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Cryopreservation of rainbow trout *Oncorhynchus mykiss* spermatozoa: Effects of extender supplemented with different antioxidants on sperm motility, velocity and fertility ☆

Filiz Kutluyer [a,*], Murathan Kayim [a], Fatih Öğretmen [b], Serhat Büyükleblebici [c], P. Barbaros Tuncer [d]

[a] Tunceli University, Fisheries Faculty, 62000 Tunceli, Turkey
[b] Muğla Sıtkı Koçman University, Faculty of Fisheries, 48000 Muğla, Turkey
[c] Aksaray University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Aksaray, Turkey
[d] Aksaray Vocational School, Aksaray University, Aksaray, Turkey

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ABSTRACT

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 12 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): catalase (250 U/l), superoxide dismutase (250 U/l), peroxidase (250 U/l), oxidized glutathione (1.5 mmol/l), reduced glutathione (1.5 mmol/l), L-methionine (1.5 mmol/l), uric acid (0.25 mmol/l), L-ascorbic acid (0.5 mmol/l), α-tocopherol (2.0 mmol/l), β-carotene (0.5 mmol/l) and carnitine (0.5 mmol/l). After dilution the semen was aspirated into 0.25 ml straws, the straws were placed on the tray, frozen for 10 min, and plunged into liquid nitrogen. Our results indicated that the post-thaw motility rate increased in extenders supplemented with uric acid, L-methionine, SOD, L-carnitine, α-tocopherol and L-reduced glutathione ($p < 0.05$). The motility duration of frozen thawed semen increased in extenders supplemented with uric acid, L-methionine, SOD, α-tocopherol and L-reduced glutathione ($p < 0.05$). Fertilization rate and hatching rate of frozen-thawed semen was not affected by the tested antioxidants. Consequently, the tested antioxidants affected the motility parameters and cryopreservation extenders could be supplement with antioxidants. This study suggested usage of antioxidants in the cryopreservation of rainbow trout.

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Introduction

The cryopreservation of fish semen is a valuable technique due to transport of genetic material between facilities, optimal utilization in aquaculture, reducing the risk of spreading infections, performing hybridization studies in species that breeding seasons are different, protection of gene pool, biodiversity conservation, selective breeding activities, protection of endangered species [8,34,29]. Furthermore, it is an important technique in terms of increasing the total volume of available semen, avoiding aging of sperm and long-term preservation of germplasm [32]. In addition, this technique provides synchronization of gamete availability from both males and females [20]. Larvae production is increased by optimizing reproduction [23].

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 [30,33] when there is a high production of reactive oxygen species (ROS) in gametes, which are aerobic cells [18,22,28]. Moreover, unsaturated fatty acids in plasma membranes of spermatozoa are very sensitive to free radical attack [1,18,30]. Antioxidants are molecules protecting against free radical damage and inhibited oxidation [8].

Thus far, a great deal of past research has focused on cryopreservation of some fish species, mainly salmonids, sturgeons, carps and catfishes and in recent decades, considerable research has been performed in marine species and various finfish species [6].

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* Corresponding author. Fax: +90 0 428 213 18 61.
E-mail address: filizkutluyer@hotmail.com (F. Kutluyer).

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Most of the studies in salmonids were performed in rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*) and char (*Salvelinus alpinus*) [4,15,21]. Recently, studies about the use of extenders supplemented synthetic and natural antioxidants for cryopreservation in fish sperm have been conducted by researchers [16–24,33]. However, the antioxidant capacity of semen is insufficient in preventing lipid peroxidation, and that there is a need to extenders, which are supplemented with antioxidants.

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 [25]. As concerns *O. mykiss*, the knowledge about the use of extenders containing antioxidants is limited. Ubilla and Valdebenito [33] stated that the sperm viability of rainbow trout semen stored with different antioxidants is significantly prolonged. On the other hand, Lahnsteiner et al. [18] determined that effects supplementation of extender with antioxidants were not detectable in rainbow trout. Therefore, to obtain more information about effect of supplementation of extender with antioxidants (ascorbic acid, α-tocopherol, β-carotene, carnitine, catalase, superoxide dismutase, peroxidase, reduced glutathione, oxidized glutathione, methionine, uric acid) on motility and fertility of sperm in rainbow trout, the present study was conducted. Also motility parameters were investigated in fresh and in cryopreserved semen.

