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Date Palm Extract Effect on Quality of Mahseer Fish (*Tor Soro*) Spermatozoa after Frozen Storage

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Abstract: *Tor soro* is one of the endemic fishes used in traditional ceremonies. Its natural population is threatened with extinction due to environmental damage and overfishing. One of the efforts to overcome the population decline is carried out through the sperm cryopreservation process, which is influenced by a factor called cryoprotectant. Generally, cryoprotectants such as date palm extract are useful for protecting spermatozoa against damage during this process, and they are selected with natural ingredients to reduce toxicity effects. Therefore, this research aims to evaluate the effect of date palm extract with a combination of 10% methanol on spermatozoa quality (motility, viability, and abnormalities) and the percentage of *T. soro* fertility at 48 hours after cryopreservation. The extract concentrations used were 0%, 5%, 10%, 15%, 20%, and 25%, the dilution ratio was 1:10, while storage was carried out in the freezer at -10 °C. The results of the one-way ANOVA test showed that the various concentrations with 10% methanol had a significant ($P < 0.05$) effect on the spermatozoa motility, abnormalities, and fertilization ability. Hence, the combination of 10% methanol and 10% date palm extract is the optimum concentration to maintain the 81.29 ± 1.01% highest motility, 21.5 ± 1.29% lowest abnormality, and 88.50 ± 1.73% highest fertility.

Keywords: cryopreservation, date palm extract, fertilization, Masheer fish.

椰枣提取物对冷冻贮藏后马西鱼（托索罗）精子质量的影响

摘要: 托索罗是传统仪式中使用的特有鱼类之一。由于环境破坏和过度捕捞，其自然种群面临灭绝的威胁。克服人口下降的一项努力是通过精子冷冻保存过程进行的，该过程受到一种称为冷冻保护剂的因素的影响。通常，冷冻保护剂如枣椰树提取物可用于保护精子在此过程中免受损害，并且它们选用天然成分以减少毒性作用。因此，本研究旨在评估海枣提取物与 10% 甲醇的组合对冷冻保存 48 小时后精子质量（活力、活力和异常）和吨.索罗生育力百分比的影响。所用提取物浓度为0%、5%、10%、15%、20%和25%，稀释比例为1:10，同时在-10℃冰箱中保存。单因素方差分析结果表明，不同浓度的10%甲醇对精子活力、异常和受精能力有显着影响（磷<0.05）。因此，10% 甲醇和 10% 椰枣提取物的组合是维持 81.29 ± 1.01% 最高运动性、21.5 ± 1.29% 最低异常率和 88.50 ± 1.73% 最高生育率的最佳浓度。

关键词: 冷冻保存，枣椰树提取物，施肥，马西尔鱼。

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1. Introduction

The *Tor* genus includes a freshwater fish spread in several regions in Indonesia, namely in Sumatra, Java, and Kalimantan [1], [2]. Four species identified from this genus in the country are *T. douronensis*, *T. Tambra*, *T. tambroides*, and *T. soro* [3], which is endemic in Toba Lake and the Cimanuk river, Garut [4]. *T. soro* differs from the other species because of the presence of the median lobe [5]. Based on IUCN (International Union for Conservation of Nature and Natural Resources) and CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), *T. soro* status has not been evaluated [6], [7].

Tor soro is omnivorous with a diet of phytoplankton, crustaceans, insects, rotifers, and Oligochaeta, while the active hunting time is in the morning [8]. It lives in upstream river habitats with the characteristics of fast currents, rocky riverbeds, the water of 26-27.8 ℃, pH 7.65-7.97, with 5.4 -6.7 mg L^{-1} dissolved oxygen content [4]. The selling price of this fish is classified as high, around IDR. 250,000 - IDR. 500,000/kg [8]. Besides being used in cultural activities such as traditional ceremonies, *T. soro* is also consumed because it has thick meat, delicious taste, and good nutritional content for body health [3]. The high selling price and over-exploitation have caused the population in its habitat to be threatened with extinction [5]. One of the efforts to overcome the population decline is cultivation using fish seeds obtained through spawning, but this process is naturally hampered by gonad synchronization. For overcoming this problem, an alternative reproductive technology is needed, such as cryopreservation [9].

Cryopreservation is a technique for storing animal and plant cells or other genetic materials at low temperatures by reducing metabolic activity without affecting their organelles; therefore, physiological, biological, and morphological functions remain [10]. The temperature used to store a cell varies, and the one commonly used in this process is -80 ℃ [11], with the lowest reaching -196 ℃ in liquid nitrogen [9].

Cell stored at a very low temperature lasts for a long time due to its suppressed metabolic activity [11]. Cryopreservation is generally used for spermatozoa storage because of resistance to low temperatures compared to embryos or ovum [10]. The basic principle of cryopreservation involves dehydration and rehydration. Dehydration is a condition of drawing out of the cell, while rehydration is drawing fluid into it. These two processes occur due to the osmotic pressure of the fluid that moves between the cell plasma and the cryoprotectant solution used [12].

