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Supplementation of *Nigella sativa* Oil and Honey Prolong the Survival Rate of Fresh and Post-Thawed Goat Sperms

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ABSTRACT

Some chemical compositions of polyphenol antioxidants found in *Nigella sativa* and honey have potential to protect sperm membrane from lipid peroxidation. Therefore, we study the effect of *in vitro* supplementation of *Nigella sativa* oil and honey on sperm parameters; motility, progressive score, and abnormalities in fresh and post-thawed goat semen. Pooled semen from five sexually matured males Jermasia goats between aged 2 to 3 years old, with average live weight of 35.9±5.7 kg and average body condition score (BCS) of 1.7±0.45 were used in this study. The semen were added with tris-egg yolk extender and combination of supplements (*Nigella sativa* oil or/and honey), and divided into groups; Control (no supplementation), Treatment 1 (0.5% v/v *Nigella sativa*), Treatment 2 (2% v/v honey), and Treatment 3 (0.5% v/v *Nigella sativa* and 2% v/v honey). All parameters of fresh semen were immediately assessed after semen collection and parameter of post-thawed semen were assessed after 48 hours (2 days) of semen cryopreservation. The fresh sperm parameters were observed at 0, 0.5, 1, 1.5, and 2 hours after semen collection. Results showed that the motility of fresh semen at 1.5 and 2.0 hours after collection treated with *Nigella sativa* oil (Group 1; 73.8% and 72.0% respectively) and honey (Group 2; 73.3% and 72.0%; respectively) were 20% (1.5 h) and 8% (2.0 h) lower than Controls (93.0% and 79.8% of motility) (P<0.05). The progressive score and sperm abnormality were not significant between groups. While, the motility of post-thawed semen was higher (P<0.05) in treatment 3 (60.33±10.08 of motility; supplemented with *Nigella sativa* and honey) compare to Controls (24.33±8.17 of motility) at 0 and 0.5 hour after thawing. Thus, we suggested that *Nigella sativa* oil and honey show a potential as a supplement to goat's extender with the ability to protect sperm membrane from damage due to oxidative stress and could prevent ice crystal formation during cryopreservation.

Keywords: Cryopreservation, goat, sperm motility, Nigella sativa, honey

INTRODUCTION

In small ruminants, semen extender has been used to maximize the preserve quality of sperms such as viability, motility, acrosome, and membrane integrity prior to insertion into female by using Artificial Insemination (AI) technique. The diluent of extender is responsible for energy metabolism, metabolic activity of sperms, and protection of sperm cells from damage during preservation process (Salamon & Maxwell, 2000; Andrabi & Maxwell, 2007; Barbas & Mascarenhas, 2009; Pamungkas *et al*., 2014).

However, it is quite a challenge to maintain the quality of sperms by solely depending on based extender. The quality of semen is often reduced after semen collection and cryopreservation due to oxidative stress. Impact of oxidative stress in reducing sperm quality has been reported in human (Saleh *et al.,* 2003), rat (Chitra *et al.,* 2003) horse (Ball *et al.,* 2008), and bovine (Barbato *et al.,* 2017). These results show that the basic components in semen extender is not sufficient to protect sperms from oxidative damage. Therefore, over a few decades, researchers have studied the potential of antioxidant supplementations to the semen extender to overcome this problem and several studies have proved that varieties of antioxidant rich content such as ascorbic acid (Hu *et al.,* 2010), L-Arginine and glutathione (Salmani *et al.,* 2013), β-carotene (Eriani *et al*., 2018), moringa leaves (Syarifuddin *et al*., 2017) as well as a-tocopherol (vitamin E) (Shikh Maidin *et al.,* 2014) have abilities to improve the semen performance by reducing the effects of oxidative stress.

In this study, we interested to determine the potential of *Nigella sativa* oil and honey as an antioxidant supplements to semen extender of goat. *Nigella sativa* or known as black seed from Ranunculaceae family has been known as natural remedies for over 2000 years in Middle Eastern and Far Eastern countries. *Nigella sativa* has been found to be able to improve sperms quality of rabbit (Riad *et al.,* 2004) and rat (Mansour *et al.,* 2013). The therapeutic properties of black seed are due to the presence of this bioactive compound known as thymoquinone. Previous study by Salem (2005) reported that thymoquinone was able to increase the oxidant scavenger system and affect anti-inflammatory mediator such as prostaglandins and leukotrienes in the body.

