Reproduction in Domestic Animals

Reprod Dom Anim **49**, 746–752 (2014); doi: 10.1111/rda.12359
ISSN 0936–6768

Comparison of Cryoprotective Effects of Lycopene and Cysteamine in Different Cryoprotectants on Bull Semen and Fertility Results

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Contents

The objectives of this study were to compare glycerol and ethylene glycol at different concentrations as cryoprotectants and lycopene or cysteamine (with/without) as antioxidants in Tris extender for bull semen. Twenty-four ejaculates were obtained from three bulls. Each ejaculate was split into four equal aliquots and diluted using both of the Tris extenders with glycerol (5% or 7%) or ethylene glycol (3% or 5%). After that, each extenders were split into three equal aliquots and added using both of the cysteamine 5 mM or lycopene 500 μg/ml, and control (without additives). The addition of 7% glycerol with cysteamine, 5% ethylene glycol with cysteamine and 3% ethylene glycol with cysteamine groups gave the lowest CASA motility than the other groups. However, 7% glycerol and 7% glycerol with lycopene resulted in a better rate of CASA progressive motility compared with that of other groups. Generally, all the lycopene groups signed better protective effects on acrosome and total morphology than the other groups. Glycerol 7% and 3% ethylene glycol with lycopene groups yielded to slight higher percentages of membrane integrity assessed by HOST than that of the other groups, but 7% glycerol with cysteamine and 3% ethylene glycol with cysteamine showed the worst percentages of membrane integrity. Glycerol 7% and 5% glycerol with lycopene gave rise to a higher value of VAP, VSL and VCL compared with that of the other groups. On the contrary, adding to 5% glycerol with cysteamine showed negative effect for VAP, VSL, VCL and ALH values. All cryoprotectant groups with lycopene decreased chromatin damage than the other groups. Ethylene glycol 3% led to lower non-return rates of inseminated cows. However, this result was not considered to be statistically important.

Introduction

Success of artificial insemination (AI) is dependent on the quality of fresh semen and its capacity for dilution and storage with minimum loss of fertilizing ability. The best preservation technique to date of post-thaw survival is restricted to approximately 50% of the sperm population (Watson 1995). The final cryopreservation goal of semen is not only to maintain the initial motility but also to maintain the necessary metabolism to produce energy, plasma proteins to survive in the female reproductive tract at the time of fertilization and acrosomal enzymes for the penetration of the ovum, capacity of progressive movement and to prevent any damage which reduce lifespan of spermatozoa and its fertilizability (El-Harairy et al. 2011). The major factor affecting the results of insemination with frozen–thawed semen is the addition of cryoprotectants (Aboagla and Trade 2004). In order to improve the media and to protect spermatozoal damage due to the internal ice crystal formation, the solute concentration should be increased in the media (Lebouef et al. 2000). The cryoprotectants are added to extenders to protect the sperm from damage during freezing process (Singh et al. 1995). The level 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). The optimum concentration of G for freezing bull sperm is influenced by other components in the overall sperm diluent or extender (Woelders et al. 1997). Specifically, when used in high 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). Some researchers believe that a low molecular weight cryoprotectant, such as ethylene glycol (EG), may cause less damage to spermatozoa than G because its low molecular weight allows it to cross the plasma membrane more easily (Moore et al. 2006). EG has also shown better post-thaw motility results for bull sperm when compared with both G and dimethyl sulphoxide (DMSO), which may be due to a reduction in the osmotic stress (Taşdemir et al. 2013). Differences in fatty acids composition and lipid class ratios in spermatozoa among species are important factors in freezing spermatozoa. Oxidative stress significantly damages sperm functions due to lipid peroxidation (LPO) induced by reactive oxygen species (ROS). Lipid peroxidation occurs readily in the tissues rich in highly oxidizable polyunsaturated fatty acids (PUFA) (Halliwell 1994; Chatterjee et al. 2001). Every stage of cryopreservation (cooling, freezing and thawing) causes physical and biochemical stress on sperm plasma membrane and structure, which results in a subsequent loss of motility, membrane integrity, fertilizing capability and metabolic changes of sperm (Wang et al. 1997). Sperm cells, when freezed and thawed for AI, have been exposed to oxygen and light radiation which could irreversibly affect sperm functions. Under these conditions, it was stated that supplementation of antioxidants improved the post-thaw motility, viability, membrane integrity and fertility of boar (Funahashi and Sano 2005), bull (Sarıozkan et al. 2009), ram (Uysal and Bucak 2007) and goat (Ateşşahin et al. 2008) sperm

