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Positive effects of trehalose and cysteine on ram sperm parameters

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ABSTRACT: The aim of this study was to determine the effects of trehalose and cysteine on sperm motility, viability, mitochondrial activity and acrosome integrity during liquid storage of Merino ram semen. Ejaculates were collected using artificial vaginas from five Merino rams, microscopically evaluated and pooled at 37 °C. The pooled semen samples were diluted in a Tris-based extender, including cysteine (2 mM and 4 mM), trehalose (10 mM and 25 mM) and no antioxidant (control). Diluted semen samples were kept in tubes and cooled from 37 to 5 °C in a cold cabinet, and maintained at 5 °C. Cooled samples were evaluated for sperm motility, viability, mitochondrial activity and acrosome integrity at 0, 24, 48, 72 and 96 h. Extender supplemented with trehalose (10 and 25 mM) and cysteine (2 and 4 mM) led to higher motility in comparison to the control at 24, 48, 72 and 96 h of liquid storage ($P < 0.05$). Trehalose at the doses of 10 mM, 25 mM and 2 mM cysteine led to higher viability between 24–48–72 h and at 96 h of liquid storage ($P < 0.05$). Further, 4 mM of cysteine improved sperm viability rates at 24 and 48 h of storage compared to the control group ($P < 0.05$), and resulted in improved acrosome integrity rates compared to the control group at 72 and 96 h of storage ($P < 0.05$). Extender supplemented with 10 and 25 mM trehalose at 24 and 72 h and 4 mM cysteine at 24 and 96 h of storage led to higher sperm mitochondrial activity rates when compared to the control group ($P < 0.05$). In conclusion, the findings of this study show that trehalose and cysteine provided significant protection to ram sperm parameters during liquid storage.

Keywords: antioxidant; amino acids; cooling process; egg yolk-based extenders; fluorescent staining; motility

In sheep breeding, artificial insemination (AI) serves as an important tool for reproduction. The most important element in AI is the preservation of ram semen through liquid storage or freezing (Evans and Maxwell 1987). Freezing ram semen generally results in poor pregnancy rates in cervical insemination, compared to pregnancy rates with fresh semen when AI is performed within a short time after collection (King et al. 2004; Camara et al. 2011). The success of this protocol in the sheep industry is related to the ram semen quality. Because ram semen cannot be stored in a liquid state for a long time, its suitable storage relies upon the use of suitable diluents. Satisfactory pregnancy rates were obtained with diluents at 5 °C up to 24 h after semen collection (O'Hara et al. 2010; Mehr et al. 2013).

The sperm of small ruminants is sensitive to reactive oxygen species (ROS) damage due to the relatively high content of unsaturated fatty acids in the phospholipids of the sperm membrane (Ashrafi et al. 2011). A close relationship between the overproduction of ROS and various types of male infertility has been described and, it is now widely accepted that oxidative stress (OS) significantly contributes to sperm structural damage (Tvrda et al. 2015). Lipid peroxidation may represent one of the negative biochemical and physiological changes associated with storage (Cerolini et al.

