Introduction

In the last decade, the evaluation of oxidative stress (the imbalance between the production of free radicals and the efficiency of antioxidant defence systems in the body) has become increasingly important in ruminant health and animal production as complementary tools in evaluation of the nutritional and metabolic status of the animals. Free radicals are believed to play important roles in regulating the metabolic activity of some organs and productivity in farm animals [16]. However, they are capable of oxidizing various macromolecules and in amounts exceeding the capacity of antioxidant mechanisms they cause oxidative stress, which potentially leads to pathological changes [28]. All major classes of biomolecules are sensitive to oxidative stress, although lipids are particularly susceptible [29]. Several defence mechanisms are available to prevent oxidative damage [28] such as the superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzymes. Thiobarbituric reactive substances (TBARS) can be measured for assessing lipid peroxidation and oxidative stress of the animal [31].

Although oxidative stress has been implicated in numerous disease processes [12], it can be particularly dangerous because it usually leaves no clinical signs and the condition is diagnosed by means of dedicated analytical methods to evaluate the integrity of the defence mechanisms and the end products of oxidative stress [13]. In ruminants, only a limited number of conditions have been investigated in regard to the effects of oxidative stress. Studies in cattle have been sporadic and mainly concerned mastitis, pneumonia and retained placenta [12]. A number of studies in ruminants have focused on...
the peripartum period and the associated metabolic diseases [12]. Some studies in dairy cows indicate to the occurrence of oxidative stress during the peripartum period [8, 11, 19], which may be influenced by nutrition and body condition [7, 8] and associated with metabolic diseases [29]. Peripartum oxidative stress has also been reported in sheep [25]. The periparturient period, however, is a stage associated with rapid and drastic metabolic changes and increasing nutritional demands. Based on limited published data, ewes [18] and goats [15] may suffer from oxidative stress in other stages of reproductive / productive cycle that have 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 [18]. Mid-lactation dairy goats have been reported to experience moderate oxidative stress during hot seasons [15].

Compared with the ewes in the peripartum and lactation periods, non pregnant or pregnant- non lactating ewes have low nutritional requirements [34]. Under natural seasonal breeding, the nutrition of the flock is usually improved prior to and during breeding. After that, the flock may receive feeds of lower quality compared with the previous rations [21]. The aim of the present study was to evaluate the oxidative status of fat-tailed ewes under natural breeding and a routine feeding program during drought months in Fars province, Iran. The activities of antioxidant superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzymes as well as the amounts of thiobarbituric reactive substances (TBARS) in erythrocytes were assessed in the present study as sensitive indices for studying the oxidative status of the animal as reported by BERNABUCCI et al. [7].

Material and Methods

ANIMALS AND SAMPLING

The study was conducted in a sheep farm, 150 km north of Shiraz, southwest Iran, from late August to late December 2009 on 105 cross-bred fat tailed ewes, 3-5 years old with body condition scores (BCS) between 2.5 and 3.5. The selected ewes were stained on their back for easy recognition among the flock (450 ewes). The flock was grazing on meadow-to-low quality pastures and cereal stubble (typical feeds for natural grazing in the area during the dry summer) shifting for 51 days. The rams, the ewes were kept in close proximity with rams separated by tight fences. After this period, 18 fertile rams, 4 to 5 years old, were released into the ewe flock (1 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.

Whole blood samples (10 mL) from ewes were collected by jugular venipuncture into sterile EDTA tubes on days 1, 7, 21 and 120 after ram introduction for measuring the activities of SOD, GPX and the TBARS concentrations in erythrocytes. To evaluate the pregnancy status of the ewes, plasma progesterone concentration was also determined on day 120. No clinical disease was reported in the ewes during the study.

BIOCHEMICAL ANALYSES

Whole blood samples (0.5 mL) were centrifuged for 10 minutes at 700 g at room temperature and plasmas were separated. Erythrocytes were washed four times with 0.9% NaCl solution (3 mL) and were centrifuged (700 g, 5 minutes, room temperature) each time to separate the supernatant. The washed erythrocytes were diluted with cold distilled water (2 mL).

The SOD activity was measured with a commercial kit (RANSOD kit, Randox Com, UK) according to the manufacturer's instructions in hemolysates diluted to 1: 200 with 10 mM phosphate buffer (pH: 7). 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 SOD activity 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 haemoglobin which was measured by the cyanmethemoglobin method [23].

The activity of GPX was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia and Valentine [36] on hemolysates diluted to 1:80 with phosphate buffer. 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 haemoglobin.