Cryoprotectants utilization affects cryopreservation, and they are non-electrolyte chemical substances that reduce the lethal effects during the freezing process due to the solution influence or ice crystals presence, therefore maintaining cell viability [13]. They are divided into intracellular and extracellular cryoprotectants. The intracellular one penetrates cells, and it is also amphipathic, effective at reducing cell damage due to freezing, has hydrophilic, hydrophobic surfaces, and small molecular weight [14], [15]. Furthermore, the commonly used ones are glycerol [9], [16], methanol [10], [17], [18], [19], [20], [21], [22], dimethyl sulfoxide (DMSO) [9], [10], [15], dimethylacetamide (DMA) [10], and ethylene glycol (EG) [9]. The extracellular has a large molecular size, hence having difficulty penetrating the cell membrane and maintaining outer cell membrane stability [9]. In addition, the common ones include soybean milk [21], honey [23], quail egg yolk [24], chicken egg yolk [25], skim milk [26], carboxyl methylcellulose (CMC) [27], and date palm extract [28].

The success of cryopreservation is influenced by cryoprotectants, extender composition, sperm collection method and storage, solution and dilution ratio, equilibration temperature and duration, as well as freezing and thawing rate [9], [10], [11], [15]. However, apart from protecting spermatozoa from cold and hot temperatures during the freezing and thawing processes, cryoprotectants have several drawbacks, causing toxicity in the cellular system [10]. Cryoprotectants utilization with not optimal or excessive concentrations causes cellular toxicity, which reduces cryopreservation success rate [10], [12]. Extracellular cryoprotectants generally have a lower toxicity effect when compared to the intracellular counterpart in equal concentration [10], [15].

Methanol is the simplest form of alcohol, which is liquid, colorless, volatile, and flammable. Furthermore, it has 32.04 g/mol molecular weight, a freezing point (97.5 ℃) lower than that of water, and a 64.7 ℃ boiling point [14]. The relatively small molecular size permits methanol penetration into the cell to replace the plasma fluid content. This process causes cells to become dehydrated, therefore inhibiting the formation of intracellular ice crystals [10], [12], [19].

Methanol has toxic properties that inhibit ATP formation [9], [10], and this is influenced by the storage temperature and concentration used during the cryopreservation process [9], [10]. When compared to DMSO and glycerol, methanol is a cryoprotectant with higher toxicity [10]. Cryopreservation research previously conducted by [18] stated that 10% methanol as intracellular cryoprotectant significantly maintained post-cryopreserved spermatozoa motility reaching 96.43±1.49%. Follow-up research by [19], [37] on *Oreochromis niloticus* sperm stated that methanol

utilization was more effective in maintaining sperm motility than DMSO.

The toxicity effect is potentially reduced by using cryoprotectants from natural ingredients, such as date palm extract [28]. Dates are one of the high-energy foods that contain sugar levels of about 63.35% [29]. These are simple sugars such as sucrose, glucose, and fructose [30]. Dates contain 70% sugar; therefore, they easily replace the body's lost energy [30]. Dates also contain antioxidants [30] that prevent spermatozoa damage due to pathogens and other internal disorders. The utilization of their extract as a cryoprotectant has been rarely researched. However, [28] conducted such on *Epinephelus lanceolatus* sperm, while Widodo et al. used the extract in combination with Ringer's lactate solution on *Cyprinus rubrofuscus* sperm [31].

The methanol and date palm extract combination is expected to maintain spermatozoa quality during cryopreservation [32]. The extract's sugar content, such as glucose and fructose, provides additional energy to protect spermatozoa from cold shock, extends the storage period, keeps them in a stable condition, and maintains their motility [10]. The fructose provides energy for them, therefore maintaining motility and prolonging movement duration [17]. The use of natural ingredients of date palm extract is expected to minimize the cryoprotectant's toxic effects. The parameters for the success of the cryopreservation process are determined by the spermatozoa motility, viability, and abnormalities, while these qualities subsequently determine their ability to fertilize an ovum [33]. The success of fertilization by fresh spermatozoa was higher than using a cryopreserved one because cryopreservation decreases their quality, causing changes in motility [33]. Fertilization is a fusion process between male and female gamete cell nuclei [44] which is occasionally carried out by mixing cryopreserved spermatozoa that have gone through thawing with broodstock ovum cells [34]. Research using post-cryopreserved spermatozoa with date palm extract as a cryoprotectant has been carried out on *C. rubrofuscus* [31], where the successful fertilization percentage reached 84.91%.

Information regarding post-cryopreservation of *T. soro* spermatozoa quality using a combination of 10% methanol and date palm extract at various concentrations is still unknown. Therefore, this research aims to evaluate 1) The combined effect of 10% methanol with various date palm extract concentrations as a cryoprotectant on *T. soro* spermatozoa motility and abnormalities, as well as 2) The ability to fertilize female egg cells at 48 hours post-preservation.