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2000). Antioxidants inhibit OS damage caused by free radicals; they can interfere with the oxidation process in the cell membrane, chelating catalytic metals and also by acting as oxygen scavengers (Buyukokuroglu et al. 2001). Cysteine is a low-molecular weight amino acid containing a thiol group; it is an intracellular glutathione precursor. In addition, cysteine consists of thiol groups, which individually penetrate into the sperm and act as non-enzymatic antioxidants (Coyan et al. 2011; Topraggaleh et al. 2014). Cysteine can enhance intracellular glutathione (GSH) biosynthesis, which protects membrane lipids through indirect radical scavenging properties (Bansal and Bilaspuri 2011). It is frequently involved in electron transfer reactions and assists in enzymatic catalytic reactions (Piste 2013). Cysteine has been shown to have beneficial effects on viability, chromatin structure and membrane integrity in boar sperm during liquid preservation (Szczesniak-Fabianczyk et al. 2003). It also has a cryoprotective role with respect to total abnormality, acrosome damage, and viability and prevents the loss of sperm motility in freeze-thawed bull semen (Uysal and Bucak 2007). Trehalose is a white, odourless powder with a relative sweetness, that is 45% that of sucrose. It is a bisacetal, nonreducing homodisaccharide in which two glucose units are linked together in a α-1,1-glycosidic linkage (α-D-glucopyranosyl-α-D-glucopyranoside; mycose, mushroom sugar). Because of the inherent properties of trehalose, namely, prevention of starch retrogradation and stabilisation of proteins and lipids, it has proved quite useful in a number of industries including food processing, cosmetics, and pharmaceutics (Komes et al. 2003; Richards et al. 2003). Trehalose modulates membrane fluidity by inserting itself into membrane phospholipid bilayers thereby rendering membranes more stable during freezing (Aboagla and Terada 2003). Studies on ram semen have also shown that diluents including trehalose improved motility, viability and membrane integrity of spermatozoa (Bucak and Tekin 2007; Tonieto et al. 2010). Aisen et al. (2005) reported that extender containing trehalose increased antioxidant activity and decreased the OS. Ram semen extenders with trehalose resulted in 45–47% higher lambing rates by cervical insemination in two sequential studies; i.e. the fertility rate was 2.5 times greater in the experimental group than in the control group (Aisen et al. 2000; Aisen et al. 2002). However, the preservation of ram sperm has suffered from inefficiency over the past few decades (Maxwell and Stojanow 1996; Watson 2000; Camara et al. 2011).

Previous reports evaluated the effects of either trehalose or cysteine on the liquid storage of ram semen. Based on the available literature (Szczesniak-Fabianczyk et al. 2003; Bucak and Tekin 2007; Tonieto et al. 2010) our hypothesis was that different concentrations of trehalose and cysteine would reduce the damage caused by oxidation and have favourable effects on the motility, viability, acrosome integrity and mitochondrial activity of sperm cells. Therefore, the aim of our study was to evaluate and compare the effects of these antioxidants on ram spermatogical parameters during liquid storage at 5 °C for periods of up to 96 h.

MATERIAL AND METHODS

Chemicals. The chemicals used in this study were all obtained from Sigma-Aldrich (St Louis, USA).

Animals and semen collection. Semen samples from five mature Merino rams (3-year-olds) were used in this study. The rams belonged to the Selcuk University Research and Experimental Farm, Konya-Turkey. The animals were healthy and maintained under uniform optimal nutritional conditions. Ejaculates were collected twice a week from the rams, with the aid of an artificial vagina during the natural breeding season (autumn to early winter). Immediately after the collection, the ejaculates were incubated in a water bath at 33 °C, until microscopic sperm quality assessments were performed in the laboratory. All semen analyses were performed within approximately 20 min of semen collection.

Semen extending. The volume of the ejaculates was measured in a conical tube, graduated at 0.1 ml intervals, and the ejaculate concentration was determined using a haemocytometer. Sperm motility was determined using phase-contrast microscopy (200 × magnification) at 37 °C. Only ejaculates between 1 and 2 ml in volume, containing sperm with more than 80% motility and an ejaculate concentration of higher than 2.5×10^9 sperm/ml were pooled. Pooling of semen was performed to balance the sperm contribution of each male and eliminate individual ram differences. In total, five pooled ejaculates were included in the study. A Tris-based extender (Tris 297.58 mM, citric acid 96.32 mM,

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fructose 82.66 mM, egg yolk 15% (v/v), penicillin 500 IU/ml, and streptomycin 500 IU/ml, pH 6.8) was used as the base extender. Each pooled ejaculate was split into five equal aliquots and diluted at 37 °C with base extenders containing trehalose (10 and 25 mM), cysteine (2 and 4 mM) or no antioxidant (control), respectively. Specific trehalose and cysteine concentrations were selected based on results obtained from a previous trehalose and cysteine standardisation study with ram and bull semen (Aboagla and Terada 2003; Sariozkan et al. 2009; Hu et al. 2010; Coyan et al. 2011; Jafaroghli et al. 2011). The final semen concentration was 4×10^8 sperm/ml (single step dilution), in 15-ml polypropylene centrifuge tubes.

