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Comparison of different freezing techniques, extenders, and cryoprotectants on quality and fertility of cryopreserved *Salmo trutta f. fario* sperm

Yusuf Bozkurt[1]* and **İlker Yavaş[2]**

[1]Department of Aquaculture, Faculty of Marine Sciences and Technology, İskenderun Technical University, İskenderun, Hatay, Türkiye. [2]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, Antakya, Hatay, Türkiye. *Author for correspondence. E-mail: yusuf.bozkurt@iste.edu.tr

ABSTRACT. There is still a lack of standardized methodology in the cryopreservation of *Salmo trutta f. fario sperm*. Thus, the present study was designed to compare current freezing protocols to improve and as well as to examine the post-thaw quality and fertilizing ability of cryopreserved sperm in *Salmo trutta f. fario*. Sperm samples were diluted at a 1:10 ratio in one of three extenders (Alsever's solution, glucose-based, and ionic-based) containing four types of cryoprotectant (DMSO, DMA, MeOH, glycerol) at 10% concentration and frozen in 0.1 mL pellets on surface of the dry ice (solid carbon dioxide, 79°C) or in 0.25 mL straws 2 cm above of the liquid nitrogen (LN$_2$) surface at a rate of ~30°C for 10 min.[1] before storage in a mL cryotank (-196°C). The frozen sperm cells in straws and pellets were thawed in a water bath at 25°C for 30 s and at 20°C for 6 s respectively. Fertilization was carried out using a ratio of 5x10^5 sperm/egg in both freezing methods. The glucose-based solution including glycerol produced the highest post-thaw progressive motility (62.5 ± 1.24%), motility duration (57.2 ± 0.46 s), and viability (56.4 ± 1.57%) (p < 005) in the straw method. In breeding trials, similarly, sperm frozen-thawed with the glucose-based solution including glycerol produced the highest fertilization (54.2 ± 0.36%) and hatching (30.6 ± 0.28%) in the straw method. Fresh sperm used as control produced 82.6 ± 0.45% and 78.4 ± 1.27% fertilization and hatching respectively. It was concluded that sperm frozen in straws produced higher post-thaw sperm motility and fertility of eggs than those frozen in pellets. Also, the results suggest using of glycerol-supplemented glucose solution because of producing better results in both freezing techniques.

Keywords: brown trout, cryopreservation, extender, straw, pellet.

Introduction

Conservation of aquatic genetic resources by cryopreservation biotechnology has an important role in increasing of aquaculture production and in preserving of the genome, mostly sperm cells, concerning threatened or endangered species as well. On the other hand, despite the progress in sperm cryopreservation of aquatic species, the implemented protocols differ greatly in terms of freezing techniques, extender compositions, cryoprotectant types, packaging methods, as well as cooling and freezing rates (Bozkurt & Yavaş, 2021).

Among factors affecting cryopreservation success, the interactions between extenders and cryoprotectants are important for spermatozoal resistance against freezing injuries and this interaction may also yield interesting results concerning motility, viability, and fertility of frozen-thawed sperm (Akcay, Bozkurt, Seçer, & Tekin, 2004). In many studies, there are highly variable results in cryopreservation of salmonid sperm using different extenders and cryoprotectants (Yamano, Kasahara, Yamaha, & Yamazaki, 1990; Wheeler & Thorgaard, 1991; Lahnsteiner, Weismann, & Patzner, 1997; Babiak et al., 2001; Bozkurt et al., 2019). On the other hand, several cryoprotectants s uch as dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), methanol (MeOH), and glycerol are considered to provide efficient results in salmonid sperm cryopreservation (Gallant & McNiven, 1991; McNiven, Gallant, & Richardson, 1993; Babiak et al., 2001; Bozkurt, Yavaş, Bucak, Kıran, & Gül, 2021).

Therefore, cryopreservation methods need to be improved in terms of field applications. In this regard, the straw method is more popular all over the world since it is an inexpensive and simple method. Although most of the cryopreservation experiments have focussed on the packaging of sperm in straws, packaging of sperm in pellets using dry ice is another alternative freezing method in which liquid nitrogen is unavailable to

improve reproduction efficiency from frozen-thawed sperm in aquaculture (Bozkurt, Akcay, Tekin, & Seçer, 2005; Viveiros, Orfao, Nascimento, Corrêa, & Caneppele, 2012). The freezing procedure for the pellet method is relatively simple and does not require expensive equipment. It is based on rapid cooling through direct plunging of sperm droplets into a block of dry ice (-79°C) to form frozen pellets (Tekin, Seçer, Akcay, Bozkurt, & Kayam, 2007; Dziewulska, Rzemieniecki, Czerniawski, & Domagała, 2011). Additionally, the pellet method only takes a few seconds to cool and warm resulting in fast freezing rates achieved compared to the straw method (Tselutin, Seigneurin, & Blesbois, 1999).

