CRYOPRESERVATION OF KAMPUNG ROOSTER SEMEN USING EGG YOLK DILUENT FROM FOUR TYPES OF POULTRY WITH DIFFERENT CONCENTRATIONS

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ABSTRACT

The aim of this study was to determine the type and the best concentration of egg yolk in maintaining the quality of kampung rooster spermatozoa during cryopreservation. This study used a completely randomized factorial pattern design with the first factor was the type of egg yolk (purebred chicken, kampung chicken, duck, and quail) and the second factor was the concentration of egg yolk (5%, 10%, and 15%). Semen was collected from twelve kampung roosters using massage method. Immediately after collection, the semen was evaluated macroscopically and microscopically. Semen with more than 70% motility was used in this study. The semen was diluted, packed in a ministraw, equilibrated, and frozen using liquid nitrogen vapor and stored in a liquid nitrogen container for 24 hours. Observation of spermatozoa motility was carried out in fresh semen, diluted semen, after equilibration and after thawing with four replications. The results showed that the type of egg yolk treatment had no effect (P>0.05) on the recovery rate and motility of spermatozoa before and after cryopreservation, but egg yolk concentration had a highly significant effect (P<0.01) on the quality of spermatozoa. Egg yolk in 10-15% concentration had spermatozoa motility and recovery rate higher than egg yolk with 5% concentration. In conclusion, purebred chicken egg yolk, kampung chicken egg yolk, duck egg yolk, and quail egg yolk each in diluent can be used to maintain the quality of kampung rooster spermatozoa at a concentration of 10-15% during cryopreservation.

Key words: cryopreservation, egg yolk, kampung rooster, spermatozoa

INTRODUCTION

Semen freezing (cryopreservation) is a reproductive biotechnology that utilizes spermatozoa cells from superior males to be frozen and stored for a long time. Semen cryopreservation is commonly used to provide animal seed continuously, and can also be used in the preservation of germ plasm. Cryopreservation stop the activity of spermatozoa until thawing process is started.

Chicken spermatozoa are very susceptible to damage due to cryopreservation which affects its motility (Fattah et al., 2017). The addition of diluent is absolutely necessary to protect spermatozoa from the effects of freezing. Egg yolk is an organic diluent that has been used extensively in previous studies. Low density lipoprotein (LDL) is one of the components in egg yolk that contains lipids and protein. Low density lipoprotein is known to protect spermatozoa during storage (Manjunath, 2012). Differences in poultry species produce variations in lipid content (Polat et al., 2013) and protein content of egg yolks (Liu et al., 2018).

Several researches have shown the effects of egg yolks towards spermatozoa. The use of chicken egg yolk diluents results in better semen quality of deer spermatozoa after thawing compared to egg yolks from other poultry species (Sowelum et al., 2018). Panahi et al. (2017) stated that the progressive motility of camel spermatozoa in pigeon yolk diluent was as good as in duck yolk and quail yolk, but was higher than in chicken yolk and guinea fowl yolk.

Kulaksiz et al. (2010) stated that there was no difference in the membrane integrity of sheep spermatozoa in several yolk diluents, namely from goose, turkey, duck, and chuker; however, there was difference in quail egg yolk. Santiago-Moreno et al. (2008) stated that quail egg yolk offer no advantages compared to chicken egg yolk in cryopreservation of Ibex spermatozoa. The use of 5% quail yolk and 10% turkey yolk is superior to 20% chicken egg yolk in terms of the quality of post-thawing buffalo semen, both in vitro and in vivo (Akhter et al., 2016). A research by Santiago-Moreno et al. (2012) stated that the addition of 15% chicken or quail egg yolks could
protect chicken spermatozoa cells from the effects of cold shock during freezing and thawing.

Level of protection of yolk from four types of poultry, namely kampung chicken, purebred chicken, duck and quail may be different towards the quality of kampung chicken spermatozoa. This study aimed to determine the best type and concentration of egg yolk in maintaining the quality of kampung chicken spermatozoa during cryopreservation.

