The effects of apple juice (*Malus sylverstris*-Mill) as a natural antioxidant on spermatozoa viability of *Tor soro* 24 hours postcryopreservation

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Abstract. The population of *Tor soro* in Indonesia continues to decline due to overfishing. In *T. soro* farming, the gonad maturity between males and females tends to be asynchronous. Thus, it is necessary to have a stock of milt that can be used at any time for fertilization of the eggs by cryopreservation. Cryopreservation can cause cell damage due to oxidative stress and can be reduced by the addition of antioxidants in the cryopreservation medium. Apple (*Malus sylverstris*-Mill) can act as an additional antioxidant to increase the chance of spermatozoa survival post-cryopreservation. This study aims to study the influence of adding various concentrations of apple juice (0 %, 10 %, 20 %, and 30 %) and determine which is most optimal in maintaining the viability of *T. soro* spermatozoa 24 hours post-cryopreservation with a 1:9 milt-diluent ratio. The mean viability of *T. soro* spermatozoa post-cryopreservation in 0 %, 10 %, 20 %, and 30 % concentrations are 12.01 ± 2.24 %, 8.10 ± 4.53 %, 53 ± 5.14 %, and 6.23 ± 4 % respectively. The results of the one-way ANOVA test showed that the apple juice in various concentrations did not affect the viability of *T. soro* spermatozoa 24 hours post-cryopreservation (P>0.5).

Keywords: Antioxidant, apple juice, *Malus sylvertris*-Mill, *Tor soro*

1. Introduction

Indonesia has a large potential for freshwater fish aquaculture. One species of freshwater fish that has great potential for further farming is *Tor soro* (*kanca* fish) [1] *T. soro* can be found in several islands in Indonesia, such as Sumatera and Java [2]. This fish is popular with people for consumption because it has a thick and juicy texture. *T. soro* also has its religious value and is used in traditional ceremonies [3]. Due to its high economic value, *T. soro* has experienced population decline by human activities such as overfishing [4]. Therefore, it is necessary to make efforts to conserve *T. soro*, both in-situ through re-stocking and ex-situ through manual breeding. To produce large quantities of *T. soro* is not easy if it only depends on the natural spawning process. The biggest challenge in *T. soro* farming is asynchronous of gonad maturity between males and females [5]. Thus, it is necessary to have milt stock with good quality that can be used at any time for fertilization of the eggs by cryopreservation.
Cryopreservation can cause cell damage (cryoinjury) such as ice crystal formation, osmotic stress, and oxidative stress if it is not carried out with proper protocols. Oxidative stress is caused by increased production of Reactive Oxygen Species (ROS). One of the impacts caused by oxidative stress can lead to damaged spermatozoa plasma membrane which results in reduced plasma membrane integrity and permeability. Both of these factors play a role in the survival of post-cryopreservation spermatozoa and can be measured by a viability test [6]. Oxidative stress can be minimized by adding antioxidants to the cryopreservation medium so that it can maintain the quality of spermatozoa during freezing. Antioxidants can work by capturing and eliminating formed ROS (scavenger antioxidants) or preventing the formation of ROS (prevention antioxidants) [7, 8].

Apples (Malus sylverstris) are a fruit that is widely consumed in various regions of Indonesia. Apples are easy to get and are very popular among people because they have certain benefits and a sweet taste. Regular consumption of apples can reduce various diseases such as heart disease, cancer, and diabetes. This is because apples are rich in antioxidants [9]. Research using apple juice as a natural antioxidant has been conducted by Adekunle [10] on cryopreservation of goat spermatozoa. The reports showed that the addition of 10% apple juice was able to maintain viability of spermatozoa up to 83% 24 hours post-cryopreservation. Another study using natural antioxidants in fruit was conducted by Adeyemo [11] using tomato juice in cryopreservation of African catfish (Clarias gariepinus) spermatozoa. The reports showed that the addition of 10% tomato juice was able to maintain spermatozoa motility only up to 60% against the control (85%) 24 hours post-cryopreservation.

