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Spermatozoa Cryopreservation of Sex-Reversed Rainbow Trout (*Oncorhynchus mykiss*): The Effect of Dilution Rate and Supplementation of a N-(2-Mercaptopropionyl)-Glycine -Based Extender on Sperm Motility and Fertilizing Capacity

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ABSTRACT

For commercial aquaculture, the obtainment of all-female salmonid populations is important in fish farms. After freezing process, variable spermatozoa maturity, higher sperm concentration, low sperm quality, and reduced fertilization success have been observed in sex-reversed female rainbow trout. For these reasons, the objective of this study was to assess effect of dilution rate and supplementation of a N-(2-Mercaptopropionyl)-Glycine (MPG)-based extender on sperm motility and fertilizing capacity of sex-reversed rainbow trout (*Oncorhynchus mykiss*). The supplementation of MPG [0 (control, 0), 1 mM; 2 mM; 4 mM] to extenders and dilution ratio (1:9, 1:15 and 1:25) were tested in sex-reversed female rainbow trout spermatozoa during cryopreservation. For thawing, the straws (0.5 ml) were placed in a water bath at 37°C for 30 s. Our results showed that the best concentration of MPG was 2 mM for post-thaw motility duration (120.67±9.07%), fertilization (62.67±3.10%) and hatching rate (54.33±3.10%) at 1:15 dilution rate. Overall, MPG provided improvements during cryopreservation process and could be used as protective agent.

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Introduction

The applications and importance of sperm cryopreservation in fish and its benefits to the aquaculture industry have been comprehensively explained in earlier studies (Suquet et al., 2000; Cabrita et al., 2010; Islam and Akhter, 2011; Martinez-Paramo et al., 2017). Significant advances have been performed in the development of this technology by modifying various factors such as the type and concentration of cryoprotectants, dilution ratio, freezing/thawing ratios, and packaging systems (Viveiros et al., 2015). However, post-thaw sperm motility and fertilization rates is still low in some fish species compared to fresh sperm (Asturiano et al., 2016; Purdy et al., 2016; Bozkurt et al., 2021). The formation of ice crystals, osmotic shock and cryoprotectants toxicity are the main factors for cold storage damage during freezing and thawing process (Meyers, 2005). Additionally, cryopreservation process increases the production of Reactive Oxygen Species (ROS) (Sandoval-Vargas et al., 2021). Although protection is provided by antioxidant agents in biological systems, the low amount of antioxidants in sperm cells cause an imbalance between the antioxidant defense system and ROS production during cryopreservation process (Lahnsteiner and Mansour, 2010; Silva et al., 2011; Birben et al., 2012; Shaliutina-Kolesova et al., 2019; Bozkurt et al., 2021). ROS is produced during cryopreservation promote changes in sperm cells, resulting in inactivation or lacking of enzymes associated with lipid peroxidation, DNA damage, mitochondrial damage and dysfunction, protein oxidation and sperm motility (Klaiwattana et al., 2016). Therefore, the addition of enzymatic and non-enzymatic antioxidants to the cryopreservation medium has been applied in the sperm

cryopreservation of various fish species (Li et al., 2018; Kutluyer, 2021; Bozkurt et al., 2021).

The obtainment of all-female salmonid populations is important in fish farms. Sex-reserved females (masculinized females, neomals) have a female genotype (XX), even if they have a male phenotype. Sex-reserved female trout is produced sperm cells with a male phenotype. Sex-reserved females usually lack spermatid ducts and sperm is obtained from the testicles and fish must be killed. Previous studies showed variable spermatozoa maturity, higher sperm concentration, low sperm quality, and reduced fertilization success after freezing process. The reasons for the poor characteristics of the sperm of sex-reserved females after cryopreservation are not fully understood. For these reasons, it is crucial to determine the effects of antioxidant supplementation on cryopreservation of sperm from sex-reserved females with respect to cryoinjury.

