Jurnal Kedokteran Hewan
Indonesian Journal of Veterinary Sciences

p-ISSN : 1978-225X
e-ISSN : 2502-5600

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ISSN
1978-225X | 2502-5600

Jurnal Kedokteran Hewan
Indonesian Journal of Veterinary Sciences

p-ISSN : 1978-225X
e-ISSN : 2502-5600

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Jurnal Kedokteran Hewan - Indonesian Journal of Veterinary Sciences

Jurnal Kedokteran Hewan (J. Kedokt. Hewan) or Indonesian Journal of Veterinary Sciences is a scientific journal field of veterinary sciences published since 2007, published **FOUR** times a year in **March, June, September, and December** by Universitas Syiah Kuala (Syiah Kuala University) and Indonesian Veterinary Medical Association (PDHI).

Jurnal Kedokteran Hewan is a double-blind review process journal that has been **accredited** National Journal Accreditation (ARJUNA) managed by the Ministry of Research, and Technology of the Republic of Indonesia, with second grade (Sinta 2), **Number: 200 / M / KPTS / 2020**.

This journal has been registered in Indonesian Publication Index (IPI), Google Scholar, Sinta, World Cat, Directory of Open Access Journals (DOAJ), EBSCO, Copernicus, Microsoft Academic, and other scientific databases.

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Jurnal Kedokteran Hewan
Indonesian Journal of Veterinary Sciences
p-ISSN : 1978-225X
e-ISSN : 2502-5600
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Indexed by:

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Mobile Phone: +6281362693330
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ISSN
1978-225X | 2502-5600

Jurnal Kedokteran Hewan
P-ISSN : 1978-225X; E-ISSN : 2502-5600
September 2022, 16(3):110-114
DOI: https://doi.org/10.21157/j.ked.hewan.v16i3.24317

DATE EXTRACT SUPPLEMENTATION IN RINGER LACTATE-EGG YOLK EXTENDER ON POST-THAWING QUALITY OF PELUNG CHICKEN SPERMATOZOA

Nu'man Hidayat[1]*, Ismoyowati[1], Elly Tugiyanti[1], Imam Suswoyo[1], Rosidi[1], Ibnu Hari Sulistyawan[1], and Aras Prasetyo Nugroho[2]

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ABSTRACT

The research purpose was to evaluate the influence of date extract supplementation in lactate ringer-egg yolk extender with 0.025% sodium dodecyl sulfate (LREYS) on post-thawing quality of Pelung chicken spermatozoa. Completely randomized design with 5 treatments and 4 replications was used in this study. Semen was collected from three Pelung chickens once in three days using the dorsal-abdominal massage method. Semen was divided into 5 treatment groups of date extract supplementation that were 0% as control (LREYSDE0), 0.5% (LREYSDE1), 1% (LREYSDE2), 1.5% (LREYSDE3), and 2% (LREYSDE4). The post thawing liquid semen was observed for sperm motility, viability, plasma membrane integrity and acrosome integrity. The result showed that the motility, viability, membrane plasma integrity and acrosomal integrity of spermatozoa in LREYSE extender with 1.5% date extract supplementation (30.44±1.02%; 49.83±0.91%; 43.26±1.02%; and 45.33±0.72% respectively) was significantly higher (P<0.05) than others treatment that were 0% (18.21±0.43%; 35.62±0.51%; 30.54±0.60%; and 31.49±0.71% respectively), 0.5% (20.45±0.72%; 40.72±0.87%; 36.81±0.55%; and 38.56±0.63% respectively), 1% (25.68±0.93%; 46.27±0.75%; 40.52±1.02%; and 42.83±1.09% respectively) and 2% (22.55±0.92%; 43,71±0,74%; 38,84±0,71%; and 41,39±0,86% respectively) of date extract supplementation. It is concluded that the 1.5% date extract supplementation in extender can maintain the best post-thawing sperm quality.

