



## RESEARCH ARTICLE

### Does natural honey have beneficial effect on Honamlı buck semen during liquid storage?

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### Doğal balın honamlı teke spermasının kısa süreli saklanması yararlı etkisi var mıdır?

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#### Öz

**Amaç:** Bal, bal arıları tarafından üretilen bileşiminde yüksek oranda şeker ihtiva etmesi sayesinde spermatozoa üzerinde yararlı etkisi olabileceği düşünülen bir besin maddesidir. Bu çalışmadaki temel amaç doğal bal ilavesinin Honamlı teke spermasının kısa süreli saklanması 4 °C 96 saat süresince spermatozoa parametreleri üzerine etkinliğinin belirlenmesidir

**Gereç ve Yöntem:** Çalışmada üreme sezonunda 3 baş Honamlı tekesi kullanıldı. Tris-yumurta sarılı sulandırıcı ile sulandırılan spermalar ile kontrol (bal eklenmeyen), %1, 3 ve 6 oranlarında bal eklenen gruplar olacak şekilde 4 grup oluşturuldu. Spermatozoa parametrelerinden subjektif motilite, membran bütünlüğü ve anormal spermatozoa oranı 0, 24, 48, 72 ve 96. saatlerde değerlendirildi.

**Bulgular:** Honamlı teke sperma motilitesi kontrol ve %1 bal ilave edilen gruplarda %3 ve %6 bal içeren gruplara göre daha yüksek bulundu (p<0,05). Bunun yanı sıra %3 ve 6 oranında bal ilave edilen gruplarda sperma motilitesi 24. saatin sonunda diğer gruplara kıyasla düşük bulundu (p<0,05). Spermatozoon membran bütünlüğünün %1 bal ilaveli grupta, %3 ve %6 bal ilaveli gruplardan daha yüksek olduğu gözlemlendi.

**Öneri:** Çalışmada elde edilen bulgulara göre Honamlı teke spermasının kısa süreli saklanması temel tris sulandırıcısına %1 oranında bal ilavesinin sperma kalitesini artırabileceği kanısına varıldı.

**Anahtar kelimeler:** Bal, spermanın kısa süreli saklanması, teke

#### Abstract

**Aim:** Honey has been produced by honey bee comprises high level of sugars which could be beneficial effects on spermatozoa. The main goal of this study was to determine the natural honey addition on the spermatological parameters of Honamlı buck spermatozoa during liquid storage at 4 °C up to 96 hours.

**Materials and Methods:** At the breeding season a total of three Honamlı bucks were used in this study. Four extenders were prepared with tris-egg yolk first was as a control (no honey added) and others had concentrate as 1, 3 and 6% honey into the base extender. Sperm parameters were assessed including subjective motility, membrane integrity and abnormality at 0, 24, 48, 72 and 96 th hours.

**Results:** The motility of Honamlı buck spermatozoa was higher control and 1% honey added group compared the 3% and 6% up to 96 hour (p<0.05). On the other hand 3 and 6% honey supplemented groups motility was getting worse until 24th hour (p<0.05). While membrane integrity results have shown that only 1% honey addition group higher than the other honey groups (p<0.05).

**Conclusion:** Our data suggested that 1% honey could be added to Tris based extender for improving the Honamlı buck sperm quality in short term storage.

**Keywords:** Honey, semen liquid storage, buck





## Introduction

Small ruminant's spermatozoa has higher levels of polyunsaturated fatty acids in the cell membrane so that, oxidative damage is very common problem. Subsequently, higher amounts of (ROS) reactive oxygen species are produced. Due to oxidative stress, lipid peroxidation could be occurred in spermatozoa. High amounts of ROS, results in damage of spermatozoon membrane, loss of motility, and finally spermatozoa apoptosis were seen (Aitken and Fisher 1994). Although spermatozoa has a defense mechanism which stand to LPO including reduced glutathione, superoxide dismutase (SOD), glutathione peroxidase and catalase, these substances are not enough to protect the semen when during short or long term storage (Fridovich 1995). Cytoplasm is the protection dynamic of spermatozoa against ROS. During maturation sperm cell lose cytoplasm volume. Wherefore, small ruminant spermatozoa does not have significant cytoplasmic component for ROS and LPO. LPO is formed during the short term storage of semen and increases with time (Eslami et al 2017, Zadeh et al 2017). Substances with antioxidative properties can be useful to reduce the harmful effects of LPO during in vitro storage. (Donnelly et al 2000).

