998; Mihailovic et al. 2000; Arıkan et al. 2001). Conversely, the activities of antioxidant enzymes [superoxide dismutase (SOD), glutathione-peroxidase (GSH-Px)] and their related substances [selenium, glutathione (GSH)] are usually decreased (Zachara et al. 1993; Mihailovic et al. 2000; Arıkan et al. 2001). Erişir et al. (2009) have reported that although plasma lipid peroxidation level does not show

any significant change, erythrocyte GSH level and GSH-Px activity significantly increase during pregnancy and erythrocyte catalase (CAT) activity significantly decreases after the first month of the pregnancy in Awassi ewes. Öztabak et al. (2005) have reported that while plasma GSH-Px activity was higher and plasma CAT activity was lower in pregnant Chios ewes on days 148 of pregnancy than in the non-pregnant ewes, plasma lipid peroxidation level on days 105 and 148 of pregnancy and plasma GSH-Px and CAT activities on day 105 of pregnancy were not different. It is clearly seen that there is a conflict regarding the changes in oxidative stress parameters during pregnancy in both ewes and women according to literature information mentioned earlier. However, there is no data regarding biomarkers of oxidative stress during normal gestation with multiple foetuses in early pregnancy in sheep. Therefore, this study was conducted to determine the changes in oxidant/antioxidant balance along with diameter of CL and P concentration during first half of the gestation in normal pregnant ewes bearing a single foetus or twin foetuses.

Material and Methods
Animals
This study was conducted in Elazığ province of Turkey located at latitude of 38°40′N during breeding season (between September and December). Seventy-two Akkaraman ewes, approximately 2 years of age, were used in this study. The age of ewes was estimated by examining their teeth and also by taking information from the animal owners. The ewes were selected from healthy animals which were systematically examined by the expert veterinarian before and after slaughter. According to the information obtained from animal owners, the ewes used in this study were generally fed on grass supplemented with alfalfa hay and mated by ram introduction method. The ewes were divided into three groups: Group 1 (non-pregnant, non-oestrous, n = 30), Group 2 (pregnant bearing a single foetus, n = 30) and Group 3 (pregnant bearing twin foetuses, n = 12) after they were slaughtered. The pregnant ewes were selected from animals that they were in the first half of the pregnancy. This stage was determined by measuring the crown-rump length (CRL) of the foetuses by callipers after slaughtering according to findings of Aksakal (1993). It has been reported that CRL is 3.0 cm in 30th day of the pregnancy and is 20 cm in 76th day of the pregnancy in ewes. Minimum and maximum CRL values were 4.2 and 20 cm, respectively, in pregnant ewes bearing a single foetus and were 3.3 and 13.6 cm, respectively, in pregnant ewes bearing twin foetuses used in this study.

Blood collection
The animals were numbered, and blood samples were collected by a sterile syringe and injector from jugular vein before the slaughtering. Blood samples were then dumped into the sterile tubes and brought to the laboratory in appropriate conditions. The samples were centrifuged at $4500 \times \boldsymbol{g}$ for 5 min; sera were removed and stored at $-20°C$ in a deep freezer until assayed.

Measurement of diameter of CL
The uterus and ovaries were gently removed from the body after slaughter. Diameter of CL was measured by callipers and measurements were recorded.

Progesterone assay
The serum progesterone concentration was determined by coated-tube radioimmunoassay method using Active® Progesterone RIA DSL – 3900 kit (Diagnostic System Laboratories Inc., Webster, TX, USA) in gamma counter (LKB-Wallac Multigamma, Oy, Turku, Finland) according to the kit manufacturer's report. The progesterone concentration was expressed as ng/ml. The calibration range and sensitivity of kit were 0.30–60 and 0.12 ng/ml, respectively. The intra-assay and interassay variation coefficient of kit were 4.8% and 9.2%, respectively.

Lipid peroxidation (MDA) assay
Lipid peroxidation (as MDA) level in serum samples was measured with the thiobarbituric-acid reaction by the method of Placer et al. (1966). The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption to the standard curve of MDA equivalents generated by acid catalysed hydrolysis of 1,1,3,3 tetramethoxypropane. The values of MDA were expressed as nmol/ml.