The research hypothesis is that 10% methanol and 10% date palm extract combination provides the optimum effect in maintaining the highest percentage of motility, viability, and fertility and the lowest percentage of spermatozoa abnormalities at 48 hours post-cryopreservation. The results are to be used as a guideline in applying date palm extract as a natural cryoprotectant to maintain *T. soro* spermatozoa quality.

2. Method

2.1. Location and Time of Research

The research was conducted for eight months from September 2019-April 2020 at the Installations for Freshwater Fish Genetics Resources, Ministry of Marine Affairs and Fisheries, Bogor, Indonesia.

2.2. Fish Ringer's Preparation

A new Fish Ringer solution was conducted based on [35].

2.3. Activator Preparation

The activator solution was prepared according to [18].

2.4. Eosin Y Preparation

The 0.5% eosin-Y solution was carried out based on [26].

2.5. 0.15 Phosphate Buffer pH 6.8 Preparation

$Na_2HPO_4.2H_2O$ solution was prepared according to [35].

2.6. Giemsa Solution Preparation

Giemsa solution was conducted based on [35].

2.7. Date Palm Extract Preparation

The date palm extract used was derived from marketed commercial date extracts, while the concentrations were 0%, 5%, 10%, 15%, 20%, and 25%. The solution was prepared by dissolving 0 mL, 2.5 mL, 5 mL, 7.5 mL, 10 mL, and 12.5 mL of date palm extract in fish ringers until the volume reached 50 mL. The diluted extract was stored in a cryotube as a mixture of a sperm-thinning solution.

2.8. Diluent Solutions Preparation

The diluent consisted of fish Ringer's solution, 10% methanol, and various date palm extract concentrations. The ratio between the diluent solution and sperm determined the success of the cryopreservation process, which was 1:10 [36].

2.9. Male Broodstock Selection and Sperm Collection

The *T. soro* broodstock used was obtained from the Installations for Freshwater Fish Genetics Resources, Ministry of Marine Affairs and Fisheries, Bogor, Indonesia. The gonads had an average age of 1 year and weighed more than 300 g [21]. The sperm were obtained by stripping the *T. soro* abdomen [20], [21]. The sperm that came out were collected and kept dry in

a disposable syringe without a needle with a scale of 3 mL as a temporary shelter [20], [21].

2.10. Cryopreservation Treatment

This experimental research used a completely randomized design (CRD), and the treatments were in the form of 0%, 5%, 10%, 15%, 20%, and 25% concentrations of the date palm extracts (DPE). The concentration was determined based on a modification from [28], and each treatment was added with an intracellular cryoprotectant of 10% methanol [18].

2.11. Equilibration

Before freezing, equilibration was carried out to adapt the spermatozoa to low temperatures by storing cryotubes containing diluted spermatozoa in a refrigerator at 5 ° C for 10 min (Modification [18]).

2.12. Freezing

The equilibrated spermatozoa were stored at – 10°C for 48 h [32].

2.13. Thawing

The thawing process was conducted by immersing the cryotube at 40 °C for 60 sec [32].

2.14. Semen (Sperm) Volume Observation

The semen (sperm) volume without bubbles or urine was measured in a scale cryotube [35].

2.15. Semen (Sperm) pH Measurement

The pH of semen (sperm) was measured based on [18].

2.16. Semen (Sperm) Color Observation

The spermatozoa were observed visually and subjectively to determine the color of the sample collected into the cryotube [35].

2.17. Spermatozoa Concentration Counting

The concentration was counting according to [10], [20], [37].

2.18. Spermatozoa Motility

The fresh spermatozoa and post-cryopreserved motility percentage were measured based on [10], [18].

2.19. Spermatozoa Abnormalities

The abnormalities were observed in fresh and post-cryopreserved spermatozoa by counting normal and abnormal spermatozoa [18].

2.20. Female Broodstock Preparation and Selection

The female *T. soro* broodstock was obtained from the Installations for Freshwater Fish Genetics Resources, Ministry of Marine Affairs and Fisheries, Bogor, Indonesia.

2.21. Spermatozoa and Ovum Collection

The mature gonadal spermatozoa were collected by stripping [38]. The collection was carried out using a disposable syringe without a needle [38]. The ova were collected 14 hours after the second hormone induction. The method used was also stripping, which was carried out in the shade because ovum damages when directly exposed to sunlight [38].

2.22. Fertilization Using Fresh and Post-Cryopreserved Spermatozoa

The fresh sample used for cryopreservation and observation were taken partly for fertilization which was then carried out by mixing them with ova in a plastic basin [39]. Fertilization using post-cryopreserved spermatozoa was conducted by mixing them with 50 ova and were stirred for 3 min [39].

2.23. Calculation of the Percentage Fertility

The percentage fertility was calculated after the fertilized ovum was incubated and left for 30 minutes [38].

2.24. Data Analysis

The collected data were presented in the form of tables. The data analysis was performed on fresh and post-cryopreserved spermatozoa. The fresh semen (sperm) data was a macroscopic evaluation that included color, pH, volume, and microscopic evaluation, which included spermatozoa concentration, the percentage motility, abnormalities, and the percentage of successful *T. soro* fertilization using fresh and 48 hours post-cryopreserved spermatozoa. The normally distributed and homogeneous data were subjected to a parametric test with variance (ANOVA) analysis and followed by the Tukey multiple comparison tests [38].

