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Bal Arısı Sperma Sulandırıcısına Katılan Shilajitin Spermanın Dondurulması Üzerine Etkileri

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ÖZET

Bu çalışmanın amacı, bal arısı (*Apis melifera*) sperma sulandırıcısına katılan farklı dozlardaki (%0, 5, 10, 15, 20) shilajitin (S) motilite, plazma membran bütünlüğü ve akrozom bütünlüğü parametreleri üzerine etkilerini belirlemektir. Çalışmada, 5 grup ve her bir grupta 5'er adet koloni olmak üzere toplam 25 adet koloni kullanıldı. Kontrol grubu (S-0), bal arısı sulandırıcısına shilajit ilavesi yapılmayan grubu oluşturmaktadır. S-1, S-2, S-3 ve S-4 grupları ise bal arısı sulandırıcısına sırasıyla %5, 10, 15 ve 20 shilajit ilaveli gruplar olarak oluşturuldu. Gün aşırı beslenen yaklaşık 25 adet koloni içerisinde 14-21 günlük yaşta bulunan yaklaşık 400 erkek arıdan sperma toplandı ve sıvı azot buharında dondurularak, -196°C sıvı azot içerisinde yaklaşık 7 ay süre ile saklandı. Bu süre sonunda dondurulan numuneler 37°C' de çözdürülerek ilgili parametreler yönünden incelendi. Diğer gruplar ile karşılaştırıldığında, S-3 ve S-4 gruplarının sperma motilite değerini arttırdığı ($p<0.05$); S-2, S-3 ve S-4 gruplarının plazma membran bütünlüğünü arttırdığı tespit edildi. Sulandırıcıya farklı dozlarda shilajit ilave edilen gruplarda, kontrol grubuna kıyasla akrozom bütünlüğününün önemli derecede korunduğu belirlendi ($p<0.01$). Sonuç olarak, bal arısı sulandırıcısına farklı dozlardaki shilajitin spermatolojik parametreler üzerine olumlu etkileri olduğu saptandı.

Zootekni

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Bal arısı
Dondurma
Shilajit
Sperma

Effects of Shilajit Added to Honeybee Sperm Extender on Sperm Freezing

ABSTRACT

The aim of this study was to determine the effects of shilajit (S) at different doses (0, 5, 10, 15, 20%) added to honey bee (Apis melilifera) semen extender on motility, plasma membrane integrity and acrosome integrity parameters. In the study, a total of 25 colonies, 5 groups and 5 colonies in each group, were used. The control group (S-0) constitutes the group where shilajit was not added to the honeybee extender. S-1, S-2, S-3 and S-4 groups were formed as groups with 5, 10, 15 and 20% shilajit added to the honey bee extender, respectively. Sperm were collected from approximately 400 drones aged 14-21 days in approximately 25 colonies that were fed every other day. Then they were frozen in liquid nitrogen vapor and stored in liquid nitrogen at -196°C for approximately 7 months. At the end of this period, the frozen samples were thawed at 37°C and examined in terms of relevant parameters. Compared with other groups, S-3 and S-4 groups increased sperm motility ($p<0.05$); It was determined that S-2, S-3 and S-4 groups increased plasma membrane integrity while the acrosome integrity was significantly preserved in the groups with different doses of shilajit added to the diluent compared to the control group ($p<0.01$). As a result, it was concluded that different doses of shilajit to honey bee extender had positive effects on spermatological parameters.

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INTRODUCTION

Storage of honeybee semen is important for preserving racial characteristics and increasing yield characteristics. With the widespread use of artificial insemination in bees, studies on the storage of bee semen have been carried out. Honeybee semen can be stored at 15-28°C or 11 -13°C for a short time, protected from light (Collins, 2000). Drone semen is suitable for storage at temperatures from 13°C to 25°C (Harbo and Williams, 1987). It has been stated that the most suitable semen storage temperatures are between 12°C and 16°C. Moreover, it has been reported that the semen viability rate was above 70% when kept at 12°C for 6 weeks (Locke and Peng, 1993; Collins 2000). Fresh bee semen can be stored in a capillary glass tube. A small amount of diluent on both sides of the tube, diluted semen in the middle and the two open ends of the tube can be sealed and stored (Burley et al., 2008). Such capillary tubes can be stored in special incubators at 16 ±°C, protected from light (Paillard et al., 2017). For long-term storage, various cryoprotectants (DMSO (Dimethyl sulfoxide), glycerol) can be added to the semen diluent and stored in a liquid nitrogen tank (Harbo, 1979). It has been reported that DMSO is the cryoprotectant agent that causes the least harm among other cryoprotectants (Hopkins and Herr, 2010). It was determined that the rate of male eggs, that is, unfertilized eggs, increased in queen bees artificially inseminated with long-term stored honeybee semen. It is also thought that genetic damage may occur during the freezing of honeybee semen (Harbo, 1981).