Materials and methods

Collection of semen

Experiments were conducted with gametes of +2 and +3 year old rainbow trout (male: 36.6 ± 2.23 cm, 666.42 ± 129.15 g; female: 43.3 ± 2.10 cm, 1498 ± 25 g). Rainbow trout semen was obtained from the fish farm Keban Trout Production Facility (Elazığ, Turkey) between November and January. The males were anesthetized with 2-phenoxyethanol (0.6 ml L^{-1}) before stripping. The sperm was collected by a gentle abdominal massage, collected into glass vials and stored on ice until use. Caution was exercised to prevent contamination of the semen with urine, feces, blood, mucus or water. Sperm samples with a motility rate ⩽90% were excluded from the experiment and the percentage of motile sperm was checked using a microscope (Eclipse E50; Nikon Corporation, Tokyo, Japan) at 10 magnification with SCA. An activating solution composed of 50 mM/L $NaHCO_3$ and 60 mM/L Tris, pH 9 was used to freshly collected and cryopreserved samples.

Semen cryopreservation

For freezing, an adjustable insulated box was used and the straws were placed horizontally oriented tray. The tray consisting of a perforated stainless steel plate was adjusted at 3 cm (about −180 °C) above the surface of liquid nitrogen for freezing [14]. During freezing the box was covered with an acrylic glass plate. Straws with a volume of 0.25 ml were pre-cooled to 4 °C before use. Semen was diluted in the different types of 4 °C cold extender at a rate of 1:10 (semen:extender). Two to 10 min after dilution the semen was aspirated into 0.25 ml straws; the straws were placed on the tray for 10 min, and plunged into liquid nitrogen. After a week, the frozen straws were thawed in a 40 °C water bath for 5 s immediately before used for motility measurements and fertilization assays.

The quality of semen frozen in the standard extender was compared with that of semen frozen in extenders containing different types of antioxidants and oxidant defensive enzymes. The standard extender consisted of 6.52 mg/ml NaCl, 0.8 mg/ml KCl, 2 mg/ml $NaHCO_3$, 2 mg/ml glucose, 4 mg/ml bovine serum albumin, 7.5% (v/v) egg yolk and 10% (w/v) DMSO [7]. The enzymatic and non-enzymatic antioxidants listed below were tested. They were separately added to the extenders (one per experimental group): (a) 250 U/l catalase, (b) 250 U/l superoxide dismutase, (c) 250 U/l peroxidase, (d) 1.5 mmol/l oxidized glutathione, (e) 1.5 mmol/l reduced glutathione, (f) 1.5 mmol/l L-methionine, (g) 0.25 mmol/l uric acid, (h) 0.5 mmol/l L-ascorbic acid, (i) 2.0 mmol/l α-tocopherol, (j) 0.5 mmol/l β-carotene and (k) 0.5 mmol/l carnitine. The selected types and concentrations of antioxidants were based on previous studies [17,18]. Each experiment was repeated 3 times with different semen samples.

Measurement of sperm motility

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). The duration of motility was determined as the time until forward movement stops. The negative phase contrast lens of 10 magnification and intermediate optic 2.5 were used to trace sperm head. Half-second films (25 frames) were recorded at 7, 10 and then at each 5 s intervals until the motility completely stopped. Undiluted milt (1–2 μl) was added to 400 μl while diluted milt (2 μl) was added to 50 μl of the activating solution in a polyethylene Eppendorf. After rapid pipetting mixing, a portion of 1.2 μl of this mixture was placed directly in one well of a 12-well multitest glass slide with coverslip (MP Biomedicals LLC, Germany). The activation and film recording of undiluted milt were repeated triplicate. These operations of diluted milt were repeated five times in order to capture at least 50 sperm at beginning of recording. Eight sperm motility parameters were chosen for analysis: MOT (percent of motile sperm, %), VCL (curvilinear velocity, >20 μm s^{-1}), VAP (average path velocity, μm s^{-1}), VSL (straight line velocity, μm s^{-1}), LIN (linearity, %), STR (straightness), ALH (amplitude of lateral head displacement, μm) and BCF (beat cross frequency, Hz) [10].