Honey contains a variety amount of simple sugars (Fuller, 2004) that can be function as a source of nutrition and act as the cryoprotectant to protect the spermatozoa during cryopreservation. Honey also contains several antioxidant compounds such as flavonoid, chrysin, vitamin C, pinobanksin, catalase, and pinocembrin (Bogdanov *et al.,* 2008; Fakhrildin & Alsaadi, 2014). There is also a study done in human sperm showing that honey possess a potential ability to increase sperm quality (Fakhrildin & Alsaadi, 2014).

Nigella sativa and honey contain antioxidants that may help in inhibiting the occurrence of lipid peroxidation by binding of the reactive oxygen species (ROS) free radicals so that they cannot damage the lipid membrane of the sperm. However, research on the utilization of these antioxidants from *Nigella sativa* and honey is limited in goat sperm. Therefore, the objective of this study is to examine the effect of supplementation of *Nigella sativa* and honey on Jermasia goat sperm parameters; sperm motility, progressive score, and abnormalities in both fresh and post-thawed semen.

MATERIALS AND METHODS

Location of Experiment

The semen collection and assessment of semen parameter were done at Ladang Mini Institut Sains Biologi (ISB), Universiti Malaya (UM), Kuala Lumpur. While the assessment of sperm abnormalities was observed at Anatomy and Histology Laboratory, Department of Biology, Faculty of Science, Universiti Putra Malaysia (UPM), Serdang Selangor.

Experimental Animals

Five sexually mature Jermasia male goats with age ranged from 2 to 3 years old, range of live body weight between 32.0 to 37.5 kg, and range of body condition score (BCS) between 1.0 to 2.5 were used in this study. The semen samples were collected for three times, once a week, and live weight and BCS were measured after semen collection. The bucks were fed according to their MEm to ensure the maintenance of live weight and water was available *ad libitum*. The semen was collected by using artificial vagina (AV). The collection was done once a week for three consecutive weeks started at 8.00 a.m. to 9.30 a.m. before bucks were fed. To determine the effect of *Nigella sativa* oil, honey, and their combination on semen parameters (motility, progressive score, and abnormality) the pooled semen was divided into four groups; Control (no supplementation), Treatment 1 (0.5% v/v *Nigella sativa*), Treatment 2 (2% v/v honey), and Treatment 3 (0.5% v/v *Nigella sativa* and 2% v/v honey). The semen parameters were observed at 0, 0.5, 1.0, 1.5, and 2.0 hours after semen collection and 0, 0.5, 1.0, 1.5 and 2 hours after thawing of 48 hours cryopreserved semen.

Extender and Supplements

Tris-citric acid yolk extender (TCAYE) was obtained from Animal Biotechnology and Embryology Laboratories, University of Malaya (UM). Virgin *Nigella sativa* oil and pure honey were obtained from natural product and used as supplements in experimental Treatment groups. The supplements were added to the TCAYE extender for both fresh and frozen preservation before the pooled semen was added.

Assessment of Semen

All semen collected from each individual bucks was analyzed to ensure the pooled semen is prepared from semen with consistent quality (percentage of morphology >80% and progression score >3.5). The volume of semen to be pooled was selected in accordance to the individual with the lowest volume to ensure that all semen pooled was of the same volume. Extender was then added to the pooled semen for dilution using the ratio of 1:9. Pooled samples (n=5) were divided into two fractions; fresh and thawed-frozen assessments and each fraction was allocated into four aliquots (Control and three Treatment groups). Assessment for fresh semen was conducted immediately after collection. The ejaculates were immersed in a warm water bath at 37°C. Sperm motility and progresive score were assessed by light microscope with 40x magnification. Assesement of sperm morphology (normal and abnormal spermatozoa) was prepared using eosin-Y staining and around 350 spermatozoa in total was counted on each slide at a 40× magnification. There were two replicates for each assessment. The semen parameters in fresh semen were evaluated 0, 0.5, 1.0, 1.5, and 2.0 hours after semen collection.

