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Research Article

The Effect of Ascorbic Acid on the Freezability of Ram Semen Diluted with Extenders Containing Different Proportions of Glycerol*

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Abstract: This study was conducted to investigate the effects of ascorbic acid on the freezability and spermatological characteristics of ram semen diluted with Tris-glucose-egg yolk based extenders containing different proportions of glycerol.

Six Akkaraman rams were used. Semen was collected by electroejaculator once every 3 days. The spermatological characteristics of the collected semen samples were determined. Pooled and diluted semen was cooled slowly to + 4 ºC over 2 h. Cooled semen was divided into 20 groups. Each of the 20 semen groups was diluted at a 1:1 ratio with one of the extender groups containing different proportions of glycerol (0%, 1%, 3%, 5%, and 7%) and ascorbic acid (0, 0.5, 1 and 2 mg/ml). Diluted semen was packaged in 0.25 ml French straws and equilibrated for 4 h before freezing. The semen in straws was frozen in liquid nitrogen vapor. The semen was thawed for 25 s at +38 ºC.

There was no significant difference in the spermatological characteristics depending on the increase in the proportions of acorbic acid after glycerolization-equilibration and freezing-thawing. While the increase in glycerol level in diluted semen reduced motility ($P < 0.05$), it raised the dead spermatozoa rate, and damaged the acrosome rate and total abnormal spermatozoa rate ($P < 0.05$) after glycerolization and equilibration. However, the increase in the glycerol level in diluted semen raised motility, and damaged the acrosome rate and total abnormal spermatozoa rate ($P < 0.05$), while it reduced the dead spermatozoa rate ($P < 0.05$) after freezing and thawing compared to the control diluent.

In conclusion, the best results after freezing and thawing were observed in diluted semen groups containing 5% glycerol although the increase in the glycerol level (3%-7%) after glycerolization and equilibration in diluted semen had negative effects on spermatological characteristics. Moreover, the increase in the proportions of ascorbic acid in extended semen did not change any spermatological characteristics.

Key Words: Ram, semen, freezing, ascorbic acid, glycerol

Farklı Oranlarda Gliserol İlave Edilen Sulandırıcılarla Sulandırılan Koç Spermasının Dondurulabilirliği Üzerine Askorbik Asitin Etkisi

Özet: Bu çalışma, farklı oranlarda gliserol içeren Tris–glikoz–yumurta sarısı sulandırıcısıyla sulandırılmış koç spermasının dondurulabilirliği ve spermatolojik özellikleri üzerine askorbik asit'in etkisini araştırmak amacıyla yapılmıştır.

Çalışmada altı Akkaraman koç kullanıldı. Sperma, elektroejakülatör yardımıyla 3 günde bir alındı. Alınan sperma örneklerinde spermatolojik özellikler incelendi. Pooling yapılan sperma örnekleri sulandırıldıktan sonra 2 saat içerisinde +4 ºC'ye düşürüldü. Soğutulmuş sperma 20 eşit kısma ayrılıp, her bir kısım farklı oranlarda gliserol (%0, 1%, 3%, 5% and 7%) ve askorbik asit (0, 0,5, 1, 2 mg/ml) içeren 20 farklı sulandırıcıdan biriyle 1:1 oranında sulandırıldı. Sulandırılmış sperma 0,25'lik Fransız payetlerinde 4 saat bekletildikten sonra sıvı azot buharında dondurudu. Dondurulan spermalar, +38 ºC'de 25 saniyede çözdürüldü.

Gruplar arasında, askorbik asit miktarının artışına bağlı olarak spermatolojik özelliklerde herhangi bir farklılık görülmedi. Gliserolizasyon ve ekilibrasyon sonrası, gliserol oranının artışına bağlı olarak akrozoma bağlı anormal spermatozoon, toplam anormal spermatozoon ve ölü spermatozoon oranları yükselirken ($P < 0,05$), spermatozoon motilitesinin düştüğü ($P < 0,05$) görüldü. Spermanın dondurulup çözdürülmesi sonucu gliserol oranının artışına bağlı olarak akrozoma bağlı anormal spermatozoon oranı, toplam anormal spermatozoon oranı ve spermatozoon motilitesi yükselirken ($P < 0,05$) ölü spermatozoon oranı azaldı ($P < 0,05$).

