Selenium, Copper, Zinc, Manganese and Their Relevant Antioxidant Enzymes in Plasma of Grazing Pregnant Ewes during Dry Season

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ABSTRACT

The plasma levels of copper (Cu), zinc (Zn), manganese (Mn) and selenium (Se) were measured in pregnant fat tailed ewes in relation to the activities of the relevant antioxidant enzymes as well as the oxidative status of the animals. Whole blood samples of 47 ewes were taken at the first day of a 51-day breeding period (before pregnancy) and 120 days later (70-120 days of pregnancy). Pregnancy was confirmed in 43 ewes by measuring plasma progesterone in the samples of day 120. For the whole period of the study, the ewes were grazing on medium-to-low quality pastures and cereal stubble and were offered about 1 kg/head/day of a mixture of alfalfa hay (40%) and wheat straw (60%) plus at least 100g barley grain/head/day. A trace mineralized supplement was available free choice. The level of malondialdehyde (MDA; an index of lipid peroxidation) and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were measured in erythrocytes as well as the concentrations of Cu, Zn, Mn and Se in plasma. In the ewes with confirmed pregnancies the results were compared between days 1 and 120. MDA level increased (P≤0.05) at day 120, revealing the presence of oxidative stress. The activities of SOD and GPX and the concentrations of Cu and Zn decreased (P≤0.05), revealing antioxidant depletion in peripheral blood. Increased metabolic functions of reproductive tissues during pregnancy along with some nutritional insufficiencies could be the underlying reasons of these findings. Fat tailed ewes may experience oxidative stress during pregnancy when they are mainly fed medium to low quality forages. Further investigations are suggested to assess if the correction of such conditions is effective in reducing the proportion of barren ewes and improving the performance of the born lambs.

Key Words: Copper, zinc, selenium, antioxidant enzymes, sheep

ÖZET

KURU DÖNEMDE OTLATILAN GEBE KOYUNLARIN PLAZMALARINDA SELENYUM, BAKIR, ÇİNKO, MANGAN VE ONLARLA İLİŞKİLİ ANTIÖKSİDAN ENZİMLER

Yağlı kuyruklu gebe koyunlarda, hayvanların oksidatif durumunun yanı sıra ilişkili antioksidan enzimlerin aktiviteleri hakkında Bakır (Cu), Çinko (Zn), Mangan (Mn) ve Selenyum’un (Se) plazma seviyeleri ölçüldü. 47 adet koyunun tüm kan örnekleri, 51 günlük üreme periyodlarının ilk günlünde (gebelik öncesi) ve 120 gün sonra (gebeliğin 70-120 günleri) alındı. Gebelik, 120 günlük örneklerde plazma progesteronu ölçülen 43 koyunda
Introduction

Trace elements have prominent roles in the antioxidative systems involved in controlling reactive oxygen species (ROS) - the byproducts of aerobic respiration and metabolism - which may initiate some drastic changes within the body if they are not neutralized. Although ROS have important roles in regulating the metabolic activities of animals (Droge, 2002), they can oxidize various macromolecules and in amounts exceeding the capacity of antioxidant mechanisms they cause oxidative stress (the imbalance between ROS and the antioxidant systems) which potentially leads to pathological changes (Lykkesfeldt and Svendsen, 2007).

Trace elements have indirect antioxidant properties and are essential in the enzymatic defense systems against ROS-induced cellular damage. Copper (Cu), zinc (Zn) and manganese (Mn) are essential constituent of superoxide dismutase (SOD) which catalyses superoxide ions into H₂O₂ and oxygen. Selenium also is a part of glutathione peroxidase (GPX) which converts H₂O₂ into water (Al-Gubory et al., 2010). Decline in the reserves of body’s trace elements is anticipated if the animal is deprived of good mineral sources and/or supplements. However, these declines may not result in a disease condition with distinct clinical signs (Mohebbi-Fani et al., 2010). Oxidative stress also leaves no clinical signs (Celi et al., 2010). So, both mineral deficiencies and oxidative stress may be left undiagnosed under routine management practices.

