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Cryopreservative Effect of Adding a Honey Solution to Native Chicken Spermatozoa

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Abstract | The current study was conducted to determine the quality of semen a native chicken Kalimantan breed. A total of four cocks were used in this study. Semen was collected twice per week via the dorsal-abdominal massage method. Semen qualities were very important to the success of fertility in the chicken. The semen was then assessed macroscopic and microscopic to determine viability, motility, integrity of the membrane plasma and to look for abnormalities. There were no significant differences (P>0.05) in sperm viability, abnormality or integrity of the membrane plasma between treatments and the control before freezing. However, sperm motility before freezing was significantly different (P<0.05) between the added 4% of honey (P4 treatment) semen and the controls. Semen qualities post-thawing such as viability, motility, abnormalities and integrity of the membrane plasma were not significantly different (P>0.05) between the controls and all treatments. The motility of spermatozoa before freezing for the P4 treatment was significantly lower than that control and all treatments, and the addition of honey with 2, 4, 6, 8% has no effect on semen qualities in post-thawed chicken spermatozoa.

Keywords | Honey, Native chicken, Semen qualities, Cryopreservation, Post-thawing

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INTRODUCTION

Semen cryopreservation is an invaluable tool for the poultry industry because it affords the ability to preserve the genetics of poultry resources. Additionally, cryopreserving semen also protects germ plasma in danger of loss and supports artificial insemination technology programs in livestock. In general, chicken sperm is more sensitive to the freezing process, and their quality post-thawing is also relatively low compared with that of other domestic livestock like mammalian species (Mosca et al., 2016). Therefore, it is necessary to improve protocols for freezing chicken semen for artificial insemination programs. The primary cause of avian spermatozoa death during the process of freezing is a resistance of osmotic stress and the membrane fluidity of the sperm (Blesbois et al., 2005; Morris et al., 2011). Overall, avian spermatozoa are recognized to be more delicate to cryopreservation than mammalian sperm (Long, 2006). The difficulty of freezing chicken spermatozoa was due to specific reproductive physiology and high variability from species and breed/strains (Rakha et al., 2016). Nevertheless, freezing-thawing processes result in dramatic damage to avian spermatozoa membranes, resulting in the loss of more than 50% of spermatozoa (Lemoine et al., 2011; Long, 2006). The cryopreservation of semen typically involves the use of a cryoprotectant to defend sperm from cold shock and cell damage due to the development of ice crystals. Cryopreservation of semen was procedures involving diverse cryoprotective agents have been studied. Procedures using cryoprotectants such as glycerol, N, N-*dimethyl acetate-amide* (DMA) and *dimethyl-sulfoxide* (DMSO) have been employed to cryopreserve chicken sperm (Blesbois et al., 2005). In general, cryoprotectants can be classified into two categories: penetrating and non-penetrating mediators of the membrane plasma of spermatozoa (Lemma, 2011). While Fuller (2004) revealed that sugars are

a type of non-penetrating cryoprotectant that acts extra cellular and change the osmotic gradient of the extended semen and allow water from the sperm to diffuse out.

Honey is a natural material rich in nutrients such as simple sugars, antioxidants, proteins, vitamins, and minerals, all of which are beneficial to improving sperm quality (Saxena et al., 2010). Furthermore, honey comprises a large variety of simple sugars that serve both as a source of nutrition and a non-penetrating cryoprotectant (Fuller, 2004). Based on the description, honey is can be used to be added to the semen extender for the freezing of sperm of chicken. Because no prior research has tested the effects of supplementing honey in vitro into a skim milk-egg yolk extender as a chicken spermatozoa-freezing medium. Therefore, this research was designed to evaluate the effects of adding various concentrations of honey to a skim milk-egg yolk extender on the cryopreservation of chicken spermatozoa.

MATERIALS AND METHODS

Animals

A total of four adults male native chickens from Kalimantan Indonesia were used this study. The averages aged about 1.5 years old. All samples were housed in individual cages maintained at 32˚C at the poultry unit faculty of the agriculture Islamic University of Kalimantan-Banjarmasin in South Kalimantan, Indonesia. All of the cocks were fed a commercial chicken diet consisting of 18% crude protein. The animals were allowed water *ad libitum*. After a noneweek collection of semen training period, semen from all of the chickens were routinely collected twice every weekas long as three weeks (replicates 6 per cock). The semen collection method followed by technique initially described by Malik et al. (2013). After the semen was collected, it was stored in a water bath (37˚C) and then analyzedboth macroscopically and microscopically. The macroscopic analysis included measurements of the semen's volume, pH, and color. The microscopic analysis included analyses of sperm concentration, viability, motility and plasma membrane integrity. The basic extender used in this research contained skim milk, glucose, egg yolks, glucose, penicillin or streptomycin, and glycerol adopted from Malik et al. (2017). Honey was added inextender with concentrations of 0% (control), 2%, 4%, 6% and 8% modification with Yimer et al. (2015). The diluent for the control was the same as that for the treatment group without the addition of honey.

