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SHORT COMMUNICATION

Effect of supplementations of docosahexaenoic acid (DHA) into a Tris-glucose based extender on the post-thaw sperm quality, fertility and hatching rates in brown trout (*Salmo trutta macrostigma*) following cryopreservation

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Abstract The supplementation of extenders with PUFAs has not been investigated in fish by now. Thus, the present study examined whether different levels of docosahexaenoic (DHA), which is the predominant PUFAs in the sperm plasma membrane, in Tris-glucose based extender could increase the quality and fertilizing ability of brown trout (*Salmo trutta macrostigma*) sperm following cryopreservation were evaluated. Sperm samples diluted (1:10) with the base extender were supplemented with 1, 2.5, 5, 7.5, and 10 ng mL^{-1} concentrations of DHA. In the control group, sperm was diluted in the base extender, without DHA. Following dilution, the sperm was aspirated into 0.25 mL straws, frozen 3 cm of above of the liquid nitrogen (LN_2) surface, and plunged directly into the LN_2. Based on the results, increasing of DHA supplementations in the Tris-glucose based extender improved post-thaw progressive motility, progressive duration of motility, viability, and fertility compared to the control group ($P<0.05$). Additionally, the results indicated that supplementation of 7.5 ng mL^{-1} of DHA significantly exhibited the best cryoprotective effect in terms of progressive motility (62.4±1.73%), progressive duration of motility (46.8±1.32s), viability (56.2±1.20%), fertility (52.6±1.48%), and hatching (28.6±1.45%). The results showed that supplementation of DHA to the Tris-glucose based extender can be beneficial for the cryopreservation of brown trout sperm.

Keywords cryopreservation . Sperm . Extender . DHA . Brown trout

Introduction

Cryopreservation biotechnology is widely applied not only to the spermatozoa of many commercial animal species but also are applied to those of cultured and endangered aquatic species as well. Using of cryopreserved sperm can evolve broodstock management by providing sperm throughout the year in hatcheries, as well as protect the genomes of endangered species (Cabrita et al. 2010).

Despite the progress in sperm cryopreservation in salmonids, there is still a lack of standardized methodologies in terms of freezing techniques, extender compositions, cryoprotectant types, packaging methods, as well as cooling and freezing rates (Cabrita et al. 2001; Sarvi et al. 2006; Ciereszko et al. 2014; Nynca et al. 2016; Judycka et al. 2018).

Although cryopreservation of sperm offering many advantages such as effective breeding programs, transportation of sperm, and conservation of genetic structure, it is also a complex process causing several forms of cellular damages (Purdy 2006; Bozkurt 2019). These damages are associated with cold shock, intercellular ice crystal, membrane alteration, and osmotic changes (Isachenko 2003; Khalili et al. 2009),

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which may lead to a decrease in motility and fertilizing ability of sperm following thawing (Matsuoka et al. 2006; Bozkurt et al. 2019).

According to previous studies, there is a remarkable statement that the lipid composition of the sperm plasma membrane is an important indicator of cold sensitivity, viability (Hammerstedt et al. 1990), motility, and membrane integrity (Robinson et al. 2006) leading to fertilization of eggs (Yildiz et al. 2015; Zaniboni et al. 2006). From this point of view, it is clear that polyunsaturated fatty acids (PUFAs) play important roles in terms of enhancing the fluidity of the sperm plasma membrane and increasing the resistance of sperm against cold shock (Stubbs and Smith 1984; Wathes et al. 2007). Additionally, it is well known that maintenance sperm membrane fluidity and fertilization capacity of sperm requires a significant amount of PUFAs.

However, the level of PUFAs in the sperm membrane decrease significantly because of lipid peroxidation (Kaeoket et al. 2008) and a decrease in motility and viability of stored frozen/thawed spermatozoa observed due to sperm are attacked by reactive oxygen species (ROS) during the cryopreservation process (Martinez-Soto et al. 2013). Therefore, supplementation of PUFAs, especially docosahexaenoic acid (DHA), to the freezing extenders to cope with the detrimental effects of ROS on post-thaw sperm quality and sperm plasma membrane has been suggested (Kaeoket et al. 2010). Additionally, it was reported that the addition of DHA to the extender improves the integrity of the spermatozoa cell membrane and sperm motility during cryostorage (Nasiri et al. 2012; Towhidi and Parks 2012; Takahashi et al. 2012; Schmidt-Lausigk and Aurich 2014).

