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Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm

A.D. Omur[1], K. Coyan[2]

[1]Faculty of Veterinary Medicine, Ataturk University, Erzurum, Turkey
2Faculty of Medicine, Pamukkale University, Denizli, Turkey

ABSTRACT: The aim of this study was to determine the effects of curcumin, ellagic acid and methionine on sperm parameters following the freeze-thawing of Merino ram semen. Ejaculates were collected via an artificial vagina from four Merino rams, evaluated microscopically and pooled at 37 °C. The pooled semen samples were diluted in a Tris-based extender and separated into groups containing curcumin (1, 2, 4mM), ellagic acid (1, 2, 4mM), methionine (1, 2, 4mM) and no antioxidant (control). The diluted semen was cooled to 5 °C slowly and equilibrated for 3 h. After the equilibration, the samples were frozen in liquid nitrogen vapour, and plunged into liquid nitrogen (–196 °C) for storage. Frozen straws were thawed at 37 °C for 30 s in a water bath for microscopic sperm evaluation, individually. All antioxidants led to a higher percentage of sperm motility in comparison to the control group. The freezing extender supplemented with methionine (1mM), curcumin (1 and 2mM) and ellagic acid (1 and 2mM) led to higher percentage of sperm plasma membrane integrity when compared to other groups ($P < 0.05$). Antioxidant supplementation also resulted in a higher percentage of sperm acrosome integrity in comparison to the control. Methionine, curcumin and ellagic acid (1mM: 27.7 ± 2.4, 28.0 ± 2.1 and 26.8 ± 2.0) groups provided higher protection in terms of sperm mitochondrial activity when compared to other groups ($P < 0.05$). The findings of this study show that varying concentrations of curcumin, methionine and ellagic acid have markedly different effects on the spermatological variables under study.

Keywords: curcumin; ellagic acid; methionine; ram semen; semen cryopreservation; spermatological parameters

Cervical artificial insemination with frozen semen is restricted by low fertility rates as a result of structural and functional damage to sperm during the freeze-thaw process (Salamon and Maxwell 2000). The production of reactive oxygen species (ROS) resulting from the process of cryopreservation and lowering in antioxidant enzyme activities in semen after freezing may contribute to the biochemical and functional damage of cryopreserved sperm (Maia et al. 2010). Reactive oxygen species (ROS) are produced by spermatozoa in the male reproductive tract (Alvarez et al. 1987). Under physiological circumstances, spermatozoa produce small amounts of ROS (Gomez et al. 1998). In minimal amounts, ROS are needed for regulation of sperm function, sperm capacitation and acrosome reaction. Nevertheless, ROS are toxic to normal cells at high concentrations (Moein et al. 2007). The addition of various antioxidants to ram semen diluents improves the motility, acrosomal integrity, viability and fertilisation ability of the sperm (Ollero et al. 1996).

Curcumin is a natural antioxidant, and has efficiency as an anti-apoptotic, cryoprotective, anti-oxidative, anti-inflammatory, anti-toxic and anti-cancer agent (Surh et al. 2001; Mathuria and Verma 2008; Glombik et al. 2014; Rashid and Sil 2015). Further, many studies in the literature have

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concluded that curcumin has potential in the prevention and treatment of a variety of diseases.

Methionine can act as a precursor amino acid for glutathione. It protects cells from oxidative damage and has a vital role in detoxification (Reed 1990). Methionine also plays an important role in the antioxidant defence system by readily reacting with oxidants to form methionine sulfoxide (Livine et al. 1999).

Ellagic acid has potent antioxidant activity, radical scavenging capacity, as well as chemo-preventive and anti-apoptotic characteristics (Turk et al. 2008; Ceribasi et al. 2010). It contains four hydroxyl groups and two lactone groups, and protects cells from oxidative damage (Pari and Sivasankari 2008).

Curcumin and methionine are used as antioxidants in semen extenders (Bucak et al. 2010; Coyan et al. 2010; Bucak et al. 2012).

This research was conducted to determine the effect of supplementation of the freezing extender with the antioxidants curcumin, methionine and ellagic acid on sperm motility and fluorescent staining parameters in post-thawed Merino ram semen.

