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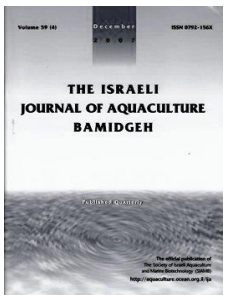

Effect of Different Antioxidants on Motility, Viability and Fertilizing Capacity of Cryopreserved Scaly Carp (*Cyprinus carpio*) Semen

İlker Yavaş[1], Yusuf Bozkurt[2*], Cengiz Yıldız[1]

[1]Mustafa Kemal University, Faculty of Veterinary Medicine,Department of Reproduction and Artificial Insemination,Antakya, Hatay/Turkey

[2]Mustafa Kemal University, Faculty of Marine Science and Technology, Department of Aquaculture, İskenderun, Hatay / Turkey

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Abstract

The present study investigated the effect of different antioxidants such as taurine (25 mM, 50 mM, 75 mM and 100 mM), bovine serum albumine (BSA) (1%, 2.5%, 5%, 7.5%), and vitamin C (ascorbic acid) (2.8 mM, 5.6 mM, 8.4 mM and 11.2mM), on the motility and fertilizing ability of cryopreserved scaly carp (*Cyprinus carpio*) semen. The results demonstrated that addition of taurine at doses of 50 mM and 75 mM, and also BSA at doses of 2.5% and 5%, improved fertilization ability of cryopreserved scaly carp semen. The highest post-thaw motility (42.4±1.5%), viability (67.8±2.6%), fertilization rate (59.3±0.5%), and also hatching rates (68.2±3.4%) were determined by supplementation of 75 mM taurine ($p<0.05$). These results suggest that supplementation of ionic extender with taurine is suitable for scaly carp semen cryopreservation.

*Corresponding author: email: yfbozkurt06@gmail.com

Introduction

The technique of cryopreservation is beneficial in many areas such as conservation of genetic diversity, selective breeding, hybridization, and maintaining a continuous and stable supply of gametes for hatchery seed production or laboratory experimentation (Lubzens et al., 1997). Therefore, development of procedures for sperm cryopreservation will aid the recovery of threatened and endangered species as well as genetic selection and maintenance of selected stock lines (Yavaş and Bozkurt, 2011).

However, the problem of sublethal damage of spermatozoa during cryopreservation needs to be resolved. Ice crystallization and lipid peroxidation induce oxidative stress, which leads to the formation of reactive oxygen species (ROS) during cryopreservation. Increased production of ROS increases sensitivity of sperm resulting in damage to sperm structure, morphology, and function (Bilodeau et al., 2001). In fish sperm ROS is produced during cryopreservation and this results in reduced post-thaw motility, viability, membrane integrity, acrosome functionality, antioxidant status, and fertility (Zilli et al., 2003). For this reason extenders have been supplemented with antioxidants but due to the antioxidant capacity of semen, these are insufficient to prevent lipid peroxidation during the cryopreservation process. Antioxidants play an important role in sperm motility, integrity, metabolism, and function, by protecting the cells against oxidative damage (Alvarez and Storey, 1983). Damage to sperm function has been successfully minimized in several mammalian species by the addition of antioxidants to the extender media prior to cryopreservation (Bucak et al., 2007; Thuwanut et al., 2008). Taurine (sulfonic amino acid) is a non-enzymatic antioxidant which can traverse the sperm plasma membrane and inhibit lipid peroxidation and also protects the cells against the accumulation of ROS (Chen et al., 1993). Vitamin C (ascorbic acid) is most effective as an antioxidant in the aqueous phase, and it may act as a free radical scavenger (Niki, 1987). Bovine Serum Albumine (BSA) enhances the motility and viability of sperm cells following freeze-thawing process (Uysal et al., 2007).

Data related to the effect of antioxidants on fish spermatozoa are limited. To the best of our knowledge, the present study is the first to examine the protective effect of three different antioxidant concentrations on cryopreservation of scaly carp spermatozoa. The present work was designed to assess the influence of taurine (sulfonic amino acid), vitamin C (ascorbic acid), and bovine serum albumine (BSA) incorporated with an ionic extender on post-thaw motility, viability, and fertilizing ability of cryopreserved scaly carp sperm.