Fertilization and hatching assays

Fertilization experiments were conducted at 8–10 °C. The fertilization solution consisted of 60 mmol/l $NaHCO_3$ and 50 mmol/l Tris (pH 9.0). 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: $\times 10^5$: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 percentage of eyed embryos in relation to the total number of eggs 28–30 d after fertilization.

Statistical analysis

Statistical analysis was performed using the software package SPSS 14.0 for Windows and results were expressed as means ± standard error. Differences among the treatments were tested by one-way ANOVA. The Duncan test was used for all *post hoc* comparisons. Significance was set at $p < 0.05$.

Results

Sperm post-thaw motility

Effect of extenders on the motility rate of frozen sperm was shown in Fig. 1. Uric acid, L-methionine, SOD, L-carnitine,

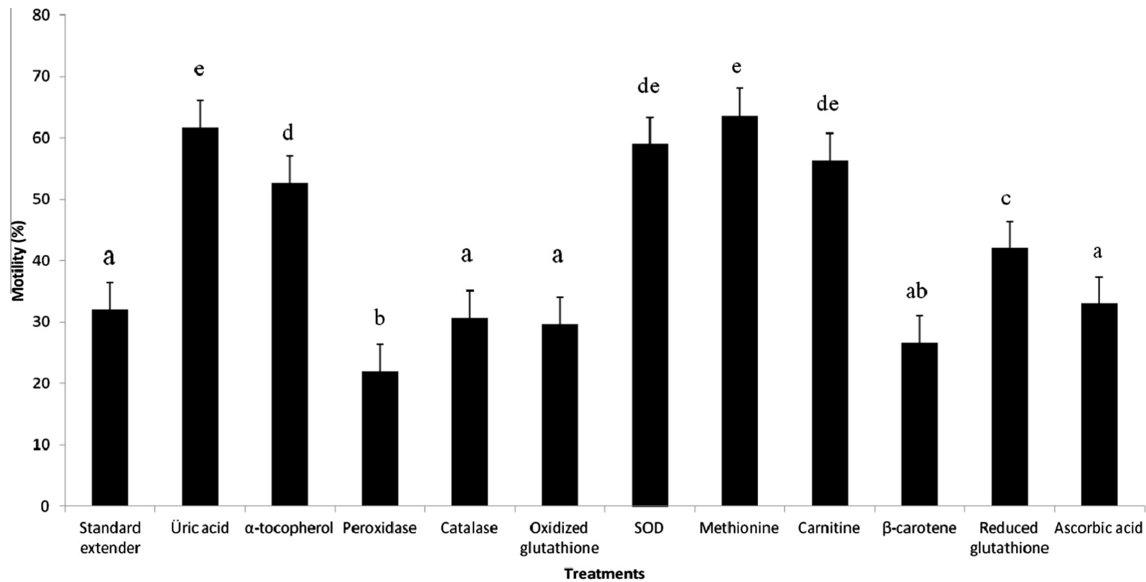

Fig. 1. Effect of antioxidants and oxidative defensive enzymes on the motility rate of frozen-thawed rainbow trout sperm ($n = 3$). Different letters show differences between treatments ($p < 0.05$).