Sonuç olarak koç spermasında gliserolizasyon ve ekilibrasyon sonrası, gliserol oranının artışı (%3-%7) spermatolojik özellikleri olumsuz yönde etkilemiş olmasına karşın dondurup çözdürme sonucu en iyi sonuçlar %5 gliserol katılan gruplardan elde edilmiştir. Dondurup çözdürme sonucu askorbik asit'in spermatolojik özellikler üzerine önemli bir etkisinin olmadığı bulunmuştur.

Anahtar Sözcükler: Koç, sperma, dondurma, askorbik asit, gliserol

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Introduction

The success of long-term storage of spermatozoa depends on the dilution of semen with a medium containing cryoprotectants to protect the cells from the stresses associated with freezing and thawing. Glycerol has been used successfully as a cryoprotectant in the freezing of diluted ram semen (1,2). Freezing semen in a diluent without glycerol may allow the formation of intracellular ice crystals that could damage the sperm structure during thawing (3,4). However, the addition of glycerol to diluents for the frozen storage of ram semen is ultimately limited by its toxicity (5,6).

Ram semen is routinely diluted with an extender without glycerol soon after collection and slowly cooled to +5 °C to maintain the viability of healthy spermatozoa, but the addition of glycerol to cooled semen for freezing reduces motility and acrosomal integrity (7-10). Therefore, the maintenance of the acrosomal integrity of ram spermatozoa requires lower glycerol levels (11,12).

An important reason for the decrease in motility during the storage of semen is the formation of lipid peroxides from oxygen radicals. The sperm plasma membrane contains a high amount of unsaturated fatty acids. Therefore, it is particularly susceptible to peroxidative damage. The lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa, and it is associated with a loss of motility and membrane integrity (13-15). Furthermore, deep freezing increases sensitivity to lipid peroxidation, and frozen-thawed semen is more susceptible to lipid peroxidation than fresh semen (16,17).

Semen contains appreciable amounts of antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (18). However, the endogenous antioxidative capacity of semen may be insufficient during storage or dilution (19). In vitro studies (20,21) suggested that the addition of some antioxidants to diluted semen could improve the motility and survival of spermatozoa, and it might allow the use of lower glycerol concentrations.

Ascorbic acid (vitamin C) is an antioxidant substance. It is present in the epididymal fluid and seminal plasma of several species including the ram, and it is a protective vitamin in the epididymis (22). Ascorbic acid may play a role in protecting sperm from reactive oxygen species (23) and in maintaining the genetic integrity of sperm cells by preventing oxidative damage to sperm DNA (24).

The objective of this study was to investigate the effects of ascorbic acid on the freezability and spermatological characteristics of ram semen diluted with extenders containing different proportions of glycerol.

Materials and Methods

Animals and Semen Collection

Six Akkaraman rams aged 2 years were used. The experiments were performed around Elazığ province in Turkey (latitude 38° 40'N), where October and November are included in the breeding season. Semen was collected from the rams by electroejaculator once every 3 days.

Semen Processing

All semen samples showing less than 70% motility were discarded. After the spermatological characteristics of each ram were determined, the ejaculates were pooled and diluted at a 1:4 ratio (semen:diluent) at +37 °C with Tris extender. The diluent contained tris (hydroxymethyl) aminomethane (3.63 g), glucose (0.50 g), citric acid (1.99 g) and egg yolk (15%). All chemicals used were purchased from Merck Chem. Company (Maslak, İstanbul, Turkey). Diluted semen was cooled gradually to +4 °C within 2 h.

In a preliminary study, the concentration of ascorbic acid to be investigated in detail in the main experiment was determined. Cooled semen was split into 6 parts and different amounts of ascorbic acid (0 (control), 0.5,1, 2, 5 and 10 mg/ml) were added to each group. Motility and pH were evaluated 0, 2, 4, 8, 16 and 24 h after dilution.

Main Experiment

Diluted and cooled semen was split into 20 parts. Each part was diluted at a 1:1 ratio with one of the extender groups containing different proportions of glycerol (0%, 1%, 3%, 5% and 7%) and ascorbic acid (0, 0.5, 1, 2 mg/ml). Extended semen was packaged in 0.25 ml French straws. The semen was allowed to equilibrate for 4 h before freezing. The semen in straws was frozen in liquid nitrogen vapor. They were thawed in a water bath at +38 °C for 25 s.

