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The effect of tomato juice (*Lycopersicon esculentum*) as natural antioxidant to fertilization rate spermatozoa of kancra fish (*Tor soro*) 24 hours postcryopreservation

I Muhiardi[1], Abinawanto[1,3], J Subagja[2], O Z Arifin[2], N A Anabella[1], M Z Arief[1]

[1]Department of Biology, Faculty of Mathematics and Natural Science, Universitas Indonesia, 16424 Depok, Indonesia
[2]Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java, Indonesia

E-mail: abinawanto.ms@sci.ui.ac.id

Abstract. Kancra fish (*Tor soro*) is one of endemic freshwater fish which is originated from Sumatera island. Nowdays, the population of kancra fish is declining because of over fishing in the habitat. Therefore, it is necessary to preserve kancra fish by doing spermatozoa cryopreservation. The objective of present study is to evaluated the effect of tomato juice on fertilization rate 24 hour after spermatozoa cryopreservation. The study used a completely randomized design with four treatments and six replications. Four treatments consisted of 0% tomato juice + 10% DMSO (0% SBt); 10% tomato juice + 10% DMSO (10% SBt); 20% tomato juice + 10% DMSO (20% SBt); and 30% tomato juice + 10% DMSO (SBt 30%). Data was analysed by One Way Analyses of Variance test followed by Tukey test. The results showed that there was the tomato juice effect ($P<0.05$) on the fertilization rate after spermatozoa was deep frozen in liquid nitrogen (LN) for 24 hours has significant difference ($P<0.05$) in the average value of the percentage of fertilization rate. The results showed that the 10% of tomato juice was optimum concentration that showed the highest fertilization rate ($81.25 \pm 6.07\%$).

1. Introduction

Indonesia is a country that has local fish that has the potential to be developed in supporting the fish industry in Indonesia. Fish that can be developed in the industry is *Tor* [1,2]. *Tor* in Indonesia consist of four types, such as *Tor tambra*, *Tor tambroides*, *Tor duronensis*, and *Tor soro* [3,4]. *Tor soro* is fish of family Cyprinidae, known in the area of West Java as kancra, dewa, dan kermat fish [5]. *Tor soro* in North Sumatera known as ihan batak or batak fish [2]. The habitat of kancra fish in area of upsteam and the characteristic of upstream is fast flowing, rocky riverbed, clear water, high oxygen content, and cool water temperatures [4,6].

Kancra fish is much in demand by the public for consumption because it has good taste and thick meat and good nutritional content for body health. One of the ingredients contained in kancra meat is Fish Serum Albumin protein (FSA) which is useful for treating hypolalbuminemia. In addition to consumption, kancra fish has a religious value because it is used by the people of North Sumatra for traditional marriage ceremonies. The use of this causes fish kancra has a high economic value, the

consequences of high economic value causes fish exploitation of kancra in the wild is not controlled. Current information statuss that uncontrolled fishing causes threatened fish populations in extinction [4,7,8,9].

In order not to extinction, it is necessary to have a solution. The solution is cryopreservation of fish spermatozoa [10]. Cryopreservation with a temperature of -196^{o}C causes the spermatozoa that are preserved to have damage to cell function. Cell function can changes due to osmotic stress, cold shock, formation of ice crystals, and excessive production of reactive oxygen species (ROS) [11]. Therefore, to reduce damage to cryopreservation of spermatozoa cryoprotectants are needed [12]. The cryoprotectants commonly used are dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), glycerol (Gly) 1-2 propanediol, ethylene glycol (EG), and methanol (MeOH) [13]. DMSO is a good intracellular cryoprotectant because it has a low level of toxocity [14].

The function of spermatozoa due to the excessive production of ROS can be reduced by the addition of an amount of anticosidan which acts to protect intracellular damage [15]. In addition, antioxidants also role in fighting free radicals [16]. Tomatoes are known to contain many antioxidants, including lycopene, flavonoids, vitamins C and E [17,18,19]. Research on cryopreservation of fish spermatozoa with various cryoprotectant combinations has been done. The cryopreservation study of African catfish (*Clarias gariepinus*) by Adeyemo [20] using 10% tomato juice into a diluent solution resulted motility is 60%. Cryopreservation of spermatozoa using tomato juice (*Lycopersicon esculentum*) and dimethyl sulfoxide (DMSO) has not been reported. This study aims to determine the effect of giving tomato juice as a natural antioxidant on fertilization rate of spermatozoa.