Glutathione (GSH), glutathione-peroxidase (GSH-Px) and catalase (CAT) assay
The GSH level in serum was measured at 412 nm using the method described by Sedlak and Lindsay (1968). The samples were precipitated with 50% trichloracetic acid and then centrifuged at $1000 \times \boldsymbol{g}$ for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of Tris–EDTA buffer (0.2 M; pH 8.9) and 0.1 ml of 0.01 M 5,5′-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 min and then read at 412 nm on the spectrophotometer. The GSH level was expressed as nmol/ml. Glutathione-peroxidase activity in serum samples was measured at 37°C and 412 nm according to Lawrence and Burk (1976). The GSH-Px activity was expressed as U/g prot. The method described by Goth (1991) was used for the determination of CAT activity in serum. The yellow complex of molybdate and hydrogen peroxide was measured at 405 nm against blank using a spectrophotometer. The CAT activity was expressed as kU/l.

Protein determination
The protein content in serum was measured by method of Lowry et al. (1951) using bovine serum albumin as the standard.

Plasma vitamins A and E analyses
Vitamins A (retinol) and E (alpha-tocopherol) were determined in serum samples by a modification of

the method described by Desai (1984). One hundred microlitres of plasma was saponified by the addition of 0.3 ml 60% (w/v) KOH in water and 2 ml of 1% (w/v in ethanol) ascorbic acid, followed by heating at 70°C for 30 min. Twenty microlitres portions of the methanol extracts were chromatographed on high-performance liquid chromatography. For fluorimetric detection of vitamin A, excitation and emission wavelengths of 330 and 480 nm were used, respectively. The values of vitamin A were expressed as μg/dl. The relevant wavelengths for alpha-tocopherol detection were 292 and 330 nm. Calibration was performed using standard solutions of all-trans retinol and alpha-tocopherol in methanol. The values of vitamin E were expressed as mg/dl.

Statistical analyses

The data are presented as mean ± SEM. A $p < 0.05$ value was considered statistically significant. One-way analyses of variance (ANOVA) and post hoc Tukey-HSD test were applied to determine the differences between the groups. Independent samples t-test was used to determine between pregnant ewes bearing female foetus and pregnant ewes bearing male foetus. Pearson correlation test was used to determine relationship between the parameters. All the data were analysed by using the SPSS (Version 10.0, Chicago, IL, USA).

Results

The mean diameters of CL in all groups are shown in Fig. 1. The diameters of CL in pregnant ewes bearing a single foetus ($p < 0.001$) and twin foetuses ($p < 0.05$) were found larger than in non-pregnant ewes. Additionally, six of 12 ewes bearing twin foetuses had only one CL and the remaining six had two CL.

The mean serum P concentrations in all groups are shown in Fig. 2. The P concentrations in pregnant ewes bearing a single and twin foetuses were found higher than in non-pregnant ewes ($p < 0.001$). Similarly, the P concentration in pregnant ewes bearing twin foetuses

Fig. 2. Serum progesterone concentrations in ewes with non-pregnant, pregnant bearing a single foetus and pregnant bearing twin foetuses (values are presented as means ± SEM). *Different from non-pregnant ($p < 0.001$). [a]Different from pregnant bearing a single foetus ($p < 0.01$)

was higher than in pregnant ewes bearing a single foetus ($p < 0.01$).

The lipid peroxidation, non-enzymatic antioxidant levels and enzymatic antioxidant activities in ewes that non-pregnant, pregnant bearing a single foetus and twin foetuses are presented in Table 1. There was no significant difference in MDA levels between non-pregnant and pregnant ewes bearing a single foetus. However, MDA level in pregnant ewes bearing twin foetuses was higher ($p < 0.05$) than that found in both non-pregnant ewes and pregnant ewes bearing a single foetus. The GSH level and GSH-Px activity in pregnant ewes bearing twin foetuses were found lower ($p < 0.05$) than in non-pregnant ewes. Additionally, the GSH-Px activity in pregnant ewes bearing twin foetuses was found lower ($p < 0.05$) than in pregnant ewes bearing a single foetus. With respect to serum vitamins A and E levels, and CAT activity, no significant differences were determined between the groups.

