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RESEARCH ARTICLE

The Comparison of Three Different Cryoprotectants in Cryopreservation of Angora Goat Semen [1][2]

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Summary

The objective of this study was to evaluate glycerol (G), ethylene glycol (EG) and dimethylsulfoxide (DMSO) which were used two different doses on in vitro semen parameters, antioxidant enzymes activities and DNA damage after the freeze-thaw process in Angora goat semen. Semen samples from 5 mature Angora goats were used in this study. A total number of 40 ejaculates were collected twice a week from the goats using an artificial vagina and the semen pooled to minimize individual variation. Each pooled ejaculate was split into 6 equal aliquots and diluted with tris base extenders supplemented with two different doses of cryoprotectants (G 3%, 6%; EG 3%, 6%; DMSO 3%, 6%). G 3% and 6% was added as a cryoprotectant had better CASA motility ($P<0.01$) and progressive motility ($P<0.001$) values when compared to EG and DMSO groups. On the other hand, EG 6% showed the best values of preserved membrane integrity ($P<0.01$). The evaluation of CASA sperm motions parameters, adverse effects were procured in the groups with DMSO groups when compared to the other groups ($P<0.05$; $P<0.001$). G 6% group was the greatest VAP, VSL and VCL values than the other groups ($P<0.05$; $P<0.001$). DNA damage was not affected by supplemented different doses of cryoprotectants as well as antioxidant activity ($P>0.05$). In conclusion, no advantages were found in using EG or DMSO to replace G for freezing of Angora goat sperm.

Keywords: *Angora goat, Antioxidant activity, Cryoprotectans, DNA integrity, Sperm freezing*

Ankara Keçisi Teke Spermasının Dondurulmasında Üç Farklı Kryoprotektanın Karşılaştırılması

Özet

Bu çalışmanın konusu, iki farklı dozlarda kullanılan gliserol (G), etilen glikol (EG) ve dimethil sulfoksidin (DMSO) Ankara keçisi teke spermasının dondurma-çözdürme sonrasındaki in vitro sperma parametrelerinde, antioksidan enzim aktivitelerinde ve DNA hasarındaki etkilerini değerlendirmekti. Çalışmada 5 yetişkin Ankara keçisinden (3 ve 4 yaşlı) spermalar kullanıldı. Haftada iki kez suni vajen kullanılarak alınan 40 ejakülat kullanıldı ve bireysel farkları azaltmak için spermalar birleştirildi. Her birleştirilen sperma 6 eşit kısma bölündü ve içerisinde iki farklı dozda kryoprotektan (G %3, %6; EG %3, %6; DMSO %3, %6) katılmış tris sulandırıcısıyla sulandırıldı. Kriyoprotektan olarak G'ün %3 ve %6 oranında eklenmesi EG ve DMSO gruplarıyla kıyaslandığında en iyi CASA motilitesi ($P<0.01$) ve progressif motilite ($P<0.001$) değerleri verdi. Diğer taraftan, membran bütünlüğünün korunmasında EG %6 en iyi sonucu verdi ($P<0.01$). CASA sperma hareket parametrelerinin değerlendirilmesinde DMSO gruplarında diğer gruplara göre daha olumsuz etkiler gözlendi ($P<0.05$; $P<0.001$). G'ün %6'lık grubu VAP, VSL ve VCL değerlerinde digger gruplara gore en yüksek değer verdi ($P<0.05$; $P<0.001$). Antioksidan aktivitesinde olduğu gibi farklı dozda kriyoprotektanların eklenmesi DNA hasarının korunmasında etkili olmadı ($P>0.05$). Sonuçta, Ankara keçisi teke spermalarının dondurulmasında G yerine EG ve DMSO'nun kullanılmasının avantajı bulunmadı.

