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Cryopreservation of bull sperm: Effects of extender supplemented with different cryoprotectants and antioxidants on sperm motility, antioxidant capacity and fertility results

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ABSTRACT

The objectives of this study were to compare glycerol (G) and ethylene glycol (EG) at different concentrations and trehalose (T) or cysteine (C; with/without) in Tris extender for cryopreservation of bull semen. Twenty-four ejaculates obtained from three bulls were included in the study. Each ejaculate was divided into four equal aliquots and diluted using both of the Tris extenders with G (5% or 7%) or EG (3% or 5%). After that, each extenders were divided into three equal aliquots and diluted using both of the 5 mM C or 25 mM T, and control (without additives) was cooled to 4 °C and frozen in 0.25 ml French straws. The addition of 3% and 5% EG without antioxidants resulted in the least Computer-Assisted Sperm motility Analysis (CASA) motility as compared with the other groups. Treatment with 25 mM T in 3% EG beneficially effected acrosome morphology as compared with the other groups. Also, treatment with 3% EG with 25 mM T and 5% EG resulted in a greater rate of total abnormalities. Treatment with 3% G yielded a slightly greater percentage of membrane integrity by Hypo-Osmotic Swelling Test (HOST) assessment than that of the other groups. Treatment with 3% EG with 5 mM C resulted in the greatest concentration of malondialdehyde (MDA). The glutathione peroxidase (GPx) antioxidant activity was increased in the C-treatment groups when compared to the other groups. Treatment with 5% EG and 5 mM C resulted in less chromatin damage and detrimental impacts on tail moment. Treatment with 5% EG led to greater non-return rates of inseminated cows. However, this result was not considered to be statistically important.

1. Introduction

Artificial insemination (AI) is the first generation biotechnological advancement that has made a profound contribution to the genetic improvement, particularly in dairy bulls through which a single ejaculate from males

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is used for insemination of multiple females. This impact could not have been possible without long standing freezing of bull semen. Freezing–thawing processes lead to the generation of reactive oxygen species (ROS) that impair sperm motility, membrane integrity, and fertilizing potential (Hu et al., 2010). The success of cryopreservation depends not only on preserving the motility of the spermatozoa but also on maintaining their metabolic function (Watson, 2000). The major factor affecting the results of insemination with frozen–thawed semen is the addition of cryoprotectants and spermatozoal damage due to the formation of internal ice crystals due to the increase in solute concentration in the extension media or interaction of both physical factors (Aboagla and Trade, 2004; El-Harairy et al., 2011). The cryoprotectants are added to extenders to protect the sperm from damage during freezing process (Singh et al., 1995). The amount and type of cryoprotectants in semen diluent influence these events and their effects on the sperm cells during freezing (El-Harairy et al., 2011). Glycerol (G) is the most widely used cryoprotectant for bull sperm because it reduces the mechanical damage to spermatozoa during the freezing process (De Leeuw et al., 1995). Specifically, when used in greater than typical concentrations, G can cause great osmotic damage to spermatozoa because G passes through the sperm membrane much slower than other cryoprotectants (Garner, 1991; Guthrie et al., 2002). Other researchers have surmised through previous studies that a low molecular weight cryoprotectant, such as EG, may cause less damage to spermatozoa than when G is used because its low molecular weight allows it to cross the plasma membrane more easily (Moore et al., 2006). In recent years, G and EG have been used as cryoprotectant in bulls (Tasdemir et al., 2013) and goats (Tuncer et al., 2013). Cryopreservation reduces the functional and structural integrity of bull spermatozoa, and is associated with reactive oxygen species (ROS) production. Oxidative stress during freezing of mammalian semen can cause functional and structural damage to spermatozoa involving ROS-mediated pathways (Baumber et al., 2005). Although bull semen has a natural defense system against the ROS, it is considered insufficient in protecting spermatozoa under cryopreservation mediated stress (Nichi et al., 2006). Therefore, the addition to semen extenders of suitable antioxidants is suggested to reduce oxidative damage during freeze–thawing of bull spermatozoa (Ansari et al., 2011).