Cryopreservation is one of the efficient biotechnological tool for the conservation of aquatic biodiveristy. From this point of view, *Salmo trutta f. fario* is one of the important freshwater fish species in terms of biodiversity and economical value and, consequently, it has been cultivating for the purposes of enhancement and protection of the wild population in nature and also its maintenance for aquaculture. However, the population of *Salmo trutta f. fario* declines day by day because of illegal practices and heavy fishing pressure, degradation of spawning habitats, and river damming (Bozkurt & Seçer, 2006; Arndt et al., 2019). In this regard, cryopreservation of *Salmo trutta f. fario* sperm is an effective strategy for protecting the biodiversity of the populations of this species.

However, it is to be highlighted that salmonid semen is among the most difficult to cryopreserve because of its peculiar features such as short duration of motility, low adenosine triphosphate (ATP) production, and its high sensitivity to osmotic stress, which make it more sensitive to the cryopreservation process (Martínez-Páramo et al., 2009).

Therefore, the development of effective cryopreservation protocols for *Salmo trutta f. fario* sperm is needed to improve reproduction efficiency in hatcheries and establish cryobanks. From these points of view, the main objective of this study was to compare two freezing techniques (straw or pellet), three extenders (Alsever's solution, glucose-based solution, and ionic-based solution), and four cryoprotectants (DMSO, DMA, MeOH, and Glycerol) to improve cryopreservation of *Salmo trutta f. fario* sperm. The comparison was made considering sperm motility, and sperm viability after thawing. Also, the fertilization rates of eggs and the hatching of larvaes were evaluated from both cryopreservation protocols.

Material and methods

Chemicals

All chemicals and reagents were obtained from Sigma-Aldrich (Germany).

Broodstock and gamete collection

In spawning season, mature male (486.2 ± 5.7 g, n = 16) and female (648.4 ± 2.8 g, n = 7) *Salmo trutta f. fario* broodstock (2- to 4 years old) were obtained from a commercial aquafarm located in Gaziantep, Türkiye. The broodstock was maintained in 750 L indoor tanks which were supplied with constant hatchery water (11.8 ± 0.4°C; 10.5 ± 6.2 mg L^{-1} O_2) flow at the rate of 3.2 L s^{-1} during the experimental period.

For gamete collection, male and female broodstock were anesthetized in a 50 L tank with 100 mg L^{-1} tricaine methanesulphonate (MS-222) (Sigma, Germany) for a few minutes (Sarvi et al., 2006). The urinary bladder was gently emptied and the genital area was dried. The sperm was collected separately into a 50 mL sterile falcon tubes by abdominal massage and maintained in a styrofoam box including crushed ice (4 ± 2°C). Then, the fresh sperm in falcon tubes were transferred to the laboratory immediately to evaluate its quality in terms of motility, density, pH, and colour. For egg collection, mature females were wiped dry and stripped by gentle abdominal massage into a 1000 mL round-bottomed bowl. The collected eggs were evaluated in terms of homogenous shape, colour, and size, and those are used for fertilization within 30 min. of collection.

Sperm quality evaluation

The motility of sperm samples were evaluated subjectively as reported in our previous papers (Bozkurt et al., 2021; Bozkurt & Yavaş, 2021). For this aim, 1 µL of sperm suspension was placed on a glass slide and activated by adding 1000 µL activation solution (AS) (125 mM NaCl, 20 mM Tris-HCl, 30 mM glycine pH 9; Billard, 1992) and sperm motility was determined using a CCD video camera (model SPC-2000P; Japan) mounted on a phase-contrast microscope at 100x magnification (BX43; Olympus, Tokyo, Japan). The observed video records were scanned to evaluate the percentages (%) and duration (s) of motility at least three times

for each sample. Sperm motility was evaluated as the percentage of cells exhibiting progressive forward movement, whereas the duration of motility was evaluated until forward movement stopped.