Anahtar sözcükler: *Ankara keçisi, Antioksidan aktivite, DNA bütünlüğü, Kriyoprotektanlar, Sperma dondurma*

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INTRODUCTION

The combinations of storage temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition and hygienic control are the key factors that affect the fertilizing capacity of spermatozoa [1]. In general, a goat sperm cryopreservation medium includes a non-penetrating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant [glycerol (G), ethylene glycol (EG), or dimethyl sulfoxide (DMSO)], a buffer (Tris or Test), one or more sugars (glucose, lactose, raffinose, saccharose, or trehalose) [2]. A cryoprotectant is included in a cryopreservation medium to minimize the physical and chemical stresses resulting from the cooling, freezing, and thawing of sperm cells. Since Polge et al.[3] reported that glycerol is beneficial to sperm preservation, it has been routinely inclueded in most cryopreservation protocols for many types of cells, including mammalian spermatozoa [4,5]. Addition of G can induce osmotic damage to spermatozoa, but the extent of the damage varies according to the species. However, goat spermatozoa are reasonably tolerant to these osmotic conditions and can withstand a rapid exposure to G. G (molecular weight: 92.10) is a penetrating cryoprotectant universally used for sperm freezing [6]. Similarly to G [7], EG is an alcohol-based cryoprotector with a low molecular weight (62.07) [8]. G causes membrane lipid and protein rearrangement, which results in increased membrane fluidity and permeability for ions and increase ATP consumption, greater dehydration at lower temperature and therefore an increased ability to survive cryopreservation [9]. Moleculer weight value of DMSO (78.13) ranks among G and EG values. Since Lovelock and Bishop [10] first reported DMSO was superior to G for protecting erythrocytes during freezing, it has been widely used as a cryoproservative agent alone or in combination with other cryoprotectants [11,12]. DMSO penetrates in cells more quickly [5] and therefore, will dehydrate the spermatozoa and minimize the intracellular ice formation [12]. DMSO has a beneficial effect for sperm cryopreservation of bovine, goat and rabbit, it cause a damaging effect during freezing-thawing process of buffalo semen [13].

The objective of this study was to compare the effects of type and concentration of cryoprotectants glycerol, ethylene glycol and dimethyl sulfoxide on the plasma membrane and DNA integrity as well as antioxidant activity of cryopreserved Angora goat sperm.

MATERIAL and METHODS

Animals, Semen Collection and Chemicals

Semen samples from 5 Angora goats (3 and 4 years of age), were used in this study. The bucks, belonging to the Livestock Central Research Institute were maintained under uniform breeding conditions. A total number of 40 ejaculates were collected twice a week intervals from the goats using an artificial vagina, during the breeding season and the semen mixed to minimize individual variation. Ejaculates which met the following criteria were evaluated: volume of 0.5-2 ml; minimum sperm concentration of 3×10^9 sperm/ml; motility of 80%. Immediately following collection, the ejaculates were placed in a water bath (35°C), until evaluation in the laboratory. Semen assessment was performed within approximately 5 min following collection. Each group was replicated eight times. The experimental procedures were approved by the Animal Care Committee of Lalahan Livestock Central Research Institute. All chemicals used in this study were obtained from Sigma-Aldrich Chemical Co. (Interlab Ltd., Ankara, Turkey).

Semen Processing

A Tris-based extender (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 15% (v/v), pH 6.8) was used as the base extender. After the extender is divided into 6 equal proportions. G, EG and DMSO cryoprotectants were added into each of them at 3% and 6% rates and extenders were prepared. Then the collected ejaculates were divided into 6 equal proportions and they were diluted 1:1 (v/v) with a basic extender that 3 different cryoprotectant were added at 2 different doses. After being stored at 35°C water bath for 10 minutes the final dilution was performed to be $200x10^6$ spermatozoa/ml in plastic centrifuge tubes. Actual sperm concentrations were calculated with the aid of an accuel photometer (IMV, France). Diluted samples were equilibrated at 5°C for a period of 4 h and then loaded in 0.25 ml French straws automatically and were frozen which was described as Taşdemir et al.[14]. After being stored for at least 24 h, straws were thawed individually (37°C), for 30 s in a water bath for sperm evaluation. Sperm evaluation was performed on all semen samples immediately after thawing.

