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The Effects of Brown Sugar as a Natural Cryoprotectant on *Tor Soro* (Valenciennes 1842) Spermatozoa Quality

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Abstract: The endemic kancra fish (*Tor soro*, Valenciennes 1842) is one of Indonesia's endemic species whose population is dwindling as a result of environmental deterioration and overfishing. Cryopreservation is a method for preserving *T. soro* genetic resources and overcoming population decline. The success of cryopreservation in maintaining the spermatozoa quality depends on cryoprotectants. One of the obstacles in cryopreservation is reducing toxicity, which may be overcome with the inclusion of natural cryoprotectants. This researchinvestigated the effects of brown sugar as a natural cryoprotectant in combination with 10% methanol on *T. soro* spermatozoa quality after 48 hours post-cryopreservation. The sperm was collected by stripping and was then diluted. The concentrations of brown sugar used in this study were 5%, 10%, 15%, 20%, and 25%. Storage was carried out in the freezer at -10 °C for 48 hours. One-way ANOVA and followed by Tukey test showed that various concentrations (5-20%) of brown sugar in combination with 10% methanol had a significant ($P < 0.05$) effect on post-cryopreserved spermatozoa motility, abnormality, and fertilization rate. The 15% brown sugar showed the highest percentage of motility (81.85 ± 1.11%), the highest percentage of fertilization ability (89.75 ± 1.71%), and the lowest percentage of abnormality (14.50 ± 1.73%) on *T. soro* post-cryopreserved spermatozoa. The overall results showed that the 15% brown sugar in combination with 10% methanol is the optimum concentration to maintain the spermatozoa quality of *T. soro* 48 hours post-cryopreservation.

Keywords: brown sugar, cryopreservation, kancra fish, spermatozoa quality, *Tor soro.*

红糖作为天然冷冻保护剂对托索罗(瓦朗谢讷 1842)精子质量的影响

摘要：**地方性坎克拉**鱼（**托索**罗，**瓦朗**谢讷 1842）**是印度尼西**亚的特有物种之一，**由于**环**境**恶化和过度捕捞，**其种群数量正在减少**。**冷**冻保存是保存托索罗遗传资源和克服种群下降**的一种方法**。**冷**冻保存在维持精子质量方面的成功取决于冷冻保护剂。**冷**冻保存的障碍之一**是降低毒性**，这可以通过加入天然冷冻保护剂来克服。**本研究**调查了作为天然冷冻保护剂的红糖与 10% **甲醇**组合对冷冻保存 48 **小**时后托索罗精子质量的影响。**通**过剥离收集精子，**然后稀**释。**本研究中使用的**红糖浓度分别为 5%、10%、15%、20% **和** 25%。**在**-10°C **的冰箱中**储存 48 **小**时。单因素方差分析和随后的图基检验表明，**不同**浓度（5-20%）**的**红糖与 10% **甲醇**组合对冷冻保存后的精子活力、**异常和受精率具有**显着（P < 0.05）**的影响**。 15%红糖对托索罗冷冻保存后的蠕动百分比最高（81.85±1.11%），**受精能力百分比最高**（89.75±1.71%），**异常百分比最低**（14.50±1.73%）**精子**。总体结果表明，15% 红糖与 10% **甲醇的**组合是在冷

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冻保存 48 小时后保持托索罗精子质量的最佳浓度。

关键词：红糖，冷冻保存，坎克拉鱼，精子质量，托索罗。

1. Introduction

Kancra fish (*Tor soro*, Valenciennes 1842) is a freshwater fish species of Cyprinidae family spread in several regions in Indonesia such as Java, Sumatra, and Kalimantan [1]. Kancra fish grows in water conditions with a high oxygen content and a rocky substrate [2]. Kancra fish in West Java is also known as dewa fish [3], as well as batak fish or ihan fish in North Sumatra [4]. *Torsoro*or kancra fish is an endemic species in Toba Lake and several water reservoirs in West Java as are the other three species identified from genus *Tor*, which are *T. douronensis, T. tambra, and T. tambroides*, [5].