cells. Oxidative stress is the result of an excessive production of ROS and/or a decrease in the antioxidant defence system, and this oxidative stress targets lipids, proteins and DNA, decreasing sperm motility and mitochondrial activity and damaging the paternal genome (Gong et al. 2012).

Cysteamine (C) stimulates glutathione (GSH) synthesis during the *in vitro* maturation of ovine oocytes, promoting embryonic development and quality (Matos et al. 2002). Additionally, it enhances post-thaw motility, elevating the antioxidant capacity of ram sperm (Bucak et al. 2007). Low molecular weight thiol compounds such as mercaptoethanol (P3-ME) and cysteamine are reported to promote cell viability (Takahashi et al. 1993). Lycopene, an aliphatic hydrocarbon, has received particular attention as a result of studies indicating that it has highly efficient antioxidant and free radical scavenging capacity (Türk et al. 2006).

The objective of this study was to evaluate the effects of the addition various ratio of two cryoprotectants (5% or 7% glycerol or 5% or 3% ethylene glycol) on bull spermatozoa cryopreserved in a Tris extender supplemented with or without lycopene and cysteamine on post-thawing microscopic sperm parameters (motility, acrosome and total abnormalities, HOS test), antioxidant activities (MDA and GPx) and DNA integrity of frozen–thawed semen. Conception rates of the cows artificially inseminated with frozen–thawed semen with the best results in all extenders were also assessed.

Material and Methods

Animals, semen collection and semen processing

Three Holstein bulls (3–4 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 with the aid 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 microscope (200×). The ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher 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 in the laboratory. A Tris-based extender (T) (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 split into four equal aliquots and diluted using both of the T extenders with G (5% or 7%) or EG (3% or 5%). After that, each extenders were split into three equal aliquots and diluted using both of the cysteamine 5 mM (C) or lycopene 500 μg/ml (L), and control (without additives) to a final concentration of approximately 60×10^6 spermatozoa per ml (15×10^6 sperm in each 0.25 ml straw). Diluted semen samples cooled down to 4°C in 4 h. After that they were 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). Thereafter, the straws were plunged into liquid nitrogen. After at least 24 h, the frozen straws were thawed in a 37°C water bath for 20 s immediately before use.

Post-thaw semen evaluation

Analysis of microscopic sperm parameters

A computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyse sperm motion characteristics. CASA was pre-adjusted for bovine 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 pre-warmed chamber slide (20 mm; Leja 4; Leja Product B.V., the Netherlands), 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 analysed 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 microscope (×1000, oil immersion, Olympus BX43, Tokyo, Japan).

The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoal membranes. The HOS test 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 coverslip and then was examined using a phase-contrast microscope (×400, Olympus BX43) (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 analysed using a

fluorescent microscope (×400; Zeiss, Jena, 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 using Comet Assay III image analysis system (Perceptive Instruments, UK). Analysis was performed blindly by one slide reader.

Biochemical assays of post-thaw semen

Malondialdehyde (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.

GPx activity: GPx activity was measured as previously described by Tüzün et al. (2010). Briefly, a reaction mixture containing 1 mM Na_2EDTA, 2 mM reduced glutathione, 0.2 mM NADPH, 4 mM sodium azide and 1000 U glutathione reductase in 50 mM TRIS buffer (pH 7.6) was prepared. Twenty microlitre 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 mM hydrogen peroxide, and the decrease of absorbance recorded at 340 nm for 3 min. GPx activity was expressed in U/ml.

