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

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Abstract. Kancra fish (Tor soro) is one of endemic freshwater fish which is originated from Sumatera island. Nowadays, 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 ± 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 upstream 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°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 toxicity [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 CaCl₂, 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°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
Solvant 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].

| Experimental group | DMSO (µL) | Extender (µL) | 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°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 aluminium 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 (N₂) for one day or 24 hours [5].

2.10 Thawing
The thawing process is come out of cryotube from liquid nitrogen canister. The aluminium foil was wrapped in cryotube must be released and put cryotube into waterbath at 40°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 agitation 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 was calculated as shown at equation:

\[
FR = \frac{\text{Number of fertilized eggs}}{\text{Number of eggs counted}} \times 100\%
\]

2.11 Statistical analysis
Data analysis was conducted by statistical program of Statistical Product and Service Solution (SPSS) version 22. Observation data obtained will be tested with normality and homogeneity tests. The normality test used the Shapiro 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 shown 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)](image)

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 spermatozoa 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 cryopreserved spermatozoa. 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 %         |

![Bar chart of the average of the percentage of fertilization rate of kancra fish 24 hours post cryopreservation](image)

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

Based on research, the data was analysed by One Way ANOVA test and Tukey test. The data was analysed 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 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 (H2O2) 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%.

References
[1] Radona D, J Subagia, Arifin O Z 2015 Peforma reproduksi induk dan pertumbuhan benih ikan tor hasil persilangan (Tor soro dan Tor douronensis) Secara Resiprokal (Performance of parent reproduction and growth of cross-bred Tor fish (Tor soro and Tor douronensis) reciprocally) J. Riset Akuakultur 10 335 – 343. [In Indonesian].
[2] Wahyuningsih H, M Z Junior, Sudrajat A O, Tumbelaka L I T A, Manalu W 2012 Perubahan plasma darah dan kematangan gonad pada ikan betina Tor soro di kolam pemeliharaan (Changes in blood plasma and gonad maturity in female Tor soro fish in rearing ponds) J. Ikhtiolgi Indonesia 12 25 – 34. [In Indonesian]
[3] Haryono 2006 Aspek biologi ikan tambra (Tor tambroides Bikr.) yang ekosotik dan langka sebagai dasar domestikasi (Biological aspects of Tambra fish (Tor tambroides Bikr.) Which are exotic and rare as the basis for domestication) Biodiversitas 7 195 – 198. [In Indonesia]
[4] Haryono and J Subagia 2007 Pertumbuhan ikan tambra (Tor tambroides) dan kancera (Tor soro) pada proses domestikasi dengan jenis pakan yang berbeda (Growth of tambra fish (Tor tambroides) and kancera (Tor soro) in the domestication process with different types of feed) J. Biologi Indonesia 4 167 – 175. [In Indonesian]
[5] Junior M Z, Handayani S, Supriyatnya I 2005 Kualitas ikan batak (Tor soro) hasil kriopreservasi semen menggunakan dimetil sulfoksida (DMSO) dan gliserol (Quality of Batak fish (Tor soro) from cryopreservation of cement using dimethyl sulfoxide (DMSO) and glycerol) J. Akuakultur Indonesia 4 145 – 151. [In Indonesian]
[6] Haryono and Subagia J 2008 Populasi dan habitat ikan tambra, Tor tambroides (Bleeker, 1854) di perairan kawasan pegunungan Muller Kalimantan Tengah (Population and habitat of tambra fish, Tor tambroides (Bleeker, 1854) in the waters of the Muller mountain region of Central Kalimantan) Biodiversitas 9 306 – 309. [In Indonesian]
[7] Kristanto A H, Asih S, Winarlin 2007 Karakterisasi reproduksi dan morfometrik ikan batak dari dua lokasi (Sumatera Utara dan Jawa Barat) (Reproductive and morphometric characterization of batak fish from two locations (North Sumatra and West Java)) J. Riset Akuakultur 2 59 – 65.
[8] Siregar B, Barus T A, Ilyas S 2013 Hubungan antara kualitas air dengan kebiasaan makanan ikan batak (Tor soro) di perairan sungai Asahan Sumatera Utara (The relationship between water quality and the dietary habits of Batak (Tor soro) fish in the waters of the Asahan River, North Sumatra) J. Biosains Unimed 1 1 – 11. [In Indonesian]
[9] Arifin O Z, Subagia J, Asih S, Kristanto A H 2019 Budidaya Ikan Dewa. IPB Press Bogor Indonesia: xix + 101 pp. [In Indonesian]
[10] Kurniawan I Y, Basuki G, Susilowati T 2012 Penambahan air kelapa dan gliserol pada penyimpanan sperma terhadap motilitas dan fertilitas spermatozoa ikan mas (Cyprinus carpio L.) (The addition of coconut water and glycerol to sperm storage on motility and fertility of carp spermatozoa (Cyprinus carpio L.)) J. of Aquaculture Management and Technology 2 51 – 56. [In Indonesian]
[11] Chatterjee S and Gagnon C 2001 Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing Mol. Reprod. and Dev. 59 451 – 458.

[12] Muchlisin Z A 2005 Review: Current status of extenders and cryoprotectants of fish spermatozoa cryopreservation Biodiversitas 6 66 – 69.

[13] Magnotti C, V Cerqueira, Lee-Estevez M, Farias J G, Valdebenito I, Figueroa E 2016 Cryopreservation and vitrification of fish semen: A review with special emphasis on marine species Review in Aquaculture. 1 – 11.

[14] Fathi R, Valejordi M R, Salehnia M 2013 Effect of different cryoprotectant combinations on primordial follicle survivability and apoptosis incidence after vitrification of whole rat ovary CryoLatters 34 228 – 238.