However, there may be some difficulties in improving ram semen quality during liquid and cold storage. Honey composition depends on the species of plants visited by honey bees and the environmental, processing and storage conditions (Bertoncelj et al 2007). Mutlu et al (2017) mentioned that honey produced by the honey bee is a natural solution of supersaturated sugar, which consists mainly of carbohydrates, enzymes (catalase, glucose oxidase, phosphatases), proteins, organic acids, amino acids and lipids, vitamins, phenolic acids, volatile chemicals, minerals and flavonoids. Commonly added substances in semen dilutions are simple sugars such as fructose and glucose (Bearden et al 2004). Inadequate additives in the semen solution will reduce the metabolic activity of spermatozoa and lead to more dead spermatozoa (Yimer et al 2015). Honey also contains several compounds that are thought to function as antioxidants such as chrysin, vitamin C and catalase (Bogdanov et al 2008, Fakhrildin and Alsaadi 2014).

Erejuwa et al (2012) has identified honey as a new antioxidant considering the effect of honey on the health of various organs of the body. Due to the various properties of honey, the addition of sperm to the extender is expected to help protect spermatozoa during the cooling phase. There are only a few studies have been conducted to test the effects of the in vitro effects of the supplemental honey on the Tris extender as the buck semen medium (Cetin and Karaca 2019). Therefore, this study was designed to determine the effects of the addition of different concentrations of honey to the Tris extender on buck semen during short-term storage.

## Material and Methods

### *Animal management*

In this study, semen was collected from three mature Honamlı bucks (1-3 years old). And the animals were housed at the under uniform optimal nutritional conditions.

### *Semen collection*

Ejaculates were collected from the Honamlı bucks with an artificial vagina in the breeding season twice a week. Totally 21 ejaculates were collected. For sperm quality, spermogram examination of ejaculates were performed. This procedure was performed in a water bath at 33 °C about 20 min after semen handling. Macroscopic analysis of ejaculate volume was recorded in a graduated tube, and the concentration was determined using a haemocytometer. In the study, ejaculates which were more than 1 ml semen volume, >70% motility and at least 1 billion sperm concentration per ml were used.

### *Semen extending and chilling*

A sperm samples were pooled for each buck's sperm to eliminate individual animal differences. In total 21 pooled ejaculates were used in the study. A Tris-based composed of (tris (Trizma® Base T1503, Sigma-Aldrich Co., St. Louis, MO, USA) 297.58 mM, citric acid (Citric acid C0759, Sigma-Aldrich Co., St. Louis, MO, USA) 96.32 mM, fructose (D-Fructose F0127, Sigma-Aldrich Co., St. Louis, MO, USA) 82.66 mM, egg yolk 15% (v/v), penicillin-streptomycin (Penicillin-Streptomycin P4333) 500 IU/mL, pH 6.8) was prepared as the main extender. Pooled ejaculate at 37°C was divided 4 equal aliquots and diluted with the base extender containing 1% (group 1), 3% (group 2) and 6% (group 3) honey and no addition was control, (group 4), respectively. The final semen concentration was diluted to 400×10<sup>6</sup> sperm/mL. Than semen samples were stored into 0.25 mL French straws and cooled from 37°C to 4°C in a refrigerator, it was established 0th hour. After that 24, 48, 72 and 96th hour evaluations were performed for each group.

### *Assessment of sperm motility*

Subjective motility was evaluated using a phase-contrast microscope (400× Magnification, Nikon Eclipse E-400, Japan), at 37°C. Sperm motility scores were performed in at least five different parts for each sample. The mean of the five successive evaluations was kept as the motility score.

### *Assessment of sperm membrane functional integrity*

For analyzing the intact sperm ratio Hypo-Osmotic Swelling (HOS) test was used, based on curled and swollen tails. This was determined by incubating 10 µL of semen with 100 µL of

100 mOsm hypoosmotic fructose (D-Fructose F0127) solution at 37 °C for 60 min. Totally 200 spermatozoa were calculated under 400× magnification and spermatozoa with coiled or swollen tails were recorded (Nikon Eclipse E-400, Japan) (Revell and Mrode 1994).

### Assessment of sperm morphology

Abnormal spermatozoon rate was determined by liquid fixation method. Spermatozoon were fixed with Hancock solution (Hancock 1952) and abnormalities (head, tail) detected under 1000× magnification with phase-contrast microscopy (Nikon Eclipse E-400, Japan) by evaluating at least 200 sperm cells (Schafer and Holzmann 2000).