Semen cooling. Diluted semen samples were aspirated into 0.25 ml French straws, sealed with polyvinyl alcohol powder, cooled from 37 °C to 5 °C in a cold cabinet and equilibrated at 5 °C, and maintained for up to 96 h. Sperm parameters were determined during liquid storage at 5 °C.

Assessment of sperm motility. Motility was assessed using a phase-contrast microscope (400 × magnification), with a warm stage maintained at 37 °C. A wet mount was made using a 5 µl drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score.

Assessment of sperm plasma membrane integrity. This assessment was performed using sperm plasma membrane staining with the Sperm Viability Kit (SYBR-14/PI Molecular Probe: L 7011 Invitrogen). The staining protocol was modified from Garner and Johnson (1995). A working solution of SYBR-14 was diluted 1 : 10 with DMSO (Appli-chem A3006), then divided into equal aliquots (30 µl) after filtering with a 0.22 µm Millipore Millex GV filter, and stored at –20 °C. The propidium iodide was dissolved in distilled water at 2 mg/ml, divided into equal aliquots (30 µl) after being filtered with a 0.22 µm Millipore Millex GV filter, and stored at –20 °C. The semen sample was diluted 1 : 3 with Tris stock solution without egg yolk, and then 30 µl of diluted semen were mixed with 6 µl SYBR-14 and 2.5 µl propidium iodide. The mixed sample was gently shaken and incubated at 37 °C in the dark for 20 min; then, 10 µl Hancock solution were added to stop sperm movement. A wet mount was made using a 2.5 µl drop sample placed directly on a microscope slide and covered by a cover slip. At least 200 spermatozoa were examined at 400 × magnification under a fluorescence microscope (Leica DM 3000 Microsystems GmbH, Ernst-Leitz-Strase, Wetzlar, Germany; excitation at 450–490 nm, emission at 520 nm, SYBR-14 stained only living sperm resulting in the nuclei fluorescing bright green when excited at 488 nm) to assess sperm membrane integrity. Sperm displaying green-red or red colouring were considered as membrane-damaged, while those with green colouring were considered to be membrane intact.

Assessment of sperm acrosome integrity. Sperm acrosome status was assessed using fluorescein isothiocyanate conjugated to *Arachis hypogaea* (peanut) (L7381 FITC-PNA, Invitrogen) and by propidium iodide staining as described by Nagy et al. (2003) with modifications. Approximately 120 µg of FITC-PNA were added to 1 ml PBS (15630056, Invitrogen) to make the staining solution, which was then divided into equal aliquots (100 µl) after filtering and stored at –20 °C. The semen sample was diluted 1 : 3 with Tris stock solution without egg yolk, and then 60 µl diluted semen were mixed with 10 µl FITC-PNA and 2.5 µl propidium iodide. The mixed sample was gently shaken and incubated at 37 °C in the dark for 20 min, and then 10 µl Hancock solution was added to stop sperm movement. A wet mount was made using a 2.5 µl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 spermatozoa were examined at × 400 magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm, FITC-PNA fluorescence was detected at 515 nm) to assess acrosome integrity. Spermatozoa displaying bright green or patchy green fluorescence were considered as acrosome non-intact or damaged, whereas cells which did not exhibit green fluorescence in the acrosomal cap region were regarded as acrosome-intact.

Assessment of sperm mitochondrial activity. Sperm mitochondrial activity was assessed using a staining protocol with JC-1/propidium iodide modified from Garner et al. (1997). A stock solution of 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl-carbocyanine iodide (T3168 JC-1, Invitrogen, 1.53 mM) was prepared in DMSO solution and then divided into equal aliquots (100 µl) after filtering and stored at –20 °C. The semen

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Table 1. The mean (± SD) motility (%) of Merino ram sperm supplemented with different doses of cysteine and trehalose for different storage times at 5 °C