T. soro have economic value as a consumption fish [6]. The cost of *T. soro* ranges IDR 250,000-500,000 per kg, moreover it can even reach IDR 1,500,000 rupiah for only one fish [7]. Besides the big body size of *T. soro*up to 20 kg and having a good taste, the high price of *T. soro* are caused bythe exploitation of *T. soro* in wild. Due to overfishing and environmental problems, *T. soro* is becoming increasingly difficult to find [1, 8, 9]. The deterioration of the T. soro habitat is influenced by anthropogenic activities and the construction of hydropower dams [10]. Furthermore, the population of *T. soro* in North Sumatra's Asahan River is declining at a rate of 4.09 percent per year [6]. The population decline of *T. soro* must be stopped promptly. One of the efforts to overcome it is cultivation using fish seeds obtained through spawning, but this process is naturally hampered by gonad synchronization. Thus, an alternative reproductive technology is needed, one of them is by doing cryopreservation [3, 5].

Cryopreservation is a method for maintaining genetic material [11]. Storage by cryopreservation has the advantage of being more efficient in terms of cost, time, storage space, and energy than other methods. The temperature at which a cell is stored varies, although the most frequent temperature is 0 °C, with the lowest temperature reaching -196 °C in liquid nitrogen [11, 12]. Cryopreservation is generally used for sperm storage because of its resistance to low temperatures when compared to embryo or ovum [13]. Meanwhile, very low temperature during freezing results in leakage of vital substances in sperm so that intracellular enzymes, lipoproteins, ATP, and intracellular potassium are reduced, thus causing damage to the cell so that the viability value decreases [14].

In cryopreservation, cell damage during freezing can be prevented [15]. The addition of cryoprotectant to sperm diluent improves cell survival after the freezing process. Moreover, utilization of a suitable cryoprotectant is one of the key factors for the success of the cryopreservation protocol [16], especially in long-term cryopreservation.

Good cryoprotectants are environmentally friendly, non-toxic, easily prepared, and available at affordable prices [17]. Due to long term storage in low temperature which is potentially increasing the chilling injury, additional extracellular cryoprotectant that have low toxicity is needed, such as a natural cryoprotectant [18]. A natural cryoprotectants are defined as materials originating from nature that do not contain artificial chemical compounds, and it has low toxicity [19].

In Indonesia, there are traditional sugars which are obtained from heating the palm sap to crystallize or commonly called as brown sugar [20]. The color formed in brown sugar is caused by Maillard's non-enzymatic browning reaction [21]. Brown sugar has a higher sucrose content when compared to some other natural extracellular cryoprotectants that are often used for cryopreservation. Brown sugar also contains higher total phenol than white sugar and refined sugar. According to Ondho [22], phenol as an antioxidant in diluents can break the lipid peroxidation chain of cell plasma membranes.

Brown sugar has been shown to be able to maintain sperm quality in cow and sheep [23-25]. However, the utilization of brown sugar in fish sperm cryopreservation has not been well studied. Therefore, this study is needed to evaluate the effects of brown sugar as a natural cryoprotectant on the *T. soro* spermatozoa quality, including motility, abnormality, and fertilization rate.

2. Methods

2.1. Location and Time of Research

The research was conducted for 8 months atthe Installations for Freshwater Fish Genetics Resources, Ministry of Marine Affairs and Fisheries, Bogor, Indonesia.

2.2. Male Broadstock Selection

Male *T. soro*broodstock was obtained from Installation for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, Bogor, West Java, Indonesia. The males had an average age of 1 year and weighed more than 300 g [26].

2.3. Sperm Sampling

Sperm sampling was done in February–September 2021 according to the natural spawning time. Sperm was sampled from matured gonad fish by stripping the abdomen and aspirating the sperm using a 3 mL disposable syringe without a needle. Urine and other contaminations were avoided, taken together with the next preparation steps [27].

2.4. Dilutions

Before equilibrating and freezing, the collected sperm was diluted in diluent solutions; fish Ringer (3.25 g NaCl, 0.125 g KCl, 0.175 g CaCl2.2H_2O, and 0.1 g NaHCO3 in 500 mL of distilled water) and 10% methanol according Abinawanto & Pramita [27] together with various concentrations of liquid brown sugar (5% 10%, 15%, 20%, or 25%) or without brown sugar (0%) as a control.

2.5. Cryopreservation and Evaluation

The steps of cryopreservation and evaluation are shown in Figure 1 as a flowchart.

