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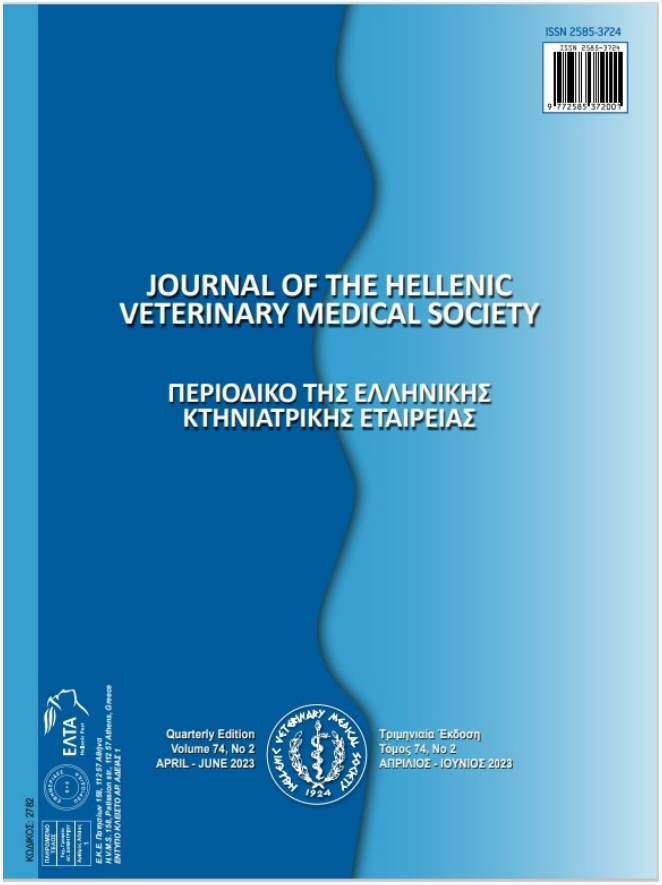

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Research article
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The influence of various rates of royal jelly enriched extenders on frozen/thawed sperm quality and fertility in goats

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ABSTRACT: The goal of this study was to detect the influence of royal jelly (RJ) as a strong antioxidant supplement at different concentrations (0.25, 0.50, 0.75, 1%) in TRIS egg yolk (TEY) and TRIS soybean lecithin (TSL) extenders, on semen quality and fertility. Sperm was collected from 5 Damascus bucks via an electro ejaculator during the breeding season. After dilution and cooling, the lowest motility and membrane integrity were detected in TEY-1RJ, in terms of abnormal spermatozoon rate ($P<0.05$). Also, osmolality decreased significantly when the RJ ratio increased ($P<0.05$). After freezing-thawing, the lowest sperm quality was detected in the 1% RJ groups of both extenders. In terms of intact acrosome ratio, the highest value was recorded in the 0.50% RJ group in TEY and TSL diluents ($P<0.05$). Fertility assessments were conducted on 150 goats synchronized with intravaginal sponges and inseminated transcervical. No difference in pregnancy rates between TEY, TSL-control, or RJ groups were evident ($P>0.05$). As a result, it was deduced that RJ additions to TEY and TSL extenders did not affect sperm quality at freezing in Damascus goat semen except for the intact acrosome ratio and had no positive effect on fertility.

***Keywords*:** Artificial Insemination, Cryopreservation, Goat, Royal Jelly, Semen Extenders

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INTRODUCTION

Artificial insemination offers goat breeders the potential for widespread use of genetically superior breeders and provides desirable new herds, reducing inbreeding and minimizing animal movement, thereby preventing the spread of diseases (Youngquist and Threlfall, 2006). Sperm can be held on for a long-time using cryopreservation. During the storage of sperm, egg yolk, or high molecular weight compounds such as lipoprotein, lecithin, cephalin, and protein are used. The TRIS-egg yolk is widely used in the freezing of buck sperm. It is reported that egg yolk protects the spermatozoon membrane and spermatozoa against cold shock during freezing and thawing (Aboagla and Terada, 2004; Barbas and Mascarenhas, 2009; Bergeron and Manjunath, 2006; Purdy, 2006). But the content of egg yolk is variable, and sterilization problems and risk of microbial contamination are limiting factors for use (Gamal et al., 2016; Masoudi et al., 2016). Soy lecithin, which has been used in place of egg yolk in recent years, stabilizes cells with elements such as phosphatidylcholine, stearic, oleic, and palmitic fatty acids (Baruah et al., 2019; Bhai et al., 2015; Vidal et al., 2013). Soy lecithin has a different fatty acid content and lipid composition than egg yolk and may be resistant to egg yolk coagulating enzyme (EYCE; Bhai et al., 2015).

The capacity of naturally occurring seminal antioxidants is impaired following cryopreservation. Antioxidant administration in semen extenders has offered a significant potential to expand potential fertility performance and diminish the oxidative damage (Bansal and Bilaspuri, 2011). Antioxidant substances added to extenders during the dilution, cooling, equilibration, and freezing of sperm have protective properties against oxidative stress (Alcay et al., 2017; Moradi et al., 2013; Shahzad et al., 2016). Antioxidants improve post-thawing sperm quality against membrane lipid phase change, osmotic-mechanical stress, lipid peroxidation, extracellular crystal formation, denaturation in membrane proteins, structural deformation in cell organelles during freezing and thawing (Bilodeau et al., 2001; Bucak and Tekin, 2007). Royal jelly has a strong antioxidant capacity and radical scavenging ability (Ghanbari et al., 2015; Silici et al., 2009) and very valuable product for health. RJ is made up of varied carbohydrates, proteins, lipids, vitamins, free amino acids, peptides, adenosine monophosphate N1-oxide, acetylcholine, fatty acids, as well as trace minerals and enzymes, and is released by the worker honeybees' hypopharyngeal and mandibular glands (Abd-Allah, 2012; Elnagar, 2010). RJ has a variety of biological activities including the cure of sexual impairment (Azad et al., 2018). Testosterone, estradiol, prolactin, progesterone, and insulin-like growth factor-1 have been found in RJ (Moghaddam et al., 2013). The supplementation of RJ is crucial for sperm viability, motility, and fertility (Abdelhafiz and Muhamad, 2008; Abdelnour et al., 2020; Ahmed et al., 2018; Khazaei et al., 2018; Mahdivand et al., 2019; Zahmatkesh et al., 2015; Zahmatkesh et al., 2014). There is no other study examining the effect of RJ supplementation of extenders on fertility in goats. The study aimed to evaluate the influence of the supplementation of Royal jelly in Tris egg yolk (TEY) and Tris soybean lecithin (TSL) extenders on Damascus goat's sperm quality and fertility parameters.

MATERIAL AND METHODS

Hatay Mustafa Kemal University's Scientific Ethical Committee accepted the experimental sets and assessment methodologies (No: 2018/5-2). The research was managed throughout the mating season. Bucks were provided at the Pan Goat Farm in Gaziantep, and goats were supplied from the farm in Hatay in Turkey. Five healthy Damascus bucks were used to collect semen ranging in age from 2 to 5 years. Artificial insemination was applied to 150 goats aged 2-4 years. Sigma Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany) provided the chemicals utilized in this research.

Sperm collection: Sperm was collected twice a week via an electro ejaculator (Ruakura Ram Probe, Shoof International Ltd, New Zealand) and repeated ten times. The semen was instantly transferred to a water bath after collection (37°C) and then the ejaculates having a volume of more than 0.5 mL, a mass activity greater than 3, and motility greater than 70% were pooled. Semen was analyzed for volume, motility, mass activity, concentration, dead and morphological abnormal sperm rates, membrane integrity, and pH in the pooled sperm. Semen was evaluated for motility, dead and abnormal spermatozoa rates, membrane integrity, pH, and osmolarity after dilution, after cooling to 4-5°C for 2 h, after equilibration for 4 h, and after thawing. In addition, an acrosome integrity test was performed after thawing.