In ruminants, the studies focusing on the conditions associated with oxidative stress are limited. Sporadic studies in cattle have mainly concerned with mastitis, pneumonia, and retained placenta. A number of studies have focused on the peripartum period and the associated metabolic diseases (Celi, 2008). Peripartum oxidative stress has been reported in dairy cows (Bernabucci et al., 2002; Bernabucci, 2005; Castillo et al., 2005; Gaál et al., 2006), sheep (Kamiloglu et al., 2006) and goat (Celi et al., 2010) and may be influenced by nutrition and body condition (Bernabucci et al., 2002; Bernabucci, 2005; Celi et al., 2010). Limited published data show that, in addition to peripartum period, oxidative stress may occur in small ruminants during other stages of reproductive/productive cycle with lower metabolic changes and nutritional requirements. Short term energy deficiency induced by 3 days of fasting in non-pregnant ewes resulted in increased lipid peroxidation (Gaál et al., 1993). Mid-lactation dairy goats have been reported to experience moderate oxidative stress during hot seasons (Di Trana et al., 2006). Oxidative stress, coincided with decline in vitamins A, E and C (parts of the non-enzymatic antioxidant system), has been reported in sheep grazing on natural pastures during breeding and pregnancy (Mohebbi-Fani et al., 2012).

Pregnant ewes have lower nutritional requirements than peripartum and lactating ewes (NRC, 1985). Under routine rearing systems, the pregnant ewes mostly receive medium to low quality forages (Glimp, 1991) although their ration is usually improved for a while prior to- and during breeding. The aim of the present study was to assess the changes in
oxidative status and the trace element-containing antioxidant enzymes in pregnant fat-tailed ewes grazed mainly medium to low quality pastures during dry months in Fars province, Iran. The level of malondialdehyde (MDA) was measured as an index for the oxidative status of the animals. Copper, zinc, manganese, selenium and the activity of their relevant antioxidant enzymes (SOD and GPX) were investigated.

Materials and Methods

Animals and Sampling

This study was conducted on 47 cross-bred fat-tailed ewes, 3–5 years old with body condition scores (BCS) of 2.5 to 3.5. The animals were reared in a 450-ewe flock, in a farm 150 km north of Shiraz, southwest Iran. The selected ewes were stained on their back for easy recognition among the whole flock. The ewes were isolated from the rams during July and August prior to the breeding season. One week before the beginning of the breeding time (early September), the ewes were kept in close proximity of the rams separated by tight fences (to apply the ram effect for flock synchronization). Then, the rams were released into the ewe flock (1 fertile ram per 25 ewes) for 51 days. The rams were serologically negative for brucellosis and were selected following clinical examination and measurement of the scrotal girth.

The ewe flock was grazing on medium-to-low quality pastures and cereal stubble - typical feeds for natural grazing in the area during the dry summer - shifting to low quality pastures within the succeeding months. Three weeks before the breeding time the ration of the flock was improved in a flushing program: about 1 kg/head/day of a mixture of alfalfa hay (40%) and wheat straw (60%) plus 300 g barley grain/head/day in the afternoons in addition to the pasture. The crude protein contents of wheat straw, alfalfa hay and barley grain were 2.5, 13.5 and 11.9%, respectively. This program was continued for the first month of the breeding period, after which the supplemental roughage was continued but the daily amount of barley was reduced to 100 g/head/day. White salt and a trace mineralized supplement were available free choice. Alfalfa stubble was also available for less than 20 days during October-November. Whole blood samples (10 ml) were collected by jugular venipuncture of ewes into EDTA tubes on days 1 and 120 after ram introduction for measuring the level of MDA and the activities of SOD, GPX in erythrocytes as well as the concentrations of Cu, Zn, Mn and Se in plasma. Plasma progesterone (P4) was measured in the samples of day 120 to confirm the pregnancy of the ewes. No clinical disease was reported in the ewes during the study.