The mean diameters of CL, P concentrations and oxidative stress parameters in groups including both types of pregnant ewes bearing female or male foetuses are shown in Table 2. No significant difference was
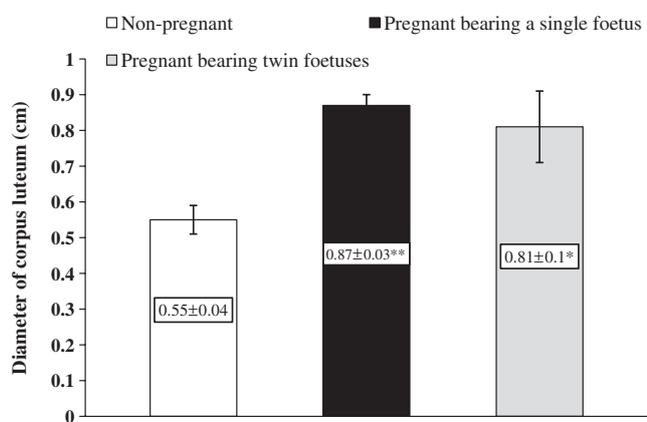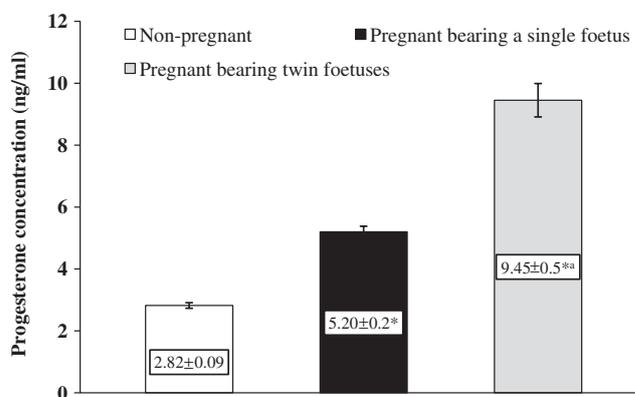

Fig. 1. Diameters of corpus luteum in ewes with non-pregnant, pregnant bearing a single foetus and pregnant bearing twin foetuses (values are presented as means ± SEM). *Different from non-pregnant ($p < 0.05$). **Different from non-pregnant ($p < 0.001$).

Table 1. The serum lipid peroxidation, non-enzymatic antioxidant levels and enzymatic antioxidant activities in ewes with non-pregnant, pregnant bearing a single foetus and pregnant bearing twin foetuses (values are presented as means ± SEM)

	Ewes		
Parameters	Non-pregnant (n = 30)	Pregnant bearing a single foetus (n = 30)	Pregnant bearing twin foetuses (n = 12)
MDA (nmol/ml)	1.83 ± 0.09[a]	1.91 ± 0.08[a]	2.80 ± 0.17[b]
GSH (nmol/ml)	0.34 ± 0.01[a]	0.33 ± 0.02[ab]	0.21 ± 0.02[b]
Vit A (μg/dl)	17.01 ± 1.80	19.24 ± 3.52	24.46 ± 5.76
Vit E (mg/dl)	0.40 ± 0.06	0.28 ± 0.04	0.26 ± 0.04
GSH-Px (U/g prot.)	3.95 ± 0.13[a]	3.83 ± 0.22[a]	2.37 ± 0.25[b]
CAT (kU/l)	16.45 ± 1.48	15.26 ± 2.24	20.90 ± 5.51

GSH, glutathione; GSH-Px, glutathione-peroxidase; MDA, malondialdehyde. The differences between the values bearing different superscript letters (a and b) in the same line are statistically significant ($p < 0.05$).

Table 2. Diameters of corpus luteum (CL), levels of progesterone, serum lipid peroxidation and non-enzymatic antioxidants and activities of enzymatic antioxidant in pregnant ewes bearing female or male foetus (values are presented as means ± SEM)

Parameters	Pregnant ewes bearing a single foetus			Pregnant ewes bearing twin foetuses		
	Female (n = 15)	Male (n = 15)	p-value	Female (n = 6)	Male (n = 7)	p-value
Diameter of CL (cm)	0.82 ± 0.17	0.80 ± 0.15	0.820	0.86 ± 0.04	0.88 ± 0.03	0.711
Progesterone (ng/ml)	5.29 ± 0.23	5.11 ± 0.26	0.588	9.28 ± 0.81	9.62 ± 0.75	0.745
MDA (nmol/ml)	1.82 ± 0.11	2.00 ± 0.15	0.367	2.79 ± 0.23	2.80 ± 0.24	0.980
GSH (nmol/ml)	0.32 ± 0.02	0.34 ± 0.02	0.710	0.20 ± 0.02	0.21 ± 0.02	0.400
Vit A (μg/dl)	18.41 ± 2.93	20.07 ± 6.77	0.813	27.40 ± 2.93	21.52 ± 6.77	0.351
Vit E (mg/dl)	0.22 ± 0.04	0.34 ± 0.07	0.620	0.21 ± 0.04	0.31 ± 0.07	0.630
GSH-Px (U/g prot.)	3.74 ± 0.28	3.92 ± 0.36	0.746	2.50 ± 0.18	2.24 ± 0.19	0.372
CAT (kU/l)	16.02 ± 2.96	14.56 ± 3.47	0.674	16.98 ± 3.10	24.82 ± 7.97	0.448

