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HONEY SUPPLEMENTATION IN LACTATE RINGER-EGG YOLK EXTENDER ON QUALITY OF PELUNG CHICKEN SPERMATOZOA POST-CHILLING

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ABSTRACT

The purpose of this research was to determine the influence of honey supplementation in lactate ringer-egg yolk extender with 0.025% sodium dodecyl sulfate and 2% vitamin E addition (LREYSE) on the quality of Pelung chicken spermatozoa preserved at 5° C for 72 hours. Semen was collected from three Pelung chickens once per day over a course of three days using the dorsal-abdominal massage method. Semen was divided into 5 treatment groups of honey supplementation that are 0% as control (LREYSEH0), 1% (LREYSEH1), 2% (LREYSEH2), 3% (LREYSEH3), and 4% (LREYSEH4). This liquid semen was observed for sperm motility and viability every 12 hours. Complete random design repeated measurement with 4 replications was used in this study. The results showed the motility and viability of spermatozoa in LREYSE extender with 2% honey supplementation (61.25±1.25% and 71.50±0.74%) was significantly higher (P<0.05) than other treatments that are 0% (51.25±1.25% and 61.88±1.36%), 1% (52.50±1.44% and 63.25±1.38%), 3% (51.25±1.25% and 61.63±1.48%), and 4% (50.00±2.04% and 60.63±2.29%) of honey supplementation in extender at 36 hours of storage until the end of the observation at 72 hours of incubation. According to the results of this study, it can be concluded that the 2% honey supplementation in extender is the best treatment to maintain sperm motility and viability for 72 hours of storage.

Key words: honey, motility, Pelung chicken, sperm viability

ABSTRAK

Tujuan penelitian ini adalah untuk mengetahui pengaruh suplementasi madu dalam pengencer ringer laktat-kuning telur dengan penambahan sodium dodesil sulfat 0,025% dan vitamin E (RLKTSE) 2% terhadap kualitas spermatozoa ayam pelung yang ipreservasi pada suhu 5° C selama 72 jam penyimpanan. Semen diambil dari tiga ekor ayam pelung setiap tiga hari sekali dengan metode dorsal-abdominal massage. Semen dibagi menjadi 5 kelompok perlakuan suplementasi madu yaitu 0% sebagai kontrol (RLKTSEM0), 1% (RLKTSEM1), 2% (RLKTSEM2), 3% (RLKTSEM3) dan 4% (RLKTSEM4). Semen cair tersebut diamati motilitas dan viabilitas spermatozoa setiap 12 jam. Rancangan Acak Lengkap dengan 4 ulangan digunakan dalam penelitian ini. Hasil penelitian menunjukkan bahwa motilitas dan viabilitas spermatozoa pada pengencer RLKTSE dengan suplementasi madu 2% (61,25±1,25% dan 71,50±0,74%) secara signifikan lebih tinggi (P<0,05) dibandingkan perlakuan 0% (51,25±1,25% dan 61,88%±1,36%), 1% (52,50±1,44% dan 63,25±1,38%), 3% (51,25±1,25% dan 61,63±1,48%), dan 4% (50,00±2,04% dan 60,63±2,29%) suplementasi madu pada 36 jam penyimpanan sampai akhir pengamatan. Dapat disimpulkan bahwa suplementasi madu 2% dalam pengencer merupakan perlakuan terbaik untuk menjaga motilitas dan viabilitas spermatozoa selama 72 jam penyimpanan.

Kata kunci: madu, motilitas, ayam Pelung, spermatozoa viabilitas

INTRODUCTION

Pelung chickens have great potential to be developed as broilers because these chickens are classified by their weight, and they are very useful for artificial insemination (AI) programs. Artificial insemination is a process that is used to accelerate the development of the population of a species. The quality of spermatozoa must be maintained as it greatly affects the success of AI. The sperm quality can be maintained by suppressing the level of spermatozoa metabolism through storage at low temperatures (2-5° C). During the storage process, spermatozoa will adapt to the shock of the cold by changing the order of fatty acid chains and proteins in the plasma membrane. This causes leakage or selectivity of the damaged plasma membrane which then allows ions such as calcium and other substrates to freely enter the cell membrane. Cryoprotectant, in addition to the extender, can minimize damage of the cell membrane due to the shock of the cold. There are two types of cryoprotectants: penetration agents (intracellular) and non-penetrating agents (extracellular) of plasma membranes (Lemma, 2011).

