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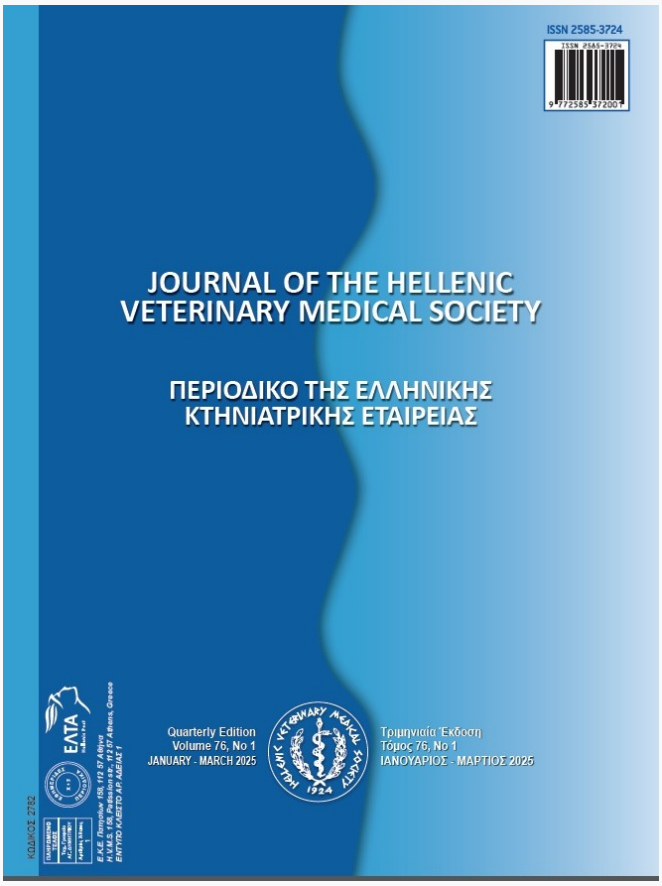

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Research article
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Effects of high and low glucose DMEM extenders on sperm cryopreservation in honey bees

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ABSTRACT: Honey bee sperm can be stored and used at room temperature for up to a week. However, freezing storage methods used for long-term storage negatively affect the sensitive honey bee semen. In order to reduce this negative effect, many different diluents and various freezing protocols have been investigated. This study aims to evaluate the effect of Dulbeco's Modified Eagle Medium (DMEM) diluent with two different glucose concentrations on the spermatological parameters of frozen honeybee semen. The study used two different DMEM diluents containing high and low concentration of glucose. Honey bee sperm samples, diluted 13 -fold with high glucose DMEM (4.5 g/L glucose, L glutamine and pyruvate) and low glucose DMEM (1 g/L glucose, L-glutamine and sodium bicarbonate) at a ratio of 1:12, were frozen in liquid nitrogen. For both groups, 10% (Dimethyl sulfoxide) DMSO was used as a cryoprotectant agent during freezing. After three months of storage at -196° C, samples were thawed and sperm parameters were evaluated (sperm motility, viability, plasma membrane functional integrity, and morphology). Motility, viability, and plasma membrane functional integrity parameters were high in honeybee semen frozen with DMEM diluent with higher glucose content ($P<0.001$). In conclusion DMEM diluent with high glucose content can be used effectively in the cryopreservation of honey bee semen.