Evaluation of semen quality: The volume of sperm was defined in collection tubes as mL. A drop of fresh semen was analyzed to determine mass activity and scored between 0 and 5 (Tekin, 1990). Motil-

ity was assessed subjectively, using a warmed (37°C) glass slide under a phase-contrast microscope (×400; Hafez and Hafez, 2013). The concentration of spermatozoa was evaluated using the Hayem solution and the hemocytometric technique (Hafez and Hafez, 2013). Sperm viability was assessed by the eosin-nigrosin staining (Jeyendran et al., 1984). Briefly, 5 µL of sperm sample were mixed with 5µL of eosin-nigrosin, and the smear was prepared without waiting on a glass slide and stained at 60 °C. At least 400 spermatozoa were counted. Spermatozoa with stained heads were considered dead, while those with unstained heads were alive. The morphological examination was carried out using the eosin-nigrosin staining method. The smears were examined under ×1000 magnification of the light microscope and the sperm abnormalities of the head, midpiece, and tail were evaluated for a total of 400 spermatozoa. Membrane integrity was assessed with Hypoosmotic Swelling Test solution-HOST (1.1 g fructose, 0.55 g sodium citrate, 100 mL distilled water, adjust to 100 mOsm/l), following Jeyendran et al. (1984) technique with minor changes. Ten µl of sperm was mixed with 100 µl of HOST solution at 37 °C for 60 min. After incubation, the mixture was spread with a coverslip on a glass slide and a total of 200 sperm cells were evaluated with a phase-contrast microscope (×400). Spermatozoa with intact plasma membranes were provided with the swollen tail. The pH value of the sperm was evaluated using a pH meter (Hanna HI 83141, Country of origin). PSA-FITC (L0770, Sigma, USA) was used for acrosome integrity evaluation and prepared according to Nur et al. (2010). The prepared samples were viewed with a fluorescence microscope (Nikon, Eclipse Ni, Makato Kimura, Tokyo, Japan). The spermatozoa, whose entire acrosome appeared green, were evaluated as intact and determined as % (*Fig. 1*) (Masoudi et al., 2016; Nur et al., 2010). The osmolarity of the semen is determined as mOsm/kg using an osmometer (Osmomat 3000, Gonotec, Germany) operating according to the freezing point principle.

Dilution and storage of semen: TRIS-egg yolk (TEY) extender was prepared with 3.605 g TRIS, 1,488 g fructose, 2,024 g citric acid, 100,000 IU Penicillin G (İ.E. 1,000,000 IU, Istanbul, Turkey), 100 mg Streptomycin (İ.E.Ulagay, 1 g, Istanbul, Turkey), 12% egg yolk and made up to 100 mL with distilled water according to Camara et al. (2011). TRIS-soybean (TSL) extender was arranged as 3,605 g TRIS, 1,488 g fructose, 2,024 g citric acid, 100,000 IU Penicillin G, 100 mg Streptomycin, 1,5% soybean lecithin (P3644, 25 g, Sigma) and made up to 100 mL with distilled water. The sperm was divided into ten parts in the water bath at 37 °C (Msb 30, Mikrolab, Country of origin) and diluted with TEY and TSL extenders added to 0.25, 0.50, 0.75, and 1% RJ (Fanus, 20,000 mg pure royal jelly, Trabzon, Turkey). To prepare the royal jelly stock solution, 2g royal jelly (RJ) was dissolved in 100 mL distilled water, mixed by using a vortex and experimental groups were formed using 2% RJ stock solution. The stock solution was used to obtain various levels of royal jelly. Each group's spermatozoa concentration was adjusted to 200×10^6 spermatozoa/mL. After dilution, sperm were placed in 0.25 mL straws and gradually chilled to 4°C within 2 h and 5% glycerol was added to the chilled sperm according to Üstüner et al. (2014) and stored at 4°C for 4 h in a cooled incubator for equilibration (Câmara et al., 2011; Üstüner et al., 2014). Semen straws were frozen in a 35×30×22 styrofoam. Straws were arranged in a single row on a steel shelf in styrofoam and placed at 5 cm above the nitrogen level for 10 min. Also, goblets were kept in nitrogen for 5 min and then transferred to the liquid nitrogen tank. The straws were thawed at 38°C for 25 sec and the examinations were carried out. This procedure was repeated 10 times in all groups. After the sperm variables examination and based on the results after the statistical analysis a total of 6 batches with the best spermatological quality belonging to the 2 main extender groups, one of the controls, and 0.25 and 0.50% royal jelly added groups were selected. The prepared straws were kept in the nitrogen tank until the artificial insemination.

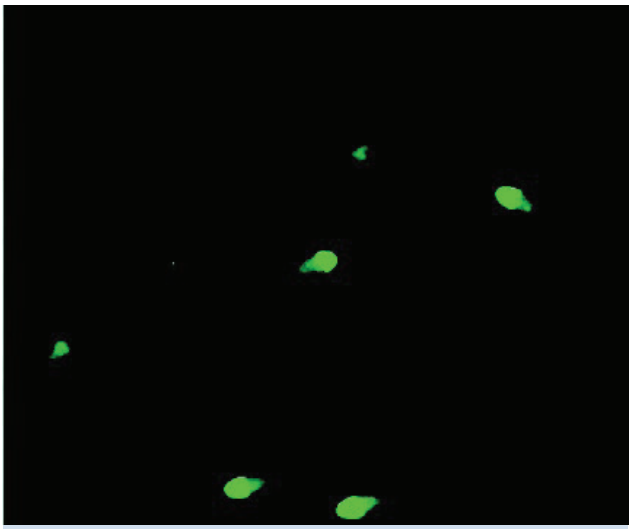

Fig. 1. Detection of acrosome integrity with FITC-PSA. (a: spermatozoon with intact acrosome, b: spermatozoon with damaged acrosome).

Synchronization and artificial insemination of goats: Sponges containing 60 mg medroxyprogesterone acetate (Esponjavet, Hipra, Turkey) were administered intravaginally to 150 goats for synchronization. The sponges were kept in the vagina for 10 days. On the 9th day of application, goats were injected with 200 IU PMSG (Oviser, 5000 IU, Hipra, Turkey) and 250 mcg PGF_2 alpha intramuscularly. Estrus was detected with teaser bucks after 24 h of sponges retrieve and 25 goats were inseminated in each extender group. The goat was fixed by two-person, and the anus area and the lips of the vulva were dry cleaned. One assistant bent the hind legs of the goat from the articulation genu joint and lifted the back part of the goat. The tip of the transparent speculum with a light source mounted inside was inserted through the vulva after being lightly lubricated with non-spermicidal gel. After the cervix was found, the catheter was passed through the speculum and transmitted from the orifice uteri external to the cervix, and insemination was completed by passing through the cervix.

Fertility evaluation: Pregnancy diagnoses were done at 21 and 50 days after artificial insemination. Blood samples 21 days after artificial insemination were collected to determine the early period in pregnancy diagnosis. Blood was collected from the v. jugularis, and after centrifugation at 2000 rpm for 10 min, the plasma was removed and stored in the deep freezer (-20°C) until progesterone analysis. Plasma progesterone measurements were made with the Direct Chemiluminescence method (ADVIA Centaur Ready Pack TM, Siemens, USA), and progesterone levels ≥3 ng/mL were considered as pregnant (Al-Merestani et al., 2003). At 50 days after artificial insemination, transabdominal ultrasound scanning was applied with an ultrasound device (DP-2200 Vet, Mindray, China) using a convex probe at a frequency of 5 MHz. The formula of the pregnant goats/inseminated goats × 100 (Ahmed et al., 1998) was used to determine the pregnancy rate.

Statistical analysis: The Shapiro-Wilk test has been used for the determination of normal distribution. The data were analyzed with SPSS 23 and detected mean ± standard error values, with significant variations defined as $P<0.05$. The data were evaluated using ANOVA, and the Duncan test was utilized to identify the differences between the groups for spermatological testing. The Chi-square test was beneficial in assessing pregnancy rates, and Pearson-Chi-square was applied to determine statistical significance.

RESULTS

Following mean values in pooled fresh semen samples were detected for volume 6.3±0.63 mL, mass activity 3.85±0.08, motility 77±0.82 (%), dead spermatozoa ratio 16.2±1.40 (%), membrane integrity 78.3±1.63 (%), abnormal sperm ratio 12.3±0.94 (%), pH 6.47±0.06, and concentration 2.375±57.60 × 10^6/mL.