Materials and Methods

*Broodstock Management and Gamete Collection***.** The experiment was carried out during spawning season of scaly carp (*Cyprinus carpio*). The broodstock were kept in earthen ponds under the natural photoperiod regime. In these ponds, water temperature varied between 20-22ºC during spawning season. The broodstock were fed ad libitum twice a day. State of maturity was controlled from about 2 weeks before the expected date of spawning until they were in the running stage. The broodstock were collected from wintering ponds by seining, and transported to the hatchery 48 h prior to gamete collection. In the hatchery, the ponds were supplied with a continuous supply of refreshed water through porous plastic pipes to maintain adequate dissolved oxygen.

The broodstock were anesthetized in a solution containing 100 ppm of MS 222 (Argent Labs., Redmond, WA, USA). They were then removed from the water and their genital apertures were wiped dry. Semen was collected from ten males by gentle abdominal massage directly into 20 ml glass tubes 12 h after a single injection of 2 mg/kg of carp pituitary extract (CPE). In the females, ovulation was induced by intramuscular double injection of a total of 3.5 mg /kg CPE. The first injection of CPE (0.35 mg/kg) was given 10 h before the second (3.15 mg/kg) (Bozkurt et al., 2012). Care was taken to avoid contamination with urine, mucus, feces, or water. Sperm samples were not pooled and the beakers were covered with parafilm and stored on ice in aerobic conditions. Only transparent, well rounded, and unwrinkled eggs were used for fertilization experiments.

Evaluation of Semen Quality and Dilution. Spermatozoa motility was determined using a phase contrast microscope (Olympus, Japan) with 400x magnification. Sperm was activated by mixing 1 µl of sperm with 200 µl activation solution (0.3% NaCl) on a glass slide. Spermatozoa density was determined using a hemocytometer. Sperm was diluted at a ratio of 1:1000 with Hayem's solution (5 g Na_2SO_4, 1 g NaCl, 0.5 g $HgCl_2$, 200 ml bicine) and density was determined using a 100 µm deep Thoma hemocytometer (TH-100, Hecht-Assistent, Sondheim, Germany) at 400x magnification with Olympus BX50 phase contrast microscope (Olympus, Japan) and expressed as spermatozoa $x10^9$/ml (three replicates). Counting chambers were kept in a moist atmosphere for at least 10 min before cell counting. Sperm pH was measured using indicator papers (Merck, 5.5-9) within 30 min of sampling. 300 sperm cells were counted on each slide at 1000x magnification to assess live/dead sperm percentage, using eosin-nigrosin solution according to the method described by Björndahl et al. (2003).

Collected sperm showing >70% motility was pooled into equal aliquots and chosen for cryopreservation experiments. Semen and extenders were kept at 4ºC prior to dilution. Pooled semen was diluted at 1:3 (v/v) ratio with an extender composed of 75 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Tris, 0.5% glicine, and 10% DMSO at pH 7.8 which contained four concentrations of three different antioxidants: taurine (25 mM, 50 mM, 75 mM, 100 mM); bovine serum albumine (BSA) (1%, 2.5%, 5%, 7.5%); or vitamin C (ascorbic acid) (2.8 mM, 5.6mM, 8.4mM, 11.2 mM).

Semen Cryopreservation and Thawing. Diluted semen density was around (1.0-2.5) x 10^8 cells/ml spermatozoa. The diluted samples were drawn into 0.25-ml plastic straws (IMV, France) and were sealed with polyvinyl alcohol (PVA). Before freezing, the semen samples were equilibrated at 4ºC for 10 min to reach the appropriate temperature. A styrofoam box (inner dimensions 54 x 22 cm, height 33 cm) was used for the cryopreservation experiments. The interior of the styrofoam box was filled with liquid nitrogen. The tray floating on the surface of the liquid nitrogen in the styrofoam box was adjusted according to the desired freezing level and equilibrated for 5 min to reach the appropriate temperature. The straws were placed 4 cm above the liquid nitrogen surface in the box and were frozen in liquid nitrogen vapour (-140ºC) for 10 min. The straws were then kept in liquid nitrogen (-196ºC) in a container until thawing. To thaw, the straws were removed from the liquid nitrogen and submerged in a water bath at 25ºC for 30 s. Sperm motility measurements and viability tests were performed immediately after thawing.

