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Comparing ethylene glycol with glycerol and with or without dithiothreitol and sucrose for cryopreservation of bull semen in egg-yolk containing extenders ☆

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ABSTRACT

There are few studies performed for investigating the roles of different ratio and cryoprotectants with dithiothreitol or sucrose on sperm motility characteristics and antioxidant capacities of post-thawed bull spermatozoa. The objectives of this study were to compare glycerol (G) and ethylene glycol (EG) at different concentrations as cryoprotectants and dithiothreitol (D) or sucrose (S) (with/without) as antioxidants in Tris extender for cryopreservation of bull semen. Twenty-four ejaculates obtained from three bulls were included in the study. 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 diluted using both of the dithiothreitol 5 mM or sucrose 25 mM, and control (without additives) was cooled to 4 °C and frozen in 0.25-ml French straws. when compared to control, different doses cryoprotectants and antioxidants addition no significantly increased the percentages of post-thaw sperm progressive and motitilities, acrosome abnormality and plasma membrane integrity ($P > 0.05$). However, EG3 + S yielded the greatest percentages of the total abnormality ($P < 0.05$). As regard to antioxidant activities G7 and EG5 led to lowest MDA activity with or without D or S but, these results were not supported to the GPx activity ($P < 0.01$). The sperm motion characteristics such as VAP, VCL, ALH and BCF gave significantly different results ($P < 0.05$). When compared the DNA integrity, different doses cryoprotectants without antioxidants addition significantly increased the percentages of the tail intensity and tail moment ($P < 0.05$). There were no significant differences observed in non-return rates among all treatment groups ($P > 0.05$).

Introduction

The success of cryopreservation depends on the specific susceptibility of sperm cells at low temperatures. Changes in sperm cell and it's function associated to cold shock have often been reported [5,29]. Cryopreservation processes lead to the generation of reactive oxygen species (ROS) that impair sperm motility, membrane integrity, and fertilizing ability [7,18,20]. Although bovine semen has natural defense system against the ROS attack, it is insufficient under cryopreservation due to stress [28]. Sperm plasma and acrosomal membranes are especially important with regard to survival following thawing and it is considered that there are changes on the primary organelles caused by the cryopreservation process. These changes are due to oxidative and osmotic stresses [19,43]. The level and type of cryoprotectants influence the sperm cells during freezing in semen extender [17]. In recent years, many cryoprotective agents have been used as a cryoprotectant in bull [37] and goat [40]. Glycerol (G) can cause chemical and osmotic deleterious effects on sperm cells and changes in the structure of the plasma membrane, these effects and changes can result in a lower fertility

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rate when AI is used, which demonstrates the need for alternative cryoprotectants [6]. Besides, improvement of semen extenders with suitable antioxidant is suggested to reduce oxidative damage during freeze–thawing of bull spermatozoa [1]. Dithiothreitol (D) is known as an antioxidant and it decreases protamine disulfide bond [42]. It prevents the oxidation of sulfhydryl groups, also it has a mucolytic effect on mucoprotein disulfide bonds, which may probably damage the frozen membranes [29]. It is stated that D provides a protective effect against apoptosis and oxidative damage [15]. The addition of D seems to improve bull and human sperm motility during liquid storage or in the frozen state [14]. Sugars are known to maintain the osmotic pressure of extenders by inducing cell dehydration and less ice crystal formation into the sperm cells [30]. They also interact with phospholipid membranes at low hydration and thus cause stabilization of the membranes. Furthermore, sugars are utilized by spermatozoa as an energy source during glycolysis and mitochondrial oxidative phosphorylation to support sperm motility [27]. Addition of sugars in extenders, such as sucrose, tends to protect the sperm cells. This sugar, as non-permeating cryoprotectant, is not able to diffuse across the plasma membrane, but creates an osmotic pressure that induces cell dehydration before freezing. Thus it decreases the extent of cell injury by intracellular ice formation [21].