Spermatologic values after dilution

As stated in Table 1, the lowest motility, membrane integrity, and the number of dead spermatozoa were higher in the 1% RJ group than in the other groups of TEY extenders ($P<0.05$). In terms of abnormal spermatozoa ratio and pH, no noticeable differences were noted. The osmolality of the control group was the highest, and the 1% RJ group was the lowest within the group in TEY extender. In the TSL extender, only the difference in terms of osmolality was significant ($P<0.05$). Osmolality was found to be higher in the TSL-control than in the experimental groups, and osmolality values decreased significantly as the amount of RJ increased.

Between the TEY and TSL groups (Table 2), only the pH of the TEY-0.50RJ group was significantly lower than the TSL-0.50RJ group ($P<0.05$). During equilibration, the addition of cryoprotectant to the diluent and the freezing process change the osmolality and thus long-term storage of the cell is achieved.

Spermatologic values after cooling

In the TEY extender groups (Table 3), motility and sperm membrane integrity of the control group was superior compared to the TEY-1RJ group ($P<0.05$), while they were similar to the rest TEY extender groups. For the dead sperm and pH, no differences were observed between groups ($P>0.05$). Values of abnormal spermatozoa were higher ($P<0.05$) in the TEY-1RJ group than in TEY-0.25RJ and TEY-0.5RJ groups. The Control group provided the highest value of osmolarity, while it decreased proportionally as the RJ concentration was increased. No differences were observed for any variable of the TSL groups, except for osmolarity, in which the same situation as TEY groups were noticed. There were no differences among TEY and TSL extender groups.

Spermatologic values post equilibration

Spermatological values after equilibration in the experimental groups are shown in Table 4. In the TEY extender, there were no differences for the examined

sperm variables except for membrane integrity which was remarkably higher in the control (P<0.05) than in the TEY-1RJ group while it was similar to the other experimental groups. In the TSL extender, control and RJ groups did not differ in any sperm variable. When TEY and TSL extender groups were compared no difference was found between groups (P>0.05).

Spermatological values after freezing-thawing

Spermatological parameters obtained in frozen-thawed semen in TEY and TSL extender groups are shown in Table 5. Motility in the TEY-control group was higher than 1 and 0.75% RJ groups. The lowest value of motility was found in the TEY-1RJ group (P<0.05). Dead spermatozoa rate in the control was similar to 0.75, 0.50, and 0.25% RJ added groups while lower than the 1% RJ added group (P<0.05). Membrane integrity in the control was similar to the 0.50 and 0.25% RJ groups and it was considerably higher compared to the 0.75 and 1% RJ groups (P<0.05). Among the experimental groups, the membrane integrity declined in the 1% RJ group (P<0.05), while the 0.25% RJ group had the highest membrane integrity. Regarding osmolality, the control was superior to the RJ-added groups (P<0.05). In terms of intact acrosome ratio, while the value of control was higher than the 1% RJ group (P<0.05), it was similar to the other groups.

In the TSL extender, the motility in the control was higher than the 1 and 0.75% RJ added groups (P<0.05) and was similar to the 0.50 and 0.25% RJ added groups. In terms of the dead spermatozoa rate, the control was provided lower values than the experimental groups with 1% and 0.75% RJ (P<0.05), and no differences were observed with 0.50% and 0.25% RJ groups. Values of membrane integrity in the control group was higher than in the 1% and 0.75% RJ supplemented groups (P<0.05), and similar to the 0.50% and 0.25% RJ added groups. In terms of osmolality, the control was similar to the 0.25% RJ group, while it was higher than the other RJ-added groups (P<0.05). When the experimental groups were evaluated, the lowest osmolality was in the 1% RJ group, while the other experimental groups were found to be similar to each other. In terms of intact acrosome ratio, the highest acrosome integrity was determined in the 0.50% RJ group, and the lowest value was determined 1% RJ group (P<0.05). In the evaluation of TEY and TSL extenders (Table 6), it was determined that the TEY-0.75RJ group had a higher number of intact plasma membranes compared to the TSL-0.75RJ group (P<0.05). The number of spermatozoa with intact acrosome rate noted in the TEY-0.50RJ was lower compared to the TSL-0.50RJ group (P<0.05).

Pregnancy rates

In the study, the best spermatological results were recorded in control, 0.25, and 0.50% RJ groups of TEY and TSL-based extenders. The pregnancy rates are given in Table 7. No difference was observed between the control and RJ-supplemented groups in extenders (P>0.05).

In terms of pregnancy rate on the 21st day, the TEY-0.50RJ group was found to be notably lower than the TSL-0.50RJ group as stated in Table 8 (P<0.05). According to the pregnancy rate on the 50th day, pregnancy rates did not vary among the groups (P>0.05).

Table 1: Spermatological values post-dilution in TEY and TSL extender groups (Mean ± Std. Error)

Groups	Motility (%)	Dead Sptz (%)	HOS Test (%)	Abnormal Sptz (%)	pH	Osmolality (mOsm/kg)
TEY Control	74.50±1.17^{a}	16.90±1.19^{b}	73.60±2.07^{a}	12.40±1.19	6.49±0.05	349.40±1.51^{a}
TEY 1RJ	60.00±4.15^{b}	28.50±3.67^{a}	59.80±3.51^{b}	13.70±1.58	6.48±0.04	210.40±4.94^{e}
TEY 0.75RJ	73.00±2.00^{a}	18.30±1.54^{b}	72.80±1.66^{a}	13.30±1.01	6.45±0.05	249.10±4.28^{d}
TEY 0.50RJ	74.00±1.63^{a}	19.10±1.94^{b}	73.70±2.01^{a}	12.90±1.45	6.47±0.04	278.40±3.67^{c}
TEY 0.25RJ	73.50±2.48^{a}	19.10±1.85^{b}	72.60±2.70^{a}	12.80±1.63	6.52±0.03	315.30±1.95^{b}
P	**0.001****	**0.004****	**0.001****	**0.967^{-}**	**0.826^{-}**	**0.000*****
TSL Control	70.00±3.42	20.50±2.92	69.00±3.28	14.60±1.77	6.57±0.03	352.10±3.87^{a}
TSL 1RJ	61.50±4.95	25.90±4.23	62.00±4.43	16.50±2.33	6.55±0.05	222.30±3.52^{e}
TSL 0.75RJ	69.50±2.73	20.00±2.29	69.30±2.45	13.50±1.79	6.57±0.04	251.30±4.99^{d}
TSL 0.50RJ	73.50±1.30	17.20±2.18	73.50±1.73	12.00±1.15	6.61±0.04	280.90±3.99^{c}
TSL 0.25RJ	73.50±1.98	16.20±1.76	73.33±1.92	12.90±1.55	6.58±0.06	312.80±3.76^{b}
P	**0.060^{-}**	**0.143^{-}**	**0.054^{-}**	**0.434^{-}**	**0.902^{-}**	0.000***

$^{-}$P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001

a,b,c,d,e: Differences between means with different letters in the same column are significant.

Table 2: Intergroup spermatological values post-dilution in TEY and TSL extender groups (Mean ± Std. Error)

Groups	TEY Control	TSL Control	P	TEY 1RJ	TSL 1RJ	P	TEY 0.75RJ	TSL 0.75RJ	P	TEY 0.50RJ	TSL 0.50RJ	P	TEY 0.25RJ	TSL 0.25RJ	P
Motility (%)	74.50±1.17	70.00±3.42	**0.228**-	60.00±4.15	61.50±4.95	**0.819**-	73.00±2.00	69.50±2.73	**0.315**-	74.00±1.63	73.50±1.30	**0.813**-	73.50±2.48	73.50±1.98	**1.000**-
Dead Sptz (%)	16.90±1.19	20.50±2.92	**0.269**-	28.50±3.67	25.90±4.23	**0.648**-	18.30±1.54	20.00±2.29	**0.546**-	19.10±1.94	17.20±2.18	**0.524**-	19.10±1.85	16.20±1.76	**0.271**-
HOS Test (%)	73.60±2.07	69.00±3.28	**0.251**-	59.80±3.51	62.00±4.43	**0.702**-	72.80±1.66	69.30±2.45	**0.252**-	73.70±2.01	73.50±1.73	**0.941**-	72.60±2.70	73.33±1.92	**0.835**-
Abnormal (%)	12.40±1.19	14.60±1.77	**0.317**-	13.70±1.58	16.50±2.33	**0.334**-	13.30±1.01	13.50±1.79	**0.924**-	12.90±1.45	12.00±1.15	**0.586**-	12.80±1.63	12.90±1.55	**0.965**-
pH	6.49±0.05	6.57±0.03	**0.203**-	6.48±0.04	6.55±0.05	**0.242**-	6.45±0.05	6.57±0.04	**0.054**-	6.47±0.04	6.61±0.04	**0.033***	6.52±0.03	6.58±0.06	**0.363**-
Osmolality (mOsm/kg)	349.40±1.51	352.10±3.87	**0.524**-	210.40±4.94	222.30±3.52	**0.065**-	249.10±4.28	251.30±4.99	**0.742**-	278.40±3.67	280.90±3.99	**0.650**-	315.30±1.95	312.80±3.76	**0.562**-

*: P<0.05, The difference between groups is statistically significant.