N-(2-Mercaptopropionyl)-Glycine (MPG) is a reducing and complex thiol. MPG is a widely used antioxidant with a free mercapto group. Some reports have shown that it is a free radical inhibitor and prevents cell membrane damage. MPG is widely used in the treatment of various diseases (Li et al., 2015; Li et al., 2018). As a possible antidote, MPG has many advantages over other sulfhydryl compounds and is used clinically in the treatment of various liver diseases, nephrotoxicity and cystine urolithiasis (Fetoni et al., 2004; Li et al., 2015; Li et al., 2018). This greatly facilitates its use as a preventive drug. Thus far, MPG has caused very few side effects in clinical use. In contrast to previous studies in animals and humans, no studies have been conducted on how the addition of MPG in fish affects cells after sperm cryopreservation. Çakır Sahilli et al. (2019) evaluated the effects of the supplementation of MPG [0 mM (Control), 0.25 mM, 0.5 mM, and 1 mM] to activation solution on motility and duration of rainbow trout (*O. mykiss*) sperm and, they determined the increases in the percentage of sperm motility rate (91.80±6.42%) and duration (29.40±5.86 s) at concentration of 0.25 mM. Within this context, we evaluated MPG-induced impacts and dilution ratio on sperm quality and fertility ability of sex-reserved rainbow trout (*O. mykiss*) spermatozoa.

Material and Methods

Fish and Sperm Collection

The masculinization (sex-reversed females) was conducted at Abalıoğlu Production Facility (Kayseri, Türkiye) using 17α-methyltestosterone (MT). The exogenous feeding began in November 2019 with hormone administered feed (2 mg/kg MT/Feed) and, maintained for a period of 60 days at 10°C. Milt from sex-reversed females (n=6, 1334.17±84.76 g, 46.00±3.63 cm) was collected postmortem by cutting testes and gently squeezing through a double-layer gauze to remove any testicular tissue (Nynca et al., 2012a, 2012b). All experiments were approved by Munzur University Animal Experiments Local Ethics Committee (22-05/14).

Cryopreservation Process

The extenders were supplemented with MPG at the following concentrations: 0 (control, 0), 1 mM; 2 mM; 4 mM. Pooled sperm were diluted at ratios of 1:9, 1:15 and 1:25 (semen:extender) in an extender composed of Glucose (0.3 M), DMSO (10%), egg yolk (10%), Penicillin/Streptomicyn (0.3%) (Tekin et al., 2003). After cryopreservation, NaCl (52 mM) was used for sperm activation. Each sperm suspension sperm was aspirated into 0.5-mL straws. For equilibration, straws were placed 3 cm above the liquid nitrogen (LN_2) surface in a Styrofoam box during 10 min. followed by floating on liquid nitrogen for 5 min and then plunging into liquid nitrogen. The straws were then thawed by immersion in a water bath at 37°C for 30 s. Analyses were performed in triplicate.

Sperm Quality Evaluation

Sperm motility and duration were analyzed in samples of fresh and post-thawing using ZEN Imaging Software by ZEISS with a microscope (Zeiss Primo Star, Germany). solution of NaCl (0.3%) was used to activate sperm. The progressive movement of the sperm was determined as the motility rate. The survival period of forwarding motility was evaluated as the time complete stopping movement of active sperm. Sperm quality assessment was done in triplicate.

Egg Collection and Fertilization

After the egg and sperm are collected from mature fish (n=6, 3256.67±420.22 g, 53.17±4.79), the same amount of sperm and eggs were taken and, then the activation solution was added to the mix. After fertilization, the eggs were washed with hatchery water for 3 to 5 minutes to eliminate debris. Fertilized eggs were incubated under natural photoperiod using spring water at 10±0.5°C". Eggs will be transferred to separate plastic baskets and incubated at 10°C. Dead eggs (opaque eggs) were removed at regular intervals. Fertilization rate and development of the embryo observed 25 days after fertilization and the hatch rate 26 days after fertilization were calculated. In the calculation of the fertilization rate; FR (%) = (Number of fertilized eggs/Total number of eggs) × 100 formula were used.