Semen Examination

Motility, dead spermatozoa rates, damaged acrosome rates and total abnormal spermatozoa rates were determined following cooling to +4 °C, glycerolization-equilibration and freezing-thawing.

Individual sperm motility was assessed subjectively under a phase-contrast microscope (magnification X400) equipped with a heated stage adjusted to +37 °C. Motility estimations were performed from 5 different fields in each sample by the same person throughout the study; the mean value averaged from 5 successive estimations was used as the final motility score.

To evaluate the live/dead spermatozoa rate, eosin-nigrosin preparations were made according to the method described by Bearden and Fuquay (25). A total of 100 sperm cells were counted on each slide at X400 magnification.

The total abnormal spermatozoa rate and the proportion of the sperm cells with damaged acrosomes were determined with Hancock solution (26). For morphological assessment, a drop of semen fluid was added to 500 µl of Hancock solution. One drop of this mixture was put on a slide and covered. A total of 400 sperm cells were counted on each slide. The morphology of the spermatozoa was assessed under phase contrast microscopy (magnification X1500, oil immersion).

Statistical Analyses

All values were calculated as means ± standard errors of the means (± SEM), and P value < 0.05 was considered significant. The data for motility, dead spermatozoa rate, damaged acrosome rate and total abnormal spermatozoa rate were examined by Fisher's post hoc test following one-way analyses of variance (ANOVA) using the SPSS/PC computer program.

Results

In the preliminary study, ascorbic acid at concentrations of 0.5, 1 and 2 mg/ml in diluent during the storage of semen at +4 °C did not affect the motility of spermatozoa or pH (P > 0.05) compared to the control group. However, ascorbic acid at concentrations of 5 and 10 mg/ml in the diluted semen significantly decreased (P < 0.05) motility (Figure 1) and pH (Figure 2).

In the main experiment there was no significant difference in motility, acrosomal integrity, total abnormal spermatozoa rate or dead spermatozoa rate depending on the increase in the proportions of ascorbic acid in the diluted semen groups containing the same glycerol levels after equilibration (Table 1).

The percentage of progressively motile spermatozoa in the A1 control group (without glycerol and ascorbic acid) was 79.0 ± 0.77% after equilibration, and the increase in the glycerol level significantly decreased motility in the C4, D4 and E4 groups compared to the A1 control group (Table 1).

The lowest total abnormal spermatozoa rate and damaged acrosome rate were 8.96 ± 0.04% and 2.84 ± 0.03% in the A3 group (0% glycerol-1 mg ascorbic acid) after equilibration. The increase in the glycerol level significantly raised (P < 0.05) the total abnormal spermatozoa rate and damaged acrosome rate in the C, D

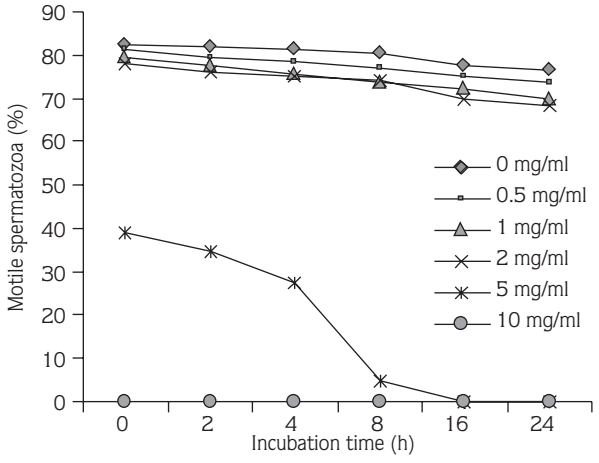

Figure 1. Changes in the percentage of motile spermatozoa during liquid storage at +4 ºC following dilution of semen in extender containing different proportions of ascorbic acid.