Cryoprotecting ability of DHA enriched sperm was reported by feeding studies in some domestic animals such as boars (Mitre et al. 2004), cockerels (Cerolini et al. 2006), rams (Esmaeili et al. 2014), and stallions (Brinsko et al. 2005). On the other hand, the mechanism by which DHA preserves the sperm during cryopreservation is not known in fish species. There are only a few reports that have studied the effect of PUFAs in extenders on the post-thaw viability and fertility of mammalian sperm such as boar (Kaeoket et al. 2010), bovine (Abavisani et al. 2013), and ram (Abdi-Benemar et al. 2015). To the best of our knowledge, there is a lack of information regarding the biological role of DHA supplementation in extenders on post-thaw quality and fertilizing ability following cryopreservation in fish sperm.

Thus, the present study was performed to investigate the protective effect of Tris-glucose based freezing media supplemented with different levels of DHA on post-thaw sperm quality, fertilization, and hatching rates of brown trout eggs following cryopreservation.

Materials and methods

Ethical approval and reagents

This study and experimental design was approved by the Local Animal Experiment Ethics Committee of the Mustafa Kemal University in Antakya, Hatay (MKU-BAP-1005-M-0111). All chemicals and reagents including n-3 polyunsaturated fatty acid capsules (containing 25 mg of DHA) were purchased from Sigma-Aldrich (Germany). The fatty acids were dissolved in 0.05% ethanol (Kaka et al. 2015).

Broodstock and collection of gametes

Mature male (527.4±3.8 g, n=25) and female (745.2±4.6 g, n=10) brown trout broodstock (3-years old) were provided by a commercial aquafarm located in Gaziantep (Turkey) during their spawning season. During the experimental period, the broodstock was maintained in 1000-L indoor tanks which were supplied with constant hatchery water (12.4±1.2°C; 9.2±7.2 mg L^{-1} O_2) flow at a rate of 2.5 L s^{-1}. Broodstock was fed twice daily with a commercial pelleted diet containing 35% protein.

For gamete collection, male and female broodstock were anesthetized in a 50 L tank with 15 mg L^{-1} quinaldine (Sigma-Aldrich, Germany) for a few minutes. The urogenital pore was dried to avoid water, urine, and feces contamination. The sperm was collected separately into 50 mL sterile falcon tubes by abdominal massage and maintained at a temperature of 4±1°C in a styrofoam box including crushed ice and immediately transferred to the laboratory. Mature females were also wiped dry and stripped by 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 stripping.

Evaluation of sperm

Sperm motility and duration

To evaluate the sperm samples, a 10 µL drop of sperm dilution was placed on a microscope slide, 20 µL activation solution (AS) (45 mM NaCl, 5 mM KCl, and 30 mM Tris–HCl, pH 8.2; Horvath et al. 2003) were added and then the sperm suspensions were thoroughly mixed for 2 s. The motility and motility duration of spermatozoa were immediately recorded for 1 min following activation using a CCD video camera (CMEX-5, the Netherland) mounted on a phase-contrast microscope (100X, Olympus BX43, Tokyo, Japan) until the spermatozoa trajectories become tight concentric circles (Rurangwa et al. 2004). The obtained video records were scanned to determine the percentage of progressive motility (%) and the duration of progressive motility (s). Sperm motility was evaluated from sperm performing progressive forward movement, while motility duration was determined by counting the time from activation until it stopped moving (Horvath et al. 2003). Concerning post-thaw evaluation, at least three straws were used for the evaluation of each parameter and it was performed three times for each treatment.

Sperm density and viability

Sperm density was determined with a 100 µm deep Thoma hemocytometer (TH-100, Hecht-Assistent, Sondheim, Germany) at 400x magnification with Olympus BX50 phase-contrast microscope (Olympus, Japan) in Hayem non-activating solution (35.2 mM Na_2SO_4, 17.1 mM NaCl, 1.8 mM $HgCl_2$, 200 mL bicine) with a dilution ratio of 1:1000 and expressed as spermatozoa $x10^9$ mL^{-1} (three replicates) (Bozkurt et al. 2019).

Sperm viability was assessed as described by Bjorndahl et al. (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). A mixture of 5 µL of semen with 5 µL of the stain was smeared on a clean slide and allowed 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 bright field microscope. For this aim, unstained spermatozoa were considered alive, while stained spermatozoa were considered as dead.

Experimental design

Preparation of extender

The base extender was prepared by the addition of 30 mM Tris, 350 mM glucose, 20 mL fresh egg yolk, and 10% DMSO in 100 mL of distilled water (Bozkurt et al. 2014). The experimental extenders were supplemented with the 0 (control), 1, 2.5, 5, 7.5, and 10 ng mL^{-1} of DHA, which were adapted from our pilot experiments. In this way, six experimental extender groups were prepared. All sperm extenders were prepared on the day before sperm collection.