Assessment of in vitro Sperm Quality

Progressive motility was assessed using a phase-contrast microscope (×100 magnification), fitted with a warm stage maintained at 37°C. Sperm motility estimations were performed in 3 different microscopic fields for each semen sample and the mean of the 3 successive estimations recorded as the final motility score. Besides recording the subjective sperm motility, a computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyze sperm motility, progressive sperm motility and sperm motion characteristics. The method described by Bucak et al.[15]. For the evaluation of sperm abnormalities and the hypo-osmotic swelling test (HOST) were performed in the semen samples. These tests were performed by Taşdemir et al.[14] as described.

Assessment of Biochemical Assays

Semen samples were thawed in 37°C water for 20 s and they were centrifuged at 4°C at 1.000 g for 15 min in order to separate spermatozoa. Pellet was washed 3 times with a 0.5 ml of PBS. This final solution was homogenized 5 times by sonication in cold for 15 s for the Lipid Peroxidation Analysis (LPO), 120 µl of homogenate was mixed with 10 µl 0.5 mM butyl hydroxyl toluene (BHT) and kept in –80°C until the analysis. The rest of the homogenate was centrifuged at 8.000 g for 15 min and the supernatant was separated and kept in –80°C for the other enzyme analysis. Enzyme levels were determined using commercial kits by spectrophotometry (Cintra 303-UV, GBC, Australia). Biochemical assay kits were obtained from Sigma-Aldrich Chemical (Interlab Ltd., Ankara, Turkey).

Determination of Sperm DNA Damage using Comet Assay

In our study, the most commonly used alkaline comet assay parameters have been used, which are; Tail intensity (percentage of DNA in the tail compared to the percentage in the 'head' or unfragmented DNA); Tail length (the length of the tail measured from the leading edge of the head) and Tail moment (percentage of DNA in the tail - tail DNA - times the distance between the means of the tail and head fluorescence measures). Each of these parameters describes endogenous DNA damage corresponding to DNA strand breakage and/or alkali-labile sites. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. The method described by Tuncer et al.[16].

Statistical Analysis

Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared. The test revealed that the variances were homogeneous. After that, comparisons between the groups were made using analysis of variance with Duncan post hoc test. The results are expressed as means or proportions (±S.D.). P>0.05, not significant; P<0.05; P<0.01 and P<0.001.

RESULTS

As shown in *Table 1*, G 6% led the highest values CASA (P<0.01) and progressive (P<0.001) motility when compared to EG and DMSO groups. But, EG 6% showed the best values of preserved membrane integrity (P<0.01). Acrosome and total morphology were affected by the type of cryoprotectant used; DMSO (3 and 6%) resulted in greater damage than the other groups (P<0.001; P<0.01). Additionally in *Table 1*, at the evaluation of CASA sperm motions, adverse effects were procured in the groups with DMSO groups when compared to the other groups with cryoprotectants (P<0.05; P<0.001). G 6% group was the greatest values (VAP and VCL) than the other groups (P<0.05; P<0.001). Also G 3% and 6% had better VSL sperm motion parameter than the other groups (P<0.001).

As shown in *Table 1* and *Table 2*, DNA damage and antioxidant activities were not affected by supplemented different doses and different cryoprotectants as well as antioxidant activity (P>0.05).

Table 1. *Mean (±SEM) sperm parameters, CASA sperm motion parameters and chromatin damage values of detected by Comet Assay in frozen–thawed Angora goat semen*

Tablo 1. *Dondurulmuş çözdürülmüş Ankara keçisi spermasının ortalama spermatolojik parametreleri, CASA spermatozoon hareket parametreleri ve Comet testi ile tanımlanan kromatin hasarı*

