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Original article

The addition of duck egg yolk to skim milk-fructose extender maintained the motility and viability of Muscovy duck (*Cairina moschata*) spermatozoa at 5°C storage

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ABSTRACT

This study aims to determine the effect of adding duck egg yolk to skim milk-fructose extender on the motility and viability of Muscovy duck (*Cairina moschata*) spermatozoa at 5°C storage. Muscovy duck ejaculate was divided into four equal portions. The first portion was for the control group, which was extended in skim milk-fructose, while the second, third, and fourth portions were for treatment groups T1, T2, and T3, which were extended in skim milk-fructose with the addition of 2.5, 5.0, and 7.5% (v/v) duck egg yolk. Sperm motility and viability were evaluated every two hours until the motility percentage reached 40%. Data were analyzed using ANOVA and Duncan's test. The results showed that spermatozoa motility and viability were higher ($p < 0.05$) in groups T1, T2, and T3 compared to those of group T0 after 10 hours storage at 5°C. The highest percentage of spermatozoa motility and viability was in the T2 group compared to other groups ($p < 0.05$). It could be concluded that the Muscovy duck semen extended in skim milk-fructose containing 2.5 to 7.5% (v/v) duck egg yolk met the requirements for artificial insemination when stored for up to 10 hours at 5°C.

Keywords: duck egg yolk, motility, Muscovy duck, skim milk, viability

INTRODUCTION

Muscovy ducks are one of the meat ducks that have the largest body size compared to other types of ducks, so they have great potential as a source of meat (Fitasari *et al*., 2022). In addition, Muscovy ducks have better meat quality with lower fat content than other ducks (Ismoyowati *et al*., 2012; Kokoszyński *et al*., 2020). However, Muscovy duck farming faces several obstacles, including low population and low productivity. The application of artificial insemination is expected to increase the productivity and genetic quality of Muscovy ducks. The artificial insemination technique allows the use of one ejaculate from a superior male Muscovy duck to be added with an extender, and stored for a certain period of time to inseminate several

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female Muscovy ducks (Fitasari *et al*., 2022).

Extenders function as an energy source for spermatozoa, as a protective agent against cold shock, as a buffer when changes in pH occur, maintaining osmotic pressure, increasing volume, balancing electrolytes, and preventing the growth of germs (Rizkallah *et al*., 2022). Muscovy duck spermatozoa will die quickly if stored without extender (Blesbois and Brillard, 2007). In this study, skim milk and fructose extender were used with the addition of various concentrations of duck egg yolk. Skim milk can provide energy for spermatozoa, but it needs to be supplemented with other ingredients as protective agents. Skim milk contains protein and energy sources that can be used to maintain spermatozoa viability during storage (Hoesni, 2016; Khaeruddin *et al*., 2020). Fructose can be used as an extender because it is an energy source that supports spermatozoa motility. Fructose was chosen as an extender because semen plasma biochemically contains various certain organic compounds, one of which is fructose (Tsujii *et al*., 2006).

Egg yolk added to semen extender functions as a medium to provide an energy source, and extracellular protection for spermatozoa. Egg yolk contains lecithin and lipoprotein which can protect and maintain the integrity of the protein layer on the spermatozoa cell membrane (Widiastuti *et al*, 2018). The egg yolk that is commonly used as an extender is chicken egg yolk. However, semen quality in extender supplemented with duck egg yolk was better than that supplemented with chicken egg yolk (Clulow *et al*., 2007). Storing semen at a temperature of 5°C is expected to inhibit the metabolic rate of spermatozoa so that spermatozoa can survive longer (Azzam *et al*., 2022). Based on the description above, this study aims to determine the effect of using skim milk and fructose with the addition of duck egg yolk in various concentrations as an extender on the motility and viability of Muscovy duck spermatozoa at 5°C storage.

MATERIALS AND METHODS

The sample used in this study was collected from one male Muscovy duck that was approximately one year old, had a healthy appearance, normal genitals, and good sexual libido. Female Muscovy duck was used as teasers. Male and female Muscovy ducks were kept in separate cages to increase their libido.