Shilajit is a brown-blackish substance extracted from the rock layer of the mountainous region of the world, especially from some parts of India (Agarwal et al., 2007). Shilajit contains antioxidant-rich compounds in its structure (Rege et al., 2012). In particular, it is a substance rich in humic and fulvic acids (Ghosal et al., 1990). It is known that shilajit is very effective against the damage of free radicals thanks to the antioxidants it contains (Bhattacharya et al., 1995). It has been reported that shilajit shows strong antioxidant properties, as well as increasing the effectiveness of vitamin C, which has antioxidant properties (Agarwal et al., 2007). In a study on rats, it was stated that shilajit decreased the symptoms of fatigue by increasing mitochondrial activity (Surapaneni et al., 2012). It is also known that shilajit, which has more than 200 mineral substances in its structure, is effective in eliminating reproductive problems in many Asian countries. In studies on rats and humans, it has been reported that shilajit increases semen density and serum testosterone ratio (Zubair, 2016). In a study that was conducted on humans, shilajit was found to significantly reduce the harmful effects of oxidative stress. It has also been stated that shilajit contributes to increasing the motility, semen density and serum testosterone level, which are important semen parameters (Biswas et al., 2010).

The aim of this study was to determine the effects of shilajit, which is known to have significant positive effects on semen, on long-term storage by adding it to honeybee semen diluent.

MATERIAL and METHODS

Preparation of diluents and freezing of semen

All colonies used in the study were fed with syrups prepared with sugar at a ratio of 1:1 every other day. A modified Kiev diluent (D+-glucose 0.3 g, potassium chloride 0.41 g, 2.43 g disodium citrate dihydrate per 100 ml) and 10% DMSO was used as semen diluent (Rosseau and Giovenazzo, 2016). While shilajit was not added to the diluent prepared for the control group (S-0) in the colonies used in the study, semen diluents were prepared by adding 5, 10, 15, and 20% shilajit to the S-1, S-2, S-3, and S-4 groups, respectively. Sperm was collected from approximately 400 drones aged 14-21 days in 25 colonies. Fresh semen collected from drones that have reached sufficient maturity were examined in terms of motility, and it was observed that the average motile spermatozoa rate was above 80%. Also, sperm with a motility evaluation of less than 80% in fresh semen were not included in the study. Each group was separetely diluted to final concentration of approximately 100 $x10^6$ (spermatozoa/mL) with control or shilajit supplemented extenders. After the diluted semen was transferred into 0.25 ml straws in accordance with the procedure (diluent-air-space-semen-air-space-diluent), the openend of the straw was pressed and closed. Straws were kept at 4 ºC for 2 hours for equilibration. Then, it was kept in liquid nitrogen vapor 5 cm below the straws on the special wire grid in the liquid nitrogen setup for 10 minutes, and after freezing, it was stored in liquid nitrogen. Straws stored by freezing were thawed at 37 ºC for 30 seconds and evaluated.

Evaluation of semen

Motility

Semen straws were thawed at 35-36°C for 30 seconds. Afterwards, 5 µl was taken from it and placed on the slide and covered with a coverslip. Motility evaluation was done with a computer-assisted semen analyzer

(CASA) (SCA®, Microptic, Barcelona, Spain). The results were evaluated as 20%, 40%, 60%, 80% and 95% and above, in particular for honey bee semen (Taylor et al., 2009).

Plasma membrane integrity

To evaluate the integrity of the plasma membrane 100 µl of 100 mOsm HOST (Hypo-Osmotic Swelling Test) solution was used. After 10 µl of thawed semen was left at 37 °C for 30 minutes, 200 spermatozoa were counted. Coiled and bent-tailed spermatozoa were detected (Kumar et al., 2018).

Acrosome integrity

In the study, 100 ml of phosphate buffer solution (PBS) was added to 10 µl of semen. After centrifugation at 100 RCF for 5 minutes, semen was separated from PBS. After the part was completed by adding 100 ml of PBS, it was centrifuged again for the second time. After removing the supernatant PBS, the smear was taken. After staining with PSA-FITC (L0770) solution in the darkroom, it was kept in the dark at 37°C for 1 hour. At least 200 spermatozoa were evaluated under fluorescence microscopy and stained ones were expressed as a percentage (Alçay et al., 2019).

Statistical analysis

The statistical significance level for each group used in the study was made with 'One-Way Analysis of Variance'. Duncan's multiple range test was used to investigate the differences between group means. The effects of the groups were evaluated as significant at the at the P<0.05 level. SPSS 20.0 (IBM Corp., 2011) package program was used for variance analysis of the data obtained from the study.

