The motility of *Tor soro* fish (Valenciennes, 1842) using post cryopreservation sperm: the effect of grape juice (*Vitis vinifera*) as a natural antioxidant

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Abstract. *Tor soro* is an endemic fish found on several islands in Indonesia including Java, Sulawesi and Kalimantan. *Tor soro* has decreased in number due to overfishing, habitat destruction and asynchronous maturation time of male and female gonad. One of the alternative solutions to solve the problem is sperm cryopreservation. Therefore, the objective of this study is to evaluate the effect of grape juice as a natural antioxidant on sperm motility of *Tor soro* 24 hours post cryopreservation. The sperm was collected through stripping method, and was diluted using 1:9 comparison into the soluble liquid (Fish Ringer + DMSO 10% + grape juice). The concentrations of the grape juice were 0%, 10%, 20%, and 30%, respectively. The equilibration was carried out at 4°C for 3 h, sample were then frozen at -196°C for 24 h in liquid nitrogen and were thawed at 40°C for 1 min. The sperm motility percentage was analyzed using ANOVA and was followed by Tukey test. The result showed that there was an effect (p<0.05) of grape juice on the post cryopreservation spermatozoa motility. The 10% grape juice showed the highest sperm motility (41.8 ± 11.2 %) 24 h post cryopreservation.

1. Introduction

*Tor soro* is an endemic fish that only found in Indonesia. The distribution of *Tor soro* includes Sumatera, Kalimantan and Java [1]. *Tor soro* has a high economic value because it is large in size and has a thick flesh that is popular for consumption [2]. In North Sumatera, *Tor soro* is considered a sacred fish and has a high cultural value because it is often used for traditional ceremonies. *Tor soro* has been collected from the wild because of their high demand value and now is becoming rare. In addition, high exploitation and habitat degradation is another problem and makes its population downgrading in number [3]. Besides that, the maturation time of male and female gonads does not occur simultaneously so there is little possibility of fertilization [4]. Sperm cryopreservation would contribute immensely to this [5].

Sperm cryopreservation is a technique for storing genetic material (spermatozoa) at low temperatures usually ranging from 4°C to -196°C in liquid nitrogen which aim to maintain cell life [6,7]. The cryopreserved spermatozoa can be used for artificial spawning to increase the availability of male gamete resources throughout the year to optimize the period time use of spermatozoa and
facilitate the process of \textit{in vitro} fertilization without the limitation of distance and time [8]. The principle of cryopreservation is storing of genetic material at low temperatures so the biological activity of cells is temporarily suppressed or inhibited and can be stored for a long time [9]. During spermatozoa cryopreservation (the freezing process) can cause cryoinjury due to oxidative stress. Oxidative stress is caused by cold shocks and osmotic stress that occur during cryopreservation [10].

Oxidative stress is a condition that occurs due to increased reactive oxygen species (ROS) beyond the ability of spermatozoa biological systems to detoxify [11]. ROS are free radicals that have oxygen atom and are very reactive. ROS has unpaired electrons so it tends to bind electrons to other compounds [12]. ROS attack the bonds of poly unsaturated fatty acids (PUFA) in the spermatozoa plasma membrane and cause lipid peroxidation that can inhibit oxidative metabolism in spermatozoa and decrease spermatozoa motility [13]. Therefore, the addition of antioxidants in cryopreservation of spermatozoa needs to be done to protect spermatozoa from the adverse effects of ROS so that the quality of spermatozoa is maintained [14].

The role of grape juice is a natural antioxidant to reduce cell damage because of oxidative stress during the freezing process. One of the antioxidants found in grapes is flavonoids [15]. Flavonoids are antioxidants that can neutralize free radicals by chelating metal ions to prevent oxidation. Flavonoids also have the ability to scavenge the free radicals to reduce lipid peroxidation [16]. DMSO is an intracellular cryoprotectant that commonly use in fish spermatozoa cryopreservation [17]. DMSO can reduce the freezing of intracellular ice crystals so that the cells do not experience damage [18]. This study therefore assesses as the preliminary project to know the efficacy of readily available semen extenders (DMSO and grape juice) for spermatozoa preservation of \textit{Tor soro} at liquid nitrogen.