Semen collection

Female duck was put into male duck cage to stimulate the male duck's libido. The male duck will try to mate with the female Muscovy duck by pecking at the female's head while trying to mount the female, but was not allowed to copulate. After the male duck tried to mate with the female duck three times, the semen was collected by massaging the back near the base of the tail, then the semen was collected in a beaker (Figure 1). Semen collection was carried out five times as replicates.

Figure 1 Muscovy duck penis in semen sample collection

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The ejaculate was examined macroscopically and microscopically before the semen was extended. Macroscopic examination included evaluation of semen volume, smell, colour, consistency, and pH. Microscopic examination of semen included examination for spermatozoa concentration, mass movement, and individual movement, as well as viability.

Extender

The extender was made from a mixture of 10 grams of powdered skim milk with distilled water up to a volume of 100 ml, heated in a water bath until the temperature reached 92-95°C for 10 minutes, then cooled slowly to room temperature (20-23°C). Fructose (7.5 grams) was added to the skim milk solution and then stirred homogeneously. Fresh duck egg was cleaned with 70% alcohol cotton, the eggshell was cracked, the egg white was completely removed, the whole egg yolk in the vitelline membrane was transferred onto filter paper to remove the remaining egg white, and the vitelline membrane was discarded. The groups in this study were T0 (control), T1, T2, and T3, each of which was extended in skim milk-fructose extender, without and with 2.5, 5, and 7.5% (v/v) duck egg yolk. Penicillin and Steptomycin, 1000 IU and 1 mg respectively were added for each mL of extender and mixed homogenously.

Each ejaculate was divided into four equal portions, and extended in each group's extender at a 1:10 ratio (semen/extender). Extended semen was stored at 5°C and assessed for spermatozoa motility and viability every two hours until the motility percentage reached 40%.

Evaluation of spermatozoa motility and viability

A drop of extended semen was placed on an object glass, covered, and examined under a light microscope (Nikon Eclipse E100 LED) with 400x magnification (Gitayana *et al*., 2023). The percentage of motility was calculation was calculated by counting spermatozoa that move progressively in three fields of view and then averaging them.

The percentage of viable spermatozoa was calculated using eosin nigrosin staining. One drop of eosin nigrosin and one drop of semen were mixed on a clean glass slide, smeared and flame dried. Examination was performed under a light microscope (Nikon Eclipse E100 LED) at 400x magnification. Dead spermatozoa appeared purplish red and live spermatozoa appeared transparent (Gitayana *et al*., 2023). Live and dead spermatozoa were counted from a minimum of one hundred spermatozoa from five fields of view.

Data analysis

Data were analyzed using ANOVA followed by the Duncan's multiple range test at a significance level of 5% using SPSS for Windows version 23.0.

RESULTS

The fresh semen of Muscovy duck used in this study had a specific smell, milky white colour, thick consistency, and normal pH (pH= 7), plus-three mass movements, and progressive individual movements. The quantitative characteristics of Muscovy duck ejaculate were shown in Table 1.

Table 1 Quantitative characteristics of fresh Muscovy duck semen

	range	average
volume (mL)	0.70-1.10	0.94 ± 0.18
concentration (10^6/mL)	1080-1560	1284 ± 253.14
viability (%)	92.00-95.00	93.4 ± 1.14
motility (%)	85.00-90.00	88.00 ± 2.74

The percentage of spermatozoa motility decreased consistently during the storage period. In the T0 group, spermatozoa motility was the lowest ($p < 0.05$) compared to the other groups. The addition of duck egg yolk in groups T1, T2, and T3 showed an increase in spermatozoa motility ($p < 0.05$) compared to group T0 stored for 8 and 10 hours. The highest percentage of

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spermatozoa motility ($p < 0.05$) was found in the T2 group compared to other groups. In groups T1, T2, and T3, the percentage of motility was maintained at more than 40% at storage for up to 10 hours (Figure 2).

