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Effect of Skim Milk on Sperm Motility of Brek Fish, *Systomus orphoides* (Valenciennes, 1842) for Short Term Preservation

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ABSTRACT

The purpose of this research was to evaluate the effect of skim milk on sperm motility of Brek fish, *Systomus orphoides* (Valenciennes, 1842) after 48 hours in 4˚C. The method in this research is sperm was collected by hand stripping and was diluted by diluent, which consisted of the fish ringer, skim milk and methanol 10%. The ratio of sperm to diluent is 1:9. The concentrations of skim milk used in this research were 0%, 5%, 10%, 15%, and 20%, respectively. The sperm was stored at 4 ˚C for 48 hours after diluted sperm is thawed in room temperate for 1 minute and sperm motility was observed in improved Neubauer with a light microscope.The motility percentage was analyzed using ANOVA and Tukey test. According to the Annova test (P>0.05), 10% of skim milk showed the highest sperm motility (92.18 $\pm$ 3.05) % after five replication per dose. Ten percent of skim milk was the optimum concentration to preserve sperm motility at 4˚C for 48 hours compared to 0%, 5%, 15%, and 20%. The result of each concentration is 90,85%, 84,68%, 79,54%, and 74,14% respectively.

Keywords: *motility, preservation, skim milk, sperm, Systomus orphoides*

1. INTRODUCTION

The population of Brek fish *S. orphoides* in habitat is getting decreased. Environmental changes resulting from anthropogenic disturbances are a major threat to species extinction [1]. Besides, the other factors causing the extinction of freshwater fish include habitat destruction, introduce of foreign fish species, overfishing, and use of fishing gear that is not environmentally friendly in natural habitat [1,2]. Accordingly, the efforts to keep the species to still exist in their natural habitat is by ex-situ conservation.

The main problem in the breeding of a type of fish is maturity of gonad time does not coincide between female and male brood stock [3]. To offer come this problem can be conducted by sperm storage. Sperm preservation methods usually use protective agent to protect cells from low temperature.

Technique for storing animal, plant cells, or other genetic material (including cement) in a frozen state by reducing metabolic activity without affecting the organelles in the cell so that physiological, biological, and morphological functions remain is preservation [4]. The main purpose of preservation in sperm is to conserve rare germplasm and support artificial insemination technology programs. Preservation techniques are good supporting factors for conservation [5].

Besides protective agent, some materials are also needed to preserved sperm such as extenders, intracellular protectant, extracellular protectants. Intracellular protectants are compounds with small molecules that can penetrate into cells. One example of intracellular protectant is methanol [6].

Methanol can be used as an intracellular protectant because it has a smaller molecule size so that it can easily penetrate into cell membranes, and

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methanol also has lower toxic properties than dimethyl sulfoxide (DMSO) and dimethyl acetamide (DMA) [7].

Extracellular protectant has a relatively large molecular size so that it cannot penetrate into cells, an example of a potential and non-toxic protectant is skim milk. Skim milk is one of the extracellular cryoprotectants which is widely used in the cryopreservation of spermatozoa [6]. Skim milk is produced from the centrifugation of whole milk at low speed. Skim milk is formed in the middle layer as a result of centrifugation, namely the aqueous supernatant. The substances contained in whole milk are the same as in skim milk except for fat and vitamins that are dissolved in fat [8]. The fat contained in skim milk isn't more than 0.5%, while the whole milk ranges from 2.4 to 5.5% [8].

Skim milk contains nutrients that can be utilized by spermatozoa as an energy source [9]. This nutrient is lactose which can be broken down into glucose and galactose [10]. Skim milk contains protein which can be divided into two, namely casein and whey protein [11]. The content of casein protein in skim milk plays a role in protecting spermatozoa from cold shock [12].

The previous study has been conducted to protect sperm using skim milk as extracellular protectants for example cryopreservation of *Barbonymus gonionotus* [13]. Further, the effect of skim milks as natural supplement preserved Brek fish sperm has not been conducted yet. Accordingly, the aims of study are to evaluate Brek fish sperm motility after preservation at 4 °C 48 hours.

2. MATERIALS AND METHODS

2.1. Research Design

The research was made experimentally using a completely randomized design (CRD). The study consisted of 5 treatments and 5 repetitions. 5 treatments consisted of spermatozoa with the addition of various concentrations of skim milk (0%, 5%, 10%, 15%, and 20%), Fish ringer, and 10% methanol. The concentration determination of skim milk is based on a modification of previous research [14]. The number of repetitions is determined based on Frederer's formula, namely (t-1) (r-1)> 15, r is the number of repetitions and t is the number of treatments [15].

Table 1. Various concentration of skim milk, fish ringer and 10% methanol.