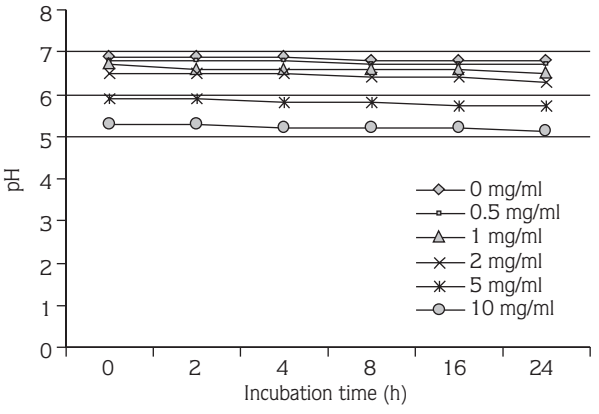

Figure 2. Changes in pH during liquid storage at +4 ºC following dilution of semen in extender containing different proportions of ascorbic acid.

Table 1. Spermatological characteristics of ram semen extended in Tris-based diluents containing varying concentrations of ascorbic acid and glycerol after glycerolization-equilibration.

Groups	Motility (%)	Damaged Acrosome Rate (%)	Total Abnormal Spermatozoa Rate (%	Dead Spermatozoa Rate (%)
A1 (0% G - 0 mg/ml Aa)	79.0 ± 0.77^{a}	2.90 ± 0.04^{a}	8.98 ± 0.06^{a}	6.62 ± 0.16^{a}
A2 (0% G - 0.5 mg/ml Aa)	78.8 ± 0.68^{a}	2.88 ± 0.03^{a}	8.96 ± 0.06^{a}	6.78 ± 0.15^{a}
A3 (0% G - 1 mg/ml Aa)	78.0 ± 0.70^{ab}	2.84 ± 0.03^{a}	8.96 ± 0.04^{a}	6.84 ± 0.17^{a}
A4 (0% G - 2 mg/ml Aa)	77.2 ± 0.70^{abc}	3.00 ± 0.04^{a}	9.10 ± 0.05^{a}	7.08 ± 0.16^{a}
Average (0% Glycerol)	78.25 ± 0.36^{1}	2.91 ± 0.19^{1}	9.00 ± 0.03^{1}	6.83 ± 0.08^{1}
B1 (1% G - 0 mg/ml Aa	77.8 ± 0.72^{ab}	3.08 ± 0.06^{a}	9.12 ± 0.08^{a}	8.86 ± 0.22^{ab}
B2 (1% G - 0.5 mg/ml Aa	77.6 ± 0.67^{abc}	3.12 ± 0.05^{a}	9.13 ± 0.05^{a}	8.70 ± 0.23^{ab}
B3 (1% G - 1 mg/ml Aa	76.4 ± 0.74^{abcd}	3.01 ± 0.05^{a}	9.11 ± 0.06^{a}	8.94 ± 0.22^{ab}
B4 (1% G - 2 mg/ml Aa	74.0 ± 0.81^{abcd}	3.34 ± 0.07^{a}	9.28 ± 0.05^{a}	9.22 ± 0.22^{ab}
Average (1% Glycerol	76.45 ± 0.38^{1}	3.14 ± 0.22^{1}	9.16 ± 0.31^{1}	8.93 ± 0.11^{12}
C1 (3% G - 0 mg/ml Aa	77.0 ± .71^{abc}	5.83 ± 0.09^{a}	11.47 ± 0.63^{a}	14.24 ± 0.28^{bc}
C2 (3% G - 0.5 mg/ml Aa	76.2 ± 0.69^{abcd}	5.96 ± 0.11^{a}	12.00 ± 0.14^{a}	14.04 ± 0.24^{bc}
C3 (3% G - 1 mg/ml Aa	74.8 ± 0.71^{abcd}	5.77 ± 0.07^{a}	11.91 ± 0.12^{a}	13.80 ± 0.19^{bc}
C4 (3% G - 2 mg/ml Aa	71.8 ± 0.62^{bcd}	6.22 ± 0.09^{a}	12.50 ± 0.20^{a}	14.38 ± 0.26^{bc}
Average (3% Glycerol	74.95 ± 0.37^{1}	5.94 ± 0.33^{1}	11.97 ± 0.18^{1}	14.12 ± 0.12^{23}
D1 (5% G - 0 mg/ml Aa	74.2 ± 0.81^{abcd}	13.51 ± 0.24^{b}	19.60 ± 0.38^{b}	15.02 ± 0.28^{c}
D2 (5% G - 0.5 mg/ml Aa	75.4 ± 0.71^{abcd}	13.56 ± 0.17^{b}	19.56 ± 0.25^{b}	14.74 ± 0.25^{c}
D3 (5% G - 1 mg/ml Aa	72.6 ± 0.69^{abcd}	12.90 ± 0.18^{b}	19.02 ± 0.23^{b}	14.64 ± 0.22^{c}
D4 (5% G - 2 mg/ml Aa	70.4 ± 0.70^{cd}	14.04 ± 0.12^{b}	20.08 ± 0.35^{b}	16.58 ± 0.28^{c}
Average (5% Glycerol	73.15 ± 0.39^{1}	13.50 ± 0.11^{2}	19.57 ± 0.17^{2}	15.25 ± 0.18^{23}
E1 (%7 G - 0 mg/ml Aa	72.6 ± 0.89^{abcd}	16.04 ± 0.37^{b}	23.90 ± 0.43^{b}	16.58 ± 0.29^{c}
E2 (%7 G - 0.5 mg/ml Aa	73.2 ± 0.78^{abcd}	16.08 ± 0.39^{b}	23.92 ± 0.49^{b}	15.94 ± 0.18^{c}
E3 (%7 G - 1 mg/ml Aa	74.2 ± 0.71^{abcd}	15.60 ± 0.35^{b}	23.24 ± 0.43^{b}	15.74 ± 0.16^{c}
E4 (%7 G - 2 mg/ml Aa	69.4 ± 0.78^{d}	16.58 ± 0.38^{b}	24.80 ± 0.47^{b}	16.30 ± 0.24^{c}
Average (7% Glycerol	72.35 ± 0.41^{1}	16.08 ± 0.19^{2}	23.97 ± 0.24^{2}	16.14 ± 0.12^{3}