Cysteine (C) is a non-essential amino acid, scavenger of free radicals and precursor molecule of glutathione and enhances intracellular glutathione production both *in vivo* and *in vitro* (Malo et al., 2010; Tuncer et al., 2010) and to prevent hydrogen peroxide-mediated loss of sperm motility in bulls (Bilodeau et al., 2001). Additionally mammalian cells can only utilize C, which has been shown to penetrate the cell membrane easily (Sarıözkan et al., 2009a,b). In recent years, C has been used as an antioxidant in the cryopreservation of boar (Taylor et al., 2009), bull (Tuncer et al., 2010) and dog sperm (Michael et al., 2007). Sugar maintains the osmotic pressure of the diluents by inducing cell dehydration and less ice crystal formation into the spermatozoa (Purdy, 2006).

Sugar is utilized by spermatozoa as an energy source through glycolysis and mitochondrial oxidative phosphorylation to support sperm motility and movement (Naing et al., 2010). Many researchers have studied the effect of sugar supplementation in semen extender on the quality of cryopreserved spermatozoa. Trehalose (T) is a nonreducing disaccharide which is able to protect the integrity of cells against a variety of environmental stresses such as dehydration, heat, cold and oxidation (Chen and Haddad, 2004). Furthermore, T had been extensively used to improve sperm quality variables in semen cryopreservation and its protective effects improved the freezing capacity of goat spermatozoa due to increase in membrane fluidity resulting from the depression of membrane transition temperature, allowing the sperm membrane to tolerate low-temperature effects (Tasdemir et al., 2013; Tuncer et al., 2013). The extender containing T improved antioxidant action and reduced the oxidative stress induced by cryopreservation (Aisen et al., 2005; Hu et al., 2010).

The objectives of the present study were to compare G and EG at different concentrations as cryo-protectants and T or C (with/without) as antioxidants in Tris extender for cryopreservation of bull semen and to compare microscopic sperm variables (motility, acrosome and total abnormalities, HOS test, sperm motion characteristics), concentrations of malondialdehyde (MDA) and glutathione peroxidase (GPx) and DNA integrity of frozen–thawed semen. Conception rates of the cows artificially inseminated with frozen–thawed semen were also assessed.

2. Materials and methods

2.1. Animals, semen collection and semen processing

Three Holstein bulls (3 or 4 years of age) were housed at the Lalahan Livestock Central Research Institute (Ankara, Turkey), and maintained under uniform feeding and housing conditions. A total number of 24 ejaculates were collected from the bulls by using of an artificial vagina twice a week, according to AI standard procedures. The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals and sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). Sperm motility was estimated using phase-contrast microscopy (200×). The ejaculates containing spermatozoa with >80% forward progressive motility and concentrations greater than 1.0×10^9 spermatozoa/ml were used in the study. Immediately after collection, the ejaculates were immersed in a warm water bath at 35 °C until their assessment. A Tris-based (T) extender (189.5 mM of Tris, 63.2 mM of citric acid, 55.5 mM of fructose, 20% (v/v) egg yolk, and 1000 ml of distilled water at a pH of 6.8) was used as the base for the experimental extenders. Each ejaculate was divided into four equal aliquots and diluted using both of the T extenders with G (5% or 7%) or EG (3% or 5%). Subsequently, each of the extenders was divided into three equal aliquots and diluted using both of 5 mM C or 25 mM T, and control (without additives) to a final concentration of approximately 60×10^6 spermatozoa per ml (15×10^6 total spermatozoa in each 0.25 ml straw). Diluted semen samples were cooled to 4 °C in 4 h. Subsequently semen was

frozen at a programmed rate of −3 °C/min from +4 to −10 °C; −40 °C/min from −10 to −100 °C; and −20 °C/min from −100 to −140 °C in a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen. After at least 24 h of storage, the frozen straws were thawed in a 37 °C water bath for 20 s immediately before use.