One ingredient that can be used as an alternative to cryoprotectant is honey. Honey contains carotenoids, phenolic acids, flavonoids, ascorbic acid, peroxidase, and catalase enzymes, which function as antioxidants (Sergiel *et al*., 2014). Honey also possesses antibacterial properties and contains high concentrations of D-glucose and D-fructose that act as sources of energy (Nayik *et al*., 2019). Honey has been used as a cryoprotectant and a source of fructose in the semen extender for cryopreservation in bulls as well (Yimer *et al*., 2015). Honey has been studied previously as a supplement for different aims like antioxidants, cryoprotectants, and as an energy source for the improvement of quality during the preservation of semen for turkeys (Sari *et al*., 2015), goat (Maidin *et al*., 2018), stallion (El-Sheshtawy *et al*., 2016), bull (El-Nattat *et al*., 2016), and also human (Fakhrildin *et al*., 2014).

Olayemi *et al*. (2011) stated that the addition of honey in egg yolk extender could increase the motility and viability of post-thawing buck spermatozoa. Based on research by Malik *et al*. (2017), the use of glycerol-substituted honey as a cryoprotectant in an egg yolk extender can increase the motility and viability of bovine

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spermatozoa. The addition of honey was also able to maintain the motility and viability of catfish spermatozoa for up to 48 hours (Rahardhianto *et al*., 2012).

Various studies on the benefits of honey in semen preservation for different species have been done, but there is not as much research on its use for chicken semen. Therefore, it is necessary to do research on the benefits of honey on the extenders of chicken semen. This study aims to know the effects of honey supplementation in lactate ringer-egg yolk extenders with 0.025% sodium dodecyl sulfate and 2% vitamin E addition (LREYSE) on the quality of Pelung chicken spermatozoa that is preserved at 5° C during 72 hours storage.

MATERIALS AND METODHS

The experiments were performed at the Teaching and Experimental Farm and the Poultry Production Laboratory, Animal Science Faculty, at Jenderal Soedirman University. Three Pelung Chickens aged about 1.5 years were used in this study and all of the male chickens were fed a commercial chicken diet (PT Mulia Harvest Agritech, Grobogan, Indonesia) consisting of 10-12% crude protein as much as 150 g/day. All chickens were given water ad libitum. Semen from all of the chickens was routinely collected once per day for three days with 4 replications.

Extender Preparation

The basic extender adopted from Hidayat *et al*. (2016) used in this research contained 90% lactate ringer (PT Widatra Bhakti, Pasuruan, Indonesia), 10% egg yolks, 0.025% sodium dodecyl sulfate (catalog number: 8.17034.1000, Merck KGaA, Germany), 2% vitamins E (tocopherol acetate, Healthylife), 1000 IU/mL penicillin-G Meiji (PT Meiji Indonesian, East Java, Indonesia), and 1 mg/mL streptomycin sulfate Meiji (PT Meiji Indonesian, East Java, Indonesia). Alas Roban Honey (Apiari Pramuka, Batang, Indonesia) was added to this basic extender with concentrations of 0% (control), 1%, 2%, 3%, and 4%. The solution homogenized with the styrer for 5 minutes and was then centrifuged at 3000 rpm for 10 minutes. Supernatants were used as the semen extender. Tris hidroxymethyl aminomethane (product code: 998660, Central Drug House Ltd., New Delhi, India) was added into the supernatant to reach a pH of 6.7.

Semen collection, Chilling, and Evaluation

Semen was collected from three male pelung chickens and repeated four times at three-day intervals using the massage method. Semen collection was done by massaging the lower part of the pubic bone until the male responded by producing the papillae. After the papillae appeared, the lower part of the pubic bone was pressed until the ejaculation reflex subsided. This was done to ensure the maximum amount of semen could be collected.

Collected semen was divided into 5 treatment groups based on honey concentration. All of the treatments were stored in the refrigerator for 72 hours at 5° C and every 12 hours were observed for sperm motility and viability. Evaluation of sperm motility was carried out by placing a drop of semen on object glass, which was then covered and observed with a 400× magnification microscope using the methodology of Malik *et al*. (2017). The motility percentage of spermatozoa was assessed from 0 to 100% subjectively. The sperm viability was evaluated with eosin-nigrosin staining. The eosin-nigrosin solution as defined by Arifiantini (2012) was prepared from 20 g nigrosin (catalog number: 1.15924.0025, Merck KGaA, Germany) and 1.5 g sodium citrate (catalog number: 1.06448.1000, Merck KGaA, Germany) and was mixed into 300 ml distilled water. This solution was stirred until dissolved and then was added to eosin yellow (catalog number: 1.15935.0100, Merck KGaA, Germany). The pH was adjusted to 7 using tris hidroxymethyl aminomethane (Central Drug House Ltd, New Delhi, India). The sperm viability was then evaluated by placing one drop of semen and two drops of eosin-nigrosin solution on object glass, and then observed by a microscope at 400x magnification. Uncolored sperm cells were used to calculate the percentage of sperm viability from a total number of 200.