GSH, glutathione; GSH-Px, glutathione-peroxidase; MDA, malondialdehyde.

found between pregnant ewes bearing female foetus and male foetus with respect to all these studied parameters.

There were significant positive correlations between foetal number (0, 1, 2) and diameter of CL ($r = 0.528$, $p = 0.000$), P concentration ($r = 0.903$, $p = 0.000$), MDA level ($r = 0.358$, $p = 0.003$). However, significant negative correlations were found between foetal number (0, 1, 2) and GSH level ($r = -0.307$, $p = 0.012$), GSH-Px activity ($r = -0.308$, $p = 0.012$). Additionally, there were significant positive correlations between P concentration and diameter of CL ($r = 0.421$, $p = 0.000$), MDA level ($r = 0.294$, $p = 0.016$). However, a significant negative correlation was determined between P concentration and GSH-Px activity ($r = -0.311$, $p = 0.011$).

Discussion

The primary function of the CL in pregnancy is the production of P. The development of the CL begins immediately after ovulation. After an ovum is extruded at ovulation, the follicle collapses; this is followed by haemorrhage into the follicular cavity within a matter of hours. Over the next 2–4 days, the wall is penetrated with thin-walled blood channels that pass centrally towards the lumen with fresh blood. This neovascularisation is essential for the delivery of cholesterol, a principal substrate for P synthesis within the wall of the CL. As the CL matures, blood is gradually resorbed from within the cavity, and the overall structure enlarges. If conception is not achieved, the CL regresses. If pregnancy occurs, the CL continues to enlarge (Hafez and Hafez 2000). Although the sheep CL is necessary to maintain pregnancy at least during the first 60 days, it remains functional throughout the pregnancy. In addition, CL together with placenta secretes more P than required for pregnancy maintenance after day 60 (Al-Gubory et al. 1999). The presence of high levels of luteal tissue P until day 142 of pregnancy in sheep indicates that luteal cells have a sustained and high steroidogenic capacity (O'Shea and McCoy 1988). Many authors have reported that maternal plasma P concentration of different pregnant ewe breeds such as Menz (Mukasa-Mugerwa and Viviani 1992), Javanese thin-tail (Manalu and Sumaryadi 1998) and Awassi (Kaskous et al. 2003) is higher than in non-pregnant ewes. It has been reported that pregnant Awassi (Kalkan et al. 1996; Kaskous et al. 2003), German blackheaded mutton ×

long-wool Merino crossbred (Müller et al. 2003) and prolific Merino (Kulcsar et al. 2006) ewes bearing multiple foetuses have higher P concentration than that found in ewes bearing a single foetus until the last week of pregnancy. Additionally, Mukasa-Mugerwa and Viviani (1992) and Kalkan et al. (1996) have reported that maternal P concentration is not affected by the sex of the foetuses. In this study, it was found that diameter of CL and maternal serum P concentration of pregnant ewes were higher than in non-pregnant ewes, and ewes bearing a single foetus had lower P concentration than that found in ewes bearing twin foetuses. In addition, there was no significant difference between ewes bearing female and male foetuses with respect to diameter of CL and P concentration in this study. Our findings are in agreement with those previous studies. Kaulfuss et al. (2003) have reported that corpora lutea of sheep formed after multiple ovulations produce more P than CL formed after one ovulation and also reported that there is a positive correlation between total volume of CL and P concentration in cyclic and pregnant sheep. In addition, Manalu et al. (1996) have alleged that increased number of corpora lutea contributes more P secretion in pregnant sheep and goats bearing more than one foetus. In this study, a significant positive correlation was found between foetal number (0, 1, 2) and diameter of CL, maternal P concentration, and between diameter of CL and P. The enlargement in diameter of CL in pregnant ewes versus non-pregnant ewes may be explained by increase in luteal cell volume to provide more P production to maintain pregnancy at least during early pregnancy. The increase in P concentration in ewes bearing twin foetuses versus one foetus is mainly because of the increased number of corpora lutea in this study.