Keywords: Cryoprotectant; DMEM; honey bee; semen; extender

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INTRODUCTION

Appropriate breeding studies are needed to preserve and improve existing genetic lines in honey bees. For this purpose, it is crucial to freeze honey bee sperm. There is an increasing interest in developing appropriate methods for cryopreservation of bee semen to support a long-term storage condition and honey bee sperm feritlizing capacity after thawing (Smilga-Spalvina et al., 2023). It is reported that the honey bee population must be protected due to the decrease in the number of insects due to the deterioration in environmental conditions in recent years (Gulov et al., 2023). New approaches regarding the cryopreservation of bee semen are essential and they could support the honey bee breeding, and enhance the genetic diversity in honey bees that could be related to resistance to various diseases (Wegener & Bienefeld, 2012). Thanks to cryopreservation of sperm, it is possible to protect endangered honey bee populations and develop desired traits so that breeders can increase productivity. Recently, significant advances have been made in the cryopreservation of honeybee semen (Auth & Hopkins, 2021). However, it is known that to increase and preserve the current quality of honeybee semen, better protocols and appropriate extenders are needed for freezing and cryopreservation (Dadkhah et al., 2016).

Cryopreservation of bee semen can be affected by different factors such as diluents, cryoprotectant agents (CPAs), equilibrium time, freezing rate, and thawing temperatures. Considering the species-specific effects of these factors, it is necessary to conduct studies to determine the optimum type and concentration of extenders and CPAs to develop a semen cryopreservation protocol suitable for the honey bee (Machebe et al., 2015). Cryoprotectant agents protect cells against damage that may occur during the freezing and thawing of semen, but attention should be paid to the amount added to the extender. Otherwise, it may have a toxic effect (Sieme et al., 2016). Extenders contain appropriate cryoprotectants, buffering agents, and necessary nutrients to maintain the optimal conditions of the semen after dilution (Machebe et al., 2015). The quality of the extender used to preserve honeybee semen indirectly affects the viability and motility of spermatozoa (Ioan Bratu & Pătruică., 2021.; Wegener et al., 2014). Therefore, cryopreservation protocols and appropriate extenders are critical for the post-thaw quality of honeybee semen (Yániz et al., 2019).

Some CPAs used during cryopreservation of bee semen cause various adverse effects on spermatozoa and a decrease in semen quality (Gulov & Bragina, 2022). Dimethyl sulfoxide (DMSO) has the least toxic effect compared to other cryoprotectant agents used in honey bee studies (Hopkins & Herr, 2010). In addition, DMSO is the most frequently used cryoprotectant agent in studies on the cryopreservation of honeybee semen (Wegener & Bienefeld, 2012). However, DMSO may damage the queen bee's reproductive tract during artificial insemination of the queen bee. For this reason, to eliminate the effect of the cryoprotectant agent in frozen and thawed honeybee semen, the sperm is separated by centrifugation after the thawing process. It has been observed that with this application, the viability of the queen bee and sperm is significantly preserved and does not negatively affect the spermatozoa concentration (Paillard et al., 2017).

The sugars added to the extender content are the energy source for merabolilc activity of spermatozoa (El-Sheshtawy et al., 2016). Honey bee sperm requires energy within the spermatheca of the queen bee. Thanks to the sugars in the spermatozoa, spermatozoa can use the glycolytic pathway. In this way, spermatozoa stored in the spermatheca produce ATP (adenosine triphosphate) using anaerobic metabolism (Slater et al., 2021). It has been reported that simple sugars (fructose, glucose, sucrose) in honey are essential for the energy metabolism of semen (Hashem et al., 2021). ATP, the energy source of sperm, is directly related to the motility of spermatozoa. Decreased ATP consumption causes fertility problems as it slows down motility during sperm competition (Kairo et al., 2016).

Dulbecco's modified Eagle's medium (DMEM) is a commercially available solution in many different forms. Cell culture studies are among the most commonly used areas in the clinical field. High glucose (4.5 g/L) or low glucose (1 g/L) solution forms are widely used. However, most DMEM formulations also contain pyruvate downstream of glucose in the glycolytic pathway and functions as an additional energy source (Babich et al., 2009).

In this study, the effect of DMEM supplemented with low or high glucose concentrataion on frozen-thawed honey bee spermatozoa parameters was evaluated.