The influence of apple juice addition on the cryopreservation of Tor soro spermatozoa is currently unknown. The antioxidants contained in apples can provide an additional supply to maintain the quality of spermatozoa post-cryopreservation [12]. Therefore, it is necessary to conduct cryopreservation research of T. soro spermatozoa by adding apple juice to the cryopreservation medium. This study was conducted to study the influence of adding various concentrations of apple juice (0%, 10%, 20%, and 30%) and determine which is the most optimal concentration in maintaining the viability of T. soro spermatozoa 24 hours post-cryopreservation. This research is expected to provide additional information in the conservation and cultivation of T. soro as well as a preliminary study in optimizing the cryopreservation technique of T. soro spermatozoa.

2. Experimental method

2.1. Preparation of the fish ringer solution
Fish Ringer’s extender solution is composed of various compounds, such as 750 mg NaCl, 20 mg KCl, 20 mg CaCl₂, 20 mg NaHCO₃ dissolved in 450 ml ddH₂O. Then ddH₂O was added until the total volume reaches 100 mL as a stock solution. The stock solution was stored in a refrigerator at 4°C in a closed glass bottle. The maximum storage time for this solution is 3 days [13].

2.2. Preparation of the activator solution
The activator solution was prepared by dissolving 45 mM NaCl, 5 mM KCl and 30 mM Tris in 100 mL distilled water [14].

2.3. Preparation of apple juice
Apples (‘Fuji’ variant) are washed with clean water, cut into small pieces with a knife. About 200 grams of apple is processed using a juicer, then the juice is filtered two times with filter paper number 1. The maximum storage time for the juice is 2 days [10].

2.4. Preparation of eosin-Y 0.5 % solution
The eosin-Y solution was prepared by dissolving 0.5 g of eosin-Y in 100 mL of distilled water. The stock solution was stored in a place protected from direct sunlight [15].
2.5. **Milt collection**
Milt sampling was carried out by stripping or spawning method through one-way massaging of the fish’s abdominal to the urogenital opening [16]. Artificial spawning was carried out by technicians of the Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk.

2.6. **Milt dilution**
Milt dilution was carried out at milt:diluent ratio of 1:9 in cryotube. The diluent solution was determined according to table 1.

2.7. **Equilibration and freezing**
Milt was equilibrated in a refrigerator at 3–4 °C for 3 hours [5] and froze in liquid nitrogen for 24 hours.

2.8. **Thawing**
Milt was thawed at 40 °C for 90 seconds [5].

2.9. **Milt macroscopic evaluation**
The macroscopic parameters of spermatozoa that were evaluated were volume, pH, and color.

2.10. **Spermatozoa viability evaluation**
The spermatozoa viability was observed by dropping 5 μL of eosin-Y on the slide and 5 μL of milt which had been diluted 10 times. Observations were conducted under a microscope 400 times magnification.

2.11. **Statistical analysis**
The study was conducted with 4 treatments and 6 replications. The data were tested by one-way ANOVA with α = 5 %.

3. **Results and discussion**
The physical properties of the fresh milt were milky white color, 7.5–8 pH, and 2.5 ± 0.7 mL volume per ejaculate (table 2). The percentage of viability of fresh milt obtained was 90.56 ± 5.87 %.

The percentage of viability was estimated based on the integrity of the plasma membrane [17]. According to Cabrita [18], the minimum viability value to meet the standards in cryopreservation of spermatozoa is 80 %. Dead and survived spermatozoa can be distinguished by eosin-Y staining (figure 1). The dead spermatozoa will be colored red by eosin because it cannot maintain the integrity of the plasma membrane and lose its selective permeable properties so eosin can penetrate the cell. The survived spermatozoa do not appear to be red and tend to be green. This can occur because it can maintain the integrity of the plasma membrane and prevent eosin from entering the cells [19].

| Experimental group | DMSO 10 % (µL) | Fish ringer (µL) | Milt (µL) | Apple juice (µL) |
|--------------------|---------------|-----------------|-----------|-----------------|
| Apple 0 % (Control)| 50            | 400             | 50        | 0               |
| Apple 10 %         | 50            | 350             | 50        | 50              |
| Apple 20 %         | 50            | 300             | 50        | 100             |
| Apple 30 %         | 50            | 250             | 50        | 150             |
Table 2. Physical properties and spermatozoa viability of fresh milt in 6 replications.

| Parameter   | Results                      |
|-------------|------------------------------|
| Volume (mL) | 2.5 ± 0.7                    |
| pH          | 7.5–8                        |
| Color       | Milky white                  |
| Viability (%)| 90.57 ± 5.87               |

*The value is in mean ± standard deviation

Figure 1. Viable spermatozoa (A), and non-viable spermatozoa (B).