Sperm Parameters	G 3%	G 6%	EG 3%	EG 6%	DMSO 3%	DMSO 6%	P
Subjective Mot. %	50.0±5.09c	58.8±2.27c	36.3±4.30b	31.3±6.03b	8.8±2.46a	15.0±4.23a	***
CASA Mot. %	25.4±4.87^{b}	36.9±4.62^{c}	27.0±2.78^{b}	30.4±4.03^{b}	11.0±2.43^{a}	14.6±1.64^{ab}	**
Prog. Motility %	5.9±1.27^{b}	9.3±1.63^{c}	5.3±1.29^{b}	5.4±0.91^{b}	0.9±0.23^{a}	2.0±0.46^{a}	***
VAP (µm/s)	86.4±3.57^{b}	92.2±2.38^{c}	77.9±2.81^{b}	80.6±1.79^{b}	51.9±2.21^{a}	57.3±1.26^{a}	*
VSL (µm/s)	66.3±2.68^{c}	70.4±2.57^{c}	59.5±2.57^{b}	58.2±1.50^{b}	36.5±2.02^{a}	41.1±0.70^{a}	***
VCL(µm/s)	178.9±7.98^{bc}	192.1±5.75^{d}	164.0±6.00^{b}	175.8±3.45^{bc}	118.1±5.12^{a}	128.7±3.20^{a}	***
ALH (µm)	8.9±0.28^{b}	9.4±0.27^{b}	8.8±0.29^{b}	8.6±0.28^{b}	7.2±0.50^{a}	7.3±0.29^{a}	***
STR	73.1±0.52^{c}	72.6±1.18^{bc}	72.1±0.72^{bc}	71.3±0.73^{bc}	66.1±1.48^{a}	69.0±0.71^{a}	***
LIN (%)	36.4±0.63^{c}	36.1±0.81^{c}	35.1±0.72^{c}	33.9±0.44^{ab}	30.3±0.84^{a}	31.3±0.53^{ab}	***
HOST %	48.0±4.39^{b}	49.9±3.22^{b}	50.1±3.61^{b}	56.0±2.77^{c}	34.3±2.89^{a}	41.9±2.14^{a}	**
Acrosome %	4.0±0.57^{b}	3.1±0.52^{ab}	2.8±0.37^{ab}	2.3±0.25^{a}	6.5±0.63^{c}	6.0±0.54^{c}	***
Total Morphology %	13.1±0.52^{b}	12.9±1.11^{b}	8.9±0.69^{a}	10.1±0.81^{a}	21.0±1.49^{c}	20.3±0.75^{c}	**
Tail Length (µm)	101.86±8.42	90.22±10.73	88.01±11.84	93.77±9.23	98.25±6.82	95.31±8.05	N.S
Tail Intensity (%)	19.14±2.56	16.34±2.65	16.95±3.85	17.43±3.05	19.36±3.99	15.79±1.81	N.S
Tail Moment (µm.%)	11.83±2.52	9.40±2.15	10.04±2.88	11.85±3.11	11.69±3.27	9.60±1.87	N.S

a-d: *Different superscripts within the same row demonstrate significant differences among groups, * P<0.05, ** P<0.01, *** P<0.001,* ***NS:*** *No significant difference (P>0.05)*

Table 2. *Mean (±SEM) glutathione peroxidase (GPx), lipid peroxidation (LPO), glutathione (GSH), catalase (CAT) and total antioxidant levels in frozen–thawed Angora goat semen*

Tablo 2. *Dondurulmuş çözdürülmüş Ankara keçisi spermasında ortalama glutatyon peroksidaz (GPx), lipid peroksidasyonon (LPO), glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Antioxidant	G 3%	G 6%	EG 3%	EG 6%	DMSO 3%	DMSO 6%	P
GPx (mU/ml-10^9 cell/ml)	11.8±0.42	11.6±0.13	11.7±0.15	12.4±0.53	11.7±0.20	11.3±0.13	N.S
LPO (mU/ml-10^9 cell/ml)	4.1±1.48	2.1±0.45	1.8±0.43	1.9±0.47	1.9±0.63	2.0±0.53	
GSH (mU/ml-10^9 cell/ml)	37.2±3.97	46.7±8.78	45.1±4.61	40.9±5.92	43.4±4.44	38.2±4.33	
Catalase (mU/ml-10^9 cell/ml)	23.4±5.13	14.5±1.05	14.9±1.11	17.0±2.91	15.7±1.20	16.2±1.05	
Total Antioxidant (mmol/trılox/ml-10^9 cell/ml)	0.9±0.28	0.4±0.19	0.7±0.29	0.4±0.26	0.5±0.26	0.6±0.33	