Data Analysis

Sperm qualities including viability and motility were shown as means ± standard error (SE) for chilling semen incubation. One-way analysis of variance (ANOVA) was used to analyze the data and was followed by Duncan's Multiple Range Test to determine differences between the treatment means. Significant differences were statistically based on a probability of $P<0.05$.

RESULTS AND DISCUSSION

Based on the results of the study, motility of spermatozoa after dilution (0 hours) and 12 hours of storage showed all treatments were not significantly different ($P>0.05$). Sperm motility in LREYSEH2 was significantly higher ($P<0.05$) than LREYSEH3 and LREYSEH4, but was not significantly different ($P>0.05$) with LREYSEH0 and LREYSEH1 after 24 hours of incubation. At 36, 48, 60, and 72 hours, the sperm motility in the LREYSEH2 was significantly higher ($P<0.05$) than LREYSEH0, LREYSEH1, LREYSEH3, and LREYSEH4. Motility of spermatozoa in LREYSEH4 was significantly lower ($P<0.05$) than LREYSEH0 at 72 hours of storage (Table 1).

Table 2 showed no significant difference ($P>0.05$) on sperm viability between all groups until 12 hours of storage. After 24 hours, sperm viability in LREYSEH2 was significantly higher ($P<0.05$) than LREYSEH3 and LREYSEH4, but was not significantly different ($P>0.05$) with LREYSEH0 and LREYSEH1. Sperm viability in the LREYSEH2 was significantly higher ($P<0.05$) than LREYSEH0, LREYSEH1, LREYSEH3, and LREYSEH4 after 36, 48, 60, and 72 hours of

storage. At 72 hours, viability of spermatozoa in LREYSEH4 was significantly lower (P<0.05) than LREYSEH0.

In the present study, the sperm motility and viability of LREYSEH2 was significantly (P<0.05) higher than LREYSEH0, LREYSEH1, LREYSEH3, and LREYSEH4. Honey at 2% concentration might have maintained a nearly isotonic environment around the sperm and provided energy, as it is a rich source of fructose and glucose. These results are in line with the research of El-Nattat *et al*. (2016) who also obtained better post-thaw sperm motility and viability with honey at the rate of 2% as compared to 3%, 4%, and 5%. Based on Malik *et al*. (2019) research, the addition of 2% honey in the skim-egg yolk extender can also maintain the best motility of native chicken spermatozoa before freezing as compared to 0%, 4%, 6%, and 8% honey addition. Kandiel and Elkhawagah (2017) stated that the addition of 2% honey in skim milk extender can maintain the quality of buffalo spermatozoa after 2 hours with 15% difference out of the control.

Sperm motility and viability in LREYSEH3 and LREYSEH4 is significantly (P<0.05) lower than LREYSEH2. The decrease of sperm motility and viability post-chilling in LREYSEH3 and LREYSEH4 treatment may be caused by honey concentrations that are too high. The higher concentration of honey might induce a hyperosmotic extracellular and which then leads to sperm dehydration and disruption in plasma membrane integrity, resulting in the death of spermatozoa (Yimer *et al*., 2015). Furthermore, according to Maidin *et al.* (2018), the addition of 2% honey could inhibit the decrease in motility of goat spermatozoa up to 2 hours after semen collection. Honey can protect the cell of spermatozoa from damage during freezing by being an intracellular agent (Fakhrildin *et al*., 2014), but it can also be effective at low concentrations which might have maintained a nearly isotonic environment around the spermatozoa (Banday *et al*., 2017).

The results of the present study are not different from Yimer *et al*. (2015) study that showed the supplementation of 2.5% honey in tris extender can maintain the quality of bull's spermatozoa after chilling and thawing. Sari *et al*. (2015) reported that the addition of 3% honey in egg yolk phosphate extender can maintain the motility and viability of turkey spermatozoa for 36 hours at a storage of 5° C more so than 4% and 5% honey additions, while Olayemi *et al*. (2011) reported that the addition of 5% honey in egg yolk extender can maintain the quality of goat spermatozoa better than 10% and 20% honey additions. Honey can also be a substitute for glycerol as a cryoprotectant in egg yolk extenders to maintain the quality of spermatozoa of Bali cattle post-thawing (Malik *et al*., 2017).

Honey contains simple sugars such as disaccharides, monosaccharides, polysaccharides, and oligosaccharides. The spermatozoa use fructose and glucose as a source of energy (Rahardhianto *et al*., 2012). Honey has a lower activity because most of the water molecules bind to monosaccharides. These conditions can suppress microbial growth, which maintains the quality of spermatozoa (Kumar *et al.*, 2010).

