



Influence of Honey on Sperm Traits in KUB Rooster Concerning Cold Storage

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Abstract | Damage due to oxidative stress during semen preservation can cause a decrease in motility, viability, and damage to plasma membranes that can reduce fertility rates. The use of antioxidants in semen preservation is very important to suppress lipid peroxidation so it can maintain the quality of the sperm. This study aimed to determine additional honey to the quality of KUB rooster sperm during cold storage. The semen with superior quality was diluted into four different treatments, lactate ringer egg yolk without honey as control (LREY0), LREY with 0.2% honey (LREY2), LREY with 0.4% honey (LREY4), and LREY with 0.6% honey (LREY6). Semen was stored at 5°C for 48 hours. The evaluation of semen quality includes an examination of spermatozoa motility using a microscope, the viability with the eosin-negrosin staining method, and membrane integrity using the hypoosmotic swelling test (HOST) at the fresh semen, 5th, 24th, and 48th hours post-cold storage. The results revealed that in the 5th hour, percentage of motility, viability, and membrane integrity were not significantly different. However, at the 24th and 48th-hour results revealed significant differences by adding honey ($P < 0,05$). The 0.4% honey supplementation was the best to maintain KUB rooster quality until the 48th hour with motility of up to $41.50 \pm 1.19\%$, the viability of up to $59.75 \pm 0.63\%$, and membrane integrity of up to $66.50 \pm 1.32\%$. It can be concluded from the current study that adding 0.4% honey can preserve the quality of KUB rooster sperm during cold storage.

Keywords | Antioxidant, Chilling storage, Honey, KUB rooster, Sperm membrane integrity

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INTRODUCTION

Statistical data on livestock and animal health states that the consumption of non-purebred chicken meat and eggs in Indonesia from 2016 to 2017 has increased by 24,9% and 14,7% (Anonim, 2018). Ayam Kampung Unggul Badan Penelitian dan Pengembangan Pertanian (Kampung Chicken KUB) is known as one of the Indonesian selected-

native chickens with a high-productivity of eggs and high-quality meats (Krista and Harianto, 2013; Udjianto, 2016) which can meet people's needs efficiently. The increase in the efficiency and productivity of livestock can be achieved through artificial insemination (AI) technology. The AI implementation can improve the effectiveness and efficiency of superior males (Mohan et al., 2018). Rooster semen has a high concentration value, but its volume is

low, so it needs to be diluted to increase the volume so that it can be used for AI in large quantities (Bebas and Laksmi, 2015; Hafez, 2000). The viability and quality of spermatozoa stored at room temperature cannot last long, which is necessary to carry out storage in cold temperatures (Kusumaningrum et al., 2002).

Rooster spermatozoa have a high arrangement of polyunsaturated fatty acids (PUFA), which are then easily damaged via lipid peroxidation and free radicals such as reactive oxygen species (ROS). Uncontrolled ROS production can damage the membrane and the mitochondria of spermatozoa (Shah et al., 2016). The addition of antioxidants into the diluent can eliminate free radicals to maintain sperm quality during the storage process (Herdis, 2007; Hendiyani et al., 2018). Antioxidants are molecules that can end the chain of free radical reactions before the occurrence of damage to main molecules (Burgos et al., 2006).

Honey, as an antioxidant, contains vitamin C and E, phenolic components, flavonoids, ascorbic acid, glucose, oxidase enzyme, and catalase enzyme (Hidayaturrehman, 2007). The study of honey in semen extender had carried out on different species as turkeys (Sari et al., 2015), cattle (Yimer et al., 2015; Malik, 2018), stallions (Sheshtawy et al., 2016), and fish (Rahardhianto et al., 2012; Condro et al., 2012; Nainggolan et al., 2015; Martin et al., 2018). The addition of 3% honey can maintain the quality of turkey sperm (motility and viability) during storage at 5°C (Sari et al., 2015). In Arab stallions, the addition of honey can maintain the motility, viability, and membrane integrity of spermatozoa during cryopreservation (Sheshtawy et al., 2016). There are no reports on the sperm quality of KUB roosters during preservation at 5°C in an extender with honey supplementation as an antioxidant which is the main reason conducted the current study. The honey supplementation is used as an antioxidant for preserving the sperm quality of KUB roosters during storage at 5°C. We evaluated the sperm characteristics such as motility, viability, and membrane integrity.