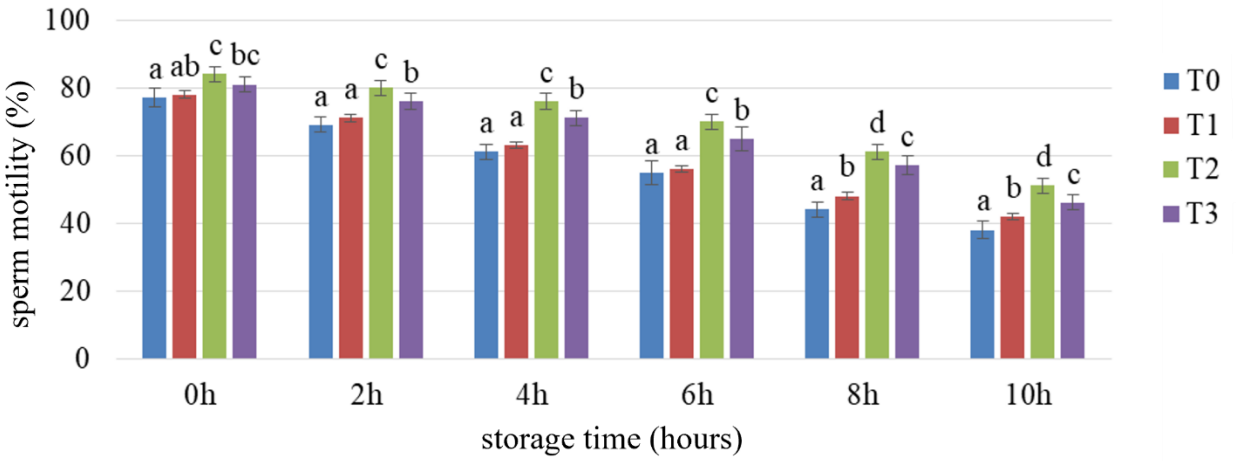

Figure 2 Muscovy duck spermatozoa motility at 5°C storage; T0: Muscovy duck semen in skim milk-fructose extender; T1, T2, and T3: Muscovy duck semen in skim milk-fructose extender contains 2.5, 5, and 7.5% (v/v) duck egg yolk; different superscripts in the same cluster show significant differences ($p < 0.05$).

The results of the spermatozoa viability examination showed live and dead spermatozoa (Figure 3). The percentage of spermatozoa viability decreased consistently during the storage period. In the T0 group, spermatozoa viability was the lowest ($p < 0.05$) compared to the other groups. The addition of duck egg yolk in groups T1, T2, and T3 showed an increase in spermatozoa viability ($p < 0.05$) compared to group T0 stored for 8 and 10 hours. The highest percentage of spermatozoa viability ($p < 0.05$) was in the T2 group compared to the other groups (Figure 4).

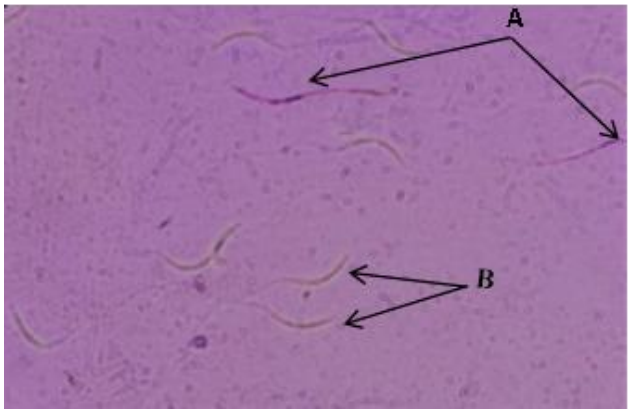

Figure 3 Results of the viability examination of Muscovy duck spermatozoa after treatment; A: dead spermatozoa appear red-headed; B: live spermatozoa appear to have transparent heads; magnification 400 times.

DISCUSSION

Poultry semen volume is influenced by age, breed, and the amount of fluid consumed (Mussa *et al*., 2023). The semen volume obtained in this study was considered normal because the volume of fresh Muscovy duck semen is 0.5-2.0 mL (Chen *et al*., 2016). Fresh semen in this study had a distinctive Muscovy duck smell. This smell indicates that the semen is in normal condition and there is no contamination. Semen in normal conditions generally has a distinctive smell that matches the smell of the animal (Fitriani and Sugiarti, 2019).

