

Effects of shilajit addition to honey bee diet on semen freezing

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Abstract

This study aimed to investigate the effects of adding shilajit to the diet of honey bees on semen freezing. A total of 5 groups were formed in the research, one of which was a control group (SH-0) and the other four were an experimental group (SH-1, SH-2, SH-3, SH-4). A total of 25 study colonies were used, 5 in each group. While the SH-0 group was formed without using any additives, the experimental groups were fed with the addition of shilajit (1/1 sugar/water) in different doses (5, 10, 15, 20 mg/L) to the honey bee diet. The collected semen samples were frozen in liquid nitrogen vapor and then stored in liquid nitrogen at -196°C. Then, semen samples were thawed at 37°C and evaluated to determine spermatological parameters (motility, acrosome integrity, plasma membrane integrity, spermatozoa concentration). It was also examined in terms of total oxidant and total antioxidant. Compared to the control group, it was determined that all shilajit doses significantly increased spermatological parameters such as motility, hypoosmotic swelling test (HOST), acrosome integrity ($p < 0.001$), but did not significantly affect the spermatozoa concentration value ($p > 0.05$). Although there is no statistical difference between the groups in terms of semen TAS (Total Antioxidant Status) and TOS (Total Oxidant Status) values, which are oxidative stress parameters, the numerical increase in TAS values in SH-2 and SH-3 groups; striking. As a result, it was determined that adding shilajit to the honey bee diet positively affected the post-thawing spermatological parameters of frozen bee semen.

Keywords: Antioxidant, Freezing, Honey bee, Shilajit, Semen

INTRODUCTION

To increase the yield characteristics of honey bees (*Apis mellifera*), appropriate colonies should be obtained to benefit from artificial insemination, to provide genetic improvement and to carry out breeding studies. It is known that gene errors that cause offspring death may occur as a result of the increase in the consanguinity status of the colonies (Danka et al., 1986). To prevent inbreeding and to prevent diseases caused by the queen bee, it is necessary to add blood from colonies with appropriate breeds and desired characteristics. For this purpose, it is very important that honey bee semen can be stored. It has been reported that great success has been achieved in artificial insemination applications recently (Cobey, 2007).

Cryopreservation methods are used for the long-term storage of honey bee semen. For this purpose, semen can be stored in liquid nitrogen for the short or long term by using various cryoprotectants such as dimethyl sulfoxide (DMSO) and glycerol (Harbo, 1979). However, it has been determined that queen bees fertilized with semen stored for a long time lay a large number of unfertilized

eggs. This situation is thought to be due to genetic damage (oxidative stress) during the freezing of semen (Harbo, 1981). Oxidative stress is defined as the harmful effects of ROS (reactive oxygen species) due to antioxidant deficiency, which is the body's natural defense mechanism (Tremellen, 2008). Petruska et al. (2014) emphasized that antioxidants added to semen used in artificial insemination in farm animals are important for reducing the negative effects caused by ROS.

Shilajit has been widely used in medicine for many years. Shilajit has antioxidant and anti-inflammatory properties with the active ingredients it contains. It has been stated that fulvic acid in the structure of shilajit causes a decrease in the abnormal semen rate and malondialdehyde (MDA) level, in addition, it protects the integrity of the membrane and acrosome and causes an increase in motility (Xiao et al., 2018). Stohs (2014) stated that shilajit has antioxidant, anti-inflammatory, immunomodulatory, adaptogenic effects as well as positive effects on spermatogenesis. Moreover, Biswas et al. (2010) reported that shilajit had a positive effect on oxidative stress in oligospermic patients, causing a decrease in MDA levels and a positive effect on motility, spermatozoa concentration and semen volume. Furthermore, Mishra et al. (2018) stated that shilajit is effective against the negativities caused by toxic substances.

In this study, it was aimed to determine the effects of shilajit added to the honeybee diet on the long-term storage of honeybee semen.