Groups	0 h	24 h	48 h	72 h	96 h
Cysteine (2 mM)	82.00 ± 2.24	74.00 ± 4.18^{b}	66. 00 ± 4.18^{b}	59.00 ± 5.47^{b}	48.00 ± 7.58^{b}
Cysteine (4 mM)	83.00 ± 2.74	75.00 ± 3.54^{b}	68. 00 ± 2.74^{b}	61.00 ± 4.18^{b}	49.00 ± 2.23^{b}
Trehalose (10 mM)	82.00 ± 2.74	73.00 ± 2.74^{b}	68. 00 ± 4.47^{b}	63.00 ± 4.47^{b}	57.00 ± 2.74^{c}
Trehalose (25 mM)	83.00 ± 2.74	74.00 ± 4.18^{b}	69. 00 ± 4.18^{b}	64.00 ± 4.18^{b}	61.00 ± 4.18^{c}
Control	81.00 ± 2.24	71.00 ± 4.18^{a}	59. 00 ± 6.52^{a}	50.00 ± 7.91^{a}	36.00 ± 4.18^{a}
P-value	ns	< 0.05	< 0.05	< 0.05	< 0.05

^{a,b,c}Different superscripts within the same column demonstrate significant differences

sample was diluted 1 : 3 with Tris stock solution without egg yolk, and then 300 µl diluted semen were mixed with 2.5 µl JC-1 and 2.5 µl propidium iodide. The mixed sample was gently shaken and incubated at 37 °C in the dark for 20 min. Thereafter, 10 µl Hancock solution was added to stop sperm movement. A wet mount was made using a 2.5 µl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 sperm cells were examined at 400 × magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm, JC-1 exists as a monomer with excitation and emission peaks in the green wavelengths of 510–520 nm) to assess the mitochondrial activity. A high level of yellow/orange fluorescence associated with the sperm mid-piece (where mitochondria are located) indicated high mitochondrial activity. Mitochondria with low activity stained green.

Statistical Analysis. The experiment was replicated five times. The results were expressed as mean ± SD. Data were analysed using one-way analysis of variance (ANOVA), followed by Duncan's post hoc test to determine significant differences in all parameters among all groups. Statistical tests were run using the SPSS/PC computer program (IBM SPSS Statistics version 21.0), and differences with values of $P < 0.05$ were considered statistically significant.

RESULTS

The cooled extender supplemented with trehalose (10 and 25 mM) and cysteine (2 and 4 mM) led to higher subjective motility in comparison to the control during the period 24 – 48 – 72 h and at 96 h of liquid storage ($P < 0.05$; Table 1). In Table 2, trehalose at doses of 10 and 25 mM and 2 mM cysteine led to higher viability during the period 24 – 48 – 72 h and at 96 h of liquid storage ($P < 0.05$). Also, 4 mM cysteine improved sperm viability rates at 24 and 48 h compared to the control group ($P < 0.05$), and, as shown in Table 3, 4 mM cysteine resulted in a significant improvement in acrosome integrity rate compared to the control group at 72 and 96 h of storage ($P < 0.05$). As shown in Table 4, extender supplemented with 10 and 25 mM trehalose at 24 and 72 h and with 4 mM cysteine at 24 and 96 h of storage led to higher sperm mitochondrial activity rates when compared to the control group ($P < 0.05$).

Table 2. The mean (± SD) viability (%) of Merino ram sperm supplemented with different doses of cysteine and trehalose for different storage times at 5 °C

Groups	0 h	24 h	48 h	72 h	96 h
Cysteine (2 mM)	83.30 ± 1.93^{ab}	77.42 ± 3.54^{b}	70.72 ± 1.61^{b}	64.65 ± 2.71^{b}	57.25 ± 3.57^{b}
Cysteine (4 mM)	83.83 ± 1.56^{ab}	76.03 ± 3.43^{b}	69.95 ± 3.20^{b}	61.27 ± 6.98^{ab}	49.08 ± 4.50^{a}
Trehalose (10mM)	82.90 ± 2.08^{ab}	75.00 ± 3.79^{b}	69.12 ± 4.89^{b}	65.15 ± 4.10^{b}	60.85 ± 4.73^{b}
Trehalose (25 mM)	84.52 ± 1.70^{b}	75.57 ± 1.19^{b}	70.13 ± 2.34^{b}	64.32 ± 2.77^{b}	61.45 ± 2.94^{b}
Control	81.92 ± 0.98^{a}	71.07 ± 2.54^{a}	60.55 ± 5.70^{a}	55.47 ± 4.86^{a}	46.98 ± 4.08^{a}
P-value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

^{a,b}Different superscripts within the same column demonstrate significant differences