Measurement of MDA

The lipid peroxidation level of the RBC membrane was evaluated by a modified HPLC method (Lykkesfeldt, 2001) based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colored MDA–TBA adduct. Erythrocytes were washed three times with phosphate-buffered saline. Then, 40 μL of sample was diluted with 100 μL of H2O and mixed with 20 μL of 2.8 mmol/L butylated hydroxytoluene (BHT) in ethanol, 40 μL of 81 g/L sodium dodecyl sulfate and 600 μL of TBA reagent (8 g/L TBA diluted 1:1 with 200 mL/l acetic acid adjusted to pH 3.5 with NaOH). The mixture was immediately heated (60 min at 95°C) and cooled with running water; 200 μL of H2O and 1000 μL of butanol–pyridine (15:1, v/v) were then added. After vigorous mixing, the organic layer was separated by centrifugation (3 min at 16,000 g). The supernatant was analyzed on a UV-visible spectrophotometer fitted with an 80μL flow cell. The absorbance was measured at 532 nm (the mobile phase was consisted of 300 mL/L methanol in 50 mmol/L potassium dihydrogen phosphate buffer, pH 7.0). 1, 1, 3, 3-tetraethoxypropane was used as a standard, and MDA-TBA reactive substances values were expressed as MDA nano-moles per grams of hemoglobin (nmol/g Hb). The HPLC system was consisted of a solvent delivery pump (JASCO 980-PU, Tokyo, Japan), a reversed-phase column (Luna C18, 250 mm × 4.6 mm, Phenomenex, CA, USA), and a UV–Vis
detector (Jasco, UV-975, Tokyo, Japan) operated at 532 nm.

**Measurement of trace elements**

Plasma minerals were determined using atomic absorption spectrophotometry (apparatus: Shimadzu AA-670) after wet digestion of samples in a 7:3 mixture of nitric acid and perchloric acid; and diluting to the analytical range of the apparatus with deionized water.

**The activities of SOD and GPX**

The activity of SOD was measured with a commercial kit (RANSOD kit, Randox Com, UK). In this method, xanthine and xanthine oxidase are employed to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The activity of SOD in hemolysate was determined by the degree of inhibition of this reaction as one unit of SOD corresponded to 50% inhibition of INT reduction under assay condition. Finally, the enzyme activity was expressed as units/g of hemoglobin.

The activity of GPX was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia and Valentine (1967). The GPX present in the hemolysate catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The absorbance was measured at 340 nm and the enzyme activity was expressed as units/g of hemoglobin.

**Measurement of hemoglobin**

Hemoglobin concentration was measured by cyanmethemoglobin method.

**Measurement of plasma progesterone**

Plasma progesterone (P4) was measured in the samples of day 120 for assessing pregnancy status of the ewes. A commercial ELISA kit (DRG Instruments GmbH, Germany), was used, which detects P4 concentrations as low as 0.045 ng/ml and has coefficients of variation of 6.86 and 5.59% for the intra- and inter-assays, respectively.

**Statistical analysis**

All ewes with P4 concentrations ≥2.5 ng/ml at day 120 were considered to be pregnant (Boscos et al., 2002). Accordingly, 4 barren ewes were not included in the statistical study and the remainders (43 ewes) were studied using paired t-test (non-pregnant and pregnant). The results were analyzed at P≤0.05 using the SPSS statistical software (Version 15.0, SPSS Inc, Chicago, Illinois).

**Results**

The differences between the results of day 1 of the study (start of breeding) and day 120 (when the days in pregnancy could be 70 to 120) are shown in Figures 1 and 2. The concentration of MDA increased at day 120 compared with day 1. The activities of SOD and GPX declined at pregnancy. The levels of Cu and Zn decreased but those of manganese and selenium increased at pregnancy.