The density of sperm cells were determined according to the hemocytometric method. For this aim, sperm were diluted at a ratio of 1:1000 with Hayem solution (35.2 mM Na_2SO_4, 17.1 mM NaCl, 1.8 mM $HgCl_2$, 200 mL bicine), and density was determined using a 100 µm deep Thoma hemocytometer (TH-100; Hecht-Assistant, Sondheim, Germany) at 400x magnification with an Olympus BX50 phase contrast microscope (Olympus) and expressed as sperm x $10^9\,mL^{-1}$ (three replicates). Standard pH electrodes were used to measure sperm pH, and semen colour was evaluated visually within 30 min. of sperm collection. mL

Sperm viability was evaluated according to Bjorndahl, Söderlun, and Kvist (2003) using eosin-nigrosin stain (0.67 g eosin Y, 0.9 g of sodium chloride, and 10 g nigrosin dissolved in 100 mL of distilled water). For this aim, a mixture of 5 µL of sperm with 5 µL of the stain was spread on a clean slide and remained to air dry in a dust-free environment. The percentage of live sperm cells was calculated from a total of 300 sperm cells examined under ×100 oil immersion with a phase-contrast microscope (Olympus). In this way, unstained sperm cells were considered alive, while stained sperm cells were considered as dead (Bozkurt & Yavaş, 2021).

Supplementation of sperm with extenders and cryoprotectants

Sperm obtained from 14 males exhibiting > 80% motility and having approximately 12.5×10^9 sperm mL^{-1} density were used for each protocol. Two sperm samples showing below 80% motility were discarded and the remaining samples were pooled in equal volume and used for experiments. Sperm were individually split into three sub-pools and each of them was diluted at a ratio of 1:10 (v:v) with three different extenders, which were supplemented with 10% of DMSO, DMA, Glycerol, or MeOH, respectively composing (E-I: Alsever's solution; 0.4 g NaCl, 0.8 g sodium citrate, pH 7.9; Sarder, Sarker, & Saha, 2012), (E-II: glucose-based solution; 300 mM glucose, 10% egg yolk; Tekin, Seçer, Akcay, & Bozkurt, 2003), (E-III ionic-based solution; 103 mmol L^{-1} NaCl, 40 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM Hepes, pH. 7.8; Lahnsteiner & Mansour, 2012). Before the freezing process, diluted sperm were equilibrated in a cool chamber at +4°C for 10 min. (Tekin et al., 2007). Resultingly, 12 treatments (each treatment includes 10 straws or pellets) with nine replicates were applied to each of both freezing methods.

Sperm cryopreservation techniques

Straw method

Sperm was packaged in 0.25 mL straws and sealed with clay. Sperm was frozen 2 cm (freezing rate mL 30°C min.$^{-1}$) above of the liquid nitrogen (LN_2) surface inside a polystyrene box for 10 min. (Cabrita, Robles, Alvarez, & Herráez, 2001) Resultingly, twelve straws per sample were frozen. Then, the frozen samples in each experiment were plunged into the liquid nitrogen (-196°C). After two weeks storing of samples in cryotank, thawing was performed with at least three straws per treated group in a water bath at 25°C for 30 s for 0.25 mL straws in terms of evaluation of post-thaw sperm characteristics (Jawahar & Betsy, 2020).

Pellet method

Sperm samples (100 µL) were drawn into small hemispherical indentations (approximately 1 cm diameter) on the surface of dry ice (solid CO_2, -79°C) within 90 s of dilution and were frozen in pellet form (freezing rate mL 30°C min.$^{-1}$) for 10 min. (Jawahar & Betsy, 2020). Frozen pellets were collected using forceps, each pellet was rapidly placed in cooled cryovials and stored in a cryotank containing liquid nitrogen at -196°C. Resultingly, thirty pellets per treated group were frozen. After two weeks storing of samples in the cryotank, thawing of sperm was performed with at least three pellets per treated group by immersing the bottom section of the cryovial in a water bath at 20°C for 6 s for the evaluation of post-thaw sperm characteristics (Dziewulska et al., 2011).

Fertilization and hatching experiments

The good-quality eggs from three females were pooled and divided into aliquots of about 100 eggs. Each egg batch was fertilized with 100 µL frozen-thawed sperm from straws or pellets (sperm to egg ratio: ~5×10^5:1) in 500 mL round-bottomed bowls in all experimental (straw and pellet) groups. Also, fertilization was carried out using 25 µL of fresh sperm (motility > 80; sperm to egg ratio: ~5×10^5:1).