2. Material and methods
2.1. Time and place
The study was conducted from September to December 2019 at the Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java.

2.2. Collection of sperm
The sperm collected by massaging the abdominal part of male fish with mature gonad (stripping method). The sperm that comes out was taken with a needleless disposable syringe and stored in 1,5 ml Eppendorf tube. The blood and feces contaminated sperm are removed [19].

2.3. Semen dilution
Sperm was diluted in a dilution solution consists of extender Fish Ringer + 10% DMSO + grape juice using a dilution ratio of 9: 1 of dilution solution and sperm. The concentration of grape juice used in this study were 0%, 10%, 20%, and 30% [20].

2.4. Cryopreservation process
The samples were equilibrated at a temperature of 4°C for 3 hours, frozen at the temperature of -196°C for 24 hours in liquid nitrogen and thawed at 40°C for 1 minute [21].

2.5. Parameters examined
The sperm macroscopic evaluation had conducted includes the measurement of color, pH, and volume of fresh sperm. The sperm microscopic evaluation had conducted includes observation and calculation of both fresh sperm and post cryopreservation sperm, using a light microscope with the image driving software; Image Focus 4.

2.6. Observation of motility
Total of 10 µl of fresh and post-cryopreservation semen was diluted using Ringer fish in a ratio of 1: 9 to obtain 10 times dilution. From the mixture, 10 µl is taken and added to 390 µl activator so that the total dilution is 400 times. The mixture is homogenized by up and down 10 times. Observation of
motility was done by counting the number of immotile spermatozoa and the total number of spermatozoa made in an improved Neubauer chamber and observed under a microscope with a magnification of 10 x 40. The formula for calculating the percentage of motility is as follows [22,23]:

\[
\% \text{ Motility} = \frac{\Sigma \text{motile spermatozoa}}{\Sigma \text{total spermatozoa}} \times 100\%
\]

2.7. Statistical analysis
Sperm motility percentage was evaluated using the analysis of variance (ANOVA) and also the Tukey test [24], which requires the SPSS software version 22.0.

3. Results and discussions
3.1. Analysis of fresh sperm
Fresh semen was obtained from 1 to 3 Tor soro fish with each individual body weight of 0.74 - 1.69 kg and an average fish length of 45.8 cm. Fresh semen characteristic were observed to have volume of 3.1 ml per individual, with pH of 8, a milky white coloration and 91.8 ± 5.2% fresh sperm motility (Table 1).

| Macrophscopic analysis  | Value              |
|-------------------------|--------------------|
| Volume                  | 3.1 ± 1.1 ml       |
| pH                      | 8                  |
| Color                   | Milky White        |

| Microscopic analysis     | Value              |
|-------------------------|--------------------|
| Sperm motility (%)       | 91.8 ± 5.2         |

The average volume of sperm obtained was 3.1 ml. In general, the semen volume of fish from the Cyprinidae family ranges from 0.2 to 3.55 ml per individual [25]. The research results obtained are also in accordance with research conducted by Junior et al. that the average volume of semen of individual Tor soro was ± 3.9 ml [26]. The difference in the volume of fresh semen can be caused by environmental conditions during maintenance such as temperature, season, water quality, and feed quality [27]. The pH value obtained is normal compared with the pH of fish semen from the Cyprinidae family which has a pH range between 6.2 up to 8.2 [28]. The results of the study are also in accordance with the research of Junior et al. that Tor soro has a pH ranging from 7.6 to 7.9. The fresh semen color obtained was milky white. The results of the study are the same when compared to the cement colors of other Cyprinidae [29]. The results of the percentage of sperm motility from fresh semen obtained was 91.8 ± 5.2%. Fish sperm that is suitable for use in cryopreservation is fish cement which has a motility value ≥ 80% [30]. Based on the obtained data, the fresh sperm can be used for cryopreservation.