Cryopreservation and thawing of sperm

Samples (n=23) exhibiting high progressive motility (>80%) and having approximately 15×10^9 spermatozoa mL^{-1} sperm concentration were used in this study. Sperm samples were individually split into six subsamples and each of them diluted at a ratio of 1:10 (v:v) with the base extender which composing 30 mM Tris, 350 mM glucose, 20 mL fresh egg yolk, 10% DMSO in 100 mL of distilled water. Following, the base extender was supplemented with 1, 2.5, 5, 7.5, and 10 ng mL^{-1} of DHA in test tubes and were incubated in a water bath for 15 min for the absorption of DHA by sperm membrane (Kaka et al. 2015).

Diluted sperm was equilibrated at 4 °C for 10 min and then drawn into 0.25 mL straws (IMV, France) and sealed with polyvinyl alcohol (PVA). Sperm concentration was approximately $12.5x10^6$ in per 0.25 mL straw. Before freezing, diluted sperm were maintained in a cool chamber at 4 °C to obtain isothermal

conditions. Sperm samples were frozen 5 cm above of the liquid nitrogen (LN_2) surface inside a polystrene box for 20 min. Then, the frozen samples in each experiment were plunged into the liquid nitrogen (LN_2) for 1 min and finally, nine straws per sperm sample were frozen. After two weeks of storing samples in an LN_2 tank, rewarming was performed with three straws per treated group in a water bath at 30 °C for 20 s for the evaluation of post-thaw sperm characteristics.

Fertilization and hatching

The good quality eggs pooled from four females were divided into batches of about 100 eggs and fertilized with 100 µL frozen-thawed sperm or 25 µL fresh sperm (sperm to egg ratio: ~$5x10^6$:1) in 500 mL round-bottomed bowls. Following, fertilization solution (125 mM NaCl, 20 mM Tris–HCl, 30 mM glycine, pH 9) was added onto the eggs in a ratio of 1:2 (fertilization solution/eggs) (Lahnsteiner 2000). The eggs were slightly stirred for 30 min and rinsed with hatchery water at the end of stirring and finally transferred into the labeled vertical incubators supplied with flow-through hatchery water. Eggs were incubated for about 40 days until the eyed stage. The fertilization (the number of 4-cell stage embryos/number of total eggs) rates were determined under a stereomicroscope at 20-fold magnification. Three replicates were performed for each treatment with frozen/thawed and fresh sperm as well.

Statistical analyses

All analyses were repeated three times for each treatment. Results were presented as mean±SD. Motility values were normalized through arcsine transformation and differences among the parameters were analyzed using one-way ANOVA. Duncan 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.

Results

In fresh sperm, the mean percentage (%) and duration (s) of progressively motile spermatozoa were 84.6±5.72 % and 57.8±4.37 s, respectively. The post-thaw progressive motility percentages (%), durations (s), viability (%), fertilization (%) and hatching (%) rates of the brown trout spermatozoa cryopreserved in DHA added Tris-glucose based extender were presented in Fig.s 1 and 2 for all concentrations.

The findings of the present study showed that there were significantly higher percentages in terms of post-thaw progressive motile sperm (%), duration of progressive sperm motility (s), and viability (%) in DHA supplemented groups compared to the control group ($P < 0.05$).

Similarly, increasing of DHA supplementations into the Tris-glucose based extender caused an increase both in post-thaw fertilization ability of sperm ($P < 0.05$), and also hatching of eggs compared to the control group but there were no statistical differences among the all tested concentrations of DHA in terms of hatching rates($P > 0.05$).

The highest post-thaw average progressive motility (%), progressive motility duration (s), fertilization (%), and hatching (%) values were determined with the 7.5 ng mL^{-1} of DHA concentration in all tested treatments ($P < 0.05$) (Fig. 1). On the other hand, a slight decrease was observed after 7.5 ng mL^{-1} of DHA addition in all tested parameters.

Discussion

Sperm cells are subjected to structural and chemical changes associated with a loss of motility and viability during the cryopreservation process (Watson 2000). However, it is proven that a decrease in viability and fertility occurs due to the damage on sperm plasma membrane controlling extra and intracellular processes and also plays an important role during fertilization (Abavisani et al. 2013).