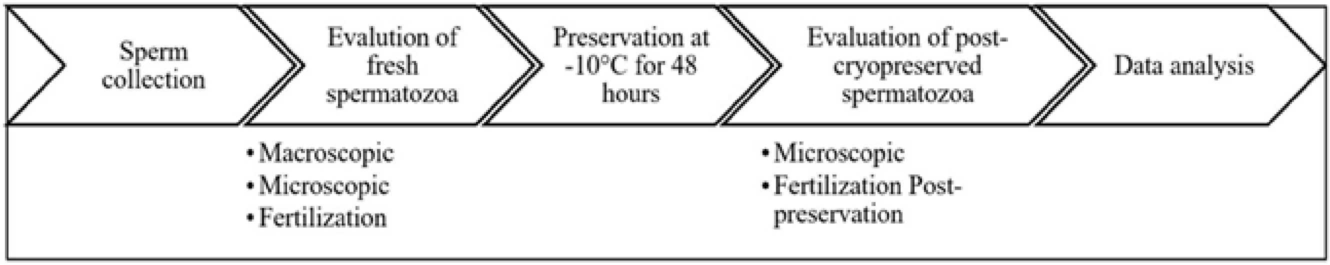


Fig. 1 Research flowchart

2.5.1. Equilibration, Freezing, and Thawing

Equilibration was carried out by storing the diluted sperm at 4 °C for 10 minutes [18]. Freezing was done at -10 °C for 48 Hours, and the thawing process was conducted by immersing the cryotube in a water bath at 40 °C for 60 seconds [28, 29].

2.5.2. Macroscopic Evaluation

The sperm was evaluated macroscopically by observing the volume, color, and pH before cryopreservation. The volume of sperm (semen) was measured in a scale cryotube. The color of sperm collected in a cryotube was observed visually. The pH was measured using a universal pH meter [29].

2.5.3. Microscopic Evaluation

Microscopic analysis was done by measuring spermatozoa motility and abnormality before and after cryopreservation. Before the analysis, the sperm was diluted with Ringer (3.25 g NaCl, 0.125 g KCl, 0.175 g $CaCl_2$.2H_2O) based on reports in previous studies [29, 30]. The spermatozoa motility was then analyzed by using Improved Neubauer. The abnormality of fresh and post-cryopreserved spermatozoa was analyzed by using Giemsa solution [31].

2.5.4. Female Broodstock Selection

Female *T. soro* broodstock was obtained from Installation for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, with hormones twice to induce the ovulation [32].

2.5.5. Ova Collection

The ova were collected 12 hours after the second hormone induction. The method used was stripping carried out under the shade [33].

2.5.6. Fertilization

The fresh sperm used for cryopreservation and observation were taken partly for fertilization. Fertilization was carried out by mixing them with ova in a plastic basin. Fertilization using post-cryopreserved spermatozoa was conducted by mixing them with 50 ova and were gently stirred for 2 min [34].

2.5.7. Fertility Rate Observation

The fertility rate was observed two hours post-fertilization. A fertilized egg forms a cleavage shoot and shows bright color [35].

2.5.8. Data Analysis

Data in this study were obtained from macroscopic and microscopic evaluations with 6 treatments and 4 replications. The data analysis was performed on microscopic evaluation results, which are percentage of motility, abnormality, and fertilization rate of fresh and 48 hours post-cryopreserved spermatozoa quality. The normally distributed and homogeneous data were subjected to a parametric test with one-way analysis of variance (ANOVA) and followed by the Tukey multiple comparison test [36]. The analyzed data were then presented in the form of tables.

3. Results and Discussion

3.1. Macroscopic Evaluation of Fresh Semen

Initial examination of spermatozoa quality is very important to determine whether the semen used is feasible or not to be used for cryopreservation. Fresh semen of *T. soro* was obtained from fish with an average weight of 0.85 kg. Macroscopic evaluation data can be seen in Table 1.

Table 1 Macroscopic analysis of fresh semen (sperm)

Parameter	Results
Color	Milky white
Average volume (mL)	1.95 ± 0.64
pH range	8 – 8.5

The volume of fresh *T. soro* semen ranged from 1.5 to 2.4 mL/ejaculate, with an average of 1.95 ± 0.64 mL/ejaculate. The semen volume was lower than the semen volume obtained by Junior et al. [3] that is 3.92 ± 1.44 mL/ejaculate. Semen volume can be influenced by several factors such as age, feeding management, and the frequency of ejaculation [37].