The colour of the semen obtained in this study was milky white with a thick consistency. The white colour mixed with reddish and brownish indicated that the semen was

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contaminated with blood or there was an infection in the genitals. The pH of semen in this study was 7, in accordance with the pH of semen obtained in the study of Etuk *et al.* (2006) which ranged from 7.0 to 7.53. The percentage of viability of Muscovy duck spermatozoa obtained in this study ranged from 92-95%, higher than the results reported by Fitriani and Sugiarti (2019), i.e. 70%. The mass movement of Muscovy duck spermatozoa in this study showed a three-plus value, which meant that the mass movement of semen formed large, fast, and numerous waves. The motility percentage ranged 85-90% with progressive individual movements. Fresh semen must have a minimum motility value of 80% so that it can be processed further for artificial insemination (Chen *et al.*, 2016).

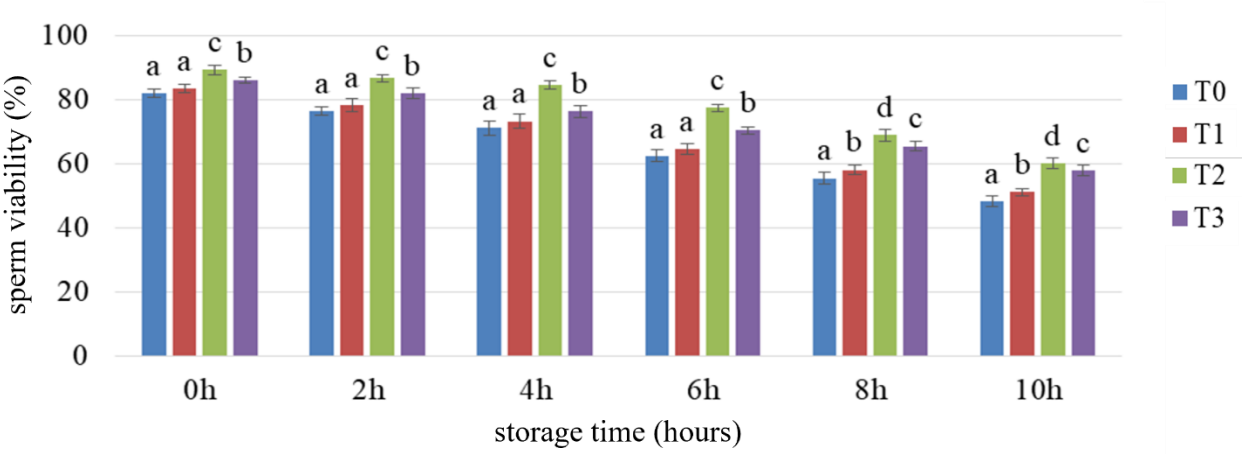

Figure 4 Muscovy duck spermatozoa viability at 5°C storage; T0: Muscovy duck semen in skim milk-fructose extender; T1, T2, and T3: Muscovy duck semen in skim milk-fructose extender contains 2.5, 5, and 7.5% (v/v) duck egg yolk respectively; different superscripts in the same cluster show significant differences ($p < 0.05$).

The motility and viability of spermatozoa from the 0 hour of observation to the 10th hour of observation showed a decrease. The highest spermatozoa motility and viability occurred at the beginning of the examination, due to the availability of energy sources needed for the survival of spermatozoa. Spermatozoa motility and viability decreased because the energy sources used were increasingly depleted (Sushadi *et al.*, 2023). The percentage of live spermatozoa decreased due to the large amount of lactic acid resulting from anaerobic fructose metabolism which was toxic to spermatozoa (Rochmi and Sofyan, 2019).

Spermatozoa motility

Spermatozoa motility is one of the criteria for determining semen quality. Spermatozoa motility describe the number of spermatozoa that move progressively to reach the fertilization site. Spermatozoa motility is used as a measure of the ability of spermatozoa to fertilize an egg (Fitriani, 2019). Spermatozoa motility gradually decreased with storage. Even though semen was stored at cold temperatures (3-5°C), spermatozoa metabolism continued so that energy supply became increasingly limited, and spermatozoa motility decreased (Haq *et al.*, 2020).