2.2. Post-thaw semen evaluation

2.2.1. Analysis of microscopic sperm variables

A Computer-Assisted Sperm motility Analysis (CASA; IVOS Version 12; Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyze sperm motion characteristics. CASA was pre-adjusted for bull sperm analysis. Thawed semen was diluted (5 ml semen + 95 ml extender) in a Tris based extender (without egg yolk and cryoprotectant) and evaluated immediately after dilution. A 4-ml sample of diluted semen was put onto a prewarmed chamber slide (20 mm; Leja 4; Leja Products Luzernestraat B.V., Holland), and sperm motility characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded; CASA motility (%), progressive motility (%), VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), ALH (amplitude of lateral head displacement, μm), LIN (linearity index; LIN = [VSL/VCL] × 100), BCF (beat cross frequency, Hz), STR (straightness, VSL/VAP). For each evaluation, 10 microscopic fields were analyzed to include at least 300 cells.

For the assessment of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 ml Hancock solution [62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml double-distilled water] (Schafer and Holzmann, 2000). One drop of this mixture was put on a slide and covered with a coverslip. The percentages of total sperm abnormalities (acrosome and other abnormalities) were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (1000×, oil immersion, Olympus BX43, Tokyo, Japan).

The Hypo-Osmotic Swelling Test (HOST) was used to assess the functional integrity of the spermatozoal membranes. The HOST was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution at 37 °C for 60 min. After incubation, 0.2 ml of the mixture was spreading on a warm slide with a cover slip and then was examined using a phase-contrast microscope (400×, Olympus BX43, Tokyo, Japan) (Revel and Mrode, 1994). The number of swollen spermatozoa out of 200 was counted; swelling is characterized by a coiled tail, indicating that the plasma membrane is intact.

For the assessment of sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay by the method of previously described by Tuncer et al. (2013). Analysis of comet was performed at high alkaline conditions. After analysis, the images of 100 randomly chosen nuclei were analyzed using a fluorescent microscope (400×, Zeiss, Germany). Nucleotide DNA extends under electrophoresis to form "comet tails," and the relative intensity of DNA in the tail images DNA break frequency. The percentage of the total DNA in the comet tail was taken as a measure of DNA break frequency. Tail DNA (%) was assessed in 100 cells by using Comet Assay III image analysis system (Perceptive Instruments, UK). Analysis was performed blindly by one slide reader.

2.2.2. Biochemical assays of post-thaw semen

2.2.2.1. MDA concentrations. Lipid peroxidation was estimated by the measuring of MDA in sperm samples by the method of previously described by Eken et al. (2012). After the reaction of MDA with thiobarbituric acid reactive substances (TBARS), the reaction product was followed spectrophotometrically at 532 nm, using tetramethoxypropane as a standard. The results were expressed as nmol/ml.

2.2.2.2. GPx activity. GPx activity was measured as previously described by Tüzün et al. (2010). Briefly, a reaction mixture containing 1 mmol/l Na_2EDTA, 2 mmol/l reduced glutathione, 0.2 mmol/l NADPH, 4 mmol/l sodium azide and 1000 U glutathione reductase in 50 mmol/l Tris buffer (pH 7.6) was prepared. 20 μl of sperm samples and 980 μl of the reaction mixture were mixed and incubated for 5 min at 37 °C. The reaction was initiated by adding 8.8 mmol/l hydrogen peroxide and the decrease of absorbance recorded at 340 nm for 3 min. GPx activity was expressed in U/ml.

2.2.3. Evaluation of in vivo fertility

In fertility trials, the fertilizing ability of spermatozoa was calculated based on the non-return rates at 60 days. A total of 967 cows were artificially inseminated with one insemination dose (15×10^6) of frozen samples with antioxidants and control samples, each comprising the semen of one bull. The effective non-return rates (NNR) were determined at 60 days post-insemination by transrectal palpation.

2.3. Statistical analysis

The experiment was repeated eight times. The results were expressed as mean ± SEM. Means were analyzed using a repeated measurement model of ANOVA, followed by LSD *post hoc* test to determine significant differences for all parameters between all groups using the SPSS/PC software package (version 13.0; SPSS Inc., Chicago, IL, USA). Differences with values of $P < 0.05$ were considered statistically significant. Pregnancy rates were evaluated using the chi-square test.