Cryopreservation

Diluted pooled semen were stored in screw cap cryoprotectant vials and placed in ice box at 5ºC for two hours. Later, vial tubes were placed close to liquid nitrogen's vapor (approximately 5 cm) above the surface of liquid nitrogen for 10 minutes before the vials were immersed completely in liquid nitrogen for 48 hours for sperm assessments. After 48 hours of storage, the vial tubes were removed from liquid nitrogen tank. Immediately, the vial tubes were thawed at 37ºC in two minutes before being transferred into micro-centrifuge tubes (have been immersed in 37ºC water bath). Then, the semen parameters were observed at 0, 0.5, 1.0, 1.5, 2.0 hours after thawing.

Statistical Analyses

The data for fresh and post-thawed semen parameters (sperm motility, sperm progressive score and abnormality) were analysed by using univariate (ANOVA) using SPSS statistical software version 20.0. The interaction (time/treatment x semen parameters) was included in the model. The data were presented as mean ± S.E and differences were regarded as significant at P<0.05.

RESULTS

Sperm Motility and Progressive Score

The sperm motility and progressive score for fresh sperm within groups were significant throughout the observation of 0 to 2 hours after semen collection (P<0.05; Tables 1 and 2). For fresh semen, at 0 hour, all groups showed the sperm motility above 90% except in sperms treated with 2% v/v honey. After 2 hours observation of sperm motility, the sperms sustain the motility above 70% in all groups except in semen treated with combination mixed of 0.5% v/v Nigella sativa and 2% v/v honey (Table 1; Treatment 3; about 24%; P<0.05). The progressive score for fresh semen in all groups were maintained above 3 during 0 to 1 hour after semen collection and decreased to the level of 2 at 2 hours after semen collection (Table 2; P<0.05).

The sperm motility in post-thawed semen was significantly different between groups and throughout time of observation after thawing (Table 1; P<0.05). In Table 1, at 0 hour of motility observation, the sperm motility of post-thawed semen treated with combination mixed of 0.5% v/v Nigella sativa and 2% v/v honey (Treatment 3; sperm motility was 60%) was higher compared to other groups [Treatment 2 (46%), Treatment 1 and Control (below 30%); P<0.05]. While for progressive score in post-thawed semen, Treatment groups had scores above 2 during 0 to 0.5 hour after thawing (P<0.05 only at 0 hour observation).

Sperms Abnormality

In general, the total number of sperm morphology (normal and abnormal sperms) both in fresh and post-thawed semen were similar between groups and time of observation (Tables 3 and 4). Numerically, the number of lower sperms defect was found at midpiece/neck of sperms in both fresh and post-thawed semen.

DISCUSSION

Overall, semen treated with honey (Treatment 2) and combination of honey and *Nigella sativa* (Treatment 3) had improved sperm motility both in fresh and post-thawed semen. The present study also reveals that addition of combination of honey and *N. sativa* (Treatment 3) likely gives a better effect on post-thawed sperms compared to fresh sperms. This result was clearly seen in semen 0.5 hour after thawing when the sperm membrane

Table 1. Sperm motility (%) of fresh and post-thawed semen of goats between groups and within time interval

Group	Semen motility (%mean±SE) within time interval (h)									
	Percentage of spermatozoa in fresh semen					Percentage of spermatozoa in post-thawed spermatozoa				
	0	0.5	1	1.5	2	0	0.5	1	1.5	2
Control	90.17±0.73^{by}	88.50±3.00^{by}	79.67±3.61^{ax}	79.67±0.93^{aw}	78.50±1.04^{aw}	24.33±8.17^{bwx}	16.00±8.69^{abx}	5.33±3.24^{awx}	6.83±5.34^{awx}	3.50±1.26^{aw}
T1	93.00±1.04^{cy}	91.83±0.33^{cy}	82.33±2.20^{bx}	73.83±2.52^{awx}*	72.00±2.47^{aw}*	26.00±2.47^{abw}	12.50±4.08^{bw}	17.50±3.82^{abw}	23.00±10.50^{abw}	1.67±0.93^{aw}
T2	79.83±3.00^{bx}*	87.67±1.45^{cy}	72.67±2.68^{awx}	73.33±2.68^{abwx}*	72.00±2.29^{aw}*	46.83±16.72^{bx}	19.00±2.04^{abx}	34.67±1.63^{bwx}	25.67±16.73^{abwx}	3.00±0.50^{aw}
T3	94.50±0.29^{cz}	89.33±0.33^{cz}	79.00±2.50^{by}	77.83±0.73^{bx}	24.33±2.96^{aw}*	60.33±10.08^{cy}*	61.83±7.22^{cy}*	30.00±11.46^{bx}	22.33±9.71^{abw}	4.50±3.51^{aw}