Fertilization Experiments. Eggs from 10 scaly carp females were collected and one mililiter of thawed cryopreserved sperm was poured on 1 g eggs (about 1000 eggs) (Magyary et al.,1996). The spermatozoa were activated by adding 10 ml of 0.3% NaCl solution followed by 30 ml fertilization solution (3 g of urea, 4 g of NaCl in 1l of water) to prevent stickness of eggs (dry fertilization). Fertilization experiments were replicated three times and eggs were incubated in Zuger-type hatching jars. The fertilization ratios were counted in the 4-cell stage under a stereomicroscope at 20X magnification and again 3-4 days later to record hatching ratios.

Statistical Analysis. All data were expressed as mean±standard deviation. Relative quantities were transformed by angular transformation and metric data were tested for normality. Data were analyzed with one-way variance (ANOVA) with subsequent Tukey's b-test. Results are presented as mean±SD and significance was considered at the level of α=0.05. Statistical analyses were performed with SPSS 10 for Windows statistical software package.

Results

Fresh Sperm Quality Parameters. In ten male scaly carp, fresh semen volumes were rather variable and ranged from 5.2-17.8 ml (mean volume was 12.4±2.37 ml). Motility values ranged from 80-100%. The mean motility value of fresh sperm samples was 88.4±2.27 %. In addition, individual mean spermatozoa movement duration (s), sperm density ($x10^9$/ml), and pH values were 48.9±3.2s, 24.2$x10^9$/ml, and 7.2±1.25, respectively.

Cryopreservation and Fertilization Experiments. Mean post-thaw sperm motility, motility period, and viability results are presented in Table 1. The highest post-thaw spermatozoa motility (42.4±1.5%) and cell viability (67.8±2.6%) were determined by supplementation of 75mM taurine ($p<0.05$). In other words, supplementation of taurine to the extender resulted in a better cryoprotective effect for sperm viability against freezing damage than other tested groups.

Table 1. Effect of antioxidants on post-thaw motility, motility period and viability rates of cryopreserved scaly carp (*Cyprinus carpio*) semen.

Antioxidants	Antioxidant Concentrations	Post-thaw Motility (%)±S.D.	Post-thaw Motility Period (s)	Post-thaw Viabile Cells (%)±S.D.
Taurine	25 mM	31.4±2.3^{b}	23.4±1.4^{b}	51.6±1.4^{b}
	50 mM	33.2±0.4^{b}	25.6±1.5^{b}	56.4±1.2^{b}
	75 mM	42.4±1.5^{a}	38.2±2.4^{a}	67.8±2.6^{a}
	100 mM	32.4±0.7^{b}	36.4±0.8^{a}	65.9±2.4^{a}
BSA	1 %	20.3±0.9^{c}	14.1±1.2^{c}	39.8±1.4^{c}
	2.5 %	33.6±1.2^{b}	24.6±1.4^{b}	57.5±2.8^{b}
	5 %7.5 %	32.1±2.1^{b}	21.8±0.9^{b}	56.2±1.4^{b}
		21.4±1.4^{c}	15.2±1.4^{c}	42.4±0.7^{c}
Vitamin C	2.8 mM	17.2±0.5^{c}	22.3±1.4^{b}	32.4±2.5^{cd}
	5.6 mM	23.1±1.5^{c}	16.8±2.1^{c}	37.2±1.4^{c}
	8.4 mM	24.1±1.2^{c}	23.8±1.2^{b}	43.5±1.2^{c}
	11.2 mM	19.2±2.1^{c}	18.8±0.9^{c}	29.2±1.4^{d}

Data are means±SD of three replications. Data within columns followed by different superscript are significantly different ($P<0.05$, ANOVA).B SA = Bovine serum albumin.

Mean post-thaw fertilization and hatching rates are presented in Table 2. The highest fertilization (59.3±0.5%) and hatching rates (68.2±3.4%) of frozen-thawed sperm were obtained with supplementation of 75 mM taurine ($p<0.05$).

Table 2. Effect of antioxidants on fertilization and hatching rates of eggs fertilized with cryopreserved scaly carp (*Cyprinus carpio*) semen.