MATERIALS AND METHODS

Preparation of study colonies

Five study colonies, each one with a population of at least 7 slatted bees, were used in the study. However, the colonies used in the study do not have a queen bee. Additionally, a support colony with 4 queen bees was used to meet the worker bee needs of the working colonies. Frame with drone larvae taken from support colonies were given to colonies without queen bees. These colonies were also supplemented with worker bees by support colonies at regular intervals. The male bees that emerged from the pupa were marked. When drones were 14 days old and went on a mating flight, they were collected with the help of a trap set at the entrance of the hive.

Collection of sperm

For each sample, 100 µl of sperm was collected from 120-130 drones (N=10). Before the experiment, the homogeneity of the groups in terms of spermatological parameters (sperm motility, viability, plasma membrane functional integrity, and morphology) was tested ($p > 0.05$). It was observed that the average viability and motility of fresh semen was above 90%. In order to collect sperm from male bees, the bees must complete the maturation process. For this reason, male bees are required to be at least 14 days or older (Cobey et al., 2013). The drone was kept in the appropriate position. Pressure was applied to the head and abdomen for eversion of the endophallus. Eversion was completed in 2 stages. In the first stage, orange bursal cornu appeared. In the second stage, complete eversion was achieved to collect semen by increasing the pressure. Semen was seen above the mucus secretion. A honey bee artificial insemination syringe Schiley model 1.02 (Peter Schley, Germany) was used for sperm collection. It was collected according to the technique with the help of a special syringe (Cobey et al., 2013).

Sperm cryopreservation protocol

The study used two different Dulbecco's modified Eagle's medium (DMEM) diluents with low (5mM D-glucose) and high (25mM D-glucose) glucose concentration. Honeybee sperm samples diluted at the same rate with the two different diluents, were frozen and stored at -196° C for 90 days. Semen collected from captured drones was diluted 13-fold with the appropriate diluent containing 10% DMSO in a 37°C hot water bath without delay after collection, and then was drawn into 0.25µl straws, 40µL per pipette (Taylor et al., 2009). The diluted honey bee sperms were kept in the refrigerator for 2 hours at +4°C for equilibration, then frozen at 4 cm above the surface of liquid nitrogen for 10 minutes and finally transferred to a liquid nitrogen tank (-196°C). Frozen semen samples were thawed in a water bath at 37 °C for 30 s and then sperm parameters were assessed (Sharaf et al., 2022). Frozen semen samples were thawed after being stored for 3 months.

Assessment of sperm variables

Spermatological parameters were evaluated under the phase-contrast microscope (Nikon, Eclipse E200, Tokyo, Japan) equiped with a heating stage at 37 °C.

Assessment of motility

For motility evaluation, 5 µL of the thawed semen sample was placed on a preheated (37°C) cover slide and evaluated at 100× magnification. Sperm motility was subjectively classified from 0% to 100% (Morais et al., 2022).

Assessment of viability

Each sperm sample was mixed with 5% eosin and 10% nigrosin at 37 °C, it was dried quickly and evaluated by counting at least 200 spermatozoa at 400×magnification. The spermatozoa with red colored heads were counted as dead, while those with white one were counted as alive. The results were expressed as percentages of dead and alive (Moreira et al., 2022).

Evaluation of plasma membrane functional integrity

Typically, sperm plasma membrane functionality is assessed by HOST. Since the sperm membrane reacts when spermatozoa exposed to a hypoosmotic solution, this considers as functional integrity. A HOST (9g fructose and 4.9g sodium citrate/1L distilled water) solution was prepared to evaluate plasma membrane functional integrity. A volume of 10 µL of the sperm sample was gently mixed with 100 µL of HOST solution and kept at 37 °C for 30 minutes. Finally, at least 200were counted. Those with curled tails were evaluated as HOST positive (H^+), and those with uncurled tails were evaluated as HOST negative (H^-) (Alcay et al., 2019).

