

Cryopreservation of Nile Tilapia (*Oreochromis niloticus*) Sperm

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Abstract

The main aim of this study is to determine the effect of the straw volume (0.25 vs. 0.5 mL) on Nile tilapia sperm quality after cryopreservation. Sperm was frozen according to conventional slow freezing procedure and diluted at ratio of 1:3 with ionic extender containing 350 mM glucose and 30 mM Tris containing 10% dimethylacetamide. Diluted semen was equilibrated at 4°C for 10 min and drawn into 0.25-mL or 0.5-mL plastic straws and sealed with polyvinyl alcohol. Samples were frozen 3 cm above of the liquid nitrogen surface and exposed to the liquid nitrogen vapor ($\approx -140^\circ\text{C}$) for 10 min. After this, frozen sperm cells were kept into the liquid nitrogen container (-196°C). The frozen sperm in different volume of straws were thawed in a water bath at 30°C for 20 s (0.25-mL straws) or at 30°C for 30 s (0.5-mL straws), respectively. Fertilization was conducted using 1×10^5 spermatozoa/egg ratio with each straw type. The findings of the present study indicated that cryopreservation of sperm in glucose-Tris-based extender using 0.5-mL straws improved post-thaw progressive motility, duration of progressive motility, and fertilization results ($P < 0.01$). On the other hand, differences in term of post-thaw cell viability was not significant among the treatments ($P > 0.01$). In conclusion, our results suggest that Nile tilapia sperm can be successfully cryopreserved in Tris-based extenders supplemented with glucose containing 10% dimethylacetamide in 0.5-mL straws.

Keywords: *Oreochromis niloticus*, sperm, cryopreservation, straw volume, dimethylacetamide

1. Introduction

Cryopreservation biotechnology has important roles for aquaculture industry and also for conservation of aquatic genetic resources. In this field, sperm cryopreservation has been used

for transporting of genetic material between facilities, optimal using of gametes in aquaculture, reducing risk of spreading infections, performing of hybridization studies, conserving of protecting endangered species, and also for conserving of biodiversity [1, 2].

Cryopreservation technique involves addition of cryoprotectants to the extender and freezing and thawing of sperm samples, which may result in some damage to the spermatozoa and may decrease egg fertilization rate. Therefore, before cryopreservation of spermatozoa, a thorough evaluation of different extender solutions, cryoprotectants, and cooling and thawing rates are essential to develop optimum cryopreservation protocol for various species [3–5].

During the cryopreservation process, some factors may change the physiological status of sperm. The success of cryopreservation depends not only on preserving the motility of the spermatozoa but also on maintaining their metabolic functions [6]. Extender composition and cryoprotectant concentration are the main factors affecting cryopreservation success [7]. Extenders are required for dilution of fish sperm prior to cryopreservation and are generally designed to be compatible with the physiochemical composition of the fish seminal plasma. Most important function of the extenders is to maintain the spermatozoa in immotile state until required [8].

Cryoprotectants are added to the extenders to protect the cells against ice crystal formation during freezing and thawing [9]. Although cryoprotectants help to the prevention of cryoinjuries during freezing and thawing, they may become toxic to the cells when exposure time and concentration are increased [10, 11]. Thus, one of the most critical steps in successful cryopreservation of fish semen is the choice of the cryoprotectant and its ratio in the extender during the process.

Another important problem is the handling of sperm produced in small volumes by some fish species such as tilapia. In spite of packaging of sperm in traditional 0.25-mL and 0.5-mL straws has been successfully applied to freeze semen of the most fish species and to fertilize small egg batches [9], there is a lack of information regarding their usage in cryopreservation of Nile tilapia (*Oreochromis niloticus*) semen.

The Nile tilapia is one of the most cultivated freshwater fish species in the world aquaculture [12]. This species has great breeding potential due to its hardiness against worse environmental conditions, fast growth rate, adaptation to different environmental conditions (e.g. salinity, temperature), and also good organoleptic characteristics of its flesh [13, 14]. On the other hand, most of studies related with fish sperm cryopreservation have focused on some freshwater species, such as cyprinids [15, 16], salmonids [17, 18], catfishes [19, 20], and loach [21].