The color of *T.soro* semen is milky white, as well as reported by Junior et al. [3] and reports in several freshwater fishes. The color of semen is influenced by concentration, thus the fewer spermatozoa the color of the semen will be clear, while the more the number of spermatozoa the semen will be whitish like milk.

The pH value of T. *soro* semen in this study was 8–8.5. The pH values tend to be alkaline and higher when compared to the pH of fresh *T.soro*semen that previously reported by Junior et al. [3], which is equal to 7.6–7.9. The normal pH interval for fish of the Cyprinidae family is 7.5–8. The difference in pH values indicates that variations in pH values can also occur within the same species [38].

3.2. Microscopic Evaluation of Fresh Spermatozoa: Motility and Abnormality

The results of motility and abnormality evaluation in fresh spermatozoa (before cryopreservation) can be seen in Table 2. The evaluation showed that the percentage of fresh spermatozoa motility was 93.23 ± 1.31%. The average percentage of fresh spermatozoa motility in this study was found relatively higher than that of Junior et al. [3], which is 76.67 ± 5.37%. The motility of fish spermatozoa can be different, even though they come from the same individual or species. This is because the quality of spermatozoa, including motility, is influenced by several factors, such as age, size, and physiology. The high concentration of K+ ions in the plasma fluid causes the spermatozoa to become immotile. In addition, inside the fish male reproductive organs or in an environment containing the same osmolality as the semen, spermatozoa do not move [38]. Thus, to observe the spermatozoa motility, the semen was diluted first with an activator and fish Ringer to induce the movement of spermatozoa.

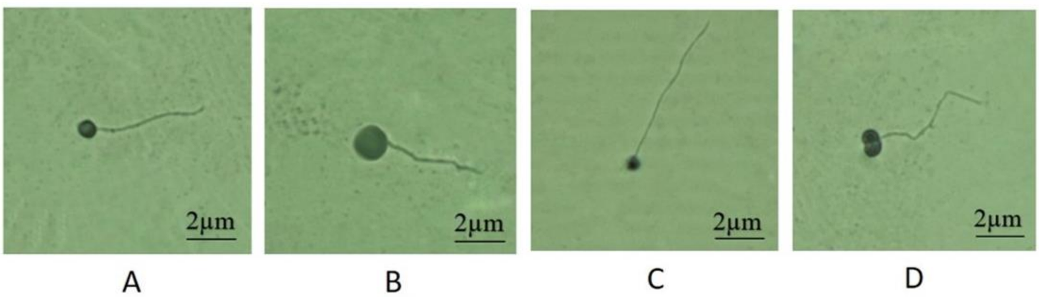
Table 2. Microscopic analysis of fresh spermatozoa

Parameter	Value (%)	Standard (%)
Motility	93.23 ± 1.31	> 70 [39]
Abnormality	12.50 ± 1.73	< 50 [40]

Fresh semen obtained in this study were eligible to be used in cryopreservation because the motility value of spermatozoa is above the standard or more than 70% [41].

The average percentage of abnormality in fresh spermatozoa obtained in this study was 12.50 ± 1.73% (see Table 2). Based on this parameter, fresh sperm of *T.soro* obtained in this study can be said to be eligible for cryopreservation because the average percentage of abnormality is less than 50% [42] or nearly 88% of evaluated fresh spermatozoa had normal structure. This value is nearly similar with other values in previous reports, especially in other freshwater fishes, such as baung fish (13.96 ± 4.86%), gouramy (13.33 ± 2.58%), and botia fish (16.00 ± 3.46%) [24].

The types of abnormal structure found in fresh spermatozoa of *T. soro* can be seen in Fig. 2. During observation, spermatozoa with macrocephaly, microcephaly, and double heads were mostly found. This kind of abnormalities is classified into primary abnormalities. According to Zulyazaini et al. [42], the primary abnormalities in fresh spermatozoa can occur because of some disruptions during spermatogenesis in the seminiferous tubules and after the spermatozoa leave the seminiferous tubules.