3. Results and Discussion

3.1. Macroscopic Observation of Fresh Semen (Sperm)

The observation data are presented in Table 1.

Table 1 Macroscopic observation of fresh semen

Observation	Parameter	Results
Macroscopic	Color	Milky white
	Volume (mL)	2.38 ± 1.11
	pH	8 — 8.5

Based on Table 1, *T. soro*'s spermatozoa volume in one ejaculation had an average of 2.38 ± 1.11 mL, obtained from the males with a bodyweight of 700-1000 g each. Other research has been conducted by Devi et al. on fish from the *Cyprinidae* family, namely *B. gonionotus*. The volume of *B. gonionotus* spermatozoa produced in one ejaculation was 3 mL [39]. The previous study has also shown a variation of

fresh semen volume based on the species, for example, 9 mL in *C. rubrofuscus* [31], 4.03 mL in *C. Carpio* [40], 0.63±0.24 mL in botia, *C. macracanthus* [18], 0.25-0.5 mL in *V. variegatus* [9], and 40-46 mL in grouper, *E. lanceolatus* [26]. The *H. nemurus* spermatozoa volume in one ejaculation had an average of 1.5 mL which was obtained from the males with a weight of 800 g [4] Semen volume is also influenced by hormone. Ovaprim injection 0.6 mL/kg BW can increased semen volume of *Anabas testudineus* up to 0.09 mL [42]. Other macroscopic characteristics observed were color and pH. Based on observations, *T. soro*'s spermatozoa were milky white with a pH ranging from 8 to 8.5, and previous research in grouper *E. lanceolatus* has also reported this same color. The pH value was 7 [27], while the color of the *T. soro* sperm is similar to that of the fish in the *Cyprinidae* family, namely *C. Carpio*. The pH owned by *C. Carpio* and *O. vittatus* is not much different from that of the results, which tends to be alkaline with a value of 7 [40], [43]. The variation of pH was also shown in different species, such as pH 8 in Java Barb, *B. gonionotus*, and *C. rubrofuscus* [31], [39]; pH 7.5 in *H. nemurus* [41]; pH 8.2-8.55 in European burbot (*Lota lota* L.) [12]; pH 7.2-7.5 in Tilapia, *Oreochromis mossambicus* [20]; pH 7.9 in botia fish, *C. macracanthus* [18] and in spotted halibut, *V. variegatus* [9]. The pH condition also affects the spermatozoa movement duration, which, when below 4, causes the duration to be lower than when at 5-13 [12]. Differences in the macroscopic characteristics are influenced by various factors, such as fish conditions, feed management, and environmental conditions [44], [50]. Moreover, environmental conditions that change drastically and are unsuitable cause stress for fishes [44], [50].

3.2. Microscopic Evaluation of Fresh Spermatozoa

The microcopic evaluation of fresh semen included observation of concentration, motility, and abnormalities (Tables 2, 3, and 4).

3.2.1. Spermatozoa Concentration

Based on Table 2, *T. soro* spermatozoa concentration was $15.32 \pm 3.11 \times 10^9$ cells/mL.

Table 2 Spermatozoa concentration of fresh semen

Species	Concentration (x 10^9 cells/mL)	Author
Tor soro	15.32 ± 3.11	Present paper
Lota lota	39.2 ± 12.2	[12]
Salmo cettii	10.6 ± 1.4	[10]
Cyprinus Rubrofuscus	9.58	[31]
Piaractus Mesopotamicus	21.09	[33]

According to Widodo et al., the spermatozoa concentration of fish from the Cyprinidae family was 9.58×10^9 cells/mL [31]. Generally, the concentration varied in different males and the same male fish of the same species. Judhyka et al. reported that the optimal sperm concentration with high post-thaw sperm motility was 2.0×10^9 spermatozoa/mL for brook trout, 3.0×10^9 spermatozoa/mL for brown trout and sea trout, and 4.0×10^9 spermatozoa/mL for Atlantic salmon [51]. According to Sukendi et al., the concentration value does not affect fertilization success. This is because an ovum is only fertilized by one spermatozoa cell [42]. The spermatozoa motility and viability were considered to have more effect on cryopreservation success.

3.2.2. Spermatozoa Motility

Based on Table 3, the percentage of *T. soro* fresh spermatozoa motility was 89.00 ± 3.20%.