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 979 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.

Statistical analysis

The experiment was repeated eight times. The results were expressed as mean ± SEM. Means were analysed 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.

Results

Microscopic sperm parameters (percentages of motility, acrosome, total abnormalities, HOS test and *in vivo* fertility)

The influence of glycerol (G) (5% or 7%) or ethylene glycol (EG) (3% or 5%) in the Tris extender with or without 500 μg/ml lycopene (L) or 5 mM cysteamine (C) on standard semen parameters and sperm motion characteristics after the freeze–thawing process is shown in Tables 1 and 2.

The addition of G7+C, EG5+C and EG3+C groups gave the lowest CASA motility than the other groups ($p < 0.001$). However, G7 and G7+L resulted in a better rate of CASA progressive motility (49.13 ± 3.52% and 44.75 ± 2.32%, respectively) compared with that of other groups ($p < 0.001$). Generally, all the lycopene groups signed better protective effects on acrosome and total morphology than the other groups ($p < 0.05$ and $p < 0.01$, respectively). G7 and EG3+L groups yielded to slight higher percentages of membrane integrity assessed by HOST than that of the other groups (47.50 ± 0.28% and 48.38 ± 2.85%). But G7+C and EG3+C showed the worst percentages of membrane integrity (34.50 ± 1.63% and 35.50 ± 2.15%, respectively) ($p < 0.001$). Lycopene groups with G7 and G5 gave rise to a higher value of VAP, VSL and VCL compared with that of the other groups ($p < 0.001$). On the contrary, adding to G5+C showed negative effect for VAP, VSL, VCL and ALH values (57.30 ± 1.45%, 47.19 ± 0.69%, 89.03 ± 1.42% and 4.73 ± 0.37 μm, respectively). EG5+C gave the lowest values of BCF

Table 1. Mean (±SEM) 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	22.00 ± 1.46^{a}	44.75 ± 2.32^{ab}	3.13 ± 0.44^{b}	13.00 ± 1.36^{c}	47.50 ± 0.28^{a}	1.84 ± 0.11^{c}	7.44 ± 0.16^{bc}
7% G + 500 μg/ml L	24.38 ± 3.17^{a}	49.13 ± 3.52^{a}	2.50 ± 0.27^{c}	12.25 ± 0.77^{c}	42.00 ± 2.17^{bc}	2.39 ± 0.09^{bc}	9.10 ± 0.36^{ab}
7% G + 5 mM C	8.75 ± 1.19^{d}	20.88 ± 1.69^{e}	4.13 ± 0.23^{a}	19.00 ± 0.53^{a}	34.50 ± 1.63^{d}	2.78 ± 0.30^{ab}	1.90 ± 0.25^{d}
Control 5% G	15.75 ± 2.52^{bc}	37.00 ± 4.05^{cd}	3.38 ± 0.42^{b}	15.38 ± 1.61^{b}	38.13 ± 4.30^{cd}	2.66 ± 0.16^{ab}	9.50 ± 0.30^{ab}
5% G + 500 μg/ml L	19.88 ± 1.57^{b}	41.63 ± 2.79^{bc}	2.63 ± 0.42^{bc}	13.25 ± 1.25^{c}	41.50 ± 2.67^{bc}	3.60 ± 0.31^{a}	10.32 ± 0.36^{ab}
5% G + 5 mM C	13.38 ± 0.56^{c}	27.50 ± 1.88^{de}	2.88 ± 0.23^{bc}	14.00 ± 0.50^{bc}	40.50 ± 1.54^{bc}	3.80 ± 0.30^{a}	2.09 ± 0.25^{d}
Control 5% EG	15.00 ± 1.68^{bc}	34.75 ± 3.18^{d}	3.00 ± 0.31^{bc}	13.50 ± 0.79^{c}	45.38 ± 3.51^{ab}	3.15 ± 0.21^{a}	13.16 ± 0.45^{a}
5%EG + 500 μg/ml L	17.75 ± 1.64^{b}	43.13 ± 3.03^{ab}	2.75 ± 0.53^{bc}	12.13 ± 1.15^{c}	47.75 ± 2.83^{a}	1.25 ± 0.08^{c}	13.77 ± 0.20^{a}
5% EG + 5 mM C	8.25 ± 0.80^{d}	16.00 ± 0.96^{f}	4.00 ± 0.27^{a}	17.50 ± 0.38^{ab}	38.50 ± 0.65^{cd}	2.67 ± 0.16^{ab}	2.88 ± 0.34^{d}
Control 3% EG	14.13 ± 1.85^{bc}	31.63 ± 3.30^{d}	2.88 ± 0.40^{bc}	14.00 ± 1.44^{bc}	44.88 ± 3.68^{ab}	1.51 ± 0.28^{c}	11.22 ± 0.45^{ab}
3% EG + 500 μg/ml L	15.13 ± 2.13^{bc}	34.50 ± 3.39^{d}	2.75 ± 0.25^{bc}	12.75 ± 1.22^{c}	48.38 ± 2.85^{a}	1.33 ± 0.95^{c}	13.44 ± 0.61^{a}
3% EG + 5 mM C	8.38 ± 0.82^{d}	18.38 ± 2.44^{ef}	4.00 ± 0.27^{a}	18.38 ± 0.50^{a}	35.50 ± 2.15^{d}	1.90 ± 0.02^{c}	2.58 ± 0.37^{d}
p	<0.001	<0.001	<0.05	<0.01	<0.001	<0.05	<0.001

