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EFFECTS OF TREHALOSE SUPPLEMENTATION ON POST-THAW SPERM QUALITY OF HONEY BEE DRONES

Zekariya NUR[1], Selvinar SEVEN ÇAKMAK[2], İbrahim ÇAKMAK[3]✉, Nail Tekin ONDER[1], Elif GOKÇE[4], Burcu USTUNER[1], Selim ALCAY[1], Mehmet Berk TOKER[1], Mustafa Kemal SOYLU[1]

[1]*Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Bursa Uludag University, 16059, Turkey*
[2]*Department of Biology, Faculty of Science, Ankara, Turkey*
[3]*Beekeeping Development-Application and Research Center, Department of Animal Science, Faculty of Agriculture, Bursa Uludag University, 16059, Turkey*
[4]*Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Tekirdag Namık Kemal University, 59030, Turkey*

✉Email: icakmak@uludag.edu.tr; ORCID: 0000-0002-8000-5770

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ABSTRACT: Sperm cryopreservation has led to an increase in widespread use and has it made it more practical to use artificial insemination not only for domestic animals but also for non-mammalian species and humans. Dimethyl Sulfoxide (DMSO) was the most frequently used cryoprotectant by protecting honey bee drone semen when freezing it. The objective of this study was to determine the effects of Trehalose (0.05M, 0.1M or none at all) on extending the viability of semen with 12% DMSO that was based on sperm motility and plasma membrane functional integrity of frozen drone semen. Three different freezing extender solutions were designated as follows; the 0.05M Trehalose, 0.1M Trehalose and Trehalose free (control group). Semen motility and plasma membrane functional integrity were evaluated under phase-contrast microscopy (400X). We found that in control group, DMSO is a critical substance in freezing extender and supports post-thaw sperm motility (53%) and plasma membrane functional integrity (79%) to some extent. Addition of 0.05M Trehalose to the extender leads to a small recovery of post-thaw motility (55%) and plasma membrane integrity (89%), but when Trehalose is added at 0.1M concentration, this led to significantly better post-thaw motility (62%) and plasma membrane integrity (91%). In conclusion, the freeze-thaw process is detrimental to post-thaw drone semen viability. The addition of 0.1 or 0.05M Trehalose to the freezing media containing 12% DMSO has been seen better post-thaw cell motility and plasma membrane integrity of spermatozoa.

Keywords: Honey bee, Drone, Semen, Cryoprotectant, DMSO, Trehalose

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INTRODUCTION

Honey bee contribution in pollination of crops (*Apis mellifera* L.) and end products (honey, royal jelly, wax, pollen, and propolis) are crucial for agriculture and beekeepers. Recently the variety of subspecies and populations of honey bees have declined dramatically by virtue of man-made (habitat destruction, usage of pesticide, transport stress, management failures and poor queen health) and natural factors (starvation, parasites, winter colony losses) (Çakmak and Çakmak, 2016; Moritz and Erler, 2016). The losses of honey bees vary from region to region, but in general recently there has been large scale colony losses (35% to 75%) observed (Neumann and Norman, 2010). Therefore, the ability to preserve honey bee genetic material and to artificially inseminate for breeding different honey bee genetic lines are regarded as a critical tool for improving bee health and producing the beekeeping products mentioned above. Maintenance of genetic diversity, protection of desirable genetic lineages, prevention of colony loss, and increasing the productivity of bee colonies are possible if honey bee drone sperm preservation and artificial insemination of queen bees are probable with frozen thawed sperm (Collins, 2000; Cobey et al., 2013; Pallard et al., 2017).

Semen extender solution composition has a pivotal effect when overcoming the deleterious effects of ultra-low temperatures and sustaining the fertilization viability of cryopreserved semen (Gul et al., 2017; Pallard et al., 2017; Alcay et al., 2019a,b). Cryoprotectants are known as one of the essential ingredient to extend the shell-life of semen and are used for preventing the detrimental effects of freezing, intracellular ice crystallization, and the effects of freeze-thaw cycles (Watson and Fuller, 2001; Domingo et al., 2019). These molecules are classified as internal or penetrating and external or non-permeating cryoprotectants (Salamon and Maxwell, 2000). Internal cryoprotectants increase the plasma membrane fluidity, enhance the dehydration of spermatozoon, and reduce the intracellular ice formation, thereby improving the freezing ability and survivability of spermatozoa (Holt, 2000).

