DETERMINATION OF THE EFFECT OF L-CARNITINE, β-CAROTENE AND ASCORBIC ASID ON THE QUALITY OF CRYOPRESERVED SEMEN IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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ABSTRACT
Cryopreservation of sperm is common procedures in aquaculture, particularly used for routine in artificial insemination. However, these application cause damages and adversely affected sperm motility, viability and consequently lower hatching rates. In present study, it was examined whether addition of different antioxidants to the cryopreservation extenders had an effect on semen post-thaw fertility and motility in rainbow trout (Oncorhynchus mykiss) and also it was investigated the sperm characteristics post-thaw sperm characteristics and fertility. The collected semen was pooled to minimize individual variation. Each pooled ejaculate was split into 4 equal aliquots and diluted with base extenders supplemented with the antioxidants, and a base extender with no additives (control). The pooled semen samples diluted at the ratio of 1:10 by the extenders were subjected to cryopreservation. Antioxidants were separately added to the extenders (one per experimental group): 0.5 mmol/l L-carnitine, 0.5 mmol/l β -carotene and 0.5 mmol/l L-ascorbic acid. After dilution the straws were placed on the tray, frozen for 10 min, and plunged into liquid nitrogen. L-carnitine increased the post-thaw sperm motility rate in comparison to the standard extender. ($p < 0.05$). There were significantly differences among the treatments in sperm motion characteristics (VAP, VCL, VSL, LIN,

STR and WOB) of frozen-thawed sperm ($p < 0.05$). Fertilization rate and hatching rate of frozen-thawed semen was not affected by the tested antioxidants ($p > 0.05$).

INTRODUCTION

The cryopreservation of fish semen is an important technique in terms of increasing the total volume of available semen, avoiding aging of sperm and long-term preservation of germplasm. Rainbow trout, O. mykiss, is one of the most important fish species in world due to its aquaculture potential, economic value and wide consumer demand (Kutluyer et Al., 2014). The cryopreservation methods offer several benefits such as stock protection from being totally eliminated species, supplying of sperm for optimal utilization in hatchery production and laboratory experiments. However, the methods of sperm cryopreservation induced some problems, such as considerable low motility after thawing fertilizing and hatching rates as a result of cryoinjuries (Kopeika and Kopeika, 2008). The cryoinjuries occur at the defined temperature ranges by the procedures in relation to process during cryopreservation, particularly in cold and hot shocks throughout freezing and thawing (Chao and Liao, 2001). The cryopreservation procedures that allow preserving sperm cells have been applied for sperm of many fish species. Cryopreservation of common carp sperm has also been well established by many researchers (Li et al., 2010). As concerns O. mykiss, the knowledge about the use of extenders containing antioxidants is limited. Lipid peroxidation of sperm cell membranes, damage of midpiece, axonemal structure, and DNA, malfunctions of capacitation and acrosomal reaction, loss of motility, and infertility may carry out (Ubilla and Valdebenito, 2011) when there is a high production of reactive oxygen species (ROS) in gametes, which are aerobic cells (Lahnsteiner, 2011). Moreover, unsaturated fatty acids in plasma membranes of spermatozoa are very sensitive to free radical attack (Sikka, 2004). Antioxidants are molecules protecting against free radical damage and inhibited oxidation Therefore, to obtain more information about effect of supplementation of extender with antioxidants on motility and fertility of sperm in rainbow trout. In present study, it was examined whether addition of antioxidants (0.5 mmol/l L-carnitine, 0.5 mmol/l β -carotene and 0.5 mmol/l L-ascorbic acid) to the cryopreservation extenders had an effect on semen post-thaw fertility and motility in rainbow trout and also the sperm kinematic characteristics post-thaw.

MATERIALS AND METHODS

Experiments were conducted with gametes of +2 and +3-year-old rainbow trout were obtained from the fish farm Keban Trout Production Facility (Elazığ, Turkey). The sperm was collected by a gentle abdominal massage, collected into glass vials and stored on ice until use. Semen samples with a motility rate ≥90% were excluded from the experiment. Antioxidants were separately added to the extenders. The collected semen was pooled to minimize individual variation. Each pooled ejaculate was split into 4 equal aliquots and diluted with base extenders supplemented with the antioxidants, and a base extender with no additives (control). The pooled semen samples diluted at the ratio of 1:10 by the extenders were subjected to cryopreservation. Antioxidants were separately added to the extenders (one per experimental group): 0.5 mmol/l L-carnitine, 0.5 mmol/l β -carotene and 0.5 mmol/l L-ascorbic acid. After dilution the semen (1:10) with 3 extenders was aspirated into 0.25 ml straws, the straws were placed on the tray, frozen for 10 min, and plunged into liquid nitrogen. The straws were thawed in 40°C water for 5 sec. Motility parameters were measured using an automated system, SCA (Sperm Class Analyzer v. 4.0.0. by Microptic S.L., Barcelona, Spain). The spermatozoa movement was monitored using a camera (Basler A312fc, with sensor type CCD) at 50 Hz mounted on a Nikon Eclipse 50i microscope, coworking with SCA, at room temperature (20°C). Fertilization experiments were conducted at 8–10°C. One homogenous egg pool was used for the fertilization experiments. From the eggs the ovarian fluid was drained off and the eggs were placed in fertilization solution a ratio of 1:2 (eggs:fertilization solution), then the semen was added and the components were mixed with each other. 100±5 eggs were fertilized with 100 µl cryopreserved semen or 25 µl untreated semen (sperm to egg ratio: $x10^5$:1). Three to 5 min after fertilization the eggs were