3. Results

3.1. Microscopic sperm variables

The values for influence of G (5% or 7%) or EG (3% or 5%) in the Tris extender with or without 25 mM T or 5 mM C on standard semen variables and sperm motion characteristics after the freeze–thawing process are shown in Tables 1 and 2.

The addition of EG 3% and EG 5% without T or C resulted in lesser CASA motility values than that of the

Table 1
Mean (±SEM) Computer-Assisted Sperm motility Analysis (CASA) progressive motility, CASA sperm motility, acrosome, total abnormalities, Hypo-Osmotic Swelling Test (HOST), glutathione peroxidase (GPx) and malondialdehyde (MDA) in frozen–thawed bull semen.

Groups	Progressive motility (%)	Motility (%)	Acrosome (%)	Total abnormality (%)	HOST (%)	MDA (nmol/ml)	GPx (U/ml)
Control 7% G	20.75 ± 1.46^{ab}	46.63 ± 2.40^{ab}	2.25 ± 0.25^{ab}	11.13 ± 0.77^{ab}	41.50 ± 2.32^{ab}	2.01 ± 0.26^{b}	8.88 ± 0.44^{a}
7% G + 25 mM T	19.75 ± 2.48^{bc}	49.75 ± 4.67^{a}	2.88 ± 0.30^{a}	13.38 ± 0.96^{a}	39.13 ± 3.17^{b}	2.27 ± 0.23^{b}	8.66 ± 0.30^{a}
7% G + 5 mM C	21.88 ± 1.79^{a}	49.00 ± 3.10^{a}	2.50 ± 0.33^{ab}	12.25 ± 1.21^{ab}	37.75 ± 2.38^{c}	3.21 ± 0.27^{b}	1.05 ± 0.08^{c}
Control 5% G	23.00 ± 3.30^{a}	47.50 ± 3.87^{ab}	2.38 ± 0.32^{ab}	11.88 ± 1.04^{ab}	41.50 ± 2.04^{ab}	1.71 ± 0.17^{c}	6.76 ± 0.35^{ab}
5% G + 25 mM T	18.13 ± 1.33^{cd}	43.38 ± 2.24^{b}	2.38 ± 0.38^{ab}	11.63 ± 0.87^{ab}	39.25 ± 1.76^{b}	1.36 ± 0.09^{c}	8.00 ± 0.29^{a}
5% G + 5 mM C	21.63 ± 1.84^{a}	49.13 ± 4.01^{a}	2.63 ± 0.32^{ab}	11.13 ± 1.04^{ab}	40.25 ± 3.06^{ab}	2.70 ± 0.23^{b}	0.75 ± 0.07^{cd}
Control 5% EG	15.88 ± 1.86^{e}	41.88 ± 1.54^{c}	2.00 ± 0.19^{b}	10.38 ± 0.75^{bc}	42.00 ± 2.66^{ab}	1.27 ± 0.07^{c}	7.56 ± 0.23^{ab}
5% EG + 25 mM T	19.38 ± 1.72^{bc}	44.88 ± 2.52^{b}	2.25 ± 0.25^{ab}	11.13 ± 0.92^{ab}	44.75 ± 4.57^{ab}	1.03 ± 0.07^{c}	8.29 ± 0.27^{a}
5% EG+ 5 mM C	17.75 ± 1.46^{d}	46.00 ± 2.15^{ab}	2.38 ± 0.26^{ab}	12.13 ± 0.95^{ab}	42.88 ± 3.18^{ab}	2.40 ± 0.10^{b}	1.78 ± 0.09^{c}
Control 3% EG	15.25 ± 1.91^{e}	39.13 ± 2.33^{d}	2.13 ± 0.23^{ab}	11.38 ± 0.75^{ab}	46.75 ± 2.46^{a}	2.50 ± 0.16^{b}	7.71 ± 0.44^{ab}
3% EG + 25 mM T	17.63 ± 2.22^{d}	41.00 ± 3.25^{c}	1.88 ± 0.23^{bc}	10.13 ± 0.74^{bc}	45.75 ± 3.20^{ab}	2.47 ± 0.17^{b}	7.13 ± 0.22^{ab}
3% EG+ 5 mM C	17.88 ± 2.36^{d}	44.88 ± 3.98^{b}	2.50 ± 0.42^{ab}	12.38 ± 1.09^{ab}	40.63 ± 2.58^{ab}	5.96 ± 0.42^{a}	2.93 ± 0.27^{c}
P	<0.001	<0.05	<0.05	<0.05	<0.05	<0.01	<0.001

[a–e] Different superscripts within the same column demonstrate significant differences among groups.