Note: Control (no supplementation), T1 (0.5% v/v *Nigella sativa*), T2 (2% v/v honey), and T3 (0.5% v/v *Nigella sativa* and 2% v/v honey)
^{abcd} Means in the same column with different superscripts differ significantly (P<0.05)
^{wxyz} Means in the same row with different superscripts differ significantly (P<0.05)
*Mean difference is significantly different when compared between control and treatment groups (P<0.05).

Table 2. Sperm progressive score (%) of fresh and post-thawed semen of goats between groups and within time interval

Group	Progressive score (mean±SE) within time interval (h)									
	Fresh semen					Post-thawed semen				
	0	0.5	1	1.5	2	0	0.5	1	1.5	2
Control	3.30±0.19^{bx}	3.22±0.15^{bx}	3.08±0.22^{abx}	2.62±0.22^{aw}	2.57±0.08^{aw}	1.67±0.33^{ax}	1.55±0.58^{ax}	0.90±0.25^{awx}	1.25±0.60^{awx}	1.42±0.58^{aw}
T1	3.50±0.00^{bx}	3.33±0.17^{bx}	3.37±0.17^{bx}	2.60±0.15^{aw}	2.33±0.26$_{aw}$	2.25±0.14^{bw}	2.13±0.62^{bw}	1.33±0.33^{abw}	1.57±0.29^{abw}	0.47±0.12^{aw}
T2	3.07±0.28^{abwx}	3.33±0.17^{bx}	2.88±0.22^{abwx}	2.58±0.26^{aw}	2.38±0.36^{aw}	2.43±0.50^{by}	2.12±0.72^{by}	1.80±0.48^{abxy}	1.60±0.48^{abx}	0.80±0.20^{aw}
T3	3.60±0.05^{dy}	3.23±0.12^{cdxy}	3.07±0.12^{cx}	2.50±0.25^{bw}	1.98±0.27^{aw}	2.83±0.38^{cxy}*	2.20±0.47^{bcy}	1.58±0.22^{abxy}	1.55±0.33^{abx}	1.05±0.33^{aw}

Note: Control (no supplementation), T1 (0.5% v/v *Nigella sativa*), T2 (2% v/v honey), and T3 (0.5% v/v *Nigella sativa* and 2% v/v honey)
^{abcd} Means in the same column with different superscripts differ significantly (P<0.05)
^{wxyz} Means in the same row with different superscripts differ significantly (P<0.05)
*Mean difference is significantly different when compared between control and treatment groups (P<0.05).

Table 3. The sperm morphology of fresh and post-thawed semen of goats between groups and within time interval