Afterward, the eggs were slightly stirred for 30 min. by adding the fertilization solution (125 mM NaCl, 20 mM Tris-HCl, 30 mM glycine, pH: 9) onto the eggs in a ratio of 1:2 (fertilization solution/eggs) (Lahnsteiner,

2000). Then, the eggs were rinsed with hatchery water and left for 45 min. to swell the eggs. Finally, the eggs were gently transferred into the labeled vertical incubators supplied with flow-through hatchery water at about 12°C.

Fertilization success was evaluated as the percentage of eyed eggs ~35 days following incubation. The hatching rate was determined as the number of individuals as a proportion of the total number of eggs, by counting the individuals at hatching (Dziewulska et al., 2011). Three replicates were performed for each egg batch with frozen-thawed sperm from both cryopreservation protocol and with fresh sperm as well.

Statistical analyses

Results were presented as mean ± S.D. Motility values were normalized through arcsine transformation and differences among the parameters were tested with 2-way ANOVA. Duncan's *post-hoc* test was implemented for all comparisons among the treatments at a level of $P < 0.05$. All statistical analyses were carried out using the SPSS 17.0 software package program.

Ethical statements

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Results

Fresh sperm

In order to determine spermatological properties in fresh sperm 16 males were used. The males had a sperm volume higher than 15 mL with a sperm motility > 70%. The mean percentage (%) and duration (s) of progressive motile sperm cells are 86.2 ± 3.46% and 64.8 ± 2.35 s, and sperm density varied between 10.6 and $22.4x10^9 \, mL^{-1}$ (n = 16). Fresh sperm used as control produced mean 82.6 ± 0.45% and 78.4 ± 1.27% fertilization and hatching respectively. Mean spermatological properties of fresh sperm are given in Table 1.

Table 1. Mean sperm quality parameters of fresh sperm of *Salmo trutta f. fario* (n=16).

Volume (mL)	Motility (%)	Motility Duration (s)	Density (x10^9 mL)	Total Density (x10^9)	pH	Colour
19.25±2.42	86.2±3.46	64.8±2.35	18.4±2.27	331.2±5.27	7.28±0.27	Milky White

Cryopreservation methods

For all cryopreservation methods, all tested parameters are found significantly lower in thawed samples than in fresh sperm (p < 0.05). Addition of different cryoprotectants to the all extender types caused statistical differences in motility, duration, viability, fertility, and hatching parameters of frozen-thawed sperm in comparison to conrol values (fresh sperm) in two compared cryopreservation methods (p < 0.05). Proportion of all tested parameters are significantly higher in the straw method than from that of pellet method (p < 0.05) (Figure 1 and 2).

Straw method

As shown in Figure 1, the findings of the present study indicated that the highest mean post-thaw progressive motility (62.5 ± 1.24%), motility duration (57.2 ± 0.46s), and viability (56.4 ± 1.57%) parameters are observed with the glucose-based solution containing glycerol (p < 0.05). On the other hand, whereas the lowest post-thaw mean progressive motility (40.2 ± 0.45%), and viability (34.6 ± 0.28%) are observed with the Alsever's solution, the lowest mean post-thaw motility duration (27.3 ± 1.4%) was obtained with the ionic-based solution when DMA was used in both solutions (p < 0.05).

Among the cryopreserved samples, the highest mean post-thaw fertilization (54.2 ± 0.36%) and hatching (30.6 ± 0.28%) values were observed with the glycerol containing glucose-based solution (p < 0.05). However, post-thaw fertilization (25.4 ± 0.48%) and hatching (8.24 ± 0.17%) results were found as the lowest in DMA and MeOH containing Alsever's solution respectively (p < 0.05) (Figure 1).

Pellet method

The highest results were observed with the glucose-based solution containing glycerol in terms of post-thaw progressive motility (56.2 ± 1.45%), motility duration (42.5 ± 1.27 s), and viability (50.6 ± 1.54%)

($p < 0.05$). In contrast, while the lowest post-thaw progressive motility ($32.5 \pm 0.45\%$), and viability ($27.3 \pm 0.28\%$) observed with the ionic-based solution, the lowest post-thaw motility duration ($19.7 \pm 1.8\%$) was obtained with the Alsever's solution when DMA was used in both solutions ($p < 0.05$).