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Table 3. The mean (± SD) intact acrosome integrity (%) of Merino ram sperm supplemented with different doses of cysteine and trehalose for different storage times at 5 °C

Groups	0 h	24 h	48 h	72 h	96 h
Cysteine 2 mM	57.30 ± 2.71	52.38 ± 2.23	49.00 ± 4.30	46.50 ± 4.99^{ab}	41.70 ± 5.16^{ab}
Cysteine 4 mM	61.38 ± 3.60	58.53 ± 4.37	55.52 ± 3.76	52.58 ± 4.03^{b}	49.72 ± 4.97^{b}
Trehalose 10 mM	61.28 ± 5.75	57.63 ± 7.91	54.00 ± 7.96	50.18 ± 7.03^{ab}	47.55 ± 5.91^{ab}
Trehalose 25 mM	59.88 ± 1.88	53.75 ± 3.53	52.15 ± 3.43	50.37 ± 3.96^{ab}	47.55 ± 4.42^{ab}
Control	60.17 ± 2.94	54.88 ± 4.75	51.06 ± 4.92	44.88 ± 4.40^{a}	41.70 ± 3.98^{a}
P-value	ns	ns	ns	< 0.05	< 0.05

^{a,b}Different superscripts within the same column demonstrate significant differences

DISCUSSION

In this study, we investigated the effects of trehalose (10 and 25 mM) and cysteine (2 and 4 mM) on sperm motility, viability, acrosome integrity, and mitochondrial activity of ram semen. We stored ram spermatozoa at 5 °C for up to 96 h with the addition of these antioxidants to the extender in order to support the antioxidant defence system of spermatozoa. Because of the structure of the spermatozoa membrane, ram semen storage is more difficult than in other species. In addition, fertility rates do not reach the desired levels (Lopez-Saaz et al. 2000; Bucak and Tekin 2007; Bucak et al. 2007). The sperm structure is comprised of unsaturated fatty acid which is very susceptible to lipid peroxidation (LPO). ROS are beneficial for sperm membrane fluidity and permeability under normal conditions (Agarwal et al. 2014) but, uncontrolled ROS generation causes loss of sperm motility, viability, membrane integrity, mitochondrial activity and low fertility (Watson 2000; Agarwal et al. 2008).

Trehalose had positive effects on motility, mitochondrial activity, sperm viability, and acrosome integrity of ram (Molina et al. 1994; Aisen et al. 2005; Bohlool et al. 2013), bull (El-Shestawy et al. 2015), boar (Athurupana et al. 2015), and rabbit spermatozoa (Dalimata and Graham 1997). Trehalose protects sperm cells from the damaging effects of dehydration and rehydration during temperature changes (Crowe et al. 1998). Cold shock affects cellular functions, causing loss of selectivity in plasma membrane and acrosome membrane permeability (Medeiros et al. 2002). In this state, the observed injuries to the plasma membrane in the anterior segment included swelling and breaking, whereas injuries to the acrosome included swelling or acrosome reaction (Aisen 2004). Trehalose-based diluents are hypertonic, and can protect the cell against osmotic changes and against cellular damage caused by changes in temperature. However, the inclusion of trehalose in extenders without glycerol may improve sperm motility during the cooling process (Sanchez-Partida et al. 1992). In this study, addition of trehalose (10 and 25 mM) resulted in improved motility compared to the control group. These findings are confirmed by the following studies performed with different doses and in different species: ram (Bucak and Tekin 2007; Cirit et al. 2013), rat (Sariozkan et al. 2012), and bull semen

Table 4. The mean (± SD) total mitochondrial activity (%) of Merino ram sperm supplemented with different concentrations of cysteine and trehalose for different storage times at 5 °C