MATERIAL AND METHODS

Chemicals. The antioxidants (curcumin, ellagic acid and methionine) and other chemicals used in this study were all obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals and semen collection. Semen samples from four mature Merino rams (four years of age) were used in this study. The rams**,** belonging to the Selcuk University Research and Experimental Farm, were maintained under uniform optimal nutritional conditions. Ejaculates were collected twice a week from the rams, via an artificial vagina, during the natural breeding season (for four weeks). Eight experimental replicates were run. After collection, the ejaculates were incubated in a water bath at 33 °C, until microscopic sperm quality assessments were performed in the laboratory. All semen analyses were performed within approximately 20 min of semen collection.

Semen freezing and thawing. The volume of the ejaculates was gauged in a conical tube, graduated at 0.1 ml intervals, and the ejaculate concentration was determined by way of a haemocytometer (Smith and Mayer 1955). Sperm motility was estimated with phase-contrast microscopy (magnification × 100) at 37 °C. Ejaculates between 1 and 2 ml in volume, containing sperm with > 80% progressive motility and an ejaculate concentration of higher than 2.5×10^9 sperm/ml were pooled. Sperm balance for each male was performed and individual ram differences were eliminated by semen pooling. In total, six pooled ejaculates were included in the study**.** A Tris-based extender (Tris 297.58mM, citric acid 96.32mM, fructose 82.66mM, egg yolk 15% (v/v), glycerol 6% (v/v), penicillin 500 IU/ml, streptomycin 500 IU/ml, pH 6.8) was used as the base extender (cryopreservation diluent). The osmolarity of the extender was 300 mOsmol. Each pooled ejaculate was split into 10 equal aliquots and diluted at 37 °C with base extender containing curcumin (1, 2, 4mM), methionine (1, 2, 4mM), ellagic acid (1, 2, 4mM) or no antioxidant (control), respectively. Each aliquot was diluted to a final semen concentration of approximately 4×10^8 sperm/ml (single step dilution), in 15-ml polypropylene centrifuge tubes. The diluted semen samples were aspirated into 0.25 ml French straws, sealed with polyvinyl alcohol powder and equilibrated at 5 °C for 3 h. After equilibration, the straws were frozen in liquid nitrogen vapour (4 cm above the liquid nitrogen) for 15 min and then plunged into liquid nitrogen for storage. After storage for one month, the frozen straws were thawed individually (37 °C for 25 s) in a water bath for microscopic semen evaluation.

Semen evaluation. Subjective motility was assessed using a phase-contrast microscope (magnification × 100), with a warm stage maintained at 37 °C. A wet mount was made using a 5 µl drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score.

Assessment of the integrity of sperm plasma membrane was performed by staining with the Sperm Viability Kit (SYBR-14/PI Molecular Probe: L 7011 Invitrogen). This staining protocol was modified from Garner and Johnson (1995). A working solution of SYBR-14 was diluted 1 : 10 with DMSO (Appli-chem A3006), then divided into equal aliquots (30 µl) after filtering through a 0.22 µm Millipore Millex GV filter, and stored at –20 °C. Propidium iodide (PI) was dissolved in distilled water at 2 mg/ml, divided into equal

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aliquots (30 µl) after filtering through a 0.22 µm Millipore Millex GV filter, and stored at –20 °C. The thawed straw was diluted 1 : 3 with Tris stock solution without glycerol and egg yolk, and then 30 µl of diluted semen was mixed with 6 µl of SYBR-14 and 2.5 µl of PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min, and then 10 µl of Hancock solution was added to stop sperm movement. A wet mount was made using a 2.5 µl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 spermatozoa were examined at 400 × magnification under a fluorescence microscope (Leica DM 3000 Microsystems GmbH, Ernst-Leitz-Strase, Wetzlar, Germany; excitation at 450–490 nm, emission at 520 nm) to assess the sperm membrane integrity. Sperm displaying green–red or red colourisation were considered as having damaged membranes, while green colourisation was considered as indicating an intact membrane.