MATERIAL AND METHODS

In this study, a total of 5 groups were generated, one of which was the control (SH-0) and the other four (SH-1, SH-2, SH-3, SH-4) were the experimental groups. A total of 25 study colonies, 5 in each group, were used. In the present study, 10 support colonies were used to provide drone larvae of the colonies and to meet the worker bee needs. The control group was fed with no additives, and the experimental groups were fed with the honey bee diet (1/1 ratio sugar/water) with the addition of shilajit in different doses (5, 10, 15, 20 mg/l). Drone slats from support colonies were transferred to working colonies, where the mature drones were collected to collect semen when they were 2-3 weeks old. By applying pressure to the thorax and abdomen of the drones, the endophallus was exposed and semen was collected. At the beginning of the study, a motility evaluation was performed in native semen, and it was observed that the mean of motile spermatozoa rate was above 80%. To dilute semen, Modified Kiev Solution (0.3 g D⁺-glucose, 0.41 g KCl, 2.43g sodium citrate dihydrate per 100 ml) (Rosseau and Giovenazzo, 2016), was prepared containing 10% DMSO. Reconstituted semen was drawn into 0.25 mL straws in accordance with the diluent-air-space-semen-air-space-diluent procedure, and the tip of the straws was pressed and closed. Straws were kept at 4°C for 2 hours for equilibration. Straws were frozen in liquid nitrogen vapor for 10 minutes at approximately 5 cm above the liquid nitrogen assembly. The frozen straws were transferred to the liquid nitrogen tank for long-term storage. Then, thawing was performed at 37 °C for 30 seconds to evaluate the semen samples. Thawed semen were examined in terms of motility, plasma membrane integrity (HOST), spermatozoa concentration, acrosome integrity, total antioxidant level (TAS) and total oxidant level (TOS) parameters. Motility was determined as 20%, 40%, 60%, 80%, taking into account the intensity of the circular movement specific to honeybee semen (Taylor et al., 2009., Gontarz, et al 2016). To determine the HOST positive rate, 10 µl of semen were kept in 100 µl of 100 mOsm HOST solution at 37°C for 30 minutes. 200 spermatozoa were counted and spermatozoa with coiled, bent tails were evaluated as HOST positive (Alçay et al., 2019). In order to evaluate the concentration, of spermatozoa, 1 µl of semen was diluted with 1 ml of Kiev diluent, then placed on a Thoma counting chamber and counted 5 middle squares at 400x magnification under the microscope and calculated according to the formula below (Tekin, 1994; Cobey, 2013). To determine the acrosome integrity, 10 µl of semen was added to 100 ml of phosphate buffer saline (PBS), it was centrifuged at 100 RCF for 5 minutes and the supernatant was separated and discarded. PBS in 100 ml was added to the remaining semen and centrifuged at 100 RCF for 5 minutes. Again, the supernatant was discarded. A smear was taken from the semen below and stained with PSA-FITC solution in the darkroom. After keeping it in the dark at 37°C for 1 hour, at least 200 spermatozoa were examined under a fluorescent microscope and the acrosome integrity was determined as % (Alçay et al., 2019). Spectrophotometric (Thermo®) measurements were made using special kits (REL Assay Diagnostics®) to determine TAS and TOS values, which are oxidative stress parameters.

Statistical analysis of data obtained in the present study (One-way ANOVA) and comparisons between groups (Duncan's test) were performed using SPSS package program (SPSS Inc., Chicago, IL, USA). The effects (significance) of the groups were evaluated at P<0.05 level (IBM., Corp., 2011). The number of colony used in the study was determined by G*Power (Faul et al., 2007) software package (version 3.1.4).

RESULTS AND DISCUSSION

Motility, spermatozoa concentration, HOST, acrosome integrity (Table 1), total antioxidant and total oxidant (Figure 1) values were determined by adding different doses of shilajit (0, 5, 10, 15, 20 mg/l) to bee diet in honey bee colonies.

The effects of shilajit in different amounts added to the honey bee diet on the cryopreservation of bee semen were

examined. In our study, the average motility values of the groups (SH-0, SH-1, SH-2, SH-3, SH-4) were 20%, 56%, 52%, 60%, and 64%, respectively (Table 1). There was a significant difference between the groups in terms of motility values (%). It was determined that adding different doses of shilajit to the honey bee diet significantly increased the motility percentage ($P < 0.001$).

Findings regarding the effects of shilajit at 0, 5, 10, 15, 20 mg/l levels on the average spermatozoa concentration, in honey bee semen are given in Table 1.