2. Materials and methods

2.1 Time and research location

The study was conducted in September 2019 - December 2019 at the Installations for Freshwater Fish Genetic Resources (BRPBATPP), Ministry of Marine Affairs and Fisheries, Cijeruk, West Java.

2.2 Spermatozoa collection

Spermatozoa of kancra fish get from Installations for Freshwater Fish Genetic Resources. The Sperm and fish eggs are obtained from kancra fish that have matured gonads. The sperm were collected by abdominal gently striped method and place in 1,5 ml Eppendorf tube [21]

2.3 Eggs collection

Mature gonad in female of kancra fish are done by giving human chorionic gonadotropin (hCG) or ovaprim to induce ovulation. Hormone induction is done 16 hours before artificial spawning. The dose of hormones given is 0.8 ml/kg. The female of kancra fish Fish eggs were collected by striped method and 40 eggs place in container with dry condition [1].

2.4 Preparation of the extender fish ringer

Extender fish ringer is made by mixing 0,750 g NaCl, 0,02 g KCl, 0,02 g $CaCl_2$, dan 0,02 g NaHCO3. The ingredients are put into a 100 ml beaker glass and dissolved with 100 ml of distilled water and stirred using a stirring glass. The finished fish ringer is then placed in a dark glass bottle and stored in a refrigerator at 4^{o}C with a maximum storage time of three days [12,22].

2.5 Preparation of the tomato juice

Tomato juice is done based on Daramola et al. [23] which has been modified. Making tomato juice is done by cutting tomatoes into small pieces. Small pieces of tomatoes that have been cut are put into the juicer to be mashed. Water of tomato obtained is then transferred into a 100 ml glass beaker. Water of tomato that still contains fruit fiber is filtered using filter paper twice as much filtering. During filtration, filtered water is placed in another glass beaker. Filtering is done using Whatman filter paper

number one. The last step is to move the tomato juice into a dark glass bottle and stored at room temperature.

2.6 Preparation of the solvent solution

Solvent solution was made with mixed by three components and have three steps. The component is dimethyl sulphoxide (DMSO), extender fish ringer, and tomato juice. First step is mixed DMSO with extender fish ringer. The second step was made tomato juice, and than mixed all of component and added into cryotube with used micropippet 20 – 200 µL [21].

2.7 Spermatozoa dilution

Spermatozoa dilution was mixed with solvent solution. The solvent or dilution solution containing extender fish ringer, 10% of DMSO, and tomato juice. The composition of the solvent solution can be seen of Table 1. The composition of spermatozoa dilution based studied was modified by [20]. The dilution ratio for fresh sperm with dilution was 1: 9 based on [24,25].

Table 1. The composition of solvent solution for each experimantal group

Experimental group	DMSO 10% (µL)	Extender	Semen (µL)	Tomato juice (µL)
Control	50	400	50	0
Tomato juice 10%	50	350	50	50
Tomato juice 20%	50	300	50	100
Tomato juice 30%	50	250	50	150

2.8 Equilibration

Semen was mixed solvent dilution on cryotube then entered into refrigerator at 4^{o}C with 3 hours. Furthermore, the cryotube is then removes and then evaporated in the mouth of a liquid nitrogen canister for 3 minutes [5].

2.9 Freezing

The freezing process is carried out by wrapping the cryotube by using parafilm. The whole cryotube is wrapped with alumunium foil. The cryotube that has been wrapped placed in the canister. The cryotube is entered into a canister that has been filled with liquid nitrogen (N_2) for one day or 24 hours [5].

2.10 Thawing

The thawing process is come out of cryotube from liqiud nitrogen canister. The alumunium foil was wrapped in cryotube must be released and put cryotube into waterbath at 40^{o}C for 60 second [5].