Sperm acrosome status was assessed using fluorescein isothiocyanate conjugated to Arachis hypogaea (peanut) (L7381 FITC-PNA, Invitrogen) and PI staining as described by Nagy et al. (2003). About 120 µg of FITC-PNA was added to 1 ml of PBS (15630056, Invitrogen) to make staining solution, and then divided into equal aliquots (100 µl) after filtering and stored at –20 °C. The thawed straw was diluted 1 : 3 with Tris stock solution without glycerol and egg yolk, and then 60 µl of diluted semen were mixed with 10 µl of FITC-PNA and 2.5 µl of PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min, and then 10 µl of Hancock solution were added to stop sperm movement. A wet mount was made using a 2.5 µl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 sperm cells were examined at 400 × magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm) to assess the sperm acrosome integrity. Spermatozoa displaying bright green or patchy green fluorescence were considered as acrosome non-intact or damaged, whereas cells that did not stain green in the acrosome cap were regarded as acrosome-intact.

Sperm mitochondrial activity was assessed using a staining protocol with JC-1/PI modified from Garner et al. (1997). A stock solution of 5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl-benzimidazolyl-carbocyanine iodide (T3168 JC-1, Invitrogen, 1.53mM) was prepared in DMSO solution and then divided into equal aliquots (100 µl) after filtering, and stored at –20 °C. The thawed straw was diluted 1 : 3 with Tris stock solution without glycerol and egg yolk, and then 300 µl of diluted semen were mixed with 2.5 µl of JC-1 and 2.5 µl of PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min, and then 10 µl of Hancock solution was added to stop sperm movement. A wet mount was made using a 2.5 µl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 sperm cells were examined at 400 × magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm) to assess the activity. A high level of yellow/orange fluorescence associated with the sperm mid-piece (where mitochondria are located) indicated high mitochondrial activity. Mitochondria showing low mitochondrial activity were stained green.

Statistical analysis. The study was repeated eight times. The results were expressed as mean ± SEM. Means were analysed using a one-way analysis of variance, followed by Duncan's post hoc test to determine significant differences in all parameters among all groups using the SPSS/PC computer program (version 12.0; SPSS, Chicago, IL). Differences with values of $P < 0.05$ were considered as statistically significant.

RESULTS

Merino ram sperm parameters following the freeze-thaw are set out in Table 1. All groups supplemented with antioxidants exhibited a higher percentage of sperm motility in comparison to the control group. The freezing extender supplemented with methionine (1mM), curcumin (1 and 2mM) and ellagic acid (1 and 2mM) resulted in a higher percentage of sperm plasma membrane integrity when compared to the other groups ($P < 0.05$). Groups supplemented with antioxidants exhibited a higher percentage of sperm acrosome integrity in comparison to the control. Methionine, curcumin and ellagic acid (1mM: 27.7 ± 2.4, 28.0 ± 2.1 and 26.8 ± 2.0) groups provided higher protection in terms of sperm mitochondrial activity when compared to other groups ($P < 0.05$). Thus, the findings of this study show that different antioxidants (curcumin, methionine and ellagic acid) have protective effects on ram sperm variables.

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Table 1. Mean (±SE) of evaluated parameters in the pooled semen of four Merino rams supplemented with different antioxidants following the freeze-thawing process

	Control	Methionine (mM)			Curcumin (mM)			Ellagic acid (mM)		
		1	2	4	1	2	4	1	2	4
Motility (%)	45.0 ± 2.1^{b}	58.7 ± 3.0^{a}	51.2 ± 3.2^{a}	53.1 ± 4.3^{a}	53.7 ± 2.6^{a}	55.0 ± 1.3^{a}	50.6 ± 1.7^{a}	54.3 ± 1.9^{a}	56.2 ± 2.9^{a}	54.3 ± 2.9^{a}
Membrane integrity (%)	52.0 ± 1.8^{b}	64.2 ± 2.3^{a}	57.1 ± 1.6^{ab}	58.5 ± 3.4^{ab}	60.1 ± 20.14^{a}	61.6 ± 1.1^{a}	58.5 ± 0.9^{ab}	63.0 ± 1.7^{a}	61.6 ± 3.7^{a}	57.7 ± 2.2^{ab}
Acrosome integrity (%)	45.6 ± 1.9^{b}	58.8 ± 1.9^{a}	53.0 ± 1.3^{a}	56.5 ± 3.3^{a}	54.8 ± 2.0^{a}	56.6 ± 1.1^{a}	55.8 ± 1.1^{a}	57.8 ± 1.4^{a}	57.3 ± 2.0^{a}	58.7 ± 1.5^{a}
Mitochondrial activity (%)	23.5 ± 2.2^{abc}	27.7 ± 2.4^{a}	23.5 ± 1.6^{abc}	24.6 ± 2.8^{abc}	28.0 ± 2.1^{a}	20.2 ± 2.0^{bcd}	20.2 ± 1.6^{bcd}	26.8 ± 2.0^{ab}	19.7 ± 2.4^{bc}	16.2 ± 1.7^{c}