MATERIALS AND METHODS

This study used a completely randomized factorial pattern design with the first factor was the type of egg yolk (purebred chicken, kampung chicken, duck, and quail) and the second factor was the concentration of yolk (5%, 10%, and 15%). Each treatment was repeated four times.

The semen diluted used consisted of ringer lactate, egg yolk as treatment material, 7% dimethyl sulfoxide, 100 IU/mL of penicillin, 1 mg mL\(^{-1}\) of streptomycin, and tris hydroxyl aminomethane to increase the pH of the diluent according to the pH of the semen.

The semen was collected from twelve 1-year-old kampung roosters with body weight of ± 2 kg which were kept in individual cages measuring 40 x 50 x 70 cm. The chicken was given complete diet at rate of 150 g/individual/day and drinking water was given ad libitum. The semen collection was carried out by massage the cloaca until stimulation occurred which was marked by the lifting of the tail and papillae of the proctodeum until the semen was ejaculated. The semen was put in 1 mL syringe.

The obtained semen was evaluated macroscopically and microscopically. Semen that showed spermatozoa motility of more than 70% with minimum spermatozoa concentration of 2500 x 10\(^6\) were used in this study. The semen was divided into twelve tubes which were diluted according to each treatment with spermatozoa concentration of 50x10\(^6\) to 0.25 mL\(^{-1}\).

The liquid semen were then packed in ministraws, equilibrated at 5°C for two hours, and then frozen using liquid nitrogen vapor for 10 minutes and stored in a container filled with liquid nitrogen for 24 hours. Thawing of semen was conducted in a water bath with water temperature of 37°C for 10 seconds. Observation of spermatozoa motility was carried out using a light microscope with 16 times ocular lens magnification and 40x10 objective lens magnifications on fresh semen, after dilution semen, after equilibration semen, and after thawing semen.

RESULTS AND DISCUSSION

The macroscopic characteristics of fresh kampung rooster semen showed that it had milky white color, thick consistency, average volume of 0.24 mL, and pH of 7.6. Meanwhile, the microscopic characteristics of fresh kampung rooster semen showed that it had average spermatozoa concentration of 2.81 billion mL\(^{-1}\), very good mass movement (+++), motility of 84%, viability of 93.2%, and abnormalities of 9.09%. The volume of semen obtained in this study was higher than in previous study by Murcahyana et al. (2016) which was 0.19 mL. However it was within the range obtained by Malik et al. (2018) which was 0.2-0.4 mL. Spermatozoa concentration found in this study was similar to that obtained by Malik et al. (2018) which was 2.81 billion mL\(^{-1}\), but it was higher than the reports of Lubis (2011) and Ulus et al. (2019) which were 1.60 billion mL\(^{-1}\) and 1.37 billion mL\(^{-1}\), respectively. The motility of spermatozoa was almost similar to the previous studies by Murcahyana et al. (2016) and Malik et al. (2018) which found motility of 83.7% and 82.31%, respectively. Viability obtained in this study was also higher than that of Lubis (2011) and Murcahyana et al. (2016) which found viability of 83.7% and 85.3%, respectively. The average abnormalities found in this study was 9.09±6.28% which was higher than previous reports of 1.3% (Ulus et al., 2019) and 8.70% (Murcahyana et al., 2016). Most of spermatozoa abnormality in this study was in the form of mid-piece bending (Figure 1).

