

## Combination of 5% Dextrose Ringer's solution and egg yolk extender maintained the motility and viability of kampung rooster spermatozoa in chilled temperature

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### ABSTRACT

This study aims to determine the motility and viability of kampung rooster (*Gallus domesticus*) spermatozoa in 5% Dextrose Ringer's solution as an extender with or without the addition of various egg yolk concentrations at 5°C during storage. Semen was collected by massage in the morning. In this study, four types of extenders were used, i.e., 5% Dextrose Ringer's extender without egg yolk, 5% Dextrose Ringer's extender with 5, 7.5, and 10% egg yolk for T0, T1, T2, and T3, respectively. Then, 1000 IU Penicillin and 1 mg Streptomycin were added per mL extender. Fresh semen from each rooster was evenly divided into four volumes to be diluted 11 times in each group extender. Extended semen from all groups was stored at 5°C. Data were analyzed using ANOVA and Duncan's new multiple range test. The result showed a significant difference ( $p < 0.05$ ) between each treatment group for spermatozoa motility and viability. In conclusion, this study revealed that the addition of various concentrations of egg yolk into 5% Dextrose Ringer's solution as an extender maintained the motility and viability of kampung rooster spermatozoa.

**Keywords:** chilled temperature, kampung rooster, Dextrose Ringer's, spermatozoa motility, viability

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### INTRODUCTION

Kampung rooster (*Gallus domesticus*) is native free-range rooster in Indonesia that is easy to care for, tolerant of diseases, and easy to adapt to the environment and food (Prayogi, 2011). Kampung rooster as an organic farm product is popular with the public because its meat and eggs have a distinctive taste compared to purebred chicken, even though the price on the market is higher. Raising kampung chicken has a dual-purpose, i.e., meat and eggs for

consumption (Aedah *et al.*, 2016). However, there were problems with low productivity with free ranging and natural mating systems (Hadi *et al.*, 2021). The application of the artificial insemination (AI) technique was expected to increase the productivity and genetic quality of kampung chicken (Kharayat *et al.*, 2016).

In applying AI techniques, it is necessary to preserve semen. Fresh rooster semen only survived in the range of 4-7 hours at room temperature (Sutiyono *et al.*, 2021). The addition of extender and storage at cold temperatures was

expected to prolong the eligibility of semen for AI for a longer time. Semen extender could maintain spermatozoa metabolism, pH, controlled bacterial contamination, and reduced damage due to lipid peroxidation during storage. Thus, semen extender could maintain the life of spermatozoa and fertility (Bustani and Baiee, 2021). Egg yolk contains lipoproteins and lecithin as extracellular cryoprotectant ingredients that protect the extracellular spermatozoa from cold shock (Anand *et al.* 2017). Semen damage due to cold shock can be reduced by using diluents containing lecithin and lipoproteins (Widiastuti *et al.*, 2018). Egg yolk-based semen extenders usually used a concentration of 5% (v/v) in 10% (w/v) skim-milk solution in distilled water (Susilowati *et al.*, 2021). Simple carbohydrate such as 5% Dextrose Ringer's solution could be added to meet the energy needs of spermatozoa. Semen storage at 5°C was expected to reduce the metabolic rate of spermatozoa so that spermatozoa could survive longer. Therefore, this study aims to determine the use of various concentrations of egg yolk in 5% Dextrose Ringer's as an extender of the motility and viability of kampung rooster spermatozoa at 5°C storage.

## MATERIALS AND METHODS

The research sample used was semen collected from two, one year old kampung roosters weighing approximately two kilograms. Semen was collected in the morning with back massage technique. Ejaculate was collected into a microfuge tube.

### Semen extender

Fresh chicken eggs for laboratory use (produced by CV. Redjo, Surabaya, Indonesia) were cleaned with 70% alcohol cotton ball, then the shells were broken. Then the egg white liquid was removed, while the whole egg yolk enclosed in vitelline membrane were transferred to a filter paper to remove the remaining egg white liquid. The vitelline membrane was removed, and the egg yolk was poured into a measuring glass. Four types of extenders were used in this study, i.e., 5% Dextrose Ringer's (PT. Widatra Bhakti, Pasuruan, Jawa Timur, Indonesia) extender without egg yolk, 5% Dextrose Ringer's

extender with 5, 7.5, and 10% egg yolk (Hernawati and Safitri, 2020), respectively for T0, T1, T2, and T3. Each semen extender was added with 1000 IU Penicillin and 1 mg Streptomycin per mL. Fresh semen from each rooster was divided evenly into four volumes to be diluted 11 times in each group extender. Extended semen of all groups was stored at 5°C.