Group	Abnormality of spermatozoa between time interval (h); mean±SE									
	Fresh semen					Post-thawed semen				
	0	0.5	1	1.5	2	0	0.5	1	1.5	2
Head										
Control	33.67±12.34[a]	39.83±12.92[a]	36.00±13.58[a]	32.17±11.97[a]	31.83±12.06[a]	39.00±15.33[ab]	50.33±4.75[a]	42.83±17.23[a]	41.83±13.61[a]	40.00±15.02[a]
T1	32.50±11.90[a]	31.50±10.75[a]	28.17±11.20[a]	37.33±11.53[a]	33.67±11.85[a]	41.00±16.35[a]	51.00±19.53[a]	42.33±12.65[a]	54.17±19.54[a]	46.17±10.59[a]
T2	31.33±12.25[a]	38.67±12.25[a]	35.83±13.87[a]	32.50±12.62[a]	38.50±15.10[a]	41.17±17.36[a]	52.33±7.76[a]	46.00±14.44[a]	47.50±18.41[a]	41.67±12.41[a]
T3	34.17±12.42[a]	28.00±12.09[a]	28.33±9.38[a]	31.17±13.71[a]	38.63±13.39[a]	45.67±17.33[a]	35.00±13.43[a]	39.67±15.09[a]	42.17±14.35[a]	44.33±14.92[a]
Midpiece/Neck										
Control	6.67±2.91[a]	6.50±2.75[a]	4.33±1.59[a]	8.17±3.18[a]	4.83±1.30[a]	4.33±1.42[a]	3.50±1.32[a]	1.83±0.88[a]	1.67±0.93[a]	2.33±1.36[a]
T1	6.33±2.47[a]	9.67±4.33[a]	8.50±2.00[ab]	4.33±1.36[a]	4.50±1.89[a]	3.50±1.00[a]	3.33±0.88[a]	5.33±3.38[a]	2.67±2.42[a]	1.50±0.76[a]
T2	6.67±2.17[a]	7.17±3.77[a]	7.50±0.76[ab]	6.33±1.59[a]	3.83±0.73[a]	3.17±2.46[a]	4.00±3.50[a]	1.83±1.36[a]	2.83±0.83[a]	1.67±0.17[a]
T3	6.67±1.09[a]	5.17±1.92[a]	10.83±0.93[b*]	7.83±1.59[a]	6.33±1.01[a]	3.17±1.48[a]	10.33±4.92[a]	4.17±2.05[a]	3.00±2.50[a]	1.33±0.67[a]
Tail										
Control	55.67±17.13[a]	56.17±19.59[a]	57.67±6.75[a]	66.83±7.19[ab]	63.00±13.53[ab]	37.67±4.91[a]	46.33±11.51[a]	43.33±5.73[a]	35.17±7.54[a]	36.50±7.65[a]
T1	61.50±11.82[a]	63.17±13.45[a]	62.67±15.43[a]	52.33±15.60[ab]	45.17±10.58[ab]	38.00±10.77[a]	34.00±4.75[a]	30.67±8.17[a]	40.83±8.01[a]	35.83±8.90[a]
T2	76.50±13.61[a]	73.17±24.35[a]	76.83±5.25[a]	78.00±14.29[ab]	70.50±15.49[b]	45.67±1.64[a]	33.50±7.09[a]	31.50±6.11[a]	32.83±8.12[a]	30.83±8.57[a]
T3	70.00±6.54[a]	84.50±14.95[a]	75.67±14.41[a]	73.33±5.75[ab]	50.17±9.33[ab]	46.67±5.86[a]	38.50±9.10[a]	30.17±5.05[a]	33.00±9.57[a]	32.50±9.26[a]
Normal sperm morphology; P>0.05	244.58±8.53	239.17±7.13	241.79±7.34	242.04±7.92	251.83±6.94	262.75±8.55	260.58±10.19	270.08±7.66	267.67±8.98	271.33±8.88
Abnormal sperm morphology; P>0.05	105.42±8.53	111.67±7.42	108.21±7.34	107.96±7.92	98.17±6.94	87.25±8.545	89.42±10.19	79.92±7.66	82.33±8.98	78.67±8.88

Note: Control (no supplementation), T1 (0.5% v/v *Nigella sativa*), T2 (2% v/v honey), and T3 (0.5% v/v *Nigella sativa* and 2% v/v honey)
[ab] Means in the same column with different superscripts differ significantly (P<0.05)
*Mean difference is significantly different when compared between control and treatment groups (P<0.05).

was able to sustain about 60% of motility compared to the other groups. The supplementation seems to protect the sperms membrane from cryopreservation damage that often causes detrimental effect on the sperm due to the oxidative stress by lipid peroxidation and ice crystal formation.