To evaluate lipid peroxidation in erythrocytes a modified HPLC method was used which is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a coloured MDA-TBA adduct [27]. Erythrocytes were washed three times with phosphate-buffered saline and then sample (40 μL) was diluted with 100 μL of distilled water and was added to a mixture containing 2.8 mM butylated hydroxytoluene (BHT) in ethanol (20 μL), 81 g/L sodium dodecyl sulfate (40 μL) and TBA reagent (600 μL) (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 and thereafter butanol–pyridine (15:1, v/v) (1 mL) was added and final volume was adjusted.
to 2 mL with distilled water. After vigorous mixing, the organic layer was separated by centrifugation (16 000 g, 3 minutes, at room temperature). The supernatant was analyzed on a UV-visible spectrophotometer fitted with an 80 μL flow cell. The absorbance was measured at 532 nm (the mobile phase was consisted of 300 mL/L methanol in 50 mM KH₂PO₄, pH: 7.0), 1, 1, 3, 3-tetraethoxypropane was used as a standard, and MDA-TBA reactive substances values were expressed as MDA nmol/g of haemoglobin. 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.

Plasma progesterone was measured in the samples of day 120 for assessing pregnancy status of the ewes. Plasmas were achieved by blood centrifugation at 750g for 15 minutes at room temperature and stored at -20°C until assessed. Plasma progesterone concentrations were determined using an ELISA kit (DRG Instruments GmbH, Germany), which detects concentrations as low as 0.045 mg/L with intra- and inter-assay coefficients of 6.86% and 5.59%, respectively.

STATISTICAL ANALYSIS

The results were analyzed at $P < 0.05$ using the SPSS statistical software (Version 15.0, SPSS Inc, Chicago, Illinois). With the observed means, the effect of time and age, as well as the effect of time and BCS on changes of antioxidant enzyme activities and TBARS contents were analyzed with ANOVA for repeated measures using the GLM procedure. The analyses were performed in I) ewes with thin and good body condition (BCS < 3 and ≥ 3, respectively); II) ewes at different ages (3, 4 and 5 years old); and III) all 105 ewes. Differences among various steps of sampling were studied by Bonferroni test for multiple comparisons. The data of thin and good condition ewes were compared at each step of sampling using student's $t$-test. The data of the ewes of different ages were compared at each step using one way ANOV A. The correlations of the studied parameters were studied by Pearson's correlation test separately at each step of sampling as well as pooled data of all samplings ($n = 420$).

Results

As shown in figure 1, the SOD and GPX activities declined gradually and significantly (day 1 vs. day 21 and day 120: $P < 0.05$) in the ewe flock according to time after ram introduction. It was observed that respectively 49.1% and 81% of the total declines in SOD and GPX activities were recorded at day 21. For the 2 enzyme activities, the lowest mean values were noticed on day 120 (day 120 vs. other days: $P < 0.05$). However, the GPX activity showed a rapid and significant drop within the first week (day 1 vs. day 7: $P < 0.05$) whereas the decrease in SOD activity began more slowly (day 1 vs. day 7: $P < 0.05$). A positive and significant correlation was evidenced between the 2 antioxidant enzyme activities considering all samplings in all ewes ($n = 420$) ($r = 0.441, P < 0.01$) although no significant association between SOD and GPX activities was obtained at separate days of sampling except on day 1 ($r = 0.209, P < 0.05, n = 105$).

In parallel, the TBARS concentrations in erythrocytes significantly increased ($P < 0.05$) on day 21 continuing to day 120 although the difference between days 21 and 120 was not significant. Most of the total increase in TBARS (71%) occurred within the first 21 days of the study. For each separate sampling day, TBARS concentrations were not significantly correlated

FIGURE 1: Variations of SOD and GPX enzyme activities and TBARS concentrations in erythrocytes from ewes ($n = 105$) according to time after ram introduction in the flock.
with SOD or GPX activities but, considering all time points for all ewes (n = 420), negative and significant correlations were recorded between the TBARS concentrations and the SOD activities (r = -0.271, P < 0.01, n = 420) or the GPX activities (r = -0.259, P < 0.01, n = 420).

Similar profiles of SOD and GPX activities and of TBARS concentrations in erythrocytes according to time were obtained in ewes with a correct or a thin body condition compared to the all ewes (figure 2). No significant differences in SOD and GPX activities and in TBARS concentrations at each sampling day were evidenced between ewes with a good conformation and thin females, showing that the daily variations of erythrocyte antioxidant enzyme activities and TBARS concentrations were not affected by the body condition. Furthermore, no significant difference in SOD and GPX activities and in TBARS concentrations was noted between the 3 age groups at any sampling days and variations of the biochemical markers according to time were similar between the 3 groups and to those noted for all ewes (figure 3).

In 90.5% of ewes (95 out of 105 ewes), plasma progesterone concentrations were above 2.5 μg/L on day 120 after ram introduction into the flock, indicating that these females were pregnant [10]. This percentage of pregnant females was closely related to performance of the same flock in the previous years (based on the 3-year history of the farm, data not shown). No correlations were detected between plasma progesterone concentrations and SOD or GPX activities and TBARS concentrations in erythrocytes from pregnant ewes (n = 95) and all studied ewes (n = 105).