The percentage of viability of *Tor soro* spermatozoa 24 hours post-cryopreservation (table 3) shows that the addition of various concentrations of apple juice did not give any significant difference (P > 0.05). The mean viability of *T. soro* spermatozoa 24 hours post-cryopreservation in 0 %, 10 %, 20 %, and 30 % concentrations were 12.01 ± 2.24 %, 8.10 ± 4.53 %, 53 ± 5.14 %, and 6.23 ± 4 % respectively. These results indicate that the mean viability of spermatozoa 24 hours post-cryopreservation is lower than the mean viability of spermatozoa pre-cryopreservation and seems very low compared to previous studies. The presumption is because the fish spermatozoa have less cryostability especially in mitochondrial membrane rather than mammalian spermatozoa. Thus, the mitochondrial apparatus of the mammalian spermatozoon is expected to be more stable than the fish spermatozoon [20]. The other reason is due to the damaged plasma membrane by the formation of intracellular ice crystals. According to Jun [21], the range of viable spermatozoa with intact membrane and a functional mitochondrion is about 0–18 %. The cell membrane became unstable and lost its osmoregulation because of the rupture of plasmalemma through intracellular ice-crystal formation during freezing or recrystallization during thawing.

The addition of fruit juice provides various kinds of protection for spermatozoa from the negative effects of cryopreservation because they contain several important compounds such as antioxidants and sugar. Sugar compounds in fruit (mainly fructose) can provide the supplements needed for spermatozoa respiration and maintain osmotic balance [22, 23]. Apple juice on the cryopreservation of *Tor soro* spermatozoa for 24 hours was not better than the control because the presence of apple juice can reduce the amount of antioxidants due to an antagonistic effect [24]. However, the influence of apple juice in long term *T. soro* spermatozoa cryopreservation is still unknown. For instance, the best concentration of apple juice on goat spermatozoa cryopreservation for 240 hours is 10 %. Meanwhile, the best results for 24 hours long cryopreservation of goat spermatozoa was shown at 7.5 % [10].
Table 3. Viability of postcryopreservation spermatozoa.

| Treatment | Spermatozoa viability (%) |
|-----------|---------------------------|
| Apple 0 % | 12.01 ± 2.24              |
| Apple 10 %| 8.10 ± 4.53               |
| Apple 20 %| 7.47 ± 4.13               |
| Apple 30 %| 5.96 ± 4.10               |

No significant difference based-on ANOVA test (P > 0.05)

The highest antioxidants in apples are vitamin C and phenolic compounds in the form of quercetin which can work as scavenger antioxidants [9]. According to Tavadyan [24], the antioxidant activity between quercetin and vitamin C has an antagonistic effect based on the rate of reaction which indicates that the antioxidant activity of quercetin is depleted faster at the presence of vitamin C. Ascorbyl radicals produced from vitamin C tend to be more reactive with the OH group in quercetin which does not have a glucosyl group, so that the antioxidant activity of the flavonoids is used up more quickly. In cryopreservation of spermatozoa, a rapid decrease in antioxidant activity is thought to cause an imbalance between the number of antioxidants and free radicals that are formed. A high concentration of free radicals (in this case ROS) can result in decreased viability of spermatozoa by damaging plasma membranes via Lipid Peroxidation (LPO) cascade. ROS comprises an oxidative attack of bis-allylic methylene groups of spermatozoa phospholipid-bound polyunsaturated fatty acids (PUFAs) and leads plasma membrane permeability and fluidity to be modified [7, 25]. The highest percentage value of post-cryopreserved spermatozoa viability was found in the control treatment (12.01 ± 2.24 %). This shows that the addition of apple juice in various concentrations (10 %, 20 %, 30 %) has not been able to maintain the viability of T. soro spermatozoa.

4. Conclusion
The addition of various concentrations of apple juice (0 %, 10 %, 20 %, and 30 %) did not have an effect in maintaining the viability of Tor soro spermatozoa 24 hours post-cryopreservation.

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