Additionally, the highest mean post-thaw fertilization ($42.6 \pm 1.50\%$) and hatching ($25.7 \pm 1.62\%$) values were observed with the glycerol containing glucose-based solution ($p < 0.05$). However, the lowest mean post-thaw fertilization ($20.5 \pm 1.56\%$) and hatching ($4.5 \pm 1.27\%$) results were obtained with the Ionic-based solution containing DMA ($p < 0.05$) (Figure 2).
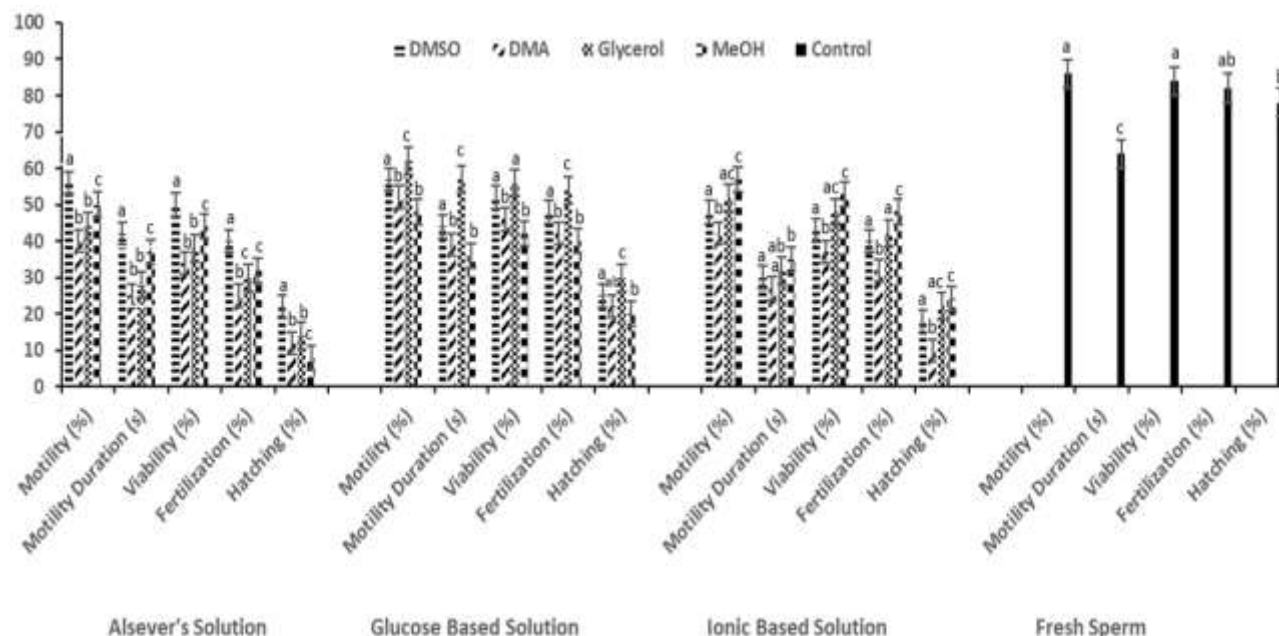