3.2. Analysis of sperm after 24 hours cryopreservation
The mean percentage of motility of spermatozoa 24 hours post cryopreservation using the grape juice at a concentration of 0%, 10%, 20%, and 30%, were 26.3 ± 8.9%, 41.8 ± 11.2%, 19.93 ± 5.1%, and 8.2 ± 3.8%, respectively, as shown in Table 2.

| Treatment       | % Motility         |
|-----------------|--------------------|
| 0% grape juice  | 26.3 ± 8.9%        |
| 10% grape juice | 41.8 ± 11.2%       |
| 20% grape juice | 19.93 ± 5.1%       |
| 30% grape juice | 8.2 ± 3.8%         |

Based on the obtained data, the results of the analysis of variance analysis ANOVA indicated that there were significant differences between treatment groups (p<0.05). Tukey’s further tests showed that there were significant differences between the treatments of 0% grape juice with 10% and 30% grape juice as well as treatment with 10% grape juice with all treatment. Treatment with 10% grape juice exhibited the highest average motility percentage, which was 41.8 ± 11.2%.
Table 2. Percentage of sperm motility 24 hours post cryopreservation using grape juice as natural antioxidant (the mean value ± SD out of six repetition).

| Treatments       | Sperm Motility (%) |
|------------------|--------------------|
| Grape juice 0%   | 26.3 ± 8.9 %       |
| Grape juice 10%  | 41.8 ± 11.2 %      |
| Grape juice 20%  | 19.9 ± 5.1 %       |
| Grape juice 30%  | 8.2 ± 3.8 %        |

Figure 1. Percentage of spermatozoa motility after 24 h of cryopreservation using grape juice as a natural antioxidant

The results of this study indicate that there was an increase in motility of the treatment group of 10% grape juice which was 15.5% compared to control group (grape juice 0%). The research on the effect of grape juice on the quality of fish spermatozoa 24 hours post-cryopreservation has never been done before but the research result is similar range of the result conducted by Al-daraji that there was an increase in motility of post cryopreserved sperm roaster which was 13% [31]. The research result is similar range of the result conducted by Al-daraji. The difference in results can be due to the species used in the study are different and the type of extender used is different [32]. Based on the data obtained, the addition of grape juice 10% used as natural antioxidant in this study succeeded in maintaining the ability of post-cryopreservation spermatozoa motility in Tor soro with a yield of 41.8%.

Grape juice contains antioxidants in the form of flavonoids. Flavonoids work as free radical scavenger that causes lipid peroxidation in spermatozoa. Flavonoid is able to stop the lipid peroxidation chain reaction that causes damage to the spermatozoa membrane [33]. Flavonoids act as scavenger by donating hydrogen atoms from hydroxyl groups (-OH) to free radicals to form flavonoid phenoxy radicals (FIO). FIO is a more stable radical compound [34]. In the grape seed section, there is a strong antioxidant namely oligomeric proanthocyanin (OPC’s) which is a powerful antioxidant and has a function as a scavenger. OPCs in grape seeds can reduce the lipid peroxidation process and protect cells from free radicals [35,36]. OPC can also protect cells from oxidative stress and DNA fragmentation [37,38]. The content of vitamin C and vitamin E in grapes is also circulating as an antioxidant that has the ability to scavenging free radicals. Vitamin C and vitamin E can reduce
damage to cell membranes and increase the motility of spermatozoa [39]. Flavonoids can also increase the effectiveness of vitamin C, vitamin E and vitamin A [40].

This percentage results are lower when compared with the percentage value of spermatozoa motility by fresh semen. These results are consistent with research by Li Jun et al. which stated that the percentage of post-cryopreservation motility is lower than the motility of fresh semen spermatozoa [41]. The decreasing of post cryopreservation sperm motility can be caused by the process during cryopreservation such as dilution ratio, type of extender used, low sperm motility after thawing and decreased percentage of motile sperm during freezing and thawing [42,43].

4. Conclusions
In conclusion, it was established that combination of 10% DMSO and 10% grape juice as natural antioxidant shows the highest percentage of spermatozoa motility 41.8 ± 11.2%.

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