Key words: date extract, extender, pelung chicken, post-thawing, sperm quality

ABSTRAK

Tujuan penelitian ini adalah untuk mengetahui pengaruh suplementasi ekstrak kurma dalam pengencer ringer laktat-kuning telur dengan penambahan sodium dodesil sulfat 0,025% (RLKTS) 2% terhadap kualitas spermatozoa ayam pelung post-thawing. Semen diambil dari tiga ekor ayam pelung setiap tiga hari sekali dengan metode dorsal-abdominal massage. Rancangan Acak Lengkap dengan 5 perlakuan dan 4 ulangan digunakan dalam penelitian ini Semen dibagi menjadi 5 kelompok perlakuan suplementasi ekstrak kurma 0% sebagai kontrol (RLKTSEK0); 0,5% (RLKTSEK1); 1% (RLKTSEK2); 1,5% (RLKTSEK3); dan 2% (RLKTSEK4). Semen cair tersebut diamati kualitasnya yang meliputi motilitas, viabilitas, integritas membran plasma dan integritas akrosom spermatozoa post-thawing. Hasil penelitian menunjukkan bahwa motilitas, viabilitas, integritas membran plasma dan integritas akrosom spermatozoa pada pengencer RLKTSE dengan suplementasi ekstrak kurma 1,5% (berturut-turut 30,44±1,02%; 49,83±0,91%; 43,26±1,02%; and 45,33±0,72%) secara signifikan lebih tinggi (P<0,05) dibandingkan perlakuan 0% (berturut-turut 18,21±0,43%; 35,62±0,51%; 30,54±0,60%; dan 31,49±0,71%); 0,5% (berturut-turut 20,45±0,72%; 40,72±0,87%; 36,81±0,55%; dan 38,56±0,63%); 1% (berturut-turut 25,68±0,93%; 46,27±0,75%; 40,52±1,02%; dan 42,83±1,09%); dan 2% (berturut-turut 22,55±0,92%; 43,71±0,74%; 38,84±0,71%; and 41,39±0,86%) suplementasi ektrak kurma. Dapat disimpulkan bahwa suplementasi ekstrak kurma 1,5% dalam pengencer dapat mempertahankan kualitas spermatozoa terbaik post-thawing.

Kata kunci: ekstrak kurma, pengencer semen, ayam pelung, post-thawing, kualitas spermatozoa

INTRODUCTION

Pelung chicken is a typical chicken from Cianjur, West Java which is classified into heavy type with an adult body weight of 52 weeks reaching 3.51±0.21 kg in males and 2.045±0.18 kg in females (Nataamijaya *et al*. 2003). The chicken has the potential to be used as a male to get offspring that have a high body weight. Artificial insemination (AI) is the right alternative for efficient use of superior males. Spermatozoa quality is very influential on the success of AI, so the quality must be maintained since semen collection. Spermatozoa quality can be maintained by suppressing the metabolic rate of spermatozoa through storage at low temperatures. During the storage process, spermatozoa will experience cold shock. Cold shock occurs due to changes in the order of fatty acid chains and proteins in the plasma membrane, causing leakage or damaged plasma membrane selectivity, which causes ions such as calcium ions and other substrates to freely enter the cell (Martinenaite and Tavenier 2010).

Effort that can be made to minimize damage to the plasma membrane of spermatozoa cells due to the influence of cold shock is by adding a cryoprotectant to the extender. There are two types of cryoprotectants, namely penetrating agents (intracellular) and non-penetrating agents (extracellular) of the plasma membrane (Lemma 2011). One of the extracellular cryoprotectants is a simple sugar that forms a balanced extracellular concentration by changing the osmotic gradient of the semen extender and allowing water in the spermatozoa plasma membrane to diffuse out (Fuller 2004). One of the materials that can be used as an alternative to cryoprotectants is dates. Dates are a source of energy for spermatozoa (Al Juhaimi *et al*. 2014) and function as antioxidants (Al Farsi *et al*. 2005) and antimicrobials (Taleb *et al*. 2016). The date extract supplementation in extender up to 1% can maintain the plasma membrane integrity of Aceh cattle spermatozoa (Dwitya *et al*. 2019).