HOST (the Hypo-Osmotic Swelling Test), known as the plasma membrane integrity test, is an important test for evaluating the functional integrity of the membrane structure of spermatozoa. The increase in the positive rate of the test is an indication that the spermatozoa have a solid membrane structure. In our study, the average positive percentages of the groups (SH-0, SH-1, SH-2, SH-3 and SH-4) of frozen honey bee semen were 46.20%, 67.20%, 72.20%, 74.20%, 79.20% was determined as 79.00% (Table 1). It was also observed that the addition of shilajit to the honey bee diet significantly increased the HOST positive percentage ($P < 0.001$). In a study, it was emphasized that Reactive Oxygen Species (ROS) cause lipid peroxidation in the lipid sperm membrane and that this may lead to deterioration of the sperm membrane and decrease in motility, and that the negative effects of ROS on the sperm cell can be prevented by the use of antioxidants (Liu et al., 2015). When the statistical average values were examined, it was found important that as the amount of shilajit used increased, the percentage of membrane permeability also increased. In the study, it was also found remarkable that there was a general increase in the rate of motile spermatozoa in parallel with the increase in host values. This shows that shilajit improves plasma membrane integrity as well as sperm motility.

There was a significant difference between the groups in terms of positive values of acrosome integrity ($P < 0.001$). SH-0 (96.60%), SH-1 (97.80%), SH-2 (98.20%), SH-3 (98.80%) and SH-4 (99.20%) average as can be seen from the values, acrosome integrity values gradually increase as the amount of shilajit added to the syrup increases (Table 1). Therefore, it was observed that the addition of shilajit significantly increased the acrosome integrity percentage ($p < 0.001$).

Table 1. Post-thaw spermatological parameters in study groups

Groups	Motility (%)	Spermatozoa Concentration ($\times 10^4/\mu\text{l}$)	HOST (%)	Acrosome Integrity (%)
SH-0 (0mg/L)	20.00 \pm 0.00 ^b	425.00 \pm 48.70	46.20 \pm 2.15 ^c	86.60 \pm 0.24 ^d
SH-1 (5mg/L)	56.00 \pm 4.00 ^a	400.00 \pm 32.55	67.20 \pm 2.05 ^b	87.80 \pm 0.20 ^c
SH-2 (10mg/L)	52.00 \pm 4.89 ^a	515.00 \pm 76.85	72.20 \pm 0.66 ^{ab}	88.20 \pm 0.20 ^{bc}
SH-3 (15mg/L)	60.00 \pm 0.00 ^a	590.00 \pm 38.40	74.20 \pm 1.71 ^{ab}	88.80 \pm 0.20 ^{ab}
SH-4 (20mg/L)	64.00 \pm 7.48 ^a	485.00 \pm 16.95	79.00 \pm 5.83 ^a	89.20 \pm 0.20 ^a
P	<0.001	0.051	<0.001	<0.001

a,b,c,d: Differences between means with different letters in the same column are significant. SH-0: Control; **SH-1:** 5 mg/L shilajit; **SH-2:** 10 mg/L shilajit; **SH-3:** 15 mg/L shilajit; **SH-4:** 20 mg/L shilajit. **HOST:** Hypoosmotic Swelling Test; \pm : Standard Deviation

The effects of shilajit, which was added to the honey bee diet at different levels, on semen TAS values are given in Figure 1. When the data were examined, the mean TAS values of the groups were determined as 11.50, 4.50, 14.50, 14.50, and 8.00, respectively. It was noteworthy that the semen TAS values of the SH-2 and SH-3 groups were numerically higher than the other study groups. TOS values of the groups were found as 98.45, 87.69, 123.07, 110.76, 55.38, respectively (Figure 1).

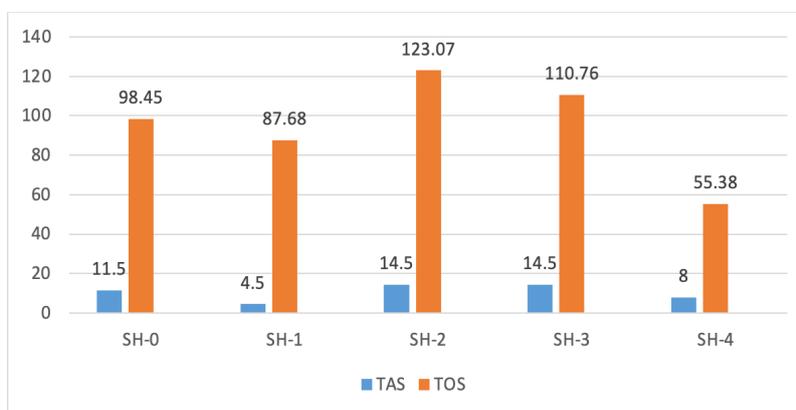


Figure 1. Post-thaw TAS and TOS parameters in semen in study groups

SH-0: Control; SH-1: 5 mg/L shilajit; SH-2: 10 mg/L shilajit; SH-3: 15 mg/L shilajit; SH-4: 20 mg/L shilajit.