2.11 Observation of fertilization rate

Observation of the fertilization rate is based on the modified studied by Pertiwi et al. [26].Observations were made by taking 40 eggs of fish that have been spawned. Furthermore, 40 eggs are then mixed with 50 µl of fresh semen or spermatozoa post cryopreservation in a plastic container and stirred using feathers. The next step, the mixture of semen and eggs is activated by using pondfish water that is added slowly, and agitated for two minutes, and then removed pondfish water. Furthermore, the container containing the egg is refilled with fishpond water, and incubated for 1 hour or 60 minutes. Eggs that have been incubated for 1 hour were observed using a stereo microscope by

counting the number of fertilized eggs. Fertilized egg by spermatozoa cells are characterized by the presence of animal poles [9,27]. The fertilization rate wa calculate as shown at equation:

$$FR = \frac{Number\ of\ fertilized\ eggs}{Number\ of\ eggs\ counted} \times 100\%$$

2.11 Statisctical analysis

Data analysis wa conducted by statistic program of Statistical Product and Service Solution (SPSS) version 22. Observation data obtained will be tested with nomality and homogeneity tests. The normality test used the Saphiro and Wilk test, while the homogeneity test used Levene. Data that have a normal and homogeneous distribution are tested by the One Way ANOVA test, then followed by Tukey test [28].

3. Result and discussion

3.1 Analysis of fertilized eggs from fresh semen

The average value of the percentage of fertilization rates on the spermatozoa of fish from fresh semen obtained was 97.5 ± 2.23%. Observation of the fertilization rate is observed by counting the number of eggs fertilized by spermatozoa that have been incubated for one hour. Fertilized egg are characterized by the division of the bud at the animal pole, as shwon in figure 1 [9]. The results of research conducted have differences with the literature. Results of research conducted [26] showed that the percentage of spermatozoa fertilization of lukas fish (*Puntius bramoides*) was 78.92 ± 1.81%.

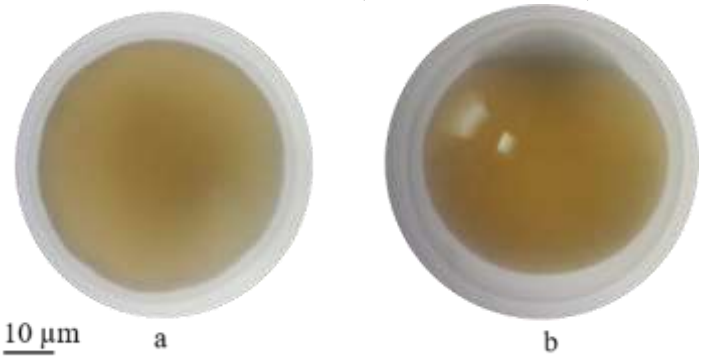

Figure 1. Fertilized (a) and unfertilized egg cell(a) (magnification of 10 x 4)

Fertilization is the fusion of male and female gametes so as to form diploid cells [29]. The fertilization rate is influenced by the concentration of spermatozoa which can affect the number of fertilized eggs. Spermatozoa with high levels of concentration cause a large density of spermatozoa, making it difficult for spermatozoa to enter the microphiles. Therefore, in carrying out artificial fertilization it is necessary to add a medium to thin the semen in spermatozoa fertilization, in addition to that dilution can also activate spermatozoa motility [26]. The fertilization is greatly influenced by the quality of spermatozoa especially the level of spermatozoa motility, if the level of spermatozoa motility is high then the level of fertilization rate is also increasing [30].

3.2 Analysis of fertilized eggs from cryopreserved spermatozoa

The results obtained by the fertilization rate of fresh spermatozoa is 97.5 ± 2.23%. The results of fertilization rate in post cryopreservation spematozoa were 70.41 ± 3.67% (SBt 0%), 81.25 ± 6.07% (SBt 10%), 58.33 ± 4.65% (SBt 20%), and 59.58 ± 6.00% (SBt 30%), as shown in table 2 and figure 2. The results of study prove the difference between fresh spermatozoa and post cyopreserved spermatazoa. The fertilization rate of post cryopreservation of spermatozoa has decreased compared to

fresh spermatozoa. That is because the cryopreservation of spermatozoa has decreased motility caused by damage to the cell membrane. In addition, the addition of high cryoprotectant concentrations causes the death of spermatozoa [31,32].