There are few studies performed for investigating the roles of different ratio and cryoprotectants with D or S on sperm motility characteristics and antioxidant capacities of post-thawed bull spermatozoa. Thus, the aim of this study is to determine the effects of G or EG (with or without D or S) on post-thaw sperm parameters (motility, acrosome and total abnormalities, plasma membrane integrity), antioxidant activities (MDA and GPx) and DNA integrity as well as conception rate.

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 (8 ejaculates for each Holstein bull) 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 microscopy (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 25 mM sucrose (S) or 5 mM dithiothreitol (D), 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, 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, 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 analyze sperm motion characteristics. CASA was pre-adjusted for bovine sperm analysis. Thawed semen was diluted (5 µl semen + 95 µl extender) in a Tris based extender (without egg yolk and cryoprotectant) and evaluated immediately after dilution. A 4-µl 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 spermatological characteristics 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] [35]. 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 (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 cover slip and then was examined using a phase-contrast microscope (×400, Olympus BX43, Tokyo, Japan) [31]. 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. [40]. 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.

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. [16]. 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. [41]. 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.

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 935 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 8 times. The results were expressed as mean ± SEM. Means were analyzed using a repeated measurement model of ANOVA, followed by Duncan 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

As shown in Table 1, when compared to control, addition of different doses of cryoprotectants and antioxidants did not significantly increased the percentages of post-thaw sperm progressive and CASA motitilities, acrosome abnormality and plasma membrane integrity ($P > 0.05$). However, EG3 + S yielded the greatest percentages of the total abnormality ($P < 0.05$). As regard to antioxidant activities G7 and EG5 led to lowest MDA activity with or without D or S but, these results were not supported to the GPx activity ($P < 0.01$).

Sperm motion characteristics such as VAP, VCL, ALH and BCF gave significantly different results except for VSL, STR, LIN and elongation ($P < 0.05$) (Table 2).

As shown in Table 3, when compared the DNA integrity, different doses of cryoprotectants without addition of antioxidants significantly increased the percentages of the tail intensity and tail moment ($P < 0.05$). There were no significant differences observed in non-return rates among all treatment groups ($P > 0.05$).

Discussion

The semen cryopreservation process, which includes the decrease in temperature, causes oxidative stress on the sperm. This, respectively, results in irreversible damage to the sperm organelles and changes in enzymatic activity, associated with a reduction in sperm motility, membrane integrity and fertilizing ability [9,10]. Frozen–thawed bull semen is more easily peroxidized than fresh sperm. Besides, intracellular antioxidant capacity in sperm decreases following freeze–thawing [33]. Progressive and CASA motilities positively correlate with the resistance of the spermatozoa exposed to cold shock and their fertility [26]. It has been reported that EG quickly penetrates the sperm cell during freezing and is easily removed during thawing due to its low molecular weight, causing less osmotic stress [25]. In current study, addition of different doses of cryoprotectants and antioxidants did not significantly increased the percentages of post-thaw sperm progressive and CASA motitilities and plasma membrane integrity ($P > 0.05$). Consistent with our results, Başpınar et al. [4] reported that when D was supplemented to semen extender, it did not produce a beneficial effect on sperm motility. Several studies have been reported contradict with our findings. In a Holstein breed bull study, using of EG in Tris extender improved spermatozoa viability and plasma membrane integrity [36]. According to Bucak et al. [12] 2mM D gaved better membrane integrity than 0.5 mM D in bull frozen-thawed semen. In a boar study, addition of trehalose as a main sugar to the extender, obtained better post-thawed sperm viability and fertilizing ability [24]. Besides, addition of trehalose and sucrose to the extender enhanced sperm motility and acrosome integrity [44]. It has been reported that the supplementation of sucrose decreased the sperm motility [13]. The addition of 3.5% G and 3.5% DMSO together exhibited higher motility [17]. In terms of post-thaw sperm motion characteristics (VAP, VSL, VCL, ALH and BCF), our results were compatible with those reported by Tasdemir et al. [37] who demonstrated that using EG and DMSO as a cryoprotectant instead of G didn't give marked effect on sperm motion characteristics. In contrast with our study, Tris-based media containing sucrose decreased sperm motion parameters [13]. Based on our findings, we can hypothesize that these differences among the studies, may be due to the turbidity of other substances in Tris based extender.