Even though many successes have been achieved in fish semen cryopreservation, the technique remains as a method that is difficult to standardize and use in all types of fishes. This is due to the fact that cryopreservation of sperm from different fish species required different conditions, where the protocol needs to be established individually [22]. To the best of our knowledge, there is limited information regarding cryopreservation of Nile tilapia sperm. In this concept, the effect of cryoprotectants and packaging methods on freezability and also on post-thaw quality of Nile tilapia sperm still remains unclear. Thus, standardization and

simplification of cryopreservation procedure for Nile tilapia sperm are needed for commercial and gene bank applications.

The main aim of this study was to establish an efficient method for cryopreservation of Nile tilapia sperm that can be applied to aquaculture of this species. The present experiment was designed to study the straw volume (0.25 vs 0.5 mL) on Nile tilapia sperm quality after cryopreservation using glucose-Tris-based solution containing 10% dimethylacetamide.

2. Materials and methods

2.1. Reagents

The additives and other chemicals used in this study were obtained from local representative of Sigma-Aldrich Chemicals Company (St. Louis, MO, USA).

2.2. Broodstock handling

The experiments were carried out spawning season of the Nile tilapia. In the pre-spawning period, sexually mature male (n=15) and female (n=5) Nile tilapia were pit-tagged and kept separately in 150 L indoor tanks under constant environmental conditions. The broodstock tanks were provided with freshwater constantly at ratio of 1.5 L/s, while compressed air was provided through air stones. The water temperature ranged from 27.2°C to 30.5°C, and salinity was maintained at 1.5 ppm. Nile tilapia was fed with floating pellets twice daily (1–5% body weight per day).

2.3. Gamete collection

Gametes were collected from healthy mature males and females following immersion anesthesia with 10 ppm quinaldine (Reanal Ltd., Budapest, Hungary) for a few minutes. For sperm collection, 1-mL tuberculin plastic syringe, without needle, was used to aspirate sperm released by gentle abdominal massage to eliminate urine in the ducts. Following, sperm samples were transferred individually into 1.5-mL Eppendorf tubes on ice (0–4°C). A 10-μL pipette tip connected to a mouth pipette was used to extract sperm cells, which were diluted 1:1 in Hanks' balanced salt solution (HBSS) (280 mOsm/kg, pH 7.0) in 1.5-mL microcentrifuge tubes and placed on ice until analysis. Eggs were also collected by gentle abdominal massaging and stored in HBSS at 25°C and used for fertilization within 30 min following stripping [23].

2.4. Gamete quality determination

One microlitre sperm of each sample was placed on a microscope slide and observed under a phase-contrast microscope (Olympus, Japan) at 100× magnification. The motility characteristics of the collected sperm samples were evaluated by adding activation solution (AS) (45 mM NaCl, 5 mM KCl, and 30 mM Tris-HCl, pH 8.2) [24] at a ratio of 1:100. Sperm cells that vibrated in place were considered as immotile. Only samples whose quality parameters ranging

between the following values were used for the cryopreservation experiment: osmolality 50–100 mOsm/kg, pH 7.0–8.0, and progressive motility 80–100% [25]. The quality of ova was determined from their morphological features seen under a dissecting microscope as described in the study of Fauvel et al. [26].

2.5. Cryopreservation and thawing experiments

Semen samples showing ≥ 80 motility was pooled into equal aliquots and chosen for cryopreservation experiments. Semen and extenders were kept at 4°C immediately under aerobic conditions prior to dilution. Pooled semen was diluted at a ratio of 1:3 with an extender composing 350 mM glucose and 30 mM Tris [27] containing 10% dimethylacetamide (DMA). The diluted semen were drawn into 0.25-mL or 0.5-mL plastic straws (IMV France) and sealed with polyvinyl alcohol (PVA). Following equilibration of semen for 10 min at 4°C, the straws were placed on a styrofoam rack floating on the surface of liquid nitrogen in a styrofoam box. Samples were frozen 3 cm above of the liquid nitrogen surface and exposed to the liquid nitrogen vapor ($\approx -140^\circ\text{C}$) for 10 min [28]. Following, frozen sperm cells were kept into the liquid nitrogen container (-196°C) until analyses for a few days.

