ARTICLE / INVESTIGACIÓN

# Effect of addition of honey and skim milk and cooled cauda epididymal spermatozoa of Awassi ram

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**Abstract:** The current study intends to assess the effectiveness of combining honey and skim milk in an extender on individual motility, livability, and abnormalities of cauda epididymal Awassi ram spermatozoa after diluting and cooling. Nine pairs of testicles Awassi rams were collected after the slaughter at the abattoir. Honey and skim milk combined were prepared. The cauda epididymal spermatozoa were divided into four equal parts and diluted in a Tris-based extender. (Control, basic diluents), HSM1 (basic diluents containing 0.5 ml honey and 9.5ml skim milk), HSM2 (basic diluents containing 1 ml honey and 9ml skim milk), HSM3 (basic diluents containing 1.5 ml honey and 8.5ml skim milk) and cooled 4ºC for evaluation of the percentages of sperm individual motility, live and abnormalities spermatozoa (including head, midpiece, and tail) at 0, 24, 48, and 72h. Results showed that individual motility spermatozoa preserved (P<0.05) in HSM1 and HSM2 groups at 24h and 48h. Livability spermatozoa were increased (P<0.05) in the HSM1 group at 48h and 72h. Groups HSM1, HSM2, and HSM3 decreased total abnormalities (P<0.05) at 48 h and 72h while decreasing tail abnormalities (P<0.05) at 24h and 48h than control. The HSM2 group was lower (P<0.05) in head abnormality of spermatozoa at 48 h, whereas the HSM1 group was at 72 h. In conclusion, the nourishing and protective effects of lower honey concentrations in extender favorably impact cauda epididymal spermatozoa of Awassi rams.

**Key words:** Cauda epididymal spermatozoa, honey, skim milk, motility, livability, and abnormalities.



## Introduction

Honey is a famous natural substance, not just for its flavor but also for its nutritional properties[1]. It has several chemical substances[2], including protein and amino acids, vitamins, enzymes, minerals, and phenolic chemicals[3]; Compounds are the fundamental constituents contributing[4] due to their physicochemical properties[5], which demonstrated beneficial biological activities[6] to the antioxidant and antimicrobial, antiviral, anti-inflammatory, immunomodulatory, anti-fungal, healing, and cardio-protective properties[7]. With advances in research techniques and a greater understanding of biological processes, the primary active components[2] has been shown to have a wide range of biological effects due to its physicochemical properties[5]. Honey could be a potential source of bioactive substances with functional qualities[8]. Another intriguing line of research is to look at the synergistic benefits of honey[9] that are responsible for the potential medicinal effect on reproduction[10] to enhance sperm, maintain a constant sperm percentage, minimize sperm head and tail abnormalities, and preserve the deterioration[7].

Researchers nowadays are making efforts to use different additives in semen extenders to preserve during short storage at 4ºC. Therefore, researchers used honey due to a sugar-rich natural substance[11] that provides energy to enhance sperm motility[7]. Furthermore, honey contains bioactive chemicals, including phenolic compounds that have intriguing biological effects[12] and a good source of natural antioxidants that can help prevent oxidative damage[13]. On the other hand, the addition of honey to skim milk extender improves the properties of chilled sperm[14] and leads to improved sperm motility and can be stored at 5C for up to 96h[15]. Therefore, the present study determined the effect of honey and skim milk combined in an extender on some characteristics of cauda epididymal spermatozoa of Awassi rams.

## Materials and methods

### Collection of testicles

Nine pairs (n= 18 testes) of testicles Awassi rams (means age 2 years old) were collected after the slaughter at an abattoir in Baghdad from February to April 2021. Each animal's testicles were removed and placed in a plastic bag that was then placed in an ice box of 4-8°C and transported to the laboratory of Biotechnology and Environmental Center/ University of Fallujah as soon as possible after slaughter. In the lab, physiological saline solution (0.9 % NaCl) containing antibiotics was used to wash the testes at room temperature and the surrounding tissues were removed by scalpel blade.

