The Quality of Ettawa Crossbreed Sperm: The Treatment with a Combination of Cryoprotectant in Tris Diluents

Dwi Cahya Prastya Rini, Siti Zaenab, Samsun Hadi, Fuad Jaya Miharja*
Department of Biology Education, Faculty of Teacher Training & Education, University of Muhammadiyah Malang, Jl. Raya Tlogomas No. 246 Malang. Telp (+62341) 464318

*Corresponding author: fuad.jayamiharja@umm.ac.id

Abstract. The formation of ice crystals easily damages the sperm of goats during the freezing process. This study aimed to determine the optimum treatment of cryoprotectant DMSO, glycerol, and a combination of DMSO with glycerol in Tris-egg yolk diluents to maintain the quality of frozen sperm of ettawa breeds. This study used four male goats of which semen was collected twice a week using an artificial vagina. The independent variables in this study were 6% DMSO, 6% glycerol, and a combination of 3% DMSO: 3% glycerol (without treatment), and positive control (7% glycerol). The sperm quality parameters include motility, viability, and abnormalities. Data collection techniques used observation techniques before and after freezing. The data were tested statistically using one-way ANOVA and Duncan test. The results showed that the combination treatment of 3% DMSO: 3% glycerol produced significant motility, viability, and abnormalities (P <0.05) compared to other treatments. These results indicate that the addition of a combination of DMSO and glycerol in egg yolk thinners successfully protected sperm from various stresses during the cryopreservation process to maintain the sperm quality.

Keywords: Ettawa crossbreed, quality of sperm, DMSO, glycerol

1. Introduction
Goats are one of the livestock commodities that are used by the meat. Goat meat is considered as a substitute for beef as a food source [1–4]. This case is to anticipate the increasing needs and demands of the community for products animal foods [2]. However, in 2015 there was a decline in the production of mutton as much as 0.3% when compared to 2014 [5].

The Ettawa is one of Indonesia's local livestock genetic resources assets that are very important to be conserved and developed so that the benefit of production is having economic value [6]. However, breeders generally do not have the proper knowledge to improve the reproductive capacity of livestock so that it can affect the quality of livestock breeds. On the other hand, livestock breedings are an essential means to increase livestock production [7–10]. One of the efforts to maximize its potential by improving the genetic quality of goats in Indonesia is by utilizing artificial insemination technology [11]. Artificial insemination also plays an essential role in preventing the spread of sexually transmitted diseases transmitted through the marital process, increasing the efficiency of the use of
superior males, lowering the cost of raising male cattle, with artificial insemination males that can fertilize many females without sexual intercourse [12,13].

The factors for the successful artificial insemination also depend on the ability to maintain quality and increase the volume of cement by adding cryoprotectants in cement diluents. The diluent substance acts as a buffer solution to prevent pH changes during the cement freezing process [14–16]. The diluents commonly used are egg yolks, milk, and coconut water [11,17,18]. The use of egg yolks in Tris diluents functions as extracellular cryoprotectants for sperm because they contain lipoproteins and lecithin [19,20].

The cryoprotectants commonly used in freezing sperm are glycerol and DMSO. Both are used in specific concentrations, both separately [21–24] and combined [25,26]. Glycerol with a concentration of 5-7% was reported to be able to protect sperm optimally during the freezing process [13,23,24,27]. Meanwhile, DMSO was reported to be able to provide optimal protection during re-dilution so that the sperm motility and sperm viability could be maintained [10,28]. The combination of the two compounds was also reported to have a positive effect on sperm because it could reduce the formation of ice crystals by stabilizing sperm membranes during the freezing process [29–31]. However, not many researchers that reported the concentration of both of them optimally protecting sperm during the freezing phase, because at the same time DMSO is potentially toxic [29,32,33]. This study aimed to determine the concentration of cryoprotectant glycerol, DMSO, and the combination of glycerol-DMSO which optimally protects sperm during the freezing and post-thawing period.