MATERIALS AND METHODS

ANIMAL TRIAL AND ETHICS

The procedures were approved by the Ethics Committee of Research at the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta number 0041/EC-FKH/Int/2022. Five KUB roosters aged 48 weeks were placed in a 90 x 56 x 67 cm of individual cage. The roosters were daily fed with 150 grams of commercial feed (Multifeed BR 1-7 RT, Indonesia) and ad-libitum water. A poultry house, 3x9 meters, was used to place the individual cage with steady light and circulation. The rooster cages were cleaned and disinfected every day. The roosters were

treated with anthelmintics before the study began, and the vitamins were given every two days. The semen collection habituation was done two weeks before the research to create an adaptable environment.

EXTENDER PREPARATION

The basic diluents used in this study were a lactated ringer and 20% egg yolk (LR-EY) with penicillin and streptomycin antibiotics. The lactate ringer and egg yolk are homogenized using a stirrer, then centrifuged for 15 min at 3000 rpm. Supernatants were harvested as a basic diluent, then penicillin 1000 IU/ml and streptomycin 1 mg/ml were added. The basic diluents were divided into four solutions then honey was added at rates of 0.2% (LREY2), 0.4% (LREY4), and 0.6% (LREY6) to the 1st, 2nd, and 3rd solutions respectively, while the 4th was kept without honey as a control (LREY0).

SEMEN COLLECTION AND DILUTION

We collected semen from five healthy KUB roosters aged 48 weeks. KUB rooster semen was collected twice a week by using the dorso-abdominal massage method (Tabatabaei et al., 2011), with a total of four times repetitions. Fresh semen was evaluated macroscopically (volume, color, pH, and consistency) and microscopically (motility, mass movement, concentration, and viability). Semen with spermatozoa motility of more than 70% and concentration of more than 2x10⁹/ml was divided into four tubes. Each tube was diluted with a lactate ringer egg yolk extender with honey supplementation at rates of 0.2% (LREY2), 0.4% (LREY4), 0.6% (LREY6), and without honey as control (LREY0), then stored in a temperature of 5°C for 48 hours.

SPERMATOZOA QUALITY EVALUATION

Semen was stored in four different storage conditions, fresh, 5th, 24th, and 48th hours post-storage. Straws were incubated in a water bath at 37 °C for 30 sec. Semen quality evaluation included motility (%), viability (%), and membrane integrity (%).

SPERM MOTILITIES

A coverslip slide was used to cover a glass object containing 20 µl of collected sperm and 80 µl of saline solution. Sperm motility was evaluated under a microscope (XSZ-107BN, BW Optics China) with a 40x magnification in five fields.

SPERM VIABILITIES

The eosin-negrosin staining method was used to examine the viability (Telnoni et al., 2017). A bunsen burner was used to heat a mixture of 50 ml of eosin-negrosin staining (1:5) and 10 ml of semen on the microscope slide. The slides were evaluated under a microscope with a 40x a microscope (XSZ-107BN, BW Optics China) with a 40x magnification. In a sample stained with eosin-negrosin, morphometric measurements involved 200 sperm cells. A living sperm is

characterized as a white non-colored head and a dead sperm revealed as a red head (Susilawati, 2011; Telnoni et al., 2017).

Membrane integrity. Following Akcay et al. (2012), the membrane integrity was evaluated using a modified version of the Hypoosmotic Swelling Test (HOST). A fixed volume of 10 ml of sperm was dissolved in 100 µl of HOST solution (0.9 g of fructose and 0.49 g of sodium citrate dissolve equates to a final volume of 100 ml) and incubated for 30 min at 37°C. Under a microscope with a microscope 40x magnification (XSZ-107BN, BW Optics China), 200 spermatozoa were counted after the mixture was spread on a glass object, dried, and fixed. Spermatozoa with a normal plasma membrane are characterized as swollen or curled tails, while the damaged membrane is revealed as a straight tail. Percentage calculation of the percentage of the normal plasma membrane (NPM) was carried out using the formula:

$$NPM(\%) = \frac{\text{Total number of swollen or curled tail sperms}}{\text{The total number of sperms counted}} \times 100$$

DATA ANALYSIS

The motility (%), viability (%), and membrane integrity

(%) of KUB rooster sperm were statistically processed by the Analysis of Variance (ANOVA) method and a Duncan post hoc multiple ranges to determine the differences between treatments.