### Measurement of spermatozoa motility and viability variables

Semen quality was evaluated every two hours until the percentage of motility reached 40% (minimum percentage of spermatozoa motility for AI). Spermatozoa motility and viability were assessed with three replicates and then averaged.

### Motility of spermatozoa

Semen sample was dripped onto an object glass and then covered with a cover slip. Progressive spermatozoa motility was observed on 100 spermatozoa under a light microscope (Olympus BX-53) with 400x magnification (Retta *et al.*, 2022).

### Viability of spermatozoa

Sample semen was dripped onto an object glass, added with eosin nigrosin, homogenized, smeared, and dried over the flame quickly. The slides were examined under a light microscope with a magnification of 400x. Spermatozoa with transparent heads were counted as live spermatozoa, while spermatozoa with reddish heads were counted as dead (Retta *et al.*, 2022) (Figure 1). Viability count was performed on 100 spermatozoa within 5 different fields.

### Data analysis

The data obtained were analyzed using ANOVA. The results of the significantly different analysis were further analyzed using Duncan's New Multiple Range Test using SPSS (Statistical Product and Service Solutions) software for Windows version 20.

## RESULTS

Examination of fresh kampung rooster semen included volume, odor, color, pH, and consistency. The fresh semen of a kampung

rooster was milky white in color, had a characteristic smell, pH of 7.35, thick consistency, and a mass movement of three plus. Quantitative parameters of fresh kampung rooster semen are shown in Table 1.

**Table 1** Quantitative parameters of kampung rooster fresh semen

volume (ml)	0.64 ± 0.13
concentration (million/mL)	1272 ± 262.91
progressive motility (%)	87.6 ± 1.67
viability (%)	94.4 ± 1.34

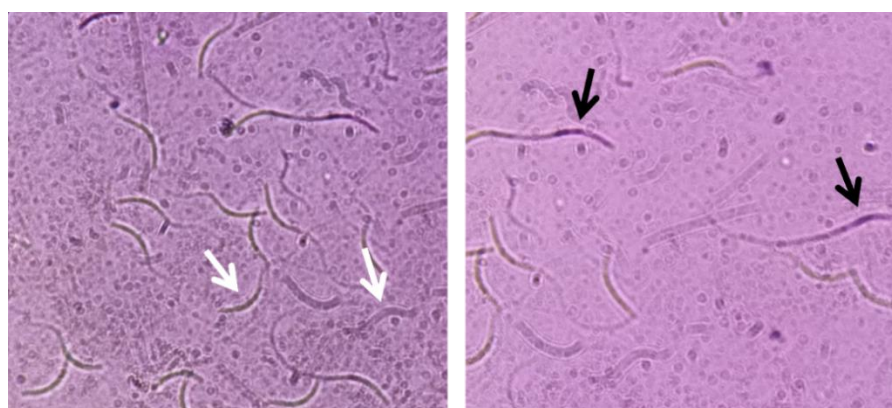
Spermatozoa in the control group (T0, without the addition of egg yolk), showed the

lowest spermatozoa motility and viability ( $p < 0.05$ ). Spermatozoa motility in the control group (T0) decreased ( $p < 0.05$ ) to less than 40% on storage for ten hours at 5°C. In the T2 and T3 groups, spermatozoa viability was not significantly different ( $p > 0.05$ ) between 0 and 2 hours of storage. However, there was a decrease ( $p < 0.05$ ) in all groups during ten hours of storage. The addition of egg yolk increased ( $p < 0.05$ ) spermatozoa motility and viability at 0, 2, 4, 6, 8, and 10 hours of storage. The addition of 7.5% egg yolk was the optimum dose to obtain the best spermatozoa motility and viability on storage up to the tenth hour.