The epididymis from each testis was carefully removed using a scalpel blade. The spermatozoa were collected by the flushing method. A cut near the corpus and the proximal side separated each cauda epididymis. Then, the cauda epididymis was injected with 3-5ml normal saline and

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a blunted 23G needle. Following that, several longitudinal incisions were made, and it was kept in a 35-mm Petri dish for 15 min in a water bath at 37°C. Finally, the spermatozoa were harvested in Petri dishes in a glass tube.

### Extender preparation

The basic extender used in the current study was concentration (Tris 2.42g, citric acid 1.26g, fructose 1.25g, egg yolk 15ml and gentamycin 0.5 mg/ml). To produce 100 ml, diluent was mixed with distilled water and (pH=6.8).

### Preparation of honey and skim milk

The honey used in the present study was produced from an apiary north of Baghdad. Skim milk was prepared by dissolving skim milk powder (10%, w/v) in 100 ml distilled water, heating it to 95°C for 10 minutes, and then allowing it to cool to room temperature. Therefore, three tubes extenders were prepared by adding 0.5 ml honey and 9.5ml skim milk (HSM1), adding 1ml honey and 9ml skim milk (HSM2) and adding 1.5 ml honey and 8.5ml skim milk (HSM3). Tubes were placed in a refrigerator after centrifugation (4°C).

### Experimental Design

One sample of semen from each cauda epididymis was divided into 4 equal parts and was diluted 1:10 fold with a Tris-based extender according to the concentration. Therefore, control and 3 parts-containing extenders were prepared for semen dilution. Groups were control, basic diluents), HSM1 (basic diluents containing 0.5 ml honey and 9.5ml skim milk), HSM2 (basic diluents containing 1 ml honey and 9ml skim milk), HSM3 (basic diluents containing 1.5 ml honey and 8.5ml skim milk), Then diluted semen cooled gradually by addition a piece of ice till it reached 5ºC and maintained at 4ºC in a refrigerator. Semen parameters were measured after cooling at 0, 24, 48 and 72 h.

### Semen evaluation

Using a microscope at a magnification of 400, determine the percentage of individual motility spermatozoa at 37ºC 35. The percentage of live and abnormal spermatozoa was assessed using the eosin-nigrosin staining; abnormalities were recorded (including head, midpiece, and tail) by a microscope at X100; the sperm concentration was using a hemocytometer[16].

### Statistical analysis

The data are presented as the mean ± S.E. Comparisons were conducted by one-way analysis of variance (ANOVA) followed by the Duncan Multiple Range Test. The significance level was set at P< 0.05and data were analyzed using the S.P.S.S. Statistics 24.0 (2016).

## Results

The percentage of individual motility spermatozoa observed a significant effect (P<0.05) when Tris-based extender was used during storage at 4ºC at 0h, 24h, 48h, and 72h. Therefore, the percentage of individual motility spermatozoa was higher (P<0.05) in groups HSM3 and HSM2 compared to the control group, while the HSM1 group did not significantly influence within groups Table 1. However, the percentage of individual motility spermatozoa in control, HSM1, and HSM2 groups remained maintained during storage at 24h and 48h, except for the HSM3 group, which gradually decreased at this time. Moreover, at 72h of storage, the HSM3 group had a lower significance (P<0.05) compared to the control and then the HSM2 group, while the HSM1 group did not differ between the control and HSM2 groups (Table 1).

In the current study, the percentage of live spermatozoa was more significant (P<0.05) when compared to the control group in the HSM3 group at 0h and the percentage of live spermatozoa in HSM2 and HSM1 groups that similar findings between HSM3 and control groups at this time Table 2. However, the effect of honey and skim milk additive on the percentage of live spermatozoa in the HSM1 group appeared to maintain livability spermatozoa during storage at 48h and 72h compared to the HSM3 group at the same time. During storage at 48h and 72h, the HSM2 group was similar to the control group, and these groups (control and HSM2) did not differ from the HSM1 and HSM3 groups (Table 2).

