

MOTILITY EVALUATION AND CRYOPRESERVATION OF FISH SPERM EXPOSED BY WATER-BORNE AND FOOD-BORNE BORON

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ABSTRACT

Boron (B) is required for organisms as an essential nutrient, whereas could be toxic in definite doses. In this study, for the first time, the effects of water-borne and food-borne B on fish sperm motility were initially determined and these sperm samples were cryopreserved in liquid nitrogen aiming to reveal how the quality of these samples are affected by cryopreservation. 12 mature male goldfish (*Carassius auratus*) individuals for each treatment performed in triplicate for 45 days were used in the study. Fish were fed with diets containing 1 mg, 5 mg and 10 mg of B per kg feed for foodborne experiments. In waterborne experiments, fish were kept in aquariums containing 1 mg/L, 10 mg/L and 20 mg/L of B. In both fresh and cryopreserved samples, the motility durations (s) and percentages of the sperm samples, vitality of spermatozoa (%) were determined. In the fresh samples, percentages of sperm motility and viability in the both B treatments decreased compared to the control, while increasing B concentration. After cryopreservation, the sperm samples in 5 mg of B per kg feed treatment has shown higher duration of sperm motility. Therefore, it is shown that feed-borne B could improve sperm resistance to cold shock.

Keywords: Boron, *Carassius auratus*, Sperm motility, Sperm vitality, Cryopreservation

Introduction

Boron (B) is a micro-mineral and a trace element, with properties between that of a metal and a nonmetal which is present in the environment (sedimentary rocks, soils, coal, seawater, etc.) in different forms such as borates, boric acid, boric oxide and salts (Samman *et al.*, 1998; Bustos-Obregon and Olivares, 2012; Kabu and Akosman, 2013). Commercially important deposits of boron-containing minerals are found in the United States, Turkey, South America, Russia, and China (Woods, 1994). There has been a long-standing usage of B. For instance, it was used by the ancient Egyptians in mummification and medicine. Nowadays, B minerals are used manufacture of glass fibers, ceramics, cleaning materials (detergents), bleaches, alloys, fire retardants, fertilizers, wood preservatives, cosmetics antifreeze, brake fluids and motor oil, particularly in the United States, China and Europe (Scialli *et al.*, 2010).

A limit of 0.3 mg B/L could be acceptable for drinking water (Simonnot *et al.*, 2000). Generally, surface and ground waters are below this limit but in the regions of borate mines and some industrial discharges B levels were determined around 7 mg/L. (Okay *et al.*, 1985; Recepoglu and Beker, 1991). On the other hand, the average concentration of B in seawater is approximately 5 mg/L (Song and Huang, 1987). Boron as borate compounds or boric acid is an essential element for plant growth, an essential nutrient in some animals and humans at defined concentrations (Blevins and Lukaszewski, 1994; Devirian and Volpe, 2003; Nielson, 2008). The toxicological studies in mammals have shown that the toxic effects of B on central nervous system, cardiovascular system, and axial skeleton. But the most sensitive endpoints originated from B toxicity are observed on the reproductive system where effects are detected at much lower dose levels (Ku *et al.*, 1993).

Cryopreservation as an assisted reproduction method is the use of very low temperatures (typically in liquid nitrogen) to preserve structurally intact living cells like spermatozoa (Pegg, 2007). Moreover, cryopreservation of sperm offers many advantages such as improving artificial reproduction, facilitating genetic manipulation (Chao and Liao, 2001). Conceptually, the procedure of cryopreservation could be divided into two main processes; the freezing and the thawing. These processes have potential to damage sperm cells especially associated with loss of integrity of cell membrane and oxidative stress (Suquet *et al.*, 1998). B has a part in cell membrane functions, anti-oxidative defense systems, structure of cell transporter, and cations metabolism, which are very critical for sperm quality (Goldbach *et al.*, 2001; Park *et al.*, 2004; Alavi and Cosson, 2006; Kabu and Akosman, 2013). Taking in consideration B functions in organisms, it

is motivated to study on the effects of B on sperm quality of fish, and cryopreservation of this sperm. Thus, this study aims to investigate the effects of water-borne and food-borne B at different concentrations on fish sperm quality, vitality of spermatozoa and mainly resistance to cryopreservation of sperm obtained from fish exposed by B.

