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RESEARCH ARTICLE

L-Carnitine Supplemented Extenders Improve Post-Thawing Quality of Honey Bee Drone *(Apis mellifera)* Spermatozoa [1]

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Abstract

The study aimed to evaluate the effects of L-carnitine on honey bee *(Apis mellifera)* drone spermatozoon at post-thaw. Semen samples obtained from sexually mature drones were pooled. Then, pooled semen was diluted with different concentrations of L-carnitine (2.5 mM/ 5 mM/ 10 mM) and without L-carnitine (control) supplemented extenders. Motility, plasma membrane functional integrity, acrosomal integrity and mitochondrial function were negatively affected by the cryopreservation process but DNA integrity was not affected. Malondialdehyde (MDA) concentration was used for analyzing the membrane lipid peroxidation status and a better result was obtained in the LC5 group compared with the control group at post-thaw. L-carnitine groups had a positive effect on sperm motility, plasma membrane integrity, and mitochondrial function compared to the control group ($P<0.05$). Acrosomal integrity was better preserved in the LC5 group compared to the control group. The study shows that LC supplemented extenders have beneficial effects on honey bee drone sperm parameters. The results of the present study demonstrated the beneficial effect of using a 5 mMol LC supplemented extender.

Keywords: *Apis mellifera, Cryopreservation, Drone semen, Honey bee, L-carnitine*

L-Karnitin İlave Edilmiş Sulandırıcılar Bal Arısı *(Apis mellifera)* Spermatozoası'nın Çözdürme Sonrası Kalitesini Arttırır

Öz

Bu çalışmada, L-karnitinin eritme sonrası bal arısı *(Apis mellifera)* spermatozoonu üzerindeki etkilerini değerlendirme amaçlandı. Cinsel olarak olgun erkek arılardan elde edilen sperm örnekleri birleştirildi. Daha sonra birleştirilen semen, farklı konsantrasyonlarda L-karnitin içeren (2.5 mM/5 mM/10 mM) ve içermeyen (kontrol) sulandırıcılar ile seyreltildi. Motilite, plazma membranı fonksiyonel bütünlüğü, akrozomal bütünlük ve mitokondriyal fonksiyon kriyoprezervasyon sürecinden olumsuz etkilenmiş, ancak DNA bütünlüğü etkilenmemiştir. Malondialdehit (MDA) konsantrasyonu, membran lipid peroksidasyon durumunu analiz etmek için kullanıldı ve çözdürme sonrası kontrol grubuna kıyasla LC5 grubunda daha iyi bir sonuç elde edildi. L-karnitin grupları, kontrol grubuna kıyasla sperm motilitesi, plazma membran bütünlüğü ve mitokondriyal fonksiyon üzerinde olumlu bir etkiye sahipti ($P<0.05$). Akrozomal bütünlük, kontrol grubuna kıyasla LC5 grubunda daha iyi korunmuştur. Çalışma, LC takviyeli sulandırıcıların bal arısı sperm parametreleri üzerinde faydalı etkilere sahip olduğunu göstermektedir. Mevcut çalışmanın sonuçları, 5 mMol LC takviyeli sulandırıcı kullanmanın faydalı etkisini gösterdi.

Anahtar sözcükler: *Apis mellifera, Bal arısı, Erkek arı semen, Kriyoprezervasyon, L-karnitin*

INTRODUCTION

Beekeeping is indispensable in ensuring the integration of animal and plant production models. As a result of a better understanding of the contribution of honey and other bee products (royal jelly, propolis, pollen, beeswax, and bee venom) to human health in recent years, the areas of use for food, treatment, and cosmetic purposes have

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expanded [1]. Increasing honey production in the world can be achieved through productive and genetically improved colonies [2,3]. It is known that drones have an important effect on colony productivity in terms of genetic potential as well as that of the queen. Queen bees that cannot mate with a sufficient number of qualified drones will not be able to store enough sperm, so their economic use will be shorter [4]. Therefore, colony productivity can be increased by artificial insemination of queens with the genetically superior drone semen.