Figure 1. Effect of extenders (Alsever's, glucose-based, ionic-based) and cryoprotectants (DMSO, DMA, Glycerol, MeOH) on the mean post-thaw progressive motility (%), duration of progressive motility (s), viability (%), fertilization ability (%) and hatching of larvae (%) from frozen–thawed *Salmo trutta f. fario* sperm cryopreserved by straw method. Different letters indicate differences among treatments (ANOVA, $p < 0.05$, $n = 3$).
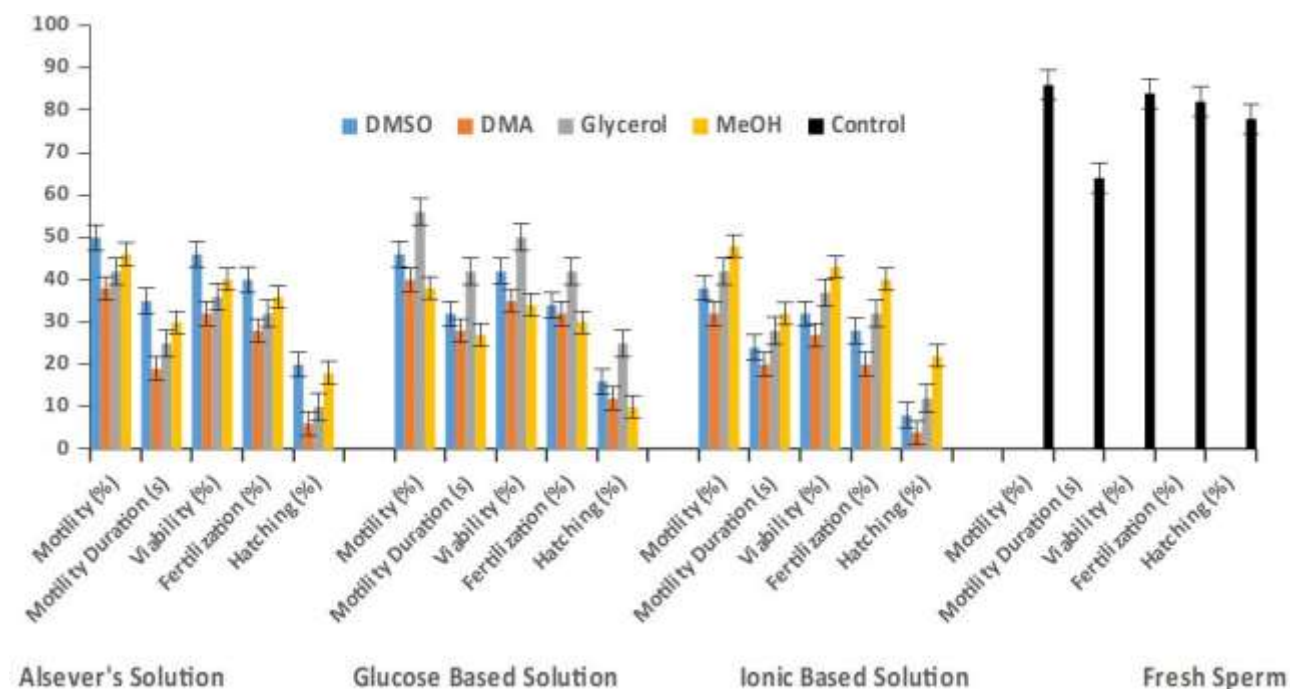

Figure 2. Effect of extenders (Alsever's, glucose-based, ionic-based) and cryoprotectants (DMSO, DMA, Glycerol, MeOH) on the mean post-thaw progressive motility (%), duration of progressive motility (s), viability (%), fertilization ability (%) and hatching of larvae (%) from frozen-thawed *Salmo trutta f. fario* sperm cryopreserved by pellet method. Different letters indicate differences among treatments (ANOVA, $p < 0.05$, $n = 3$).

Discussion

The present study demonstrated that the highest post-thaw quality, fertility, and hatching parameters were obtained with different extender and cryoprotectant compositions regardless of freezing methods. This situation shows that interaction among extender ingredients is an important factor to pay attention. The present study also reveals that there are differences in the permeability of sperm cells in terms of CPA and extender interactions.

In this study, when the post-thaw performance of sperm cryopreserved with three diluents including four cryoprotectants was compared, significant differences were observed concerning post-thaw motility, fertilization, and hatching results ($p < 0.05$). Among the twelve combinations of extenders and cryoprotectants, glucose solution supplemented with glycerol provided the best post-thaw motility and fertility in both straw (62.4 ± 0.45% motility; 54.2 ± 0.27% fertility) and pellet (56.2 ± 0.28% motility; 42.6 ± 0.38% fertility) method.

According to the results of this study, the glucose and glycerol cryosolution combination proved their suitability for the cryopreservation of *Salmo trutta f. fario* sperm. The success of carbohydrates can be explained by their cryoprotective effect. It is well known that saccharides usually serve as extenders in the cryopreservation of fish sperm (Stoss & Holtz, 1983; Ciereszko & Dabrowski, 1996). On the other hand, these compounds have a certain protective effect against freezing damage as membrane stabilizers (Maisse, 1994).

In this regard, several authors have reported the combined usage of different sugar types as part of CPA agents in semen cryopreservation extenders. For instance, Nynca, Dietrich, Dobosz, Grudniewska, and Ciereszko (2014) have demonstrated that using an extender containing 0.18 M glucose in 9% methanol resulted in remarkably high 73.8% post-thaw sperm motility in *Salmo trutta morpha fario*. Conget, Fernández, Herrera, and Minguell (1996) reported a range of 47-85% fertilization rates with cryopreserved semen of *Oncorhynchus mykiss* with an extender containing 0.6 M sucrose and 10% DMSO. Furthermore, it should also be noted that sugar-based extenders have improved post-thaw sperm quality and fertility of cryopreserved sperm in several salmonid species such as Mediterranean brown trout (*Salmo cettii*) (Rusco et al., 2019), Caspian brown trout (*Salmo trutta caspius*) (Sarvi et al., 2006), sea trout (*Salmo trutta m. trutta* L.) (Dziewulska & Domagała, 2013).