It is well known that lipids are the major component of sperm membrane and these compounds are also important for sperm maturation, viability, and membrane functions. (Towhidi and Parks 2012). Additionally, PUFAs share a major portion of the membrane lipids and protect sperm cell membranes

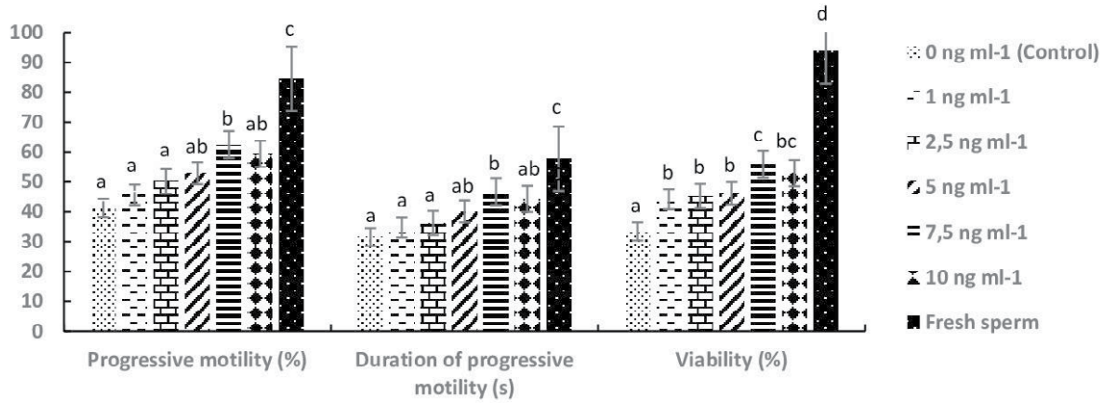

Fig. 1 The mean post-thaw progressive motility (%), duration of progressive motility (s) and viability (%) of frozen–thawed brown trout (*Salmo trutta macrostigma*) sperm exposed to different concentrations (0-10 ng ml^{-1}) of n-3 PUFAs in Tris-glucose based extender. Different letters indicate differences among treatments (ANOVA, $P < 0.05$, $n = 9$).

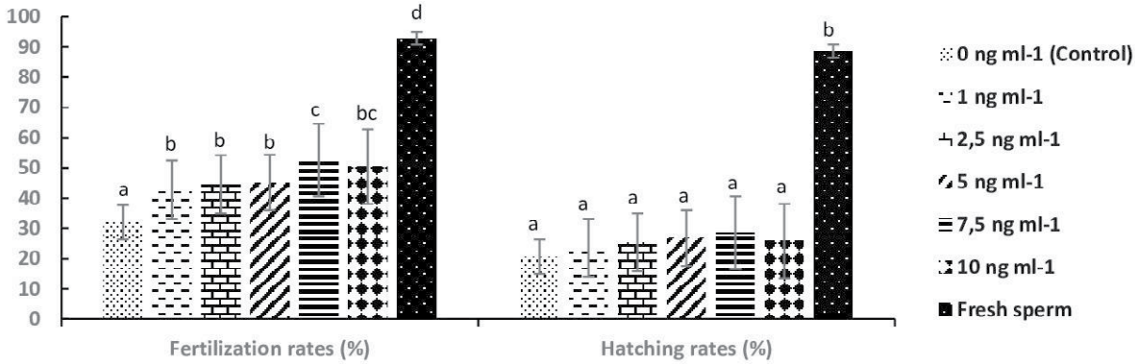

Fig. 2 The mean post-thaw fertilization (%) and hatching rates (%) of frozen–thawed brown trout (*Salmo trutta macrostigma*) sperm exposed to different concentrations (0-10 ng ml^{-1}) of n-3 PUFAs in Tris-glucose based extender. Different letters indicate differences among treatments (ANOVA, $P < 0.05$, $n = 9$).

during the cryopreservation from the harmful effect of oxidative stress through changes in sperm membrane fluidity (Kaeoket et al. 2008; Wathes et al. 2007). Maldjian et al. (2005) have reported docosahexaenoic (DHA) is the predominant PUFAs within the sperm plasma membrane, but the level of PUFAs decreases significantly because of lipid peroxidation during the cryopreservation process (Cerolini et al. 2001).

Although the importance of PUFAs is emphasized in many studies regarding improving the health status and immune functions in humans (Neuringer et al. 1988; Wathes et al. 2007; Martinez-Soto et al. 2013; Shahidi and Ambigaipalan 2018; Kalupahana et al. 2020), most of them are associated with its supplementation of PUFAs in diets regarding its protective effect on sperm quality in domestic animals recently (Blesbois et al. 2004; Maldjian et al. 2005; Brinsko et al. 2005; Samadian et al. 2010; Jafaroghli et al. 2014).

