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Effect of additives on the post-thaw quality of Aksaray Malaklı dog semen

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Abstract

The goal of this study was to determine effects of trehalose (T) and Trolox (TX) additives on post-thaw viability of canine sperm after freezing. The sperm-rich portions of ejaculates obtained from 15 dogs were divided into three aliquots, which were diluted with Tris-based extender (TBE) (control), TBE + T (25 mM), and TBE + TX (1 mM) and put into straws. The samples were equilibrated at 4 °C for 1.5 hours, then frozen in liquid nitrogen vapour for 15 minutes and plunged into liquid nitrogen. Plasma membrane and acrosome integrity (PMAI) and high mitochondrial membrane potential (HMMP) were evaluated after thawing the straws in a water bath at 37 °C for 30 seconds. Motility, percentage of live spermatozoa, and hypo-osmotic swelling (HOS) were determined using phase-contrast microscopy. Motility (53.00 ± 6.46%), percentage of live spermatozoa (62.97% ± 6.84%), and HOS (40.40 ± 7.11%) of T were significantly higher (P <0.05) than the control (40.50 ± 4.76%, 50.63% ± 4.78%, and 30.47% ± 5.59%) and TX (40.50 ± 4.76%, 50.96 ± 5.84%, and 33.02% ± 8.77%) respectively. The PMAI and HMMP levels were higher (P <0.05) in T (35.51±8.72 and 24.36±7.15%) than in the control (19.18 ± 3.49 and 12.04 ± 3.83%). The TX group (35.62 ± 8.21%) had significantly higher PMAI (P <0.05) than the control (12.04 ± 3.83%). Thus, addition of T protected canine spermatozoa against cryogenic injury. Nonetheless, additional dose-response trials are recommended to determine the effects of Trolox on the quality of canine semen more accurately.

Keywords: acrosome integrity, cryoprotection, mitochondrial membrane potential, plasma membrane, trehalose, Trolox

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Introduction

Semen cryopreservation is applied routinely in canine species. With the high demand for pure dog breeds, assisted reproduction techniques are used increasingly in dog species (Van den Berghe *et al.,* 2018). However, a dearth of studies on artificial insemination and sperm cryopreservation in dogs limits assisted breeding practices in dog species (Gobello & Corrada, 2003).

Sperm cells may be damaged by changes in osmotic pressure and intracellular ice formation during freezing, a condition referred to as 'cold shock injury', which occurs when living cells are cooled to 0 °C (Hagiwara *et al.,* 2009). The freezing process exerts a negative effect on sperm vitality, both physically and chemically, by damaging the sperm membrane. The free radicals formed during the freezing process lead to oxidative stress, which creates cold shock in the cells (Salvador *et al.,* 2006). Antioxidants can neutralize the free radicals, as demonstrated in several studies (Abadjieva *et al.,* 2020; Rostami *et al.,* 2020).

Sugars may cause membrane damage during freezing and thawing. On the other hand, they may interact with the phospholipids in the membrane and thus protect by increasing the surface area, preventing cell damage during thawing (Bucak & Tekin, 2007). Trehalose (T) is one of the most frequently used disaccharides, which is reported to protect the sperm cells from cryodamage by regulating the osmotic pressure during preservation (El-Badry *et al.,* 2017). Trehalose can balance the extracellular and intracellular osmotic pressures during freezing (Pan *et al.,* 2017). Sugars, including T, play an important role in maintaining sperm viability after thawing by serving as an energy source and reducing intracellular ice crystal formation (Zhang *et al.,* 2016). Trehalose has been demonstrated to exert a cryoprotectant effect

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successfully during semen freezing when added to the sperm extender for freezing goat (Aboagla & Terada, 2003), ram (Gungor *et al.,* 2018), and dog (Yamashiro *et al.,* 2007) sperm.