On the other hand, external cryoprotectants act as additional solute and decrease the freezing temperature of extender solution, reduce extracellular ice formation, and protect spermatozoon against cryopreservation as well (Amann, 1999). Out of all of these cryopotectants, dimethyl sulfoxide (DMSO) has attracted the most attention because to date it provides the best post-thaw motility and viability results for honey bee spermatozoa cryopreservation (Taylor et al., 2009; Hopkins ad Herr, 2010; Wegener et al., 2014; Alcay et al., 2016, Alcay at al., 2019a, b); Yaniz et al., 2019). However, there are other cryoprotective agents (CPAs), which are commonly used to protect survival of spermatozoa at low

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temperatures. Due to toxicity concerns of the permeable CPAs, disaccharides (trehalose, maltose and sucrose), amino acids (EDTA, Proline, Glycine, Glutamic acid, Aminobutyric acid, Glutaric acid, Ammonium acetate), and proteins (Bovine serum albumin, gelatin, peptones and sericin) have been recently considered instead (Hubalek, 2003; Igbal et al., 2016; Mosca et al., 2016). Considering how other insect species have successfully used Trehalose for, sperm preservation, Threhalose was selected as a good candidate to test to see if it can increase preservation of honey bee sperm as well. The objective of the ongoing experiments was to evaluate the effects of various concentrations of Trehalose (0.05M, 0.1M or none at all) as viable freezing extenders that are also supplemented with 12% DMSO. The effectiveness of treatments on extending the shelf-life of honey bee spermatozoa was evaluated based on post-thaw drone semen motility and plasma membrane functional integrity.

MATERIALS AND METHODS

Animals

Apis mellifera anatoliaca drones were collected from the Beekeeping Development-Application and Research Center (AGAM) colonies and semen freezing was carried out at the Laboratory of Andrology within the Faculty of Veterinary Medicine, located in Bursa Uludag University, Turkey between May and July 2017. Mature drones that were 16 days age or older chosen from 5 different colonies that were established with drone wax foundations. The colonies used in this experiment were managed using standard beekeeping practices and were only treated for Varroa mite infestations using amitraz strips.

Chemicals

Catalase, DMSO (D2650), Trehalose and Amoxicillin were bought from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA). The other chemicals (Na citrate, $NaHCO_3$, KCl) used were purchased from Merck (Merck & Co., Inc. Germany).

Semen collection and dilution

Semen was collected by squeezing drone abdomens as described by Collins and Donoghue (1999) using a Schley syringe tip 1.10 (Schley Instrumental Insemination equipment, Lich, Germany) under a stereo microscope (Collins and Donoghue, 1999). A total of 0.8 μL of saline solution was drawn into a capillary, followed by 3μL of semen (approximately 3-4 mature drones) for each freezing group.

Sperm motility

The diluted and thawed sperm motility was assessed under a phase-contrast microscope, at 400 X magnification. Observed sperm motility was scored on a scale of 0 to 5 corresponding to 0%, 20%, 40%, 60%, 80% and ≥80% amount of motility respectively (Nur et al., 2012).

Plasma membrane integrity

Sperm plasma membrane functional integrity was evaluated by the Water test (Nur et al., 2012). The 1.0 μL of diluted semen was added to 250 μL of distilled water and rested for 5 min at room temperature. One drop of incubated semen was placed on a microscope slide and the percentage of coiled tail spermatozoa was determined under a phase-contrast microscope (400X) (Nur et al., 2012). Sperm plasma membrane integrity was also assessed at the post-thaw stage as well.

Semen dilution and freezing

As suggested by Taylor et al. (2009) extender solutions IV (Na Citrate 2.43g, $NaHCO_3$ 0.21g, KCl 0.04g, Amoxicillin 0.03g, Catalase 200μL in a volume 100ml) were used. The extender's Ph was fixed to 8.1 and split into three groups I: Trehalose free (control group), II: 0.05 M Trehalose and 0.1M Trehalose. Obtained semen (3μL) was diluted (1 part semen to10 parts extender solutions) with one of the freezing extender group solutions at room temperature and loaded into a 0.25ml straw (Alcay et al., 2015). The semen filled straws were cooled to 5°C within 1 h in a cold cabinet and then were equilibrated for 2 h. Equilibrated straws were then frozen in liquid nitrogen vapor (5cm above the level of LN2, −80°C-120) for 10 min and then dipped into liquid nitrogen at roughly -196°C. From each group two straws were thawed at 37°C for 30 s in a water bath which then followed with a post-thaw semen motility and plasma membrane integrity evaluation. The procedure was repeated 5 times for each group (5x2=10straws).