In this study, spermatological parameters (motility, sperm concentration, HOST, acrosome integrity) and oxidative stress parameters (TAS, TOS) were investigated to determine the effects of shilajit added to the honey bee diet at doses of 0, 5, 10, 15, 20 mg/L on long-term storage of honeybee semen.

Taylor et al. (2009), in their study on the comparison of different diluents in the freezing storage of semen, defined the motility values as 0, 20, 40, 60, 80 and above 95, but expressed them with values between 0 and 5. Diluent 3 and diluent 4 used in the study are hypotonic. Diluent 3 is a Tris extender containing amino acids. Diluent 4 is a modified Kiev extender containing antioxidants. It was observed that the motility values of diluent 4, which was more successful than other diluents, were above diluent 3. It has been emphasized that this situation may be due to the antioxidants used. Shilajit used in different doses in our study showed improvement in spermatological parameters with increasing doses due to its antioxidative effect. It has been emphasized that this situation may be due to the antioxidants used. The use of Kiev solution and a diluent containing 10% DMSO in the study is similar to our study. Additionally, the study showed that DMSO was significantly more effective than other cryoprotectant agents. Therefore, the Kiev solution and 10% DMSO used in the study coincide with our current study. The results of experimental group 4, whose motility percentage was successful compared to the other groups in the study, are similar to the average findings of the SH-3 and SH-4 groups (60%, 64%) in our current study.

Biswas et al. (2010) in their study examining the effect of shilajit on motility by spermiogram test, they emphasized that the motility values of patients with oligospermia who received oral shilajit treatment for 3 months increased significantly. In the study, the importance of the effect of shilajit on motility was emphasized by comparing the motility values before the treatment with the sperm results collected at 0.5, 1 and 2 hours after the treatment. While an average increase of 12.4% in motility was observed in the first half hour, an increase of 13.2% in 1 hour and 1.4% in 2 hours was observed, and it was stated that shilajit had a significant effect on motility.

It has been reported that selenium and various mineral substances in the structure of shilajit, dibenzo-alpha pyrons, as well as humins containing humus, humic acid and fulvic acid have regulatory and antioxidant effects (Carrasco-Gallardo et al. 2012). It has been stated that shilajit has an aphrodisiac effect (Ghosal, 1990). Moreover, it has been emphasized that fulvic acid caused a decrease in abnormal spermatozoa and malondialdehyde levels at a certain rate, so the use of shilajit caused an increase in motility (Xiao et al., 2018). It has been reported that shilajit increases semen motility by increasing the testosterone level and the number of spermatozoa in the epididymis (Park et al., 2006). Kreuter et al. (2002) stated that fulvic acid plays an important role in providing energy to spermatozoa, therefore it is an element that increases motility. Studies on shilajit-related motility show that shilajit has a positive effect on motility values, which seems consistent with our study.

Studies in mammals have shown that shilajit increases semen concentration. Park et al. (2006) emphasized the importance of shilajit increased the number of epididymal spermatozoa in rats compared to the control group, thus contributing significantly to the spermatogenesis process.

As a result of the increase in polyunsaturated fatty acids in the spermatozoa plasma membrane, the increase of free radicals and the creation of a suitable environment for reactive oxygen species cause the structure of the spermatozoa to deteriorate, resulting in a decrease in the number of spermatozoa (Alvarez and Storey, 1995). It has been emphasized that shilajit significantly decreases the MDA level in semen and improves semen quality (Biswas et al., 2010). It is also known that shilajit increases the testosterone level required for spermatozoa production (Park et al., 2006).

It is thought that the reason why no statistically significant difference was found between the concentration values of honey bee sperm in the studies is due to the unique structure of honey bee sperm and the evaluation of bee sperm by creating a sperm pool rather than individually for each bee.