Discussion

In the present study the decline in the activity of erythrocyte antioxidant enzymes started soon after the ram introduction. The major part of decline occurred during the first 21 days. Concentration of TBARS showed a significant increase at day 21 with a further non-significant increase at day 120, which could indicate a continuous lipid peroxidation. Ewes of various ages and body conditions were affected almost similarly throughout breeding and pregnancy. The changes of antioxidant defence systems are influenced by the metabolic status of the tissues. Antioxidant depletion could be the consequence and not the cause of oxidative stress [44]. Increased oxidative conditions within the reproductive tissues along with dietary protein deficiency in the present study could explain the decreased activity of antioxidant enzymes and increased concentrations of TBARS.

Both reactive oxygen species (ROS) and antioxidants have major physiological roles in all reproductive processes [4] and numerous studies have analysed the oxidative conditions in ovaries and placenta during reproductive functions [1-6, 39-43]. In rat ovaries, ROS are generated in luteal tissue during natural regression to inhibit steroidogenesis and in follicles to induce oocyte maturation and ovulation [6]. In human, an oxidative stress condition is established in the mid corpus luteum, coinciding with the maximal steroidogenic capacity [43]. Antioxidant enzymes are involved in the protection of corpus luteum from luteolysis and continuation of steroidogenesis when pregnancy occurs in various mammalian species. The activities of SOD and GPX change in the ovine corpus luteum throughout the oestrous cycle [2] and pregnancy [1] probably linked to ROS generated in the luteal cells, and may be involved in the inhibition of apoptosis and maintenance of luteal...
steroidogenesis. The activity of SOD in the rat corpus luteum changes in a manner similar to the change in serum progesterone concentrations throughout pregnancy [41] and pseudopregnancy [40]. High level of SOD expression is reported in the bovine [39] and human [42] corpus luteum during early pregnancy. Superoxide dismutase has been isolated and identified from the sheep corpus luteum of pregnancy [3]. Antioxidant enzymes are important components of the developing pre-implantation mouse, cow, porcine and human embryos and its receptive uterine endometrium [9, 17, 22, 35]. Throughout gestation the uterine blood flow increases to supply placental and foetal nutrient and oxygen requirements. The sheep placenta [5] has predominant roles in progesterone production early in pregnancy. Oxidative stress increases during early pregnancy because of high metabolic rate of the placenta and increased generation of ROS [4] and may be linked to foetal programming [32, 33]. The depletion of placental antioxidant systems has been suggested as a key factor in early human pregnancy failure [24, 26]. Adequate placental antioxidant status is essential for proper placental function and development and its effectiveness against oxidative stress varies with the stage of placental development in sheep [20] and humans [37, 38].

In the present study, the ewes were subjected to the ram effect and intensive breeding after a period of seasonal anoestrus. It is concluded that the commencement of the ovarian cyclic activity during the breeding season, followed by pregnancy (confirmed in 90.5% of the ewes by measuring plasma progesterone concentrations on day 120), has resulted in oxidative conditions and increased demands for antioxidant enzymes within the reproductive tissues. This, together with dietary protein deficiency has probably resulted to oxidative conditions in the ewes.

Protein deficiency may impair cellular antioxidant capacities because proteins provide the amino acids needed for the synthesis of antioxidant enzymes [4]. The sheep in the present study were mainly fed on medium-to-low quality pastures and cereal stubble. The supplemental feed of the flock consisted of wheat straw, alfalfa hay and small amounts of barley grain. According to the local feed analysis and calculation of the amount of daily allocated feeds (about 1 kg/head/day roughages plus 100-300 grams of barley grain), the protein content of the supplemental feed could reach maximally to 8% DM which was less than the minimum requirements of non-lactating and pregnant ewes during the first 15 weeks of gestation (9% of DM; NRC [34]). Taking into account the feed consumed on the pasture, the actual crude protein of the whole ration could be still lower. While the ration was deficient in protein, with the re-commencement of reproductive functions, a major proportion of the available amino acids would have continuously been conducted from plasma to the reproductive tissues to support the synthesis of antioxidant enzymes. These have probably resulted in less production of SOD and GPX in non-reproductive tissues (including erythrocytes) rendering them susceptible to lipid peroxidation with consequent elevation in TBARS. Many micronutrients such as copper, zinc, manganese and selenium, form part of the active site necessary for the antioxidant enzyme function or act as cofactors in the regulation of antioxidant enzymes. Although these micronutrients were not studied here, it is worth noting that absorption, metabolism, and homeostasis of minerals need synchronization and coordination among the various hormones, enzymes, receptors, and those present in blood and various organs [30]. These may not be achieved when the general metabolism is hampered due to protein deficiencies.
As a conclusion, under the conditions of the present study, fat tailed ewes at various ages and body conditions may suffer from oxidative stress during breeding and pregnancy; the breeding time may be a more challenging period than pregnancy. This may be of special importance since preconception nutrition plays a major role in programming the offspring susceptibility to disease, which may be mediated by macro- and micronutrient deficiencies and oxidative stress [14]. Research is essential to determine the optimum maternal antioxidant status needed to improve foetal survival and pregnancy outcomes.

References