The frozen sperm in different volume of straws were thawed in a water bath at 30°C for 20 s (0.25-mL straws) or at 30°C for 30 s (0.5-mL straws). Thawed semen was activated using activation solution (AS) (45 mM NaCl, 5 mM KCl, and 30 mM Tris-HCl, pH 8.2) [24] and observed under a phase-contrast microscope (Olympus, Japan) for progressive motility (%), progressive motility duration (s), and viability (%) evaluations (three replicates). At least five straws were used for each parameter evaluation with three replications.

2.6. Post-thaw sperm quality determination

The percent of motile spermatozoa and motility duration was immediately recorded following activation using a CCD video camera (CMEX-5, Netherland) mounted on a phase-contrast microscope (100 $\times$, Olympus BX43, Tokyo, Japan) at room temperature (20°C). Progressive spermatozoa motility and duration of progressive spermatozoa motility were evaluated from sperm with forward movement. Immotile spermatozoa were defined as spermatozoa that did not show forward movement after activation. Percentage of spermatozoa motility was determined within 30 s post-activation. Motility duration was evaluated by counting the time from spermatozoa activation until spermatozoa stopped moving. In order to assess viable sperm percentage, eosin-nigrosin preparations were made according to the method described by Bjorndahl et al. [29] and totally 300 sperm cells were counted on each slide at 1000 $\times$ magnification. At least five straws were used for each evaluation parameter, and analyses were repeated three times for each treatment.

2.7. Fertilization experiments

Pooled eggs from five mature females were used to assess fertilization rates. In this stage, most of the HBSS was decanted from the eggs, and fertilization was carried out in dry Petri dishes (10 cm diameter). Fresh or thawed sperm was added over the eggs and gently mixed before

activation with 20 mL of fertilization solution (3 g urea and 4 g NaCl in 1 L distilled water) [30]. Following fertilization process, 2 mL embryo buffer medium (EBM) (13.7 mM NaCl, 5.40 mM KCl, 0.25 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 1.30 mM CaCl_2 , 1.00 mM MgSO_4 , 4.20 mM NaHCO_3 at 52 mOsm/kg and pH 7.0) was added for activation as described by Westerfield [31]. After 10 min, 100 mL EBM was added again over the eggs and was left undisturbed without movement in a convection type incubator at 27°C (Panasonic MCO-19M-PE, Japan). Unfertilized eggs were removed, and EBM was changed twice daily. After 48 h, the eggs were evaluated for fertilization results. Eggs that developed to stage 11 (embryonic keel and somite formation) were recorded as fertilized eggs, described by Galman and Avtalion [32].

Fertilization experiments were carried out using 1×10^5 :1 spermatozoa/egg ratio with each straw types (0.25 or 0.5 mL) for the each aliquot of eggs (containing 100 eggs). Three straws were thawed for each fertilization treatment (three replications). For the control, another three aliquot of eggs (containing 100 eggs) were fertilized with fresh semen collected from two other males. Eggs were fertilized with fresh semen samples using the same number of sperm cells (1×10^5 cells) similar to treatments with frozen semen.

2.8. Statistical analysis

A two-way analysis of variance (ANOVA) including the straw volumes (0.25 and 0.5 mL) as fixed effects was used. Means were separated by Duncan's multiple range test and were considered at 5% level of significance. Results are presented as mean $\pm$ S.D. All analyses were carried out using SPSS 17 for Windows statistical software package.

