Ovozoa 12 No. 1, April 2023 p-ISSN 2302-6464 e-ISSN 2722-967X
Available at https://e-journal.unair.ac.id/OVZ/index

Original article

The effect of addition of melon (*Cucumis melo* L.) flesh juice into Lactated Ringer's-egg yolk extender on spermatozoa plasma membrane integrity and spermatozoa morphological abnormalities of semen of native rooster

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Open access under CC BY – SA license, DOI: 10.20473/ovz.v12i1.2023.49-58
Received June 16 2022, Revised March 25 2023, Accepted April 4 2022
Published online April 2023

ABSTRACT

This study aims to determine the effect of adding melon (*Cucumis melo* L.) juice to Lactated Ringer's-egg yolk extender on the intact plasma membrane (IPM) and morphological abnormalities of native rooster spermatozoa. Five ejaculate each from two native roosters (*Gallus gallus domesticus*) were divided into four equal volumes. Ejaculate was extended 1:10 (v/v) in Lactated Ringer's-egg yolk without or with the addition of of 2, 4, and 8% (v/v) melon flesh juice, respectively for groups T0, T1, T2, and T3. Extended semen was stored in a refrigerator (5°C) and evaluated every two hours. The results showed that IPM of all groups decreased ($p < 0.05$) consistently, lasting more than 40% for six hours in the T0 and T1 groups and four hours in the T2 and T3 groups. The percentage of spermatozoa morphological abnormalities in all groups stored for six hours was higher ($p < 0.05$) than before storage. However, it was not significantly different ($p > 0.05$) from that which had been stored for two and four hours. It was concluded that native rooster semen extended in Lactated Ringer's-egg yolk without (group T0) or with 2% melon flesh juice (group T1) and stored at 5°C retained spermatozoa plasma membrane integrity of more than 40% and morphological abnormalities of less than 20% for six hours. While the addition of 4 % (T2) and 8% (T3) melon flesh juice maintained the percentage of IPM and spermatozoa morphological abnormalities for only four hours.

Keywords: melon flesh juice, morphological abnormality, native rooster, spermatozoa membrane integrity

INTRODUCTION

Artificial insemination in poultry aimed to improve genetic quality, productivity, and reproductive efficiency (Kharayat *et al*., 2016; Malik *et al*., 2018). The application of artificial insemination in roosters was highly dependent on the rooster strain and age, extender used,

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insemination dose, semen quality, semen deposition, and time of insemination. Artificial insemination of roosters usually used fresh semen with or without extender (Getachew, 2016).

A good extender had to be non-toxic to spermatozoa and able to provide energy substances for spermatozoa as well as other materials as buffers and prevent cold shock (Najafi *et al*., 2022). In prolonged storage of rooster semen, spermatozoa damage often occurred due to the respiration process in the spermatozoa mitochondria which produced free radicals (Zong *et al*., 2023). The cell plasma membrane was the part most easily damaged by free radicals. This was because the plasma membrane was composed of phospholipids and glycolipids which contained unsaturated fatty acids. Free radicals took electrons from unsaturated fatty acids (Martemucci *et al*., 2022). This process destroyed all phospholipids in the plasma membrane of spermatozoa. Damage to the plasma membrane of spermatozoa could interfere with the active transfer of substances which were a source of energy for spermatozoa, so the livability of spermatozoa would decrease (Partyka and Niżański., 2021).

Lipid peroxidation could be overcome by protecting the spermatozoa membrane with antioxidants. The antioxidants are abundant in melon (*Cucumis melo* L.) flesh (Kowalczyk, 2021). Melon flesh juice could better maintain the quality of spermatozoa, partly because melon flesh juice contains vitamin C (Malathi *et al*., 2021). Superior melon varieties grown in Indonesia are Sunrise meta, Orange meta, Sky rocket, Action 434, Mai 119, and Apollo varieties. Among the six varieties of melon, it was reported that the concentrations of ascorbic acids (vitamin C) in Sky Rocket melon was 30.84 ± 0.31 mg/100 g (Evana and Barek, 2021). Vitamin C was an antioxidant that could break free radical reaction chains. Vitamin C could strengthen the plasma membrane's stability against lipid peroxides, thereby maintaining the quality and fertility of semen (Akbari *et al*., 2016). Melon flesh juice also contained carbohydrates (Mallek-Ayadi *et al*., 2022), which are thought to be used as an energy source for spermatozoa. The study of the utilization of antioxidant potential of melon flesh juice for preserving native rooster semen has not yet been published. Therefore, this study aims to determine the effect of melon (*Cucumis melo* L.) flesh juice in Lactated Ringer's-egg yolk extender on the integrity of the plasma membrane and morphological abnormalities of native rooster spermatozoa.