Statistical Analyses

Six rainbow trout males were used for sperm quality analysis. 5000 ± 5 eggs were used in each group of eggs fertilized by fresh, control and the treated groups to determine fertilization ability and hatching rate. Before analysis, data were tested for normality using the Shapiro-Wilk test. If normality was not accepted, the Kruskal-Walli's test and Kruskal–Wallis all-pairwise comparisons tests were applied. At any statistical test the level of significance (α) was set to 0.05.

Results

Sperm concentration, pH, motility rate and duration of fresh sperm in sex-reversed females were 12.5×10^9/ml, 8.4±0.13, 97.67±0.58%, 159.67±21.73 s, respectively. Mean values for measured sperm quality parameters, fertility and hatching rates in rainbow trout are given in Table 1 for different MPG concentrations and dilution rates. The dilution of sperm at ratios of 1:15 and 1:25 of caused a significant increase of percentage of sperm motility, fertility and hatching compared to control group ($P<0.05$). The post-thaw motility duration (120.67±9.07%), fertilization (62.67±3.10%) and hatching rate (54.33±3.10%) were significantly higher at the concentrations of 2 mM MPG ($P<0.05$).

Table 1. Effects of N-(2-MercaptopropionyL)-glycine and different dilution ratios on motility rate and duration, fertility and hatching rates of sex-reversed females.

Treatments	Dilution					
	1:9				1:15	
	Motility rate (%)	Motility duration (s)	Fertility rate (%)	Hatching rate (%)	Motility rate (%)	Motility duration (s)
Fresh sperm	97.67±0.58^{a}	159.67±21.73^{a}	92.00±2.00^{a}	88.67±1.20^{a}	97.67±0.58^{a}	159.67±21.73^{a}
Control (0 mM)	66.00±2.54^{b}	95.67±3.21^{bc}	57.67±2.50^{b}	35.00±2.10^{b}	65.00±6.56^{b}	97.00±2.78^{b}
1mM	29.33±4.04^{c}	63.33±6.35^{d}	24.00±3.60^{c}	16.00±3.00^{c}	39.33±3.06^{c}	69.00±4.00^{c}
2 mM	70.33±5.51^{d}	112.33±4.04^{b}	35.33±2.00^{d}	25.67±2.50^{d}	75.33±5.51^{d}	120.67±9.07^{d}
4 mM	54.33±5.86^{b}	91.00±3.00^{c}	36.67±2.60^{d}	28.00±3.80^{d}	68.67±4.04^{bd}	97.33±5.51^{b}
χ2 value	12,654	13,257	12,833	13,099	12,165	12,879
P value	0.013	0.010	0.012	0.011	0.016	0.012
Treatments	Dilution					
	1:15				1:25	
Treatments	Fertility rate (%)	Hatching rate (%)	Motility rate (%)	Motility duration (s)	Fertility rate (%)	Hatching rate (%)
Fresh sperm	92.00±2.00^{a}	88.67±1.20^{a}	97.67±0.58^{a}	159.67±21.73^{a}	92.00±2.00^{a}	88.67±1.20^{a}
Control (0 mM)	57.67±2.50^{b}	35.00±2.10^{b}	66.00±5.45^{bc}	92.67±1.89^{b}	57.67±2.10^{b}	35.00±2.10^{b}
1mM	51.00±3.80^{c}	37.67±3.10^{b}	53.33±18.93^{c}	94.33±17.93^{b}	45.00±3.60^{c}	29.67±3.60^{c}
2 mM	62.67±3.10^{d}	54.33±3.10^{c}	76.33±3.21^{b}	118.67±3.21^{b}	56.67±2.90^{b}	41.00±2.90^{b}
4 mM	63.33±1.50^{d}	53.33±1.00^{c}	70.33±5.51^{bc}	105.33±5.03^{b}	58.67±5.30^{b}	44.00±5.30^{b}
χ2 value	12,648	12,870	9,769	11,379	13,016	13,016
P value	0.013	0.012	0.045	0.023	0.011	0.011

*The same column shows significant differences among proportions. Different superscript letters^{a,b,c,d} show differences between treatments (P<0.05).