Statistical analyses

The obtained data were subjected to the Kruskal Wallis –Mann Whitney U test for the motility and plasma membrane integrity tests, comparing across the different extender solutions for cryopreservation of spermatozoa. The SPSS statistical package (SPSS 10.0 for Windows, SPSS, Chicago, IL, U.S.A), was used for all statistical tests.

RESULTS

The semen motility and plasma membrane integrity as a function of Trehalose content at the two different stages were presented in Figures 1 and 2. There were no differences among diluted semen motility between control and the treatment

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groups. No differences were observed across the diluted semen plasma membrane functional integrity of used groups (Figure 1).

Although freeze-thaw cycle affect the post-thaw sperm motility and plasma membrane integrity for used groups, spermatozoa had greater post-thaw motility and plasma membrane integrity when frozen in trehalose supplemented extender in comparison when frozen in Trehalose free extender. The freeze-thawing process was less harmful on drone semen plasma functional membrane integrity, compared to sperm motility especially in trehalose-supplemented group. Post-thaw sperm motility of Trehalose supplemented groups were better than the Trehalose free group (P>0.05). Also post-thaw plasma membrane integrity of the 0.1M Trehalose supplemented group (91%) was better than the 0.05 Trehalose (89%) (P>0.05), and Trehalose free group (79%) (P<0.05) (Figure 2).

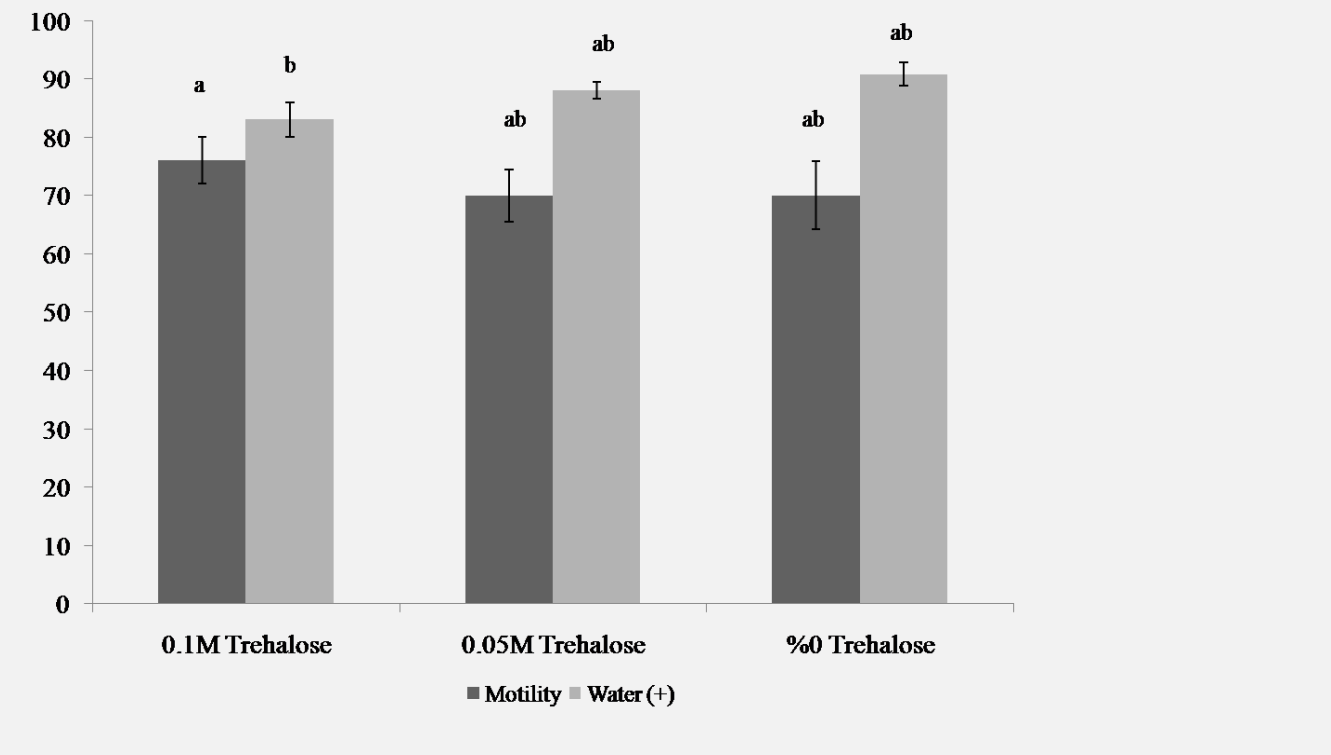

Figure 1 - Diluted semen motility (%), and plasma membrane functional integrity (%). [a,b:] Groups with different superscripts for same parameters are significantly different (P<0.05).

