The spermatozoa viability of kancra fish (Tor soro, Valenciennes 1842) 48-hour after freezing: effect of brown sugar as natural cryoprotectant

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Abstract. Kancra fish (Tor soro, Valenciennes 1842) is one of the endemic fish in Indonesia which population keeps decreasing due to degradation of the environment. Conservation is needed and cryopreservation is an effective strategy in this regard. Brown sugar as a natural cryoprotectant has the potential to substitute less eco-friendly synthetic cryoprotectant. The study was carried out to determine the viability of T. soro spermatozoa after 48 hours of cryopreservation using brown sugar. Sperm sample were collected through stripping method. The ejaculated sperm were diluted using the extender (brown sugar-fish ringer + methanol 10%, 1:9). The brown sugar concentration examined were 0%, 5%, 10%, 15%, 20%, and 25%, and the sample was equilibrated at 4 °C for 10 minutes, and frozen at -10 °C for 48 hours. The process was followed by thawing process ast 40 ºC for 60 seconds. Data were analyzed using ANOVA, followed by Tukey test. The results showed that brown sugar concentration of 15% significantly affects viability of the sperm after 48 hours of cryopreservation with 83.75±1.71% (p<0.05). However, a study is needed to determine the effectivity of brown sugar as a cryoprotectant.

1. Introduction
The kancra fish (Tor soro, Valenciennes 1842) is a species of the genus Tor fish and belongs to the Cyprinidae family which is spread in several regions in Indonesia such as Java, Kalimantan, and Sumatra [1]. Kancra fish grows in water condition that has a rocky substrate and contains high oxygen level (5.9‒6.5 mg/L) [2]. Some fishermen in the Asahan River, North Sumatra, said there was a decrease in the kancra fish population with a mortality value of 4.09% per year. Population decline can be caused by various factors such as high fishing intensity which causes the mortality value to reach 3.43% from the normal 0.5% limit, unsustainable fish use, anthropogenic activities, and land conversion [1]. The decrease in kancra fish population must be prevented immediately, one of them by doing conservation using cryopreservation methods.

Cryopreservation is a process for maintaining genetic material [3]. Storage by cryopreservation has the advantage of being more efficient in terms of cost, time, storage space, and energy [4]. Reproductive engineering technology, especially cryopreservation has been developed quite a lot for spermatozoa [5]. Sperm cryopreservation is one of the sperm storage methods using liquid nitrogen (-196 °C) as a freezing liquid with cryoprotectant fluid to protect sperm cells [6]. Very low temperature during freezing resulting in leakage of vital substances in sperm so that intracellular enzymes,
lipoproteins, ATP and intracellular potassium are reduced causing damage to the plasma membrane so that the viability value decreases [7].

In the cryopreservation technique, cell damage during freezing can be prevented [8], because almost half of the cell parts will be replaced by cryoprotectant molecules [9]. The choice of cryoprotectant is the main thing in the success of the cryopreservation process. Good cryoprotectants are environmentally friendly, non-toxic, easily prepared, and available at affordable prices. The toxicity of a cryoprotectant itself depends on the type, concentration, temperature, and period of exposure [10]. One cryoprotectant that is widely used is organic dimethyl sulfoxide (DMSO), but intrinsic toxicity and the freezing protocol of the DMSO complex are obstacles to widespread use for clinical applications [11]. Therefore, alternatives are needed in the form of cryoprotectants which have low toxicity, such as natural cryoprotectants. Natural cryoprotectants are defined as materials originating from nature that do not contain artificial chemical compounds [10].

In Indonesia, there are traditional sugars which are obtained from heating the palm sap to crystallize or commonly called brown sugar. Out of the 20% solids present in coconut neera, there are 85.76% sucrose content, 1.5% of fructose, and less than 0.82% of glucose [12]. Sugars such as glucose and fructose are the main energy sources, while high molecular weight sugars function as extracellular cryoprotectants [13]. Sucrose is a natural carbohydrate that can provide nutrients for cell preservation at low temperatures (-45 °C) [9]. Low molecular weight nonpermeable cryoprotectants, such as galactose, glucose, sucrose, trehalose or other sugars, have not been widely used in freezing spermatozoa. These compounds can support dehydration before cooling and causing the formation of intracellular ice crystals to decrease [14]. Therefore, further study is needed because there are not much data about the effect of the use of brown sugar as a natural cryoprotectant on the viability of kancra fish sperm after cryopreservation.

2. Material and methods
2.1. Sperm sampling
Sperm sampling was done at the Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java, Indonesia. Male *T. soro* sperm are taken in February‒September according to the natural spawning time. Sperm is sampled by stripping with the help of syring. Sperm is then collected in a microtube. Then the sperm is inserted into the cryotube using a micropipette.

2.2. Cryopreservation
The sperm was then diluted with diluent solution (brown sugar + fish ringer (3.25 g NaCl, 0.125 g KCl, 0.175 g CaCl\(_2\).2H\(_2\)O, and 0.1 g NaHCO\(_3\) in 500 mL of distilled water) as extender + 10% methanol) with 0% brown sugar treatment (A), 5% (B), 10% (C), 15% (D), 20% (E), and 25% (F) (the modified method from Kurokura et al. 1984) [15,16]. Each sample was then equilibrated for 10 minutes at 4 °C before being stored in the freezer at -10 °C for 48 hours. The viability of the cryopreservation sperm was evaluated and then compared with the viability of fresh sperm.

2.3. Sperm quality
The quality of the sperm was tested for macroscopically (volume, color, consistency, and pH) and microscopically (motility).