MATERIALS AND METHODS

The study was conducted at the Laboratory of Artificial Insemination, Faculty of Veterinary Medicine, Universitas Airlangga. This study has been approved by the Examining Commission including the implementation of animal ethics during the study based on Number 164/UN3.1.6/2021.

Experimental Animals

Two mature and healthy native roosters (*Gallus gallus domesticus*) aged 1 and 1.5 years, weighing 1.9 and 2.3 kg were adapted for seven days to the cage environment before semen collection. Roosters were fed pellet Boiler 2 twice daily, and water was given ad libitum. Healthy native roosters met the criteria for semen collection, namely the cloaca area and its surroundings were red, the distance between the cloaca and the two ends of the pelvic bones, and the distance between the two ends of the pelvis was greater, the tail feathers were long, the skin turgor was good, the eyes are sharp, and shiny fur, as well as a good body posture (McGary *et al*., 2003).

Extender

Sky Rocket melon was obtained from organic farming at the Furusato Gardens in Bumiaji, Batu City, East Java, Indonesia. This melon plantation is located at the foot of Mount Arjuno, at an altitude of 900-1200 m above sea level, 7°44'– 8°26' South Latitude, and 122°17'–122°57" East Longitude. The average

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temperature was 15-19 °C with humidity around 75-98%, and an average rainfall volume of 298 mm per month in the range of 6 days per month (https://furusatoindonesia.com/). Fresh melon flesh was cut into a size of about 4x4x4 cm, blended, then filtered twice to obtained fresh juice. Melon flesh juice was prepared fresh before the addition to the extender.

Lactated Ringer's-egg yolk extender was prepared by mixing 80 mL of Lactated Ringer's solution (PT Kimia Farma - Persero, Tbk. Plant Bandung) and 20 mL of egg yolk (laboratory use egg, CV Redjo, Surabaya), homogenized using a magnetic stirrer for 10 minutes, and centrifuged at a speed of 3000 x g for 15 minutes. The extender was added with penicillin 1000 IU/mL extender and streptomycin 1 mg/mL extender and stirred until homogeneous (Azzam *et al*., 2022).

Semen collection

The cloaca of the rooster was cleaned using tissue paper moistened with 70% alcohol, then rooster was massaged on the dorsal side to the base of the tail (Azzam *et al*., 2022). Semen was collected twice a week (Monday and Thursday) to obtained five ejaculates per rooster as replicates.

Each ejaculate was evaluated by macroscopic examination (smell, color, volume, thickness, and pH of semen) and microscopic examination (semen concentration, percentage of viable spermatozoa, percentage of abnormal spermatozoa, mass movement, and individual movement) (Azzam *et al*., 2022). Each ejaculate was divided into four equal volumes for each treatment groups. Extenders for all groups were Lactated Ringer's-egg yolk but for groups T1, T2, and T3, 0.04, 0.08, and 0.16 mL of fresh melon juice were added to Lactated Ringer's - egg yolk up to 2 mL. Fresh semen was added to the extenders at a ratio of 1:10 (v/v). Subsequently, the extended semen was stored in the refrigerator (5°C).

Examination of variables

Spermatozoa concentration in each ejaculate was measured with a spectrophotometer (Thermo Fisher Scientific, USA). Examination of the integrity of the spermatozoa plasma membrane and morphological abnormalities were conducted before and after storage in the refrigerator. Sample examination was carried out every two hours until the percentage of spermatozoa motility reached 40% (Danang *et al*., 2012). Examination of the integrity of the spermatozoa plasma membrane was carried using a hypoosmotic solution. The hypoosmotic solution was prepared from 0.565 grams of fructose and 0.31 grams of sodium citrate added with distilled water up to 50 mL. Sample of 0.1 mL was added to 1 mL hypoosmotic solution, incubated at 37°C for 30 minutes, then dripped onto an object glass, covered, and observed under a light microscope (Olympus BX-53) with 400x magnification. Spermatozoa with intact plasma membranes have a coiled or bent tails, while spermatozoa with damaged plasma membranes have straight tails (Silyukova *et al*., 2022).