G: Glycerol Aa: Ascorbic acid

^{a,b,c,d} Means in the same column followed by different letters are significantly different ($P < 0.05$).

^{1,2,3} Means in the same column followed by different numbers are significantly different ($P < 0.05$).

The results are expressed as average values (± SEM).

and E groups compared to the A1 control group after equilibration (Table 1).

The lowest dead spermatozoa rate was 6.62 ± 0.16% in the A1 control group after equilibration. The increase in the glycerol level significantly raised ($P < 0.05$) the dead spermatozoa rate in the C, D and E groups compared to the A1 control group (Table 1).

The addition of different proportions of ascorbic acid to the diluted semen groups containing the same glycerol levels did not affect the motility, acrosomal integrity, total abnormal spermatozoa rate or dead spermatozoa rate of frozen-thawed ram semen (Table 2).

The highest percentage of progressively motile spermatozoa was 51.4 ± 0.70% in the D3 group (5% glycerol and 1 mg/ml ascorbic acid) after thawing. The

Table 2. Spermatological characteristics of ram semen extended in Tris-based diluents containing varying concentrations of ascorbic acid and glycerol after freezing-thawing.

Groups	Motility (%)	Damaged Acrosome Rate (%)	Total Abnormal Spermatozoa Rate (%	Dead Spermatozoa Rate (%)
A1 (0% G - 0 mg/ml Aa	1.3 ± 0.31^{a}	14.41 ± 0.61^{a}	21.12 ± 0.70^{a}	97.20 ± 0.39^{a}
A2 (0% G - 0.5 mg/ml Aa	1.0 ± 0.29^{a}	14.02 ± 0.54^{a}	22.78 ± 0.57^{a}	97.48 ± 0.30^{a}
A3 (0% G - 1 mg/ml Aa	1.5 ± 0.33^{a}	12.40 ± 0.52^{a}	20.22 ± 0.58^{a}	97.00 ± 0.40^{a}
A4 (0% G - 2 mg/ml Aa	1.0 ± 0.29^{a}	16.14 ± 0.43^{a}	24.28 ± 0.50^{a}	97.64 ± 0.24^{a}
Average (0% Glycerol	1.20 ± 0.15^{1}	14.24 ± 0.33^{1}	22.10 ± 0.38^{1}	97.33 ± 0.17^{1}
B1 (1% G- 0 mg/ml Aa	15.1 ± 0.83^{b}	18.14 ± 0.43^{a}	27.14 ± 0.49^{abc}	81.32 ± 0.68^{b}