3. Results

Semen volume was rather variable and ranged from 0.9 to 7.5 mL, with a mean volume of 3.6 ± 0.40 mL. Progressive motility was ranged from 60% to 90%, and mean motility was determined as $80.4 \pm 0.15\%$. In addition, mean progressive spermatozoa motility duration (s),

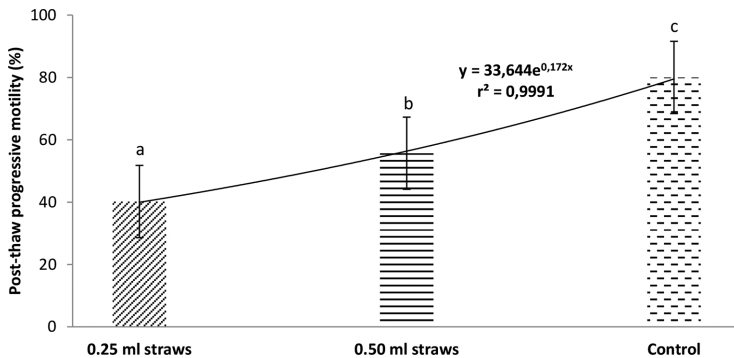


Figure 1. Post-thaw progressive motility (%) of Nile tilapia sperm cryopreserved with glucose-Tris-based extender. Columns marked with different letters are significantly different ($P < 0.01$, $n = 3$).

spermatozoa density ($\times 10^9/\text{mL}$), viability (%), and pH values were determined as 64.2 ± 0.45 s, $1.75 \times 10^9/\text{mL}$, $92.5 \pm 4.25\%$, and 7.2 ± 0.25 , respectively. In addition, mean fertilization rate was determined with fresh semen, which was $72.5 \pm 0.20\%$. The findings of the present study indicated that cryopreservation of sperm in glucose-Tris-based extender using 0.5-mL straws increased post-thaw progressive motility (**Figure 1**), duration of progressive motility (**Figure 2**), and fertility (**Figure 4**) ($P < 0.01$). On the other hand, differences in terms of post-thaw cell viability were not significant among the treatments (**Figure 3**, $P > 0.01$). The fertility of the frozen-thawed sperm showed high positive linear correlation with motility ($r^2 = 1.000$, **Figure 5**) and ($r^2 = 0.9932$, **Figure 6**) in case of using 0.25-mL and 0.5-mL straws.

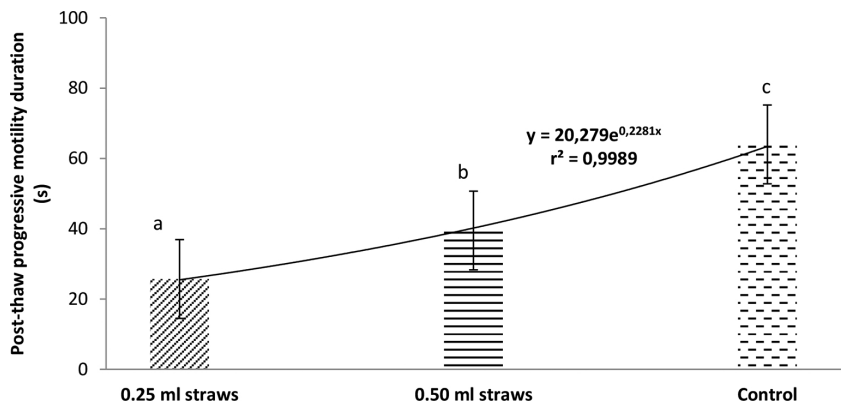


Figure 2. Post-thaw progressive motility duration (s) of Nile tilapia sperm cryopreserved with glucose-Tris-based extender. Columns marked with different letters are significantly different ($P < 0.01$, $n = 3$).