Differences in egg yolk types in diluents did not result in differences in motility of spermatozoa (P>0.05) after dilution, equilibration and thawing, as well as recovery rates of kampung chicken spermatozoa (Figure 2-5). The results of this study indicate that purebred chicken egg yolk, kampung chicken egg yolk, duck egg yolk or quail egg yolk could be used as diluent in cryopreservation of kampung chicken semen. The percentage of egg yolk in the diluent produced a very significant difference (P<0.01) in the motility of kampung chicken spermatozoa after dilution, equilibration, and thawing (Figure 2-4) as well as the percentage of recovery rate (Figure 5). Meanwhile, the association of egg yolk

| Table 1. Average characteristics of fresh semen of kampung chicken |
|---------------------------------------------------------------|
| Parameter                                                        | Average ± SEM                  |
| Semen volume (mL)                                               | 0.2 ± 0.06                     |
| Semen color                                                     | Milky white                    |
| Semen thickness                                                 | Thick                          |
| semen pH                                                        | 7.6 ± 0.24                     |
| Spermatozoa concentration per ml (x10\(^6\))                    | 2.81 ± 0.40                    |
| Spermatozoa concentration per ejaculate (x10\(^6\))             | 0.70 ± 0.26                    |
| Spermatozoa mass movements                                       | +++                            |
| Spermatozoa motility (%)                                         | 84.00 ± 5.79                   |
| Spermatozoa viability (%)                                        | 93.28 ± 2.54                   |
| Spermatozoa abnormality (%)                                      | 9.09 ± 2.61                    |
type and the percentage of yolk had no effect (P>0.05) on motility and percentage of recovery rate of kampung chicken spermatozoa during freezing.

The results of this study support previous statement that chicken egg yolk and quail egg yolk had the same protective effect in maintaining the motility and viability of chicken spermatozoa after thawing (Santiago-Moreno et al., 2012). Likewise, the results of a study by Magfira et al. (2017) found that the storage of semen at temperature of 5°C with addition of a quail egg yolk and chicken egg yolk and between chicken LDL and quail LDL in Ringer's lactate diluent did not cause differences in longevity of Merawang chicken spermatozoa. This result was also supported by a research by Widiastuti et al. (2018) which found that there was no difference in the progressive motility of pelung chicken spermatozoa using purebred chicken yolk and quail egg yolk diluents at 4°C storage temperature.

The use of 10-15% of egg yolks in diluents resulted in higher spermatozoa motility of 79.06-80% after dilution, 72.81-73.12% after equilibration, and 29.06% recovery rate of Merawang chicken spermatozoa during freezing.

Figure 1. Observation of spermatozoa abnormalities. a= Head abnormalities, b= Mid-piece abnormalities. Eosin negrosin 100x

Figure 2. Motility of rooster spermatozoa after dilution. Different letters on the same data column shows significant differences (P<0.01)

Figure 3. Motility of rooster spermatozoa after equilibration. Different letters on the same data column shows significant differences (P<0.01)
after thawing. Meanwhile, the use of 5% egg yolk concentration resulted in low motility of 72.19% after dilution, 63.75% after equilibration, and 23.75% after thawing (Figure 2-4). This showed that the increase in egg yolk concentration in the diluent increased the quality of kampung rooster spermatozoa. These results are in line with a research by Mehdipour et al. (2018) which found that the use of egg yolk plasma with 20% concentration was better than 10% concentration in maintaining the quality of frozen semen of chicken. The increase in egg yolk concentration in this study was predicted to be able to increase the protection of spermatozoa from damage during storage and freezing.

Egg yolk diluent consists of two fractions, namely insoluble granules and soluble plasma, which contains LDL as cryoprotectants (Orrego et al., 2019). Egg yolks are used as cryoprotectants to prevent damage to spermatozoa during freezing process. Protein, fatty acid, phospholipids, and cholesterol are also considered instrumental in the success of freezing process (Anand et al., 2014).

The egg yolk in this study was predicted to be able to supplement the protein in spermatozoa that was lost during storage. This was in accordance with the opinion which stated that cryopreservation may cause change in the protein of spermatozoa. Twenty-four types of protein in spermatozoa experience decrease after thawing. Egg yolk could provide 15 types of protein to sheep spermatozoa (Pini et al., 2018). The results of a study by Santiago-Moreno et al., (2012) showed that addition of egg yolk could protect chicken spermatozoa against cold shock during freezing and thawing.