$^{a, b, c, d}$different superscripts within the same row demonstrate significant differences $P < 0.05$

DISCUSSION

Leukocytes, varicocele, heat, xenobiotics, toxic metals, and reactive oxygen species – hydroxyl radicals (OH), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) are all known to elicit damage to sperm (Mieusset et al. 1987; Aitken and West 1990; Whittington and Ford 1999; Practice Committee of the American Society for Reproductive Medicine Report on varicocele and infertility 2004; Wright et al. 2014). The major ROS produced include O_2^- and H_2O_2, and antioxidants such as GPX, CAT and SOD, that neutralise these species, are present both within mitochondria and in the secretions of the reproductive tract (Vernet et al. 2004; Starkov 2008).

Peroxidation of polyunsaturated fatty acids has been implicated in a wide variety of pathological conditions including infertility (Sanocka and Kurpisz 2004). Lipid peroxidation in biological membranes impairs the functioning of membrane, and results in decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions (Esterbauer et al. 1990).

Mammalian spermatozoa contain a high concentration of polyunsaturated fatty acids linked to phospholipids, asymmetrically distributed over the lipid bilayer of the plasma membrane (Gadella et al. 1999). The Ram semen plasma membrane is rich in unsaturated fatty acids and is thus very sensitive to lipid peroxidation due to reactive oxygen species. It is well known that ram spermatozoa are more sensitive to cold shock than those of other species such as bulls, rabbits and humans (Watanabe and Fukui 2006). During the freezing process sperm freezing results in cold shock, membrane damage and oxidative stress related to phase changing in the membrane structure (Aitken 1994). The loss of sperm function is due to the peroxidation of unsaturated fatty acids in the sperm plasma membrane, because of which the latter loses its fluidity and the cells lose their function (Aitken and Sawyer 2003). For this reason, supplementation of certain cryoprotective and antioxidative additives into semen extender can minimize the effects of cold shock on spermatozoa.

Some studies have described damage caused to sperm by oxidants. Cisplatin induced incremental abnormalities in rat sperm which could be blocked by ellagic acid (Turk et al. 2008). Similarly, cyclophosphamide induced lipid peroxidation leading to structural damage to spermatozoa and the testicu-

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lar tissue of rats. Ellagic acid (2 mg/kg) also exerted a protective effect in this scenario (Ceribasi et al. 2010). Further, some studies report the beneficial effects of curcumin (2.5mM) on post-thawed goat semen (Bucak et al. 2010). Methionine has also given positive results in the liquid storage of ram (1mM) and buck (2.5 and 5mM) semen, in terms of semen quality (Coyan et al. 2010; Tuncer et al. 2010). Curcumin, especially, readily penetrates into the cytoplasm and is able to accumulate in membranous structures such as plasma membrane (Jaruga et al. 1998). Because of this characteristic, curcumin can protect the plasma membrane against lipid peroxidation.

In contrast to these results, incubation of sperm with curcumin caused a concentration-dependent (especially at ≥ 250mM concentrations) decrease in sperm forward motility, capacitation/acrosome reaction, and murine fertilisation in vitro (Naz 2011). It is likely that these toxic effects were due to overdosing of curcumin.

The effect of these antioxidants constitutes indirect evidence that ram sperm is subject to oxidative stress. This theory was confirmed in studies that detected the occurrence of lipid peroxidation in fresh and frozen ram semen as well as changes in the concentrations of antioxidant enzymes present in seminal plasma, according to the quality of the semen or after a cycle of freeze-thawing (Peris et al. 2007). In the current study, physical damage was observed due to cell volume changes associated with freezing and thawing in the control group, while detrimental effects, especially at the 2 and 4mM concentrations of all antioxidant groups, were observed with respect to mitochondrial activity. We determined that membrane integrity was preserved by 1mM methionine (64.2 ± 2.3), 1 and 2mM curcumin (60.1 ± 2.4 and 61.6 ± 1.1), and 1 and 2mM ellagic acid (63.0 ± 1.7 and 61.6 ± 3.7) when compared to the control and other antioxidant groups. All antioxidant groups exhibited positive effects in terms of acrosomal status and motility in comparison to the control. Curcumin at 0.5mM exerted protective effects with respect to functional integrity of the membrane (54.40 ± 2.09%), in comparison to the control (37.20 ± 1.77%, $P < 0.001$) in experiments of freeze-thawed bovine semen (Bucak et al. 2012). These observations are consistent with the present findings.