Freezing and Thawing

After the semen collection was evaluated and mixed with the extender, the solution was diluted to a final concentration of 25 $\times 10^9$ sperm/mL.The diluted semen was placed on 0.25-ml straws (Biovet, France). After sealing, the straws were placed on a cold rack (5˚C) and lowered into nitrogen vapors (-50˚C) 5 cm above the surface of the liquid nitrogen. When the temperature had fallen to -120˚C, the straws were moved to a liquid nitrogen tank. One week after being stored in the liquid nitrogen, straws of each treatment were randomly chosen, thawed in water at 37˚C for 30 seconds and evaluated.

Semen Quality

The quality of the sperm was measured in fresh semen, before freezing and post-thawed. The volume of semen was measured inmicroliters using a micropipette. The motility of the sperm was evaluated by placing a drop of semen sample on a pre-warmed (37˚C) glass slide under a light microscope (400×) using the methodology of Malik et al. (2017). The percentage of sperm motility was subjectively assessed on a scale ranging from 0 to 100%.

Sperm Viability

The viability of chicken spermatozoa was observed by adding eosin-nigrosin staining as defined by Malik et al. (2015). One drop of semen was located on a glass slide and mixed with one drop of eosin-nigrosin solution before freezing and post-thawed. Eosin was enters non-viable cells, which appear red, and nigrosin creates a dark background to facilitate the detection of viable, non-stained cells.

The Integrity of the Membrane Plasma

The integrity of membrane of native chicken fowl spermatozoa was evaluated using the hypo-osmotic swelling test (HOS) previously defined by Kaka et al. (2015). The HOS solution was preparedby adding 1g of sodium citrate to 100 mL of distilled water. Previously diluted semen in a volume of 25mL was varied with 500L of HOS solution (100 mOsmol/kg) and incubated at 25˚C for as long as 30 min. A drop of incubated solution was placed on a pre-warmed (37˚C) slide and fixed in buffered 2% glutaraldehyde. In all, 200 sperm were measured using a phase-contrast microscope (1000×).

Statistical Analysis

Sperm qualities including viability, motility, abnormality and integrity of membrane were provided as means ± standard error of the mean (SEM) for fresh, unfrozen sperm and post-thawed sperm. The data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc tests to determine differences among the treatment means. Differences of $P<0.05$ were considered statistically significant. Statistical analyses were done using the Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The mean volume, pH and sperm concentration recorded

Table 1: Percentages of sperm viability before freezing and post-thawing in native chicken spermatozoa

Treatments	Viability (%)	
	Before freezing	Post thawing
P0 (without of honey)	62.25±0.42	40.51±0.71
P1 (2% of honey)	61.35±1.05	42.85±1.35
P2 (4% of honey)	62.18±0.15	41.01±0.15
P3 (6% of honey)	63.21±1.30	40.15±1.30
P4 (8% of honey)	60.03±0.10	38.40±1.03

Table 2: Percentages of sperm motility before freezing and post-thawing in native chicken spermatozoa

Treatments	Motility (%)	
	Before freezing	Post thawing
P0 (without of honey)	48.33±0.76^{b}	31.27±0.62
P1 (2% of honey)	56.07±1.05^{b}	35.13±1.16
P2 (4% of honey)	51.56±0.27^{b}	34.47±0.07
P3 (6% of honey)	43.15±1.30^{b}	33.15±1.03
P4 (8% of honey)	26.51±0.12^{a}	26.67±1.12

Different superscripts along the column indicate the significant differences (P < 0.05) among groups (n= 24)

Table 3: Percentages of sperm abnormality beforefreezing and post-thawing in native chicken spermatozoa