Table 3 Spermatozoa motility of fresh semen

Species	Motility (%)	Author
Tor soro	89.00 ± 3.20	Present paper
Piaractus mesopotamicus	90.83 ± 7.32	[33]
Acipenser ruthenus	89.3 ± 6.5	[22]
Chromobotia macracanthus	91.70±6.67	[18]
Rasbora tawarensis	71.33	[15]
Osteochilus vitatus	78.33 ± 7.63	[43]
Salmo cettii	81.2±5.7	[10]
Cyprinus rubrofuscus	37%	[31]
Oreochromis mossambicus	62	[20]
Verasper variegatus	>80	[9]
Lota lota	55.0 ± 5.7	[12]
Puntius bramoides	84.71 ± 3.53	[47]
Epinephelus lanceolatus	84.18 ± 2.08	[46]
Osphronemus goramy	77.35±5.98	[26]

Previous research on the fresh sperm motility of fish from the Cyprinidae family, namely *Cyprinus rubrofuscus*, showed a lower percentage of 37% [31]. Furthermore, the suitable spermatozoa for cryopreservation need to have a motility percentage of at least 80% [50]. The minimum criterion for suitable spermatozoa motility percentage for cryopreservation was important because it was associated with damage possibility during this process [33]. Spermatozoa motility is the ability to move using their energy. Their movement was assessed based on the moving patterns, such as active, fast, or slow, moving forward, moving in the same place or a circle, and not moving at all [33]. The spermatozoa motility was affected by external, internal factors, and their tail structure. The movement duration generally lasts from 30 seconds to several minutes [33]. Spermatozoa do not move in the reproductive organs of male fish or an environment containing the same osmolality as the semen [12]. The movement is affected by temperature, which tends to be slower at 20 ºC than at 26-30 ºC [49]. The spermatozoa motility was also affected and controlled

by the concentration of potassium ions (K +) with differences in osmotic pressure [12]. The high concentration of K + ions in the plasma fluid causes the spermatozoa to become immotile [12]. An environment with hypotonic conditions reduces K + ions concentration, causing the opening of K + ion channels and K + ion efflux. The low osmolality condition, the K + ion channel opening, and the K + ion efflux lead to membrane hyperpolarization, which causes membrane depolarization, triggering the Ca2 + ions influx. The increase in Ca2 + ion activates the adenyl cyclase enzyme that converts ATP into cAMP [12]. The movement of the spermatozoa tail is affected by the axoneme activated by cAMP [44].

3.2.3. Spermatozoa Abnormality

Table 4 showed the percentage of *T. soro* fresh spermatozoa abnormality was 19.75 ± 1.71%, which was lower than the 49.89 ± 7.26% in *Piaractus mesopotamicus* [33] and 20.01 ± 1.01% in the *Epinephelus lanceolatus* [46].

Table 4 Spermatozoa abnormality of fresh semen

Species	Abnormality (%)	Author
Tor soro	19.75 ± 1.71	Present paper
Cyprinus carpio	7.68	[45]
Piaractus mesopotamicus	49.89 ± 7.26	[33]
Chromobotia macracanthus	16.00±3.46	[18]
Osphronemus goramy	18.67±3.20	[26]
Epinephelus lanceolatus	20.01 ± 1.01	[46]
Puntius bramoides	16 ± 2.07	[47]

According to [44], spermatozoa are of good quality when the abnormality percentage is ≤20%. Those with a high level of abnormality inhibit the fertilization process due to imperfect movement [33]. Good and suitable spermatozoa for cryopreservation have 19.75 ± 1.71% abnormality. *T. soro* spermatozoa abnormalities happened in the head and tail, while their morphology generally consists of the head, neck or midpiece, and tail [33]. The spermatozoa abnormalities were related to their morphological abnormalities, and these took place due to storage in the testes. Long storage time affects spermatozoa morphological changes [44], [50]. Spermatozoa abnormalities were divided into two groups: primary, which happened during the spermatogenesis process, and secondary, due to environmental effects [33], [50]. Primary abnormalities included double, enlarged, or shrunken heads [55], while the secondary ones happened in the midpiece and tail, such as the curled, split, double, and broken tail [33].

3.3. Microscopic Evaluation of Post-Cryopreserved Spermatozoa

Evaluation of *T. soro* spermatozoa at 48 hours post-cryopreservation included motility and abnormality observation can be seen in Table 5.

Table 5 The average percentage of motility and abnormality of spermatozoa 48 h post-cryopreservation

Treatment	Parameter	
	Motility	Abnormality
0% Date Palm Extract	60.55 ± 1.72^{a}	31.00 ± 1.82^{cd}
5% Date Palm Extract	77.92 ± 1.78^{c}	26.25 ± 1.70^{b}
10% Date Palm Extract	81.29 ± 1.01^{c}	21.50 ± 1.29^{a}
15% Date Palm Extract	70.24 ± 1.85^{b}	27.50 ± 2.08^{bc}
20% Date Palm Extract	69.60 ± 1.48^{b}	33.50 ± 2.38^{d}
25% Date Palm Extract	67.73 ± 3.41^{b}	33.25 ± 2.06^{d}

3.3.1. Evaluation of Spermatozoa Motility 48 h Post-Cryopreservation

The average percentage of *T. soro* spermatozoa motility at 48 hours post-cryopreservation is presented in Table 6. Furthermore, the results of statistical tests using one-way analysis of variance (ANOVA) showed a significant difference in the effect raised by combined administration of 10% methanol with various date palm extract concentrations (0%, 5%, 10%, 15%, 20%, and 25%) ($P < 0.05$). Tukey's comparison test results on the motility data showed a significant difference in the treatment of 0% with 5%, 10%, 15%, 20%, and 25% date palm extract. The combination of 10% methanol with 10% date palm extract showed the highest percentage motility (81.29 ± 1.01%) (Table 4), while the lowest (60.55 ± 1.72%) was obtained when date palm extract (0%) was not added. This confirmed that a combination of 10% methanol and date palm extract as a cryoprotectant affected the maintenance of *T. soro* spermatozoa motility at 48 hours post-cryopreservation.

