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Seasonal Variation of Oxidative Stress Parameters in Ram Seminal Plasma

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Abstract: The purpose of this study was to assess the level of oxidative stress, monitored both by lipid peroxidation, nitric oxide, protein carbonyl oxidation and total antioxidant capacity in seminal plasma from semen collected from fertile rams during the breeding and non-breeding seasons. Oxidative stress in the reproductive system is thought to affect the fertilizing ability of sperm. Seminal oxidative stress status emerges as a significant prognostic tool in assisted reproductive technology. Lipid peroxidation generates physical and chemical stress on the cell membranes of spermatozoa. Normally, there is a balance generation of free radicals between detoxification of them. However, the breakdown of balance causes poor motility viability and fertility of semen. Seminal plasma of rams show differences in before, during and after the breeding season for some oxidative stress parameters.

Key words: Oxidative stress, ram, season, seminal plasma, reproductive system, lipid peroxidation

INTRODUCTION

Small ruminants show a seasonal pattern in reproductive activity that is shaped by seasonal changes in their habitats (Gundogan, 2006). In the breeding season, superior rams are use extensively for mating or as sperm donors for genetic improvement (Jennings and Mcweeney, 1976). Photoperiod is the main environmental signal timing the reproductive cycle in several mammalian species, driving seasonal changes in testicular size, weight and secretion, sperm production, mating activity and fertility of males (Gundogan and Serteser, 2005). Seminal plasma, which is a complex mixture secreted from the testes, epididymis and accessory sex glands, can affect sperm morphology, motility, acrosome reaction and fertility (Mann and Lutwak-Mann, 1981). Several SP components prevented and reverted cold-shock damage on the sperm membrane and improved the viability and fertility of frozen-thawed sperm. Semen production in rams is influenced by many factors among which daylight length, climate temperature and humidity account for great variations in semen quality (Folch, 1984). The increase in quantity and improvement in quality of ram semen during the breeding season has been shown (Gundogan, 2006). Male infertility occurs mainly due to deficiency of active motile sperm count in semen. Reactive Oxygen Species (ROS) in semen have a potential role in normal fertilization (Dacheux *et al*., 1981; Agarwal *et al*., 2006a), but high levels of ROS in semen damage the spermatozoa, which results in infertility (Aitken, 1994). Levels of Malondialdehyde (MDA), Nitric Oxide (NO), Protein Carbonyl (PC) and Total Antioxidant Capacity (TAC) indicate damage of lipids and proteins respectively by ROS.

A simple tool to evaluate the effect of lipid peroxidation on the spermatozoa is the assay of sperm and seminal plasma Malondialdehyde (MDA), which is a stable lipid peroxidation product (Aitken *et al*., 1989). Malondialdehyde (MDA) is one of the products of lipid peroxidation and is commonly used parameter to indicate oxidative stress (Kamal *et al*., 1990). Lipid peroxidation of Polyunsaturated Fatty Acids (PUFA) can lead to diminished sperm membrane fluidity and therefore, compromise the fertilizing ability of human (De Lamirande and Gagnon, 1995), bovine (Beconi *et al*., 1991), rabbit (Alvarez and Storey, 1983), boar (Cerolini *et al*., 2000) and ram (Jones and Mann, 1977) sperm.

Nitric Oxide (NO) is a biological messenger molecule produced by one of the essential amino acids, L-arginine, by the catalytic action of the enzyme NO synthase (NOS). NO is known to be involved in diverse physiological and pathophysiological processes in various organ systems,

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including the human male and female reproductive tracts (Thippeswamy *et al*., 2006). The physiological role of NO in male reproductive process has previously been identified and expression of all isoforms of NOS in male genital tract cells suggested a definite role for NO in contractile, haemodynamic, hormonal aspects of testicular and epididymal function as well as in spermatogenesis and germ cell degeneration (O'Bryan *et al*., 2000; Lue *et al*., 2003).

Levels of Protein Carbonyl (PCO) indicate damage of lipids and proteins, respectively by ROS. A high level of ROS may also result in protein oxidation, leading to the production of carbonyl groups. Consequently, determination of carbonyl content in proteins can be used as a biomarker of oxidative protein damage and several methods have been developed for determination and quantification of protein carbonyl groups (Levine *et al*., 1990; Reznick and Packer, 1994).