RESULTS

Fresh semen evaluation of the KUB rooster indicated that the seminal quality was normal. The macroscopic evaluation revealed that the volume and pH were 0.38±0.01 ml and 7.25±0.14, respectively. Microscopic examination results recorded a concentration (2.38x10⁹±0.087 cells/ml), motility (92.00±0.82%), viability (92.00±1.22%), and membrane integrity (93.75±1.11%). The motility percentage was revealed in Table 1, the viability in Table 2, and membrane integrity in Table 3. The results discovered in Figure 1, at 5th-hour storage did not show any significant differences, while honey supplementation in the 24th and 48th hours storage indicated a significant effect (P < 0.05). The recommended honey concentration for maintaining KUB rooster semen quality in cold storage until the 48th hour was 0.4% with motility (41.50±1.19%), viability (59.75±0.63%), and membrane integrity (66.50±1.32%).

Table 1: The motility (%) of KUB rooster semen on LREY extender with honey supplementation during cold storage for 48 hours.

Treatments	Sperm motility (%)			
	Fresh semen	5 th hours	24 th hours	48 th hours
LREY0 (without honey)	92.00±0.82	73.75±6.57	55.50±1.55 ^b	33.50±1.55 ^b
LREY2 (0.2% of honey)	92.00±0.82	78.25±5.45	56.75±0.95 ^b	35.50±1.04 ^b
LREY4 (0.4% of honey)	92.00±0.82	79.25±4.78	61.25±1.65 ^a	41.50±1.19 ^a
LREY6 (0.6% of honey)	92.00±0.82	75.00±5.97	56.00±1.22 ^b	35.50±2.22 ^b

^{a,b,c} Different superscripts following the numbers within the same column indicate significant differences between treatments P<0.05.

Table 2: The viability (%) of KUB rooster semen on LREY extender with honey supplementation during cold storage for 48 hours.

Treatments	Sperm viability (%)			
	Fresh semen	5 th hours	24 th hours	48 th hours
LREY0 (without honey)	92.00±1.22	84.00±2.35	64.75±1.18 ^b	50.00±2.68 ^b
LREY2 (0.2% of honey)	92.00±1.22	85.25±1.80	69.25±1.11 ^{ab}	54.00±2.27 ^{ab}
LREY4 (0.4% of honey)	92.00±1.22	83.75±2.02	73.00±0.91 ^a	59.75±0.63 ^a
LREY6 (0.6% of honey)	92.00±1.22	81.50±2.63	67.25±2.87 ^b	50.25±2.66 ^b

^{a,b,c} Different superscripts following the numbers within the same column indicate significant differences between treatments P<0.05.

Table 3: The membrane integrity (%) of KUB rooster semen on LREY extender with honey supplementation during cold storage for 48 hours.

Treatments	Sperm membrane integrity (%)			
	Fresh semen	5 th hours	24 th hours	48 th hours
LREY0 (without honey)	93.75±1.11	81.50±3.86	73.50±0.87 ^b	59.50±0.87 ^b
LREY2 (0.2% of honey)	93.75±1.11	82.25±1.54	74.25±1.25 ^b	63.25±1.70 ^{ab}
LREY4 (0.4% of honey)	93.75±1.11	83.50±4.19	79.25±0.95 ^a	66.50±1.32 ^a
LREY6 (0.6% of honey)	93.75±1.11	81.25±4.71	73.75±2.29 ^b	62.50±1.19 ^{ab}

^{a,b,c} Different superscripts following the numbers within the same column indicate significant differences between treatments P<0.05.

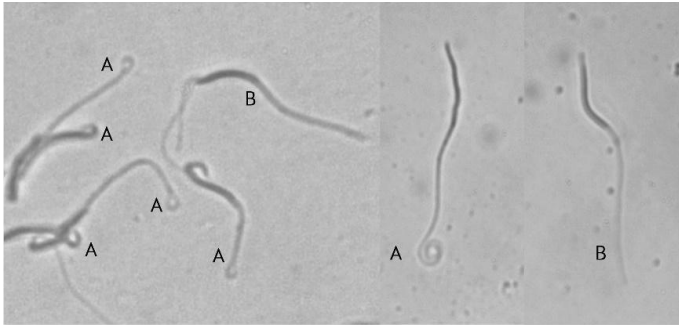


Figure 1: Spermatozoa membrane integrity observation using HOST; normal membrane of spermatozoa showed as swelling or curling-tail (A), damaged membranes showed as a straight spermatozoa tail (B).