Treatment	Methanol 10% (µL)	Extender *fish* Ringer (µL)	Skim Milk (µL)	Cement (µL)
A (0%)	500	4500	0	50
B (5%)	500	4250	250	50
C (10%)	500	4000	500	50
D (15%)	500	3750	750	50
E (20%)	500	3500	1000	50

2.2. Population and Sample

Brek fish are kept in the Freshwater Fishery Germplasm Research Installation pond, Cijeruk, West Java. Brek fish are fed 2 times per day, morning and evening. The temperature of the pond water where the rearing is between 21 ˚C - 25 ˚C with a pH of 6.5-7.8 [16]. Fish are reared in an open pond with a pool size of 10.8 x 5.2 meters and a depth of 60-80 cm. Main's selection is done by selecting the parent that has matured gonads. 20 individuals' male fish has been used for this experiment.

2.3. Sample Collection Techniques

The sperm was collected by stripping hand method, the ejaculated sperm was sucked by needless syringe and was storage in 1.5 ml micro tube. The fresh sperm were obtained then preserved at 4°C for 48 hours and analyzed macroscopic and microscopic.

Evaluation of spermatozoa is carried out macroscopic and microscopic. Macroscopic evaluation was carried out by measuring the volume, color, and pH of the semen, while microscopic evaluation of spermatozoa was carried out by observing and calculating the percentage of spermatozoa motility. Macroscopic evaluation was only performed on fresh semen while microscopic evaluation was carried out twice, namely fresh spermatozoa and pascapreservation spermatozoa.

2.4. Solution Preparation

Fish Ringer is made by dissolving 3.25 g of NaCl, 0.125 g of KCl, 0.175 g of $CaCl_2H_2O$, and 0.1 g of

$NaHCO_3$ into 500 mL of sterile distilled water [13]. Fish Ringer's solution is then put in a glass bottle and closed with parafilm.Fish Ringer solution is stored in a refrigerator at 4 ˚C for 72 hours [17]. During process of freezing and thawing, extenders are used to keep the quality of spermatozoa cells [18].

Activator solutions were prepared by dissolving 45 mM NaCl (0.2633 g NaCl), 5 mM KCl (0.0373 g KCl), and 30 mM Tris HCl pH 8.0 (0.3634 g C4H11NO3) in 100 mL sterile distilled water [20]. The activator solution is then stored in a glass bottle and covered with parafilm.

The preparation of George's solution was made by dissolving 1.5 grams of sodium citrate, 0.3 g of eosin B and 0.5 mL of formaldehyde in 50 mL of distilled water. The mixture is stirred until evenly distributed and then stored in a dark bottle glass at room temperature [19].

2.5. *Motility Observation*

The calculation of spermatozoa motility was carried out on fresh semen and post-preservation cement. The spermatozoa motility was observed twice. The first observation was made to see immotile or immobile spermatozoa by first diluting the semen 400 times with two dilutions. The first dilution was 10 times by taking 10 µL of cement into 390 µL of George's solution and then homogenized, 10 µL was taken to drop it in the improved Neubauer counting room which had been covered with a glass cover. The motility of spermatozoa was observed with a light microscope with the magnification of 10 x 40 and a camera (digital eye piece) connected to a laptop [21]. Motility calculations were carried out in 5 counting rooms R.

The percentage of motility based on [12] can be calculated using the formula:

$$\% \text{ Motilitas} = \frac{\Sigma \text{ spermatozoa total} - \Sigma \text{ spermatozoa imotil}}{\Sigma \text{ spermatozoa total}} \times 100\%$$

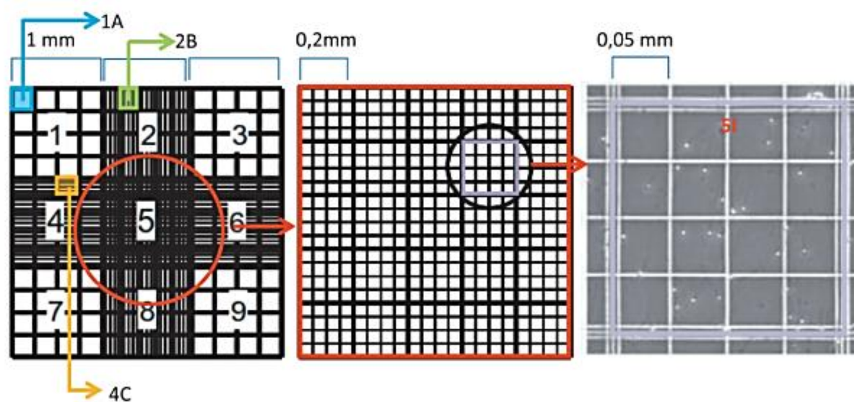

Figure 1. Line pattern diagram in a hemocytometer count room (improved Neubauer (Agustinus et al., 2019).