RESULTS and DISCUSSION

In the study investigating the effects of different levels of shilajit (0, 5, 10, 15, 20 %) added to honey bee semen diluent on long-term storage of semen, it was determined that there was a significant difference between the groups in terms of motility, HOST and acrosome integrity parameters (Table 1).

Table 1. Spermatological parameter values between groups
Çizelge 1. Gruplar arasındaki spermatolojik parametre değerleri

Groups	Total Motility (%)	HOST (%)	Acrosome Integrity (%)
S0 (Control)	28.01±4.90^{c}	56.20±2.15^{c}	87.00±0.32^{c}
S1 (%5 Shilajit)	36.06±7.48^{bc}	61.40±3.49^{bc}	88.00±0.00^{b}
S2 (%10 Shilajit)	32.00±4.90^{bc}	64.00±2.59^{ab}	87.80±0.20^{b}
S3 (%15 Shilajit)	48.71±4.90^{ab}	69.20±2.46^{a}	88.40±0.25^{b}
S4 (%20 Shilajit)	56.04±4.00^{a}	69.80±1.39^{a}	89.00±0.00^{a}
P	0.008	0.005	0.000

Data is presented in Mean ± S.D.
^{a,b,c} Values Differences between means with different letters in the same row are significant.

In our study, which investigated the effects of adding different levels of shilajit to honey bee semen on the cryopreservation of bee semen, mean motility values were determined as 28,01 ±4.90%, 36,06 ±7.48%, 32,00 ±4.90%, 48,71 ±4.90% and 56,04 ±4.00% in the groups (Control, S1, S2, S3, S4), respectively (Figure 1).

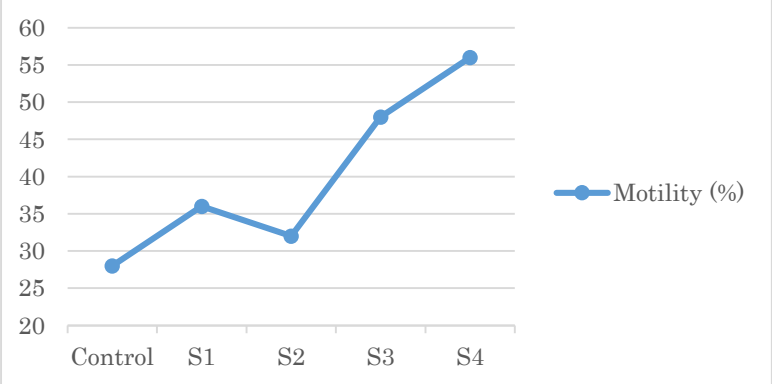

Figure 1. Semen motility distribution between groups
Şekil 1. Gruplar arasındaki sperma motilite dağılımı

There was a statistically significant difference between the groups in terms of motility values (P<0.05). Particularly, the percentage of motility was found to be the highest statistically in the S4 group,

in which shilajit added 20% to the semen diluent, compared to the other groups (P<0.05).

In the study of Ikram-ul-Haq et al., (2016) on semen parameters of shilajit in rams, 3 different groups were formed with 4 rams in each group. In the study, it was observed that shilajit administered orally at different doses for 9 weeks significantly increased the semen volume and motility values. However, it is seen that the use of shilajit is not similar to our study. In another study, the effects of shilajit treatment were observed in mice with infertility by administering cadmium, and it was reported that shilajit had a significant effect on eliminating the negative effects on semen parameters (Mishra et al., 2018). A study invested the effects of fulvic acid on semen parameters in goats observed that fulvic acid, which is found in a significant amount in the structure of shilajit applied at different doses, had a positive effect on semen motility values (Xiao et al., 2018). Fulvic acid was added to Tris-egg yolk diluent at different doses and stored in liquid nitrogen for about 2 weeks after being frozen and thawed under appropriate conditions, and its effects on goat semen parameters were observed. The presence of fulvic acid used in the study in the structure of shilajit, the addition of fulvic acid used in the study to the diluent at different doses, and the examination of frozen and thawed semen in terms of motility are in line with our study. While there was no significant difference between the different doses in the study, it was observed that fulvic acid significantly increased the percentage of motility when compared with the control group.

The mean HOST positive percentages of the groups in our study (Control, S1, S2, S3, S4) were determined as 56.20 ±2.15%, 61.40 ±3.49%, 64.00 ±2.59%, 69.20 ±2.46% and 69.80 ±1.39%, respectively (Figure 2).