- P>0.05, The difference between groups is statistically insignificant.

Table 3: Spermatological values at 4-5 °C in TEY and TSL extender groups (Mean ± Std. Error)

Groups	Motility (%)	Dead Sptz (%)	HOS Test (%)	Abnormal (%)	pH	Osmolality (mOsm/kg)
TEY Control	75.62±1.75^{a}	17.50±2.41	74.37±1.64^{a}	14.00±1.19^{ab}	6.45±0.05	360.62±9.44^{a}
TEY 1RJ	60.00±4.53^{b}	28.87±7.01	61.37±3.63^{b}	18.62±3.13^{a}	6.45±0.05	228.87±13.22^{e}
TEY 0.75RJ	71.25±2.79^{a}	20.00±1.95	71.00±2.40^{a}	14.25±1.23^{ab}	6.45±0.05	261.37±10.32^{d}
TEY 0.50RJ	73.12±2.09^{a}	17.00±1.91	72.50±2.13^{a}	11.62±0.99^{b}	6.51±0.05	292.12±11.55^{c}
TEY 0.25RJ	73.12±1.87^{a}	16.25±1.85	70.25±2.07^{a}	11.62±1.19^{b}	6.51±0.04	322.87±10.34^{b}
P	**0.003****	**0.109**-	**0.008****	**0.047***	**0.799**-	**0.000*****
TSL Control	69.37±2.39	22.00±3.44	67.00±3.16	15.25±2.44	6.53±0.04	357.12±3.24^{a}
TSL 1RJ	63.12±4.11	29.37±4.18	62.50±3.31	14.62±2.05	6.52±0.07	236.50±2.93^{e}
TSL 0.75RJ	66.87±2.09	24.50±2.37	67.37±2.54	13.62±1.36	6.50±0.05	255.37±9.35^{d}
TSL 0.50RJ	69.37±1.75	19.25±2.63	67.00±2.79	13.50±1.55	6.55±0.08	291.12±5.67^{c}
TSL 0.25RJ	70.00±1.63	19.75±2.80	66.37±2.60	13.50±2.04	6.52±0.07	320.12±5.65^{b}
P	**0.313**-	**0.168**-	**0.748**-	**0.952**-	**0.989**-	**0.000*****

-P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001

a,b,c,d,e: Differences between means with different letters in the same column are significant.

Table 4: Spermatological values after equilibration in TEY and TSL extender groups (Mean ± Std. Error)

Groups	Motility (%)	Dead Sptz (%)	HOS Test (%)	Abnormal (%)	pH	Osmolality (mOsm/kg)
TEY Control	69.50±3.37	22.90±2.79	70.70±2.83^{a}	16.20±1.21	6.53±0.05	1008.30±73.36
TEY 1RJ	55.00±5.32	33.30±4.18	56.00±3.69^{b}	17.90±1.82	6.44±0.08	1023.30±116.34
TEY 0.75RJ	65.00±3.42	25.30±3.25	65.40±2.59^{a}	16.30±1.44	6.46±0.06	957.60±54.65
TEY 0.50RJ	67.00±3.27	24.30±2.55	68.70±2.10^{a}	17.30±1.32	6.56±0.06	894.80±44.86
TEY 0.25RJ	67.00±2.38	23.20±1.60	68.30±2.26^{a}	13.90±1.20	6.51±0.05	1008.70±74.86
P	**0.068**-	**0.102**-	**0.004****	**0.339**-	**0.626**-	**0.753**-
TSL Kontrol	63.50±4.15	28.90±3.74	64.80±3.03	17.00±1.24	6.56±0.07	963.00±52.18
TSL 1RJ	55.00±5.27	37.50±5.09	56.60±4.39	17.70±1.65	6.55±0.04	839.20±55.05
TSL 0.75RJ	63.50±3.17	30.10±3.82	64.30±2.30	15.40±1.21	6.52±0.04	892.90±35.92
TSL 0.50RJ	67.50±2.39	23.80±2.87	68.60±1.83	15.50±1.01	6.56±0.05	840.40±40.60
TSL 0.25RJ	65.50±2.03	27.60±2.85	65.50±2.22	14.90±1.59	6.56±0.06	1016.50±79.61
P	**0.155**-	**0.150**-	**0.069**-	**0.557**-	**0.979**-	**0.108**-

-P>0.05, *: P<0.05, **: P<0.01

a,b: Differences between means with different letters in the same column are significant.

Table 5: Spermatological values post-thawing in TEY and TSL extender groups (Mean ± Std. Error)

Groups	Motility (%)	Dead Sptz (%)	HOS Test (%)	Abnormal (%)	pH	Osmolality (mOsm/kg)	Intact Acrosome (%)
TEY Control	47.00±3.67^{a}	48.30±3.71^{bc}	46.80±2.67^{a}	18.20±1.88	6.42±0.06	1153.00±40.92^{a}	50.90± 0.95^{ab}
TEY 1RJ	21.50±3.58^{c}	69.50±3.15^{a}	22.20±2.46^{c}	22.20±2.37	6.36±0.05	847.30±27.28^{c}	42.20± 2.00^{c}
TEY 0.75RJ	35.50±3.45^{b}	57.30±4.01^{b}	37.60±2.68^{b}	17.60±1.23	6.44±0.06	937.50±27.76^{bc}	48.10± 1.27^{b}
TEY 0.50RJ	41.00±3.79^{ab}	51.40±3.98^{bc}	42.80±3.41^{ab}	20.80±1.30	6.41±0.05	981.80±25.26^{b}	52.10± 0.77^{a}
TEY 0.25RJ	50.00±3.33^{a}	43.90±4.43^{c}	49.10±3.05^{a}	19.70±2.34	6.46±0.04	1034.00±37.06^{b}	50.90± 0.82^{ab}
P	**0.000*****	**0.000*****	**0.000*****	**0.424**$^{-}$	**0.736**$^{-}$	**0.000*****	**0.000*****
TSL Control	44.50±1.89^{a}	47.90±3.27^{c}	41.80±2.16^{a}	20.60±1.74	6.46±0.04	1165.10±26.39^{a}	49.30 ± 1.22^{b}
TSL 1RJ	15.50±2.41^{c}	74.80±3.42^{a}	16.30±1.60^{c}	21.30±2.53	6.45±0.03	907.70±42.79^{d}	42.50 ± 1.42^{c}
TSL 0.75RJ	29.50±3.11^{b}	60.60±3.53^{b}	28.10±2.22^{b}	19.30±1.93	6.47±0.04	1004.40±34.18^{c}	51.50 ± 1.73^{b}
TSL 0.50RJ	47.50±3.44^{a}	46.50±4.21^{c}	48.10±3.13^{a}	19.20±1.22	6.49±0.03	1037.90±27.77^{bc}	57.30 ± 1.85^{a}
TSL 0.25RJ	47.00±3.09^{a}	44.20±3.96^{c}	47.90±3.81^{a}	18.40±1.18	6.43±0.04	1122.20±23.89^{ab}	52.00 ± 0.93^{b}
P	**0.000*****	**0.000*****	**0.000*****	**0.789**$^{-}$	**0.816**$^{-}$	**0.000*****	**0.000*****

$^{-}$P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001

a,b,c: Differences between means with different letters in the same column are significant.