Discussion

To date, earlier studies on the analysis of sperm quality have been performed in sex-reserved females of rainbow trout (Nynca et al., 2012a, 2012b; Figueroa et al., 2013; Dietrich et al., 2014; Ciereszko et al. et al., 2015; Judyckaa et al., 2017, 2019, 2020). Previous studies have reported similar sperm motility, sperm concentration, and seminal plasma osmolality values of semen obtained from sex-reversed females and normal rainbow trout (Ciereszko et al., 2015; Judycka et al., 2017, 2019; Nynca et al., 2012b). In terms of sperm concentration, the values were 2-3 times higher for rainbow trout due to lacking sperm ducts and obtaining semen directly from the testicles (Judycka et al., 2017, 2019). In this study, the sperm concentration was determined as $12.5x10^{9}$/ml. The dilution of sperm at ratios of 1:15 and 1:25 of caused a significant increase sperm motility, fertility and hatching compared to control group.

Antioxidants provide the inhibition of ROS formation (Partyka et al., 2013; Kutluyer Kocabaş, 2022). MPG is a reducing and complex thiol with a free mercapto group. The cell membrane damage is prevented by MPG due to be a free radical inhibitor and various diseases are treated by MPG (Li et al., 2014; Li et al., 2015). As a possible antidote, MPG has many advantages over other sulfhydryl compounds and is used clinically in the treatment of various liver diseases, nephrotoxicity and cystine urolithiasis (Fetoni et al., 2004; Li et al., 2014; Li et al., 2015). This greatly facilitates its use as a preventive drug. MPG has caused very few side effects in clinical use. In contrast to earlier studies in animals and humans, no studies have been conducted on how the addition of MPG in fish affects cells after sperm cryopreservation. Thus far, Çakır Sahilli et al. (2019) added MPG to the activation solution and evaluated the effects of increasing MPG concentrations [0 mM (Control), 0.25 mM, 0.5 mM, and 1 mM] on rainbow trout (*O. mykiss*) sperm motility and duration. They determined that the increases in the percentage of motility (91.80±6.42%) and duration (29.40±5.86 s) were statistically significant at the concentration of 0.25 mM (P<0.05) and, motility could not be determined in sperm cells at 1 mM concentration. In contrast to previous studies, the best results in sperm cryopreservation were obtained at the 2 mM concentration in this study. The percentage and duration of sperm motility, fertilization and hatching rate increased compared to the control group. The motility of sperm cells is affected by the production of ATP by the enzyme succinate dehydrogenase, which is important for mitochondrial activity, and provides respiratory electron transfer chain and citric acid cycle as a result of oxidation of succinate succinate in fumarate. N-(2-mercaptopropionyl)-glycine, together with the free thiol (sulfuryl) group, is a widely used antioxidant. This molecule, in common with other sulfhydryl (SH) containing compounds, not only scavenges oxygen radicals such as superoxide (O^{2}) and hydroxyl radical (OH^{-}), but also increases the capacity of SOD to scavenge oxygen radicals; moreover, SH groups maintain intracellular glutathione level (Piste, 2013). The increase in sperm motility with the addition of MPG can be explained by the fact that MPG protects the cell due to its antioxidant properties.

In current study, the motility of sperm cells, fertilization and hatching rate decreased after 2 mM concentration. High doses of MPG have inhibiting motility in sperm cells. The reduced motility may be due to the toxic effect of MPG. This can be explained by the fact that high doses of MPG can lead to cell disruption and mitochondrial disruption for sperm motility. Apart from these, the components in the diluent may affect the bioavailability of MPG.

In conclusion, the concentration of 2 mM MPG and 1:15 dilution ratio used in the present study provided significant improvements during sperm cryopreservation. MPG could be effectively used as an alternative promoting agent for sperm cryopreservation of *O. mykiss*, and this study will be useful in evaluating the impact of MPG on sperm cells of other fish species. Regarding potential effects, further research is needed on the effects of different concentrations of MPG on subsequent fertilization ability and embryonic development.

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