A: Normal spermatozoa C: Microcephalic spermatozoa
B: Macrocephalic spermatozoa D: Double-headed spermatozoa
Fig. 2 Abnormalities found in fresh spermatozoa of *T. soro*.

3.3. Microscopic Evaluation of Spermatozoa 48 h Post-Cryopreservation

Microscopic evaluation of *T. soro* spermatozoa 48 hours post-cryopreservation included motility and abnormality observation can be seen in Table 3. Based on the observation in *T. soro* spermatozoa 48 hours post-cryopreservation using brown sugar, the highest motility was found in the 15% brown sugar treatment, which was 81.85 ± 1.11%, while the lowest motility was found in the 0% brown sugar treatment, which was 68.36 ± 1.20%. On the other hand, the highest abnormality was found in the 0% brown sugar treatment, which was 22.75 ± 0.96%, while the lowest abnormality was found in the 15% brown sugar treatment, which was 14.50 ± 1.73%.

Table 3. The average percentage of motility and abnormality of spermatozoa 48 h post-cryopreservation

Treatment	Parameter	
	Motility (%)	**Abnormality (%)**
0% brown sugar	68.36 ± 1.20[a]	22,75 ± 0.96[d]
5% brown sugar	78.15 ± 0.90[c]	20.25 ± 1.50[bcd]
10% brown sugar	80.20 ± 0.86[cd]	17.75 ± 1.71[ab]
15% brown sugar	81.85 ± 1.11[d]	14.50 ± 1.73[a]
20% brown sugar	75.09 ± 1.53[b]	19.25 ± 2.06[bc]
25% brown sugar	70.70 ± 1.87[a]	21.25 ± 0.96[cd]

3.3.1. Post-Cryopreserved Spermatozoa Motility

The results of statistical tests using one-way analysis of variance (ANOVA) showed a significant difference (P <0.05) in the effect raised by various brown sugar concentrations (0%, 5%, 10%, 15%, 20%, and 25%) in combination with 10% methanol. The results of Tukey's comparison test on the motility data showed a significant difference (P <0.05) in the treatment of 0% with 5%, 10%, 15%, and 20% brown sugar. The addition of 15% brown sugar showed the highest average percentage of motility (81.85 ± 1.11%), while the lowest (68.36 ± 1.20%) was obtained when brown sugar (0%) was not added. These results confirmed that the addition of brown sugar as a cryoprotectant affected the maintenance of *T. soro* spermatozoa motility at 48 hours post-cryopreservation.The average percentage of post-cryopreserved spermatozoa motility showed that all treatments had a relatively lower value (Table 3) than the average percentage of fresh spermatozoa motility (Table 2), which is decreased by 12%. Decreased motility value of spermatozoa after freezing can be caused by cold shock and osmotic pressure imbalance due to ongoing metabolic processes during storage, as well as the formation of ice crystals in cells [41].

The formation of intracellular ice crystals can cause damage to organelles such as lysosomes and mitochondria. Mitochondria are located at the base of the tail of the spermatozoa and are the site of the breakdown of carbohydrates through glycolysis or fructolysis to produce ATP and ADP, which are the energy source of spermatozoa. Disrupted mitochondria will cause a break in the oxidation chain [12]. As a result, the movement of spermatozoa stops because there is no longer a supply of energy from the mitochondrial organelle that functions to stimulate the function of microtubules in the tail [43]. The motility of *T. soro* spermatozoa in 48 hours post-cryopreservation increase in the use of 5%, 10%, 15%, and 20% brown sugar. At higher concentrations, both 20% and 25% brown sugar, the percentage of motility value decreases. This can be influenced with the toxic effect due to the concentration of 25% brown sugar that is too high [44]. In addition, it has been reported that the higher concentration of cryoprotectant may increase viscosity of the diluent solution that inhibits the spermatozoa movement [45].

The lethal effects during the freezing process are able to minimize by using cryoprotectants. Based on the results of post-cryopreserved spermatozoa motility evaluation, it was found that 5% to 25% brown sugar treatments showed a motility value of more than 70%. The high motility can occur because the nutrients needed are still available [23]. Brown sugar used in this study contains an average of 3.77% glucose and an average of 76.51% sucrose based on laboratory tests. Sucrose in semen diluent serves as an energy source substrate during frozen storage. Energy in the form of ATP is used by spermatozoa to move. Energy is generated through the metabolism of sucrose through the glycolysis pathway, followed by the tricarboxylic acid reaction (Krebs cycle). This can be seen based on the high motility of post-cryopreserved spermatozoa with brown sugar addition when compared to the motility of spermatozoa without brown sugar addition (0% brown sugar) [23, 46].