Based on Table 5, the average percentage of motility of 48 hours post-cryopreserved *T. soro* spermatozoa was lower than that of the fresh semen. The highest percentage of post-cryopreserved spermatozoa motility was 81.29 ± 1.01%, decreasing approximately by 8% from that of fresh spermatozoa that were 89.00 ± 3.20%. According to [53], decreased spermatozoa motility during the cryopreservation process occurs due to mitochondria disruption in cells, which also involves changes in mitochondrial membrane fluidity. Ultrastructure of the middle piece and mitochondria were altered significantly in cryopreserved spermatozoa compared to fresh spermatozoa [19], [33]. Besides this, Cosson reported that spermatozoa motility depended on various factors, including ATP production, plasma membrane channel activity, and flagellar structure or tail [44]. Table 6 shows spermatozoa motility of different species after freezing.

Table 6 Spermatozoa motility after freezing

Species	CPA + Extender	Motility (%)	Author
Tor soro	10%MeOH+ 10%Dates+Ringer	81.29 ± 1.01	Present paper
E. lanceolatus	6%Glycerol+ 10%Dates+Ringer	76.70 ± 1.54	[48]
C. macracanthus	10%MeOH+ 0.1%Honey+Ringer	89.4 ± 5.45	[36]
P. mesopotamicus	1 part semen + Three parts CPA	11.83 ± 7.41	[33]

R. tawarensis	5%DMSO+ 5%Egg Yolk	61 ± 2.64	[15]
O. mossambicus	12.5%MeOH+ 175mMGlucose	27-28	[20]
C. rubrofuscus	1mL Dates+ 99 mL Ringer Lactate	50.33	[31]
O. vitatus	30 mg/L Glutathion+ [DMSO+Egg Yolk]	63 ± 5.89	[43]
S. cettii	DMSO+Glucose	42.6 ± 3.5	[10]
E. lanceolatus	6% Glycerol+ 15%Egg Yolk+Ringer	83.64 ± 1.72	[46]

Previous research was conducted by Muchlisin et al.,-who used liquid nitrogen as a spermatozoa storage medium for 15 days [15]. This used a combination of DMSO, various concentrations of glycerol, and egg yolk [15], [46]. The observation result of post-cryopreserved *T. soro* spermatozoa showed that the percentage motility was 81.29 ± 1.01%. Widyaningsih et al. reported that the motility of *E. lanceolatus* spermatozoa in 48 hours post-cryopreservation was 76.70 ± 1.54% [28]. Cryopreservation research on freshwater fish using 10% methanol and date palm extract has not been widely carried out. Another approach is needed to determine the cryoprotectant combinations effect, one of which uses 10% methanol with various sucrose concentrations similar to the one performed by [18] on *Chromobotia macracanthus*. The results showed 10% methanol, 15% Egg Yolk and glucose extender maintained spermatozoa motility reaching 96.43±1.49%.

The motility of *T. soro* spermatozoa in 48 hours post-cryopreservation increase in the use of 5% and 10% date palm extract. At higher concentrations starting from 15%, 20% to 25%, the percentage motility value decreases, and this tends to be caused by increased viscosity of the diluent solution that inhibits the spermatozoa movement [54]. Di Iorio et al. also showed that higher glucose (one of the sugars in date palm) concentrations might harm spermatozoa due to high osmolality [10]. Moreover, Galo et al. reported that inappropriate or too high cryoprotectant concentration caused the cryoprotectant to be toxic [33].

The cryoprotectant combination of 10% methanol and 10% date palm extract was the optimum concentration to maintain motility because cryoprotectants utilization protected spermatozoa intracellularly and extracellularly. Intracellular protection by methanol happened due to its capability to enter cells, replacing some of the free water and forcing out electrolytes, thereby reducing their destructive power to spermatozoa [33]. Extracellular protection was carried out by the sugar content of date palm extract.

The date palm extract sugar content and high viscosity protected spermatozoa against cold shock during the freezing process by stabilizing their cell membrane. It contains simple sugars such as fructose, glucose, and sucrose which are used as extracellular cryoprotectants. The above-mentioned sugar content provided nutrition for spermatozoa after cryopreservation; hence their motility was maintained. Furthermore, the glucose reduced the freezing point, therefore preventing excess fluid secretion from the spermatozoa cells. This prevented damage to the cells' integrity that often affects their motility [33]. Also, the motility was inhibited by high potassium ions (K +) concentration. The physiological solution composition did not fulfill the K + and Ca2 + ions required for mobile spermatozoa. Furthermore, the date palm extract provided the ions and energy needed for their motility [12].