In conclusion, varying concentrations of curcumin, methionine and ellagic acid exhibited markedly different efficiencies in safeguarding spermatological parameters in the current study.

REFERENCES

Aitken RJ (1994): Pathophysiology of human spermatozoa. Current Opinion in Obstetrics and Gynecology 6, 128–135.

Aitken RJ, Sawyer D (2003): The human spermatozoon – not waving but drowning. Advances in Experimental Medicine and Biology 518, 85–98.

Aitken RJ, West KM (1990): Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated on Percoll gradients. International Journal of Andrology 13, 433–451.

Alvarez JG, Touchstone JC, Blasco L, Storey BT (1987): Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. Journal of Andrology 8, 338–348.

Bucak MN, Sariozkan S, Tuncer PB, Sakin F, Atessahin A, Kulaksiz R, Cevik M (2010): The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Ruminant Research 89, 24–30.

Bucak MN, Baspinar N, Tuncer PB, Coyan K, Sariozkan S, Akalin PP, Buyukleblebici S, Kucukgunay S (2012): Effects of curcumin and dithioerythritol on frozen-thawed bovine semen. Andrologia 44, 102–109.

Ceribasi AO, Turk G, Sonmez M, Sakin F, Atessahin A (2010): Toxic effect of cyclophosphamide on sperm morphology, testicular histology and blood oxidant–antioxidant balance, and protective roles of lycopene and ellagic acid. Basic Clinical Pharmacology and Toxicology 107, 730–736.

Coyan K, Baspinar N, Bucak MN, Akalin PP, Ataman MB, Omur AD, Gungor S, Kucukgunay S, Ozkalp B, Sariozkan S (2010): Influence of methionine and dithioerythritol on sperm motility, lipid peroxidation and antioxidant capacities during liquid storage of ram semen. Research in Veterinary Science 89, 426–431.

Esterbauer H, Rotheneder-Dieber M, Waeg G, Striegl G, Jurgens G (1990): Biochemical, structrural and functional properties of oxidized low-density lipoprotein. Chemical Research in Toxicology, 3, 77–92.

Gadella BM, Miller NG, Colenbrander B, Van Golde LM, Harrison RA (1999): Flow cytometric detection of transbilayer movement of fluorescent phospholipid analogues across the boar sperm plasma membrane: elimination of

doi: 10.17221/8677-VETMED

labeling artifacts. Molecular Reproduction and Development 53, 108–125.

Garner DL, Johnson LA (1995): Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of Reproduction 53, 276–284.

Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE (1997): Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biology of Reproduction 57, 1401–1406.

Głombik K, Basta-Kaim A, Sikora-Polaczek M, Kubera M, Starowicz G, Styrna J (2014):Curcumin influences semen quality parameters and reverses the di (2ethylhexyl) phthalate (DEHP) induced testicular damage in mice. Pharmacological Reports 66, 782–787.

Gomez E, Irvine DS, Aitken RJ (1998): Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. International Journal of Andrology 21, 81–94.

Jaruga E, Salvioli S, Dobrucki J, Chrul S, Bandorowicz-Pikula J, Sikora E, Franceschi C, Cossarizza A, Bartosz G (1998): Apoptosis-like, reversible changes in plasma membrane asymmetry and permeability, and transient modifications in mitochondrial membrane potential induced by curcumin in rat thymocytes. FEBS Letters 433, 287–293.

Livine RL, Berlett BS, Moskovitz J, Mosoni L, Stadtman ER (1999): Methionine residues may protect proteins from critical oxidative damage. Mechanisms of Ageing and Development 107, 323–332.

Maia MS, Bicudo SD, Sicherle CC, Rodello L, Gallego ICS (2010): Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. Animal Reproduction Science 122, 118–123.