**Table 2** Spermatozoa motility of kampung rooster semen stored in 5% Dextrose Ringer’s extender with and without egg yolk at 5°C

	T0	T1	T2	T3
0 hours	77.20 ± 1.79 <sup>Aa</sup>	79.80 ± 0.83 <sup>Ab</sup>	81.80 ± 1.64 <sup>Ab</sup>	84.00 ± 2.00 <sup>Ac</sup>
2 hours	70.80 ± 1.48 <sup>Ba</sup>	74.20 ± 1.92 <sup>Bb</sup>	77.00 ± 1.58 <sup>Bc</sup>	80.20 ± 1.30 <sup>Bd</sup>
4 hours	64.00 ± 2.12 <sup>Ca</sup>	67.00 ± 1.41 <sup>Cb</sup>	70.60 ± 1.67 <sup>Cc</sup>	77.00 ± 0.70 <sup>Cd</sup>
6 hours	52.00 ± 1.22 <sup>Da</sup>	60.60 ± 0.54 <sup>Db</sup>	63.20 ± 0.44 <sup>Dc</sup>	69.20 ± 0.83 <sup>Dd</sup>
8 hours	47.20 ± 1.09 <sup>Ea</sup>	51.20 ± 1.30 <sup>Eb</sup>	55.60 ± 1.34 <sup>Ec</sup>	60.00 ± 1.73 <sup>Ed</sup>
10 hours	39.20 ± 1.48 <sup>Fa</sup>	42.20 ± 1.64 <sup>Fb</sup>	47.40 ± 1.67 <sup>Fc</sup>	49.60 ± 1.81 <sup>Fc</sup>

Different uppercase superscripts in the same row, and lowercase superscripts in the same column were significantly different ( $p < 0.05$ ); T0: 5% Dextrose Ringer’s extender without egg yolk; T1, T2, and T3: 5% Dextrose Ringer’s extender with 5, 7.5, and 10% egg yolk respectively; hours: after semen collection



**Figure 1** Eosin nigrosine staining of kampung rooster spermatozoa showing viable spermatozoa with transparent head (white arrows), and dead spermatozoa with reddish colored head (black arrows) under a light microscope at 400x magnification.

**DISCUSSION**

The volume of kampung rooster semen obtained in this study was 0.64 ± 0.134 ml. This

volume was higher than that found in native roosters by Khaeruddin *et al.* (2020) (0.12 ± 0.03 ml). The volume of different rooster semen was influenced by several factors, namely breed, age,

frequency of ejaculation, quality of feed provided, and the health condition of the rooster (Luvanga and Kashoma, 2022). This study's fresh semen of the kampung rooster was milky white with a thick consistency and a concentration of  $1272 \pm 262,907$  million/mL, which was lower than that obtained by Khaeruddin *et al.* (2020) (2460 million/mL). Fresh semen has a characteristic odor similar to that of the animal itself. The pH of fresh kampung rooster semen obtained in this study

was classified as neutral, according to the pH of native rooster semen obtained in previous reports (Wiyanti *et al.*, 2013). The mass movement of kampung rooster spermatozoa in the results of this study showed an excellent value (three plus), meaning that the mass movement of semen formed large, numerous, and fast waves. The percentage of live spermatozoa in Kampung roosters was  $94.4 \pm 1.342\%$ , lower than that reported by Pratiwi *et al.* (2019) ( $92.61 \pm 1.93\%$ ).

**Table 3** Spermatozoa viability of kampung rooster semen stored in 5% Dextrose Ringer's extender with and without egg yolk at 5°C

	T0	T1	T2	T3
0 hours	$83.20 \pm 1.78$ <sup>Aa</sup>	$85.80 \pm 0.83$ <sup>Ab</sup>	$87.20 \pm 1.64$ <sup>Ab</sup>	$90.00 \pm 2.00$ <sup>Ac</sup>
2 hours	$77.80 \pm 1.48$ <sup>Ba</sup>	$81.20 \pm 1.92$ <sup>Bb</sup>	$84.00 \pm 1.58$ <sup>Ac</sup>	$87.20 \pm 1.30$ <sup>Ad</sup>
4 hours	$72.00 \pm 2.12$ <sup>Ca</sup>	$75.00 \pm 1.41$ <sup>Cb</sup>	$78.60 \pm 1.67$ <sup>Cc</sup>	$85.00 \pm 0.70$ <sup>Bd</sup>
6 hours	$61.00 \pm 1.22$ <sup>Da</sup>	$69.60 \pm 0.54$ <sup>Db</sup>	$71.60 \pm 1.51$ <sup>Dc</sup>	$78.20 \pm 0.83$ <sup>Cd</sup>
8 hours	$57.20 \pm 1.09$ <sup>Ea</sup>	$61.20 \pm 1.30$ <sup>Eb</sup>	$65.60 \pm 1.34$ <sup>Ec</sup>	$70.00 \pm 1.73$ <sup>Dd</sup>
10 hours	$50.20 \pm 1.48$ <sup>Fa</sup>	$53.20 \pm 1.64$ <sup>Fb</sup>	$58.40 \pm 1.67$ <sup>Fc</sup>	$60.60 \pm 1.81$ <sup>Ec</sup>