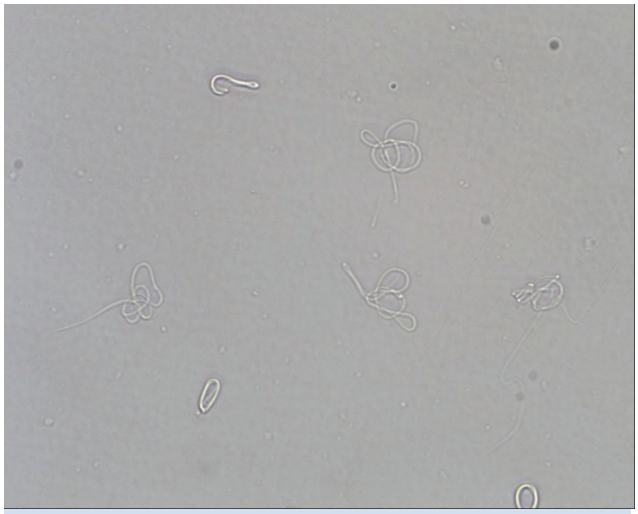

Figure 1. Evaluation of plasma membrane functional integrity in honey bee sperm (HOST positive)

Evaluation of spermatozoa morphology

For the morphological examination of bee semen, one drop of semen sample spread on the slide was stained with SpermBlue® kit (Microptic, Spain) dye and evaluated at 400× magnifications (Bratu et al., 2022). Morphological abnormalities in the head and tail were evaluated when examining abnormal spermatozoa in honey bees.

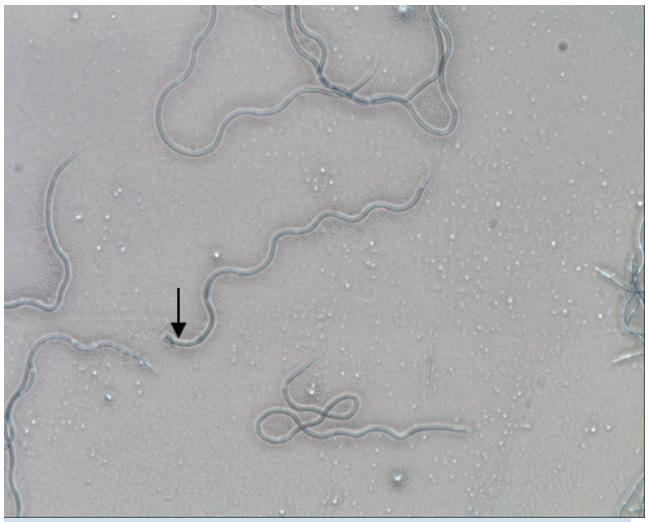

Figure 2. Curved tail abnormality of honey bee spermatozoon

Statistical analysis

This study used an independent samples t-test to statistically evaluate spermatological parameters (motility, viability, plasma membrane functional integrity, and spermatozoa morphology). The effects of the groups were considered significant at the $P<0.05$ level. SPSS 21.0 (IBM Corp., 2011) package program was used for variance analysis.

RESULTS

Effects on spermatological parameters (motility, viability, HOST, and spermatological morphology) of low/high glucose levels of DMEM extenders were given Table 1.

When the results were examined, a statistical difference was determined between the two DMEM diluents. In the evaluation made in terms of spermatological parameters, it was determined that while there was no difference in the rate of abnormal spermatozoa, all other spermatological parameters were statistically significantly affected by the glucose level in DMEM. The highest motility (76.00%), viability (70.40%) and HOST (74.10%) values were observed in the group with high levels of glucose in the DMEM diluent ($P<0.001$).