2. Methods
This study is an experimental research using a completely randomized design consisting of four treatments, namely 7% glycerol, 6% DMSO, 6% glycerol, and the combination of 3% DMSO and 3% glycerol in the Tris-egg yolk diluent. The population used in this study was male Ettawa obtained from cement storage at the Center for Artificial Insemination with motility below 60%. The sample used was fresh cement Ettawa with five replications.

The sperm quality parameters include motility, viability, and abnormalities at the time before freezing and post-thawing. Data collection on the percentage of Ettawa sperm motility was by taking frozen straw semen after diluting at ± 37 °C for ± 10 seconds. Observations were made by dipping cement into an object glass at several points then covered with glass. The observation of sperm viability was by making a test preparation with eosin staining and quickly calculating using a counter, while the percentage of sperm abnormalities was observed at the time of viability observation. The calculation of motility, viability, and abnormalities was carried out under a microscope with a magnification of 100-400 times. The data analysis used a different test of One-Way Analysis of Variance (ANOVA) and further tests using the Duncan test.

3. Results and Discussion

3.1. The Effect of cryoprotectant types on sperm motility
The sperm quality can be observed from its motility. Therefore, many researchers consider this case, how to maintain sperm motility remains optimal before freezing and post-thawing. The results of motility calculation are presented in Table 1.

Table 1. The average percentage of motility and standard deviation of sperm*

| Observation          | Cryoprotectant                  |
|----------------------|---------------------------------|
|                      | Control (+)                    | 6% DMSO | 6% Glycerol | Combination 3% DMSO and 3% Glycerol |
| Before Freezing      | 38±7.583<sup>ab</sup>          | 38±5.701<sup>ab</sup> | 40±5<sup>ab</sup> | 40±6.124<sup>ab</sup> |
| Post Thawing         | 33±7.583<sup>b</sup>           | 30±5<sup>b</sup> | 32±5.701<sup>b</sup> | 35±6.124<sup>b</sup> |

*a, b, c: the treatment with the same notation means not having a significantly different percentage
These results indicated that the combination of 3% DMSO and 3% glycerol did not differ significantly (P > 0.05) on sperm motility in all treatments before freezing. However, the combination turned out to have a significant effect (P < 0.05) on motility at post-thawing. In other words, it could be interpreted that this combination treatment was effective when the sperm was thawed again.

Furthermore, a decrease in sperm motility by 5% before freezing and post-thawing was the best among the other treatments. The combination between 3% DMSO and 3% glycerol showed that both cryoprotectants supported and complemented each other in protecting sperm [25,29]. The addition of 3% DMSO was combined with 3% glycerol into the Tris-yolk diluent protects by motivating ice crystals formed during clotting so that the cell damage could be avoided. If the sperm cells are damaged, then the organelles are also damaged, the metabolic process does not take place, and eventually, the sperm cells die [24]. Cryoprotectant added to the diluent is an essential component in the cement freezing process. Intracellular cryoprotectant can diffuse into sperm cells and can be metabolized in a process that produces energy and forms fructose. Cryoprotectant will enter the fructose overhaul cycle in triose phosphate and will then be transformed into lactic acid to be oxidized. The availability of fructose is a factor that causes sperm to keep moving because fructose acts to produce energy in the form of ATP which contains energy-rich inorganic phosphate and will be used for contracting fibrils and generating sperm motions [24].

3.2. The Effect of cryoprotectant types on sperm viability

The ability of sperm to survive after the clotting process is one indicator of the success of sperm through a critical period during the freezing process. Thus, agents need to be able to maintain and improve the survival of sperm (Figure 1). The viability of Ettawa breed goat sperm after the addition of cryoprotectant type in Tris diluent egg yolk before freezing and after being diluted again is shown in Table 2.