3.3.2. Evaluation of Spermatozoa Abnormality 48 h Post-Cryopreservation

The average percentage of post-cryopreserved *T. soro* spermatozoa abnormalities is presented in Table 7. The combination of 10% methanol and 10% date palm extract showed the lowest abnormalities percentage, namely 21.5 ± 1.29% ($p<0.05$). The percentage of abnormal spermatozoa after cryopreservation was higher than that of fresh spermatozoa.

Table 7 Spermatozoa abnormality of the different species after freezing

Species	**CPA + Extender**	**Abnormality (%)**	**Author**
Tor soro	10%MeOH+ 10%Dates+Ringer	21.5 ± 1.29	Present paper
Epinephelus lanceolatus	6% Glycerol+ 10%Dates+Ringer	21.53 ± 0.84	[48]
Chromobotia macracanthus	10%MeOH+ 15% Egg Yolk+Ringer	11.50± 1.29%	[18]
Piaractus mesopotamicus	1part semen + Three parts CPA	64.61 ± 4.48	[33]
Epinephelus lanceolatus	6% Glycerol+ 15%Egg Yolk+Ringer	21.5 ± 1.2	[46]

A previous study on post-cryopreservation by [48] used a combination of 6% Glycerol and 10% Dates with various concentrations in *Epinephelus lanceolatus*, which was carried out in the freezer at -34 ºC for 48 hours. The results showed the abnormalities percentage of *T. soro* spermatozoa at 48 hours after the cryopreservation was comparable. The combination of 10% methanol and 10% date extract is considered a cryoprotectant that maintains spermatozoa quality. This is evidenced by the less than 30% abnormality of *T. soro* spermatozoa at 48 hours after preservation. Methanol utilization as a cryoprotectant influences and also maintains the normality of post-cryopreserved spermatozoa [10].

The type of abnormality of *T. soro* spermatozoa observed at 48 hours post-cryopreservation was similar to that of the fresh sample. The abnormalities found

occurred in their head and tail regions, and these are often due to differences in osmotic pressure during the cryopreservation, which cause cells to become dehydrated [17], [33]. Furthermore, the abnormalities potentially occur during cooling or when the preparation is reviewed [33]. However, using the correct and careful techniques in the smear-making process minimize damage to spermatozoa. An abnormally high level of spermatozoa inhibits fertilization, while their imperfect movement is one of the obstacles in this process, causing a low fertilization rate [33], [42].

3.4. Fertilization

3.4.1. Fertilization Using Fresh Spermatozoa

Table 8 shows the fertilization rate of fresh semen from different species.

Table 8 Fertilization rate of fresh semen from different species

Species	Fertilization rate (%)	Author
Tor soro	94.00 ± 1.63	Present paper
Epinephelus lanceolatus	82.08 ± 1.48	[46]
Chromobotia macracanthus	100	[36]
Piaractus mesopotamicus	95.64 ± 1.7	[33]
Rasbora tawarensis	65.25 ± 4.2	[15]
Oreochromis mossambicus	56	[20]
Cyprinus rubrofuscus	70.68	[31]
Osteochilus vitatus	75.67 ± 1.15	[43]

Based on observations, the percentage of *T. soro*'s fertilization rate using fresh spermatozoa was 94.00 ± 1.63% (Table 8), but compared to the study on *C. rubrofuscus*, the value was higher. The results showed that the egg fertilization of *C. rubrofuscus* reached 70.68%. Ugwu et al., which used *Oreochromis mossambicus*, reported a much lower fertilization success value than that of *T. soro*, which only reached 56% [20]. These results were obtained using fresh spermatozoa with 27-28% motility. The success value of *T. soro* fertilization showed good results when using fresh spermatozoa because their quality supports this process. The quality was reflected in the percentage of motility, viability, and abnormalities, where their motility reached 89.00%.

The percentage of *T. soro* eggs' successful fertilization is known to be directly proportional to spermatozoa motility. Galo et al. reported that in addition to being influenced by spermatozoa quality, fertilization is also influenced by oocytes, including the properties of the ovarian fluid and the specific compounds contained in it [33]. A good oocyte has the potential to increase the success of egg hatchability and produce a good embryo. But, those of poor quality cause disrupted embryo development and decreased egg hatchability.

The female *T. soro* broodstock used for fertilization was previously injected twice with ovaprim for 8 hours. The function of this hormone addition is to stimulate ovulation. Galo et al. reported that fertilization success is influenced by the maturation rate of the parent gonads, the size of the broodstock, and the quality of the eggs produced [33]. Gonad development tends to be slow until it reaches the maturity of fish oocytes that have just been domesticated or cultivated, such as in *T. soro* [33], [56]. Slow development still occurs even though the location of the breeding grounds has been adapted to their natural habitat and quality feed is provided. This situation is due to gonadotropin hormone availability in the body or inadequate external stimulation [50].