Table 3 shows the results of the percentage of total abnormalities of spermatozoa. After 24h, 48h, and 72h storage, there was a significant (P<0.05) decrease in total spermatozoa abnormalities in groups compared to control. Furthermore, the change in total spermatozoa abnormalities after 24 hours of storage (4ºC) was significantly lower in the HSM3, HSM2, and HSM1 groups compared to the control group (P<0.05).

The percentage of abnormal head spermatozoa is shown in Table 4. During 0h and 24h storage groups, the effect was insignificant. However, the HSM3 group increased head abnormalities spermatozoa (P<0.05) at 48h and 72h storage in extender compared to the other groups. Furthermore, the HSM2 group lower (P<0.05) in head abnormality of spermatozoa at 48 h, whereas the HSM1 group decreased (P<0.05) in head abnormality of spermatozoa at 72 h.

Table 5 reveals that during storage for 0h, 24h, and 48h, the percentage of middle abnormality of spermatozoa in neither one of these groups changed significantly. However, it decreased significantly (P<0.05) in the HSM1 group compared to groups simultaneously.

The data on the effect of honey and skim milk combined on the percentage of tail abnormality of spermatozoa showed higher significance (P<0.05) in the control group than groups at 24h, 48h, and 72h Table 6. However, the preservation of the percentage of tail abnormality of spermatozoa in HSM1, HSM2, and HSM3 group than the control group at 24h and 48h storage. Moreover, the percentage of tail abnormality of spermatozoa was lower significantly (P<0.05) in the HSM3 group than in the control group at 72h.

## Discussion

Researchers nowadays are making efforts to use different additives in semen extenders to preserve during short storage at 4ºC. Therefore, the current study has assessed the effect of adding honey in skim milk on some characteristics of spermatozoa for 72h at 4ºC. However, individual motility is affected differently by varying concentrations of honey and skim milk. The values 0.5 ml and 1.0 ml of honey maintain individual motility compared to the 1.5 ml of honey. This finding agrees with (17-19). Honey, on the other hand, is a sugar-rich natural substance[11]. As a result, honey provides energy to enhance sperm motility[7].

| groups | 0h | 24h | 48h | 72h |
|---|---|---|---|---|
| control | 73.33±2.10 b | 69.72±2.47 a | 63.05±3.68 a | 58.88±3.53 a |
| HSM1 | 76.94±2.06 ab | 70.55±2.13 a | 59.72±3.55 a | 51.11±6.17 ab |
| HSM2 | 80.55±1.75 a | 63.88±2.27 a | 53.61±4.23 a | 39.72±5.79 b |
| HSM3 | 81.38±1.32 a | 49.72±4.11 b | 26.94±3.96 b | 25.00±2.99 c |

Within the same column, different superscripts (a, b, c) show a significant (P< 0.05) difference.

**Table 1.** The effectiveness of honey and skim milk combined in an extender on Awassi rams' individual motility spermatozoa % (mean S.E.).



| groups | 0h | 24h | 48h | 72h |
|---|---|---|---|---|
| control | 78.25±1.73 c | 75.00±2.39 a | 73.47±2.37 ab | 65.40±4.18 ab |
| HSM1 | 80.68±1.49 bc | 75.16±1.79 a | 74.14±1.50 a | 69.31±2.33 a |
| HSM2 | 83.64±1.12 ab | 77.59±1.45 a | 73.07±1.04 ab | 61.92±2.93 ab |
| HSM3 | 85.04±0.90 a | 73.69±1.37 a | 65.26±4.87 b | 57.01±3.55 b |

Within the same column, different superscripts (a, b, c) show a significant (P< 0.05) difference.