![Figure 1. Goat sperm with microscope 400x magnification. The dyes absorbed by the acrosome show that the sperm are dead (a), while the unstained acrosomes indicate that the sperm cells are still alive (b)](image-url)

Table 2 shows that the addition of 3% DMSO: 3% glycerol before freezing and post-thawing had a significant effect (P < 0.05) on the percentage of sperm viability. The combination of 3% DMSO and 3% glycerol resulted in 79.8% higher percentage of live sperm (P < 0.05) than the addition of 6% DMSO (68%), 6% glycerol (73.4%), and 7% glycerol of 68.8%. Duncan's test results showed that the most effective treatment for maintaining Ettawa sperm viability post-thawing was a combination of DMSO and glycerol with a decrease in sperm viability compared to before freezing of 3.4% which was the lowest decrease in viability than a decrease in other treatments.
The combination of DMSO and glycerol gave the best results because the presence of both cryoprotectants in diluents complemented each other. DMSO and glycerol are intracellular cryoprotectant agents that play an essential role in protecting cells because they can penetrate cell membranes and modify the formation of ice crystals through the prevention of increasing electrolyte concentrations that can harm cells during the clotting process. Besides, cryoprotectant decreases the freezing point of the solution, thus giving the cell an opportunity to release water and prolong the acclimatization of the cell to drastic temperature changes and reduce the amount of intracellular freezing water. Optimal cryoprotectant concentration is the amount needed for cell protection and what is needed so that the solution does not cause toxicity [9,25,32,33]. DMSO and glycerol will diffuse to penetrate and enter the sperm cell membrane and will be used for oxidative metabolism, replacing free water and pushing out electrolytes, thereby reducing the concentration of intracellular electrolytes and reducing damage to sperm cells [24]. Furthermore, Tambing et al. explained that the presence of cryoprotectants in Tris-egg yolk diluents provides a protective effect by maintaining the balance of inter and extracellular electrolytes so that the biochemical processes that occur in sperm cells remain in place and reduce unnecessary sperm cell death [24].

3.3. The effect of cryoprotectant types on sperm abnormality

The observation of sperm abnormalities after the addition of cryoprotectants in Tris-egg yolk diluent is described in Table 3. The evaluation of abnormalities was carried out before freezing and after treatment to determine the indicators of biological stress or toxic exposure. The results of the analysis in Table 3 show the value of abnormalities before freezing significantly (P < 0.05) with the other treatments, while the post-thawing abnormalities significantly affected (P < 0.05) between treatments. Based on the results test, it can be concluded that sperm abnormalities before freezing differed from other types of treatment (P < 0.05). Meanwhile, the treatment that was most effective in minimizing sperm abnormalities post-thawing was 6% DMSO and 6% glycerol by 5.8% compared to other treatments. The abnormal sperm was found in semen as shown in Figure 2.

Table 3. The average percentage of abnormality and standard deviation of sperm*
The abnormalities found in sperm were secondary upnormal in all treatments. After re-dilution, the percentage of abnormalities increased, because the secondary abnormalities occurred during the sperm storage and cryopreservation and were caused by a treatment at the time of staining in making pillow preparations [34]. The sperm abnormalities could be due to the disturbances in the seminiferous tubules of the testicular clan, the disturbances during travel in the male reproductive tract disrupting post-ejaculation handling. The secondary abnormalities found were the folded tail and formed an angle, curly tail, without a tail, and breaks tail.

Based on the results of the data and the discussion of the above studies, the hypothesis about the effect of adding cryoprotectant on Tris-egg yolk diluents before freezing and post-thawing of the quality of sperm was proven. This was evidenced by the combination treatment between 3% DMSO and 3% glycerol which was the optimal ability to maintain sperm motility and viability and minimize abnormalities at the time after freezing compared with controls (-) which did not add cryoprotectant to tris-egg yolk diluent. These results indicated that the combination of 3% DMSO and 3% glycerol could maintain the quality of sperm.

4. Conclusion
The addition of cryoprotectant to Tris-egg yolk diluent affected the sperm quality of Ettawa post-thawing. The combination of 3% DMSO and 3% glycerol could maintain the sperm motility and viability. The results of the study recommend the use of a combination of 3% DMSO: 3% glycerol in the Tris-egg yolk diluent in the cement freezing process. However, further research is required regarding the concentration of DMSO and glycerol combined with different concentrations to work optimally in maintaining the sperm quality.

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