Seminal plasma has a very effective antioxidant system that can provide the spermatozoa with a protective environment against oxidative stress. This protection compensates for the loss of cytoplasmic sperm enzymes that occur during the maturation and transportation processes, which in turn diminishes the spermatozoa's endogenous enzymatic and repair defenses. Indeed, the Total Antioxidant Capacity (TAC) of seminal plasma is due to the sum of enzymatic (e.g., superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic (e.g., ascorbate, urate, vitamin E, pyruvate, glutathione, taurine and hypotaurine) antioxidants. Low level of seminal Total Antioxidant Capacity (TAC) has a key role in male infertility. It is important to ensure that any measurement of seminal TAC is accurate and reliable and yet easy to use as a diagnostic tool in the evaluation and follow-up of male infertility (Aitken, 1994; Donnelly *et al*., 1999; Fingerova *et al*., 2007; Kolettis *et al*., 1999; Aitken, 1999; Kampa *et al*., 2002).

In this study, we determined seasonal changes in the activity of the antioxidant activity comprising lipid peroxidation, nitric oxide, protein carbonyl and total antioxidant capacity in ram seminal plasma.

MATERIALS AND METHODS

Sperm collection: Semen was collected from six sexually mature Pirlak (Daglic x Kivircik) rams using an artificial vagina at monthly intervals in each animal before (June to August), during (September to November) and after (December to February) the breeding period. The rams, which ranged from 20-24 months of age, were kept at the Research Center, Afyon Kocatepe University located in the Afyonkarahisar Province (L: 1021 m, 38°45'N, 30°32'W) of Turkey. All animals were kept outdoors with shelter during the daytime and housed in a semi-open barn at night. Animals were fed roughage and concentrate supplement and each animal also received 500 g day^{-1} of concentrate mixture and 1.0 kg day^{-1} of dry alfalfa. Chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo. USA).

Collection of seminal plasma: Seminal plasma was separated from ejaculates by centrifugation at 5000 rpm for 10 min. The supernatants were transferred into Eppendorf tubes, recentrifuged 5000 rpm 10 min to eliminate the remaining cells and the supernatant was stored at -20°C before being assayed.

Measurement of lipid peroxidation: The circulating MDA concentration, an index of lipid peroxidation, was measured by the double heating method of Draper and Hadley (1990). The method is based on the spectrophotometric measurement of the purple colour generated by the reaction of Thiobarbituric Acid (TBA) with MDA. Briefly, 2.5 mL of a trichloroacetic acid solution (10% w v^{-1}) was added to the seminal plasma (0.5 mL) and the mixture was placed in a boiling water bath for 15 min. After cooling to room temperature and centrifugation (1000 g for 10 min at 4°C), the supernatant fraction (2 mL) was transferred to a test tube containing 1 mL of the TBA solution (0.67%, w v^{-1}). Each tube was again placed in a boiling water bath for 15 min, cooled to room temperature and finally the absorbance was measured at 532 nm using a Shimadzu UV 1601 spectrophotometer. The MDA concentration was calculated based on the absorbance coefficient of the TBA-MDA complex ($\varepsilon = 1.56.10^5$/cm/M).

Measurement of NOx in seminal plasma: Nitric oxide decomposes rapidly in aerated solutions to form stable Nitrite/nitrate products (NOx). Seminal plasma nitrite/nitrate concentrations were measured by using a modified Griess assay, described by Miranda *et al*. (2001). The principle of this assay is reduction of nitrate by vanadium combined with detection by the acidic Griess reaction. Enzyme Linked Immunosorbent Assay (ELISA) was used to measure the photometric absorbance of the mixed solution at 540 nm. This would indirectly determine NO concentration in seminal plasma.

Measurement of Protein Carbonyl content (PCO): Protein carbonyls were measured by using the method of Levine *et al*. (1990, 1994). Briefly, 15 μL of seminal plasma was placed in each of the two glass tubes. Then 0.5 mL of 10 mM DNPH in 2.5 M HCl was added to one of the tubes,

while, 0.5 mL HCl (2.5 mM) was added to the second tube. Tubes were incubated for 1 h at room temperature. Samples were vortexed every 15 min. Then 0.5 mL TCA (20%, w v^{-1}) was added and the tubes were left on ice for 5 min followed by centrifugation for 5 min to collect the protein precipitates. The pellet was then washed three times with 2 mL ethanol-ethyl acetate (1:1, v v^{-1}). The final precipitate was dissolved in 1 mL of guanidine hydrochloride solution (6 M) and was incubated for 15 min at 37°C while mixing. The absorbance of the sample was measured at 365 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\varepsilon = 2.2\times10^{4}$ cm M^{-1}) and expressed as nanomoles per milligram of protein.