B2 (1% G - 0.5 mg/ml Aa	15.6 ± 0.80^{b}	17.34 ± 0.52^{a}	26.30 ± 0.47^{ab}	81.93 ± 0.56^{b}
B3 (1% G - 1 mg/ml Aa	17.0 ± 0.81^{b}	16.98 ± 0.43^{a}	25.56 ± 0.47^{a}	80.72 ± 0.52^{b}
B4 (1% G - 2 mg/ml Aa	14.2 ± 0.79^{b}	18.60 ± 0.44^{a}	27.80 ± 0.45^{abc}	83.04 ± 0.36^{b}
Average (1% Glycerol	15.48 ± 0.41^{2}	17.77 ± 0.24^{1}	26.70 ± 0.26^{1}	81.75 ± 0.29^{2}
C1 (3% G - 0 mg/ml Aa	33.0 ± 1.41^{c}	29.10 ± 0.50^{b}	35.70 ± 1.54^{d}	60.45 ± 0.60^{c}
C2 (3% G - 0.5 mg/ml Aa	34.0 ± 1.34^{c}	30.02 ± 0.69^{b}	34.60 ± 0.77^{cd}	59.96 ± 0.52^{c}
C3 (3% G - 1 mg/ml Aa	35.4 ± 1.28^{c}	28.58 ± 0.48^{b}	33.32 ± 0.68^{bcd}	58.84 ± 0.42^{c}
C4 (3% G - 2 mg/ml Aa	31.6 ± 1.29^{c}	31.00 ± 0.60^{bcd}	36.82 ± 0.99^{d}	61.10 ±0.49^{c}
Average (3% Glycerol	33.50 ± 0.67^{3}	29.68 ± 0.31^{2}	35.11 ± 0.55^{2}	60.09 ± 0.28^{3}
D1 (5% G - 0 mg/ml Aa	50.2 ± 0.78^{de}	33.12 ± 0.52^{bcd}	38.70 ± 0.93^{d}	40.80 ± 0.69^{de}
D2 (5% G - 0.5 mg/ml Aa	49.2 ± 0.75^{def}	31.54 ± 0.71^{bcd}	36.66 ± 0.79^{d}	40.06 ± 0.72^{de}
D3 (5% G - 1 mg/ml Aa	51.4 ± 0.70^{d}	31.00 ± 0.54^{bcd}	35.75 ± 0.69^{d}	38.60 ± 0.49^{d}
D4 (5% G - 2 mg/ml Aa	47.4 ± 0.85^{def}	32.29 ± 0.61^{bcd}	38.06 ± 0.99^{d}	40.32 ± 0.53^{de}
Average (5% Glycerol	49.55 ± 0.40^{4}	31.99 ± 0.32^{2}	37.29 ± 0.45^{2}	39.95 ± 0.31^{4}
E1 (%7 G - 0 mg/ml Aa	43.8 ± 0.85^{ef}	40.16 ± 0.87^{e}	48.10 ± 0.73^{e}	44.63 ± 0.87^{de}
E2 (%7 G - 0.5 mg/ml Aa	42.8 ± 0.86^{f}	38.57 ± 0.93^{de}	46.98 ± 0.94^{e}	45.62 ± 0.77^{de}
E3 (%7 G - 1 mg/ml Aa	44.6 ± 0.91^{def}	37.56 ± 0.92^{cde}	44.84 ± 0.97^{e}	46.58 ± 0.61^{e}
E4 (%7 G - 2 mg/ml Aa	43.6 ± 0.85^{ef}	42.72 ± 1.33^{e}	49.90 ± 1.36^{e}	47.72 ± 0.57^{e}
Average (7% Glycerol	43.70 ± 0.43^{4}	39.75 ± 0.59^{3}	47.46 ± 0.57^{3}	46.14 ± 0.39^{4}

G: Glycerol Aa: Ascorbic acid

^{a,b,c,d,e,f} Means in the same column followed by different letters are significantly different ($P < 0.05$).