Sucrose contains in brown sugar can also act as an extracellular cryoprotectant [44]. Sucrose as an extracellular cryoprotectant will coat and bind the spermatozoa membrane from the cold shock effect in the cryopreservation process [25]. Spermatozoa membranes are composed of a double lipid layer (bilayer). Lipids that build cell membranes include phospholipids, glycolipids, and cholesterol. The cryoprotective effect is formed by hydrogen bonds between the hydroxyl groups of sucrose and the polar heads of the cell membrane phospholipids, so that sucrose replaces the position of water molecules during the dehydration process during freezing [47].

Besides the brown sugar, 10% methanol was also used in this study as a cryoprotectant in combination with brown sugar. Methanol acts as an intracellular cryoprotectant because it has a relatively small molecular weight and the ability to penetrate into the cell to replace the plasma fluid content [48]. This process causes cells to become dehydrated, therefore inhibiting the formation of intracellular ice crystals [17]. However, the use of intracellular cryoprotectants alone can cause a toxic effect and cell death [49]. Thus, the addition of brown sugar as a natural cryoprotectant which is combined with 10% methanol in this study is very important to minimize the lethal effects of cryopreservation in spermatozoa quality.

Brown sugar in combination with 10% methanol as a cryoprotectant combination in fish spermatozoa cryopreservation has not been well studied. Several previous reports mentioned the effects of glucose or sucrose, which are the contents in brown sugar. The utilization of glucose or sucrose as extracellular cryoprotectant in fish sperm cryopreservation has been reported in several studies. The addition of 0.2 M glucose and 10% methanol showed the highest post-cryopreserved spermatozoa motility (41%) in rainbow trout [50]. In addition, Abinawanto et al. [51] has been reported that 6% glucose showed the highest post-cryopreserved spermatozoa motility (88.45%) after 24 hours cryopreservation at 34 °C. Abinawanto et al. [51] reported that 0.5% sucrose in combination with 10% methanol for 48 hours on gouramy spermatozoa cryopreservation were able to maintain spermatozoa motility to 81.62 ± 4.19%.

3.3.2. Post-Cryopreserved Spermatozoa Abnormality

The results of post-cryopreserved spermatozoa abnormality evaluation in *T. soro* show that the percentage of post-cryopreserved spermatozoa abnormality is relatively higher than the fresh spermatozoa abnormalities (Table 3). Some forms of abnormal spermatozoa after cryopreservation of kancra were not much different from fresh spermatozoa. Post-cryopreserved spermatozoa abnormalities in this study included secondary abnormalities such as curved tails and broken tails. The secondary abnormalities can be caused by several things such as shocks to the cryotube during distribution, cold shock, and thawing. According to Best [17], during the freezing and thawing process, spermatozoa experience changes in temperature and osmotic pressure which cause the plasma membrane to be damaged and the membrane integrity to decrease. Damage to the plasma membrane due to cold shock causes changes in osmotic pressure, thereby disrupting the activity of the ATPase enzyme located in the membrane and middle tail of the spermatozoa [17].

In Table 3, the highest percentage of post-cryopreserved abnormality was found at 0% brown sugar concentration, then the abnormality decreased to 15% brown sugar and began to increase at 20% brown sugar. A high percentage of spermatozoa abnormalities in other freshwater fishes was also found in the cryoprotectant treatment at 0% concentration (control). According to Abinawanto et al. [50], the highest percentage of post-cryopreservation spermatozoa abnormalities in gouramy was found in the treatment of 0% sucrose and 10% methanol, which was 19.50 ± 3.39%, similar results were found in the study of post-cryopreservation spermatozoa abnormalities in tawes fish [46], that the highest percentage of abnormalities was found in the treatment of 0% egg yolk and 10% methanol, which was 23.00 ± 2.16%.