2.6. *Data Analysis*

Pascapreservation spermatozoa analysis data is presented in tabular form in the form of the percentage of motility. The quality data of spermatozoa were analyzed using Statistical Product Software for Scientific and Social Science (SPSS) 16.0 for Windows [22].

The data were tested for normality using Saphiro-Wilk, then continued with the homogeneity test using Lavene. Data that is normally distributed and homogeneous will be analyzed using one-way ANOVA, then if it is significantly different, a further test will be carried out using the Tukey test. If the data is not normally distributed and is not homogeneous, then the Kruskal-Wallis test is performed, and then further tested using the Mann-Withney test [22].

3. RESULT AND DISCUSSION

The percentage value of fresh sperm motility *S. orphoides* in this study had an average value of 97.62 $\pm$ 1.09%. For Treatment the best motility value of *S. orphoides* in this study shows in concentration 10% that average value is 92,18 $\pm$ 3,05% and shows that the quality of *S. orphoides* sperm is of good quality (figure 2). This value is higher than previous research conducted by [14] on *Tor soro* kancra (Valenciennes, 1842), with a motility value of 89.11 $\pm$ 1.31%. The

level of motility in fish sperm is said to have a good quality for preservation if it has a value above 70% [23,24]. Microscopic observation of sperm viability can be seen in Figure 3. Even the shape of tail of the sperm is not clearly visible.

Table 2. Macroscopic evaluation Brek fish sperm

Observation	Parameter	Results
Macroscopic	Color	Milky white
	Volume (ml)	2,9
	pH	8

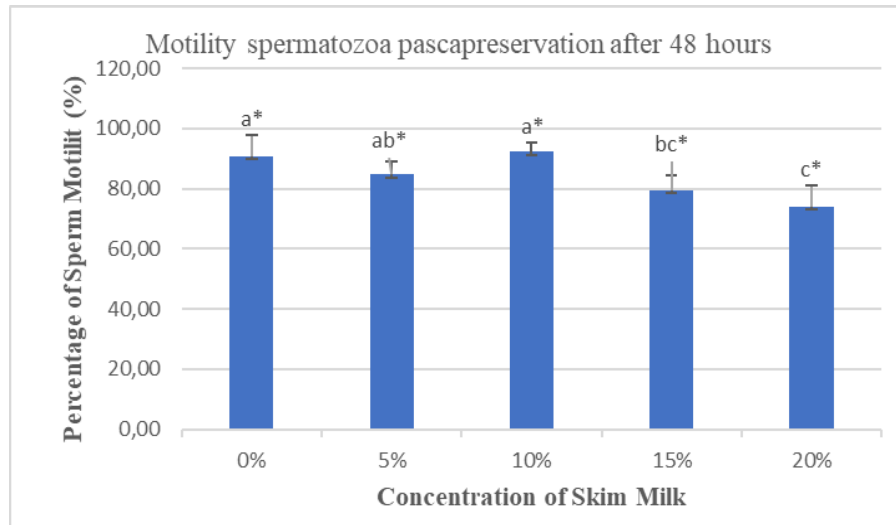

Figure 2. Graph of motility each concentration after preservation at 4 °C for next 48 hours.

Table 3. Motility of spermatozoa after preservation 48 hours

Treatment	Repitition (%) 1	2	3	4	5	Average
Fresh Sperm	97,06	96,92	98,84	98,77	96,52	97,62 ± 1,09
A (0%)	82,71	96,15	84,85	98,30	92,26	90,85 ± 6,85
B (5%)	90,80	87,69	82,54	81,73	80,65	84,68 ± 4,36
C (10%)	96,15	91,67	92,77	92,66	87,63	92,18 ± 3,05
D (15%)	87,71	78,95	77,61	76,03	77,42	79,54 ± 4,68
E (20%)	80,17	63,16	78,38	76,92	72,06	74,14 ± 6,18