Different superscripts within the same column demonstrate significant differences among groups.

Table 2. Mean (±SEM) 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	95.84 ± 2.78^{b}	74.03 ± 2.31^{cd}	170.80 ± 6.26^{c}	7.91 ± 0.27^{c}	19.21 ± 0.54^{c}	76.75 ± 1.16^{ab}	44.63 ± 1.16^{bc}	40.38 ± 0.63^{a}
7% G + 500 μg/ml L	101.53 ± 3.09^{c}	78.64 ± 2.21^{de}	179.11 ± 8.35^{cd}	8.20 ± 0.40^{bc}	18.53 ± 1.05^{bc}	77.50 ± 1.22^{ab}	46.13 ± 1.54^{bc}	42.13 ± 0.77^{b}
7% G + 5 mM C	57.30 ± 1.45^{a}	47.19 ± 0.69^{a}	89.03 ± 1.42^{a}	4.73 ± 0.37^{a}	20.01 ± 0.26^{c}	80.88 ± 0.79^{a}	50.50 ± 0.50^{a}	39.88 ± 0.30^{a}
Control 5% G	97.76 ± 4.70^{c}	76.48 ± 3.37^{cd}	174.64 ± 10.82^{c}	8.30 ± 0.59^{b}	18.36 ± 1.31^{bc}	78.25 ± 1.32^{ab}	45.63 ± 2.02^{bc}	42.13 ± 1.14^{b}
5% G + 500 μg/ml L	103.09 ± 3.12^{c}	80.96 ± 2.51^{e}	184.99 ± 7.29^{d}	8.41 ± 0.42^{b}	20.21 ± 0.86^{c}	78.38 ± 1.31^{ab}	45.13 ± 1.32^{bc}	41.50 ± 0.87^{ab}
5% G + 5 mM C	94.04 ± 1.32^{b}	76.41 ± 1.57^{cd}	182.00 ± 2.31^{d}	8.25 ± 0.11^{bc}	19.10 ± 0.16^{bc}	76.88 ± 0.35^{ab}	44.13 ± 0.44^{bc}	40.13 ± 0.30^{a}
Control 5% EG	94.85 ± 2.20^{b}	75.28 ± 2.15^{cd}	164.39 ± 6.82^{bc}	7.46 ± 0.40^{c}	19.95 ± 1.08^{c}	78.63 ± 1.80^{ab}	47.13 ± 2.29^{ab}	40.38 ± 0.53^{a}
5% EG + 500 μg/ml L	89.95 ± 3.42^{b}	71.21 ± 1.98^{c}	154.16 ± 8.61^{bc}	7.04 ± 0.50^{c}	19.54 ± 1.10^{c}	78.88 ± 1.51^{ab}	48.13 ± 2.26^{ab}	41.63 ± 0.68^{ab}
5% EG + 5 mM C	90.83 ± 1.38^{b}	66.88 ± 1.22^{b}	130.53 ± 7.37^{b}	7.21 ± 0.04^{c}	13.99 ± 0.52^{a}	72.75 ± 0.53^{c}	44.75 ± 0.65^{bc}	40.38 ± 0.50^{a}
Control 3% EG	98.24 ± 2.21^{c}	79.35 ± 2.62^{de}	172.09 ± 5.26^{c}	8.03 ± 0.36^{bc}	19.03 ± 0.93^{bc}	80.50 ± 0.91^{a}	47.50 ± 1.30^{ab}	40.63 ± 0.46^{a}
3% EG + 500 μg/ml L	98.78 ± 2.54^{c}	77.23 ± 1.79^{cd}	171.49 ± 5.48^{bc}	7.88 ± 0.30^{c}	16.85 ± 1.04^{b}	78.13 ± 0.91^{ab}	46.63 ± 0.93^{bc}	42.75 ± 1.41^{b}
3% EG + 5 mM C	97.36 ± 1.88^{c}	76.59 ± 2.90^{cd}	166.51 ± 4.18^{bc}	7.59 ± 0.36^{c}	16.88 ± 1.08^{b}	78.88 ± 1.47^{ab}	48.13 ± 1.52^{ab}	40.63 ± 0.53^{a}
p	<0.001	<0.001	<0.001	<0.01	<0.01	<0.05	<0.05	<0.05