The use of oxygen during normal metabolism in living cells can cause normal production of ROS. In a healthy body, ROS and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, oxidative stress occurs. Reactive oxygen species are a double-edged sword: they serve as key signal molecules in physiological processes but also have role in pathological processes involving the female reproductive tract. Reactive oxygen species affect multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy (Agarwal et al. 2005). Normal pregnancy is characterized by a high energy demand for many physiological functions

and an increased oxygen requirement by different organs, including the feto-placental unit. During gestation and embryogenesis, the organism of the mother and embryo is the site of physiological high rates of ROS production, primarily involved in cell signalling and control of the foetal development; replication, differentiation, and maturation of the foetal cells and organs. However, excessive ROS production can be involved in harmful effects and can lead to breakdown of vitamins and mineral trace-elements associated with enzymes specialized in the defence against ROS and can end in peroxidation of cell components and induction of pathologies and gestation failure (Aurousseau et al. 2006). It has been reported (Catalona et al. 1999; Homko et al. 2001) that physiological increases in insulin resistance during gestation result in an increase in circulating lipids [e.g. triglycerides, free fatty acids, total cholesterol and low density lipoprotein (LDL) levels]. Increased plasma LDL is associated with increased lipid hydroperoxides in normal pregnancy (Toescu et al. 2004). In sheep and cows, the blastocyst loses its zona pellucida 7 and 10 days before implantation, respectively, whereas this phenomenon occurs at the moment of implantation in women. Therefore, an extended period of high rates of cell to cell contacts and triggering of NADPH oxidases and ROS fluxes is likely to occur in the ruminants (Aurousseau et al. 2006). Moreover, NO radical production by peripheral bovine lymphocytes has been reported to be steadily increased by a factor of 3 between the non-pregnant state and seventh day after conception and by a further factor of 1.5 between day 7 and days 90–120 of gestation (Dixit and Parvizi 2001). Evidences based on the circulating biomarkers indicate that there is an increase in oxidative stress during normal pregnancy in women (Chen and Scholl 2005). Erişir et al. (2009) have reported that plasma MDA level was 2.99 ± 0.52 nmol/ml in non-pregnant healthy Awassi sheep. Şimşek et al. (2006) have reported that serum MDA level was 2.83 ± 0.42 nmol/ml in non-pregnant healthy Akkaraman ewes. Similarly, plasma MDA level was found as 1.43 ± 0.48 nmol/ml in non-pregnant healthy Anatolian goats by Kızıl et al. (2007). The serum MDA levels (1.83 ± 0.09 nmol/ml) reported here show similarity with these results. It has been reported that plasma lipid peroxidation level on days 105 and 148 of pregnancy in Chios ewes is not different from non-pregnant control ewes (Öztabak et al. 2005). Similarly, Erişir et al. (2009) have reported that no significant change is seen in plasma MDA level during early and late pregnancy in Awassi ewes compared with non-pregnant ewes. Although the MDA levels in pregnant ewes bearing twin foetuses were found higher than in both non-pregnant and pregnant with a single foetus, there were no significant differences between non-pregnant and pregnant with a single foetus in this study. In addition, a significant positive correlation was found between foetal number (0, 1, 2) and MDA level, and P concentration and MDA level. Our findings are in agreement with previous studies in ewes and women. The increased MDA level in normal pregnant ewes bearing twin foetuses may be attributed to an increased oxygen requirement and increased circulating lipids.