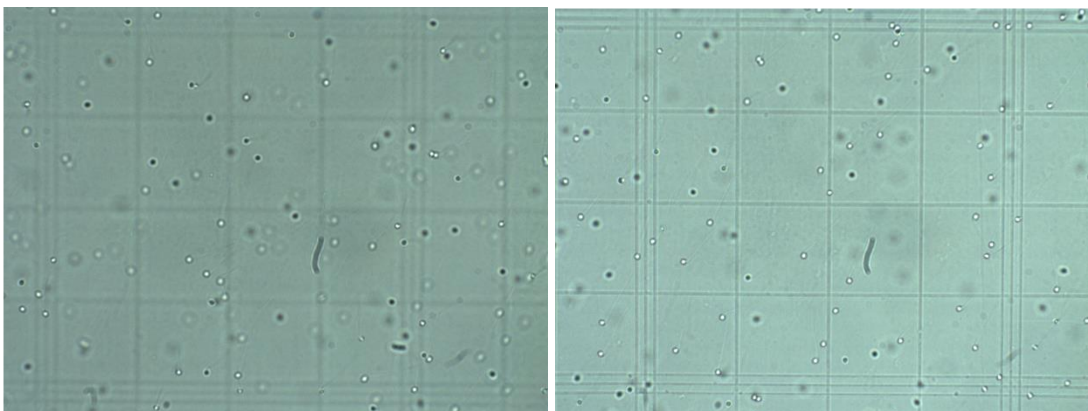

Figure 3. Motility of spermatozoa in 10% concentration skim milk after preservation 48 hours.

The differentiation about spermatozoa motility value of each sample was influenced by the differentiation concentrations of skim milk as a protectant. The skim milk was used in this study is lactenin. The content of lactenin in skim milk is thought to maintain the motility spermatozoa of *S. orphoides* during the process of preservation. In this research results obtained, the treatment with concentration 10% skim milk show the highest motility value.

The motility percentage of spermatozoa *S. orphoides* 48 hours after preservation is presented in the figures 2 and table 3. Based on the figure presented, it is known that spermatozoa stored for 48 hours using a combination of protectant methanol 10% with skimmed milk in different concentrations (0%, 5%, 10%, 15% and 20%) are able to maintain motility with an average value. The percentage of motility obtained by the control that is concentration 0% (without skim milk) is 90,85 $\pm$ 6,85%, this value is higher than the value with the addition of 5% and 20% skim milk.

The characteristics of fish spermatozoa while still in the reproductive tract are still immotile, but when the spermatozoa are released into the environment, they will be motile. In this study, for induction the movement of spermatozoa an activator solution was used. Activator solution has a lower osmolality value than fish spermatozoa plasma, which can induce spermatozoa movement [25]. The activator solution added to spermatozoa can help reduce the osmolality value so that it can help induce motile spermatozoa. This can be due to the unbalanced ion composition [26].

The condition of the fish spermatozoa plasma becomes hypotonic which results in an efflux of K^+ ions through the K^+ ion channels, which causes the membrane in the spermatozoa to hyperpolarize. Membrane hyperpolarization will then be followed by membrane depolarization which triggers the influx Ca^{2+} process and results in an increase in the concentration of Ca^{2+}. Increasing the concentration of Ca^{2+} results in the activation of adenyl cyclase which converts ATP to cAMP which helps the motility of spermatozoa [26,27].

Observation on fish spermatozoa motility is a common parameter to determine the quality of fish spermatozoa because it has an important effect on fertilization [28]. Motility in fish is influenced by several factors, including maturity of gonad and spermatozoa morphology. Spermatozoa are categorized as motile if they are stationary, move forward but not straight, move forward but slowly and move straight up quickly [29].

The component contained in milk in the form of lactenin can also inhibit the motility of spermatozoa. Lactenin is one of the compounds in milk which is toxic to spermatozoa. The toxicity of lactenin can be overcome by heating the skim milk before using it. Heating is done at an ambient temperature of 87-98 °C for 1 minute [30]. This process will cause inactivity of the enzymes contained in milk, one of which is the catalase enzyme which can carry out its metabolism so that the toxicity of methanol will be reduced [31].

Heating skim milk in addition to deactivating the enzyme catalase can also cause denaturation of serum protein in skim milk [32]. Denaturation of serum proteins will produce cysteine amino acids which have a cysteine hydrochloride group. Cysteine can inactivate formaldehyde which is produced from methanol metabolism. This inactivation occurs through the binding of formaldehyde by cysteine so that formic acid is not formed [33]. Cysteine can also play a role in neutralizing lactenin in skim milk.

ANOVA test results shows a significant difference value in the treatment of concentration 10% skim milk. The concentration is a good composition and effective in the preservation process, so that compounds that are detrimental to spermatozoa can be minimized. The right concentration will have a good effect on spermatozoa in the preservation process of *S. orphoides.*

4. CONCLUSION

The optimum concentration of skim milk and methanol after preservation 48 hours at 4°C was at concentration 10% with the results is 92,18 $\pm$ 3,05% for motility observation.

AUTHORS' CONTRIBUTION

RR carried out the literature survey. analyzed data and wrote manuscript. A conceived of the study and participated in its design and performed the statistical analysis. RG helped review to draft the manuscript. All authors read and approved the final manuscript.

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