Table 6: Intergroup spermatological values obtained post-thawing in TEY and TSL extender groups (Mean ± Std. Error)

Groups	TEY Control	TSL Control	P	TEY 1RJ	TSL 1RJ	P	TEY 0.75RJ	TSL 0.75RJ	P	TEY 0.50RJ	TSL 0.50RJ	P	TEY 0.25RJ	TSL 0.25RJ	P
Motility (%)	47.00±3.67	44.50±1.89	**0.552**$^{-}$	21.50±3.58	15.50±2.41	**0.181**$^{-}$	35.50±3.45	29.50±3.11	**0.213**$^{-}$	41.00±3.79	47.50±3.44	**0.220**$^{-}$	50.00±3.33	47.00±3.09	**0.518**$^{-}$
Dead Sptz (%)	48.30±3.71	47.90±3.27	**0.936**$^{-}$	69.50±3.15	74.80±3.42	**0.269**$^{-}$	57.30±4.01	60.60±3.53	**0.545**$^{-}$	51.40±3.98	46.50±4.21	**0.408**$^{-}$	43.90±4.43	44.20±3.96	**0.960**$^{-}$
HOS Test (%)	46.80±2.67	41.80±2.16	**0.162**$^{-}$	22.20±2.46	16.30±1.60	**0.059**$^{-}$	37.60±2.68	28.10±2.22	**0.014***	42.80±3.41	48.10±3.13	**0.267**$^{-}$	49.10±3.05	47.90±3.81	**0.808**$^{-}$
Abnormal (%)	18.20±1.88	20.60±1.74	**0.362**$^{-}$	22.20±2.37	21.30±2.53	**0.798**$^{-}$	17.60±1.23	19.30±1.93	**0.467**$^{-}$	20.80±1.30	19.20±1.22	**0.381**$^{-}$	19.70±2.34	18.40±1.18	**0.625**$^{-}$
pH	6.42±0.06	6.46±0.04	**0.556**$^{-}$	6.36±0.05	6.45±0.03	**0.154**$^{-}$	6.44±0.06	6.47±0.04	**0.699**$^{-}$	6.41±0.05	6.49±0.03	**0.195**$^{-}$	6.46±0.04	6.43±0.04	**0.600**$^{-}$
Osmolality (mOsm/kg)	1153.00±40.92	1165.10±26.39	**0.807**$^{-}$	847.30±27.28	907.70±42.79	**0.249**$^{-}$	937.50±27.76	1004.40±34.18	**0.146**$^{-}$	981.80±25.26	1037.90±27.77	**0.152**$^{-}$	1034.00±37.06	1122.20±23.89	**0.061**$^{-}$
Intact Acrosome (%)	50.90 ± 0.95	49.30 ± 1.22	**0.314**$^{-}$	42.20 ± 2.00	42.50 ± 1.42	**0.904**$^{-}$	48.10 ± 1.27	51.50 ± 1.73	**0.131**$^{-}$	52.10 ± 0.77	57.30 ± 1.85	**0.018***	50.90 ± 0.82	52.00 ± 0.93	**0.388**$^{-}$

*: P<0.05, The difference between groups is statistically significant.

$^{-}$P>0.05, The difference between groups is statistically insignificant.

Table 7: Pregnancy rates in Control, 0.50% and 0.25% RJ supplemented groups

Parameters	TEY Control	TEY 0.50RJ	TEY 0.25RJ	Pearson Chi-Square Test	TSL Control	TSL 0.50RJ	TSL 0.25RJ	Pearson Chi-Square Test
Pregnancy Rate (Day 21, %)	%44 (11/25)	%44 (11/25)	%64 (16/25)	**0.264**$^{-}$	%60 (15/25)	%72 (18/25)	%68 (17/25)	**0.657**$^{-}$
Pregnancy Rate (Day 50, %)	%36 (9/25)	%32 (8/25)	%44 (11/25)	**0.671**$^{-}$	%24 (6/25)	%28 (7/25)	%48 (12/25)	**0.156**$^{-}$

$^{-}$P>0.05, The difference between groups is statistically insignificant.

Table 8: Intergroup pregnancy rates in TEY and TSL extender groups

Parameters	TEY Control	TSL Control	Pearson Chi-Square Test	TEY 0.50RJ	TSL 0.50RJ	Pearson Chi-Square Test	TEY 0.25RJ	TSL 0.25RJ	Pearson Chi-Square Test
Pregnancy Rate (Day 21, %)	%44 (11/25)	%60 (15/25)	**0.258**$^{-}$	%44 (11/25)	%72 (18/25)	**0.045***	%64 (16/25)	%68 (17/25)	**0.765**$^{-}$
Pregnancy Rate (Day 50, %)	%36 (9/25)	%24 (6/25)	**0.355**$^{-}$	%32 (8/25)	%28 (7/25)	**0.758**$^{-}$	%44 (11/25)	%48 (12/25)	**0.777**$^{-}$

*: P<0.05, The difference between groups is statistically significant.

$^{-}$P>0.05, The difference between groups is statistically insignificant.

DISCUSSION

The data are consistent with studies reporting that the quality of sperm diluted TEY and TSL extenders were similar after dilution, cooling, and freezing-thawing in control groups (Akhter et al., 2010; Bhai et al., 2015; Forouzanfar et al., 2010; Salmani et al., 2014; Sarıözkan et al., 2010; Sun et al., 2020; Yodmingkwan et al., 2016).

El-Sherbiny (2013) reported that low concentrations of RJ at 5°C reduced morphological abnormal and dead spermatozoa rates and increased motility in TEY extender in rabbits. Moradi et al. (2013) researched the effect of adding RJ at different rates

to the TEY extender in rams and low concentrations of RJ had an affirmative influence on motility and membrane integrity at 4°C. Raeesi Dehkohneh et al. (2017) noticed that the low levels of royal jelly improved sperm motility during liquid storage in Arabi ram (Raeesi Dehkohneh et al., 2017). Iljenkaite et al. (2020) showed that motility was reduced at high doses of royal jelly supplementation and there was no positive influence in terms of viability in boar. Similarly, in our previous research, the addition of high doses of RJ to the extender negatively affects motility, membrane integrity, and viability. It is thought that as the dose of RJ added to the extender is increased, it may have a pro-oxidant effect and may cause adverse effects by reacting with some reactive oxygen species required for optimal cell functions (Iljenkaite et al., 2020). Amini et al. (2019) showed that the addition of the lyophilized RJ into the TEY extender improved quality during the chilling and cryopreservation process in rams, especially 3% RJ, but 5% RJ in the TEY extender was found harmful to motility, membrane integrity, and viability during long-term. It was similar to the spermatological quality analyses in our study; high RJ doses were more detrimental compared to low and average concentrations among the experimental groups. El-Sheshtawy (2020) determined that the quality of chilled semen increased with the low addition of royal jelly in TEY extender, but decreased at high doses in buffalo. Also, El-Sheshtawy (2020) reported that the quality of frozen sperm improved with low doses of RJ supplementation, but decreased at high doses. In our study results were similar. It was shown that high levels of RJ addition to the extender have importantly diminished the rooster sperm quality compared to low levels of RJ and the control groups (Hadavand Mirzaei et al., 2021). Alcay et al. (2019) stated that high RJ addition decreased spermatozoa motility while low concentrations of RJ (1 and 2%) increased motility and membrane integrity during semen cryopreservation in honey bees. In our study, motility and membrane integrity decreased in increased doses of royal jelly and these results were in harmony with Alcay et al. (2019).