Research on the benefits of dates in semen cryopreservation has been carried out and proven to be

Revised 25-07-2022, Accepted23-08-2022

able to maintain the quality of cattle spermatozoa, but there has been no research on chicken semen in maintaining the quality of spermatozoa during the freezing process. Therefore, it is necessary to do research on the benefits of dates in chicken semen extender during cryopreservation. This study aimed to determine the effect of adding date extract to ringer lactate-egg yolk extender with sodium dodecyl sulfate on post-thawing quality which includes motility, viability, acrosome integrity and plasma membrane integrity of pelung chicken spermatozoa.

MATERIALS AND METHODS

Animals

The experiment was performed in Teaching and Experimental Farm and the Poultry Production Laboratory, Animal Science Faculty, Jenderal Soedirman University. Three Pelung Chickens aged about 1.5 years old were used in this study and all of the cocks were fed 150 g/day of B-12L commercial chicken diet (PT. New Hope Indonesia) consisting of 16% crude protein. All chickens were given water ad libitum. Semen from all of the chickens was routinely collected once in three days with 4 replications.

Date Extraction

Date extract was obtained by the maceration extraction method of dry date palm powder in methanol solvent with a ratio of 1:2. Dates are dried and made into powder for the optimal extraction process. The maceration process was carried out for 2x24 hours at room temperature to avoid the compounds damage in the extract. During the extraction, stirring was carried out several times so that the contact between the sample and the solvent was evenly distributed. The obtained macerate was filtered and the filtrate was evaporated using a rotary evaporator to evaporate the solvent so that the extract is not damaged by high temperatures (Abdillah *et al*. 2017).

Extender Preparation

The basic extender adopted from Hidayat *et al*. (2016) used in this research contained 90% ringer lactate (PT Widatra Bhakti, Pasuruan, Indonesia), 10% egg yolks, 0.025% sodium dodecyl sulfate (catalog number: 8.17034.1000, Merck KGaA, Germany). Date extract (Ajwa, Madinah) was added in this basic extender with concentrations of 0% (control), 0.5%, 1%, 1.5% and 2%. The solution homogenized with the stirrer for 5 minutes and then centrifuged at 3000 rpm for 10 minutes. Supernatants were used as semen extender. Tris hidroxymethyl aminomethane (catalog number: 998660, Central Drug House Ltd., India) was added into the supernatant to reach pH of 7.

Semen Collection, Cryopreservation and Evaluation

Semen was collected from three pelung roosters and repeated four times at three-day intervals using the massage method. Semen collection was conducted by massaging the lower part of the pubic bone until the rooster responded by producing the papillae. After the papillae appeared, the lower part of the pubic bone was pressed until the ejaculation reflex disappeared so the semen maximally comes out.

Collected semen was divided into 5 treatment groups based on date extract concentration. Semen used for cryopreservation must have a standard quality of motility 70%, sperm concentration $800x10^6$ cells mL^{-1}, and abnormalities <15%. Semen was collected and mixed with the 5 groups of extenders that had been made. Each semen mixture was packaged using a mini straw (0.25 mL) with a concentration of 100 million motile spermatozoa and then equilibrated in a refrigerator at a temperature of about 5° C for 4 hours (Eriani *et al*. 2017). Prefreezing was conducted by placing the straws in styrofoam containing liquid nitrogen at a position of 2 cm above liquid nitrogen for 15 minutes and then freezing process was carried out by placing the straws in a liquid nitrogen container with a temperature of -196° C.

The frozen semen was thawed after 7 days in liquid nitrogen by placing the straws in a water bath containing warm water at 37° C for 30 seconds, and then the motility, viability, acrosome integrity and plasma membrane integrity of the spermatozoa were evaluated.

Spermatozoa Motility Assessment

Spermatozoa motility was evaluated under a microscope at 400x magnification by adding 1 drop of fresh semen plus 8-10 drops of physiological NaCl. The motility value was determined based on the percentage of the number of progressively moving spermatozoa from 200 cells of the total number of spermatozoa present from five fields of view.