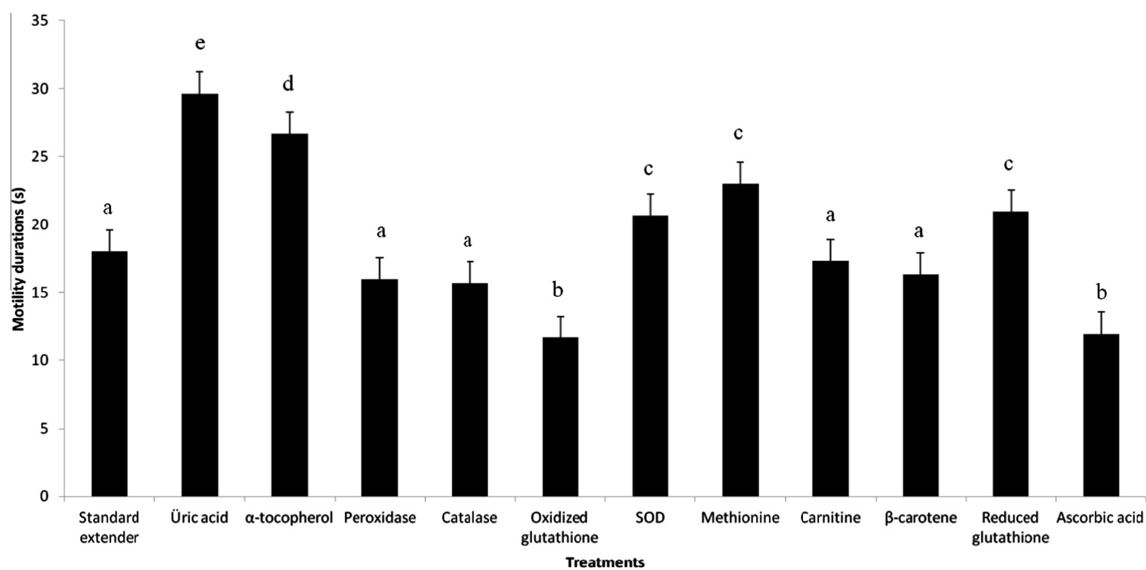

Fig. 2. Effect of antioxidants and oxidative defensive enzymes on the motility duration of frozen-thawed rainbow trout sperm ($n = 3$). Different letters show differences between treatments ($p < 0.05$).

α-tocopherol and L-reduced glutathione increased the post-thaw sperm motility rate in comparison to the standard extender. Supplementation of the extender with catalase, peroxidase, oxidized glutathione, β-carotene decreased the post-thaw motility rate. Ascorbic acid only slightly increased post-thaw motility. Differences in the motility rate of frozen-thawed semen were significant among the treatments ($p < 0.05$).

Effect of extenders (with or without antioxidants) on the motility duration of frozen sperm was shown in Fig. 2. Uric acid, L-methionine, SOD, α-tocopherol and L-reduced glutathione increased the post-thaw sperm motility duration in comparison to the standard extender. Differences in the motility duration of frozen-thawed sperm were significant among the treatments ($p < 0.05$).

Effect of extenders on the motility parameters of frozen sperm was presented in Table 1. There were significantly differences among the treatments in sperm motion characteristics (VCL, VAP, VSL, LIN, STR, ALH and BCF) of frozen-thawed sperm ($p < 0.05$). LIN and STR were significantly higher in the extenders containing carnitine and ascorbic acid ($p < 0.05$).

Fertility

Effect of extenders on the fertility was presented in Table 2. A post-thaw fertility of 86.71 ± 2.11% was obtained with the standard extender. Higher post-thaw fertility (90.01 ± 3.14%) was obtained with the extender containing carnitine and lower with the extender containing ascorbic acid.

Discussion

In the study, we compared the effects of enzymatic and non-enzymatic antioxidants on motility and fertilizing ability of