### Statistical analysis

The study was replicated seven times. The results were analyzed for normality test Shapiro Wilks and homogeneity of variance test as Levene's test. Results expressed as Mean (X) ± standard error mean (±SEM). The main effect of "treatment", "time" and the interaction term of "treatment x time" upon the spermatological parameters were modelled by

using the GLM procedure for repeated measures of SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Post hoc testing was carried out for significant interactions and was performed using simple effect analysis. A probability value of less than 0.05 was considered significant, unless otherwise noted.

### Results

Different doses of honey were investigated on Honamlı buck semen parameters through the liquid storage process evaluated in seven replications. Group 1 (31.14±6.27) and group 4 (35.16±6.88) were higher motility ( $p < 0.05$ ) than Group 3 (0.00±0.00) after 96 hour of storage. Group 2 and Group 3 motilities were significantly decreased ( $p < 0.05$ ) until 24h of liquid storage. Group 3 sperm motility was finished at 48. hour of sperm storage (Table 1). Also, in table 2, membrane intact sperm was positively correlated with motility scores. While intact sperm membrane was highest in group 1(31.40 ± 3.61 %) and group 4 (37.50 ± 5.27%), it was lowest in group 2 (2.00 ± 2.00%) and group 3 (0.00 ± 0.00%) respectively;  $p < 0.05$ ). However, there were no significant differences among groups regarding for abnormal spermatozoa rate term storage period up to 96 hour ( $p > 0.05$ ).

Table 1. Mean (±SEM) subjective motility rates of Honamlı buck semen up to 96th hour of liquid storage at 4 °C

Group	0 <sup>th</sup> hour	24 <sup>th</sup> hour	48 <sup>th</sup> hour	72 <sup>th</sup> hour	96 <sup>th</sup> hour	p-Value		
						Time	Group	Time*Group
Group 1	88.57±1.79 <sup>aA</sup>	77.85±2.14 <sup>aB</sup>	59.28±5.05 <sup>aC</sup>	45.14±6.65 <sup>aD</sup>	31.14±6.27 <sup>aE</sup>			
Group 2	83.57±2.36 <sup>aB</sup>	38.57±9.30 <sup>bB</sup>	17.14±10.97 <sup>bC</sup>	10.42±10.42 <sup>bC</sup>	7.85±7.85 <sup>bC</sup>	<0.001	<0.001	<0,001
Group 3	77.85±2.40 <sup>bA</sup>	3.42±0.78 <sup>bB</sup>	-	-	-			
Group 4	86.66±1.66 <sup>aA</sup>	78.33±2.10 <sup>aB</sup>	62.66±3.48 <sup>aC</sup>	55.50±5.09 <sup>aD</sup>	35.16±6.88 <sup>aE</sup>			
p	*	*	*	*	*			

a,b,c Different superscripts within the same column demonstrate significant differences among groups ( $p < 0.05$ )

A,B,C,D,E Different superscripts within the same line demonstrate significant differences during storage period in same group ( $p < 0.05$ )

Table 2. Mean (±SEM) membrane integrity rates of Honamlı buck semen up to 96th hour of liquid storage at 4 °C

Group	0 <sup>th</sup> hour	24 <sup>th</sup> hour	48 <sup>th</sup> hour	72 <sup>th</sup> hour	96 <sup>th</sup> hour	p-Value		
						Time	Group	Time*Group
Group 1	62.19±2.40 <sup>aA</sup>	53.60±2.67 <sup>aB</sup>	45.37±3.46 <sup>aC</sup>	38.44±3.90 <sup>aD</sup>	31.40±3.61 <sup>aD</sup>			
Group 2	57.14±1.99 <sup>bA</sup>	39.40±3.47 <sup>bB</sup>	11.33±5.60 <sup>bC</sup>	3.85±3.85 <sup>bD</sup>	2.00±2.00 <sup>bD</sup>	<0.001	<0.001	<0,001
Group 3	31.29±3.29 <sup>bA</sup>	21.90±2.18 <sup>bB</sup>	-	-	-			
Group 4	64.79±2.74 <sup>aA</sup>	55.05±3.43 <sup>aB</sup>	53.74±3.20 <sup>aB</sup>	47.25±4.04 <sup>aC</sup>	37.50±5.27 <sup>aD</sup>			
p	*	*	*	*	*			

a,b,c Different superscripts within the same column demonstrate significant differences among groups ( $p < 0.05$ ).