Table 1
Mean (±SEM) CASA progressive motility, CASA sperm motility, acrosome and total abnormalities, plasma membrane integrity, 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	21.13 ± 2.55	48.00 ± 2.44	2.63 ± 0.42	12.88 ± 0.88^{ab}	35.13 ± 3.84	1.17 ± 0.10^{a}	11.45 ± 0.35^{gh}
7% G + 5 mM D	22.88 ± 2.84	48.13 ± 3.71	2.63 ± 0.42	11.63 ± 0.89^{ab}	39.75 ± 2.38	1.31 ± 0.05^{a}	10.14 ± 0.43^{def}
7% G + 25 mM S	23.50 ± 2.53	50.88 ± 2.87	2.00 ± 0.33	10.38 ± 0.96^{b}	42.38 ± 3.39	1.14 ± 0.05^{a}	10.87 ± 0.37^{fgh}
Control 5% G	25.50 ± 3.70	49.50 ± 3.94	2.13 ± 0.55	10.50 ± 1.41^{b}	41.13 ± 2.79	1.73 ± 0.15^{bc}	10.34 ± 0.48^{efg}
5% G + 5 mM D	25.38 ± 2.97	52.25 ± 2.78	2.38 ± 0.42	11.38 ± 1.29^{ab}	39.00 ± 3.39	1.85 ± 0.13^{c}	9.46 ± 0.53^{cde}
5% G + 25 mM S	18.38 ± 2.57	44.75 ± 4.41	2.50 ± 0.27	11.38 ± 0.65^{ab}	36.63 ± 2.17	1.83 ± 0.14^{c}	10.82 ± 0.38^{fgh}
Control 5% EG	22.13 ± 2.68	50.25 ± 3.48	2.25 ± 0.45	11.75 ± 1.28^{ab}	40.13 ± 2.51	1.16 ± 0.07^{a}	8.40 ± 0.13^{bc}
5% EG + 5 mM D	21.63 ± 2.62	46.50 ± 2.97	2.38 ± 0.38	12.00 ± 0.98^{ab}	46.00 ± 2.98	1.44 ± 0.15^{ab}	11.89 ± 0.38^{h}
5% EG + 25 mM S	18.88 ± 2.04	45.50 ± 3.41	2.50 ± 0.27	12.63 ± 0.92^{ab}	42.63 ± 4.43	1.48 ± 0.09^{ab}	9.12 ± 0.45^{cd}
Control 3% EG	23.25 ± 3.33	49.00 ± 3.11	2.13 ± 0.35	10.25 ± 1.28^{b}	44.63 ± 3.44	1.74 ± 0.09^{bc}	7.66 ± 0.52^{ab}
3% EG + 5 mM D	21.88 ± 2.88	46.88 ± 4.43	2.13 ± 0.30	11.38 ± 1.34^{ab}	45.38 ± 4.55	1.95 ± 0.22^{c}	6.98 ± 0.27^{a}
3% EG + 25 mM S	17.25 ± 2.93	41.50 ± 4.43	2.75 ± 0.16	14.13 ± 0.52^{a}	40.50 ± 5.11	1.69 ± 0.08^{bc}	6.99 ± 0.23^{a}
P	N.S.	N.S.	N.S.	<0.05	N.S.	<0.001	<0.001