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

Extenders	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Standard extender	110.99 ± 15.74^{ab}	71.51 ± 13.50^{ab}	83.77 ± 10.78^{ab}	66.54 ± 18.35^{ab}	85.27 ± 11.45^{ab}	77.08 ± 14.79^{abc}	3.15 ± 1.54^{abc}	8.37 ± 2.38^{ab}
Reduced glutathione	102.16 ± 8.75^{abc}	71.06 ± 25.05^{ab}	88.75 ± 11.91^{ab}	69.36 ± 23.98^{bc}	79.11 ± 24.28^{abc}	86.89 ± 9.18^{bcd}	2.72 ± 1.12^{bc}	8.85 ± 3.65^{ab}
Oxidized glutathione	113.85 ± 7.83^{a}	40.35 ± 33.12^{a}	77.73 ± 13.92^{ab}	36.47 ± 31.11^{d}	47.99 ± 33.77^{d}	68.76 ± 14.75^{ae}	6.13 ± 2.31^{de}	6.03 ± 1.85^{ac}
Ascorbic acid	85.06 ± 12.16^{c}	82.07 ± 9.87^{a}	84.09 ± 10.25^{a}	96.49 ± 9.45^{d}	97.60 ± 6.78^{d}	98.86 ± 7.03^{abe}	0.95 ± 0.78^{abde}	2.27 ± 1.31^{d}
Methionine	120.53 ± 26.00^{a}	51.59 ± 23.24^{a}	79.62 ± 17.78^{ab}	43.34 ± 19.02^{ad}	63.25 ± 21.97^{abd}	66.96 ± 12.25^{ae}	5.73 ± 1.73^{ade}	5.88 ± 2.23^{ac}
α-Tocopherol	107.07 ± 20.39^{abc}	55.43 ± 14.07^{a}	59.49 ± 11.61^{a}	55.86 ± 29.01^{abd}	92.48 ± 4.73^{c}	59.37 ± 26.98^{e}	3.94 ± 2.33^{bcde}	8.47 ± 3.28^{ab}
Uric acid	125.46 ± 26.15^{a}	51.94 ± 37.15^{a}	86.68 ± 28.41^{ab}	46.38 ± 37.35^{abd}	62.18 ± 35.61^{abd}	70.57 ± 27.21^{ae}	4.18 ± 2.95^{abde}	3.21 ± 3.88^{cd}
Carnitine	115.63 ± 13.61^{a}	104.20 ± 16.14^{b}	107.50 ± 7.84^{bc}	91.61 ± 15.77^{c}	96.56 ± 12.17^{c}	93.95 ± 10.14^{d}	1.62 ± 1.42^{c}	11.13 ± 2.29^{b}
β-Carotene	106.09 ± 13.45^{abc}	37.48 ± 22.19^{a}	75.03 ± 15.23^{ab}	35.05 ± 19.90^{d}	48.84 ± 24.86^{d}	70.86 ± 12.85^{ae}	4.36 ± 1.15^{abde}	5.99 ± 2.98^{ac}
SOD	191.92 ± 46.69^{d}	107.95 ± 60.64^{b}	136.67 ± 49.64^{c}	55.78 ± 2635^{abd}	74.82 ± 23.25^{abc}	70.98 ± 17.13^{ae}	6.49 ± 3.16^{d}	6.23 ± 3.21^{ac}
Peroxidase	100.25 ± 9.29^{abc}	52.12 ± 20.48^{a}	77.44 ± 14.37^{ab}	52.34 ± 20.86^{abd}	66.25 ± 18.96^{abd}	77.13 ± 12.30^{abc}	3.60 ± 1.07^{bcde}	6.38 ± 2.41^{ac}
Catalase	97.19 ± 32.05^{bc}	42.28 ± 10.21^{a}	94.05 ± 9.87^{a}	43.50 ± 7.84^{abd}	44.96 ± 8.03^{cd}	96.77 ± 6.34^{cd}	1.49 ± 0.38^{bc}	10.00 ± 0.69^{ac}
p value	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a,b,c,d,e] Different superscripts within the same column demonstrate significant differences ($p < 0.05$).