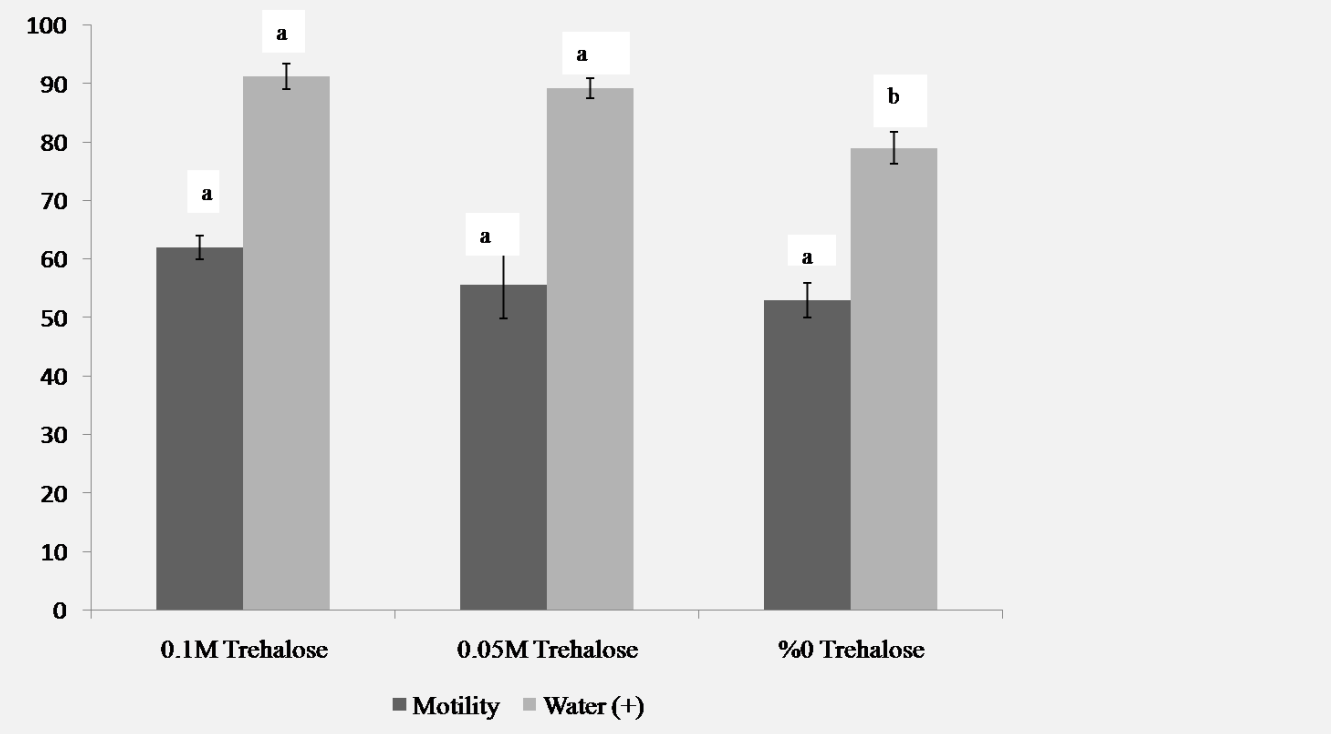

Figure 2 - Post-thaw semen motility (%), and plasma membrane functional integrity (%). [a,b:] Groups with different superscripts for same parameters are significantly different (P<0.05).

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DISCUSSION

Semen preservation requires a decrease or interruption of spermatozoa metabolism which is realized with cryopreservation. This technique includes diluting, cooling, freezing, and storing the sperm at -196°C in liquid nitrogen, followed by the thawing of the stored sperm. However, the processes of cryopreservation can affect sperm motility, viability and fertility; permanently or temporarily. Irreversible reduction of spermatozoa metabolism originates from the dilution effect, cold shock treatment, the formation of intracellular ice crystals, osmotic stress, and lipid peroxidation that can occur throughout the cryopreservation process (Isachenko, 2003; Domingo et al., 2019).