Table 2
Mean (±SEM) Computer-Assisted Sperm motility Analysis (CASA) sperm motion characteristics in frozen–thawed bull semen.

Groups	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR	LIN (%)	Elongation
Control 7% G	99.95 ± 1.85^{b}	78.59 ± 2.67^{a}	181.64 ± 5.06^{bc}	8.51 ± 0.35^{b}	20.28 ± 0.61^{a}	78.00 ± 1.86^{a}	44.63 ± 1.93^{a}	40.38 ± 0.82^{cb}
7% G + 25 mM T	95.11 ± 3.25^{bc}	74.16 ± 3.08^{a}	172.29 ± 5.57^{d}	8.46 ± 0.39^{b}	18.98 ± 0.57^{bc}	77.13 ± 1.30^{a}	44.25 ± 1.92^{a}	41.38 ± 0.38^{ab}
7% G + 5 mM C	100.98 ± 2.40^{b}	77.35 ± 2.78^{a}	187.68 ± 4.06^{b}	9.14 ± 0.26^{a}	19.78 ± 0.40^{ab}	76.13 ± 1.48^{a}	42.13 ± 1.37^{ab}	41.00 ± 0.95^{ab}
Control 5% G	101.15 ± 4.71^{b}	79.19 ± 5.08^{a}	184.86 ± 7.99^{b}	8.60 ± 0.35^{b}	18.55 ± 0.42^{bc}	77.38 ± 1.73^{a}	43.63 ± 2.07^{ab}	40.25 ± 0.88^{ab}
5% G + 25 mM T	101.66 ± 2.14^{b}	81.50 ± 2.28^{a}	181.35 ± 4.30^{bc}	8.23 ± 0.32^{b}	18.15 ± 0.85^{bc}	77.13 ± 1.51^{a}	44.38 ± 1.65^{a}	42.50 ± 1.50^{a}
5% G + 5 mM C	107.21 ± 4.50^{a}	78.95 ± 2.85^{a}	202.30 ± 8.84^{a}	9.36 ± 0.23^{a}	18.79 ± 0.37^{bc}	74.13 ± 1.51^{b}	41.00 ± 1.34^{ab}	43.25 ± 1.31^{a}
Control 5% EG	90.89 ± 3.06^{d}	71.96 ± 3.92^{b}	162.75 ± 5.47^{e}	7.94 ± 0.36^{bc}	20.34 ± 0.98^{a}	78.13 ± 2.31^{a}	45.63 ± 2.41^{a}	40.75 ± 1.07^{ab}
5% EG + 25 mM T	88.01 ± 2.21^{d}	69.13 ± 2.76^{b}	156.35 ± 3.67^{e}	7.79 ± 0.25^{bc}	19.51 ± 0.66^{ab}	77.50 ± 1.76^{a}	45.00 ± 1.56^{a}	42.38 ± 1.28^{a}
5% EG + 5 mM C	97.76 ± 2.24^{bc}	76.20 ± 3.37^{a}	176.81 ± 5.27^{cd}	8.65 ± 0.40^{b}	18.41 ± 0.48^{bc}	76.75 ± 1.78^{a}	43.63 ± 1.94^{ab}	42.63 ± 1.18^{a}
Control 3% EG	95.88 ± 3.40^{bc}	74.20 ± 3.36^{a}	173.89 ± 7.99^{d}	8.40 ± 0.54^{b}	18.05 ± 0.39^{bc}	77.13 ± 2.12^{a}	44.50 ± 2.35^{a}	42.25 ± 0.96^{a}
3% EG + 25 mM T	95.23 ± 2.49^{bc}	73.36 ± 4.04^{a}	171.11 ± 3.39^{d}	8.44 ± 0.38^{b}	17.75 ± 0.81^{cd}	76.38 ± 2.42^{a}	44.00 ± 2.36^{a}	41.63 ± 1.05^{ab}
3% EG + 5 mM C	106.58 ± 3.42^{a}	74.63 ± 3.21^{a}	203.00 ± 7.58^{a}	9.65 ± 0.26^{a}	18.33 ± 0.32^{bc}	71.00 ± 2.32^{c}	38.50 ± 1.52^{c}	42.25 ± 0.80^{a}
P	<0.001	<0.05	<0.001	<0.01	<0.001	<0.05	<0.01	<0.01