Trolox (TX) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is the water-soluble analogue of vitamin E (Giulivi & Cadenas, 1993). Trolox is a cell-permeable and water-soluble antioxidant with protective properties against oxidative stress and apoptosis (Minaei *et al.,* 2012). Vitamin E is reported to decrease lipid peroxidation by neutralizing the lipid peroxide and alkoxyl radicals, which cause loss of both motility and membrane fluidity in spermatozoa (Agarwal *et al.,* 2003). In studies conducted on human sperm, TX was demonstrated to affect the spermatological parameters positively (Taylor *et al.,* 2009; Minaei *et al.,* 2012).

The present study aimed to investigate the effects of T and TX additives in Tris extender on the spermatological parameters of Aksaray Malakli semen after the freezing–thawing process. Previous studies investigated the efficiency of TX in Rottweiler semen (Evangelista *et al.,* 2014) and the efficiency of the diluent containing T in the cryopreservation of Poodle sperm (Yamashiro *et al.,* 2007). However, studies on the freezing of Aksaray Malaklı semen are scarce. Therefore, in the present study, the spermatological parameters of Aksaray Malaklı semen were evaluated with fluorescence microscopy and flow cytometry.

Material and Methods

Ethical approval for the present study was obtained from the Ethics Committee of Mehmet Akif Ersoy University (2016/242). Fifteen fertile male Aksaray Malakli dogs (age 2–3 years) were used to obtain the ejaculates for the experiments, which were collected in graded plastic tubes with digital manipulation (Kutzler, 2005) once a week for four weeks. These fractions were evaluated. The second fraction contained more than 70% subjective motility and above 250 × 10^6 spermatozoa/mL, which was rich in sperm. The sperm volume was determined with grade semen collection glasses.

All chemicals in the present study were purchased from Applichem and Sigma Chemical Co. (St. Louis, Missouri, USA). Tris-based extender (TBE) (Tris 297.58 mM, citric acid 96.32 mM, and fructose 82.66 mM) was used as the main semen diluent. On the day of semen collection, 15% fresh egg yolk was added to TBE, followed by centrifugation at 6000 rpm for 15 min, removal of the supernatant, and addition of 6% glycerol to the precipitate. The mixed ejaculates were diluted separately with TBE (control), TBE + T (25 mM) (Gungor *et al.,* 2018), and TBE + TX (1 mM) (modified from Silva *et al.,* 2013) forming the three treatment groups, to reach a final concentration of 400 × 10^6 spermatozoa/mL. The samples were loaded into mini straws (0.25 mL) and equilibrated at 4 °C for 1.5 hours. After equilibration, the straws were frozen in nitrogen vapour (−100/−120 °C) for 15 min and then stored in liquid nitrogen. After at least three months' storage, the straws were thawed in a water bath at 37 °C for 30 seconds.

To determine semen motility, at least five microscopic fields were examined for each semen sample drop at 400x magnification under a phase-contrast microscope at 37 °C. The mean motility values were expressed as percentage motility. The concentration of the spermatozoa (in mL) was determined using the hemocytometric method (Akalın, 2015).

The viability of the spermatozoa was evaluated using Eosin-Nigrosin staining. One drop of Eosin dye and one drop of Nigrosin dye were added to the sperm sample on a slide. Three hundred spermatozoa were counted under a phase-contrast microscope at 400x magnification. Spermatozoa were characterized as live or dead based on whether their head did not (head appears white) or did absorb the stain (head appears pink) (Figure 1a). The results were expressed as percentage live ratio (Akalın, 2015).