Honey induces spermatogenesis (Abdul-Ghani *et al.,* 2008) and one of its active ingredients is phenolic compound which has high antioxidant activity (Perez & Rodriguez-Malaver, 2006). Study using semen of Arab stallion by El-Sheshtawy *et al.* (2016) found that semen supplemented with honey bee showed sperms protected from cryoprotectant damage and also suggested that this treatment was able to inhibit the disruption of the sperm DNA by free radicals induction. In addition, infertile semen supplemented with 10% of pure honey and cryoprotectant medium sustained the sperm motility about 50% after thawing compared to infertile semen treated with 5% pure honey. This result suggests that honey acts as intracellular agents and protect sperm cells from freezing damage at the intracellular level (Fakhrildin *et al.,* 2014). Similar results are also shown in goat, ram, bull, and human (Olayemi *et al.,*2011; Jerez *et al.,* 2013; El-Sheshtawy *et al.,* 2014). At low temperature, honey does not freeze and the viscosity of honey increases with the decreased temperature. This occurrence creates a low surface tension that eventually minimizing the ice crystals formation inside the cytoplasm of sperm and reduce sperm damage during cryopreservation (Fuller, 2004; Fakhrildin *et al.,* 2014; El-Sheshtawy *et al.,* 2016). Moreover, honey contains high variety of simple sugars that might serve as a nutrition source and non-penetrating cryoprotectant to sperm cells during cryopreservation (Fuller, 2004). This condition shows the potential capability of honey as an inhibitor to ROS; thus useful in sperm cryopreservation.

The present results showed that fresh semen treated with combination of honey and *N. sativa* (Treatment 3) sustained its motility up to 70% after 1.5 hours of semen collection and dropped to 24% after 2 hours of semen collection. While in post-thawed semen, only semen treated with combination of *N. sativa* showed sperm motility about 60% after 0.5 hour of thawing compared to the other groups and decreased to 30% after 1 hour of thawing. These results suggest that the protective effect of lipid peroxidation in this treatment only protects the sperm cells from oxidative stress activity and the protection seems disappeared slowly after 1.5 hours in fresh semen and 0.5 hour in post-thawed semen; finally, the sperms experience impaired motilities. However this claim needs more investigation.

The bioactive components in *N. sativa* proficient to inhibit ROS that eventually protect spermatozoa from lipid peroxidation (Houghton *et al.,* 1995; Ozugurlu *et al.,* 2005; Parandin *et al.,* 2012). *Nigella sativa* seed extract, fixed and essential oils contain thymoquinone as a phytochemical compound and active compound having a protective effect; the most prominent is antioxidant effect (Kruk *et al.,* 2000; Khader & Eckl, 2014; Gholamnezhad *et al.,* 2016). Study in Iranian infertile

men showed that oral supplementation of 5 ml *N. sativa* oil daily for two months improved their semen parameters compared to non-supplemented group (Kolahdooz *et al.*, 2014). Furthermore, histological study of testicular epididymal caudal in rat supplemented with *N. sativa* showed that the number of sertoli cells, Leydig cells, and nuclear diameter of Leydig cells increased after 53 days of supplementation (Al-Sa'aidi *et al.*, 2009).

The sustainability of sperm motility (about 70% of spermatozoa was motile) and progressive score (the score was about 2.5) in fresh semen for about 1.5 hours after semen colection as shown in the present result (Tables 1 and 2) might be related to the mitochondrial function. The results also found that the number of abnormal sperm was the lowest at midpiece of sperm compared to other part of sperms (head and tail) both in fresh and frozen semen (Tables 3 and 4). These results suggest that fundamental research has to be done on mitochondrial status and expression of specific mitochondrial proteins; thus mitochondrial functionality could be determined. Regarding the sperm abnormality, the present results give no indication of major abnormality in both fresh and post-thawed semen.

Overall, the present findings have shown indication of honey and *Nigella sativa* as an inhibitor to ROS and prevent the formation of ice crystal during sperm cryopreservation. In addition, as mentioned earlier, further studies need to focus at the cellular levels and on the potential role of mitochondria in response to semen quality.

CONCLUSION

The combination of 0.5% v/v *Nigella sativa* and 2% v/v honey as a supplement in TCAYE extender prolong the survival rate of spermatozoa; protect spermatozoa in both fresh and post-thawed semen. This result also indicates that the supplement is useful as a material to preserve the quality of fresh and frozen semen.

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REFERENCES

Abdul-Ghani, A. S., N. Dabdoub, R. Muhammad, R. Abdul-Ghani, & M. Qazzaz. 2008. Effect of Palestinian honey on spermatogenesis in rats. J. Med. Food. 11: 799-802. https://doi.org/10.1089/jmf.2008.0085

Al-Sa'aidi, J. A. A., A. L. D. Al-Khuzai, & N. F. H. Al-Zobaydi. 2009. Effect of alcoholic extract of Nigella sativa on fertility in male rats. Iraqi J. Vet. Sci. 23.