2.4. Sperm viability
Sperm viability was evaluated by eosin staining technique [17]. One drop of sperm (±0.01 mL) was placed on the glass object, then the eosin liquid dye was added. Furthermore, preparation was made by pressing and pushing using a cover glass to form an angle of 45° then observed under a microscope with 400x magnification. Live spermatozoa will be light colored on the head, while the dead will be red-purple. The calculation was done for 100 spermatozoa cells and then the percentage between the living and the dead spermatozoa was calculated with the following formula.
2.5. Statistical analysis
The study was conducted with 6 treatments and 4 replications. Data obtained in the homogeneity and normality test. Data of this study was analyzed using Analysis of Variance (ANOVA) with $\alpha = 5\%$. Further tests were done using the Tukey Test to identify which treatments were significantly different. All statistical analyses were tested using SPSS Version 16.0 of 2007.

3. Result and discussion
Based on the results of the study, it is known that *T. soro* fresh sperm is milky white, thick, and has a pH of 8.5 (Table 1). After sperm was cryopreserved using brown sugar with different concentrations for 48 hours, the difference in viability values was found in Table 2 and Figure 3. The highest viability was found in treatment C (15%) in the amount of $83.75\pm1.71\%$ ($p<0.05$) and the lowest in treatment A (0%) is $72.00\pm2.94\%$. Although the viability of treatment C was no better than the viability of fresh sperm ($89.75\pm0.96\%$), the treatment was thought to be the optimum treatment, because the viability value was considered quite close.

Microscopic observations of sperm viability can be seen in Figure 1 and Figure 2. Even though the shape of the tail is not clearly visible, live sperm are marked by a white head, whereas inactive sperm show a red-purple color on the sperm head.

**Table 1. Quality of fresh *T.soro* spermatozoa.**

| Parameter   | Zairin et al. (2005) [6] | Based on this study |
|-------------|--------------------------|---------------------|
| Volume      | 3.92±1.44 mL             | 1.95±0.64 mL        |
| Color       | Milky white              | Milky white         |
| Consistency | Thick                    | Thick               |
| pH          | 7.6–7.9                  | 8.5                 |
| Motility    | 76.67±5.37%              | 93.23±1.31%         |

**Figure 1.** Viability of fresh sperm.

**Figure 2.** Viability of sperm after cryopreservation, (a) live sperm, (b) dead sperm.
Figure 3. Viability of spermatozoa after 48 hours cryopreservation.

Table 2. Viability of spermatozoa after cryopreservation.

| Treatment | Repetition (%) | Average (%) |
|-----------|---------------|-------------|
| Fresh sperm | 89.00 90.00 91.00 89.00 | 89.75±0.96 |
| A (0%) | 69.00 72.00 71.00 76.00 | 72.00±2.94ab |
| B (5%) | 79.00 78.00 77.00 80.00 | 78.50±1.29abc |
| C (10%) | 80.00 84.00 82.00 79.00 | 81.25±2.22abcd |
| D (15%) | 83.00 86.00 84.00 82.00 | 83.75±1.71bcd |
| E (20%) | 80.00 78.00 77.00 83.00 | 79.50±2.63bcd |
| F (25%) | 76.00 75.00 78.00 75.00 | 76.00±1.41ab |

The difference in the spermatozoa viability value of each sample tested was influenced by the different concentrations of brown sugar as a cryoprotectant. The brown sugar used in this study is known to contain ±76.5% sucrose. The content of sucrose in brown sugar is thought to maintain the viability of T. soro spermatozoa during the cryopreservation process. Based on this study results obtained, treatment with 15% brown sugar concentration shows the highest viability value because more spermatozoa lived than dead.

Living spermatozoa are characterized by not absorbing the eosin dye so that the spermatozoa are not colored in the head, while the dead spermatozoa are marked by the more viscous colored head [18]. The difference in the value of viability that occurs between sperm cryopreservation using brown sugar with fresh sperm can be caused by various factors. In many fish species, sperm cryopreservation has a detrimental effect and causes a significant decrease in spermatozoa viability [19]. However, sucrose can increase sperm viability after cryopreservation, because it can prevent cell dehydration and inhibit cell apoptosis [20].

Brown sugar is a product that is already familiar to the people of Indonesia [21]. Brown sugar has a variety of sucrose contents [22]. The sucrose content in sugar functions as an energy source substrate and at the same time as extracellular cryoprotectant which has been proven to be able to improve the quality of sperm [23,24]. As an energy source substrate, sugar will be metabolized through the glycolysis or continued with tricarboxylic acid reaction (Krebs cycle), so that the energy produced in the form of adenosine triphosphate (ATP) will be utilized by spermatozoa in their movements (motility), while as extracellular cryoprotectant, sugar will protect the cell plasma membrane and spermatozoa cells as a whole from mechanical damage that occurs during the cryopreservation process [24].

From the results of the study, it was suspected that the concentration of brown sugar that was too high or too low became less effective as cryoprotectants. The higher the concentration of brown sugar...
will be negative because it can damage cells. Meanwhile, if the lower the smaller the effect. Sugar added to the sperm diluent will provide optimal protection against the integrity of the cell plasma membrane and spermatozoa cells as a whole, especially at the drastic temperature changes that are during freezing and thawing. Reparation of the cell plasma membrane will positively influence the motility and viability of spermatozoa [25].

4. Conclusion
The conclusion of this study is that the use of 15% brown sugar has the highest potential to maintain the viability of spermatozoa of *T. soro* up to 83.75±1.71%. It is interesting to find out the potential of brown sugar as a natural cryoprotectant, especially in the cryopreservation of *T. soro* sperm. To determine the effectiveness of brown sugar as a cryoprotectant, it is necessary to conduct similar studies on other fish species.

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