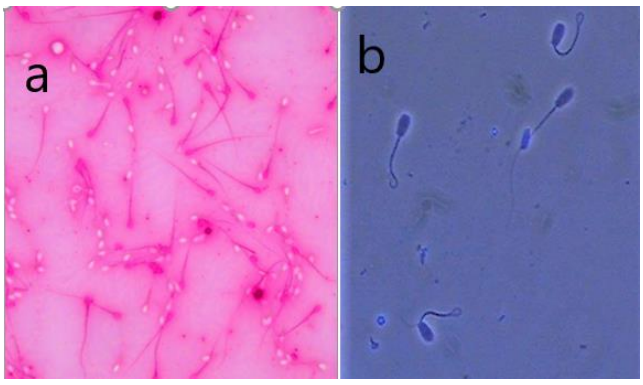

Figure 1 Images of dog sperm typical of those used to measure viability (a) and hypo-osmotic swelling (b)

To each semen sample, 40 µL was added to 400 µL of the HOS-test solution [0.9 g fructose and 0.49 g trisodium citrate per 100 mL], followed by incubation at 37 °C for 30 min (Figure 1b). Three hundred spermatozoa were counted under a phase-contrast microscope at 400x magnification. The spermatozoa with intact membranes and twisted tails were recorded, and the results were expressed as percentage ratio (Kulaksız, 2009). The Cytoflex flow cytometer (Beckman Coulter) with a 50 mW laser output (488 nm laser beam) and 610 ±20 nm, 585 ±42 nm, and 525 ±40 nm emission filters was employed for the analyses, and about 10×10^3 events were gated.

The fluorescent isothiocyanate-conjugated peanut agglutinin (FITC-PNA)/propidium iodide (PI) probes were used for multi-parameter sperm analysis and PMAI evaluation. The sperm samples were thawed at 37 °C for 30 seconds, and a concentration of 5×10^6 sperm cells (10 µL) diluted in 496 µL of PBS was used for analysis. In a dark room, 5 µL FITC/PNA and 3 µL PI were mixed and then incubated at 37 °C for 30 min. All the PMAI analyses were performed with CytExpert 2.2 software (Inanc *et al.,* 2019). Sample results have been shown in Figure 2a. The JC-1 (5,5′,6,6′-tetrachloro1,1′3,3′-tetramethyl benzimidazolyl-carbocyanine iodide)/PI molecular probes were used to determine HMMP. The sperm samples were thawed at 37 °C for 30 seconds, and a concentration of 5×10^6 sperm cells (10 µL) diluted in 487 µL of PBS was used for the analysis. In a dark room, 10 µL of JC-1 and 3 µL of PI were mixed and then incubated at 37 °C for 30 min. After the incubation, the same protocol was used as for PMAI evaluation, and the debris was gated out. The PMAI and HMMP evaluations were performed using CytExpert 2.2 software (Inanc *et al.,* 2019). Sample results for the PMAI and HMMP analysis are shown in Figure 2a and Figure 2b, respectively.