Several antioxidant compounds in honey can increase sperm quality. These include: flavonoid, chrysin, vitamin C, pinobanksin, catalase, and pinocembrin (Ahmed and Othman, 2013; Erejuwa *et*

Table 1. Percentage of sperm motility (%) with honey supplementation in lactate ringer-egg yolk extender

Time (h)	Sperm motility (%)				
	LREYSEH0	LREYSEH1	LREYSEH2	LREYSEH3	LREYSEH4
0	82.50±1.44^{a}	83.75±1.25^{a}	83.75±1.25^{a}	81.25±1.25^{a}	81.25±1.25^{a}
12	73.75±2.39^{a}	75.00±2.04^{a}	76.25±2.39^{a}	73.75±1.25^{a}	72.50±1.44^{a}
24	65.00±2.04^{ab}	66.25±2.39^{ab}	70.00±2.04^{a}	62.50±1.44^{b}	62.50±1.44^{b}
36	51.25±1.25^{a}	52.50±1.44^{a}	61.25±1.25^{b}	51.25±1.25^{a}	50.00±2.04^{a}
48	41.25±1.25^{a}	42.50±1.44^{a}	51.25±2.39^{b}	40.00±2.04^{a}	37.50±1.44^{a}
60	25.00±2.04^{a}	26.25±2.39^{a}	35.00±2.04^{b}	26.25±1.25^{a}	22.50±1.44^{a}
72	6.25±1.25^{ab}	7.50±1.44^{b}	16.25±1.25^{c}	2.50±1.44^{ad}	1.25±1.25^{d}

$^{a, ab, ad, b, c, d}$Different superscripts in the same row indicate significant differences (P<0.05). LREYSEH0= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 0% honey, LREYSEH1= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 1% honey, LREYSEH2= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 2% honey, LREYSEH3= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 3% honey, LREYSEH4= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 4% honey

Table 2. Percentage of sperm viability (%) with honey supplementation in lactate ringer-egg yolk extender

Time (h)	Sperm viability (%)				
	LREYSEH0	LREYSEH1	LREYSEH2	LREYSEH3	LREYSEH4
0	93.00±0.65^{a}	92.75±0.66^{a}	92.88±0.85^{a}	91.25±0.52^{a}	91.88±0.83^{a}
12	84.25±1.85^{a}	83.88±1.98^{a}	85.75±1.55^{a}	82.63±0.77^{a}	81.75±1.23^{a}
24	74.63±2.02^{ab}	75.75±1.98^{ab}	79.13±1.65^{a}	72.50±1.24^{b}	73.13±0.90^{b}
36	61.88±1.36^{a}	63.25±1.38^{a}	71.50±0.74^{b}	61.63±1.48^{a}	60.63±2.29^{a}
48	52.13±0.85^{ab}	53.63±1.20^{a}	63.13±1.91^{c}	50.38±2.24^{ab}	48.00±1.24^{b}
60	35.00±2.28^{a}	37.13±2.27^{a}	45.13±2.06^{b}	36.50±1.14^{a}	33.13±1.70^{a}
72	15.63±1.01^{ab}	17.13±1.70^{b}	26.38±0.97^{c}	11.75±1.33^{ad}	10.88±1.60^{d}

$^{a, ab, ad, b, c, d}$Different superscripts in the same row indicate significant differences (P<0.05). LREYSEH0= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 0% honey, LREYSEH1= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 1% honey, LREYSEH2= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 2% honey, LREYSEH3= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 3% honey, LREYSEH4= Lactate ringer-egg yolk + 0.025% SDS + 2% vitamin E + 4% honey

al., 2014). The antioxidants in honey play a role in fighting oxidative stress/reactive oxygen species that can damage the cell (Erejuwa *et al*., 2012). Honey also has a high viscosity. Low temperatures will create a smaller surface tension, thereby preventing the formation of ice crystals in the sperm cytoplasm and ultimately reducing damage during cryopreservation (El-Sheshtawy *et al*., 2016).

Honey also possesses antibacterial properties that are attributed to the presence of bee defensin-1 and methylglyoxal (Kwakman and Zaat, 2012). This is due to the high content of sugars that are present and more importantly to the generation of hydrogen peroxide when diluted due to the conversion of glucose into hydrogen peroxide by glucose oxidase enzymes to reduce cell damage (Aurongzeb and Azim, 2011).

CONCLUSON

The supplementation of 2% honey in LREYSE extenders maintains sperm motility and viability better than the control and other concentrations of honey supplementation during 72 hours of storage at 5° C. It can therefore be concluded that the 2% honey supplementation is more suitable for preserving the quality of chilling Pelung chicken semen than other concentrations.

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