3.4.2. Fertilization Using Post-Cryopreservation Spermatozoa

The percentage fertility of ovum using 48 hours post-cryopreserved *T. soro* spermatozoa is presented in Table 9.

Table 9 The average percentage of fertilization rate of fresh semen and 48 h post-cryopreservation

Treatment	Percentage of fertilization rate (%)
Methanol 10% + Date palm extract 0%	$73.75 \pm 2.63^{a*}$
Methanol 10% + Date palm extract 5%	85.25 ± 2.50^{bc}
Methanol 10% + Date palm extract 10%	88.50 ± 1.73^{c}
Methanol 10% + Date palm extract 15%	81.75 ± 1.71^{b}
Methanol 10% + Date palm extract 20%	81.25 ± 2.75^{b}
Methanol 10% + Date palm extract 25%	80.75 ± 2.22^{b}
Fresh spermatozoa	94.00 ± 1.63

* Different letters in the column indicate significant differences ($P < 0.05$)

Based on Table 9, the highest fertilization rate percentage was obtained in the treatment of stored spermatozoa with the addition of a combined 10% methanol and 10% date palm extract, reaching 88.50 ± 1.73%. Spermatozoa stored with 10% methanol without adding date palm extract showed the lowest percentage, 73.75 ± 2.63%. The percentage of successful *T. soro* fertility using 48 hours post-cryopreserved spermatozoa ranged from 73-88%, which suggests their effectiveness in fertilizing the ovum. The percentage of successful fertilization using

post-cryopreserved spermatozoa was smaller than that of the fresh semen, which reached 94.00 ± 1.63%.

Table 10 demonstrates the fertilization rate of cryopreserved spermatozoa from different species.

Table 10 Fertilization rate of various species of cryopreserved spermatozoa

Species	CPA + Extender	Fertilization rate (%)	Author
Tor soro	10%MeOH+ 10%Dates+Ringer	88.50 ± 1.73	Present paper
Epinephelus lanceolatus	10%MeOH+ 10%Dates+Ringer	66.25 ± 3.23	[48]
Chromobotia macracanthus	10%MeOH+ 0.1%Honey+Ringer	98.55 ± 1.69	[36]
Piaractus mesopotamicus	1part semen + 3 parts CPA	59.01 ± 25.69	[33]
Rasbora tawarensis	5%DMSO+ 5%Egg Yolk	55.95 ± 12.43	[15]
Oreochromis mossambicus	12.5%M ±eOH+ 175mMGlucose	49-52	[20]
Cyprinus rubrofuscus	1mL Dates+ 99 mL Ringer Lactate	84.91%	[31]
Osteochilus vitatus	50 mg/L Glutathion+ [DMSO+Egg Yolk]	51.33 ± 17.01	[43]
Salmo cettii	DMSO+ Glucose	36.5 ± 5.5	[10]
Epinephelus lanceolatus	10%MeOH+ 15% Egg Yolk+ Ringer	77.31 ± 1.9	[46]

According to Galo et al. [33], the percentage of successful fertilization using post-cryopreserved spermatozoa was generally lower than that of the fresh spermatozoa. Damage to spermatozoa during the clotting process, such as loss of movement ability or morphological damage, prevents them from fertilizing the ovum [33]. In detail, Galo et al. demonstrated that decreased value in spermatic vigor and duration of motility caused by changes in structural morphology of cryopreserved spermatozoa explained the reduced fertilization and hatching rate observed in *Piaractus mesopotamicus* [33]. Sperm damage is caused by cold shock, cooling processes, and changes in osmotic pressure which affect the cell membrane stability [44]. The fertilized egg forms a cleavage shoot, while unfertilized eggs are presented round and brightly colored [48].

Previous research on fertilization using post-cryopreserved sperm was conducted by Di Iorio et al. on *Salmo cettii* using a synthetic combination such as DMSO, DMA, glycerol, ethylene glycol, and methanol as intracellular cryoprotectants, and serum albumin, sucrose, and glucose as the extracellular [10]. The results showed a smaller percentage value when compared to that of *T. soro*'s fertility, which was 36.5 ± 5.5%. Widyaningsih's research on *E. lanceolatus* using combined glycerol cryoprotectants and date palm extract found that their fertility rate was 66.25 ± 3.23% when using post-cryopreserved spermatozoa [48]. This value has decreased compared to that of fresh sperm, which reached 83%. Fish sperm parameters such as motility, sperm concentration, volume, seminal plasma pH, osmolality, DNA integration, membrane stability, mitochondria status, enzymatic activity, and male fertility are the main sperm quality indicator and important for sperm function [50], [51], [52].

4. Conclusion

The utilization of 10% methanol and 10% date palm extract as intracellular and extracellular cryoprotectants, respectively, is the optimum combination with the highest post-cryopreserved *T.soro* spermatozoa percentage motility of 81.29 ± 1.01%. It also had the highest fertilization rate percentage of 88.50 ± 1.73% and the lowest abnormality of 21.50 ± 1.29%.

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