[a–e] Different superscripts within the same column demonstrate significant differences among groups.

other groups ($P < 0.001$) and use of 3% EG resulted in the lesser CASA progressive motility ($39.13 \pm 2.33\%$) compared with that of other groups ($P < 0.05$). The use of 25 mM T in 3% EG was beneficial to acrosome morphology ($1.88 \pm 0.23\%$) and was greater than for other groups ($P < 0.05$). Also, the use of 3% EG with 25 mM T and 5% EG resulted in fewer total abnormalities ($P < 0.05$). The use of 5% EG resulted in a slightly greater percentage of membrane integrity assessed by HOST assessment than that of the other groups ($46.75 \pm 2.46\%$) and treatment with 7% G with 5 mM C resulted in lesser membrane integrity ($37.75 \pm 2.38\%$; $P < 0.05$). Treatment with 5% G and 3% EG with 5 mM C resulted in a greater value of VAP (107.21 ± 4.50 and 106.58 ± 3.42, respectively) and VCL

Table 3
Mean (±SEM) DNA damage values and fertility results based on 60-day non-return to estrus rates after rectovaginal insemination with frozen–thawed bull semen.

Groups	Tail length	Tail intensity (%)	Tail moment (μm/s)	Non-return rates (%)
Control 7% G	72.38 ± 3.19^{a}	12.38 ± 1.09^{a}	8.30 ± 0.19^{a}	51.28 (40/78)
7% G + 25 mM T	62.41 ± 3.33^{bc}	10.71 ± 0.97^{bc}	6.38 ± 0.12^{c}	55.56 (50/90)
7% G + 5 mM C	62.08 ± 2.50^{bc}	9.70 ± 0.89^{c}	8.13 ± 0.14^{a}	45.98 (40/87)
Control 5% G	68.11 ± 3.29^{ab}	11.21 ± 1.03^{ab}	7.58 ± 0.21^{ab}	54.79 (40/73)
5% G + 25 mM T	63.91 ± 2.92^{b}	10.14 ± 1.12^{bc}	7.13 ± 0.10^{b}	53.75 (43/80)
5% G + 5 mM C	74.21 ± 4.12^{a}	10.11 ± 0.94^{bc}	7.78 ± 0.22^{a}	52.00 (39/75)
Control 5% EG	63.23 ± 2.49^{b}	12.45 ± 1.24^{a}	7.48 ± 0.12^{ab}	50.53 (48/95)
5% EG + 25 mM T	62.33 ± 3.09^{bc}	10.23 ± 1.15^{bc}	7.23 ± 0.15^{b}	48.72 (38/78)
5% EG + 5 mM C	63.01 ± 3.45^{b}	9.03 ± 0.84^{c}	6.15 ± 0.09^{c}	55.95 (47/84)
Control 3% EG	67.13 ± 2.99^{ab}	12.01 ± 1.10^{a}	7.78 ± 0.40^{a}	61.90 (52/84)
3% EG + 25 mM T	65.28 ± 3.42^{ab}	11.21 ± 0.99^{ab}	6.23 ± 0.16^{c}	58.57 (41/70)
3% EG + 5 mM C	60.14 ± 3.89^{cd}	11.32 ± 1.09^{ab}	7.48 ± 0.30^{ab}	43.84 (32/73)
P	<0.05	<0.01	<0.01	N.S.