Different superscripts within the same column demonstrate significant differences among groups.

(13.99 ± 0.52 Hz), and G7+C showed the highest value of LIN (50.50 ± 0.50%), but it signed lowest value of elongation (39.88 ± 0.30) ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively). As shown in Table 3, in terms of chromatin damage all cryoprotectant groups with lycopene decreased chromatin damage than the other groups ($p < 0.05$). 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 ($p > 0.05$).

Analysis of oxidative stress parameters

The influence of antioxidants on LPO level and GPx activity in thawed bull semen is set out in Table 1. All cryoprotectants groups (with or without antioxidants) did not prevent MDA producing, except EG 5% groups (with or without antioxidants) ($p < 0.05$). GPx activity was significantly elevated in the groups 5 mM C with 7G, 5G, 5EG and 3EG when compared to the other groups ($p < 0.001$).

Discussion

The shape and the size of the sperm head could define its cryosensitivity. Comparative studies in the boar, bull, ram, rabbit, cat, dog, horse, human have shown negative correlations between the size of the sperm head and cryostability (Gao et al. 1997). The semen freezing–thawing process is still associated with the reduction of the cellular motility, of the viability and of the fertilization capacity. All the cellular components, including lipids, proteins, nucleic acids and sugars, are potential targets of oxidative stress. The proportion of alterations induced by oxidative stress depends on the nature and concentration of ROS, on the exposure period to ROS, but also on extracellular factors such as temperature, oxygen and environmental make-up (ions, proteins, ROS scavengers) (Gadea et al. 2004). Seminal plasma contains a large number of non-enzymatic antioxidants such as ascorbate, α-tocopherol, pyruvate, glutathione, taurine. These are the most important protection elements used by sperm cells against the attack of ROS. The use of natural antioxidants in the