Spermatozoa morphology was examined by eosin-nigrosin staining. The preparations were then observed under a light microscope (Olympus BX-53) with 400x magnification. Two hundred spermatozoa were assessed and the percentage of morphological abnormalities was calculated (Azzam *et al*., 2022) with the criteria including no tail, abnormal head shape, abnormal tail shape, and the presence of proximal or distal cytoplasmic droplets in the tail (Feyisa *et al*., 2018).

Data analysis

Data were analyzed using the Multivariate Analyses of Variance (MANOVA), followed by Tukey's honestly significant difference (HSD) test at a significant level of 5%. Data were analyzed using Windows's Statistical Product and Service Solutions (SPSS) version 20.

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RESULTS

The results indicated the macroscopic and microscopic qualifications of fresh native rooster semen, as shown in Table 1. Storage at 5°C caused a decrease in spermatozoa motility and reaches 40% in six hours. Storage for six hours at cold temperature (5°C) in all groups showed a consistent decrease ($p < 0.05$) in plasma membrane integrity at two hour intervals. Plasma membrane integrity of the T0 and T1 groups was maintained by more than 40% on storage for up to six hours. However, in the T2 and T3 groups, plasma membrane integrity remained more than 40%, surviving only up to four hours of storage. Before cold storage (0 h), plasma membrane integrity was the highest in the control group, decreased ($p < 0.05$) in the T1 and T2 groups, and increased again in the T3 group so that it was not significantly different ($p > 0.05$) from T0 group. After two hours of cold storage, there was no significant difference ($p > 0.05$) in plasma membrane integrity between groups. Examination of plasma membrane integrity after four and six hours of cold storage showed the highest plasma membrane integrity was found in group T0 and the lowest in group T3 ($p < 0.05$) (Table 2).

Table 1 Macroscopic and microscopic parameters of fresh native rooster semen

macroscopic		microscopic	
volume (mL)	0.64 ± 0.11	mass motility	+++
smell	specific	individual motility (%)	87.00 ± 2.00
color	creamy white	concentration (million/mL)	1068 ± 360.99
pH	7	viability (%)	88.80 ± 2.28
consistency	thick	morphological abnormality (%)	5.60 ± 0.96

Table 2 Percentage of native rooster spermatozoa with intact plasma membranes after cold storage at 5°C

	0 h	2 h	4 h	6 h
T0	73.60 ± 3.04 ^{Aab}	64.00 ± 2.00 ^{B}	56.40 ± 1.14 ^{Ca}	42.60 ± 3.64 ^{Da}
T1	69.60 ± 1.14 ^{Ab}	60.40 ± 2.70 ^{B}	53.60 ± 2.88 ^{Ca}	42.20 ± 3.42 ^{Da}
T2	70.00 ± 5.29 ^{Ab}	57.20 ± 5.89 ^{B}	45.00 ± 6.00 ^{Cb}	38.80 ± 1.78 ^{Da}
T3	76.00 ± 1.41 ^{Aa}	59.20 ± 6.68 ^{B}	44.40 ± 1.51 ^{Cb}	32.20 ± 2.86 ^{Db}

Different lowercase superscripts in the same column and different uppercase superscripts in the same row indicated significant differences ($p < 0,05$); T0, T1, T2, and T3: native rooster semen extended in Lactated Ringer's-egg yolk without or with the addition of 2, 4, and 8% melon flesh juice respectively.

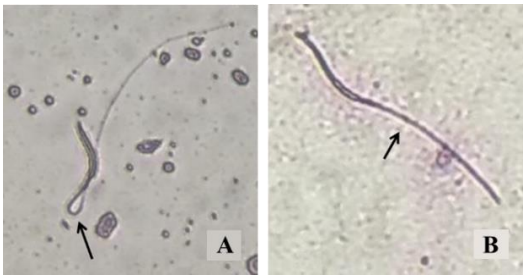

Figure 1 Plasma membrane integrity of native rooster spermatozoa after storage at 5°C under a light microscope (Olympus BX-53) at 400x magnification; A: spermatozoa with intact plasma membranes (coiled tails coiled); B: spermatozoa with damaged plasma membranes (straight tails).

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There was no significant difference (p >0.05) in the percentage of abnormal spermatozoa morphology between the treatment groups (T0, T1, T2, and T3) before cold storage (0 h) or after storage for 2, 4, and 6 hours. Storage of semen at cold temperatures for six hours resulted in a higher percentage of spermatozoa morphological abnormalities (p <0.05) than before cold storage, but not significantly different (p >0.05) from storage for two and four hours (Table 3).