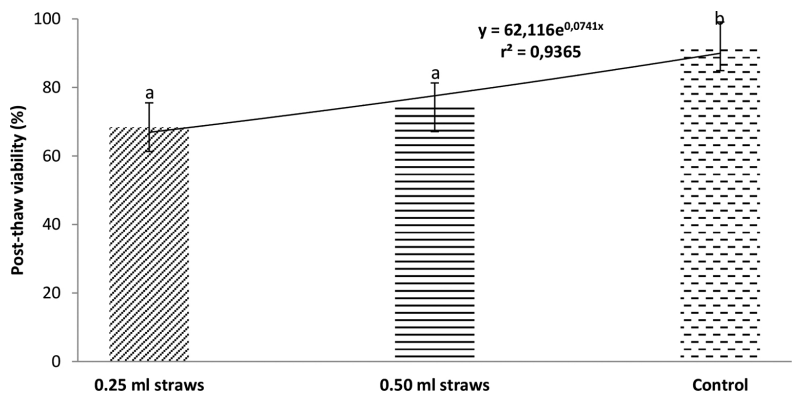


Figure 3. Post-thaw viability (%) of Nile tilapia sperm cryopreserved with glucose-Tris-based extender. Columns marked with different letters are significantly different ($P < 0.01$, $n = 3$).

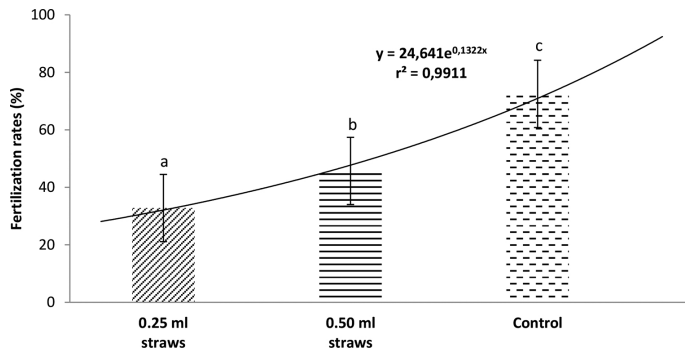


Figure 4. Post-thaw fertility (%) of Nile tilapia sperm cryopreserved with glucose-Tris-based extender. Columns marked with different letters are significantly different ($P < 0.01$, $n = 3$).

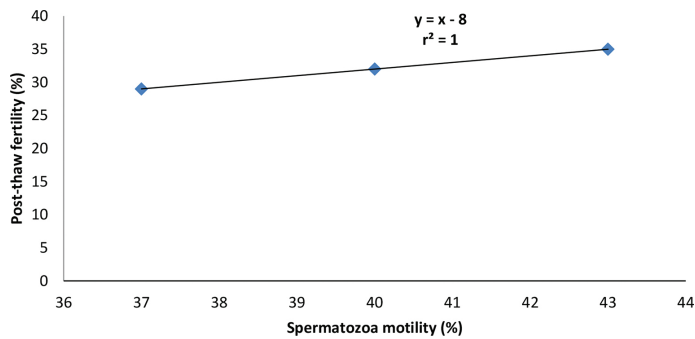


Figure 5. Relationship between post-thaw spermatozoa motility (%) and fertility (%) of Nile tilapia sperm cryopreserved with glucose-Tris-based extender using 0.25-mL straws.

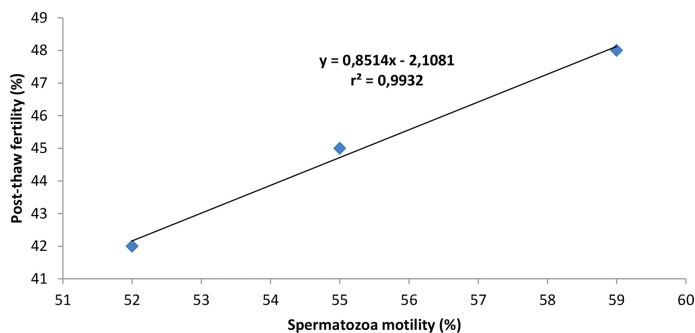


Figure 6. Relationship between post-thaw spermatozoa motility (%) and fertility (%) of Nile tilapia sperm cryopreserved with glucose-Tris-based extender using 0.50-mL straws.

4. Discussion

Tilapias are widely cultured in the tropical and subtropical regions of the world. Several species of tilapia are cultured commercially, but Nile tilapia is the predominant cultured species worldwide that its production reached 3,197,330 mt in 2012 [33].