rinsed in hatchery water and incubated in flow incubators at water temperature of 9±0.5°C. The experimental success was determined as the percentages of eyed embryos in relation to the total number of eggs 28–30 d after fertilization. Statistical analysis was performed using the software package SPSS 14.0 for Windows and significance was set at $p < 0.05$.

RESULTS

L-carnitine increased the post-thaw sperm motility rate in comparison to the standard extender. Supplementation of the extender with β -carotene decreased the post-thaw motility rate. L-ascorbic acid only slightly increased post-thawed motility. Differences in the motility rate of frozen-thawed semen were significant among the treatments ($p < 0.05$). In table 1; there were significantly differences among the treatments in sperm motion characteristics (VAP, VCL, VSL, LIN, STR and WOB) of frozen-thawed sperm ($p<0.05$). VAP, VSL, LIN and STR were significantly higher in the extenders containing carnitine and ascorbic acid ($p<0.05$). Our results indicated that the post-thaw motility rate increased in extender supplemented with L-carnitine ($p<0.05$).

Table 1. Effect of antioxidants and oxidative defensive enzymes on the motility parameters of frozen-thawed rainbow trout sperm.

Extenders	Standard extender	L-carnitine	β -carotene	L-ascorbic
VAP	83.77±10.78^{a}	107.50±7.84^{c}	75.03±15.23^{ab}	84.09±10.25^{a}
VCL	110.99±15.74^{ab}	115.63±13.61^{a}	106.09±13.45^{ab}	85.06±12.16^{c}
VSL	71.51±13.50^{bc}	104.20±16.14^{a}	37.48±22.19^{d}	82.07±9.87^{b}
LIN	66.54±18.35^{b}	91.61±15.77^{a}	35.05±19.90^{c}	96.49±9.45^{a}
STR	85.27±11.45^{ab}	96.56±12.17^{a}	48.84±24.86^{c}	97.60±6.78^{a}
WOB	77.08±14.79^{c}	93.95±10.14^{ab}	70.86±12.85^{cd}	98.86±7.03^{a}

a, b, c, d: Different superscripts within the same row demonstrate significant differences ($p < 0.05$).

A post-thaw fertility of 86.71±2.11% was obtained with the standard extender. Higher post-thawed fertility (90.01±3.14%) was obtained with the extender containing L-carnitine and lower with the extender containing L-ascorbic acid (77.23±8.71). So, fertilization rate and hatching rate of frozen-thawed semen was not affected by the tested antioxidants ($p>0.05$).

Table 2. Effect of antioxidants and oxidative defensive enzymes on the fertility and hatching of frozen-thawed rainbow trout sperm.

Extenders	Fertility (%)	Hatching (%)
Standard extender	86.71±2.11	76.75±2.16
L-carnitine	90.01±3.14	80.07±3.35
β -carotene	87.44±5.68	77.34±3.69
L-ascorbic	77.23±8.71	67.63±4.70
P	-	-

–: The same column shows no significant differences among proportions ($p > 0.05$).

DISCUSSION

Cryopreservation of sperm is common procedures in aquaculture, particularly used for routine in artificial insemination. However, these application cause damages and adversely affected sperm motility, viability and consequently lower hatching rates. Cabrita et al. (2011) reported that addition of ascorbic acid did not significantly increase the post-thaw motility parameters of motility in gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax). Martinez-Paramo et al. (Martinez-Paramoa et al., 2012) demonstrated that supplementation of freezing media with ascorbic acid improved sea bass sperm motility, resulting in higher percentages of motile spermatozoa with higher curvilinear velocity. Sperm velocities play a key role in success of fertilization (Kutluyer et al., 2014). In previous experiments, it was

reported that success of fertilization correlated with sperm motility velocities (VCL, VSL, and VAP) in rainbow trout (O. mykiss) (Lahnsteiner, 2000). The present results may be due to male and female gamete interactions and maternal genetic and non-genetic constituents, egg quality and female donor that it is important on a male's fertilization ability for fish. Carnitine is antioxidant and protects phospholipid membranes against lipid peroxidation. In addition, it provides pyruvate utilization (Kutluyer et al., 2014). In present study, supplementation of the cryopreservation extenders with carnitine did not significantly improve fertility. This result is supported by previous findings. For example, Lahnsteiner and Mansour (2010) reported that carnitine improved sperm motility parameters and membrane integrity in A. alburnus.
In conclusion, the tested antioxidants positively affected the motility parameters. The post-thaw motility rate increased in extender supplemented with L-carnitine.

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