DISCUSSION

During preservation, semen is highly susceptible to oxidative stress which causes biochemical and functional damage to the sperm. The sperm membrane plasma contains a high concentration of polyunsaturated fatty acids (PUFA) which maintains membrane stability and flexibility during the fertilization process. However, during cold storage, this plasma also makes the sperm more susceptible to lipid peroxidation through reactive oxygen species (ROS) (Sharideh et al., 2019; Vui et al., 2021). In physiological concentrations, ROS can help sperm function in hyperactivation, acrosome reaction, capacitation, and oocyte penetration, however high concentrations of ROS can cause lipid peroxidation, changes in membrane fluidity, and damage to sperm structure and death (Vui et al., 2021). Damaged membranes contribute to dead spermatozoa and disrupt membrane stability which plays an important role in fertilization (Ciftci and Aygun, 2018). This study's findings were in line with the theory that preservation of KUB rooster semen at 5°C for 48 h negatively influences the spermatozoa and causes decreased membrane integrity, motility, and viability of the spermatozoa.

In the seminal plasma, some antioxidants can prevent or minimize the effects of oxidation damage by ROS. However, antioxidants in seminal plasma are not always available compared to ROS in semen which is greater during the preservation process, so the antioxidant capacity may not be able to fight lipid peroxidation (Amstrong et al., 1999; Vui et al., 2021). To maintain spermatozoa viability and fertility, the antioxidant system and the amount of ROS production needed to be in sync (Neuman et al., 2002). In this study, honey was used as an antioxidant in semen. Honey provides exogenous molecules that can prevent auto-oxidation reactions and neutralize free radicals through various mechanisms (Otmani et al., 2021), as well as the antioxidant properties can maintain the motility of the sperm, the viability, and the membrane integrity (Sheshtawy et al., 2016). Rochmi and Sofyan (2019) stated

that spermatozoa motility is the primary determinant in pigeons, turkeys, and roosters's fertility.

The membrane integrity, motility, and viability of KUB rooster spermatozoa in extender with various concentrations of honey at the 5th hour were not significantly different, this might have happened at the start of storage, but the antioxidant capacity in seminal plasma was still able to fight lipid peroxidation during brief preservation (Amstrong et al., 1999; Vui et al., 2021). At the 24th to 48th hour, honey supplementation had a significant effect ($P < 0.05$) on motility ($41.50 \pm 1.19\%$), viability ($59.75 \pm 0.63\%$), and membrane integrity ($66.50 \pm 1.32\%$). Our results matched those reported on sperm from Pelung roosters (Hidayat et al., 2021), turkeys (Sari et al., 2015), bulls (Yimer et al., 2015; Malik, 2018), stallions (Sheshtawy et al., 2016), and fish (Rahardhianto et al., 2012; Condro et al., 2012; Nainggolan et al., 2015; Martin et al., 2018).

Honey, as an antioxidant, contains vitamins C and E, phenolic components, flavonoids, ascorbic acid, glucose oxidase enzyme, and catalase enzyme (Sari et al., 2015). Honey protein exerts its antioxidant activity as a reducing agent and free radical scavenger, transferring electrons (e^-) or hydrogen atoms (H^+) (Ramon-Sierra et al., 2022). Additionally, honey contains simple sugars (monosaccharides) like fructose and glucose, which are nutrients and cryoprotectants for spermatozoa that do not penetrate (Fuller, 2014; Sari et al., 2015). Honey is a strong antioxidant in defending cells against the damage caused by ROS (Erejuwa et al., 2012). Lestari et al. (2021) stated that antioxidants in honey significantly increase superoxide dismutase (SOD) levels in semen, which continues to counteract the increase in ROS caused by the preservation process. Minimal lipid peroxidation will protect the spermatozoa membrane from damage and in turn preserve the membrane integrity, motility, and viability of the spermatozoa. So, honey can be used as an alternative antioxidant to maintain the KUB rooster sperm quality during cold preservation at 5°C.

CONCLUSIONS

The 5th hour storage with honey supplementation had no significant effect on the spermatozoa quality. At the 24th and 48th hour significant differences were revealed and 0.4% honey was the recommended concentration in maintaining motility, viability, and membrane integrity compared to other treatments.

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NOVELTY STATEMENT

Our study is the first report on the utilization of honey in semen extender to maintain spermatozoa quality of KUB roosters in cold storage. Our research demonstrated that the supplementation of 0.4% honey to the lactate ringer egg yolk can maintain the spermatozoa quality in cold storage (5 °C) until 48 hours.

AUTHOR'S CONTRIBUTION

From the beginning of this study until the publications are published, the authors declared their participation.

CONFLICT OF INTERESTS

The authors have declared no conflict of interest.

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