Different uppercase superscripts in the same row, and lowercase superscripts in the same column were significantly different ( $p < 0.05$ ); T0: 5% Dextrose Ringer's extender without egg yolk; T1, T2, and T3: 5% Dextrose Ringer's extender with 5, 7.5, and 10% egg yolk respectively; hours: after semen collection

Progressive spermatozoa motility of fresh semen was  $87.6 \pm 1.673\%$ . Fresh semen must have a motility value of 80% to be further processed for AI purposes. Spermatozoa motility assessment was critical to determine the quality of spermatozoa. High motility of spermatozoa provided a higher chance of fertilization because only motile spermatozoa can fertilize the egg. The forward direction of movement of the spermatozoa (progressive motility) was the main criterion for semen quality and ruled out the spermatozoa that move around or non-motile spermatozoa (Chakraborty and Saha, 2022).

Eosin nigrosin staining is the standard method for determining the live or dead spermatozoa. Live spermatozoa will not be stained due to the intactness of the plasma membrane. Meanwhile, dead spermatozoa would absorb the eosin nigrosin due to the loss of the lipid layer on the cell wall so that the eosin nigrosin dye entered the cells and dyed the spermatozoa red, especially on the head (Kondracki *et al.*, 2017).

Spermatozoa motility and viability decreased with storage time. The viability of kampung rooster spermatozoa at room temperature was 60 minutes (Wiyanti *et al.*, 2013). The longer the storage time, the higher the death of spermatozoa due to cold stress, osmotic imbalance, and lactic acid due to anaerobic metabolism (Yaman *et al.*, 2021). Accumulation of lactic acid due to long storage time would be followed by decreases in the pH of the extender which subsequently reduce spermatozoa motility and viability (Donoghuea and Wishart, 2000). Storage of semen at a chilled temperature (5°C) was intended to reduce the rate of metabolism and maintained spermatozoa in a longer viability (Gączarzewicz *et al.*, 2015; Mphaphathi *et al.*, 2016).

The addition of egg yolk to the extender inhibited the rate of decline in spermatozoa motility and viability. Egg yolk has a fairly good environment for spermatozoa and maintained the integrity of the plasma membrane (Indriani *et al.*, 2013). The storage time of spermatozoa also affected the viability of spermatozoa. The

decrease in spermatozoa viability was in line with the length of storage (O'Hara *et al.*, 2010). The higher the yolk concentration, the higher the ability to maintain spermatozoa viability. The viability of kampung rooster spermatozoa was longer in extender with 10% egg yolk compared to those without or 20% egg yolk (Santoso *et al.*, 2021). Egg yolks contain fat, protein, carbohydrates, and minerals. The fat content in egg yolks served as a reservoir of cholesterol, a source of long-chain polyunsaturated fatty acids (PUFAs) and phospholipids. The abundant PUFA content in egg yolk became a target for free radicals due to storage at cold temperatures, so that spermatozoa were protected from lipid peroxidation. Meanwhile, the phospholipid content in the extender protected the membrane from loss of phospholipids to protect the stability of the spermatozoa cell membrane during the cold storage process (Bustani and Baiee, 2021).

The presence of toxic substances, both from dead spermatozoa or substances contained in the extenders which have been oxidized due to storage, could cause high levels of free radicals which could damage the integrity of the plasma membrane of spermatozoa (Wiyanti *et al.*, 2013). Therefore, additional antioxidants were needed in the extender to scavenge free radicals. In this study, no antioxidants were added; dextrose and egg yolk also did not function as antioxidants. A previous study showed that addition of 2 µg vitamin C per mL Tris-egg yolk extender maintained the eligibility of Sapera goat semen for insemination for three days at 5°C storage (Pahlevy *et al.*, 2022). Also, the addition of 0.15% (w/v) green tea extract in a skim milk-egg yolk extender maintained spermatozoa motility for up to five days at chilled storage (Khoirunnisa *et al.*, 2019).

## CONCLUSION

Addition of 5-10% egg yolk to 5% Dextrose Ringer's extender maintained the quality of Indonesian kampung rooster spermatozoa for ten hours at 5°C storage.

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