Although Nile tilapia is a freshwater fish, it can tolerate a wide range of salinity [34]. Therefore, the expansion of its culture in sea and brackishwater has attracted the attention of fish farmers in recent years. However, limited reports have addressed semen cryopreservation in the Nile tilapia. Therefore, standardization and simplification of the cryopreservation procedure for the Nile tilapia sperm is needed for commercial application. On the other hand, because of limited amounts of data are available, comparison of the methods and results have been mainly made with the cyprinid species in this research. From this point of view, findings of this research significantly contribute improving of the protocols applied for the cryopreservation of the Nile tilapia sperm.

Sperm cryopreservation is an important biotechnological technique with specific advantages to the aquaculture industry. Improvements in semen cryopreservation techniques require in-depth knowledge of gamete physiology and the biochemical processes occurring during semen collection, processing, freezing, and thawing [35].

In spite of routinely using of cryopreserved semen in aquaculture artificial insemination programs worldwide, there are inconsistencies in experimental results [36]. The success of cryopreservation mainly depends on maintaining the spermatozoal metabolic functions [37]. The major factors affecting the results of insemination with frozen/thawed sperm are the type and properties of extenders and cryoprotectants, the damage caused by the formation of internal ice crystals due to the increase in solute concentration in the extension media and the interaction of these factors [38].

In the present study, glucose-Tris-based extender supplemented with DMA was used to cryopreserve Nile tilapia sperm. In spite of using glucose mainly as energetic substrate, it has been used due to its stabilization effects on the spermatozoa liposomal membrane [39]. It should be noted that, carbohydrate-based solutions such as glucose have also been found effective in some experiments [40, 41], and Tris is the most common buffer solution, not only for cyprinidae but also for other fish species [42].

Cryoprotectants are added to the extenders in order to protect the spermatozoa from damages during the freezing/thawing process. On the other hand, cryoprotectants can suppress most cryoinjuries when used at higher concentrations but at the same time it can become toxic to the cells. In addition, the amount and type of cryoprotectants used in sperm diluent depend on fish species and can affect the physiological and metabolic structure of the spermatozoa during cryopreservation procedure in different ways [43]. Therefore, selection of suitable type and concentration of the cryoprotectant is needed for the development of an effective cryopreservation protocol. However, comparison of different cryoprotectants and freezing/thawing protocols are difficult when each treatment tested for the ability of fertilization of the eggs by spermatozoa. For this reason, the protective effect of different cryoprotectants varies

in different fish species. In this concept, several cryoprotectants have been mainly used for fish sperm cryopreservation, such as dimethyl sulphoxide (DMSO), dimethyl acetamide (DMA), glycerol (Gly), methanol (MeOH), ethylene glycol (EG), and propylene glycol (PG) [44–46].

Cryoprotectants are essential for preservation, but it is dissimilarly toxic to the cells. The toxicity tolerance level of the cells also depends on cryoprotectant concentration. Also, there are differences in permeability of the cells according to cryoprotectant types. In this study, DMA as penetrating cryoprotectant was used at 10% concentration, and diluted samples were equilibrated for 10 min at 4°C. Some authors recommend having an equilibration period following dilution, allowing cryoprotectants to penetrate the spermatozoa before cryopreservation [9, 47]. However, some authors reported equilibration process did not improve cryopreservation success in fish [48, 49].

On the other hand, the freezing conditions depended on the straw size and were also species specific. Insemination with cryopreserved semen of Arctic charr (*Salvelinus alpinus*) in 1.7-mL flat straws using 10% DMSO resulted highest percentage of eyed eggs ($57.9 \pm 11.6\%$) than with 0.5-mL and 2.5-mL straws [50]. No significant difference was obtained between fertilization percentage of blue catfish spermatozoa frozen in 0.5-mL and 1-mL straws. The larval hatch rates of striped trumpeter (*Latris lineata*) semen frozen in 0.25-mL and 0.5-mL straws ($44.3 \pm 2.9\%$ and $44.2 \pm 2.0\%$) [51]. In case of common carp, the use of conventional 0.5-mL straws resulted in $67 \pm 17\%$ hatching [24]. The findings of the present study demonstrated that progressive motility decreased for the two types of tested straws and varied from 55.7% in 0.5-mL to 40.2% in 0.25-mL straws. In addition, the 0.5-mL straws gave the best results in fertility as 45.7% when compared with 0.25-mL straws. The use of these small volumed straws during the artificial reproduction in fish can be time consuming, as many straws are needed to fertilize thousands of eggs. On the other hand, small volumed straws are more suitable for gene banking or fertilization of small amounts of eggs under laboratory conditions.