DISCUSSION

In our study, the significant impact of the different D-glucose concentrations in the DMEM extender on the cryopreservation success of honey bee semen was presented. it was observed that motility, viability, and plasma membrane functional integrity parameters were higher in honey bee semen frozen with DMEM diluent with high glucose content than in DMEM diluent with low glucose content. The optimal D-glucuse concentration in the extender was as high as 25mM for the effective freezing of honey bee sperm. We can suggest that 25mM D-glucose is successful for the cryopreservation of honey bee semen. This can be explained by the fact that since glucose is a resource that can be used directly in the cells, it meets the energy

Table 1. Effects on spermatological parameters of low/high glucose levels of DMEM (N=10)

Parameters	Groups		*P value*
	Group-1	***Group-2***	
Motility (%)	**34.00±4.702**	**76.00±4.397**	**<0.001**
Viability (%)	**46.80±5.151**	**70.40±1.928**	**<0.001**
Plasma membrane Functional integrity (%)	**34.10±3.831**	**74.10±3.857**	**<0.001**
Abnormal spermatozoa (%)	**12.50±0.764**	**12.30±0.746**	**NS**

Group-1: Low glucose levels of DMEM; Group-2: High glucose levels of DMEM, NS: Non-Significant

level of the sperm cell, and this may have a positive effect on spermatological parameters.

In some studies (Judycka et al., 2018; Judycka et al., 2024), the effect of added glucose in extenders on the motility in fresh and frozen-thawed sperm of different fish species was evaluated. Increasing the level of glucose in the current study caused a decrease in motility. However, In our study, the high glucose ratio was seen to increase honey bee sperm motility. This variability can be explained by differences in seminal plasma osmolality, which in fish is markedly. The osmolality of seminal plasma is lower in fish than in honey bees. While the average is 80 mOsm in fish, it is 430 mOsm in honey bees (Verma et al., 1973). It is thought that a high glucose ratio may adversely affect sperm parameters with low osmolarity.

We showed that the highest values of post-thaw sperm motility are found at high concentrations of D-glucose extender, approximately corresponding to the values presented previously with different species. In a 2020 study investigating the effects of semen extender and glucose concentration at storage temperature on stallion sperm quality after long-term refrigerated storage, it was reported that the absence of additional glucose in the extender reduced the motility properties of stallion sperm during long-term storage (Hernández-Avilés et al., 2020).

It demonstrates the necessity of fine-tuned cryopreservation protocols based on glucose concentrations to increase motility parameters of cryopreserved semen and recommends further studies focusing on the interaction between sperm motility properties and cryoprotectant and glucose concentration effects.

We have found that D-glucose concentration significantly affected values of sperm motility parameters in cryopreserved sperm of honey bees.Honey bee sperm is negatively affected by external factors such as the toxic effects of cryoprotectants, sensitivity to temperature changes, and cold shock. It is known that DMSO is the agent that has the least toxic effect on honey bee sperm among cryoprotectants (Hopkins and Herr, 2010). For this reason, 10% DMSO, a cryoprotectant frequently used in honey bee studies, was used in our study. In addition, in order to be less affected by external effects such as cold shock and to create the same external environmental conditions in all semen samples, a 37 °C hot water bath was used until the semen was absorbed into the pipettes. The study conducted overlaps with our current study.

Many studies have tested different substancesas diluents to preserve honey bee sperm. In a study about semen cryopreservation of semen, honey was added to the extender content. The diluent contained 10 mg sucrose, 10 mg lactose, 2.5 mL egg yolk, and 10% honey in the study. As a result, it has been observed that honey has a protective effect during the freezing process of semen. In addition, a high percentage of worker bees was determined in queen bees inseminated with frozen and thawed semen. It has been reported that honey may have significant potential in cryopreservation semen studies (Gulov et al., 2023). The honey solution used in the study contains sugar. It is thought that honey may play a protective role against external effects that may occur during the freezing of semen. In our study, the effects of a DMEM extender containing different amounts of glucose were examined, and it was observed that the extender with high glucose content had a significant effect on spermatological parameters. Additionally, the 9-fold dilution rate and the use of 10% DMSO as CPA in the current study are consistent with ours.