NS: *No significant difference (P>0.05)*

DISCUSSION

The cryoprotectants are added to extenders to protect the sperm from damage during freezing process [17]. The level and type of cryoprotectants in semen diluent influence these events and their effects on the sperm cells during freezing [18]. G can cause great osmotic damage to spermatozoa because G passes through the sperm membrane much slower than other cryoprotectants [19,20]. However, the literature suggests that other cryoprotectants, such as DMSO has been shown to protect spermatozoa against cryodamage as well as G [10]. Other researchers believe that a low-molecular-weight cryoprotectant, such as EG, may cause less damage to spermatozoa than G [21]. EG can be used as a cryoprotectant agent as opposed to G in dogs [22] and in horse sperm [23]. Researchers addded G, EG, PG, and DMSO as cryoprotectants to the extender for freezing of Red deer epididymal sperm and DMSO showed the highest toxicity and G showed the lowest [24]. G, EG, and DMSO are generally used in a range of 1-8%, but the greatest recovery of sperm post-thawing has been achieved with G [11,17,25]. This study showed that using EG and DMSO to replace G as a cryoprotectant did not improve the sperm's motility after thawing. The highest subjective sperm motility rates are obtained at the groups that 3% (50.0±5.09%) and 6% (58.8±2.27%) G was added and the groups with 3% and 6% DMSO had the lowest values (8.8±2.46%, 15.0±4.23%; respectively). Similar with our result that researchers [11] determined the highest sperm motility (35%) in the extender which 6% G was added. In the same study diffferent from ours, they found DMSO was (21%) more successful when compared with EG (13%). There is a research [18] showed that post-thawing and freezability in buffalo semen extended with G or DMSO was significantly better than EG. Other researchers [17] showed that both motility and percentage of live goat bucks spermatozoa were improved with a combination of G + DMSO. Awad [26] suggested that the CASA values (VAP, VSL, VCL and LIN) were affected by the type and concentration of the cryoprotectant. In addition, he reported that the VAP and VCL values were higher in the test groups containing G and EG and that the VSL and LIN values were higher in the high concentration of G group. However, the CASA values did not differ among any of the groups with various concentrations of EG. In our study, the CASA values were different between each group. The greater values of VAP and VCL shown in the G 6% group and VSL shown in the G 3% and 6% groups. Freitas et al.[27] and Muino et al.[28] showed that semen with rapid and progressive sperm had the best post-thaw sperm longevity. Singh et al.[17] had used varying concentrations of G, DMSO, G+DMSO and G+lactose in different extenders for freezing sperm of different goat races. Both motility and the percentage of live spermatozoa were most affected by extenders containing only DMSO. Acrosomal and tail abnormalities tended to increase between post equilibration and post thawing stage, and were higher in extenders containing the higher levels of DMSO. Significantly lower percentages of abnormalities were recorded in the G+lactose extenders. Whereas in our study, the best protection against total morphological defects were found in the group that EG 3% and 6% were added, also acrosome defect was found in the groups 6% G, 3% and 6% EG were added and there was a lot of damage in the DMSO groups (3% ve 6%).

In a study [17], G and DMSO combined use have given better results for viability and motility in a goat sperm freezing study, 2% G and 4% DMSO combined use causes less damage in the acrosome structure after freezing thawing in a rabbit sperm [29], 3% EG used in stallion sperm [23] freezing has been shown to represent better cryoprotectant effect than other concentrations (6% and 9%) and also reported that EG has better results than G. EG (0.5M) was efficient for freezing ram sperm, allowing post-thaw motility similar to G (0.72M) but with a high number of intact acrosomes [6]. EG could provide similar or better results than those obtained with G during cryopreservation of ram semen [30]. G has osmotic and toxic effects on the plasma membrane and metabolism of cryopreserved cells. It is responsible for the disorganization of sperm plasma membrane and reducing motility and fertilizing ability. Higher concentrations of G lead to cell death [6]. EG has fewer detrimental effects on the viability and motility of spermatozoa [31], providing a better protective effect to the acrosome than G. In a ram sperm freezing study [32] reported that no difference was observed between EG and G for acrossome status and sperm motility. The sperm

cells that were preserved with EG showed more integrity of the plasmatic, nuclear and mitochondrial membranes. From the viewpoint of cell membrane integrity, it can be concluded that EG gives higher protection to the sperm cell than G. According to researchers [33], G enhances preservation of the acrosome and plasma membrane of ram spermatozoa, despite being toxic when used in high concentrations. Cooling and freeze–thawing produce physical, chemical and oxidative stress on the sperm membrane, which result in reduced sperm viability and fertilizing ability [2]. Similar to the findings of the literature, plasma membrane integrity and acrosomal defects were less deteriorated at the groups that EG 6% was added and less acrosomal defects were detected at both EG groups (3-6%) and at 6% G group.