Treatments	Abnormality (%)	
	Before freezing	Post-thawing
P0 (without of honey)	15.01±1.52	16.90±0.92
P1 (2% of honey)	16.75±0.85	17.10±1.15
P2 (4% of honey)	15.09±0.96	16.98±0.56
P3 (6% of honey)	16.14±1.04	16.05±1.89
P4 (8% of honey)	15.32±0.72	16.17±0.45

Table 4: Percentages of sperm plasma membrane integrity before freezing and post-thawing in native chicken spermatozo

Treatments	Plasma Membrane integrity (%)	
	Before freezing	Post thawing
P0 (without of honey)	70.87±0.96	67.05±1.04
P1 (2% of honey)	72.25±0.95	70.13±0.35
P2 (4% of honey)	60.63±0.27	60.17±0.28
P3 (6% of honey)	60.05±1.21	60.56±1.41
P4 (8% of honey)	58.33±1.20	58.31±0.70

in the fresh ejaculates were 0.3ml ± 0.06 ml, 6.9-7.1, and 2.60 ± 0.78 ×109 sperm/ml, respectively. The percentage of semen qualities including viability, abnormality, and integrity of membrane before freezing were no significant different (P>0.05) between control and all treatments. Whereas, the percentage of sperm motility before freezing was significantly different (P<0.05) between control and the treatment of P4 (Table 2). On the other hand, the percentage of semen qualities such as viability, motility, abnormality, and integrity of membrane post-thawed were no significant different (P>0.05) between control and all treatments (Table 1, 2, 3 and 4).

DISCUSSION

In this Study, sperm quality of native chickens before freezing was exhibited an upward trend, between the control group and all of the treatments with the exception of the P4 treatment. Although, the statistical analysis was not significant, the treatment of P1 (2% added honey) exhibited better sperm motility and viability results. Decreased sperm motility and viability has occurred in proportion to the addition of honey levels in the diluent for the P2, P3, and P4 treatments post-thawed. The addition of as much as 2% honey (P1) in the extender can increase the nutrients needed by sperm during storage on 4°C before freezing and freezing process. The honey was used in this study contained as much as 67.84% reducing sugar, which consists of fructose and glucose. Fructose, glucose, and minerals in the honey are used by sperm as energy (Rahardhianto et al., 2012). Sugar is one of the important constituents of most extenders of semen (Purdy, 2006; Bearden, 2004), and honey is known to contain mainly sugars such as disaccharides, monosaccharides, polysaccharides, and oligosaccharides.

During cryopreservation, spermatozoa were exposed to a number of chemical and physical stresses. Because of the injurious effect of these stresses, it is inevitable that sperm quality will decrease after cryopreservation (Lemma, 2011; Andrabi, 2007; Watson, 2000). Base on the data of semen qualities such as viability, mortality, abnormality and sperm membrane integrity has no effect addition of honey between control and all treatments. However, it was noted of trend higher of sperm motility, and viability post-thawed on the treatment of P1. It was suspected that the provision of honey at these levels (P1) delivered nutrients contained in the honey, such as antioxidants and fatty acids that can be utilized optimally by spermatozoa. Our findings also strengthen the results of Yimer et al. (2015) who reported that the addition of a 2.5% honey solution in Tris-based extenders was optimal for obtaining higher-quality semen after chilling and thawing. These findings also support those of Asadpour and Nasrabadi (2012) and Bathgate (2011). These authors revealed that the addition *in vitro* of various agents including fatty acids and antioxidants enhances post-thawed semen qualities. Furthermore, Fuller (2014) revealed that honey contains a high volume of many simple sugars, which might serve both as a source of nutrients and as a non-penetrating cryoprotectant to spermatozoa. Moreover, Erejuwa et al. (2012) stated that honey has been acclaimed as a strong noble antioxidant in

defending cells of the numerous organs in the body from injury due to reactive oxygen species (ROS). Based on the results of these studies, honey can be used as an alternative as the cryoprotectant for a freezing of semen chicken.

CONCLUSION

Wecan conclude that the motility of spermatozoa before freezing for the P4 treatment was meaningfully lower than that of control and exposed to other treatments. The addition of honey with 2,4, 6, 8 % has no effect on any sperm parameter in post-thawed chicken sperm.

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CONFLICT OF INTEREST

We have no conflict of interest including of financial or data in the research.

AUTHORS CONTRIBUTION

AriniI and Sakiman in the experiment wrote the manuscript and superviser in the field. IrwanZakir, A. Malik and Sasongko N carried out the statistical analysis.

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