Wegener et al. (2012) emphasized that the mean value of 65.3 ± 12 in unfrozen semen and 30 ± 2.1 in frozen-stored semen was insignificant as the percentage of impermeable cells when evaluating osmotic stress in their study on honey bee semen parameters. In the present study, the gradual increase of the HOST positive rate starting from the control group is important and does not coincide with the study values. It is thought that the reason for this situation may be due to the efficacy of shilajit as well as the difference in semen dilution rate and the HOST determination method. In another study on oxidative stress in honey bees, Alçay et al. (2019) investigated the effects of different amounts of the diluent containing TL-hepes supplemented with BSA on osmotic stress in honeybee semen. Although the HOST values were close to each other in the study, the percentage values of the HOST positive rate in numerical terms increased starting from the control group ($59.20 \pm 3.58\%$), and it was determined as BSA5 (67.73 ± 4.07) was the highest group. Our current study observed that with increasing levels of shilajit added to the bee diet (5 mg, 10 mg, 15 mg, 20 mg), the percentage of HOST positivity significantly increased (67.20, 72.20, 74.20, 79.00). The study observed that the groups participating in shilajit increased significantly compared to the control group (46.20%).

In addition, the current study found that adding shilajit to the honey bee diet is important. When the current study is compared with the studies, it is seen that the statistical mean values are similar. Although there is no study on shilajit in honey bees, it is seen that the results of the studies on mammals are similar to our study. Kumar et al. (2018) emphasized that the HOST positivity rate in frozen-thawed semen in buffaloes treated with shilajit increased significantly after treatment ($57.6 \pm 0.9\%$) compared to before treatment (39.8 ± 0.57). Kumar's positive effect of shilajit on buffalo semen is similar to the effect of shilajit on bee semen in the presented study. It has been mentioned that fulvic acid has a curative effect on cell functions, especially preventing damage to mitochondria and nuclei. It does this by suppressing the effect of free radicals (Sultan et al., 2021). It has been emphasized that with components such as quinone-semiquinone-hydroquinone complex, catalase, superoxide dismutase, and glutathione peroxidase in the structure of shilajit, lipid peroxidation can be prevented that may occur during freezing and thawing of semen (Agarwal et al., 2007). It has been reported that shilajit prevents the formation of lipid peroxidation, which is known to disrupt the membrane structure of spermatozoa (Tripathi et al., 1996). In our study, it is predicted that the positive status in HOST values is due to the components that prevent the formation of lipid peroxidation and high antioxidant content in the structure of shilajit.

Although there are many publications on honey bee semen, Alçay et al. (2019) investigated the protective effects of TL-hepes based diluent supplemented with BSA in their study on acrosome defect in frozen semen. In the study, it was seen that the most successful group was $54.33 \pm 3.71\%$. In our study, it was observed that shilajit was significantly effective in preserving acrosome integrity. Kumar et al. (2018), in their study evaluating the effects of shilajit on buffalo semen, applied shilajit treatment orally to buffaloes for 2 months and acrosome integrity in frozen and thawed semen before treatment ($40.9 \pm 0.72\%$), in thawed semen ($49.6 \pm 1.51\%$) and thawed semen after treatment (48.9 ± 0.60) was found significant. Studies on acrosome integrity are similar to our study on the effectiveness of shilajit.

No studies were found on TAS and TOS in honey bee semen. It is known that enzymatic antioxidants have positive effects on semen. Studies have emphasized that catalase has positive effects on semen (Taylor et al., 2009; Weirich et al., 2002). Shilajit contains selenium and various mineral substances, dibenzo-alpha pyrons as well as humins containing humus, humic acid and fulvic acid. The fulvic acid in the structure of shilajit contains antioxidant and anti-inflammatory substances (Carrasco-Gallardo et al., 2012). It has been observed that fulvic acid protects the spermatozoon membrane structure and acrosome integrity, and causes an increase in motility. It has also been emphasized that it causes a decrease in abnormal spermatozoa rate and malondialdehyde level (Xiao et al., 2018).

CONCLUSION

Studies show that the amount of MDA (malondialdehyde), which is important for oxidative stress, is significantly reduced with the use of shilajit, and that shilajit has a positive effect against oxidative stress. As a result of the study, it was observed that adding shilajit to the honey bee diet ensures long-term storage of honey bee semen and has a positive effect on spermatological parameters (motility, plasma membrane integrity, acrosome integrity). Research results reveal that feeding bees with shilajit will provide longer storage conditions for honeybee semen. Such studies on honeybee semen may contribute to improving longer-term storage conditions. As a result, it has been understood that shilajit has distinct advantages in long-term storage of honey bee sperm, especially in preserving motility, HOST and acrosome integrity.

COMPLIANCE WITH ETHICAL STANDARDS

Peer-review

Externally peer-reviewed.

Conflict of interest

The authors declare that they have no competing, actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the text, figures, and tables are original and that they have not been published before.

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Consent to participate

Not applicable.

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