A, B, C, D Different superscripts within the same line demonstrate significant differences during storage period in same group ( $p < 0.05$ )

Table 3. Mean ( $\pm$ SE) normal sperm morphology rate of Honamlı buck semen 0. and 96th hour of liquid storage at 4 °C

Group	0 <sup>th</sup> hour	96 <sup>th</sup> hour	P-Value		
			Time	Group	Time*Group
Group 1	86.41 $\pm$ 1.34 <sup>A</sup>	82.14 $\pm$ 2.69 <sup>B</sup>	<0.001	0.005	0.597
Group 2	86.05 $\pm$ 1.65 <sup>A</sup>	82.64 $\pm$ 1.25 <sup>B</sup>			
Group 3	84.01 $\pm$ 1.73 <sup>A</sup>	77.38 $\pm$ 2.31 <sup>B</sup>			
Group 4	87.01 $\pm$ 1.85 <sup>A</sup>	79.06 $\pm$ 1.95 <sup>B</sup>			
p	*	*			

A,B: Different superscripts within the same line demonstrate significant differences during storage period in same group (p<0.05)

## Discussion

In the present study, we investigated natural honey in Honamlı buck semen extender as an energy substrate, and for its beneficial antioxidant effect on sperm membrane stability. Fructose is a known energy substance for spermatozoa (El-Sheshtawy et al 2016), and the fructose content of west Mediterranean region honeys is about 45% (Mutlu et al 2017). Honey has been used with cryoprotectant extender for Arab stallion at different increased concentrations (1-5%) act on the stallion spermatozoa positively (El-Sheshtawy et al 2016). Although El-Sheshtawy et al (2016) reported that lower dose of honey could be enhanced the quality of semen in stallion semen; honey up to 2% had worse effect in Honamlı buck sperm up to 96h. This difference could be buck semen sensitivity to heat changes compared the stallion and also in short term stored sperm needs much more energy substrate compared the freezing period. The effect of sperm life at 5 °C in short-term storage (96 hours) could be changes in micro and macro-environment by supplementation honey into semen extender. Akandi et al (2015) were found dilution of honey 1-2% in the extender would be suitable for preservation boar spermatozoa at room temperature 24h period. In the present study it was similar 1% addition of honey on Honamlı buck sperm motility up to 96h of chilled semen. Although honey contains nitric oxide metabolites, which could be responsible for some of the therapeutic and biologic effects (Abdelhafiz and Muhamad 2008), storage time and concentration of sperm can be affected negatively the level of nitric oxide and malondialdehyde and the total antioxidant capacity (Gundogan et al 2010). So the current study lower motility results of upper levels (>2%) supported the possibility of this reason.

In the current study significant decrease in membrane integrity until 96. hour in 1% honey group. An interesting finding is that, for the longer storage time, the lowest honey concentration could be an applicable approach to protect the Honamlı buck spermatozoa from cold storage-induced negative effects on sperm plasma membrane functionality,

which may explained the enhancement in the occurring of oxidative stress and their harmful effect on the membrane protein and lipid distribution and finally, defect of physiological function. Duration of storage time could be decreased the membrane integrity (Camara et al 2011). Honey, mainly composed of proteins, complex mixture of carbohydrates, enzymes, amino acids, lipids, organic acids, phenolic acids, vitamins, minerals and flavonoids (Ball 2007). It acts on spermatozoon generally positively (Fakhrildin and Alsaadi 2014, El-Sheshtawy et al 2016, Kandiel et al 2017) but also it has changed the environment like NO and pH levels so higher concentration of honey occurred adverse effect on Honamlı buck semen up to 96 h of liquid storage. Like present study, lower dose (0.1-1%) honey was increase spermatozoological quality in Bali and buffalo bull semen short and long term preservation respectively (El-Nattat et al 2016, Malik 2018). Sperm abnormalities are very important for determine the fertilization ability. In the current study, honey addition was not negatively effect on sperm abnormalities except 6% honey group. It was opposite with Fakhrildin and Alsaaidi (2014) in bull semen freeze-thawed results. This difference occurs because of preservation type also El-Nattat et al (2016) was explained that lower ratio of honey could be used for chilling but higher levels could preferred for freezing in bull semen. Yimer et al (2015) were found that 2.5% honey addition was the suitable for bull spermatozoa compared the 5-10% honey concentrations. Reduction of semen quality with the addition of more than 1% honey may only be associated with an excessively hyperosmotic extracellular environment due to high honey concentration that may lead to excessive intracellular dehydration (Moradi et al 2013).

## Conclusion

The results demonstrated that 1% honey supplemented Tris extender on Honamlı buck semen facilitates process of liquid storage up to 96 hour. Moreover, different natural honey concentrations could be investigate in freezing of buck semen and in future studies would be also evaluated in-vivo results.





### Conflict of Interest

The authors did not report any conflict of interest or financial support.

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### **Ethical Approval**

The present study was approved by the Animal Ethics Committee of the MAKUHADYEK (2016/218).

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