In a different study, the effects of Tris, Tris + egg yolk (Tris+EY), and Collins extender on the cryopreservation of honey bee sperm were examined. In that study, honey bee sperm was diluted 13-fold. No significant differences were found between the extenders used in the study regarding motility, viability, and plasma membrane functional integrity. However, in morphological evaluation, while the Tris extender had the lowest value regarding abnormal spermatozoa, the highest rate of abnormal spermatozoa was found in the semen diluted with Colin's extender (da Silva Morais et al., 2023). In our study, unlike the current study, although there was a significant difference in motility, plasma membrane functional integrity and viability rate, it was found to be insignificant in terms of abnormal spermatozoa. Therefore, when the results of the study are compared, the evaluated parameters and dilution rates are compatible with our study. However, it is thought-provoking that the obtained statistical data focus on opposite parameters.

It is known that there are limited studies on the morphological evaluation of abnormal spermatozoa in honey bees (J. L. Yániz et al., 2020). Generally speaking, tail-connected spiral tail, double tail, and frayed tail forms have been described. Three sugars (fructose, glucose, and trehalose) exist in free form within the reproductive system. Additionally, male bees in the hive contain more sugar than male bees

returning from their mating flight. It has been reported that the same sugars are also found in honey bee sperm (Birnie et al., 1957). Studies conducted in different species show that glucose added to the semen extender increases the motility rate in freeze-thawed semen (Hernández-Avilés et al., 2020). Energy production is essential for mammalian spermatozoa motility. There are two metabolic pathways for adenosine triphosphate (ATP) production in spermatozoa: glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). Since the most commonly used energy source in extender content for spermatozoa is glucose, the effect of this energy substrate was examined in our study. Some studies have observed that metabolic substrates that are readily incorporated into the mitochondrial oxidative phosphorylation (OXPHOS) pathway, such as pyruvate or lactate, may be effective in maintaining motility values when semen is incubated for one h at 37 °C or kept at room temperature for 72 h (Darr et al., 2016; Gibb et al., 2015). We observed high motility after solution in the semen diluted with a high-glucose diluent in our study, which may be due to the presence of pyruvate in this diluent. 4.5 g/L glucose and piravate are thought to may possibly protect the honey bee spermatozo against oxidative stress and support fertilization ability, especially in the high glucose DMEM structure. In addition, since glucose is a resource that can be used directly in cells, it is thought that it meets the energy level of the sperm cell, and this may have a positive effect on spermatological parameters.

In a study by Taylor et al. (2009), the authors investigated the effects of a widely used Kiev solution and five modified semen extenders on the frozen storage of honey bee semen. They established an average freezing and thawing duration of 72 hours. The results indicated that the inclusion of a combination of antibiotic antioxidants and DMSO in the extender significantly enhanced spermatological parameters, achieving the highest viability rates among the diluents assessed. Notably, this effective diluent was devoid of glucose or fructose, contrasting with some other extenders evaluated in the study. Conversely, the DMEM diluent, which demonstrated strong performance, is characterized by a high glucose content and the presence of pyruvate, a component frequently utilized in cell culture applications. Importantly, the extender identified by Taylor et al. also lacked sugars but was fortified with antibiotics and catalase, a powerful antioxidant that positively impacted sperm quality. In our study, honey bee semen was frozen and stored for three months. Based on our findings, we propose that DMEM with elevated glucose levels represents a promising option for the effective preservation of honey bee semen. The fact that both diluents are effective shows that they can increase sperm quality through different mechanisms. The fact that catalase protects cell health by reducing oxidative stress, while high glucose supports metabolic processes by providing energy, suggests that these two components may have a synergistic effect on sperm vitality. In conclusion, both the catalase-containing extender and the high-glucose extender were effective in preserving sperm cells and improving their quality. Understanding the functions of the two components may contribute to optimizing future sperm storage and freezing applications.

CONCLUSIONS

In conclusion, addition of high glucose concentration (25mM D-glucose) in DMEM extender has a more significant beneficial effect on honeybee semen characteristics. Further *in vivo* investigation is needed to confirm the present results.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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