[a–d] Different superscripts within the same column demonstrate significant differences among groups.
N.S.: no significant difference ($P > 0.05$).

(202.30 ± 8.84 and 203.00 ± 7.58, respectively) compared with that of the other groups ($P < 0.001$). For VSL, the least values (71.96 ± 3.92 and 69.13 ± 2.76) were obtained with use of 5% EG and 5% EG with 25 mM T ($P < 0.05$). Treatment with 7% G and 5% EG and 3% EG with 5 mM C resulted in more desirable values of ALH than with the other treatments ($P < 0.01$). Treatment with 7% G and 5% EG resulted in the greatest values for BCF and treatment with 3% EG with 5 mM C resulted in lesser values for STR and LIN compared with those for the other groups ($P < 0.001$, $P < 0.05$ and $P < 0.01$, respectively). For the comet test, treatment with 3% EG combined with C enhanced tail length, 7% G with C and 5% EG with C on enhanced tail movement intensity, and 7% G with T and 3% EG 3% with T and 5% EG with C on enhanced tail moment with lesser chromatin damage than the other groups ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively, Table 3). Fertility results based on 60-day non-returns after rectovaginal insemination are set out in Table 3. No significant differences were observed in non-return rates among groups.

3.2. Analysis of oxidative stress variables

The values for influence of cryoprotectants and antioxidants on LPO and GPx activity in thawed bull semen are included in Table 1. Treatment with 3% EG and 5 mM C resulted in the greatest concentration of MDA (5.96 ± 0.42 nmol/ml; $P < 0.01$). The GPx activity was elevated when there was treatment with 5 mM C with 7% or 5% G and 5% or 3% EG when compared with the other treatment groups ($P < 0.001$).

4. Discussion

The semen cryopreservation process, which includes the decrease in temperature, causes oxidative stress on sperm membranes. This, in turn, results in irreversible damage to the sperm organelles and changes in enzymatic activity, associated with a reduction in sperm motility, functional membrane integrity and fertilizing ability (Bucak et al., 2009a,b). Frozen thawed bull semen is more easily peroxidized than fresh sperm. Additionally, intracellular antioxidant capacity in sperm decreases following the freeze–thawing process (Tuncer et al., 2010). The present study was undertaken to ascertain which cryoprotectant and antioxidant would provide the most effective protection against cold shock and oxidative damages during the cryopreservation process.

The axosome and associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. These are responsible for sperm motility. Treatment with C prevents loss of motility, viability and membrane integrity during sperm liquid storage or in the frozen state (Sarıözkan et al., 2009a,b). In the present study, the addition of 5% G or 7% G with C produced more cryoprotective activity as indicated by certain sperm variables such as post thaw progressive motility, CASA motility, total abnormalities, similar to previous studies with bull (Bilodeau et al., 2001; Sarıözkan et al., 2009a,b), boar (Funahashi and Sano, 2005) and goat (Ateşşahin et al., 2008) semen.

The HOST evaluation assessed the resistance of the sperm plasma membrane to damage induced by the loss in permeability under the stress of swelling resulting from the hypo-osmotic treatment. This evaluation thus provided a form of a membrane stress-test which is particularly useful when testing the membrane stabilizing action of antioxidants (Bucak et al., 2009a,b). In the present study, addition of C or T did not provide for any positive effects to functional membrane integrity (HOST) during cryopreservation when these treatment groups were compared with the other groups and the greatest HOST rates were obtained when sperm samples were cryopreserved with 3% EG. There has been previous research with similar results in goats (Ateşşahin et al., 2008) and bulls (Tuncer et al., 2010).