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

Groups	Tail length	Tail intensity (%)	Tail moment (μm/s)	Non-return rates (%)
Control 7% G	67.28 ± 4.11^{bc}	11.47 ± 1.10^{ab}	8.15 ± 0.23^{b}	56.41 (44/78)
7% G + 500 μg/ml L	64.15 ± 3.60^{c}	9.78 ± 0.94^{c}	7.22 ± 0.13^{c}	60.00 (48/80)
7% G + 5 mM C	84.14 ± 2.85^{a}	12.70 ± 0.79^{a}	8.41 ± 0.15^{b}	49.35 (38/77)
Control 5% G	67.61 ± 3.35^{bc}	10.91 ± 1.21^{ab}	8.12 ± 0.20^{b}	60.00 (57/95)
5% G + 500 μg/ml L	64.71 ± 3.92^{c}	9.10 ± 1.10^{c}	7.13 ± 0.13^{c}	60.92 (53/87)
5% G + 5 mM C	87.18 ± 4.09^{a}	12.11 ± 0.95^{a}	8.88 ± 0.25^{b}	56.92 (37/65)
Control 5% EG	68.51 ± 4.04^{b}	11.33 ± 1.20^{ab}	8.28 ± 0.27^{b}	54.35 (50/92)
5% EG + 500 μg/ml L	64.30 ± 3.86^{c}	9.27 ± 1.01^{c}	7.30 ± 0.16^{c}	54.17 (52/96)
5% EG + 5 mM C	84.19 ± 3.63^{a}	11.95 ± 1.08^{a}	7.95 ± 0.19^{b}	57.14 (48/84)
Control 3% EG	71.13 ± 3.61^{b}	13.01 ± 1.15^{a}	10.78 ± 0.42^{a}	44.78 (30/67)
3% EG + 500 μg/ml L	66.83 ± 3.44^{bc}	10.27 ± 0.91^{bc}	8.23 ± 0.14^{b}	54.22 (45/83)
3% EG + 5 mM C	84.54 ± 3.81^{a}	11.85 ± 1.09^{a}	8.58 ± 0.36^{b}	53.34 (40/75)
p	<0.01	<0.05	<0.05	N.S.

Different superscripts within the same column demonstrate significant differences among groups; N.S.: no significant difference ($p > 0.05$).

dilution and freezing media, such as α-tocopherol and ascorbate, has protecting effects on the metabolic activity and on the cell viability of cryopreserved bovine sperm cells (Bearden and Fuquay 2000). In frozen–thawed semen, motility of sperm cells is better preserved than its morphological integrity. Plasma and outer acrosome membranes are the most cryosensitive (Barbas and Mascarenhas 2009). Oxidative stress generally leads to loss of motility, swelling and the blebbing of the acrosomal membrane and disruption or increased permeability of the plasma membrane of spermatozoa (Tuncer et al. 2011). Structural damage of the plasma membrane increases the susceptibility to LPO when high production of ROS occurs during the freeze–thawing process. This was stated for boar (Funahashi and Sano 2005), ram (Başpınar et al. 2011), bull (Sarıozkan et al. 2009; Taşdemir et al. 2013) and buck (Tuncer et al. 2013) sperm.

In this study, addition of G 7%+C, EG 5%+C and EG 3%+C groups gave the lowest CASA motility than the other groups. However, G 7% and G 7%+L resulted in a better rate of CASA progressive motility compared with that of other groups. Generally, all the lycopene groups signed better protective effects on acrosome and total morphology than the other groups. G 7% and EG 3%+L groups yielded to slight higher percentages of membrane integrity assessed by HOST than that of the other groups. On the contrary, G 7%+C and EG 3%+C showed the worst percentages of membrane integrity. In a study, usage of EG as a cryoprotectant for buffalo bull semen improved its quality significantly after thawing (high viability, acrosomal integrity, plasma membrane integrity and low abnormality) except sperm motility than usage of G in Tris extender (Swellum et al. 2011). In ram (Silva et al. 2012), after thawing, progressive sperm motility was greater in cryopreservation with G 5% and EG (3% or 5%) than with acetamide (3% or 5%). Acrosome integrity was greater with EG 5% than acetamide (3% or 5%). The percentage of sperm without oxidative stress was greater with EG (3% or 5%) than with acetamide (3% or 5%). Plasma membrane integrity was greater with glycerol 5% than with the other cryoprotectants. In boar semen cryopreservation, vitamin E supplementation resulted a positive effect on sperm motility and mitochondrial membrane potential and membrane integrity depending on the portion of ejaculate (Jeong et al. 2009).