The genetic diversity within colony populations can be increased with cryopreservation of drone semen [5-7]. Although drone spermatozoon has been successfully cryopreserved in recent years, this process has a detrimental effect on spermatozoon because of temperature change and cold shock [2,8]. These undesirable effects cause a decrease in the fertilizing ability of spermatozoon [9-11]. Besides, during the freezing-thawing process, reactive oxygen species (ROS) emerge as a result of lipid peroxidation in the cytoplasm membrane. Excessive ROS formation can induce oxidative stress, leading to cell damage that can reduce motility and fertilizing ability. Therefore, antioxidants have been used to get rid of these undesirable effects of cryopreservation in recent years [9,12].

L-Carnitine (LC) is an endogenous compound maintaining cellular homeostasis, limiting the β-oxidation pathway, and acting in the transport of fatty acids to mitochondria [13,14]. Moreover, antioxidant characteristics and anti-apoptotic activities of LC may protect the mitochondrial membrane and DNA structure against ROS [14,15]. The use of L-carnitine in the extenders for buffalo, sheep, goat, rabbit, and rooster semen cryopreservation enhanced post-thawing sperm quality.

The cryopreservation of drone spermatozoon without losing its ability of fertilization contributes to the conservation of gene lines. Our hypothesis was that LC supplementation in the semen extender could improve the post-thawing drone sperm viability and its longevity. Hence, the present study was designed to compare different concentrations of LC supplemented extenders for the cryopreservation of drone sperm using quality tests.

Material and Methods

Chemicals

The chemicals used in the study were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Experimental Design

In the design of this study, the efficiency of LC supplementation extenders for drone semen cryopreservation was evaluated. For this purpose, we used various concentrations of LC (0 mM, 2.5 mM, 5 mM, and 10 mM) supplemented extenders for post-thaw quality of drone spermatozoon.

Extender Preparation

According to the experimental design, various LC concentrations were added in certain proportions to each extender group. We prepared the experimental groups as LC 2.5 (with 2.5 mM LC), LC5 (with 5 mM LC), LC10 (with 10 mM LC), and control (non-LC). Extenders contained Na citrate (82.21 mMol), catalase (1.59 mMol), KCl (5.34 mMol), NaHCO3 (24.87 mMol), amoxicillin (0.82 mMol), and DMSO (10%). The pH value of the diluents prepared was scaled to be 8.1.

Production of Drones

Healthy and strong honey bee colonies reared in Bursa Uludag University, Beekeeping-Development-Application and Research Center (artificial insemination of queens have been performed over 10 years and drones are also reared as part of the breeding studies) were used for semen collection.

Semen Collection and Dilution

Sexually mature drones (16 days and older) were selected for semen collection and at least five colonies were used for this research. Drone semen was collected five times every other day and at least 250 bees were used in each application. During the sperm collection, pressure was applied to the thorax to induce ejaculation, and then the abdominal area was gently squeezed. Approximately 1 µL semen was collected from per drone using the Schley syringe under a stereo microscope. Besides, to eliminate individual differences all semen was pooled. The volume of each pooled semen was portioned into four equal volumes. Each group of the extender was individually diluted with control or LC supplemented extenders to a final concentration of about 150×10^6 (spermatozoa/mL).

Semen Freezing and Thawing

The method of cryopreservation and thawing was based on Alcay et al.[12]. According to this method, equilibrated drone sperm was filled into 0.25 mL straws. After the filling process, straws were frozen in a programmable freezing device (Air Liquide, Marne-la-Vallée Cedex 3, France). Then the sperm-filled straws were immersed in liquid nitrogen and then stored in a liquid nitrogen tank. In each group, three straws were used for post-thaw semen parameters.

Semen Evaluation

In the evaluation of post-thaw semen, plasma membrane integrity, acrosome integrity sperm motility, and DNA integrity parameters were examined. A hypoosmotic swelling test (HOST) was used for plasma membrane integrity. FITC-Pisum sativum agglutinin (PSA-FITC) was used for acrosome integrity. Terminal deoxynucleotidyl transferase-

mediated dUTP nick-end labeling (TUNEL) is used to assess DNA integrity. Evaluations were made by the same person during the study.

- Motility

Drone semen motility assessment was performed using a phase-contrast microscope (Olympus BX51-TF - Olympus Optical Co., Ltd., Japan) with the slide heated to 37°C.

- Membrane Functionality

For the assessment of the plasma membrane integrity, the hypo osmotic swelling test method is used, which was previously described by Alcay [12]. Following this method, the membrane integrity of the drone sperm was evaluated by observing the frizzled tails.