The average motility of spermatozoa after dilution with the addition of 10-15% egg yolk in this study was 79.06-80%. The motility rate was decreased to 72.81-73.12% after equilibration. The motility of spermatozoa after equilibration in the study was almost the same as that obtained by Junaedi et al. (2016) which found motility of 73.89% in the same type of chicken. Likewise, Santiago-Moreno et al. (2012) used chicken and quail egg yolks, each with concentration of 15%, and found motility of 70.3% and 74.2%, respectively. The result of this study was higher than that obtained by Junaedi et al. (2017) which found motility of 52.77 % in kampung broiler chicken and Telnoni et al. (2017) which found motility of 59.77% in Kedu SK chicken.

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**Figure 4.** Motility of rooster spermatozoa after thawing. Different letters on the same data column shows significant differences (P<0.01)

**Figure 5.** Recovery rate of rooster spermatozoa after freezing. Different letters on the same data column shows significant differences (P <0.01)
Semen cryopreservation caused a drastic decrease in spermatozoa motility from 65-77.5% after equilibration (Figure 3) to 21.25-31.25% after thawing (Figure 4). According to Hezavehei et al. (2018), the low quality of spermatozoa after thawing was caused by sudden changes in temperature, ice formation, and osmotic pressure. In addition, Blesbois (2012) stated that during cryopreservation, cells experience a reduction in volume due to dehydration and the influence of internal cryoprotectants. Swelling of spermatozoa occurred during thawing process, due to rehydration and secretion of cryoprotectant from intracellular space, and a temporary increase in intracellular ice particle size which caused a very high tension in the cell membrane. Cryopreservation process can also produce reactive oxygen species (ROS) and reduces the levels of antioxidants. ROS can cause loss of membrane integrity, reduce spermatozoa motility, cause intracellular enzyme leakage, and damage the DNA. This damage occurs through oxidative stress and the production of cytotoxic aldehydes in spermatozoa (Baspiner et al., 2011).

According to Bennison et al. (2016), the middle part (mid-piece) of spermatozoa contains mitochondria that produce chemical energy in the form of adenosine triphosphate (ATP) for motility. Sangani et al. (2017) also stated that the progressive motility of spermatozoa is largely dependent on production of energy in the mitochondrial compartment. Spermatozoa motility increases with decreasing ROS and increasing ATP production in the mitochondria. The low motility of spermatozoa after thawing in this study might also be due to the reduced ATP, as explained by the results of a study by Maddedu et al. (2010) which found that ATP levels in chicken semen decreased significantly after thawing. According to Davila et al. (2016), the reduction of ATP levels in spermatozoa are caused by inhibition of glycolysis by deoxyglucose. The inhibition of ATP synthesis results in the loss of the integrity of spermatozoa membrane and increase production of reactive oxygen species. Grubbs et al. (2013) stated that an increase in electron leakage in the electron transport chain in mitochondria can occur with the loss of mitochondrial regulation mechanisms. Damage in the electron transport chain can decrease the synthesis of cellular ATP (Sangani et al., 2017).

Spermatozoa motility after thawing in this study ranged from 21.25 to 31.25%. Those results were higher than the motility in the studies of Junaidi et al. (2017) and Gloizzi et al. (2011) which found motility of 16.29% and 19.8%, respectively. However, the results were almost similar to that obtained by Shahverdi et al. (2015) and Malik et al. (2018) which found motility of 22.7% and 25.71%, respectively. The recovery rate of spermatozoa in this study was 25.13-36.83 %. It was close to the result of a study by Junaidi et al. (2016) which found a rate of 39.34 % but lower than that obtained by Telnoni et al. (2017) which found rate of 41.45-49.35%. The difference in results may be due to differences in the type of chicken, the type of cryoprotectant or the concentration of cryoprotectant used.

CONCLUSION

This study found that the egg yolks of purebred chicken, kampong chicken, duck, and quail in diluents could be used to maintain the quality of kampong rooster spermatozoa during cryopreservation with best concentration of 10-15%.

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