Additionally, despite there are some researches about its protective role as supplementary in the freezing media on quality and fertility of frozen/thawed sperm in domestic animals (Kaeoket et al. 2010; Abavisani et al. 2013; Abdi-Benemar et al. 2015), there is no study in terms of its cryoprotective role on quality and fertilization ability of sperm following cryopreservation in fish species. To the best of our knowledge, this study is the first attempt regarding supplementation of DHA into a Tris-glucose based freezing media for the cryopreservation of brown trout sperm and subsequent evaluation of its protective effects on sperm quality and fertilizing ability.

The positive effects of DHA enrichment of semen have shown in the feeding trials by boar (Maldjian et al. 2005), turkey (Blesbois et al. 2004), stallion (Brinsko et al. 2005), and sheep (Samadian et al. 2010; Jafaroghli et al. 2014). Additionally, some studies are focusing on the influence of DHA enriched extenders

on cryopreservation of bull (Abavisani et al. 2013) and boar (Chanapiwat et al. 2009; Kaeoket et al. 2010) sperm. However, these studies mainly evaluated the effect of DHA supplementations on the post-thaw sperm quality of mammalian sperm. On the other hand, there is not any report regarding the effects of DHA enrichment of extenders on fish sperm freezability and post-thaw fertility.

From this point of view, the present study demonstrated that supplementations of DHA to the Tris-glucose based extender improved post-thaw quality and fertility of brown trout sperm. Specifically, 7.5 ng mL^{-1} concentration of (DHA) exhibited significantly better results ($P<0.05$) than all the other concentrations on post-thaw parameters except hatchability of eggs. However, slight decreases were observed in all tested parameters at 10 ng mL^{-1} concentration of DHA. Based on the findings of the present study, it seems that PUFA content in sperm plasma membrane increased the levels of membrane fluidity. Resultingly, higher levels of membrane fluidity caused more cryotolerance during the freezing process and better post-thaw quality.

Similar to our results, Kaeoket et al. (2008) reported that supplementation of freezing media with a high concentration of DHA by adding fish oil improved post-thaw boar sperm quality. In agreement with the results of several studies regarding DHA supplementations by adding fish oil to diets led to an increase in sperm motility of rooster (Parks and Lynch 1992), boar (Maldjian et al. 2005), goat (Dolatpanah et al. 2008), sheep (Samadian et al. 2010) and turkey (Zaniboni et al. 2006). Moreover, it has been indicated that supplementation of freezing extender with DHA alone or accompanied with L-cysteine improved the motility and viability of boar semen (Chanapiwat et al. 2009). Also, Khosrowbeygi and Zarghami (2007) reported that the proportion of PUFAs was found to be positively correlated with sperm motility, viability, semen volume, and sperm concentration.

On the other hand, Kandelousi et al. (2013) reported that the higher concentrations of n-3 FAs in freezing extender caused a more decline in bull semen characteristics following cooling and freezing, suggesting that the presence of higher levels of unsaturated fatty acids in the sperm membrane make sperm more susceptible to peroxidative breakdown. Correspondingly, Taghilou et al. (2017) reported that the addition of higher concentrations (50 ng mL^{-1}) of n-3 PUFAs in freezing extender decreased sperm total motility, viability, plasma membrane integrity, and increased the proportion of sperm with abnormal tails in ram semen.

The results of the present study show that the optimum concentrations of DHA for cryoprotection are species-dependent. Additionally, differences in PUFA composition of sperm may affect the flexibility and compressibility of the sperm membrane (Neuringer et al. 1988) resulting from a decrease in motility and fertilization ability of sperm cells.

In conclusion, the results from the present study indicated that the addition of DHA to the Tris-glucose based extender, ranging from 1 to 10 ng mL^{-1}, significantly improved the quality and fertilization ability of cryopreserved sperm in brown trout following thawing. In this regard, this study provided significant information regarding the relationship between the DHA levels in the extender and the motility, viability, and fertilization ability of frozen-thawed brown trout sperm as the first attempt in fish. Also, the findings of this preliminary study will be useful for the optimization of the cryopreservation protocol as well as to improve the reproduction efficiency from cryopreserved sperm in fish. However, further investigation is needed to clarify the precise mechanism involved by analyzing membrane lipid composition as well as electron image of the sperm to determine the changes in biochemical structure and to better understand the cryoprotective role of the n-3 PUFAs in brown trout sperm.

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Conflict of interest The authors declare that they have no conflict of interest.

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