The foetus and placenta are protected by antioxidant defence system to avoid any lethal effect of the high rates of ROS production. Glutathione production and metabolism are of the utmost importance to prevent pathologies of gestation, both for the radical scavenging properties of GSH and for its role in the control of redox status inside cells. Superoxide dismutase, CAT and GSH-Px are a set of enzymes specialized in defence against ROS (Agarwal et al. 2005; Aurousseau et al. 2006). Whole blood or erythrocytes are frequently used to measure the activities of these intracellular enzymes. However, plasma and/or serum are also used to measure enzyme activity (Öztabak et al. 2005; Şimşek et al. 2006; Kızıl et al. 2007) because activity values in these samples reflect those found in erythrocytes (Nazıroğlu and Kökçam 2000). In normal pregnancy, it seems plausible that the antioxidant defence system may be able to compensate through induction and increased activity of antioxidant enzymes as well as non-enzymatic free radical protection and scavenging (e.g. by protein thiols, and vitamins A, E and C, Morris et al. 1998; Chen and Scholl 2005). It has been reported that maternal SOD and GSH-Px activity either increases, declines, or shows no change during gestation in women (Carone et al. 1993; Zachara et al. 1993; Loverro et al. 1996; Chen et al. 2003). Zachara et al. (1993) and Mihailovic et al. (2000) have reported that maternal GSH-Px activity gradually declines with gestation, whereas Chen et al. (2003) have reported that GSH-Px activity increases by 15% between week 16 and third trimester. In addition, some researchers have reported that SOD, GSH-Px and their substrates (e.g. selenium, GSH) usually decreases during gestation in women (Zachara et al. 1993; Mihailovic et al. 2000; Arıkan et al. 2001). In a study made in sheep throughout the gestation, Al-Gubory et al. (2004) have reported that enzymatic activity of SOD, GSH-Px and glutathione-S-transferase in CL increases significantly from day 15 to day 40 of pregnancy and thereafter remains constant until day 128. Kızıl et al. (2007) have reported that plasma GSH level, GSH-Px and CAT activities were 0.244 ± 0.028 nmol/ml, 1.01 ± 0.15 U/g prot. and 28.88 ± 14.02 kU/l, respectively, in non-pregnant healthy goats. Öztabak et al. (2005) have reported that plasma GSH-Px and CAT activities were 15.91 ± 7.23 U/l and 2.06 ± 1.08 kU/l respectively, in non-pregnant healthy ewes. Similarly, Kozat et al. (2007) have reported that serum CAT activity in non-pregnant healthy Akkaraman lambs was 14.16 ± 0.99 kU/l. Our findings regarding serum GSH level (0.34 ± 0.01 nmol/ml) and CAT activity (16.45 ± 1.48 kU/l) are normal values for non-pregnant ewes, and these values show similarity with the aforementioned reports. Erişir et al. (2009) have reported that erythrocyte GSH level and GSH-Px activity significantly increase during pregnancy, and erythrocyte CAT activity significantly decreases after the first month of the pregnancy in Awassi ewes. Öztabak et al. (2005) have reported that while plasma GSH-Px activity was higher and plasma CAT activity was lower in pregnant Chios ewes on day 148 of pregnancy than in the non-pregnant ewes, plasma GSH-Px and CAT activities on day 105 of pregnancy were not different. In this study,

the serum GSH level and GSH-Px activity in pregnant ewes bearing twin foetuses were significantly lower than in non-pregnant ewes. Additionally, the GSH-Px activity in pregnant ewes bearing twin foetuses was significantly lower than in pregnant ewes bearing a single foetus. However, there was no significant difference in GSH level and GSH-Px activity between the non-pregnant and pregnant ewes with a single foetus. The reason of the discrepancy between our findings and previous reports may be because of breed differences or to the different type of samples used to measure the enzymatic activities (serum, plasma or erythrocytes). With respect to serum vitamins A and E levels, and CAT activity, no significant differences were found between the groups. Significant negative correlations were found between the foetal number (0, 1, 2) and both, GSH levels and the GSH-Px activity. The reason of the decreased antioxidants in pregnant ewes bearing twin foetuses in the first half of the pregnancy observed in this study may depend on two factors; first, it is possible that there is an insufficient increase in antioxidant defences to offset the increase in oxidative stress and lipid peroxidation in pregnant ewes bearing multiple foetuses. This may explain the reduced circulating levels of antioxidants. Second, maternal circulating lipid levels and plasma volume can influence the values of biomarkers as well. For example, increased maternal lipids potentially induce lipid peroxidation; expanded plasma volume may lower the concentration of antioxidant enzymes (Chen and Scholl 2005).

In conclusion, the results of this study show that normal gestation with single and twin foetuses causes enlargement in diameter of CL and increases maternal P concentration. Although pregnancy with a single foetus does not affect the oxidant/antioxidant balance, pregnancy with multiple foetuses causes oxidative stress by increasing serum lipid peroxidation levels and decreasing serum GSH level and GSH-Px activity. Therefore, powerful antioxidants may be administered to pregnant ewes bearing more than one foetus to protect both mother and foetuses against detrimental effects of oxidative stress.