Oxidative stress is a cellular condition generally characterized by an imbalance between the production of ROS and the scavenging capacity of the antioxidants. When the production of ROS exceeds the available antioxidant defense system, significant oxidative damage occurs to the sperm organelles through the damage of lipids, proteins and DNA [34]. Cryopreservation of spermatozoa enhances oxidative stress, which not only disrupts the motility and fertilizing ability of spermatozoa, but also increases DNA damage [35]. On the other hand, DNA integrity was not affected by type of cryoprotectant in our study. In other study [36] it is reported that, despite the temperature difference causes less change on the sperm morphology during the freezing and thawing process, it reduces the spermatozoa acrosome reaction and alters the DNA structure. In addition, 1.2-3% DNA damage is normal in high fertility characteristic bulls [37]. It is shown on various studies that, freezing and thawing process on ram [38] and bull [39] sperms cause permanent structural alterations on DNA and this causes fertility problems. According to Taşdemir et al.[40] the DNA integrity was also affected by the type of cryoprotectant used in bull. DMSO 6% and EG+DMSO 3% resulted in more sperm with damaged DNA than the other groups. In a study [41] reported that total abnormal spermatozoa frequencies are positively correlated with DNA damage. Spermatozoa plasma membrane damage is also correlated with DNA damage. In our study, in terms of the plasma membrane integrity, even though the 6% EG group has yielded statistically significant results but it could not gain an advantage over preventing DNA damage when compared with the other groups which 3 different cryoprotectants were added at different doses to the extender.

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 [42]. Ultrastructural 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 ram [43], bull [44] and goat [45] sperm. Antioxidant mechanisms exist to maintain defense against oxidative stress-induced damages in semen [46,47]. However, the antioxidant capacity of sperm cells is insufficient in preventing oxidative stress during the freeze-thawing process [48]. In our study, there were no statistically significant difference in protecting GPx, LPO, GSH, CAT and total antioxidant levels between G, EG and DMSO which are added to basic Tris extender as cryoprotectants at 2 different doses ($P>0.05$). Taşdemir et al.[40] have founded the antioxidant activities of GPx, GSH, and CAT as well as the total antioxidant activity were affected by the type of cryoprotectant; notably, G+EG+DMSO 3% yielded the lowest activities when compared to the other groups ($P<0.001$) in Eastern Anatolian Red bull sperm. Aisen et al.[49] reported that the extender containing trehalose enhanced the level of GSH and decreased the oxidative stress provoked by the freeze-thaw process in ram semen. Atessahin et al.[50] found that an extender supplemented with trehalose increased the GSH-Px and CAT activity of frozen-thawed goat semen. Increasing the doses of trehalose resulted in greater activity of CAT and a marked improvement in bovine sperm motility [51]. Those reports were in contrast with our findings, the reason of this maybe using different male animals or using different species or the differences at composition of extenders.

Many membrane permeable cryoprotectants (G, EG and DMSO), and their combinations, have been tested with different goat breed sperm [11,17,25,52-54], but the most frequently used penetrating cryoprotectant is G, as our research.

While adding G to the extender at 3% or 6% as a cryprotectant, it had positive effect on sperm motility when compared with EG and DMSO, but also DMSO caused retardation in the sperm motion (VAP, VSL, VCL ve ALH) parameters compared with the other cryoprotectants. Highest protection was provided in the group which 6% EG was added in protecting membrane integrity. When total morphological evaluations were considered, fewer morphological defects were determined in the groups which EG were added at 3% and 6% rates. All three cryoprotectants didn't outmaneuver to one another both protecting DNA damage and protecting antioxidant activities. In conclusion, no advantages were found in using EG or DMSO to replace G for freezing of Angora goat sperm.

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