Spermatozoa Viability Assessment

Sperm staining was carried out by mixing 1 drop of semen with 2 drops of eosin-nigrosin staining solution on the object glass. The eosin-nigrosin staining solution was prepared by mixing 20 g of nigrosin (catalog number: 1.15924.0025, Merck KGaA, Germany) with 1.5 g of trisodium citrate dihydrate (catalog number: 1.06448.1000, Merck KGaA, Germany) in 300 mL of distilled water. The solution was stirred and warmed until dissolved, then 3.3 g of eosin yellow (catalog number: 1.15935.0100, Merck KGaA, Germany) was added and adjusted the pH to 7 using tris hidroxymethyl aminomethane (catalog number: 998660, Central Drug House Ltd., India). The stained sperm was observed under a microscope with a magnification of 400x. Spermatozoa that do not absorb color are spermatozoa that are still alive, on the other hand, spermatozoa that absorb color are spermatozoa that are dead.

Acrosome Integrity Assessment

A hundred (100) µL of semen was mixed with 500 µL of 1% formal citrate solution {2.79% trisodium citrate dihydrate (catalog number: 1.06448.1000, Merck KGaA, Germany) and 0.37% formaldehyde

solution 37% (ACS, Reagent of Analysis) in distilled water} on the object glass. Acrosomes of 200 spermatozoa cells were observed microscopically (1000x magnification) after placing immersion oil (catalog number: 1.04699.0500, Merck KGaA, Germany). Acrosome integrity was determined by the presence of a typical normal ridge.

Plasma Membrane Integrity Assessment

Hypo-osmotic swelling test (HOST) was used to assess the integrity of the spermatozoa plasma membrane. A total of 50 µL of semen was mixed with 300 µL of hypo-osmotic solution {2.04% fructose (catalog number: 1.04007.1000, Merck KGaA, Germany) and 0.71% trisodium citrate dihydrate (catalog number: 1.06448.1000, Merck KGaA, Germany) in distilled water} at 37° C for 60 minutes. A total of 100 µL of the mixture was dropped on an object glass and covered with a cover glass, then 200 spermatozoa cells were observed under a microscope with a magnification of 1000 times. Spermatozoa with intact plasma membranes had circular tails, while spermatozoa with damaged plasma membranes had straight tails.

Data Analysis

Post-thawing qualities including motility, viability, acrosome integrity and plasma membrane integrity of pelung chicken spermatozoa were showed as means ± standard error (SE). One-way analysis of variance (ANOVA) was used to analyze the data and followed by Duncan's Multiple Range Test to determine differences between the treatments. Significant differences were statistically based on probability of $P<0.05$.

RESULTS AND DISCUSSION

Fresh Semen Characteristic of Pelung Chicken

Based on the results of the study (Table 1), the average volume of pelung chicken semen was 0.45±0.17 mL. This result was higher than the result of study by Wiyanti *et al*. (2013) where the volume of pelung chicken semen was only 0.14 mL. The result of microscopic evaluation showed that the concentration of spermatozoa (3.25±0.16 $x10^9$ cells mL^{-1}) was almost similar with the previous study (Hidayat *et al*. 2016) which amounted to 3.18±0.04 $x10^9$ cells mL^{-1}. Spermatozoa concentration is very important to determine the dose of artificial insemination. The motility of the pelung chicken spermatozoa in this study (82.12±1.32%) was still in the normal range, in accordance with Dumpala *et al*. (2006) which stated that normal spermatozoa motility was more than 70%. Spermatozoa motility is one of the factors that influence the success of artificial insemination. The spermatozoa viability of pelung chickens in this study was 92.45±1.53%, almost similar to the results of research on native chickens of 92.5±2.37% (Wiyanti *et al*. 2013). The average spermatozoa abnormality of the present research was 9.51±0.47% lower than the previous study by Widya *et al*. (2013) which reached 15.5%. This difference in semen quality results can be caused by individual differences and chicken age, environmental temperature, feed, and frequency of semen storage. This is in accordance with the opinion of Froman and Kirby (2008) which stated that semen quality is influenced by nation, individual, age, body size, feed nutrition, and frequency of semen storage.