^{1,2,3,4} Means in the same column followed by different numbers are significantly different ($P < 0.05$).

The results are expressed as average values (± SEM).

increase in the glycerol level significantly raised ($P < 0.05$) the motility in the B, C, D and E groups compared to the A1 group (without glycerol) (Table 2).

The lowest total abnormal spermatozoa rate and damaged acrosome rate were 20.22 ± 0.58% and 12.40 ± 0.52% in the A3 group (0% glycerol-0.5 mg/ml ascorbic acid) after thawing. The increase in the glycerol level significantly raised ($P < 0.05$) the total abnormal spermatozoa rate and damaged acrosome rate in the C, D and E groups compared to the A1 control group (Table 2).

The lowest dead spermatozoa rate was 38.60 ± 0.49% in the D3 group (3% glycerol-1mg/ml ascorbic acid) after thawing. The increase in the glycerol level significantly reduced ($P < 0.05$) the dead spermatozoa rate in the B, C, D and E groups compared to the A1 control group (Table 2).

Discussion

Cryopreservation causes a reduction in sperm motility and viability after glycerolization and equilibration. The reduced motility of cryopreserved sperm has been attributed to the toxicity of the cryoprotective agents used in the freezing diluent (5), to membrane changes during the freezing-thawing process (1) and to the presence of free radicals, which may induce lipid peroxidation (13,14). The present experiments examined the freezability and spermatological characteristics of ram semen diluted with Tris-based diluents containing different concentrations of ascorbic acid and glycerol.

Sanchez-Partida et al. (21) reported that the inclusion of ascorbic acid at concentrations of 50 mM or 100 mM in diluted ram semen reduced the percentage of motile spermatozoa compared to the control diluent. Similarly, Aurich et al. (27) reported that ascorbic acid at a concentration of 0.9 g/l in diluted stallion semen decreased the percentage of progressively motile spermatozoa. In this study, the addition of more than 2 mg/ml of ascorbic acid in diluted semen groups reduced the motility of spermatozoa during the liquid storage of diluted semen at +4 ^{o}C. It might be related to the decrease in pH caused by ascorbic acid since the ascorbic acid was strongly acidic (10% solution: pH 2), and a low pH may induce reversible or irreversible reductions in motility (28).

One of the major causes of reduced motility and acrosomal integrity after equilibration is the negative effect of glycerol. The addition of glycerol to diluted ram semen could cause changes in the permeability of sperm cell membranes (9,12).

Pontbriand et al. (29) reported that the addition of glycerol to diluted semen had detrimental effects on acrosomal integrity and motility. In this study, an increase in the glycerol level in diluted ram semen significantly reduced motility and raised the damaged acrosome rate after equilibration. In addition, ascorbic acid did not prevent the negative effects of glycerol.

When the glycerol level added to the diluted ram semen was considered, the highest percentage of motile spermatozoa was determined in the presence of 5% glycerol immediately after thawing. The addition of different proportions of ascorbic acid to the diluent groups containing the same glycerol levels did not affect the motility of spermatozoa, acrosomal integrity, total abnormal spermatozoa rate or dead spermatozoa rate of frozen-thawed ram semen. However, some researchers (20,21) suggested that the inclusion of antioxidants with a cryoprotective effect in the diluent might also allow the use of low glycerol concentration (3% or 1%). In this study, it was observed that ascorbic acid had no cryoprotective effect, and the addition of ascorbic acid to the diluted ram semen did not improve any spermatological characteristics after thawing.

Abdelhakeam et al. (30) suggested that it is possible to freeze ram semen in the absence of glycerol with good post-thaw motility. However, in the present study, a significant decrease was obtained in post-thaw motility and live spermatozoa rate in diluents without glycerol when compared with the other diluents groups containing glycerol. The disagreement between these studies could be due to the differences in diluent composition and dilution-freezing protocol.

In conclusion, the increase in the glycerol level (3%-7%) in diluted semen had negative effects on spermatological characteristics compared to the control group after glycerolization and equilibration. However, the best results were observed in diluted ram semen groups containing 5% glycerol after freezing and thawing. Moreover, the supplementation of ascorbic acid to the diluents did not prevent the detrimental effects of glycerol on the spermatological characteristics of diluted ram semen.

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