Andrabi, S. M. H. & W. M. C. Maxwell. 2007. A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim. Reprod. Sci. 99: 223-243. https://doi.org/10.1016/j.anireprosci.2006.07.002

Ball, B. A. 2008. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim. Reprod. Sci. 107: 257-267. https://doi.org/10.1016/j.anireprosci.2008.04.014

Barbas, J. P., & R. D. Mascarenhas. 2009. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank. 10: 49-62. https://doi.org/10.1007/s10561-008-9081-4

Barbato, V., R. Talevi, S. Braun, A. Merolla, S. Sudhakaran, S. Longobardi, & R. Gualtieri. 2017. Supplementation of sperm media with zinc, D-aspartate and co-enzyme Q10 protects bull sperm against exogenous oxidative stress and improves their ability to support embryo development. Zygote 25: 168-175. https://doi.org/10.1017/S0967199416000459

Bogdanov, S., T. Jurendic, R. Sieber, & P. Gallmann. 2008. Honey for nutrition and health: A Review. J. Am. Coll. Nutr. 27: 677-689. https://doi.org/10.1080/07315724.2008.10719745

Chitra, K. C., C. Latchoumycandane, & P. P. Mathur. 2003. Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology. 185: 119-127. https://doi.org/10.1016/S0300-483X(02)00597-8

El-Sheshtawy, R. I., D. A. El-Badry, A. Gamal, W. S. El-Nattat, & A. M. A. Almaaty. 2016. Natural honey as a cryoprotectant to improve Arab stallion post-thawing sperm parameters. Asian Pac. J. Reprod. 5: 31-334. https://doi.org/10.1016/j.apjr.2016.06.004

El-Sheshtawy, R. I., El-Nattat, W. S., Sabra, H. A. & Ali, A. H. 2014. Effect of honey solution on semen preservability of local breeds of cattle bulls. World Appl. Sci. J. 32: 2076-2078.

Eriani, K., A. Azhar, M. Ihdina, B. Rosadi, M. Rizal, & A. Boediono. 2018. Quality enhancement of Aceh swamp buffalo (*Bubalus bubalis*) frozen semen by supplementing β-carotene. Trop. Anim. Sci. J. 41: 1-7. https://doi.org/10.5398/tasj.2018.41.1.1

Fakhrildin, M. B. M., & R. A. Alsaadi. 2014. Honey supplementation to semen-freezing medium improves human sperm parameters post-thawing. J. Family Reprod. Health. 8: 27-31.

Fuller, B. J. 2004. Cryoprotectants: the essential antifreezes to protect life in the frozen state. Cryo Letters. 25: 375- 388.

Gholamnezhad, Z., S. Havakhah, & M. H. Boskabady. 2016. Preclinical and clinical effects of Nigella sativa and its constituent, thymoquinone: A review. J. Ethnopharmacol. 190: 372-386. https://doi.org/10.1016/j.jep.2016.06.061

Houghton, P. J., Zarka, R., de las Heras, B. & Hoult, J. R. 1995. Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation. Planta Med. 61: 33-36. https://doi.org/10.1055/s-2006-957994

Hu, J. H., W. Q. Tian, X. L. Zhao, L. S. Zan, H. Wang, Q. W. Li, & Y. P. Xin. 2010. The cryoprotective effects of ascorbic acid supplementation on bovine semen quality. Ani. Rep. Sci. 121: 72-77. https://doi.org/10.1016/j.anireprosci.2010.04.180

Jerez, R., Gil, L., Gonzalez, N., Luno, V., Olaciregui, M. & Martinez, F. 2013. Rosemary honey as natural energetic source on refrigeration ram semen. Reprod. Dom. Anim. 48:1-6.

Khader, M. & P. M. Eckl. 2014. Thymoquinone: an emerging natural drug with a wide range of medical applications. Iran J. Basic Med. Sci. 17: 950-957.