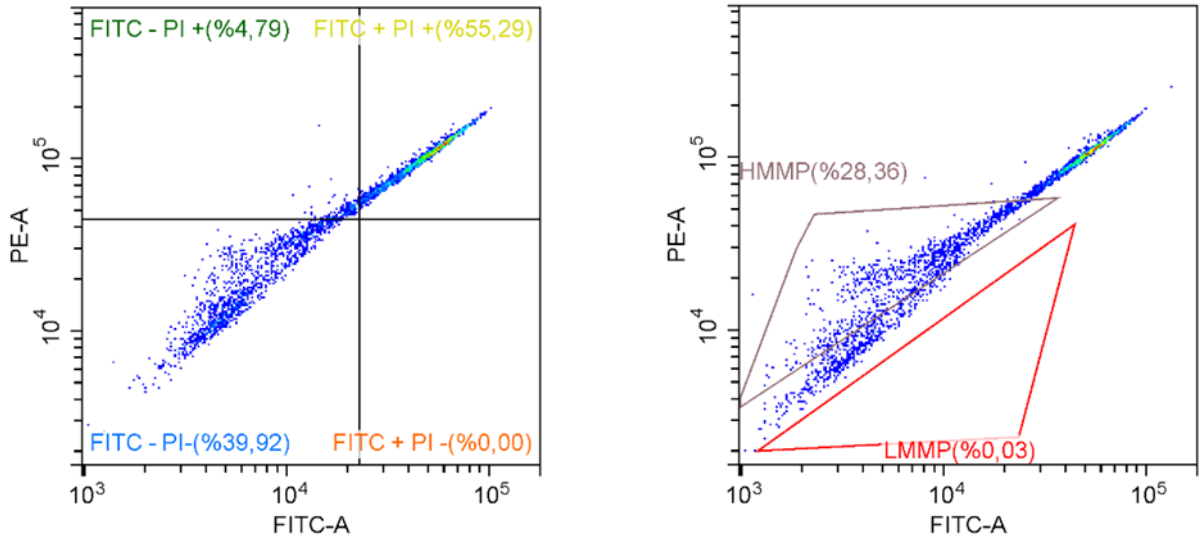

Figure 2 Flow cytometric examination of dog sperm stained with FITC-PNA/PI (a) and JC-1
a: FITC + PI - = plasma membrane intact with acrosome staining; FITC + PI + = plasma membrane damaged sperm with acrosome staining
b: HMMP = high mitochondrial membrane potential; LMMP = low mitochondrial membrane

The data obtained from the analyses were examined for normality with the Shapiro-Wilks and Kurtosis tests and for homogeneity of variance with the Levene test. The results were expressed as mean ± standard errors. ANOVA was performed, followed by Duncan's post-hoc test to determine the differences between the groups. Statistical analyses were performed for the data from post-thaw sperm samples. Treatment effects with *P*-values of <0.05 were considered statistically significant. All the analyses were performed using the SPSS (Version 21, IBM Corp., Armonk, New York, USA) software package.

Results and Discussion

The T solution had a protective effect on the viability of the spermatozoa (Table 1). In the T group, the post-thaw motility, live, and HOS were significantly higher from those in the control group (P <0.05), while no significant difference was observed in these measurements between the TX group and the control groups (P >0.05). The T group exhibited significantly higher values in the PMAI and HMMP levels compared with the control group (P <0.05). The TX group exhibited significantly higher values in the PMAI level (P <0.05) and no significant differences in HMMP levels (P >0.05) compared with the control group.

Table 1 Responses to adding trehalose and Trolox to the extender used in freezing dog semen

Response variable	Control extender	Control + trehalose	Control + Trolox
Motility, %	40.50 ± 0.61[b]	53.00 ± 0.83[a]	38.67 ± 0.78[b]
Percent live cells, %	50.63 ± 0.62[b]	62.97 ± 0.88[a]	50.96 ± 0.75[b]
Hypo-osmotic swelling, %	30.47 ± 0.72[b]	40.40 ± 0.91[a]	33.02 ± 1.18[b]
PMAI	19.18 ±1.74[b]	35.51 ± 4.36[a]	35.62 ± 4.10[a]
HMMP	12.04 ±1.92[b]	24.36 ± 4.72[a]	19.40 ± 3.51[ab]

PMAI: plasma membrane and acrosome integrity, HMMP: high mitochondrial membrane potential

Cryopreservation can cause extensive chemical and physical damage to the cells of all mammalian species by affecting the cell membranes (Watson, 2000). The mechanism underlying this damage is the lipid peroxidation (LPO) of the cell membrane induced by the ROS species during osmotic stress and transition from the lipid phase (Câmara *et al.,* 2011). In the present study, the effects of T and TX on characteristics of the sperm cells that were determined microscopically (motility, live–dead, and HOS) and using flow cytometery (HMMP and PMAI) after freezing and thawing were evaluated in dog semen.