Table 2. Percentage of fertilization rate of kancra fish 24 hours post cryopreservation

Treatments	Fertilization rate (%)
Tomato juice 0%	70.41 ± 3.67 %
Tomato juice 10%	81.25 ± 6.07 %
Tomato juice 20%	58.33 ± 4.65 %
Tomato juice 30%	59.58 ± 6.00 %

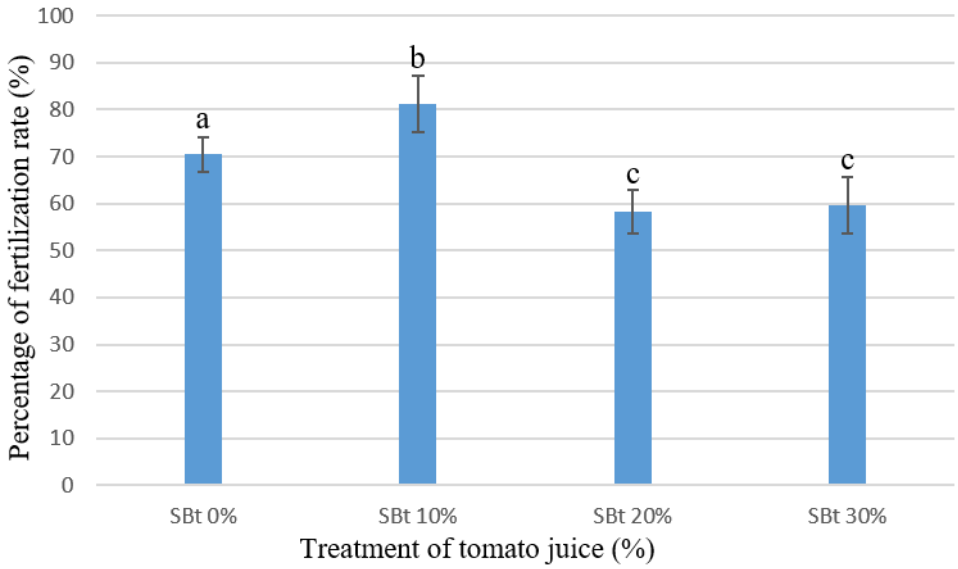

Figure 2. Bar chart of the average of the percentage of fertilization rate of kancra fish 24 hours post cryopreservation

Besed on research, the data was analysed by One Way ANOVA test and Tukey test. The data was anaylsed used One Way ANOVA test showed that the percentage of fertilization rate of kancra fish 24 hours post cryopreservation were significant different ($P < 0.05$). Furthermore, Tukey test toward the data of percentage of fertilization rate showed that indicate the treatment of tomato juice 0% (SBt 0%) and 10 % (SBt 10%) were significant different all of treatments, and then concentration of tomato juice 20% (SBt 20%) and 30% (SBt 30%) significant different to tomato juice SBt 0% and SBt 10%.

The fertilization rate is influenced by the motility and integrity of spermatozoa DNA [33]. Study on the fertilization rate obtained an average percentage higher in fresh spermatozoa compared with post cryopreservation. That is because cryopreservation can cause DNA fragmentation. DNA fragmentation occurs due to increased oxidative stress which triggers apoptosis [34]. Oxidative stress caused by changes in mitochondrial membrane fluidity that occurs during freezing can cause excessive ROS production. ROS production causes lipid peroxidation (LPO) membranes which can reduce spermatozoa motility. In addition, the effects of ROS production also cause DNA damage by break the double strand of DNA. This causes difficulty in penetration and fusion of spermatozoa with eggs [35,36].

This study on cryopreservation of spermatozoa of kancra fish using various concentrations of tomato juice. Tomato juice with the right concentration can maintain the quality of spermatozoa, which is to protect spermatozoa from damage during cryopreservation. The ability of spermatozoa fertilization with the highest percentage obtained was 81.25 ± 6.07% (SBt 10%). Tomatoes contain a variety of natural antioxidants such as lycopene, flavonoids, vitamins C and E [19]. The addition of antioxidants in the extender can reduce spermatozoa damage [37]. According to [38], vitamin C can fight free radical hydrogen peroxide (H_2O_2) which is a major factor in DNA spermatozoa damage.

4. Conclusion

The conclusion of this study is the administration of tomato juice with concentration of 10% and DMSO was optimum concentration that showed the highest fertilization rate 81.25 ± 6.07%.

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