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

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	94.10 ± 5.30^{abc}	76.31 ± 5.20	163.13 ± 8.77^{abc}	7.20 ± 0.37^{ab}	21.60 ± 1.08^{bc}	80.13 ± 1.43	47.88 ± 1.86	41.38 ± 0.63
7% G + 5 mM D	93.16 ± 5.87^{abc}	74.41 ± 5.73	162.11 ± 8.21^{abc}	7.24 ± 0.23^{ab}	20.69 ± 1.33^{abc}	78.88 ± 1.85	47.25 ± 1.69	42.38 ± 0.87
7% G + 25 mM S	102.45 ± 5.00^{bc}	79.71 ± 4.91	182.40 ± 9.29 ^{c}	8.33 ± 0.31^{ab}	19.01 ± 1.12^{abc}	77.00 ± 1.45	44.88 ± 1.16	40.88 ± 0.74
Control 5% G	99.53 ± 4.79^{abc}	80.34 ± 4.87	173.00 ± 8.96^{abc}	7.64 ± 0.36^{ab}	22.06 ± 0.79^{bc}	80.25 ± 1.66	47.88 ± 1.79	40.88 ± 0.30
5% G + 5 mM D	102.16 ± 4.43^{bc}	82.05 ± 4.15	178.80 ± 9.11^{bc}	7.76 ± 0.32^{ab}	22.24 ± 0.82^{bc}	79.75 ± 1.06	47.38 ± 1.08	41.25 ± 0.92
5% G + 25 mM S	107.20 ± 3.39^{c}	83.95 ± 2.70	186.71 ± 7.60^{c}	8.33 ± 0.33^{ab}	17.45 ± 1.25^{a}	76.63 ± 1.40	46.13 ± 1.41	43.63 ± 1.10
Control 5% EG	88.71 ± 3.08^{ab}	71.64 ± 2.84	156.00 ± 5.56^{ab}	6.96 ± 0.19^{a}	23.10 ± 0.89^{c}	80.00 ± 0.94	47.50 ± 0.65	41.13 ± 0.97
5% EG + 5 mM D	87.53 ± 4.19^{a}	70.53 ± 4.17	151.88 ± 6.39^{a}	13.69 ± 6.78^{b}	20.99 ± 0.79^{bc}	80.00 ± 1.46	47.75 ± 1.60	41.63 ± 1.12
5% EG + 25 mM S	94.86 ± 3.69^{abc}	74.99 ± 3.51	170.11 ± 6.26^{abc}	7.76 ± 0.27^{ab}	21.66 ± 0.99^{bc}	77.88 ± 1.13	45.63 ± 1.03	43.00 ± 0.98
Control 3% EG	95.76 ± 3.71^{abc}	77.75 ± 3.36	165.99 ± 7.59^{abc}	7.30 ± 0.28^{ab}	21.40 ± 0.77^{bc}	81.00 ± 1.31	48.63 ± 1.08	41.25 ± 0.70
3% EG + 5 mM D	96.70 ± 3.82^{abc}	72.21 ± 4.10	167.86 ± 7.16^{abc}	7.49 ± 0.26^{ab}	20.16 ± 1.34^{abc}	78.88 ± 1.38	47.38 ± 0.96	41.00 ± 0.75
3% EG + 25 mM S	92.39 ± 5.03^{ab}	72.83 ± 4.65	165.74 ± 9.21^{abc}	7.84 ± 0.24^{ab}	19.41 ± 1.32^{ab}	77.75 ± 0.98	44.88 ± 0.88	41.13 ± 0.67
P	<0.05	N.S.	<0.05	<0.05	<0.01	N.S.	N.S.	N.S.

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

Table 3
Mean (±SEM) DNA integrity and fertility results based on 60-days non-returns after rectovaginal insemination.