Successful fertilization of eggs using cryopreserved sperm is the final target of cryopreservation process. Fertilization ability of the cryopreserved sperm is a reliable approach to evaluate success of the cryopreservation protocol [52]. According to the results of the present study, Nile tilapia spermatozoa were influenced by cryopreservation process, and depending on this interaction, fertilization ability of frozen/thawed sperm decreased than fresh ones. The reason for the low fertility rate of frozen/thawed spermatozoa may be attributed to the changes in ultrastructural morphology, decrease in progressive motility and motility duration, and also possible toxic effects of the DMA.

Motility is one of the most important factors to assess fish sperm quality because it gives important information about the sperm cell's energy sources. In addition, better knowledge of the characteristics of fresh sperm motility is necessary to evaluate sperm quality in commercial hatcheries before artificial reproduction and also in laboratories before experiments. Spermatozoa motility is induced following releasing of the spermatozoa into the aquatic environment during natural reproduction or after transferring to an activation medium during controlled reproduction [53].

The observed decrease in sperm motility might be due to decrease in the percentage of sperm viability, high damage of sperm cells, or decrease in ATP content following cryopreservation. Similarly, Alavi et al. [54] determined that almost all studies on sturgeon sperm cryopreservation showed significant lower sperm motility and fertilizing ability of frozen/thawed sperm compared to that of the fresh sperm.

On the other hand, when fish spermatozoa are released into water or activation medium, they have a brief spermatozoal activity period [55]. For instance, in fresh sperm, the duration of spermatozoa motility in several cyprinids have been reported to last 120 s [56]. Similarly, in case of silver barb, the maximum motility period was observed until 150 s after water activation [4]. However, in case of frozen/thawed sperm, duration of mean post-thaw spermatozoa motility (32.0 ± 8.16 s) of the Nile tilapia was determined as lower than the results assessed with mirror carp [57] but higher than that of scaly carp [46] when DMSO, DMA, and glycerol were used as cryoprotectant. Similar results for the motility parameters of frozen-thawed spermatozoa were reported in fish in some experiments [48, 58, 59]. On the other hand, it is interesting to note that Godinho et al. [60] reported 241.2 ± 57.3 s post-thaw spermatozoa motility duration in glucose-based cryosolution containing 10% methanol in Nile tilapia.

In the present study, the applied sperm/egg ratio was $1 \times 10^5:1$ for fresh as well as frozen/thawed sperm, which probably resulted in excessive sperm concentrations in all batches. However, according to Lubzens et al. [48], the concentration of frozen/thawed sperm to be used to achieve optimal fertilization and hatching success is approximately 100 times higher than for fresh semen. This may be due to differences in extender compositions, cryoprotectant types, equilibration periods, egg quality, or applied protocols. In the present study, high positive correlation was determined between post-thaw spermatozoa motility and fertilization. This situation was consistent with the results that obtained from turbot (*Psetta maxima*) [61], common carp (*Cyprinus carpio*) [49], and African catfish (*Clarias gariepinus*) [62].

5. Conclusion

In conclusion, Nile tilapia sperm can be successfully cryopreserved in a glucose-Tris-based cryosolution containing 10% DMA with 0.5-mL straws. The applied protocol can be used in commercial hatcheries to facilitate artificial reproduction of Nile tilapia due to acceptable post-thaw motility and fertility results obtained. On the other hand, additional researches are needed to examine the growth and survival of the larvae originated from cryopreserved sperm.

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