Materials and Methods

Two hundred and fifty-two (1.5–2 years old, 7.8 ± 1.2 g, 18.6 ± 3.8 cm) male which were never stripped before and sexually mature sixty-three female goldfish (*Carassius auratus*) purchased from commercial dealer were used for experiments. Each treatment was done in three replicates using three aquariums of which each contains 45 liters of water with 12 male fish. The fish were kept 45 days in total in aquariums, last two weeks 3 females were put into each aquarium for matching and thereby making easier abdominal stripping of male fish. The experiments carried out the Aquarium Laboratory of Biology Department at Muğla S. K. University using with tap water (pH 7.97 ± 0.4 ; dissolved oxygen (DO) > 8 mg/L, and boron (B) concentration < 0.001 mg/L) at $16 \pm 0.5^\circ\text{C}$ on a 10-h dark and 14-h light (10D:14L) photoperiod. After putting the female fish, temperature of the aquarium water gradually increased to $22 \pm 0.5^\circ\text{C}$ on 8D:16L photoperiod during the last two weeks of the experiments. Thus, any hormone injections were avoided when obtaining sperm samples. DO and pH were measured with a portable HQ40D multimeter (Hach Lange, Germany) and a micro pH meter (WTW pH 320, Germany) respectively while B concentrations were determined by an ICP-MS procedure (Agilent 7700x, Germany) described as in Genç *et al.* (2015).

Besides the control groups which fed with B free pellets and in aquariums with tap water, the experimental design was composed of two main groups; food-borne and water-borne groups. All fish were fed daily with 2 mm feed pellets of around 2% of total fish wet weight. The water-borne B treatments were obtained adding 1, 10 and 20 mg/L of B concentrations to the aquariums. However, the pellets contained 1, 5 and 10 mg/kg of B concentrations were used in the food-borne B treatments. Each aquarium was supplied with tap or relevant B levels containing fresh water with 25% changes of it every two days and aeration to maintain enough DO. Eventually, together with the control group, there were seven treatments in the experimental design. In the food-borne B treatments, cornmeal oil (10 ml/kg feed) was used to bind B to the fish feed (Ardó *et al.*, 2008). The prepared feed was maintained at room temperature. B concentrations were adjusted using with boric acid (H_3BO_3 , Sigma-Aldrich, Germany) in all experiments. The condition factor (K)

of the experimental fish was estimated from the relationship: $K = (W/L^3) * 100$ (Bagenal and Tesch, 1978).

The fish fasted for 24 h before the start of the experiments were anaesthetized in 50 µl/L of clove oil (Öğretmen *et al.*, 2014). Sperm samples were collected by manual abdominal stripping by the help of pipette while avoiding any contamination from water, urine or faeces.

Sperm samples of 3 males were selected from each aquarium and then totally 9 samples were pooled as representative of each treatment. The motility characteristics of the pooled samples of spermatozoa (expressed as durations and percentages) were estimated by mixing an activation solution containing 45 mM NaCl, 5 mM KCl, and 30 mM Tris-HCl, pH8.2 (Horváth *et al.*, 2003) under a light microscope at 400× magnification at a semen:activation solution ratio of 1:100 on microscope slides.

Eosin-nigrosin staining for vitality of spermatozoa (%) was performed according to the concept of using eosin to mark dead cells, which take up eosin, and nigrosin as a black background (Blom, 1950). 10 µl of semen was mixed in a glass tube with firstly 50 µl of 0.5 % eosin and then 100 µL of 10% nigrosin which dissolved in 0.9 % sodium chloride in distilled water (modified from Björndahl *et al.*, 2003).

Following the stripping and pooling of the semen samples, they were placed in 12-ml glass test tubes on ice immediately under aerobic conditions. The pooled semen samples were mixed in a ratio of 1:9 (v/v) with an extender composed of modified Kurokura solution (62 mMol NaCl, 134 mMol KCl, 2 mMol CaCl₂, 1 mMol MgCl₂, and 2 mMol NaHCO₃, pH 8.2, 378 mOsm), 10% DMSO, and 10% egg yolk (Magyary *et al.*, 1996). After dilution, the samples were drawn into 0.25 ml straws (IMV, France) and sealed with polyvinyl alcohol. Next, the straws were placed on a rack 2.5 cm above a liquid nitrogen surface for 10 min and then plunged directly into the liquid nitrogen. At least seven straws per a sperm sample were frozen. After a week of storage in liquid nitrogen, the samples were thawed in a 30°C water bath for 10 s. the activation solution described above was also used to evaluate for the motility characteristics of cryopreserved samples. A 1 µl drop of sample was placed on a microscope slide, 100 µl of the activation solution was added, and then the sperm suspensions were thoroughly mixed for 2 s. The percentages of motile sperms and motility durations were immediately recorded with a CCD video camera (with a Nikon DS-U3 controller, Japan) mounted on a phase-contrast microscope (Nikon Ci-S, Japan) at 400× magnification at room temperature until the spermatozoa trajectories become tight concentric circles (Rurangwa *et*

al., 2004). The obtained video records were scanned to determine the percentages of progressive motility (%) and the durations of progressive motility (s). The sperm motility percentages were estimated as the percentage of cells that exhibited progressive forward movement (Horváth *et al.*, 2003), and the durations of motility were determined as the times until forward movement stopped and circular movement began. The percentages of sperm motility were assessed using an arbitrary scale with 10% interval increments in which non-motility was recorded as 0% (modified from Borges *et al.*, 2005). An aliquot of each straw were determined, and the average motility characteristics were then calculated.

