

Motility of Bali Sexed Sperm Following Equilibration and Cryopreservation in Different Concentrations of Ethylene Glycol

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ABSTRACT

Freezing of bovine sperm has a critical point at the time of equilibration and freezing. Therefore, the equilibration duration and concentration of cryoprotectants that can maintain sperm during freezing and storage become determining factors for the success of bovine sperm freezing. This study aims to evaluate the effect of the interaction between equilibration duration and the concentration of ethylene glycol as cryoprotectants on the sperm motility of Bali bull sexed sperm both after equilibration and after freezing (post thawing motility). The study was conducted in February-March 2021 at the UPTD of the Center for Animal Breeding and Feed, the Food Crops and Livestock Service Office of Southeast Sulawesi Province. The material used was Bali bull aged three years and weighed 410kg as a source of semen. Factorial Completely randomized design (3x3) was used in this experiment. The first factor was the equilibration duration, consisting of 3 hours, 4 hours, and 5 hours. In contrast, the second factor was the concentration of ethylene glycol, which consisted of 5, 6, and 7%. The evaluated variable was sperm motility both after equilibration and post-thawing motility. Data were collected and tabulated, then analyzed using variance analysis, while the differences between treatments were compared using Duncan's Multiple Region Test using the IBM Statistics SPSS 24 program. The results showed that the average motility of sperm after equilibration and post-thawing motility were $66,57 \pm 3,02\%$ and $47,78 \pm 4,84\%$, respectively. Based on variance analysis, it was concluded that there was no interaction effect between equilibration duration and concentration of ethylene glycol on sperm motility both after equilibration and post thawing motility of Bali bull sexed sperm.

Keywords: Bali bull, sexed sperm, equilibration, freezing, thawing

1. INTRODUCTION

Sperm sexing can improve the reproductive efficiency of cattle production because through this technology, we can manage the sex of calf to be produced. This technology tries to change the proportion of X-bearing sperm and Y-bearing sperm from the natural ratio, which has an equal portion [1]. The purpose of sexing sperm or separating sperm based on chromosome carrier type prior to artificial insemination is to produce offspring of the desired sex. Yadav *et al.* [2] stated that a direct method for sexing sperm in animals is based on sorting X and Y-chromosome-bearing sperm before insemination. Sperm

are classified as X or Y sperm based on the presence of an X or Y chromosome, respectively. If an egg that only has an X chromosome is fertilized by an X-bearing sperm, then female offspring will be produced, whereas a Y-bearing sperm produces male offspring. As a result, sperm sexing can be combined with artificial insemination to produce the desired offspring.

In the previous study [3, 4, 5], we have conducted sperm sexing using Bali bull semen and been stored in a refrigerator for 1-7 days to produce chilled semen. The motility of sperm decreased from 80% to 52.5-60.2% after sexing, but the good fertilizing ability was gained, which was indicated by 82.54-95% NRR [6].

There are two kinds of semen forms used in artificial insemination, liquid semen, and frozen semen. These two kinds of semen have different lifespans, where the liquid form allows intermediate storage of a few days only while the deep-frozen form permits storage for years without any significant decrease in semen quality. Since the discovery of cryopreservation method for bull semen, cryopreservation has become an alternative method for maintaining gamete resources of certain animals [6]. Some of the problems encountered during the freezing process are cold shock and the formation of ice crystals. To overcome this problem, a substance (cryoprotectants) is added to the diluent to reduce damage to the sperm.

The addition of cryoprotectants to frozen sperm is closely related to equilibration time, as the presence of cryoprotectants in diluents containing sperm indicates that the cells require adjustment to the cryoprotectants that enter the cells. The time required for equilibration in the semen freezing process varies depending on the type of semen, individual males, diluents, and freezing methods used [7, 8, 9]. Equilibration time is required to prevent sperm mortality and the use of some protective agents (cryoprotectants) during dilution and gradual decrease in temperature. The protective agent that can be used is ethylene glycol. Some researchers believe that low molecular weight cryoprotectants, such as ethylene glycol, can cause minor damage to sperm than glycerol because its low molecular weight allows it to cross the plasma membrane more easily [10]. Compared to glycerol and dimethyl sulfoxide (DMSO), ethylene glycol produced better post-thawing motility results for bull sperm, possibly due to reduced osmotic stress [11]. Based on this information, the researchers studied on the effect of equilibration time and ethylene glycol cryoprotectants on sexed sperm in Bali cattle.

2. MATERIALS AND METHODS

This study was conducted at the Laboratory of Animal Reproduction and Breeding Animal Breeding and Animal Feed, Food Crops, and Livestock Service Office of Southeast Sulawesi Province from February 2021 to March 2021.

The material used was bovine semen obtained from Bali bull (491kg) which were fed *ad libitum* elephant grass (10% body weight) and concentrate (1-2 kg/day). Tris-Egg Yolk (Tris-KT) medium contained tris aminomethane, citric acid, fructose, and egg yolk was used as a semen diluent. In contrast, the cryoprotectants used in the freezing process were ethylene glycol with different concentrations. The Tris-KT medium was also used for sperm sexing. Equipment used were artificial vagina for collecting semen, 10ml tube for sperm sexing, timer for keeping the equilibration time, styrofoam box containing liquid nitrogen (LN2) for sperm freezing, and LN2 container for storage of the frozen sperm.