Table 3 Percentage of morphological abnormality of native rooster spermatozoa after storage in cold storage (5°C)

	0 h	2 h	4 h	6 h
T0	7.90 ± 1.88 ^{B}	10.30 ± 2.61 ^{AB}	12.10 ± 2.60 ^{AB}	13.50 ± 2.20 ^{A}
T1	8.90 ± 1.81 ^{B}	11.00 ± 2.31 ^{AB}	13.00 ± 1.69 ^{AB}	14.20 ± 2.61 ^{A}
T2	10.00 ± 1.41 ^{B}	11.70 ± 2.43 ^{AB}	14.70 ± 2.43 ^{AB}	15.50 ± 1.83 ^{A}
T3	10.40 ± 1.47 ^{B}	12.30 ± 2.51 ^{AB}	14.70 ± 2.01 ^{AB}	15.90 ± 1.47 ^{A}

Different uppercase superscripts in the same rows indicated significant differences (p <0,05). There was no significant difference (p >0.05) between the data in the same columns; T0, T1, T2, and T3: native rooster semen extended in Lactated Ringer's-egg yolk without or with the addition of 2, 4, and 8% melon flesh juice, respectively.

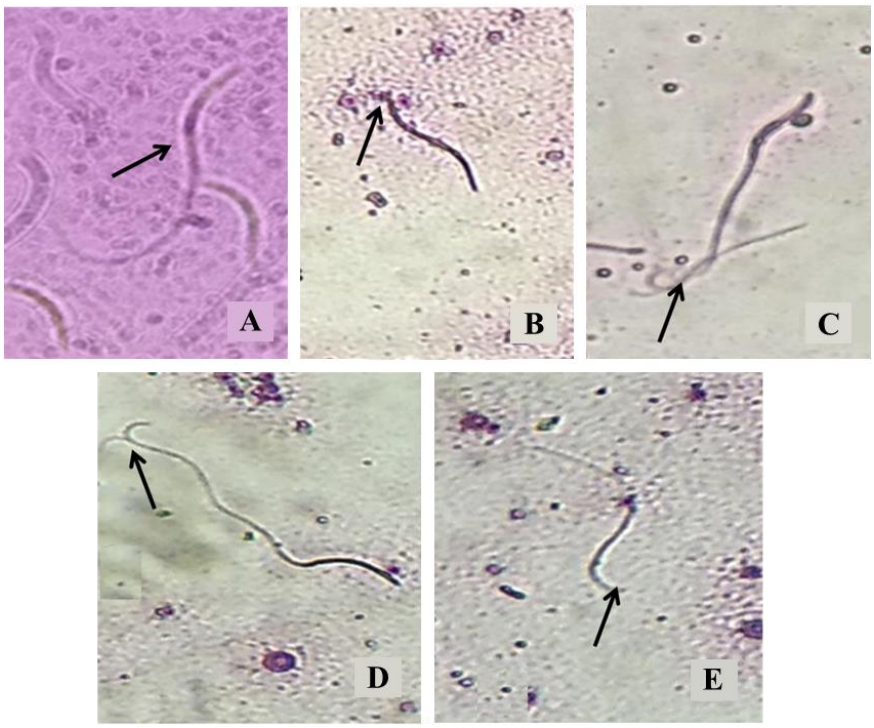

Figure 2 Morphological abnormality of native rooster spermatozoa after storage at 5°C under a light microscope (Olympus BX-53) with 400x magnification; A: spermatozoa with normal morphology; B: morphological abnormality of the spermatozoa head; C: spermatozoa with a coiled tail, D: spermatozoa with branched tail, E: headless spermatozoa.