Kolahdooz, M., S. Nasri, S. Z. Modarres, S. Kianbakht, & H. F. Huseini. 2014. Effects of Nigella sativa L. seed oil on abnormal semen quality in infertile men: A randomized, double-blind, placebo-controlled clinical trial. Phytomedicine. 21: 901-905. https://doi.org/10.1016/j.phymed.2014.02.006

Kruk, I., T. Michalska, K. Lichszteld, A. Kladna, & H. Y. Aboul-Enein. 2000. The effect of thymol and its

derivatives on reactions generating reactive oxygen species. Chemosphere. 41: 1059-1064. https://doi.org/10.1016/S0045-6535(99)00454-3

Mansour, S. W., S. Sibghatullah, H. Sree, A. K. Mueen, & A. R. N. Ibrahim. 2013. Sensibility of male rats fertility against olive oil, Nigella sativa oil and pomegranate extract. Asian. Pac. J. Trop. Biomed. 3: 563-568. https://doi.org/10.1016/S2221-1691(13)60114-8

Olayemi, F. O., D. A. Adenigi, & M. O. Oyeyemi. 2011. Evaluation of sperm motility and viability in honey included egg yolk based extenders. Global Vet. 7: 19-21.

Ozugurlu, F., S. Sahin, N. Idiz, O. Akyol, A. Ilhan, R. Yigitoglu, & B. **Isik.** 2005. The effect of Nigella sativa oil against experimental allergic encephalomyelitis via nitric oxide and other oxidative stress parameters. Cell Mol Biol (Noisy-le-grand). 51: 337-342.

Pamungkas, F., A. Batubara, & S. Sutoro. 2014. The quality of spermatozoa of Gembrong goats during cryopreservation process. Med. Pet. 37: 95-100. https://doi.org/10.5398/medpet.2014.37.2.95

Parandin, R., N. Yousofvand, & R. Ghorbani. 2012. The enhancing effects of alcoholic extract of Nigella sativa seed on fertility potential, plasma gonadotropins and testosterone in male rats. Int. J. Reprod. Biomed. 10: 355-362.

Perez, E., A. J. Rodriguez-Malaver. 2006. Vit P: Antioxidant capacity of Venezuelan honey in Wistar rat homogenates. J. Med. Food. 9: 510-516. https://doi.org/10.1089/jmf.2006.9.510

Riad, R. M., A. M. Ghoniem, & T. S. T. Seleem. 2004. Effect of *Nigella sativa* (Aquas extract) on the viability of rabbit spermatozoa, fertility traits and bacterial contamination. Assiut. Vet. Med. J. 130: 899-911.

Salamon, S., & W. M. C. Maxwell. 2000. Storage of ram semen. Anim. Reprod. Sci. 62: 77-111. https://doi.org/10.1016/S0378-4320(00)00155-X

Saleh, R. A., A. Agarwal, E. A. Nada, M. H. El-Tonsy, R. K. Sharma, A. Meyer, & A. J. **Thomas.** 2003. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril. 79: 1597-1605. https://doi.org/10.1016/S0015-0282(03)00337-6

Salem, M. L. 2005. Immunomodulatory & therapeutic properties of the *Nigella sativa* L. seed. Int. Immunopharmacol. 5: 1744-1770. https://doi.org/10.1016/j.intimp.2005.06.008

Salmani, H., M. M. Nabi, H. Vaseghi-Dodaran, M. B. Rahman, A. Mohammadi-Sangcheshmeh, M. Shakeri, & M. Zhandi. 2013. Effect of glutathione in soybean lecithin-based semen extender on goat semen quality after freeze-thawing. Small. Rumin. Res. 112: 123-127. https://doi.org/10.1016/j.smallrumres.2012.12.015

Shikh Maidin, M., N. F. Adanan, M. T. Aminudin, & A. **Tawang.** 2014. In vitro supplements improves motility and progressive score of spermatozoa in Jermasia goats. APCBEE Procedia 8: 329-333. https://doi.org/10.1016/j.apcbee.2014.03.049

Syarifuddin, N. A., A. L. Toleng, D. P. Rahardja, I. Ismartoyo, & M. Yusuf. 2017. Improving libido and sperm quality of Bali bulls by supplementation of *Moringa oleifera* leaves. Media Peternakan. 40: 88-93. https://doi.org/10.5398/medpet.2017.40.2.88