**Table 2.** The effectiveness of honey and skim milk combined in an extender on Awassi rams' live spermatozoa % (mean S.E.).

| groups | 0h | 24h | 48h | 72h |
|---|---|---|---|---|
| control | 6.49±0.57 a | 14.99±0.74 a | 22.14±2.15 a | 28.81±1.65 a |
| HSM1 | 5.96±0.50 a | 9.16±0.50 bc | 14.50±1.37 b | 20.75±2.00 b |
| HSM2 | 6.50±0.68 a | 10.96±1.38 b | 11.70±1.71 b | 20.35±1.88 b |
| HSM3 | 5.53±0.53 a | 7.93±0.50 c | 13.78±1.33 b | 19.01±0.90 b |

Within the same column, different superscripts (a, b, c) show a significant (P< 0.05) difference.

**Table 3.** The effectiveness of honey and skim milk combined in an extender on Awassi rams' total abnormality spermatozoa % (mean S.E.).

| groups | 0h | 24h | 48h | 72h |
|---|---|---|---|---|
| control | 2.92±0.22 a | 3.57±0.72 a | 5.37±0.62 ab | 10.08±0.90 bc |
| HSM1 | 2.93±0.28 a | 4.79±0.37 a | 5.13±0.61 ab | 8.43±0.46 c |
| HSM2 | 3.24±0.31 a | 5.07±0.69 a | 3.34±0.35 b | 11.65±0.55 ab |
| HSM3 | 3.03±0.24 a | 3.87±0.52 a | 7.32±1.48 a | 13.24±0.86 a |

Within the same column, different superscripts (a, b, c) show a significant (P< 0.05) difference.

**Table 4.** The effectiveness of honey and skim milk combined in an extender on head abnormality spermatozoa % (mean S.E.)..

| groups | 0h | 24h | 48h | 72h |
|---|---|---|---|---|
| control | 0.27±0.09 a | 0.12±0.05 a | 0.12±0.05 a | 0.31±0.07 b |
| HSM1 | 0.24±0.09 a | 0.15±0.05 a | 0.14±0.05 a | 0.85±0.11 a |
| HSM2 | 0.31±0.13 a | 0.15±0.06 a | 0.26±0.11 a | 0.43±0.18 b |
| HSM3 | 0.21±0.08 a | 0.04±0.03 a | 0.24±0.07 a | 0.24±0.07 b |

Within the same column, different superscripts (a, b, c) show a significant (P< 0.05) difference.

**Table 5.** The effectiveness of honey and skim milk combined in an extender on middle abnormality spermatozoa % (mean S.E.).

| groups | 0h | 24h | 48h | 72h |
|---|---|---|---|---|
| control | 3.28±0.51 a | 11.28±1.07 a | 16.64±1.79 a | 18.40±1.82 a |
| HSM1 | 2.78±0.44 a | 4.21±0.38 b | 9.22±1.30 b | 11.46±1.97 b |
| HSM2 | 2.95±0.51 a | 5.73±0.90 b | 8.08±1.53 b | 8.26±1.73 bc |
| HSM3 | 2.27±0.59 a | 4.01±0.42 b | 6.20±0.87 b | 5.52±0.78 c |

Within the same column, different superscripts (a, b, c) show a significant ($P< 0.05$) difference.

**Table 6.** The effectiveness of honey and skim milk combined in an extender on tail abnormality spermatozoa % (mean S.E.).



Furthermore, honey contains bioactive chemicals, including phenolic compounds with intriguing biological effects[12]. Honey also has antioxidant and antibacterial properties. As a result, the honey mechanism supports the idea that antioxidants protect against the damaging effects of reactive oxygen species (R.O.S.)[20]. Therefore, previous studies revealed in cold sperm, adding 10% honey to the semen extender enhanced sperm motility[17,21]. However, the individual spermatozoa motility increased in HSM2 and HSM3 groups over control and HSM1 groups during 0h because the extender contains high honey concentrations, contributing to improved individual motility at 0h. However, the HSM3 group was gradually decreased after 24h. (22) provides evidence that increased concentrations of honey impact on the characteristics of semen. On the other hand, adding honey to skim milk extender improves the properties of chilled sperm[14]. Moreover, (15) pointed out that skim milk has been shown to improve sperm motility and can be stored at 5C for up to 96h.