Kaleem et al. (2017) reported that the supplementation of 1% RJ in extenders improved motility, viability, and plasma membrane integrity in bucks after cryopreservation (Kaleem et al., 2017). Jafari and Atarchi (2008) investigated the effect of adding royal jelly to TEY extender (1, 2, 3, 4, and 5%) on sperm parameters in refrigerated and thawed sperm in rams, and recorded the highest viability and motility values were in 4% and 5% of royal jelly concentration. These results were not compatible with our data. Saberivand et. al. (2021) investigated the impact of RJ with glycerol and dimethyl sulfoxide for long time preservation and after the freeze-thaw process, moderate concentrations of RJ were found satisfactory in Romanov rams (Saberivand et al., 2021). In our study, it was determined that the addition of RJ in the extenders decreased membrane integrity, viability, and motility depending on the dose of royal jelly. It reported that the results may be different regarding the animal species, the composition of the extender, the dose, and the source of royal jelly (Amini et al., 2019; El-Sherbiny, 2013; Jafari and Atarchi, 2008; Moradi et al., 2013). Abd-Allah (2010) reported that the motility, viability, and acrosome integrity were improved with a high concentration of RJ (0.4%) during post-thaw incubation in the TRIS extender in bovine (Abd-Allah, 2010). In terms of both fertility and sperm quality, the results are contradictory. Mohammadian et al. (2016) evaluated the influence of adding RJ to the extender at different rates (0.5, 1, 1.5, and 2%) in Mahabadi bucks after freezing, and noted that motility and viability were improved after adding 1% of RJ. In our study, control groups had similar motility with low royal jelly concentrations, but high concentrations of royal jelly were harmful. It is thought that high doses of royal jelly may reduce the motility by negatively affect to osmolality and pH, while low doses were considered safer (Mohammadian et al., 2016). Masluchah and Ducha (2021) investigated the effects of RJ addition (0, 1, 2, and 3%) into a soya-based extender on the motility and viability during the equilibration, and 3% RJ had a great protective impact in Boer bucks (Masluchah and Ducha, 2021). In our previous study, the supplementation of medium and high doses of RJ in the TEY extender had a protective effect on motility and plasma membrane integrity while after liquid storage at 4°C. RJ supplementation was unsuccessful in the TSL extender (Cetin et al., 2020). The current study demonstrated that the external addition of excessive RJ in extender may be a cause of sperm quality loss by occurring antioxidant imbalance in Damascus goats supplemented with 1% RJ. Additionally, significant osmolality changes were detected in diluted and chilled semen in TEY and TSL extender groups, depending on the addition of royal jelly. Abd-Allah (2012) notified that RJ added to the fertilization medium provides an improvement in motility and acrosomal integrity in buffaloes spermatozoa. Similar to our study, the ratio of intact acrosomes

in the TSL-0.50RJ added group was superior to the control group, but the improvement of acrosome integrity was not mirrored by conception rates. It has been declared that insulin-like growth factor-1 in royal jelly provides the stability of the spermatozoon membrane (Abd-Allah, 2010). The physiological effects of 10-hydroxy-2-decanoic acid, amino acids, and vitamins may be linked to a protective effect on the membrane during the storage (Toker et al., 2016). Also, RJ is very important for cell membranes and has antioxidative activity, and hydroxyl radical scavenging (Bansal and Bilaspuri, 2011; Guo et al., 2008). Shahzad et al. (2016) noted that the addition of RJ at lower doses (0.1%) in TEY extender in Nili-Ravi buffaloes enhanced viability, plasma membrane integrity, acrosome integrity, progressive motility, viability but after freezing and thawing the high concentration of RJ had no influence on sperm quality. According to Atalla et al. (2019), the addition of 0.5% RJ enhanced plasma membrane integrity, in contrast, 1 and 2% of RJ had harmful effects on the post-thawing medium in rams. It reported that the increased royal jelly may cause biochemical changes in the semen extender and may damage spermatozoa by changing the biochemical pathways that have protective effects on the membrane (Amini et al., 2019; Moradi et al., 2013; Shahzad et al., 2016). Alcay et al. (2017) noted that the addition of 0.50% and 0.75% RJ in TSL extender had higher quality in terms of motility, membrane integrity, and acrosome damage in bucks after thawing. In the current study, high intact acrosome was found in TSL-0.50RJ but motility and membrane integrity values were indistinguishable from the control (Alcay et al., 2017). Arboud et al. (2021) reported that the supplementation of 0.05, 0.1% RJ in TRIS extender enhanced motility, membrane, and acrosome integrity after chilled and post-thawed in bulls. Optimum royal jelly supplementation may be variable due to the species-specific membrane structure and seminal plasma content. It has been noted that the addition of high RJ concentrations to extenders may have deleterious effects on the quality of spermatozoa by causing changes such as residue, increase in viscosity, and decrease in osmotic pressure. It is thought that royal jelly added in high doses may cause spermatozoa damage due to imbalances in the redox reaction and excessive use of antioxidants may lead to oxidative stress (Gawish et al., 2016; Moradi et al., 2013).

It was noted that the conception rate of goats ranges from 30 to 70% when frozen-thawed sperm is utilized for AI (Kharche et al., 2013). The pregnancy rates in our study were higher compared to some researchers (Kajaysri and Thammakarn, 2012) or lower (Al-Faruque et al., 2007; Batista et al., 2009; Dorado et al., 2007; Fathi et al., 2019; Kharche et al., 2013; Yotov et al., 2016). Sariozkan et al. (2010) in Ankara goats and Masoudi et al. (2016) reported similar pregnancy rates at the TEY and TSL extenders in rams with frozen and thawed semen. In our study, it was no variance between extender groups in terms of pregnancy rate statistics. Makawi et al. (2014) in ewes and Khalifa (2015) in goats reported higher pregnancy rates in TSL extenders. The data differ from our results. The differences between the results may be different depending on the animal species, breed, age, season, semen extenders, freezing technique, estrus synchronization protocol, time and number of artificial inseminations, and body condition score (Leboeuf et al., 2000; Martemucci and D'Alessandro, 2011; Molina et al., 2012; Nunes and Salgueiro, 2011).

There is no information about the effect of RJ enriched semen extender on pregnancy rates in goats. In our study, no variance was detected between the RJ-supplemented groups and the control groups in terms of fertility parameters. Shahzad et al. (2016) noted that embryo cleavage and pregnancy advanced with the addition of 0.1% RJ in extender in buffalos. El-Sheshtawy (2020) added royal jelly at different rates to TEY extender in buffalo (0.05, 0.1, 0.2, 0.3, and 0.4), and inseminated after freezing-thawing. The conception rate was found high in 0.05, 0.1, and 0.2% RJ added groups. Arboud et al. (2021) reported that a higher conception rate of RJ-treated semen after artificial insemination (0.05, 0.1%) compared to control in cows. However, this effect is concentration-dependent and higher concentrations of RJ were detrimental to fertilizing ability. Gimenez-Diaz et al (2012) concluded that higher conception rates were observed in ewes given 300 IU equine chorionic gonadotropin (eCG) combined with 500 mg RJ compare to only the eCG group at sponge removal. Abd-Allah (2012) showed that the in vitro fertilizing capacity of cryopreserved sperm could be improved by 0.4% RJ in combination with heparin in buffaloes. Some researchers showed that the addition of RJ improved the fertilization rate and embryonic development in sheep (Eshtiyaghi et al., 2016; Valiollahpoor Amiri et al., 2016). Veshkini et al. (2018) evaluated the RJ supplement in the oocyte maturation medium at 2.5, 5, and 10 mg/mL concentrations and concluded that 5 mg/mL RJ supplementation improved embryonic development in goats. All these researches were not

compatible with the current study.

There was no other study examining the effect of adding RJ to the extender on fertility in goats. It is considered that the difference between the 50th and 21st days pregnancy rates may be related to false-positive pregnancy diagnosis in the progesterone level on the 21st day, but also as a consequence of early embryonic death, cystic ovary, hydrometra, and pyometra (Fatet et al., 2011; Youngquist and Threlfall, 2006). During equilibration, the addition of cryoprotectant to the diluent and the freezing process changes the osmolality, and thus long-term storage of the cell is achieved.

In conclusion, our results show that the high rate (1%) of RJ addition in TEY and TSL extenders in Damascus bucks had adversely affected the semen quality while the low rates (0.25 and 0.50%) did not have any negative effects after dilution, chilling, equilibration, and freeze-thawing. It was concluded that studies should be conducted to interpret the impacts of different compositions of TEY or TSL extenders with low RJ additions on cryopreservation and fertility. More research is needed to test low RJ concentrations in goats to find the best effect on semen qualities and fertility.

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ETHICAL STATEMENT

Hatay Mustafa Kemal University's Scientific Ethical Committee accepted the experimental sets and assessment methodologies, No: 2018/5-2.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

REFERENCES

Abd-Allah, S.M., 2010. Effect of royal jelly on bovine sperm characteristics during post-thaw incubation in vitro. Revista Veterinaria, 21(2): 81-85.