- Acrosomal Integrity

For this evaluation, a sample of 10 µL spermatozoa was added into 100 mL of PBS and centrifuged for 5 min. The sperm pellet obtained after centrifugation was suspended again in 100 mL PBS. The spermatozoa obtained after these procedures were smeared. Smeared-slides were allowed to dry after being smeared. After the drying process is over, the smears were left in acetone fixation at 4°C for 15 min in a glass chalet (vertical, Hellendahl type). After fixation, smears were stained with FITC PSA solution for 1 h at 37°C in a light-proof sample kit. After the staining process was completed, at least 200 drone spermatozoa emitting fluorescent light were evaluated under a fluorescent attachment microscope [16].

- Mitochondrial Activity

Fluorescent stains, PI, and Rhodamine (R123) combination were used to examine mitochondrial integrity. For this analysis, Fareser's method was used [17]. Results are expressed as a percentage.

- DNA Fragmentation

DNA fragmentation was evaluated by the TUNEL technique using In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications [18].

- Malondialdehyde (MDA) concentrations

For the assessment of MDA concentrations, the method described by Sharafi is used [19]. As per the method, 0.25 mL of diluted semen sample was treated with 0.25 mL of cold 20% (w/v) trichloro aceticacid to precipitate the protein. During the centrifugation, the precipitated protein was pelleted and the supernatant was incubated with (w/v) thiobarbituric acid for 10 min in a 100°C boiling water bath. After the incubation in the hot water bath, the sample was allowed to cool. Absorbance was determined using a spectrophotometer (Mannheim Boehringer Photometer 4010). MDA concentrations were expressed as nmol/mL.

Statistical Analysis

All data were analyzed using IBM SPSS version 23 (Chicago, IL, USA). Shapiro Wilk test was used as normality test. Data were represented as mean ± standard deviation (SD). Statistical significance of differences between subdivided groups were analyzed with one-way ANOVA followed by Tukey.

Results

The percentages of motility, plasma membrane functional integrity, acrosomal integrity, mitochondrial function, and DNA fragmentation rates of pooled semen samples were 89.00±2.24, 93.80±1.30, 95.40±0.89, 93.00±2.00, and 0.40±0.89, respectively. In the study, sperm quality were negatively affected the cryopreservation process compared with the fresh pooled semen (P<0.05). The *Table 1* shows the effects of different concentrations of LC on drone sperm parameters at post-thawed.

The sperm motility and plasma membrane functional integrity was better-preserved in LC groups compared with the control group at post-thaw (P<0.05). Besides, the highest percentage of motility and membrane integrity rates were obtained from the LC5 group (P<0.05). The better acrosomal integrity was obtained in the LC5 group compared to the control group (P<0.05). The percentage of acrosomal integrity was not found significantly different among other groups (P>0.05). Mitochondrial function rate was higher in the LC5 group compared with the other

Table 1. *The mean of studied sperm post-thawing parameters on different extender groups*

Variable	L-Carnitine Concentrations (mM)			
	0	2.5	5	10
Motility (%)	49.67±2.96^{a}	52.67±2.58^{b}	62.00±2.54^{c}	57.66±2.58^{d}
HOST (%)	59.33±3.62^{a}	63.40±2.53^{b}	70.13±2.07^{c}	66.27±1.62^{d}
Acrosomal Integrity (%)	80.67±3.67^{a}	81.27±2.96^{a}	84.27±2.60^{b}	82.47±2.83^{ab}
Mitochondrial function (%)	58.00±3.05^{a}	61.80±2.48^{b}	67.93±3.10^{c}	64.73±2.49^{d}
DNA fragmentation (%)	0.33±0.62^{a}	0.40±0.63^{a}	0.40±0.63^{a}	0.47±0.64^{a}

Data is presented in Mean± S.D.; Different letters within the same rows show significant differences among the groups (P<0.05)

Table 2. *Malondialdehiyde (MDA) levels in frozen-thawed drone sperm*

Parameter	Groups			
	Control	LC2.5	LC5	LC10
MDA (nmol/mL)	3.22±0.67[a]	3.00±0.71[ab]	2.33±0.50[b]	2.78±0.67[ab]

Data is presented in Mean± S.D.; Different superscripts (a and b) in the same line indicate significant differences (P<0.05)

LC and control groups (P>0.05). The percentage of DNA damaged spermatozoa were not significantly different in all groups at post-thaw (P>0.05).