Groups	Tail length	Tail intensity (%)	Tail moment (μm/s)	Non-return rates (%)
Control 7% G	65.18 ± 4.19	12.38 ± 1.09^{a}	9.13 ± 0.19^{a}	55.55 (45/81)
7% G + 5 mM D	64.15 ± 3.63	10.71 ± 0.97^{bc}	7.02 ± 0.12^{b}	52.44 (43/82)
7% G + 25 mM S	64.14 ± 3.85	9.70 ± 0.89^{c}	7.48 ± 0.14^{b}	48.39 (30/62)
Control 5% G	65.86 ± 3.69	11.21 ± 1.03^{ab}	9.58 ± 0.22^{a}	45.16 (42/93)
5% G + 5 mM D	64.71 ± 3.92	10.14 ± 1.12^{bc}	7.33 ± 0.13^{b}	53.26 (49/92)
5% G + 25 mM S	65.18 ± 4.02	10.11 ± 0.94^{bc}	7.78 ± 0.27^{b}	53.42 (39/73)
Control 5% EG	64.55 ± 3.79	12.45 ± 1.24^{a}	9.18 ± 0.22^{a}	49.43 (43/87)
5% EG + 5 mM D	65.30 ± 4.02	10.23 ± 1.15^{bc}	7.23 ± 0.15^{b}	47.30 (35/74)
5% EG + 25 mM S	64.19 ± 3.85	9.03 ± 0.84^{c}	7.85 ± 0.19^{b}	50.59 (43/85)
Control 3% EG	65.13 ± 3.59	12.01 ± 1.10^{a}	9.78 ± 0.40^{a}	52.63 (30/57)
3% EG + 5 mM D	64.98 ± 3.64	11.21 ± 0.99^{ab}	7.23 ± 0.18^{b}	52.05 (38/73)
3% EG + 25 mM S	64.54 ± 3.89	11.32 ± 1.09^{ab}	7.45 ± 0.32^{b}	52.63 (40/76)
P	N.S	<0.05	<0.05	N.S.

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

Recent studies reported that the occurrences of abnormal spermatozoa are positively correlated with DNA damage [22] and spermatozoa plasma membrane damage also correlates with DNA damage [23]. The type of antioxidant which is used affects sperm DNA integrity [38]. In present study, all cryoprotectant groups (without additives) caused great damage on the tail intensity and tail moment ($P < 0.05$). These results were contrary to those reported in a previous research on bovine sperm, the antioxidants carnitine and methionine did not maintain DNA integrity [34] and the antioxidants which were used did not provide any marked improvement at DNA integrity [38]. However, according to Bucak et al. [11] and Tuncer et al. [39] addition of antioxidants in semen extender could maintain DNA integrity. According to our results, we may hypothesize that the differences among our findings may be related with the types antioxidants and antioxidant amounts that are used.

GPx is a selenocysteine containing antioxidant enzyme that plays a role in the elimination of hydrogen peroxide and is also known to be involved in the detoxification of reactive lipids [2]. In the present study, G7 and EG5 led to lowest MDA activity with or without D or S but, these results were not supported to the GPx activity ($P < 0.01$). These findings indicate that GPx activity is apparently not a major factor for eliminating to MDA level after thawing. In harmony with our findings, different doses of D (0.25, 0.5, 1 and 2 mM) had beneficial effects on endogenous antioxidant system, it also improved GPx activity [4]. This results were inconsistent with a study performed on buck [2,40] and bull [37]. Extending and freeze–thawing of semen lead to low antioxidant activity in the presence or absence of antioxidants. This may partly explain the lower fertility of frozen thawed semen in comparison to fresh semen [8]. These changes in cryopreserved semen are responsible for poor sperm viability in female reproductive tract and lower fertility rates [3]. In our study, supplementation of cryoprotectants and/or antioxidants did not present significant differences of fertility results. This results agreed with the studies which did not observe any improvement in fertility when certain additives were added to the semen extender [32,33]. Our findings indicate that 15 million spermatozoa in a straw may explain why addition of different doses of cryoprotectants and antioxidants did not increase fertility results.

In conclusion, it may be stated that, using different doses cryoprotectants and antioxidants do not have any improvement on the sperm motility after thawing. The addition of antioxidants increased the DNA integrity although there wasn't any marked effect on fertility results because of 15 million spermatozoa in a straw. The results of this study may provide an useful information for the future studies in this area.

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