Abd-Allah, S.M., 2012. Effect of royal jelly on the fertilizing ability of buffalo spermatozoa in vitro. Journal of Buffalo Science, 1(1): 1-4.

Abdelhafiz, A.T. and Muhamad, J.A., 2008. Midcycle pericoital intravaginal bee honey and royal jelly for male factor infertility. Int J Gynaecol Obstet, 101(2): 146-9.

Abdelnour, S.A. et al., 2020. Useful impacts of royal jelly on reproductive sides, fertility rate and sperm traits of animals. J Anim Physiol Anim Nutr (Berl), 104(6): 1798-1808.

Aboagla, E.M.-E. and Terada, T., 2004. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology, 62(6): 1160-1172.

Ahmed, M.M., El-Shazly, S.A., Alkafafy, M.E., Mohamed, A.A. and Mousa, A.A., 2018. Protective potential of royal jelly against cadmium-induced infertility in male rats. Andrologia, 50(5): e12996.

Ahmed, M.M., Makawi, S.l. and Jubara, A., 1998. Synchronization of oestrus in Nubian goats. Small Ruminant Research, 30(2): 113-120.

Akhter, S. et al., 2010. Cryopreservation of buffalo (Bubalus bubalis) semen in Bioxcell® extender. Theriogenology, 74(6): 951-955.

Al-Faruque, M., Bari, F., Siddiqui, M. and Shamsuddin, M., 2007. Fertilizing capacity of buck (Capra hircus) semen frozen with different concentrations of egg yolk. Journal of the Bangladesh Agricultural University, 5(452-2018-3947): 95-104.

Al-Merestani, M., Zarkawi, M. and Wardeh, M., 2003. Improving the reproductive efficiency, pregnancy diagnosis and monitoring the resumption of luteal activity in indigenous Damascus goats. Reproduction in domestic animals, 38(1): 36-40.

Alcay, S. et al., 2019. Successful cryopreservation of honey bee drone spermatozoa with royal jelly supplemented extenders. Cryobiology, 87: 28-31.

Alcay, S., Toker, M.B., Onder, N.T. and Gokce, E., 2017. Royal jelly supplemented soybean lecithin-based extenders improve post-thaw quality and incubation resilience of goat spermatozoa. Cryobiology, 74: 81-85.

Amini, S. et al., 2019. Effects of supplementation of Tris-egg yolk extender with royal jelly on chilled and frozen-thawed ram semen characteristics. Cryobiology, 88: 75-80.

Arboud, M., Waheeb, R., Sheshtawy, R. and Amrawi, G., 2021. Effect of Different Concentrations of Tris Royal Jelly-Enriched Extender on Cooled and Post-Thawed Bull Semen. Alexandria Journal of Veterinary Sciences, 68(1).

Atalla, H. et al., 2019. Impacts of supplementary royal jelly on characteristics of post-thawed ram semen. International Journal of Current Research in Biosciences and Plant Biology, 6(1): 6-11.

Azad, F., Najati, V., Najafi, G. and Rahmani, F., 2018. The Protective Effect of Royal Jelly on Testicular Tissue and Sperm Parameters in Adult Mice Treated with Nicotine. Journal of Babol University of Medical Sciences, 20(3): 50-58.

Bansal, A.K. and Bilaspuri, G., 2011. Impacts of oxidative stress and antioxidants on semen functions. Veterinary medicine international, 2011.

Barbas, J. and Mascarenhas, R., 2009. Cryopreservation of domestic animal sperm cells. Cell and tissue banking, 10(1): 49-62.

Baruah, D. et al., 2019. Effect of tris egg yolk and tris soya lecithin extenders in frozen beetal buck semen. International Journal of Current Microbiology and Applied Sciences, 8(1): 2740-2748.

Batista, M. et al., 2009. Successful artificial insemination using semen frozen and stored by an ultrafreezer in the Majorera goat breed. Theriogenology, 71(8): 1307-1315.

Bergeron, A. and Manjunath, P., 2006. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Molecular reproduction and development, 73(10): 1338-1344.

Bhai, K.L. et al., 2015. Motility and functional membrane integrity of buck spermatozoa with soyabean lecithin based extender. Journal of