Groups	0 h	24 h	48 h	72 h	96 h
Cysteine 2 mM	74.47 ± 2.96	70.89 ± 2.69^{ab}	61.01 ± 3.78	51.99 ± 3.75^{ab}	45.13 ± 4.48^{ab}
Cysteine 4 mM	73.77 ± 1.76	71.56 ± 1.82^{b}	63.44 ± 4.85	53.54 ± 5.11^{ab}	49.69 ± 5.62^{b}
Trehalose 10 mM	75.48 ± 2.57	72.62 ± 1.37^{b}	63.85 ± 1.44	56.96 ± 1.09^{b}	47.94 ± 4.36^{ab}
Trehalose 25 mM	75.32 ± 2.57	72.62 ± 2.18^{b}	61.36 ± 4.66	54.92 ± 4.82^{b}	47.71 ± 2.20^{ab}
Control	74.12 ± 3.53	65.92 ± 8.93^{a}	60.02 ± 7.08	48.87 ± 4.64^{a}	43.67 ± 4.52^{a}
P-value	ns	ns	ns	< 0.05	< 0.05

^{a,b}Different superscripts within the same column demonstrate significant differences

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(El-Shestawy et al. 2015). In addition to these findings, Lopez-Saaz et al. (2000) also reported that trehalose protects the motility rate during liquid storage of ram spermatozoa for up to 16 days. At 5 °C, the sperm cell metabolism does not stop completely, lipid peroxidation occurs, and the plasma membrane shows physical and physiological modifications (Maxwell and Stojanov 1996). The osmotic regulation in spermatozoa at low temperatures may impair motility and affect membrane integrity, owing to structural damages in the lipid bilayer of the membrane (Meyers 2005). However, trehalose acts directly on the membrane phospholipid bilayer and renders the membrane more stable. Also, owing to the fact that it is a non-permeable disaccharide, trehalose could protect cells during the cooling procedure when spermatozoa lose water (Aisen et al. 2005). Aisen et al. (2002) reported that trehalose not only renders the membrane stable, but also prevents uncontrolled ROS production and makes the extender more stable (Jafaroghli et al. 2011).

Trehalose exerted significant positive effects with respect to membrane protection and sperm viability rates in comparison to the control group in this study. Moreover, trehalose positively affected mitochondrial activity rates. Previous studies support our findings (Lopez-Saaz et al. 2000; Bucak and Tekin 2007). In addition, a cryopreservation study on bull semen reported that trehalose provided better viability and acrosome-intact spermatozoa rates compared to the control (El-Shestawy et al. 2015). On the other hand, according to the results of Motta et al. (2014), the effectiveness of a solution containing intracellular and extracellular trehalose in the cryopreservation of cord blood cells demonstrated that trehalose might be an optimal cryoprotectant when present both inside and outside the cells. Numerous authors demonstrated that post-thawed sperm characteristics improved significantly with increasing trehalose concentrations (Abdelhakeam et al. 1991; Molina et al. 1994; Aisen et al. 2002). In our study, there were no significant differences noted between trehalose at 10 and 25 mM on motility, viability, acrosome integrity and mitochondrial activity at 5 °C for up to 96 h. Thus, it can be said that lower doses of trehalose should be preferred for ram semen liquid storage.

Cysteine is an amino acid containing a thiol group; it has low molecular weight meaning that it can be easily incorporated into sperm membranes like trehalose. In addition, it is an intracellular glutathione precursor. When sperm is cooled, cysteine prevents the generation of ROS and protect the spermatozoa from LPO (Coyan et al. 2011).

Our findings showed that supplementation of 2 and 4 mM cysteine to ram spermatozoa led to better motility rates compared to the control. This finding is similar to that of Uysal and Bucak (2007), who froze goat semen with 10 mM cysteine. Funahashi and Sano (2005) also reported that 5 mM cysteine improved sperm viability rates during the liquid storage of boar spermatozoa. We found that 4 mM cysteine improved the viability at 24 and 48 h of liquid storage. In contrast to this study, Yildiz et al. (2015) found that cysteine did not have beneficial effects on motility and viability rates after the freezing of ram semen. This could be attributed to extender type, antioxidant dose differences and especially, semen preservation technique.

In terms of membrane integrity rates, a 4-mM dose of cysteine showed the best rate compared to the control. Similar results were reported in a study of liquid storage of boar semen (Szczesniak-Fabianczyk et al. 2003).

In conclusion, the supplementation of trehalose and cysteine in the extender resulted in better spermatological parameters (motility, viability, acrosome integrity and mitochondrial activation) compared to controls. The use of additives may be recommended to facilitate and improve the semen preservation systems in the sheep breeding industry.

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