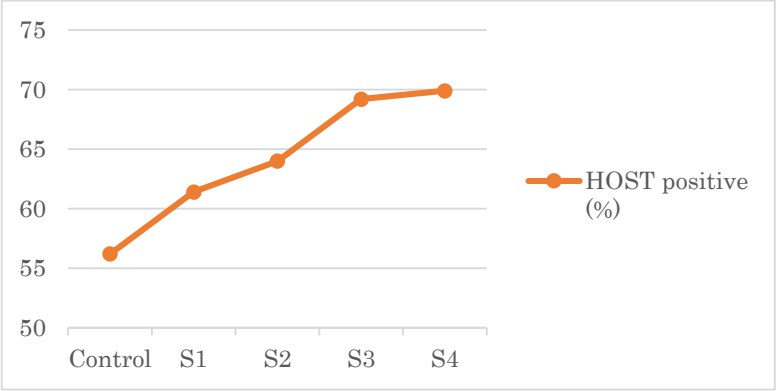

Figure 2. Distribution of HOS test positive (%) between groups
Şekil 2. Gruplar arasındaki HOS testi pozitif (%) dağılımı

It was determined that the addition of shilajit to the semen diluent significantly increased the HOST positive percentage (P<0.05). When the statistical average values were examined, it was found significant that the percentage of membrane permeability increased proportionally with the increase in the amount of shilajit used in general. The increase in the positive rate of plasma membrane integrity indicated that the membrane structure of the semen was preserved. Sultan et al., (2021), in a study in which they examined the effects of shilajit added to buffalo semen diluent at different doses, determined that 3% shilajit was more successful than other study groups. A decrease in plasma membrane integrity was found to be significant with an increase in the shilajit ratio above 3%. In a study where a special mixture containing shilajit was applied orally on buffaloes, the semen collected was diluted and frozen. In the study, a significant increase was observed in the acrosome integrity as well as the plasma membrane integrity (Kumar et al., 2018). Although the positive effect of shilajit was observed in our study, the oral administration of the application is not similar to our study. In our study, it was determined that the addition of shilajit at different doses increased acrosome integrity in the control group (P<0.01) (Table 1).

There was a significant difference between the groups in terms of positive values of acrosome integrity (P<0.05). As can be seen from the mean values (%) of control (87.00 ±0.32), S1 (88.00), S2 (87.80 ±0.20), S3 (88.40 ±0.25) and S4 (89.00), it was observed that the positive rate of acrosome integrity gradually increased as the amount of shilajit added to the semen diluent increased (Figure 3).

It was observed that the addition of shilajit statistically significantly increased the percentage of acrosome integrity (P<0.05). Acrosome integrity was found to be the highest in the S4 group, in which 20% shilajit was added to the semen diluent (P<0.05). Alçay et al., (2019) reported that the diluent had positive effects on acrosome integrity in their study on the dilution of honeybee semen with TL-Hepes based and with BSA (bovine serum albumin) added

diluent. The study is significant for determining the effectiveness of the substance added to the honeybee diluent. Although the result of the study is positive, it is not similar to our study because the content of the substance used is different. According to Tripathi et al. (1996), shilajit plays an important role in preventing lipid peroxidation, which plays an important role in the disruption of spermatozoa membrane integrity.

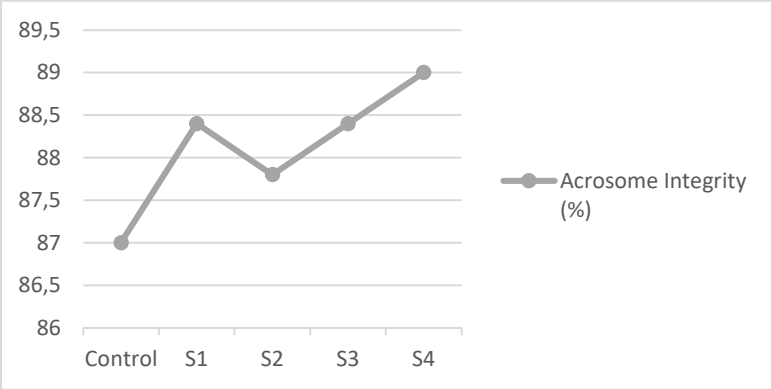

Figure 3. Distribution of acrosome integrity (%) between groups
Şekil 3. Gruplar arasındaki akrozom bütünlüğü (%) dağılımı

Regarding the storage of honeybee semen, shilajit seems to affect the storage conditions positively. As a result of the study, it was observed that the addition of shilajit to the honeybee diluent contributes to the long-term storage of honeybee semen and has a positive effect on motility, plasma membrane integrity and acrosome integrity. The results of the study reveal that the use of shilajit in semen diluent will provide long-term storage conditions in honeybee semen. It is aimed to improve long-term storage conditions with such studies on honeybee semen. As a result, it is understood that shilajit significantly improves the storage conditions by making a significant contribution to the preservation of motility, plasma membrane integrity and acrosome integrity in the long-term storage of honeybee semen.

Author's Contributions

The contribution of the athors is equal.

Conflict of Interest

The authors are declared that there is no conflict of interest.

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