Cell and Tissue Research, 15(1): 4711.
Bilodeau, J.-F., Blanchette, S., Gagnon, C. and Sirard, M.-A., 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology, 56(2): 275-286.
Bucak, M.N. and Tekin, N., 2007. Kryoprotektanlar ve gamet hücrelerinin dondurulmasında kryoprotektif etki. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 54(1): 67-72.
Câmara, D. et al., 2011. Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen. Small Ruminant Research, 100(1): 44-49.
Cetin, N.C., Kaan, O.K.O., Ates, C.T. and Karaca, F., 2020. Effect of royal jelly on quality of chilled semen from damascus buck. Journal of Veterinary Andrology, 5(1).
Dorado, J., Rodriguez, I. and Hidalgo, M., 2007. Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology, 68(2): 168-177.
El-Sherbiny, A., 2013. Effect of royal jelly in honey-included egg yolk based extenders on motility, viability and fertilizing ability of frozen rabbit spermatozoa. Egyptian Journal of Rabbit Science, 23(2): 149-160.
Elnagar, S.A., 2010. Royal jelly counteracts bucks' "summer infertility". Anim Reprod Sci, 121(1-2): 174-80.
Elsheshtawy, R., 2020. Effect of Royal Jelly Tris-enriched Extender on Buffalo Semen Cryopreservation. Egyptian Journal of Veterinary Sciences, 51(3): 405-411.
Eshtiyaghi, M., Deldar, H., Pirsaraei, Z.A. and Shohreh, B., 2016. Royal jelly may improve the metabolism of glucose and redox state of ovine oocytes matured in vitro and embryonic development following in vitro fertilization. Theriogenology, 86(9): 2210-2221.
Fatet, A., Pellicer-Rubio, M.-T. and Leboeuf, B., 2011. Reproductive cycle of goats. Animal reproduction science, 124(3-4): 211-219.
Fathi, M. et al., 2019. Soybean lecithin-based extender improves Damascus goat sperm cryopreservation and fertilizing potential following artificial insemination. Asian Pacific Journal of Reproduction, 8(4): 174.
Forouzanfar, M. et al., 2010. In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology, 73(4): 480-487.
Gamal, A., El-Nattat, W.S., El-Sheshtawy, R.I. and El-Maaty, A.M.A., 2016. Substitution of egg yolk with different concentrations of soybean lecithin in tris-based extender during bulls' semen preservability. Asian Pacific Journal of Reproduction, 5(6): 514-518.
Gawish, A.M. et al., 2016. Sperm abnormality toxicity due to cyclosporine A and the ameliorative effect of royal jelly in male rats. The Journal of Basic & Applied Zoology, 76: 60-73.
Ghanbari, E., Nejati, V., Najafi, G., Khazaei, M. and Babaei, M., 2015. Study on the effect of royal jelly on reproductive parameters in streptozotocin-induced diabetic rats. International journal of fertility & sterility, 9(1): 113.
Gimenez-Diaz, C., Emsen, B., Emsen, E., Kutluca, M. and Koycegiz, F., 2012. Improved reproductive response of sheep in intrauterine insemination program with the use of royal jelly. African Journal of Biotechnology, 11(61): 12518-12521.
Guo, H. et al., 2008. Royal jelly peptides inhibit lipid peroxidation in vitro and in vivo. Journal of nutritional science and vitaminology, 54(3): 191-195.
Hadavand Mirzaei, A., Deldar, H., Ansari Pirsaraei, Z. and Shohreh, B., 2021. Royal jelly may improve sperm characteristics during preservation of rooster semen: Gene expression of antioxidant enzymes. Reprod Domest Anim, 56(4): 658-666.
Hafez, E.S.E. and Hafez, B., 2013. Reproduction in farm animals. John Wiley & Sons.
Iljenkaite, A. et al., 2020. The effect of royal jelly on boar sperm viability and motility during liquid storage for 96 hours. Acta Veterinaria Brno, 89(1): 47-53.
Jafari, A.Y. and Atarchi, H., 2008. The effect of royal jelly in tris extender on sperm characteristics of Dallagh rams.
Jeyendran, R., Van der Ven, H., Perez-Pelaez, M., Crabo, B. and Zaneveld, L., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Reproduction, 70(1): 219-228.
Kajaysri, J. and Thammakarn, C., 2012. Estrus synchronization using intravaginal medroxyprogesterone acetate (MAP), MAP plus prostaglandin F2α, controlled internal drug release (CIDR) or CIDR plus prostaglandin F2α in Saanen dairy goats in Thailand. Agriculture and Natural Resources, 46(1): 71-79.
Kaleem, M., Rehman, A., Mehmood, U. and Sattar, M.A., 2017. Effects of royal jelly on post thaw semen quality parameters of beetal buck. Wayamba Journal of Animal Science: 1495 - 1498.
Khalifa, E., 2015. Physiological response of Zaraibi Billy goats spermatozoa to soybean lecithin addition as an alternative to egg yolk varieties in semen extenders. Egyptian Journal of Animal Production, 52(3): 155-162.
Kharche, S. et al., 2013. Fertility following frozen semen artificial insemination in Jamunapari goats. Indian J. Anim. Sci, 83(10): 1071-1073.
Khazaei, M., Ansarian, A. and Ghanbari, E., 2018. New Findings on Biological Actions and Clinical Applications of Royal Jelly: A Review. J Diet Suppl, 15(5): 757-775.
Leboeuf, B., Restall, B. and Salamon, S., 2000. Production and storage of goat semen for artificial insemination. Animal reproduction science, 62(1-3): 113-141.
Mahdivand, N., Najafi, G., Nejati, V., Shalizar-Jalali, A. and Rahmani, F., 2019. Royal jelly protects male rats from heat stress-induced reproductive failure. Andrologia, 51(3): e13213.
Makawi, S.E.A., Salim, M.A.W., Adam, A.A.G. and Elsharif, B.A.A., 2014. Freezability and fertility of frozen-thawed kabashi eco-type desert rams semen processed with tris-soybean and tris-egg yolk based extenders. Journal of Veterinary Medicine and Animal Production, 4(2).
Martemucci, G. and D'Alessandro, A., 2011. Induction/synchronization of oestrus and ovulation in dairy goats with different short term treatments and fixed time intrauterine or exocervical insemination system. Animal reproduction science, 126(3-4): 187-194.
Masluchah, M. and Ducha, N., 2021. Pengaruh Penambahan Royal Jelly dalam Pengencer Dasar Soya terhadap Kualitas Spermatozoa Kambing Boer Sebelum dan Sesudah Ekuilibrasi. LenteraBio: Berkala Ilmiah Biologi, 9(3): 218-225.
Masoudi, R. et al., 2016. Fertility and flow cytometry study of frozen-thawed sperm in cryopreservation medium supplemented with soybean lecithin. Cryobiology, 73(1): 69-72.
Moghaddam, A., Karimi, I., Borji, M., Bahadori, S. and Abdolmohammadi, A., 2013. Effect of royal jelly in ovo injection on embryonic growth, hatchability, and gonadotropin levels of pullet breeder chicks. Theriogenology, 80(3): 193-8.
Mohammadian, T., Motlagh, M. and Shahneh, A., 2016. Effect of using different levels of royal jelly in semen extender on some quality and quantity parameters of Mahabadi goat semen. Iranian Journal of Animal Science (IJAS), 46(4).
Molina, F.A.A., Pardo, B., Sánchez, M., Lopez, M. and Marín, C.C.P., 2012. Factors influencing the success of an artificial insemination program in Florida goats. Spanish Journal of Agricultural Research(2): 338-344.
Moradi, A., Malekinejad, H., Farrokhi-Ardabili, F. and Bernousi, I., 2013. Royal Jelly improves the sperm parameters of ram semen during liquid storage and serves as an antioxidant source. Small ruminant research, 113(2-3): 346-352.
Nunes, J. and Salgueiro, C., 2011. Strategies to improve the reproductive efficiency of goats in Brazil. Small Ruminant Research, 98(1-3): 176-184.
Nur, Z., Zik, B., Ustuner, B., Sagirkaya, H. and Ozguden, C., 2010. Effects of different cryoprotective agents on ram sperm morphology and DNAintegrity. Theriogenology, 73(9): 1267-1275.
Purdy, P., 2006. A review on goat sperm cryopreservation. Small ruminant research, 63(3): 215-225.
Raeesi Dehkohneh, S., Mamouei, M., Tabatabaei, S., Fayazi, J. and Naghibi Rokni, H., 2017. concentrations of Royal Jelly supplementation in tris diluent on semen quality parameters of Arabi ram. Iranian Veterinary Journal, 13(2): 13-21.
Saberivand, A., Pashapour, S., Noghani, A.E. and Namvar, Z., 2021. Syn-

ergistic effect of Royal jelly in combination with glycerol and dimethyl sulfoxide on cryoprotection of Romanov ram sperm. Cryobiology.
Salmani, H., Towhidi, A., Zhandi, M., Bahreini, M. and Sharafi, M., 2014. In vitro assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. Cryobiology, 68(2): 276-280.
Sarıözkan, S. et al., 2010. Effects of different extenders and centrifugation/washing on postthaw microscopic-oxidative stress parameters and fertilizing ability of Angora buck sperm. Theriogenology, 73(3): 316-323.
Shahzad, Q. et al., 2016. Royal jelly supplementation in semen extender enhances post-thaw quality and fertility of Nili-Ravi buffalo bull sperm. Anim Reprod Sci, 167: 83-8.
Silici, S., Ekmekcioglu, O., Eraslan, G. and Demirtas, A., 2009. Antioxidative effect of royal jelly in cisplatin-induced testes damage. Urology, 74(3): 545-51.
Sun, L. et al., 2020. Effect of substituting different concentrations of soybean lecithin and egg yolk in tris-based extender on goat semen cryopreservation. Cryobiology, 92: 146-150.
Tekin, N., 1990. Erkek üreme organlarının muayenesi (Androlojik muayeneler). Theriogenoloji evcil hayvanlarda reprodüksiyon sun'i tohumlama obstetrik ve infertilite. Nurol Matbacılık, Ankara: 53-67.
Toker, M.B., Alcay, S., Gokce, E. and Ustuner, B., 2016. Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. Cryobiology, 72(3): 205-209.
Üstüner, B., Alçay, S., Nur, Z., Sağırkaya, H. and Soylu, M.K., 2014. Effect of egg yolk and soybean lecithin on tris-based extender in postthaw ram semen quality and in vitro fertility. Kafkas Univ Vet Fak Derg, 20(3): 393-398.
Valiollahpoor Amiri, M., Deldar, H. and Ansari Pirsaraei, Z., 2016. Impact of supplementary royal jelly on in vitro maturation of sheep oocytes: genes involved in apoptosis and embryonic development. Systems biology in reproductive medicine, 62(1): 31-38.
Veshkini, A. et al., 2018. Oocyte maturation with royal jelly increases embryo development and reduces apoptosis in goats. Animal Reproduction, 15(2): 124.
Vidal, A.H. et al., 2013. Soybean lecithin-based extender as an alternative for goat sperm cryopreservation. Small ruminant research, 109(1): 47-51.
Yodmingkwan, P., Guntaprom, S., Jaksamrit, J. and Lertchunhakiat, K., 2016. Effects of extenders on fresh and freezing semen of boer goat. Agriculture and Agricultural Science Procedia, 11: 125-130.
Yotov, S.A., Velislavova, D.V. and Dimova, L.R., 2016. Pregnancy rate in Bulgarian White milk goats with natural and synchronized estrus after artificial insemination by frozen semen during breeding season. Asian Pacific Journal of Reproduction, 5(2): 144-147.
Youngquist, R.S. and Threlfall, W.R., 2006. Current Therapy in Large Animal Theriogenology-E-Book. Elsevier Health Sciences.
Zahmatkesh, E., Najafi, G. and Nejati, V., 2015. Protective effect of royal jelly on in vitro fertilization (IVF) in male mice treated with oxymetholone. Cell Journal (Yakhteh), 17(3): 569.
Zahmatkesh, E., Najafi, G., Nejati, V. and Heidari, R., 2014. Protective effect of royal jelly on the sperm parameters and testosterone level and lipid peroxidation in adult mice treated with oxymetholone. Avicenna journal of phytomedicine, 4(1): 43.