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DISCUSSION

Semen quality of roosters varied in volume, spermatozoa motility, spermatozoa concentration, pH, and color (Mussa *et al*., 2023). Meanwhile, variations in spermatozoa morphological defects of were associated with spermatozoa viability, motility, fertility, and hatchability (Feyisa *et al*., 2018). The ejaculate volume of native roosters in this study (0.64 ± 0.11 mL) was the same as in the study by Azzam *et al*. (2022) (0.64 ± 0.13 mL); higher than the ejaculate volume of rooster in the study by Woli *et al*. (2017) (0.47 mL), Sentul rooster (0.15 mL) and Nunukan rooster (0.32 mL) in Junaedi and Husnaeni's study (2019), and Bungo rooster (0.16-0.21 mL) in the province of Bengkulu, Sumatra (Putranto *et al*., 2020). Differences in rooster semen volume were influenced by genetic factor (Hermiz *et al*., 2016), age and climatic condition (Shanmugam *et al*., 2014). The concentration of spermatozoa in fresh ejaculate in this study (1,068 ± 360.99 million/mL) was lower than the concentration of spermatozoa in the study by Azzam *et al*. (2022) (1,272 ± 262.91 million/mL), Woli *et al*. (2017) (1,369.7 million/mL), Sentul roosters (3.002 million/mL) and Pelung roosters (5.043 million/mL) in the study by Junaedi and Husnaeni, (2019), and in the study by Putranto *et al*. (2020) (6,658-7,458 million/mL). Differences in the concentration of spermatozoa among roosters' ejaculates were influenced by genetic factors (Hermiz *et al*., 2016), roosters' age and environmental climate (Shanmugam *et al*., 2014). The percentage of live spermatozoa in fresh semen in this study (88.80 ± 2.28%) was higher than that of Nunukan rooster (86.29%) in the study by Junaedi and Husnaeni (2019), and lower than the percentage of live spermatozoa in the study by Woli *et al*. (2017) (99.1%), in Bangkok rooster (90.64%) in the study by Junaedi and Husnaeni (2019), and in the study by Azzam *et al*. (2022) (94.4 ± 134%). The motility of spermatozoa of native rooster in this study (87.00 ± 2.00%) was in the range of 87.60 ± 1.67% (Azzam *et al*., 2022), 75-88% (Putranto *et al*., 2020); lower than that in the study by Woli *et al*. (2017) (99.12%), and higher than that of Nunukan rooster (77.64%) and Pelung rooster (84.69%) in the study by Junaedi and Husnaeni (2019). The morphological abnormality of fresh semen of native rooster in this study (5.60 ± 0.96%) was lower than 7-8% (Putranto *et al*., 2020).

Spermatozoa with an intact plasma membrane would withstand the osmolality of the fluid in the cell so that the tail looked coiled. Meanwhile, spermatozoa with straight tails indicated that the plasma membrane has been damaged because it could not hold the fluid that entered the cell (Najafi *et al*., 2022). The integrity of the spermatozoa plasma membrane of all groups decreased consistently after six hours of storage at cold temperature. The percentage of intact spermatozoa plasma membrane decreased after cooling due to extender polarity. During cooling, the atoms or molecules making up the membrane experienced depolarization which could reduce the physiological function of the membrane (Najafi *et al*., 2022).

The percentage of spermatozoa with intact plasma membrane was maintained at 40% for six hours in Lactated Ringer's-egg yolk extender without and with 2% melon flesh juice and for four hours in Lactated Ringer's-egg yolk extender with 4% and 8% melon flesh juice. Without the addition of melon juice in the extender (T0) spermatozoa plasma integrity was maintained from free radicals by endogenous antioxidants. There are several endogenous antioxidants in rooster semen, i.e., non-enzyme antioxidants which included vitamin C and vitamin E, as well as enzyme antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase (Partyka and Niżańskiet, 2021). Vitamin C in melon juice could reduce free radicals in rooster spermatozoa. Vitamin C could strengthen the stability of the plasma membrane against peroxidation during the cold storage of semen. Direct contact of spermatozoa with oxygen could cause death (Silvestre *et al*., 2021). Vitamin C could also bind oxygen

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radicals in spermatozoa, prevent the formation of lipid peroxidation, and maintained glycolysis and spermatozoa motility (Meščić Macan *et al*., 2019). The percentage of spermatozoa with intact plasma membrane in the Lactated Ringer's-egg yolk with 8% melon flesh juice was the lowest among the groups. This decrease might be caused by the low pH of the extender. The low pH was caused by the large amount of vitamin C in the melon juice. Vitamin C is acidic, so it has to be used in relatively small doses because excessive doses could kill spermatozoa. Rapid changes in the external environment were dynamically regulated by ion channels of spermatozoa. pH played a vital role in spermatozoa viability and motility. A pH that was too-acidic caused decreased motility and even death of spermatozoa (Mishra *et al*., 2018).