Taking into account the overall results obtained from these experiments, sugars can play a dual role in semen extenders as energy sources and CPA agents. Moreover, some authors observed that disaccharides seem more effective with respect to monosaccharides when it comes to causing osmotic dehydration (Pursel, Johnson, & Rampacek, 1972). It is suggested that the cryoprotective effects of the sugars on spermatozoa may differ according to the type of buffer (Abdelhakeam, Graham, & Vazquez, 1991) used in the extender and the molecular weight of the sugars (Molinia, Evans, Casares, & Maxwell, 1994).

On this matter, some authors indicated that the protective effect of sugars as a CPA agent is related to its specific osmotic effect, which induces a decrease in the intracellular freezability of water and consequently reduces sperm injuries provoked by ice crystallization (Aisen, Medina, & Venturino, 2002; Purdy, 2006). From this point of view, it is important to note that using an appropriate cryoprotectant solution prevents cells from cellular disruption and membrane damage during freezing and thawing. According to many authors, glycerol is one of the successful CPAs for many different fish species (Piironen, 1993; Gwo, 1994; Bozkurt, Yavaş, Ogretmen, Sivaslıgil, & Karaca, 2011; Brown, Colburn, Nardi, & Berlinsky, 2013; Sanchez-Serrano, Paniagua-Chavez, Segovia, & Weirich, 2014). In the case of *Salmonidae*, Lahnsteiner, Weismann, and Patzner (1995) showed that glycerol had a positive effect on the fertilization of salmonid fish (*Oncorhynchus mykiss*, *Salmo trutta fario*, *Salmo trutta lacustris*) eggs with the cryopreserved sperm. Tekin et al. (2007) also confirmed that glycerol is a useful CPA for cryopreservation of rainbow trout semen resulting in eyed eggs (49.3%) obtained with spermatozoa frozen with glucose extender containing 15% DMSO + 1% glycerol.

The action of glycerol in reducing the freeze-thaw damage to cells is similar to that of both non-permeating (Breddermann & Foote, 1969) and permeating (Medeiros, Gomes, Carmo, Papa, & Alvarenga, 2002) CPAs. On the other hand, glycerol at high concentrations can lead to programmed cell death (Wundrich, Paasch, Leicht, & Glande, 2006). Additionally, Gao et al. (1995) reported that osmotic toxicity occurs when the permeating CP is able to penetrate the cell membrane much slower than water. According to the results obtained in this study, it is clear that the glycerol concentration (10%) used in this study did not produce osmotic stress, which induced the maintenance of membrane integrity and motility of this species.

It is assumed that the addition of 10% egg yolk to the glucose solution ensured additional protection for *Salmo trutta f. fario* sperm during the freezing and thawing processes and resulted in increased fertilization rates. Many authors also showed that the egg yolk is a valuable component in extenders for the *salmonid* sperm cyopreservation. The results obtained from the experiments showed that the addition of egg yolk in extenders significantly increased the post-thaw sperm quality of Atlantic salmon (Alderson & Macneil, 1984), rainbow trout (Lahnsteiner, Berger, Weismann, & Patzner, 1996; Babiak et al., 2001), and brown trout (Bozkurt, Yavaş, & Karaca, 2012) compared to frozen semen without egg yolk. Additionally, in mammals, many authors have attributed using of egg yolk as part of effective extener to protect spermatozoa during cryopreservation in two ways such as yolk lipoproteins binding to sperm membranes and protecting them against injuries during cryopreservation (Vishwanath, Shannon, & Curson, 1992; Maldjian et al., 2005), and yolk antioxidants such as vitamin E and carotenoids, which reduce sperm lipid peroxidation that commonly occurs during the freezing and thawing process (Chatterjee & Gagnon 2001).

A similar interaction between DMSO, which has been considered a universal CPA, and Alsever's extender was also observed resulting in higher motility and fertility in both straw ($56.2 \pm 0.27\%$ motility; $40.6 \pm 0.57\%$ fertility) and also in pellet ($50.4 \pm 0.16\%$ motility; $40.8 \pm 0.45\%$ fertility) methods respectively. Rao (1989) reported that DMSO in presence of Alsever's solution penetrates rapidly into the cell and brings a quick balance between the intra and extra-cellular fluid concentrations. The Na-citrate fraction of Alsever's solution in association with cell membrane may protect against injury during freezing of sperm.