Based on the results of the one-way ANOVA test and Tukey's follow-up test, there was a significant difference ($P<0.05$) between different concentrations of brown sugar in the abnormality of spermatozoa after 48 hours cryopreservation. The abnormality value of post-cryopreserved *T. soro* spermatozoa was higher if the concentration of brown sugar added was too little or too much. This was evidenced by the treatment of 0% brown sugar with 10% methanol and 25% brown sugar with 10% methanol, which had no significant difference ($P>0.05$). According to Junior et al. [5] and Widyastuti et al. [53], low concentrations of sucrose as a cryoprotectant are thought to be less than optimum in replacing free water and urge the release of electrolytes, while high concentrations of cryoprotectants can damage cells due to osmotic stress or the toxic effects caused by cryoprotectants.

The best concentration of brown sugar was found in this study in the 15% brown sugar in combination with 10% methanol treatment, because these treatments showed the lowest percentage of abnormalities (14.50 ± 1.73%), compared to other treatments. The difference between the treatment of 15% brown sugar in combination with 10% methanol with fresh spermatozoa (Table 2) was 2%. The low post-cryopreservation spermatozoa abnormalities showed that brown sugar treatment was sufficient to protect spermatozoa from oxidative stress due to the cryopreservation process. According to Nayaka et al. [52], brown sugar contains a total phenol of 372 ± 1.44 g GAE/g. Phenol and antioxidant activity are interrelated because phenol has a major role in the course of antioxidant activity [53].

The average percentage of spermatozoa abnormality 48 hours after cryopreservation was inversely proportional to the parameters of motility (Table 3). Post-cryopreserved spermatozoa of *T. soro* with the highest percentage of abnormalities showed the highest motility values and vice versa. This also occurs in the cryopreservation of spermatozoa of other freshwater fish, such as gouramy. According to Abinawanto et al. [50], the lower the abnormality (12.50 ± 1.52%) of gouramy post-cryopreserved spermatozoa (0% sucrose and 10% methanol), the higher the motility (81.62 ± 4.19%), while the higher the abnormality (19.50 ± 3.39%) (treatment 0.5% and 10% methanol), the lower the motility (57.43 ± 3.68%). 15% brown sugar and 10% methanol were thought to play a protective role simultaneously than 0% brown sugar and 10% methanol in reducing spermatozoa abnormalities after cryopreservation in *T. soro*. These results can be supported by the integrity and good condition of the membrane at the time of observation of spermatozoa motility.

3.4. Fertilization Ability of Fresh Spermatozoa

The evaluation of the fertilization ability showed that the fresh spermatozoa of *T. soro* has a fertility value of 90.75 ± 0.96% (Table 4). The percentage of fresh spermatozoa fertility of *T. soro* obtained in this study was not much different from other types of freshwater fish. According to Basavaraja et al. [56], the percentage of fresh spermatozoa of mahseer fish was 98.37 ± 0.19%, carp was 96.7 ± 1.40% [57], and catfish was 95.67 ± 2.67 % [18].

On the other hand, according to Adipu et al. [56], the fertilization ability of spermatozoa is influenced by the quality of spermatozoa, one of which is motility. Spermatozoa with high motility value will have high fertility value. This is evidenced in this study that evaluated fresh spermatozoa showed high percentage of motility (93.23 ± 1.31%) and fertility (90.75 ± 0.96%). It is supported by Abinawanto et al. [29], that fresh spermatozoa of botia fish which have a motility percentage of 91.70 ± 6.67% have a fertilization ability of 80.89 ± 7.46%. The ability of spermatozoa to fertilize the eggs in each fish species is different, but in general, the motility, and ability of spermatozoa to fertilize eggs have a positive correlation [40].

3.5. Fertilization Ability of 48h Post-Cryopreserved Spermatozoa

The percentage of 48 hours post-cryopreserved *T. soro* spermatozoa fertility is presented in Table 4. The average percentage of post-cryopreserved spermatozoa motility decreased in each treatment when compared to the fertility of fresh spermatozoa (90.75 ± 0.96%). According to Lismawati et al. [35], the success of the fertilization process is influenced by the ability of spermatozoa to fertilize eggs. Spermatozoa that are not stored (fresh spermatozoa) have a higher fertilization ability than cryopreserved spermatozoa. The reducing of post-cryopreserved spermatozoa fertilization ability is influenced by the effect of cold shock during freezing. The cold shock causes changes in the structural morphology of spermatozoa so that the metabolism of spermatozoa is disturbed. This resulted in decreased spermatozoa motility and increased spermatozoa abnormality [43].