Trolox is an antioxidant that is safe for use at high concentrations (Mata-Campuzano *et al.*, 2014). The production of ROS inside the sperm cell occurs mainly in the diluent and during the freezing and thawing processes, and the use of TX during freezing can prevent the oxidative stress caused by these ROS (da Silva Maia *et al.,* 2010). TX exhibits high efficacy as a free radical scavenger, even in relatively longer incubations at physiological temperatures (Domínguez-Rebolledo *et al.,* 2010). Trolox is an important additive for which positive catalysed LPO and hydrogen peroxide concentration results have been reported in ram sperm cryopreservation (da Silva Maia *et al.,* 2010). Nekoonam *et al. (*2016) reported that the addition of TX to the diluent in human sperm increased the mitochondrial membrane potential and reduced DNA damage. The production of reactive oxygen species (ROS) in the semen cell occurs mainly in the diluent and during freezing and thawing, and the use of TX during freezing can prevent the oxidative stress caused by these free radicals (da Silva Maia *et al.,* 2010). Moreover, TX exerts a positive effect on the motility parameters of human sperm after thawing (Taylor *et al.,* 2009; Minaei *et al.,* 2012). However, in the present study, the addition of TX did not exert any protective effect on the motility and HMMP levels, which represent the effect on the mitochondrial activity. In contrast, previous studies reported a positive effect on dog sperm motility (Peixoto *et al.,* 2013). Several studies reported the association of mitochondrial activity with sperm motility (Martinez-Pastor *et al.,* 2004; Espinoza *et al.,* 2009). Nekoonam *et al.* (2016) reported that motile spermatozoa and the HMMP levels in human sperm were higher in the 40 mM TX supplementation group. On the other hand, Evangelista *et al.. (*2014) reported that TX was inefficient in terms of dog sperm motility and HOS test results, which was consistent with the findings of the current study. Therefore, it may be inferred that TX exerted a protective effect only at higher amounts.

Trehalose is reported to exhibit antioxidative and cryoprotectant properties in the prevention of cold shock that occurs during freezing–thawing (Malo *et al.,* 2010). Trehalose increases the stability of the membrane phospholipid bilayer by inserting itself in the layer (Aboagla & Terada, 2003). The increase in the GSH-PX activity caused by T is thought to be an indicator of its antioxidative capacity. Diluents with hypertonic properties and high doses of T (50 and 75 mM) increased the GSH-PX activity during cryopreservation (Atessahin *et al.,* 2008). It is possible that T promotes the preservation of the functional integrity of acrosome and mitochondria, which improves sperm motility after thawing (Liu *et al.,* 2016). A study of the effects of T on ram sperm viability and membrane integrity revealed that the best results of 73.1% and 38.0%, respectively, were obtained for the 25 mM group (Gungor *et al.,* 2018). Yamashiro *et al. (*2007) examined the freezing–thawing motility results in dog semen and reported that the diluent containing T exerted protective effects. Moreover, the addition of T to canine semen extender resulted in positive effects on serum motility, vitality, and acrosome integrity (Yıldız *et al.,* 2000), which is consistent with the findings of the present study. The mitochondria responsible for the movement of spermatozoa are located in the midpiece of the cell. Freezing sperm cells increases oxygen free radical formation, causing damage to the midpiece. Lipid peroxidation also causes damage to the sperm cell and a decrease in the motion characters (Piomboni *et al.,* 2012). In the current study, the T supplementation group presented a higher value of HMMP compared with the other groups, suggesting the protective effect of T against oxidative stress.

Conclusion

Trehalose exerts a cryoprotective effect on spermatozoa motility, viability and HOS, whereas TX has no effect on any of these characteristics of preserved semen. Also, T (25 mM) imparted a protective effect on the HMMP and PMAI levels after the semen samples were thawed. Further studies are required that involve the use of various TX concentrations for cryopreservation of Aksaray Malakli semen.

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Authors' contributions

CÖ (ORCID ID: 0000-0003-0566-0684) and ŞG (ORCID ID: 0000-0003-3460-522X) designed the study, analysed the data and wrote the manuscript. CÖ was responsible for laboratory analyses (semen motility, viability and hypo-osmotic swelling test). SG was responsible for laboratory analyses (flow cytometry examination).

Conflict of Interest

The authors declare that there is no conflict of interest.

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