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

	Fertility (%)	Hatching (%)
Standard extender	86.71 ± 2.11	76.75 ± 2.16
Reduced glutathione	87.26 ± 2.05	77.62 ± 2.45
Oxidized glutathione	88.16 ± 2.15	78.66 ± 2.19
Ascorbic acid	77.23 ± 8.71	67.63 ± 4.70
Methionine	89.56 ± 4.21	79.54 ± 4.05
α-Tocopherol	88.72 ± 3.36	78.27 ± 5.16
Uric acid	88.01 ± 4.25	78.11 ± 4.65
Carnitine	90.01 ± 3.14	80.07 ± 3.35
β-Carotene	87.44 ± 5.68	77.34 ± 3.69
SOD	89.23 ± 3.49	79.25 ± 3.51
Peroxidase	88.61 ± 5.42	78.16 ± 5.22
Catalase	87.12 ± 2.16	77.32 ± 3.36
p	–	–

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

cryopreserved rainbow trout sperm. We demonstrated that supplementation of the extender with antioxidants was increased the post-thaw motility rate and duration, although fertilization process is not affected by antioxidants significantly.

In a study related to effect of antioxidants on the quality of cryopreserved semen in rainbow trout (*O. mykiss*), Lahnsteiner et al. [18] found that supplementation of the extender with catalase, peroxidase, with a mixture of reduced and oxidized glutathione, or with a mixture of reduced and oxidized methionine had no effect on the post-thaw motility rate or decreased it only slightly. Ubilla and Valdebenito [33] determined that use of vitamin C in sperm diluents for rainbow trout improved sperm motility, fertilizing capability of semen and increasing spermatozoa storage times. Cabrita et al. [5] reported that addition of ascorbic acid and tocopherol did not significantly increase the post-thaw motility parameters of motility in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). Martínez-Páramo et al. [20] demonstrated that supplementation of freezing media with α-tocopherol and ascorbic acid improved sea bass sperm motility, resulting in higher percentages of motile spermatozoa with higher curvilinear velocity. The present results showed that methionine, SOD, L-carnitine, α-tocopherol and reduced glutathione increased the post-thaw sperm motility rate in comparison to the standard extender. Supplementation of the extender with catalase, peroxidase, oxidized glutathione, β-carotene, and ascorbic acid had no positive effect. Fertilization process is not affected by antioxidants significantly.

Sperm velocities play a key role in success of fertilization [3,26,31]. In previous experiments, it was reported that success of fertilization correlated with sperm motility velocities (VCL, VSL, and VAP) in African catfish (*Clarias gariepinus*) [27], turbot (*Psetta maxima*) [9], carp (*Cyprinus carpio*) [21], and rainbow trout (*O. mykiss*) [14] and streaked prochilod (*Prochilodus lineatus*) [34]. In present study, sperm from extender treatments containing SOD had significantly higher VCL, VSL and VAP. In contrast, the best results in fertilization were obtained from extender supplemented with carnitine. BCF, LIN, STR and WOB were higher in extender including carnitine. 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 [3,28].

Uric acid has positive effect on sperm motility parameters due to its stability and to be strong reducing agent, which is formed from xanthine and hypoxanthine [2]. Additionally, uric acid can be use in sperm cryopreservation extenders of aquatic animals due to inexpensive and efficacy [16]. Lahnsteiner and Mansour [16] stated that uric acid was the major antioxidant of sperm and it improved the motility rate (%) and the rate of sperm membrane integrity in *Alburnus alburnus*, *Lota lota*, *Perca fluviatilis*, *S. trutta* and the sperm velocity in *P. fluviatilis*. Lahnsteiner et al. [17] found that uric acid had highest concentrations in spermatozoa of brown trout and play a major role in antioxidative protection of spermatozoa under *in vivo* conditions. The present results agree with these reports. Uric acid increased the post-thaw sperm motility rate in comparison to the standard extender.

Carnitine is antioxidant and protects phospholipid membranes against lipid peroxidation [13]. In addition, it provides pyruvate utilization [11,12]. 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 [16] reported that carnitine improved sperm motility parameters and membrane integrity in *A. alburnus*.

In conclusion, the present study indicates that uric acid and carnitine are the important antioxidant in sperm of rainbow trout for increase the quality of sperm and fertility. Additionally, supplementation of the extender with uric acid, L-methinoine, SOD, L-carnitine, α-tocopherol and L-reduced glutathione increased the post-thaw motility rate and duration. Fertilization process is not affected by antioxidants significantly. Further research is required in order to select the best concentration and combination of antioxidants.

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