Another interaction between MeOH and ionic based extender also demonstrated higher motility ($57.4 \pm 0.38\%$) and fertility ($48.2 \pm 0.42\%$) in both and also in pellet methods ($48.2 \pm 0.24\%$ motility) and ($40.6 \pm 0.17\%$ fertility) respectively. Methanol has been employed successfully for sperm cryopreservation in rainbow trout (Ciereszko, Dietrich, Nynca, Dobosz, & Zalewski, 2014), Atlantic salmon (Dziewulska et al., 2011), and some salmonid species (Lahnsteiner et al., 1997).

According to our findings, the ionic-based extender is less efficient compared to the sugar-based extender, which has been supported by some authors by reporting that ionic-based solutions are widely employed as immobilizers for the cryopreservation of warm water fish species (Linhart, Billard, & Proteau, 1993; Horváth, Miskolczi, & Urbányi, 2003; Kwantong & Bart, 2006).

The thawing rate is also another critical factor to maintain viability in sperm cells. In this regard, some authors reported that thawing rates should be high to avoid recrystallization (Lahnsteiner, 2000; Jawahar & Betsy, 2020). In this study, fertilization of eggs with sperm frozen in straws and then thawing at 25°C water bath for 30 s resulted in the highest eyeing and hatching rates compared to that of sperm frozen in pellets at 20°C for 6 s, which supported the findings of Dziewulska et al. (2011), Lahnsteiner et al. (1995), Cabrita et al. (2001) and Tekin et al. (2007). In this manner, in accordance with the findings of this study, research by, Lahnsteiner et al. (1995) and also Cabrita et al. (2001) demonstrated that the highest fertilization rates obtained using frozen sperm thawed at 25°C water bath for 30 s in salmonid sperm. In light of these results, we can assume that crystal formation was reduced or enzymatic activities were best reactivated during this thawing procedure.

According to literature, the sperm-to-egg ratios used in fertilization experiments with cryopreserved brown trout sperm have been ranging between 4×10^6 (Mansour, Richardson, & McNiven, 2006) and 6.2×10^6 (Ott & Horton, 1971) resulting in 45 and 66.6% fertility respectively. In this study, fertilization was carried out using ~5×10^6 sperm-to-egg ratios and the fertility ranged from 25 to 65%. In the previous studies regarding fertilization with frozen/thawed brown trout semen, the lowest sperm-to-egg ratios to assure the highest post-thaw fertilization rate (95.7%) was $1.2 - 2.4 \times 10^6$ spermatozoa per egg, as determined by Lahnsteiner, Mansour, and Kunz (2011). In the present study, observed low fertilization rates that can be explained most probably due to the comparatively low post-thaw motility of sperm cells. The reasons for this result could be owing to a decrease in the ATP stores resulting in low mitochondrial activity, damage in cell membrane integrity, or serious cryogenic DNA damage occurring during the cryopreservation process.

Another interesting point that emerged from this study that relatively poor hatching rates ranging from 4 to 25% were recorded from all cryosolution combinations and both freezing methods in this study. The reasons for the poor hatching rate in fertilized eggs can be attributed to poor aeration, lengthy in hatching duration, and most probably cryogenic DNA damage occurring during the cryopreservation process (Bozkurt et al., 2021).

In this study, all types of cryoprotectants interacted with all extenders regardless freezing method which requires different freezing/thawing rates. Based on the results of this study, glycerol addition to glucose-based extender produced the highest fertility among all cryosolution combinations. On the other hand, sperm frozen in Alsever's solution containing DMSO or ionic-based solution containing MeOH provided better

fertilization results respectively than sperm frozen in these solutions containing glycerol. On the other hand, lower post-thaw motility was obtained with DMA in almost all combinations of cryosolutions in both straw and pellet methods. The observed differences in results might be due to insufficient interactions between extender components and DMA, which induced cryoinjuries due to the low protection, and also may be owing to an increase in ROS activity in sperm frozen with cryosolutions containing DMA.

Conclusion

In conclusion, this study demonstrated that *Salmo trutta f. fario* sperm can be successfully cryopreserved in a glucose-based cryosolution containing 10% glycerol with 0.25 mL straws. The applied protocol can be used in commercial hatcheries to support the artificial reproduction of brown trout because of the acceptable post-thaw motility and fertility results provided. On the other hand, further research is needed to investigate the growth and survival of the larvae that originated from cryopreserved sperm.

Acknowledgment

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