Total Antioxidant Capacity (TAC): The total Antioxidant Activity (AOA) was determined using the method described by Koracevic *et al.* (2001). The assay measures the capacity of the seminal plasma to inhibit the production of TBA Reactive Substances (TBARS) from sodium benzoate, under the influence of the reactive oxygen free radicals derived from the Fenton's reaction. The reaction was measured spectrophotometrically at 532 nm. Antioxidants from the added sample cause suppression of the production of TBARS and the inhibition of the colour development is defined as AOA. A solution of 1 mmol L^{-1} uric acid was used as standard.

Statistical analyses: According to the prevailing climatic conditions in Turkey, the months were gathered in 3 groups: Summer (June, July, August), Autumn (September, October, November) and Winter (December, January, February). Results are shown as mean (±SE) of the number of different samples indicated in each case. Means were compared by analyses of variance tests to determine whether there were any significant differences between samples using SPSS for Windows computing program (version 11.0) and $p<0.05$ was considered to be statistically significant (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

The seasonal values in MDA, NOx, PCO and TAC during the period of 9 months are presented in Table 1. Medium values of MDA, NOx, PCO and TAC levels in monthly are shown in Fig. 1. These parameters varied significantly ($p<0.05$) between the seasons. The mean level of seminal plasma MDA was higher ($p<0.05$) in summer and autumn than in winter. NOx level was higher ($p<0.05$) in autumn than in the summer season. PCO level was higher ($p<0.05$) in autumn than in the winter season. Conversely, no significant mean level of seminal plasma TAC was found between the seasons.

Table 1: Overall mean values of MDA, NOx, PCO and TAC ram breeds during the seasons studied (X±SE)

Activities	n	Summer	Autumn	Winter
MDA (nmol mL^{-1})	18	5.11 ± 1.14^{a}	4.96 ± 1.00^{a}	3.95 ± 0.9^{b}
NOx (μmol L^{-1})	18	4.79 ± 1.39^{b}	6.62 ± 2.07^{a}	$5.79\pm1.79^{a,b}$
PCO (nmol mg^{-1} protein)	18	$2.16\pm0.45^{a,b}$	2.45 ± 0.52^{a}	1.91 ± 0.37^{b}
TAC (mmol L^{-1})	18	1.17 ± 0.22	1.28 ± 0.23	1.11 ± 0.36

Different letters in rows indicate significant differences (a, b: $p<0.05$)

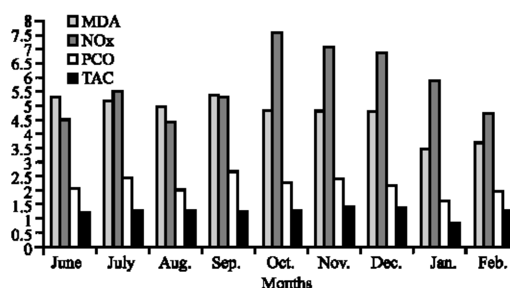

Fig. 1: Mean values of MDA, NOx, PCO and TAC in during the period of 9 months

It is well known that normal sperm counts and other variables measured in routine semen analysis do not ensure good fertility rate. Other factors not determined in routine semen analysis, such as ROS, have been related to low fertility. A fine balance of the ROS level is apparently crucial, since an inappropriate production rate leads to infertility (Iwasaki and Gagnon, 1992; Huszar and Vigue, 1994; Sharma and Agarwal, 1996; Griveau and Le Lannou, 1997; Pasqualotto *et al.*, 2001). A significant correlation between the lipid peroxide content and a severe motility loss has been proved in human spermatozoa by several researchers (Aitken, 1999; Sharma and Agarwal, 1996; Alvarez *et al.*, 1987). However, moderate lipid peroxidation is necessary to maximize the fertilizing potential of sperm by priming hyperactivation, motility and capacitation and enhancing their binding to the zona pellucid (Griveau *et al.*, 1994; Kodama *et al.*, 1996).