Alterations in the antioxidant defense system and damage to sperm membranes have been observed due to excessive generation of ROS in semen during the freeze–thawing process (Bilodeau et al., 2000; Gadea et al., 2004). These changes in cryopreserved semen are responsible for poor sperm viability in the female reproductive tract and lesser fertility rates (Bailey et al., 2000). Mammalian spermatozoa are highly sensitive to lipid peroxidation, which occurs as a result of the oxidation of the membrane lipids by the partially reduced oxygen molecules such as superoxide, hydrogen peroxide and hydroxyl radicals (Ateşşahin et al., 2008). In the present study, treatment with 5% G, 5% G + T, 5% EG and 5% EG + T had a significant effect on the prevention of MDA formation, compared with the other treatments. Treatment with 3% EG + C resulted in the greatest MDA, so this treatment did not prevent formation of MDA. Similar to the present study, Sarıözkan et al. (2009b) and Ateşşahin et al. (2008) found that C did not decrease MDA production. Also, all groups treated with 25 mM T had less MDA production than the C groups. In a previous study, however, treatment with T did not protect against the production of MDA (Ateşşahin et al., 2008).

The antioxidant system comprising GSH, GSH-PX, CAT, taurine, and superoxide dismutase have been described as functioning as a defense mechanism against LPO in semen (Bilodeau et al., 2001). GPx is a selenocysteine-containing an antioxidant enzyme that has a role in the elimination of hydrogen peroxide and is also known to be involved in the detoxification of reactive lipids (Ateşşahin et al., 2008). In the present study, the lesser concentrations of GPx were observed when there was treatment with 7% G + C, 5% G + C, 5% EG + C and 3% EG + C. Treatment with C at 5 mM in the present study had a beneficial effect on the endogenous antioxidant system, improving GPx activity (Sarıözkan et al., 2009a,b). This finding is in agreement with the results of a previous study performed with goats (Ateşşahin et al., 2008) sperm in which an increase was observed in GPx activity in the presence of 15 mM C, following the freeze–thawing process.

Cryopreservation of spermatozoa enhances oxidative stress which not only disrupts the motility and fertilizing ability of spermatozoa, but also increases DNA damage (Rajesh et al., 2002). In the present study, treatment with 3% EG + C enhanced tail length, 7% G + C and 5% EG + C enhanced intensity of tail movement, and 7% G + T and 3% EG + T and

5% EG + C on the tail moment and less chromatin damage than with the other treatments. According to Reddy et al. (2010) addition of T to the freezing extender led to the reduction of cryo-damage to buffalo sperm. The DNA integrity was also affected by the type of cryoprotectant used. Use of DMSO6 and EGDMSO3 resulted in more sperm with damaged DNA than occurred in the other groups. DNA damage of 1.2% to 3% is normal in bulls with high fertility (Tasdemir et al., 2013). The freezing and thawing process performed with ram (Peris et al., 2004) and bull (Celeghini et al., 2008; Januskauskas et al., 2003) sperm causes permanent structural alterations to DNA that, in turn, reduce fertility.

Extending and freeze–thawing of semen lead to less antioxidant activity in the presence or absence of antioxidants. This may partly explain the lesser fertility of frozen thawed in comparison with fresh semen (Bilodeau et al., 2001). In the current study, treatment with 3% EG led to greater non-return rates of inseminated cows. However, this result was not considered to be statistically important ($P < 0.05$). With respect to fertility results based on 60-day non-returns, the supplementation of antioxidants and different cryoprotectants of various ratio did not present significant differences of fertility results. This finding, however, is in agreement with previous studies where there was no improvement in fertility when certain additives were used as semen extenders (Sarıözkan et al., 2009a,b).

In conclusion, compared to the cryoprotectant groups in the present study, the use of C or T in the extender did not eliminate MDA production and adding 5 mM C in all cryoprotectant groups decreased GPx antioxidant activity during the cryopreservation process ($P < 0.001$). The sperm motion characteristics, such as VAP and VCL, were improved by adding C in EG treatment groups (3% or 5%). Sperm motility variables were influenced by adding C or T in EG (3% or 5%), but other sperm variables were not influenced by cryoprotectants and additives (with or without). However, DNA integrity was protected when 7% G + T, 5% EG + C and 3% EG + T were added ($P < 0.01$). The effects of C or T (with or without) in various cryoprotectants were not associated with fertility, although the greatest non-return rate was achieved by adding EG as a cryoprotectant without any additives (61.9%). The results of the present study provide useful information for future studies in this area.

Conflict of interest

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