Antioxidants	Antioxidant Concentrations	Fertilization Rates (%) ± S.D.	Hatching Rates (%) ± S.D.
Taurine	25 mM	48.3 ± 1.4^{b}	54.5 ± 2.2^{b}
	50 mM	50.5 ± 2.2^{b}	59.2 ± 1.^{1ab}
	75 mM	59.3 ± 0.5^{a}	68.2 ± 3.4^{a}
	100 mM	44.3 ± 2.1^{b}	52.1 ± 1.5^{b}
BSA	1 %	43.4 ± 0.7^{b}	52.4 ± 3.2^{b}
	2.5 %	51.7 ± 1.2^{b}	61.2 ± 2.1^{ab}
	5 %	51.2 ± 2.2^{b}	60.4 ± 1.7^{ab}
	7.5 %	47.1 ± 1.4^{b}	54.6 ± 1.5^{b}
Vitamin C	2.8 mM	34.5 ± 3.6^{c}	43.4 ± 1.2^{c}
	5.6 mM	42.6 ± 1.4^{b}	51.5 ± 3.2^{b}
	8.4 mM	45.1 ± 1.6^{b}	54.2 ± 1.4^{b}
	11.2 mM	35.4 ± 1.2^{c}	34.6 ± 2.6^{d}
Control	-	81.7 ± 1.4^{d}	88.2 ± 1.4^{e}

Data are means±SD of three replications. Data within column followed by different superscripts are significantly different ($P<0.05$, ANOVA). BSA = Bovine serum albumin.

Discussion

During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn increases susceptibility to lipid peroxidation that is due to increased production of ROS. Excessive production of ROS during cryopreservation has been associated with reduced post-thaw motility, viability, membrane integrity, fertility, and antioxidant status (Michael et al., 2007). Antioxidants play an important role in the protection of sperm and

can neutralize the effects caused by the cryopreservation procedure. Previous studies demonstrated reduction in total antioxidants that naturally occur in seminal plasma in post-thaw samples (Martinez-Paramo et al., 2009). Recent studies in mammalian sperm demonstrated beneficial effects of adding antioxidants to the extender media of several species for motility and cell viability (Bucak et al., 2007). However, there is a lack of information regarding the effect of commonly used antioxidants on spermatological parameters and antioxidant activity in fish.

In the present study, the highest fertilization rate was 59.3±0.5% when 75 mM taurine was used. In addition, it was shown that post-thaw motility period and cell viability increased in relation to increased taurine concentrations. On the other hand, addition of vitamin C to ionic extender resulted in some decrease in motility and cell viability. The reduction following cryopreservation was possibly due to osmotic shock. Similarly, higher concentrations of vitamin C (2.5 mM) were detrimental to sperm motility of frozen thawed bull semen (Beconi et al., 1993). Similar effects were reported in canine sperm with 1.5 mM vitamin C supplementation to the extender (Michael, 2007). However, the opposite effect was seen in sturgeon sperm regarding vitamin C supplementation (Mirzoyan et al. 2006). In these studies an increase in the motility of Russian sturgeon sperm (*Acipenser gueldenstaedti*) protected with 10 mM vitamin C as well as a reduction of chromosomal aberrations in developing embryos was seen. Bovine serum albumin (BSA) has been considered a lipid peroxidation inhibitor or an emulsifying substance (Chen et. al., 1993). It is known that BSA stabilizes sperm motility. The results of the present study showed that adding BSA to the extender medium significantly improved sperm resistance to hypoosmotic shock caused by cryopreservation. In addition, we found that motility, cell viability, and fertility of spermatozoa cryopreserved with BSA was similar to that of the other tested antioxidants. BSA protection is partial but has a synergetic effect when used in combination with lipid vesicles because of its emulsifying properties (De Leeuw, 1993).

Analyzing the sperm quality parameters such as motility, cell viability, and fertility, improves our understanding of the effect of these substances on spermatozoa. In the present study, the significant improvement in motility and fertility of the undamaged cells could be due to a high level of protection of the tested antioxidants. The freezing techniques available for scaly carp spermatozoa produce reasonable fertility rates only when a high spermatozoa/egg ratio is used. Bearing this in mind, a higher concentration of spermatozoa should be used since this would produce a higher number of viable spermatozoa and also increase the number surviving spermatozoa following cryopreservation. On the other hand, evaluation of optimal spermatozoa/egg ratio is critical for determining the fertilization capacity of thawed sperm.

Supplementation of extenders with various antioxidants at different ratios improved fertilizing capability of cryopreserved semen significantly. This study indicated that antioxidants and their concentrations are very important in scaly carp semen croyopreservation. Our results suggest a species-specific effect probably depending not only on the type of antioxidant added but also on its concentration. This experiment also demonstrated that sperm viability could be stabilized by supplementation of the storage solutions with antioxidants. The results of this study have shown that taurine was the most suitable antioxidant tested in terms of cryoprotective action against freeze damage. More research is needed to understand the precise physiological role of these antioxidants and others in sperm protection.

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