Acknowledgement

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

G Türk and E Demirci planned the study. G Türk, S Gür, M Sönmez, Ş Özer and EH Aksu collected the samples from abattoir. A Yüce made the analyses of lipid peroxidation and antioxidants in blood samples. G Türk also analysed the progesterone concentrations and wrote the first draft of the manuscript.

References

Agarwal A, Gupta S, Sharma RK, 2005: Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 3, 28, 1–21.

Aksakal A, 1993: Prenatal dönemde koyun fötuslarında ovaryum ve uterusun histogenezisi üzerinde ışık mikroskobik çalışmalar. Doktora tezi (PhD thesis), F Ü Sağlık Bil Enst (In Turkish), Elazığ.

Al-Gubory KH, Solari A, Mirman B, 1999: Effects of luteectomy on the maintenance of the pregnancy, circulating progesterone concentrations and lambing performance in sheep. Reprod Fertil Dev 11, 317–322.

Al-Gubory KH, Bolifraud P, Germain G, Nicole A, Ceballos-Bicot I, 2004: Antioxidant enzymatic defence systems in sheep corpus luteum throughout pregnancy. Reproduction 128, 767–774.

Arıkan S, Konukoğlu D, Arıkan Ç, Akçay T, Davas İ, 2001: Lipid peroxidation and antioxidant status in maternal and cord blood. Gynecol Obstet Invest 51, 145–149.

Aurousseau B, Gruffat D, Durand D, 2006: Gestation linked radical oxygen fluxes and vitamins and trace mineral deficiencies in the ruminant. Reprod Nutr Dev 46, 601–620.

Carone D, Loverro G, Greco P, Capuano 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.

Casanueva E, Viteri FE, 2003: Iron and oxidative stress in pregnancy. J Nutr 133, 1700S–1708S.

Catalona PM, Huston L, Amini SB, Kalhan SC, 1999: Longitudinal changes in glucose metabolism during pregnancy in obese women with normal glucose tolerance and gestational diabetes mellitus. Am J Obstet Gynecol 180, 903–916.

Chen X, Scholl TO, 2005: Oxidative stress: changes in pregnancy and with gestational diabetes mellitus. Curr Diab Rep 5, 282–288.

Chen X, Scholl TO, Leskiw MJ, Donaldson MR, Stein TP, 2003: Association of glutathione peroxidase activity with insulin resistance and dietary fat intake during normal pregnancy. J Clin Endocrinol Metab 88, 5963–5968.

Desai ID, 1984: Vitamin E analysis methods for animal tissues. Methods Enzymol 105, 138–147.

Dixit VD, Parvizi N, 2001: Pregnancy stimulates secretion of adrenocorticotropin and nitric oxide from peripheral bovine lymphocytes. Biol Reprod 64, 242–248.

Erişir 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.

Goth L, 1991: A Simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta 196, 143–152.

Hafez B, Hafez ESE, 2000: Anatomy of female reproduction. In: Hafez B, Hafez ESE (eds), Reproduction in Farm Animals. Lippincott Williams and Wilkins Philadelphia, Pennsylvania, USA, pp. 13–29.

Homko C, Sivan E, Chen X, Reece EA, Boden G, 2001: Insulin secretion during and after pregnancy in patients with gestational diabetes mellitus. J Clin Endocrinol Metab 86, 568–573.

Kalkan C, Çetin H, Kaygusuzoğlu E, Yılmaz B, Çiftçi M, Yıldız H, Yıldız A, Deveci H, Apaydın AM, Öcal H, 1996: An investigation on plasma progesterone levels during pregnancy and at parturition in ivesi sheep. Acta Vet Hung 44, 335–340.

Kaskous S, Gottschalk J, Hippel T, Grun E, 2003: The behaviour of growth-influencing and steroid hormones in the blood plasma during pregnancy of awassi sheep in Syria. Berl Munch Tierarztl Wochenschr 116, 108–116.

Kaulfuss KH, Moritz S, Giucci E, 2003: The influence of the ovulation rate on ultrasonically determined ovine corpus luteum morphometry and progesterone concentrations in cyclic and early pregnant sheep. Dtsch Tierarztl Wochenschr 110, 249–254.

Kızıl Ö, Özdemir H, Karahan M, Kızıl M, 2007: Oxidative stress and alterations of antioxidant status in goats naturally infected with Mycoplasma agalactiae. Rev Méd Vét **158,** 326–330.

Kozat S, Gündüz H, Değer Y, Mert N, Yörük İH, Sel T, 2007: Studies on serum α-tocopherol, selenium levels and catalase activities in lambs with white muscle disease. Bull Vet Inst Pulawy **51,** 281–284.