The decrease in the percentage of intact spermatozoa plasma membrane in Lactated Ringer's-egg yolk with 8% melon flesh juice could also be caused by the content of too many doses of antioxidants. Higher antioxidant could affect the rate of oxidation, which caused the antioxidant activity to disappear, and even excessive antioxidants can become pro-oxidants. In healthy semen, there was a balance of pro-oxidants and antioxidants (Agarwal *et al*., 2014). Pro-oxidants initiated, facilitated, and accelerated the oxidation of spermatozoa membrane lipid (Sotler *et al*., 2019). Increased lipid oxidation in the spermatozoa plasma membrane produced malondialdehyde compounds which were toxic to spermatozoa, causing damage to the spermatozoa membrane. Damaged spermatozoa membranes would cause a decrease in the integrity of the spermatozoa membrane which in turn caused a decrease in the quality of spermatozoa (Mussa *et al*., 2023).

The most common spermatozoa abnormalities found in this study were secondary abnormalities (curled tail, broken tail, and separate head and tail). Cold shock could cause spermatozoa damage resulting in spermatozoa morphological abnormalities (Zong *et al*., 2023; Ardhani *et al*., 2019). The highest percentage was found at six hours in Lactated Ringer's-egg yolk with 8% melon flesh juice. Morphological abnormalities of rooster spermatozoa that exceeded 20% were rarely used in the implementation of artificial insemination. Semen with morphological abnormalities of 20% or more reduced fertility (Yaman *et al*., 2022).

Spermatozoa morphological abnormalities before cold storage and after storage for two, four, and six hours were lower than 20%, and qualified for artificial insemination. This result was better than that reported by Kusumawati *et al*. (2020) that spermatozoa morphological abnormalities of fresh native and Arab roosters was feasible for artificial insemination for up to four hours and three hours when stored at 5°C without an extender (Kusumawati *et al*., 2019). Spermatozoa morphological abnormalities in Lactated Ringer's-egg yolk extender with or without the addition of melon flesh juice were similar. These results were different from the report of Tabatabaei (2012) that the percentage of morphological defects of spermatozoa in modified Ringer's solution supplemented with 1% ascorbic acid was significantly lower than the control. Higher doses of vitamin C in the extender for cold storage of native rooster semen were followed by lower pH, lower spermatozoa viability, and higher spermatozoa morphological abnormalities (Asmawarati *et al*., 2010). The percentage of spermatozoa with morphological abnormalities in semen stored at 5°C for six hours was higher than before cold storage. This may be due to insufficient spermatozoa metabolism during liquid storage at low temperatures. Previous study reported that cold storage of rooster semen was followed by an increase in morphological abnormalities, reduced spermatozoa motility and fertility (Vašíček and Chrenek, 2013). In this case, there was no significant difference between T0 to T2 during 6h of storage while T3 resulted in a significantly lower plasma membrane integrity at 6 h. This strongly indicated that the inclusion of 8% melon flesh juice in the extender was detrimental to rooster spermatozoa.

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CONCLUSION

Regarding the fulfillment of rooster semen quality for artificial insemination, plasma membrane integrity must be more than 40% and morphological abnormalities must be less than 20%. In this study, native rooster semen extended in Lactated Ringer's-egg yolk without or with 2% melon flesh juice maintained the quality for six hours in storage at 5°C. While the addition of 4 and 8% melon flesh juice only lasted for four hours.

ACKNOWLEDGEMENT

The authors thank Dini Salvida, Lathifa Surya Mirron, Nabila Ayu Sarasvati for their technical support in this study.

AUTHORS' CONTRIBUTIONS

Dinda Galuh Pitaloka (DGP), Mas'ud Hariadi (MH), Eka Pramyrtha Hestianah (EPH), Samuel Inioluwa Akeju (SIA), Suherni Susilowati (SS), Budi Utomo (BU), Tatik Hernawati (TH). DGP, MH and EPH conceived the proposal of the study. DGP collected-analyze data, and drafted the manuscript. SIA, SS, BU and TH had revised for substantial content. All of the authors agreed the final draft.

CONFLICTS OF INTEREST

The authors declare that they have no conflict interests.

FUNDING INFORMATION

This study was funded by the authors.

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How to cite this article: Pitaloka DG, Hariadi M, Hestianah EP, Akeju SI, Susilowati S, Utomo B, Hernawati T. 2023. The effect of addition of melon (*Cucumis melo* L.) flesh juice into Lactated Ringer's-egg yolk extender on spermatozoa plasma membrane integrity and spermatozoa morphological abnormalities of semen of native rooster. Ovozoa: Journal of Animal Reproduction. 12: 49-58.

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