When cells are frozen, they are subjected to various stresses such as cold shock and oxidative stress that arise through ice crystallization and LPO due to membrane changes (Tuncer et al. 2013). Carotenoids such as beta-carotene and lycopene are also important components of antioxidant defence. Beta-carotenes protect the plasma membrane against lipid peroxidation. In a study (Uysal and Bucak 2007), the effect of lycopene was similar to results obtained by other researchers, who reported that epididymal sperm characteristics were protected in rat testes treated by lycopene (Ateşşahin et al. 2006). The structural and functional damage in the testicular tissue and sperm quality of rats caused by oxidative stress were prevented by lycopene supplementation (Türk et al. 2006). However, a lycopene concentration of 3200 μg was determined to lead to deleterious effects on spermatological indicators including a decline in sperm motility and an increase in sperm abnormality, acrosome damage and dead sperm (Uysal and Bucak 2007). Cysteamine provided cryoprotective effect on ram (Bucak et al. 2007) and goat (Bucak et al. 2009) semen collected by AV. The addition of 5 mM cysteamine led to an increase in sperm post-thaw survival and a dramatic increase in motility (73.0% compared with 47.5; $p < 0.0.5$) of ram semen, compared with base extender (Bucak et al. 2007). According to the other study, there were no positive effects of 5 mM cysteamine on semen quality before or after freezing. Moreover, all extenders containing cysteamine had decreased motility and velocity after TST compared with control. This might suggest that addition of 5 mM cysteamine to the freezing extender decreased post-thaw resistance of EE ram semen (Cirit et al. 2013).

In frozen–thawed semen, there is a decline in motility, viability and forward progression in the female reproductive tract which causes a reduction in fertility (Salamon and Maxwell 2000). Insemination of buffalo cows with frozen semen treated by EG 5% resulted in better fertility (conception rate 46.2% and pregnancy rate 46.2%) compared to that treated by G 7% (conception rate 42.3% and pregnancy rate 38.5%) in Tris (Swellum et al. 2011). In this study, the effects of lycopene or cysteamine (with or without) in various cryoprotectants and different rates were not associated with fertility.

In conclusion, it may be stated that, compared with the cryoprotectants groups, the use of G 5%+L, G 5%+C and EG 5% gave the highest level MDA production. Also, GPx activity was significantly elevated in the all C groups with G 5%, G 7%, EG 3% and EG 5% when compared to the other groups. Additionally, G 7%+C, EG 3%+C and EG 5%+C groups did not show protective effect on the acrosome and total abnormalities. On the contrary, G 7%, EG 5%+L and EG 3%+L yielded higher percentage of membrane integrity assessed by HOST than the other groups. All cryoprotectant groups with lycopene showed better protective effect on DNA damage than the other groups. The effects of lycopene or cysteamine (with or without) in various cryoprotectants and different rates were not associated with fertility, although the highest non-return rate was achieved by adding G 5%+L (60.92%). The results of this study may provide an useful information for the future studies in this area.

Acknowledgements

This study was supported by the Republic of Turkey, Ministry of Agriculture and Rural Affairs, General Directorate of Agricultural Research (GDAR) Project number: 09/01/01/01.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

P.B. Tuncer designed the study, worked at the laboratory, involved in the evaluation and analysis of semen and wrote the manuscript; S. Büyükleblebici and U. Taşdemir worked at the laboratory, involved in the evaluation and analysis of semen; A. Eken analysed MDA and GPx activities; O. Büyükleblebici has commented antioxidant activity and helped to write manuscript; and E. Durmaz and E. Çoşkun analysed COMET.

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Submitted: 31 Mar 2014; Accepted: 21 May 2014

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