**Discussion**

**Evidence of oxidative stress**

In the present study the MDA concentration increased at day 120. MDA is an end product of polyunsaturated fatty acid oxygenation and is a reliable and commonly used biomarker for assessing lipid peroxidation (Moore and Roberts, 1998). This could be due to increased oxidative conditions within the reproductive tissues during pregnancy. Both reactive oxygen species (ROS) and antioxidants have major physiologic roles in all reproductive processes (Al-Gubory et al., 2010). High metabolic rate of the placenta increases the generation of ROS and results in oxidative stress (Al-Gubory et al., 2010) which may be linked to fetal programming (Myatt, 2006; Myatt and Cui, 2004). Placental antioxidant adequacy is essential for proper placental function and development and its effectiveness against oxidative stress varies with the stage of placental development in sheep (Garrel et al., 2010) and humans (Qanungo and Mukherjea, 2000; Qanungo et al., 1999).
depletion of placental antioxidant systems has been suggested as a key factor in early human pregnancy failure (Jauniaux et al., 2000; Liu et al., 2006). The ewes with P4 concentrations ≥2.5 ng/ml at day 120 were considered to be pregnant (Boscos et al., 2002). Accordingly, 91.5% of ewes were pregnant at day 120 of the study. This was almost the same performance of the same flock in the previous years (data not shown). A possible role for oxidative stress in 8.5% barren ewes in the studied ewes could not be overlooked. Oxidative stress coincided with rapid decline in vitamins A, E and C (parts of the non-enzymatic antioxidant system) during breeding time has been reported in grazing sheep (Mohebbi-Fani et al., 2012). In the present study, nutrition could have a part in incidence of oxidative stress (see below).

**Figure 1.** Mean ± SEM of concentration malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) in the studied ewes before and during pregnancy (a, b P<0.01).

**Şekil 1.** Incelenen koyunlarda gebelik öncesi ve sonrası malondaldehit (MDA) ortalama ± SEM konsantrasyonu ve superoksit dizmutaz (SOD) ve glutatyon peroksidaz (GPX) aktivitesi (a, b P<0.01).

**Figure 2.** Mean ± SEM of concentrations of copper (Cu), zinc (Zn), selenium (Se) and manganese (Mn) in the studied ewes before and during pregnancy (a, b P<0.01).

**Şekil 2.** Incelenen koyunlarda hamilelik öncesi ve sonrası bakır (Cu), çinko (Zn), selenium (Se) ve manganez (Mn) konsantrasyonuna ait ortalama ± SEM (a, b P<0.01).
Changes of antioxidant enzymes

Coincided with increased MDA, there was a decrease in the activity of SOD and GPX at day 120. The metabolic status of the tissues affects the antioxidant defense system and antioxidant depletion could be the consequence of oxidative stress (Venditti and Meo, 2006). Antioxidant enzymes are involved in saving corpus luteum (CL) from luteolysis and continuation of steroidogenesis during pregnancy. The activities of SOD and GPX change in the ovine CL during pregnancy (Al-Gubory et al., 2004), probably linked to ROS generation in the luteal cells and involved in the inhibition of luteal apoptosis. In the rat CL, the activity of SOD changes along with serum progesterone concentrations during pregnancy (Sugino et al., 1993) and pseudopregnancy (Shimamura et al., 1995). Expression of SOD is high in bovine (Rueda et al., 1995) and human (Sugino et al., 2000) CL during early pregnancy. SOD has been isolated and identified from the sheep CL of pregnancy (Al-Gubory et al., 2003). Antioxidant enzymes are important components of the pre-implantation embryos of mouse, cow, porcine and human and the receptive uterine endometrium (Blomberg et al., 2005; El Mouatassim et al., 1999; Harvey et al., 1995; Orsi and Leese, 2001). The sheep placenta (Al-Gubory et al., 1999) has predominant roles in progesterone production from 60 days to term of pregnancy.