Semen collecting was carried out twice a week, and the collected semen was immediately evaluated its quality after collecting. Good quality semen was then diluted with 0.9% NaCl up to the concentration of 200 million/ml sperm prior to sexing process. The sexing process was commenced by placing 1ml diluted semen on the Tris-KT medium (2 ml) in the sexing tube and maintained at room temperature (27°C) for 40 minutes. After sexing process, the upper part of the sexing medium was removed, the lower part was taken, and sperm concentration was adjusted to 20 million/ml sperm before equilibration and freezing processes. Equilibration was conducting by keeping the semen in the refrigerator for 3-5 hours according to treatments. The semen was loaded into mini tube straw (0,5ml) following equilibration and then put on a styrofoam box containing liquid nitrogen (LN2) for the freezing process.

A Factorial completely randomized design with two factors and three levels each was applied in this experiment. The first factor was the equilibration time which consists of 3 hours, 4 hours, and 5 hours. While the second factor was the concentration of ethylene glycol, which consists of 5%, 6%, and 7%. The evaluated variable was sperm motility both before and after freezing.

The sperm motility was evaluated by observing the movement of sperm under a light microscope with 400 magnifications. Good motility of sperm was indicated by progressively forward movement (0% - 100% scale). Data related to sperm motility after equilibration and thawing were tabulated and analyzed using variant analysis. At the same time, significant differences between treatments were judged using Duncan Multiple Range Test (DMRT) using the IBM Statistics SPSS 25 program.

3. RESULTS AND DISCUSSION

3.1. Post-Equilibration Motility of Bali Bull Sexed Sperm

According to [12, 13], diluted semen must be equilibrated and the temperature must be reduced during the semen freezing process. Before the sperm is frozen, equilibration is performed to allow the sperm to adapt to the diluent. If the equilibration time is too short or too long, the sperm will lose a lot of energy which in turn reduces sperm activity [14]. Sperm can be damaged by insufficient or excessive equilibration time.

The average post-equilibration motility of Bali bull sexed sperm gained in this experiment was presented in Table 1.

Based on the results of variance analysis, it was revealed that the interaction between equilibration time and ethylene glycol concentration had no significant effect ($P > 0.05$) on the percentage of sperm motility of

Table 1. The average post-equilibration motility of Bali bull sexed sperm (%)

Concentration of EG	Equilibration Duration			Averages (%)
	3 hours	4 hours	5 hours	
5%	71.67	66.67	68.33	68.89±2.55
6%	61.67	68.33	68.33	66.11±3.85
7%	65.83	63.33	65.00	64.72±1.27
Averages	66.39±5.02	66.11±2.55	67.22±1.92	66.57±3.02

Table 2. The averages post-thawing motility of Bali bull sexed sperm (%)

Concentration of EG	Equilibration Duration			Averages (%)
	3 hours	4 hours	5 hours	
5%	56.67	50.00	46.67	53.34
6%	45.00	46.67	48.33	46.67
7%	46.67	45.00	38.33	43.33
Average	49.45±6.31	47.22±2.55	44.44±5.36	47.78±4.84

Bali cattle after equilibration. The average percentage of sperm motility of Bali cattle obtained in this study ranged between 61.67% - 71.67%, with a general average of 66.57%. This value was higher than the results of a study conducted by Büyükleblebici [15], in which 41.58% sperm motility was gained when ethylene glycol using as a cryoprotectant. This result was also higher than that obtained by Seshoka *et al.* [16], namely 2.7±2.9 when using 12% ethylene glycol as a cryoprotectant in Nguni cattle in South Africa.

3.2. Post-Thawing Motility of Bali Bull Sexed Sperm

The results of variance analysis, as shown in Table 2, indicated that the treatments had no significant effect on post-thawing motility of Bali bull sexed sperm. Post-thawing motility of Bali bull sexed sperm ranged between 38.33% - 56.67% with 47.78% on average. This value was higher than that reported by [17], in which 31.40% of post thawing motility was gained when limousine bull sperm was examined. However, the result of this study was lower than those of [11] reported. They got 50% of post thawing motility of red Anatolian cattle. According to [18, 19, 20], the overall motility of sperm after thawing is lower than that of sperm motility after equilibration. This is due to the fact that the freezing process has a detrimental effect on the sperm plasma membrane, which can reduce the ability of sperm to move. Damage to the plasma membrane can occur in the head and tail. Damage to the head reduces viability; damage to the tail, particularly the midpiece, results in the loss of mitochondrial ability to produce ATP so that energy for movement is not formed and sperm stop moving; and increasing osmotic pressure in the sperm plasma reduces the permeability of the sperm membrane, resulting in increased membrane damage.

4. CONCLUSION

Based on the results and discussion in this study, it can be concluded that the interaction of equilibration time and concentration of ethylene glycol did not significantly affect the motility both of post equilibration and post thawing motilities of Bali bull sexed sperm.

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