The extenders used includes, carbohydrates (trehalose), salts (Na Citrate, $NaHCO_3$, KCl), enzyme (catalase), cryoprotectant (DMSO, trehalose) and antibiotics. These chemicals interact with different part of spermatozoa and lead to osmotic and toxic stresses, and induce biochemical changes in cell metabolism immediately after dilution (Ak et al., 2010; Domingo et al., 2019; Yániz et al., 2019). There were no differences among diluted sperm motility and plasma membrane functional integrity within studied groups generally. The extender ingredients, dilution time, rate, temperatures, cooling to 5°C and freezing curve and presence of cryoprotectant also affect post-thaw semen quality (Ak at al., 2010; Nur at al., 2012; Alcay et al., 2015; Alcay et al., 2019a,b). The cryoprotectants that were used for preventing the detrimental effects of cooling, intracellular ice crystallization throughout the freezing and thawing processes are known as one of the essential ingredients of semen extenders (Watson and Fuller, 2001; Domingo et al., 2019). Post-thaw sperm motility (53%) and plasma membrane integrity (79%) of control group were lower than diluted semen motility (70%; $P<0.01$) and plasma membrane integrity (90%; $P<0.01$). The transitional population of immotile sperm with intact plasma membranes reveals that these sperms lost mobility characteristics, but still had functional membranes. These findings indicate that using extender solutions have an ability to preserve post-thaw drone sperm plasma membrane integrity to some degree. Therefore, it appears that post-thaw drone plasma membrane integrity can withstand stress more than the motility ability of the sperm when subjected to freezing related damages (Alcay et al., 2015). Casper et al. (1996) indicated that less than 50% of sperms have intact membranes in samples with complete astenozoospermia.

Cryoprotective agents including the widely used compound DMSO that was used in the freezing media have significant cytotoxic effects on spermatozoa, both in short term and long term storage. They can induce some factors from mitochondria to nucleus and poly-(ADPribose)-polymerase (PARP) activation that cause translocation of apoptosis (Hanslick et al., 2009). In addition, DMSO affects cell plasma membrane structures and induces pore formation (Notman et al., 2006). As a cryoprotective agent DMSO yields better post-thaw sperm quality in general and for this reason it has become one of the main extender ingredients in many studies to date (Paillard et al., 2017; Gül et al., 2017; Alcay et al., 2019a,b).

The present experiments were designed to improve post-thaw drone semen quality by adding different concentrations of Trehalose (0.05M, 0.1M or none at all) in freezing extender with 12% DMSO. The addition of 0.05M Trehalose to the extender with 12% DMSO leads to a small increase in recovery of post-thaw motility ($P>0.05$) and plasma membrane integrity ($P<0.05$), but when added at a 0.1M concentration this led to even better post-thaw motility ($P>0.05$) and plasma membrane integrity ($P<0.05$). Trehalose has specific interactions with sperm membrane phospholipids and minimizes the degree of cell damage during the freeze-thaw cycle by increasing the extender solution osmolarity (Storey et al., 1998; Solocinski et al., 2017). For example, supplementation of 0.2-0.6M Trehalose to media containing 10% DMSO improves post-thaw cell viability in several mammalian cell types (Beattie et al., 1997, Buchanan et al., 2004). However, the exact mechanism(s) by which Trehalose cryoprotects the cellular structures at ultra-low temperatures remains unknown. We observed that the addition of 0.05 and 0.1M Trehalose to the freezing extender solution containing 12% DMSO, increased post-thaw drone sperm plasma membrane integrity as well (Figure 2).

CONCLUSION

In conclusion, the freeze-thaw cycle is detrimental to post-thaw drone semen viability. The addition of 0.1 or 0.05M Trehalose to freezing media containing 12% Dimethyl Sulfoxide has been demonstrated to improve both post-thaw cell motility and plasma membrane integrity. More research is needed, to improve post-thaw sperm quality with other possible candidate substances in the future for honey bee artificial insemination applications.

DECLERATIONS

Corresponding author:
Email: icakmak@uludag.edu.tr

Authors' contributions

Prof. Dr. Z. Nur performed watering, freezing, thawing, evaluation, statistical analysis and writing manuscript, PhD student S. Çakmak collected semen from drones, Prof. Dr. İ. Çakmak reared, collected drones, interpreted data, contributed to manuscript writing and revisions, E. Gokçe performed watering, freezing, thawing and evaluation, Assoc. Prof. Dr. B. Ustuner and S. Alçay performed thawing, evaluation, statistical analysis, and manuscript writing, Research assistants N. Onder and M. Toker performed freezing of semen, Prof. Dr. M Soylu contributed to manuscript writing.

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Competing Interests
Authors declare no conflict of interest.

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