[15] Gadea J, Molla M, Selles E, Marco M A, Garcia-Vazquez F A, Gardon J C 2011 Reduced glutathione content in human sperm is decrease after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders Cryobiology 62 40 – 46.

[16] Silva S V, Soares A T, Batista A M, Almeida F C, Nunes J C, Peixoto C A, Guerra M M P 2011 In Vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathion Reprod. Domest. Anim. 46 874 – 881.

[17] Toor R K, Savage G P, Lister C E 2005 Release of antioxidant components from tomatoes determined by an in vitro digestion method Int. J. Food Sci. Nutr. 60 119 – 129.

[18] Kefer J C, Agarwal A, Sabanegh E 2009 Role of antioxidants in the treatment of male infertility Int. J. Urol. 16 449 – 457.

[19] Bourguini R G, Bastos D H M, Moita-Neto J M, Capasso F S, Torres E A F S 2013 Antioxidant potential of tomatoes cultivated in organic and conventional system Braz. Arch. Biol. Tech. J. 56 521 – 529.

[20] Adeyemo O K, Adeyemo O A, Oyeyemi M O, Agbede S A 2007 Effect of semen extenders on the motility and viability of stores African catfish (Clarias gariepinus) spermatozoa J. Application Sciences Environment Management 11 13 – 16.

[21] Abinawanto, Zuraida Z, Lestari R 2016 The effect of skim milk combined with 5% of methanol on motility, viability, dan abnormality of java barb, Barbonymus gonionotus spermatozoa after 24 hours freezing AACL Bioflux 9 326 – 333.

[22] Kurokura H, Hirano R, Tomita M, Iwahashi M 1984 Cryopreservation of carp semen Aquaculture 37 267 – 273.

[23] Daramola J O, Adekunle E O, Onagbesan O M, Oke E O, Ladokun A O, Abiona J A, Abioja M O, Oyewusi I O, Oyewusi J A, Isah O A, Songunle O M, Adeleke M A 2016 Protective effects of fruit-juices on sperm viability of west african dwarf goat bucks during cryopreservation. Animal Reproductive 13 7 – 13.

[24] Horvath A, Miskolezi E, Urbanby B 2007 Cryopreservation of common carp sperm. Aquat. Living Resour. 16 457 – 460.

[25] Sunarma A, Hastuti D W B, Saleh D M, Sistina Y 2008 Kombinasi efektif ekstender dan krioprotekan pada kriopreservasi sperma ikan nilem (Osteochilus hasseltii Valenciennes, 1842) (Effective combination of extender and cryoprotecan in cryopreservation of nilem fish sperm (Osteochilus hasseltii Valenciennes, 1842)) J. Perikanan 10 76 – 84. [In Indonesian]

[26] Pertiwie P, Abinawanto, Yimastria S 2018 Fertilization rate of Lukas Fish (Puntius bromoides) AIP Conference Proceedings 2023 1 – 4.

[27] Haniffa M A, Benziger P S A, Arockiaraj A J, Nagarajan M, Siby P 2007 Breeding behaviour and embryonic development of koi carp (Cyprinus carpio) Taiwania 52 93 – 99.

[28] Zar J H 1974 Biostatistical Analysis Prantice-Hall Inc, London: xiv + 620 pp.

[29] Coward K, Bromage N R, Hibbitt O, Parrington J 2002 Gamete physiology, fertilization and egg activation in teleost fish Rev. Fish Biol. Fish. 12 33 – 58.

[30] Adipu Y, Sinjal H, Watung J 2011 Ratio pengenceran sperma terhadap motilitas spermatozoa, fertilitas dan daya tetas ikan lele (Clarias sp.) (The ratio of sperm dilution to sperm motility, fertility and hatchability of catfish (Clarias sp.)) J. Perikanan dan Kelautan Tropis 7 48 –
55. [In Indonesian]

[31] Hiemstra S J, van Der Lende T, Woelders H 2005 The potential of cryopreservation and reproductive technologies for animal genetic resources conservation strategies. The Role of Biotechnology 25 – 36.

[32] Bozkurt Y 2005 Effect of different cryoprotective on viability of mirror carp (Cyprinus carpio) spermatozoa. Cilt I Sayı 1 63 – 67.

[33] Bozkurt Y and Secer S 2005 Effect of short-term preservation of mirror carp (Cyprinus carpio) semen on motility, fertilization, and hatching rates. The Israeli Journal of Aquaculture 57 207 – 212.

[34] Thomson L K, Fleming S D, Aitken R J, de Luliis G N, Zieschang J A, Clark A M 2009 Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum. Reprod. 24 2061 – 2070.

[35] Said M, Gaglani A, Agarwal A 2010 Implication of apoptosis in sperm cryoinjury Reproductive BioMedicine Online 21 456 – 462.

[36] Amidi F, A Pazhohan, M S Nashtaei, M Khodarahmian, and S Nekoonam 2016 The role of antioxidants in sperm freezing: A review Cell Tissue Bank 1 – 12.

[37] Lahnsteiner F, Mansour N, Kunz F A 2011 The effect of antioxidants on quality of cryopreservation semen in two salmonid fish, the brook trout (Salvelinus fontinalis) and the rainbow trout (Oncorhynchus mykiss) Theriogenology 76 882 – 890.

[38] Sierens J, Hartley J A, Campbell M J, Leathem A J C, Woodside J V 2002 In vitro isoflavone supplementation reduces hydrogen peroxide-induced DNA damage in sperm Teratog, Carcinog, Mutagen. 22 227 – 234.

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