Kulcsar M, Danko G, Magdy HGI, Reiczigel J, Forgach T, Prohaczik A, Delavaud C, Magyar K, Chilliard Y, Solti L, Huszenicza GY, 2006: Pregnancy stage and number of fetuses may influence maternal plasma leptin in ewes. Acta Vet Hung **54,** 221–234.

Lawrence RA, Burk RF, 1976: Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun **71,** 952–958.

Loverro G, Greco P, Capuano F, Carone D, Cormio G, Selvaggi L, 1996: Lipid peroxidation and antioxidant enzymes activity in pregnancy complicated with hypertension. Eur J Obstet Gynecol Reprod Biol **70,** 123–127.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951: Protein measurement with folin phenol reagent. J Biol Chem **193,** 265–275.

Manalu W, Sumaryadi MY, 1998: Maternal serum progesterone concentration during gestation and mammary gland growth and development at parturition in javanese thin-tail ewes carrying a single or multiple fetuses. Small Rumin Res **27,** 131–136.

Manalu W, Sumaryadi MY, Kusumorini N, 1996: Effect of fetal number on concentrations of circulating maternal serum progesterone and estradiol of does during late pregnancy. Small Rumin Res **23,** 117–124.

Mihailovic M, Cvetkovic M, Ljubic A, Kosanovic M, Nedeljkovic S, Jovanic I, Pesut O, 2000: Selenium and malondialdehyde content and glutathione peroxidase activity in maternal and umbilical cord blood and amniotic fluid. Biol Trace Elem Res **73,** 47–54.

Morris JM, Gopaul NK, Endresen MJR, Night M, Linton EA, Dhir S, Anggard E, Redman CWG, 1998: Circulating markers of oxidative stress are raised in normal pregnancy and pre-eclampsia. Br J Obstet Gynaecol **105,** 1195–1199.

Mukasa-Mugerwa E, Viviani P, 1992: Progesterone concentrations in peripheral plasma of Menz sheep during gestation and parturition. Small Rumin Res **8,** 47–53.

Müller T, Schubert H, Schwab M, 2003: Early prediction of fetal numbers in sheep based on peripheral plasma progesterone concentrations and season of the year. Vet Rec **152,** 137–138.

Nazıroğlu M, Kökçam İ, 2000: Antioxidants and lipid peroxidation status in the blood of patients with alopecia. Cell Biochem Funct **18,** 169–173.

O'Shea JD, McCoy K, 1988: Weight, composition, mitosis, cell death and content of progesterone and DNA in the corpus luteum of pregnancy in the ewe. J Reprod Fertil **83,** 107–117.

Öztabak K, Civelek S, Özpınar A, Burçak 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.

Placer AZ, Linda LC, Johnson B, 1966: Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. Anal Biochem **16,** 359–364.

Poston L, Raijmakers MTM, 2004: Trophoblast oxidative stress, antioxidants and pregnancy outcome: a review. Placenta **25**(Suppl A), S72–S78.

Ranilla MJ, Sulon J, Carro MD, Mantecon AR, Beckers JF, 1994: Plasmatic profiles of pregnancy-associated glycoprotein and progesterone levels during gestation in Churra and Merino sheep. Theriogenology **42,** 537–545.

Sedlak J, Lindsay RHC, 1968: Estimation of total, protein bound and non-protein sulfhydryl groups in tissue with Ellmann's reagent. Anal Biochem **25,** 192–205.

Şimşek S, Yüce A, Ütük AE, 2006: Dicrocoelium dendriticum ile doğal enfekte koyunlarda serum malondialdehid seviyesinin belirlenmesi (In Turkish). FÜ Sağlık Bil Dergisi **20,** 217–220.

Spencer TE, Bazer FW, 2002: Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. Front Biosci **7,** 1879–1898.

Toescu V, Nuttall SL, Martin U, Nightingale PM, Kendall MJ, Brydon P, Dunne F, 2004: Changes in plasma lipids and markers of oxidative stress in normal pregnancy and pregnancies complicated by diabetes. Clin Sci **106,** 93–98.

Zachara BA, Wardak C, Didkowski W, Maciag A, Marchaluk E, 1993: Changes in blood selenium and glutathione concentrations and glutathione peroxidase activity in human pregnancy. Gynecol Obstet Invest **35,** 12–17.

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