Effect of Date Extract Supplementation in Extender on Post-Thawing Spermatozoa Quality

Table 2 showed that the treatment of date extract supplementation in semen extender was significantly different ($P<0.05$) on the quality of post-thawing spermatozoa (motility, viability, plasma membrane integrity, and acrosome integrity).

The results showed that the motility and integrity of the plasma membrane of spermatozoa in the extender LREYSDE3 (30.44±1.02% and 43.26±1.02%) were significantly higher ($P<0.05$) than LREYSDE2 (25, 68±0.93% and 40.52±1.02%), while LREYSDE2 was significantly higher ($P<0.05$) than LREYSDE1 (20.45±0.72% and 36.81±0.55%) and LREYSDE4 (22.55±0.92% and 38.84±0.71%). In addition, spermatozoa viability in the extender LREYSDE3

Table 1. Fresh semen characteristic of pelung chicken

Characteristics	Means ± standard error
Volume (mL)	0.45±0.17
Spermatozoa concentration ($x10^9$ sel mL^{-1})	3.25±0.16
Spermatozoa motility (%)	82.12±1.32
Spermatozoa viability (%)	92.45±1.53
Spermatozoa abnormality (%)	9.51±0.47

Table 2. Sperm quality post-thawing with date extract supplementation in lactate ringer-egg yolk extender

Parameters	LREYSDE0	LREYSDE 1	LREYSDE 2	LREYSDE 3	LREYSDE4
Motility (%)	18.21±0.43[a]	20.45±0.72[b]	25.68±0.93[c]	30.44±1.02[d]	22.55±0.92[b]
Viability (%)	35.62±0.51[a]	40.72±0.87[b]	46.27±0.75[c]	49.83±0.91[d]	43.71±0.74[bc]
PMI (%)	30.54±0.60[a]	36.81±0.55[b]	40.52±1.02[c]	43.26±1.02[d]	38.84±0.71[b]
AI (%)	31.49±0.71[a]	38.56±0.63[b]	42.83±1.09[c]	45.33±0.72[d]	41.39±0.86[c]

[a, b, bc, c, d]Different superscripts in the same row indicate significant differences ($P<0.05$). LREYSDE0= 90% lactate ringer + 10% egg yolk + 0.025% sodium dodecyl sulphate + 0% date extract, LREYSDE1= 90% lactate ringer + 10% egg yolk + 0.025% sodium dodecyl sulphate + 0,5% date extract, LREYSDE2= 90% lactate ringer + 10% egg yolk + 0.025% sodium dodecyl sulphate + 1% date extract, LREYSDE3= 90% lactate ringer + 10% egg yolk + 0.025% sodium dodecyl sulphate + 1,5% date extract, LREYSDE4= 90% lactate ringer + 10% egg yolk + 0.025% sodium dodecyl sulphate + 2% date extract, PMI= Plasma membrane integrity, AI= Akrosome integrity

(49.83±0.91%) was also significantly higher (P<0.05) than LREYSDE2 (46.27±0.75%), while LREYSDE2 was significantly higher (P<0.05) compared to LREYSDE1 (40.72±0.87%). The similar trend was also observed at the acrosomal integrity variable of spermatozoa. The acrosome integrity of spermatozoa in the extender LREYSDE3 (45.33±0.72%) was significantly higher (P<0.05) than LREYSDE2 (42.83±1.09%) and LREYSDE4 (41.39±0.86%), while LREYSDE2 and LREYSDE4 were significantly higher (P<0.05) than LREYSDE1 (38.56±0.63%). The acrosome plays an important role in the fertilization process. The initiation of the bonding of spermatozoa with the zona pellucida will trigger the acrosome reaction and cause the release and activation of acrosome enzymes, thus the spermatozoa are able to penetrate the zona pellucida (Miranda *et al*. 2009). Spermatozoa acrosome hood is a part that plays an important role in the fertilization process as a carrier of enzymes and genetic material. The acrosome is needed by spermatozoa in the fertilization process, where the nucleic acid of the spermatozoa is combined with the oocyte membrane (Susilawati 2017).