As shown in *Table 2*, it was found that MDA levels in the LC5 groups were lower than the control group (P<0.05). Also, there were no significant differences among the LC groups.

Discussion

Cryopreservation of spermatozoa ensures that genetic material is preserved for a long time. However, it is known that the freezing-thawing process has negative effects on the fertilization ability of spermatozoa [9,16,20]. These undesired effects decrease viability, motility, plasma membrane, and acrosomal integrities of spermatozoa [12]. Besides, poor sperm quality generates poorer quality of queens which is one of the main causes of colony loss [21].

LC is a water-soluble amino acid that has a beneficial effect on spermatozoa during the freeze-thaw process because of antioxidant characteristics and anti-apoptotic activities. In the study, we evaluated the effect of exogenous addition of LC in extender on drone sperm quality at post-thaw [14].

Motility is essential for sperm migration to the queen's spermathecal and subsequent egg fertilization. Therefore, it is one of the most widely used spermatological parameters to evaluate sperm quality in drone sperm similar to mammals [22]. In the study, the presence of LC in the extenders increased drone sperm motility compared to the control group at post-thaw (P<0.05). LC facilitates the transport of fatty acids across the inner membrane of mitochondria for ATP production. Therefore, this characteristic of LC may be responsible for improving the motility of drone sperm. Also, the motility values of drone spermatozoa cryopreserved with various semen extenders ranged between 25% and 62% in the different studies [1,10,12,23]. Our study shows that post-thaw motilities in the LC groups are in good agreement with the findings of the previous studies.

Plasma membrane integrity is one of the most frequently evaluated sperm quality parameters for semen analysis in the honey bee because the loss of membrane integrity is considered incompatible with sperm viability [24]. However, cold shock, ice crystallization, osmotic stress, and lipid peroxidation have negative effect on membrane permeability and integrity during cryopreservation [7,25]. Therefore, it is crucial to keep the integrity during the cryopreservation process to avoid cellular damage. HOST is the optimized test for detecting the subtle changes of spermatozoon membrane functionality [26,27]. In the study, the plasma membrane functional integrity values in the LC5 group was higher than those of in the other groups at post-thaw (P<0.05). The HOST values are in agreement with the previous studies [10,12,16,28].

During oviposition, the queen releases few spermatozoa from the spermatheca, and then the acrosome reaction releases lytic enzymes that aid in the penetration of the vitelline membrane to fertilize the egg. Therefore, acrosomal integrity is crucial for the fertility of spermatozoon at post-thawed [9,11,28]. In the honey bee, the acrosomal integrity could be evaluated by Pisum sativum agglutinin (PSA) lectin staining method [12]. In the study, there was no statistical difference among LC2.5, LC10, and the control groups. LC5 group preserved acrosomal integrity better than LC2.5 and the control groups. These results are in agreement with the previous researches [12,16].

Spermatozoon needs energy to carry out its functions and it can mostly obtain ATP through the glycolytic and oxidative phosphorylation pathways [29,30]. Mitochondria play an essential role in regulating sperm function [22]. Therefore, it is important to investigate the mitochondrial function for spermatozoon quality. In the study, mitochondrial function was better preserved in LC groups compared to the control group (P<0.05). Only one study is present evaluating the mitochondrial function of honey bee drone spermatozoa [31]. Similar results were obtained in our study.

During cryopreservation, protecting the DNA integrity also has great importance not to disrupt the early development of the embryo [18]. In this study, it was observed that drone spermatozoa were resistant to the freeze-thaw process. Besides, our DNA integrity rates statistically the same at post-thaw for all groups (P>0.05).

Oxidative damage may be evaluated by MDA levels which is a key product of polyunsaturated fatty acid's peroxidation in the cells. In our study, MDA levels in the LC5 group were lower than that of the control group (P<0.05).

The results of this study indicated that the 5 mM LC supplemented extender was the optimal for the drone semen cryopreservation process. However, the fertilizing ability of spermatozoon is crucial, and further studies must

be focusing on the effect of LC on reproductive success (viable off spring) when used to fertilize the queens.

Conflict of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author Contributions

SA, EA, MA, HS designed the experiment. SA, SC, IC, AA, MY, performed the experiment. BU, ST, ZN analyzed the data. HS, ZN made tables, and wrote the paper. AA, SA revised the manuscript. All authors reviewed and approved the final manuscript.

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