The cryopreservation process is detrimental to sperm cells. One of the reasons is the increased generation of Reactive Oxygen Species (ROS) and/or decreased antioxidant levels during and after cryopreservation. Lipid oxidation in the seminal plasma seemed to be an indicator for several levels of sperm damage (Morte *et al.*, 2008). Oxidative stress can be assessed through the direct quantification of ROS, antioxidants and by the measurement of oxidative stress end products. One product of lipid speroxidation is Malondialdehyde (MDA), which can be determined by measuring the amount of thiobarbituric acid reactive species (Sanocka and Kurpisz, 2004). Moderate lipid peroxidation is necessary to maximize the fertilizing potential of sperm by priming hyperactivation, motility and capacitation and

to enhance their binding to the zona pellucida. Literature regarding oxidative stress characteristics in ram semen is very limited; the objective of most experiments was to evaluate the effects of prooxidants or antioxidants on the concentrations of MDA, or on semen quality (Marti *et al*., 2007). Levels of MDA in seminal plasma were higher in summer and autumn than winter suggesting that there was more lipid peroxidation in ram semen during the summer and autumn months. This may be associated with the increased testicle activity in summer and autumn seasons.

Nox is a molecule of great biological significance and has long been considered to play an important role in sperm physiology such as sperm chemotaxis, sperm motility, sperm-egg interaction and spermatogenesis (Aitken and Fisher, 1994). Spermatozoa could present one of the targets of NOx and a role for NOx in controlling the function of male gametes during their maturation and migration in the genital tracts is conceivable. In fact, human and rodent sperm have been reported to be influenced by exogenous NO, as far as motility, viability and metabolism are concerned (Herrero *et al*., 1994). NOx could even participate in the mechanism leading to fertilization, as in bovine sperm it increases capacitation and acrosomal reactivity (Zamir *et al*., 1995).

Nitric oxide effects within the seminal plasma change depending on the concentration. At physiologic concentration, it functions as a mediator. It was shown that releasing component (sodyum nitropurisid) of NOx at low concentration may help for the prevention of sperm motility and longevity. Furthermore, NOx plays a crucial role in sperm hyperactivation as a stimulator in mice. Endogenous NOx appears to have an important effect in the maintenance of sperm motility, if carefully regulated (Herrero *et al*., 1994; Hellstrom *et al*., 1994). In contrast to the aforementioned studies, we found higher level of NOx levels in autumn than in summer. This finding suggests that this higher level of NOx could be necessary for the sperm quality.

Some studies have shown that infertile men have an impaired seminal plasma nonenzymatic antioxidant capacity (also called Total Antioxidant Capacity, TAC), suggesting that a decreased TAC may play a pathogenetic role in male infertility (Lewis *et al*., 1995). Total seminal plasma antioxidants are the most protective defensive mechanism available to spermatozoa against ROS. Low seminal total antioxidant capacity has been shown to be related to male infertility (Aitken, 1999; Agarwal *et al*., 2006b). Seminal plasma antioxidant capacity may be influenced by a wide range of the factors such as nutrition, vitamin supply, age, infection and so forth). Therefore, it is important to estimate accurately the total antioxidant amount of the seminal plasma (Kampa *et al*., 2002). Gil-Guzman *et al*. (2001) also observed that there is an indirect significant correlation between seminal plasma TAC and ROS levels (Gil-Guzman *et al*., 2001). They suggested that this indirect correlation between TAC and ROS might be associated with an increase in the consumption of soluble, nonenzymatic antioxidants in seminal plasma, which results from over production of ROS.

The present study was performed to establish the assay for the determination of total antioxidative capacity in seminal plasma of rams in breeding and non breeding seasons. Moreover, there were not any significant differences regarding the variabilities in TAC according to seasons. However, possible seasonal relationships amongst the levels of TAC, sperm quality, enzymatic antioxidants SOD and GPx in seminal plasma should further be studied.

A high level of ROS may also result in protein oxidation, leading to the production of carbonyl groups. Consequently, determination of carbonyl content in proteins can be used as a biomarker of oxidative protein damage and several methods have been developed for determination and quantification of protein carbonyl groups (Reznick and Packer, 1994; Levine *et al*., 1990).

We observed that PCO levels in the seminal plasma differed significantly between autumn and winter. Interestingly, a higher level of protein oxidation was found in the seminal plasma of rams in the breeding season. These results suggest that the oxidation of semen proteins may be important for sperm function. No previous observations on protein oxidation in ram semen were uncovered in the literature and to our knowledge this is the first study in the field.

CONCLUSION

Lipid and protein oxidation and nitric oxide takes place with different extents in seminal plasma from animals with varying fertility and further work will attempt to address if this is due to different susceptibility to oxidation, production of ROS or the availability of antioxidant defenses. The present study showed that variability in before, during and after the breeding season for some oxidative stres parameters of seminal plasma in rams. Further studies are needed to determine of sperm quality tests, sperm DNA damage and antioxidant enzymes with oxidative stres parameters in seminal plasma.

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