Supplementation of 1.5% date extract in semen extender resulted in the best motility, viability, plasma membrane integrity, and acrosome integrity of spermatozoa. These results are in line with the research by *Dwitya et al*. (2019) which stated that the addition of date extract in an extender of up to 1% resulted in a higher value of spermatozoa plasma membrane integrity than the control. This is due to the sucrose, glucose, and fructose content in dates which act as an energy source for spermatozoa (Al Juhaimi *et al*. 2014) and as extracellular cryoprotectant that can protect the plasma membrane from damage during cryopreservation (Mukminat *et al*. 2014). In addition, dates also contain high concentrations of antioxidants (Al Farsi *et al*. 2005) so that they can help protect plasma membrane phospholipids (Hu *et al*. 2014). Ajwa Al Madinah variety dates used in this study were date varieties with flavonoid content of 2.78 mg/100 g (Al Juhaimi *et al*. 2014). Flavonoids act as antioxidants and protect the body against reactive oxygen species (ROS). Flavonoids are oxidized by radicals, producing radicals that are more stable and less reactive, so that flavonoids can stabilize reactive oxygen species by reacting with radical reactive compounds. Flavonoids can capture free radicals directly through the donation of hydrogen atoms (Arifin and Ibrahim 2018). The mechanism of free radical prevention carried out by flavonoids can be divided into three, namely: slowing down the formation of ROS, breaking down ROS, and regulating or protecting with antioxidants. Flavonoids have a hydroxy group that plays an important role in the process of breaking down ROS, namely the hydroxy group contained in the B ring of flavonoids (Alfaridz and Amalia 2018). Increased reactive oxygen species can cause abnormalities in spermatozoa morphology, damage to the plasma membrane, decreased motility, decreased viability and ability to fertilize (Twigg and Fulton 2008).

The post thawing motility value decreased drastically, which was 52-54% of the motility of fresh semen. This result is in line with the opinion of Ozkavukcu *et al*. (2008) which stated that the decrease in spermatozoa motility after freezing ranged from 24-64%. The semen freezing process can reduce motility between 30% and 60%, changes in spermatozoa morphology, mitochondrial damage and acrosomal damage (Kim *et al*. 2013). The decrease in quality can be caused by the damage to the plasma membrane of spermatozoa during the semen freezing process. Membrane damage will affect the motility of spermatozoa. This is due to the plasma membrane as a place of exchange of substances and ions is needed for spermatozoa metabolism to produce energy for the movement of spermatozoa (Storey 2008). Eriani *et al*. (2017) reported that cryopreservation significantly reduces spermatozoa motility. The integrity of the plasma membrane is one of the determinants of semen quality because the fluid properties and flexibility of the sperm membrane are needed to assist the movement of the spermatozoa flagella (Tran *et al*. 2017). The plasma membrane of sperm has unsaturated fatty acids which are very susceptible to cryopreservation damage. Mitochondria, the energy-producing organelles for the movement of spermatozoa are the most sensitive to cellular stress induced by semen processing (Leon *et al*. 2004). According to Ferrusola *et al*. (2008), the destruction of mitochondrial structure during cryopreservation correlated with decreased post-thawing spermatozoa motility.

CONCLUSION

The supplementation of 1,5% date extract in LREYSDE extender maintain post-thawing qualities including motility, viability, plasma membrane integrity, and acrosome integrity of pelung chiken spermatozoa better than control and other concentration of date extract supplementation. It is therefore concluded that the 1.5% date extract supplementation in extender is recommended for cryopreservation of pelung chicken semen.

ACKNOWLEDGEMENT

The research was supported by BLU UNSOED (Kept. 1072/UN23/HK.02/2021). The sincerest gratitude is attended to the Head of Research Centre and Community Service of Jenderal Soedirman University for beginner lecturer research program.

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