It is concluded in the present study that pregnancy has resulted in oxidative conditions within the reproductive tissues, causing increased demands for antioxidant enzymes in these tissues. Throughout gestation the uterine blood flow increases to supply placental and fetal nutrient and oxygen requirements (Al-Gubory et al., 2010). Thus, a major proportion of the available amino acids could have been conducted from plasma to the reproductive tissues to support the synthesis of antioxidant enzymes. This has probably resulted in less production of SOD and GPX in non-reproductive tissues (including erythrocytes) rendering them to lipid peroxidation with consequent elevation in MDA. Dietary insufficiencies that could be a feature of the present study (see below) could also have a role in these findings.

Changes of trace elements

The levels of Cu and Zn decreased but those of Mn and Se increased at day 120. Copper, Zn, Mn and Se are essential components of antioxidant enzymes. They act as the active site of the enzyme or ensure the structural stability of the molecule (Shenkin et al., 2006). In mineral deficiencies, the terminal "disease" stage with reduced blood minerals is preceded by "tissue depletion" (reduction of body reserves with no sign), "marginal deficiency" (affected growth and immune functions), and "tissue dysfunction" (affected activities of mineral containing enzymes) (Suttle, 1986). Reduced activities of antioxidant enzymes (SOD and GPX) in the present study could reveal tissue dysfunction, which could partly be attributed to decreased levels of plasma Cu and Zn. This deficiency, however, could not be a primary one since a trace mineralized supplement was available to sheep free choice. Instead, a secondary deficiency due to nutritional insufficiencies could be the underlying reason.

Absorption, metabolism, and homeostasis of minerals need synchronization and coordination among various hormones, enzymes and receptors present in blood and various organs (Mohebbi-Fani et al., 2010). These may not be achieved if the general metabolism is hampered due to nutritional deficiencies. The decrease in the levels of Cu and Zn could be attributed to their lower absorption due to prolonged nutritional insufficiencies (see below). Copper-dependent proteins act as transcription factors for specific genes, such as those regulating SOD and catalase (Uauy et al., 1998). The zinc bound to albumin is in equilibrium with plasma amino acids and may be important in cellular uptake mechanisms (Shenkin et al., 2006). Thus, decreased plasma Zn may be accompanied by a shortage in plasma amino acids and impairment of protein metabolism. The increase in plasma Mn and Se cannot be explained by the experiments of the present study. It could be due to the catabolism of proteins (including metalloproteins) in response to prolonged protein deficiency. The
half-life of manganese-dependent SOD in blood serum is longer than that of Cu, Zn SOD (Shenkin et al., 2006).

Nutritional status

Nutritional condition of the sheep in this study was a sample of routine practices in Iran during drought seasons and could have a contribution in plasma antioxidant depletion. The sheep grazed mainly on low quality pastures and cereal stubble with little access to green forages. The supplemental feed was 60% wheat straw (deficient in most nutrients), 40% alfalfa hay and small amounts of barley grain. With this type of feeding protein deficiency could be a problem. The protein content of the supplemental feed could be about 9% of dry matter. Taking into account the diluting effect of the grazed feed, the actual crude protein of the ration could be lower than the minimum requirements of pregnant ewes during the first 15 weeks of gestation (9% of dry matter) (NRC, 1985). Protein deficiency may impair cellular antioxidant capacities because less amino acids may be available for the synthesis of antioxidant enzymes (Al-Gubory et al., 2010). Besides, protein deficiency could result in less absorption and/or metabolism of trace elements ending in a secondary mineral deficiency.

Conclusion

In conclusion, fat tailed ewes may experience oxidative stress during pregnancy when they are mainly fed medium to low quality forages. Further investigations are suggested to assess if the correction of such conditions is effective in reducing the proportion of barren ewes and improving the performance of the born lambs.

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