Table 4. The average percentage of fertility rate of fresh spermatozoa and 48 h post-cryopreserved spermatozoa

Treatment	Fertility rate (%)
0% brown sugar	74.25 ± 2.22[a]
5% brown sugar	82.00 ± 0.82[bc]
10% brown sugar	86.50 ± 1.73[de]
15% brown sugar	89.75 ± 1.71[e]
20% brown sugar	83.75 ± 1.50[cd]
25% brown sugar	78.75 ± 2.75[b]
Fresh spermatozoa	90.75 ± 0.96

The highest post-cryopreservation spermatozoa fertility value was found in the 15% brown sugar in combination with 10% methanol treatment, which was 89.75 ± 1.71%. The addition of 15% brown sugar in combination with 10% methanol is the best combination because it showed the highest fertility value and is not much different from that of fresh spermatozoa, which is a 1% difference. Research on the ability of spermatozoa to fertilize *T. soro* eggs has been carried out by Harjanti et al. [59], that there is a difference of 8% between the percentage of fresh spermatozoa fertility and post-cryopreserved spermatozoa fertility at optimal concentrations (10% skim milk and 10% methanol). The high percentage of fertility in *T. soro* spermatozoa which were cryopreserved using 15% brown sugar and 10% methanol concentration was thought to be because the spermatozoa had high motility values (81.85 ± 1.11%). Spermatozoa that move agile and very fast (fast progressive) are estimated to allow the highest fertilization process as increasing up to 70%. This is because the spermatozoa are actively moving, and they have a very large energy (ATP), so they can penetrate the egg cell [60].

In Table 4, it can be seen that the lowest percentage of post-cryopreserved spermatozoa was found in the 0% brown sugar treatment (74.25 ± 2.22%). The fertility value was then started to increase in the 5% brown sugar treatment (82.00 ± 0.82%) and began to decrease in the 20% brown sugar treatment (83.75 ± 1.50%). The low fertility value of 0% brown sugar treatment is thought to be due to the absence of brown sugar as an extracellular cryoprotectant and an energy source that spermatozoa should utilize during the cryopreservation process. This is supported by the research of Muchlisin et al. [61] on the cryopreservation of depik fish spermatozoa. Post-cryopreserved spermatozoa fertility of depik fish in 5% egg yolk combined with 5% DMSO treatment tend to be higher (55.95 ± 12.43%) when compared to only 5% DMSO treatment (41.66 ± 10.57%). According to Rizal et al. [42], extracellular cryoprotectants can protect and support the life of spermatozoa during the cryopreservation process, thereby minimizing problems that often arise in the cryopreservation process of spermatozoa, such as the effect of cold shock on frozen cells and changes in intracellular conditions due to the release of water associated with ice crystal formation [23, 62].

The results of the one-way ANOVA test and Tukey's follow-up test showed that there was a significant difference ($P<0.05$) between different concentrations of brown sugar on the post-cryopreserved spermatozoa fertility of *T. soro*. Several factors can influence this, one of which is the quality of post-cryopreserved spermatozoa itself. The percentage of sperm fertility after cryopreservation of *T. soro* showed a correlation with the percentage of quality of spermatozoa after cryopreservation, including motility and abnormality. This is evidenced in this study that the highest percentage of post-cryopreserved spermatozoa fertility and motility and the lowest abnormality values were found in the same treatment (15% brown sugar). It is in agreement with previous study done by Abinawanto et al. [29] that cryopreservation of botia fish spermatozoa using 15% egg yolk cryoprotectant combined with 10% methanol for 24 hours showed the highest percentage of fertility (50.64 ± 4.37%) and motility (96.43 ± 1.49%) and the lowest abnormality value (11.50 ± 1.29%).

4. Conclusion

In conclusion, the overall results suggested that 15% brown sugar as a natural cryoprotectant in combination with 10% methanol is the optimum cryoprotectant combination in maintaining the post-cryopreserved spermatozoa quality in kancra fish (*T. soro*).

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