This study used a skim milk-fructose extender. Skim milk functioned as a protein buffer and contained hydrophilic lactose to protect spermatozoa membranes from cold shock (Bustani and Baiee, 2021). Fructose is an energy source that supports spermatozoa motility (Tsujii *et al.*, 2006). Egg yolk contained nutrients such as protein, fat, vitamins, minerals, and carbohydrates which could be used as an energy source to maintain the viability of spermatozoa. In addition, egg yolk contains lecithin and lipoprotein which could protect and

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maintain the integrity of the protein layer on the spermatozoa cell membrane (Bustani and Baiee, 2021).

The percentage of spermatozoa motility in the group added 5% and 7.5% (v/v) egg yolk was higher than the control group (without the addition of egg yolk) and in the group added only 2.5% (v/v) egg yolk. Egg yolk contains glucose which is an energy source for spermatozoa motility (Bustani and Baiee, 2021). The energy available in extenders derived from 5 and 7.5% (v/v) duck egg yolk wass higher than in extenders without or with 2.5% (v/v) egg yolk. In addition, the concentration of duck egg yolk which contained higher levels of lipoprotein and lecithin was able to protect the spermatozoa plasma membrane during storage. The plasma membrane was the entrance for substances from outside into the cell, or vice versa, so that if the spermatozoa plasma membrane was damaged, metabolism would be disrupted. This could cause a decrease in spermatozoa motility (Quraini, 2021).

An extender with the addition of 5% (v/v) duck egg yolk is optimal for maintaining Muscovy duck spermatozoa motility at a temperature of 5°C. Optimal egg yolk concentration would be useful for maintaining the quality of spermatozoa because duck egg yolk contained lecithin and lipoprotein for protecting spermatozoa. Apart from that duck egg yolk also contained an energy source that spermatozoa could use to move actively forward. Egg yolk functioned as a nutrition provider, energy source, and protector for spermatozoa (Widiastuti *et al*., 2018). The addition of 7.5% (v/v) egg yolk caused the extender to become thicker, thereby inhibiting spermatozoa motility.

Spermatozoa viability

Live spermatozoa have an intact lipoid layer on their cell walls, therefore when stained with eosin negrosin spermatozoa did not absorb the dyes, while dead spermatozoa experience damage to the spermatozoa plasma membrane, so dyes easily entered the spermatozoa (Gitayana et al., 2023). Muscovy duck semen stored at cold temperature without the addition of egg yolk and with the addition of 2.5% (v/v) duck egg yolk showed lower spermatozoa viability compared to those added with 5% and 7.5% (v/v) egg yolk. Without addition or adding only 2.5% egg yolk, the availability of nutrients to maintain viability was lower than when adding 5% and 7.5% (v/v) egg yolk. In addition, the lipoprotein and lecithin content played a role in maintaining the integrity of the plasma membrane (Bustani and Baiee, 2021). Intact plasma membrane was necessary for spermatozoa to survive (Wysokińska and Szablicka, 2021). Extender with skim milk and fructose with the addition of 5% (v/v) egg yolk showed the highest percentage of spermatozoa viability. This is in accordance with the results of research by Ibrahim *et al*. (2012) where the addition of duck egg yolk with a concentration of 5% (v/v) to skim milk extender was able to maintain optimal viability compared to the addition of 2.5% and 7.5% (v/v) duck egg yolk. The addition of 7.5% (v/v) duck egg yolk caused the extender to become thicker which could disrupt osmolarity thereby reducing the viability of spermatozoa (Ananda *et al*., 2023).

CONCLUSION

The addition of duck egg yolk to skim milk-fructose extender maintained the percentage of Muscovy duck spermatozoa motility and viability. The addition of 5% duck egg yolk was the optimal treatment to maintain the percentage of motility and viability of Muscovy duck spermatozoa stored at 5°C.

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AUTHOR'S CONTRIBUTIONS

Isti Annisa Ramahtia (IAR), Tatik Hernawati (TH), Djoko Legowo (DL), Tri Wahyu Suprayogi (TWS), Tjuk Imam Restiadi (TIR), Samuel Inioluwa Akeju (SIA).

IAR, TH, DL: conceived the idea, designed the mainframe of this manuscript, acquisition, analysis, and interpretation of data, and

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manuscript drafting. TWS, TIR and SIA: critically read and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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