The current study findings revealed that adding honey and skim milk to the extender caused an increase and decrease in the percentage of live spermatozoa throughout 72h. These results agreed with the findings 23 in the present study that honey enhanced spermatozoa motility and decreased percentages of dead spermatozoa during up to 48h of chilling storages while motility of spermatozoa reduced. However, the percentages of dead spermatozoa increased as time progressed, diluted semen held at 5ºC for up to 48h. Moreover, (18) reported that adding 1% honey to the Tris-citrate semen extender during cooling improved sperm livability. In contrast, adding 1-2% honey increases the livability during cold sperm[14].

Honey concentrations, on the other hand, play a role in increasing or decreasing live spermatozoa during storage. However, the results obtained in this study in the HSM3 group at 0h disagreed with (24), who found that as the concentrations of honey increased, livability reduced considering fresh semen. On the other hand, the percentage of live spermatozoa decreased in the HSM3 group after 24h. These findings follow previous studies[21,25,26], which indicate a decline in honey concentrations in extenders. On the other hand, the results illustrated that the percentage of live spermatozoa decreased in the HSM3 group than in the HSM1 group. These results agreed with the findings[21], who found that decreased honey concentrations in extender affected sperm viability. In addition, (25) indicated that the percentage of viable sperm reduced and increased when preservation time was increased.

Furthermore, this study shows the relationship between skim milk and preservation time on the percentage of live spermatozoa. (27, 28) reported that the percentage of living spermatozoa decreased when the preservation time was increased. In contrast, adding 0.5 % Nigella-stevia and 2% honey to the extender increases the survival spermatozoa rate in fresh and post-thawed sperm[29].

Another purpose of this study was to evaluate the effect of honey and skim milk in an extender on abnormal spermatozoa during the storage time. The current study indicated a decrease in abnormal spermatozoa in the HSM1, HSM2 and HSM3 groups compared to the control during the storage time. These results disagreed with the findings (30) and (31), which reported that the percentage of abnormal spermatozoa increased gradually during storage. Twenty-three observed that the effect of honey during cooling storage for up to 48h, the percentage of sperm abnormalities decreased; however, as time passed, the abnormal spermatozoa increased. However, the results of this study indicate that abnormal spermatozoa decreased during storage time. Thirty-two indicated that milk was present in the extender, which led to the percentage of abnormal sperm being higher. In contrast, (26) reported that when compared to citrate, Tris +skim milk had the lowest percentage of abnormal spermatozoa.

During 48 and 72 hours, the percentage of abnormalities in head spermatozoa in the HSM1 and HSM2 groups reduced, respectively. Furthermore, compared to controls, abnormality tail spermatozoa reduced the duration of storage time in HSM1, HSM2, and HSM3. These results agree with the observation (33) that the proportion of abnormalities in the head and tail in the honey group is lower. (34) reported that honey could increase the percentage of normal sperm and reduce the proportion of sperm with a faulty head and tail. On the other hand, honey, like other antioxidants, helps prevent harm or injury[20] and protects against the damaging effects of reactive oxygen species (R.O.S.), which leads to decreased sperm head and tail abnormalities. Regarding the midpiece abnormality in the current study agrees with observation[29], which pointed out that in the midpiece/neck of sperm, a deficiency in the number of lower sperm was found in both fresh and post-thawed semen.

## Conclusions

In conclusion, the nourishing and protective effects of lower honey concentrations in extenders favorably impact cauda epididymal spermatozoa of Awassi rams.

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