Mathuria N, Verma RJ (2008): Curcumin ameliorates aflatoxin-induced toxicity in mice spermatozoa. Fertility and Sterility 90, 775–780.

Mieusset R, Bujan L, Mondinat C, Mansat A, Pontonnier F, Grandjean H (1987): Association of scrotal hyperthermia with impaired spermatogenesis in infertile men. Fertility and Sterility 48, 1006–1011.

Moein MR, Dehghani VO, Tabibnejad N, Vahidi S (2007): Reactive Oxygen Species (ROS) level in seminal plasma of infertile men and healthy donors. Iranian Journal of Reproductive Medicine 5, 51–55.

Nagy S, Jansen J, Topper EK, Gadella BM (2003): A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biology of Reproduction 68, 1828–1835.

Naz RK (2011): Can curcumin provide an ideal contraceptive? Molecular Reproduction and Development 78, 116–123.

Ollero M, Blanco TM, Lopez-Perez MJ, Perez JAC (1996): Surface changes associated with ram sperm cryopreservation reveled by counter-current distribution in an aqueous two-phase system: effect of differents cryoprotectant. Journal of Chromatography B 680, 157–164.

Pari L, Sivasankari R (2008): Effect of ellagic acid on cyclosporine A-induced oxidative damage in the liver of rats. Fundamental and Clinical Pharmacology 22, 395–401.

Peris SI, Bilodeau JF, Dufour M, Bailey JL (2007): Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation and functional parameters in ram sperm. Molecular Reproduction and Development 74, 878–892.

Practice Committee of the American Society for Reproductive Medicine (2004): Report on varicocele and infertility. Fertility and Sterility 82, 142–145.

Rashid K, Sil PC (2015): Curcumin ameliorates testicular damage in diabetic rats by suppressing cellular stress-mediated mitochondria and endoplasmic reticulum dependent apoptotic death. Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease 1852, 70–82.

Reed DJ (1990): Glutathione: toxicological implications. Annual Review of Pharmacology and Toxicology 30, 603–631.

Salamon S, Maxwell WMC (2000): Storage of ram semen. Animal Reproduction Science 62, 77–111.

Sanocka D, Kurpisz M (2004): Reactive oxygen species and sperm cells. Reproductive Biology and Endocrinology 2, 12.

Smith JT, Mayer DT (1955): Evaluation of sperm concentration by the hemocytometer method. Fertility and Sterility 6, 271–275.

Starkov AA (2008): The role of mitochondria in reactive oxygen species metabolism and signaling. Annals of the New York Academy of Sciences 1147, 37–52.

Surh YJ, Chun KS, Cha HH, Keum YS, Park KK, Lee SS (2001): Molecular mechanisms underlying chemopreventive activities of antiflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutation Research 480, 243–268.

Tuncer PB, Bucak MN, Sariozkan S, Sakin F, Yeni D, Cigerci İ.H, Atessahin A, Avdatek F, Gundogan M, Buyukleblebici O (2010): The effect of raffinose and methionine on frozen/thawed Angora buck (Capra hircus ancryrensis) semen quality, lipid peroxidation and antioxidant enzyme activities. Cryobiology 61, 89–93.

Turk G, Atessahin A, Sonmez M, Ceribasi AO, Yuce A (2008): Improvement of cisplatin-induced injuries to

doi: 10.17221/8677-VETMED

sperm quality, the oxidant-antioxidant system, and the histologic structure of the rat testis by ellagic acid. Fertility abd Sterility 89, 1474–1481.

Vernet P, Aitken RJ, Drevet JR (2004): Antioxidant strategies in the epididymis. Molecular and Cellular Endocrinology 216, 31–39.

Watanabe H, Fukui Y (2006): Effects of dithiothreitol and boar on pronuclear formation and embryonic development following intracytoplasmic sperm injection in pigs. Theriogenology 65, 528–539.

Whittington K, Ford WC (1999): Relative contribution of leukocytes and of spermatozoa to reactive oxygen species production in human sperm suspensions. International Journal of Andrology 22, 229–235.

Wright C, Milne S, Leeson H (2014): Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility.Reproductive BioMedicine Online 28, 684–703.

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Corresponding Author:

Ali Dogan Omur, Ataturk University, Faculty of Veterinary Medicine, Erzurum, Turkey
E-mail: alidoganomur@gmail.com