The results are shown as the means ± the standard deviation (SD). Non-parametric Mann-Whitney U tests followed by Kruskal-Wallis test were used. Prior to analysis, the original percentage data were transformed by arcsine transformation. P values less than .05 were taken to indicate significant differences the treatments in terms of motility characteristics.

Results and Discussion

As an essential nutrient, B is a necessary dietary for not just to metabolisms of bacteria, fungi, plants, animals and but also to humans (Hunt, 1994; Goldbach *et al.*, 2001). B, like other microelements, could be toxic in definite doses, depending on how organisms taken in (Blevins and Lukaszewski, 1994). The effects have been confirmed in many experiments and studies on organ systems as well as reproduction system. In some mammalian species such as rats, mice, and dogs, the negative effects of oral administration of boric acid and borax were reported on the testis and following on sperm and spermiation (Scialli *et al.*, 2010). It has been clearly indicated the nutritional essentiality of boron for normal reproduction and maturation in frog (*Xenopus laevis*) (Fort *et al.*, 1999). Also, the requirement of B during embryonic development zebrafish (*Danio rerio*) has been reported clearly. A supplementation of B concentration of 45 µmol/L of B supplementation to the water improved hatching rates of fertilized eggs, comparing to 0.1 µmol/L of B concentration in water (Rowe and Eckhert, 1999). Moreover, male reproductive studies especially about their semen characteristics of B-exposed workers, who work in boric acid production plant, B processing plants or B mine in China and Turkey, has revealed the scientific awareness of B effects on reproduction (Duydu *et al.*, 2012; Başaran *et al.*, 2012; Scialli *et al.*, 2010).

Regarding to K values, there were no significant differences among the groups (P > 0.05) at both the beginning which is

not represented and the end of the experiment (Figure 1). The average K values in the control were found 3.94 ± 0.79 . K values were calculated in water-borne treatments as 4.01 ± 1.20 , 3.91 ± 1.12 and 3.42 ± 0.65 , respectively in 1, 10 and 20 mg/L of B groups while 3.49 ± 0.98 , 4.91 ± 1.92 and 4.19 ± 1.36 were found for food-borne treatments 1, 5 and 10 mg/kg of B, respectively.

The percentages of sperm vitality obtained from samples at the end of the experiment were represented in Figure 2. Sperm vitality was found in the control as 97.2 ± 0.6 %. In contrast to K values, there were significant differences among the percentages of sperm vitality ($P \leq 0.05$). Increasing with the B concentration, a decrease in sperm vitality was observed. In water-borne groups, sperm vitality values were calculated 92.5 ± 0.4 , 88.0 ± 0.8 and 84.1 ± 0.2 % as 1, 10 and 20 mg/L of B groups, respectively. In food-borne groups (1, 5 and 10 mg/kg of B groups), the average values of sperm vitality were found 91.8 ± 2.3 , 83.0 ± 0.5 and 81.0 ± 1.6 %, respectively.

Motility percentages and durations of both fresh pooled and post-thaw sperm samples treated by boron (B) with water-

borne (1, 10 and 20 mg/L of B in water) and food-borne (1, 5 and 10 mg/kg of B in pellets) were shown in Table 1, comparing with the control. The average motility percentages and durations of the fresh sperm sample in the control were 91.7 ± 2.9 % and 58.3 ± 2.9 s, respectively. Percentages of progressive motility were recorded as 66.7 ± 5.8 and 58.3 ± 2.9 % in 20mg/L B and 10mg/kg B groups, respectively. In the treatments, motility percentages were decreased while B concentrations were increased for both water-borne and food-borne groups. This pattern was not observed in motility durations. The highest average of progressive motility percentage was found in 1mg/L of B group as 62.7 ± 1.2 %. After the application of cryopreservation, the average motility percentage and duration were recorded 48.3 ± 2.9 % and 33.0 ± 2.6 s in the control. No significant difference was found among the water-borne groups in terms of post-thaw progressive motility values. Among the cryopreserved samples obtained from the food-borne groups, the highest motility percentage and duration were in the 5 mg/kg of B as 53.3 ± 5.8 % and 40.3 ± 2.5 s. Especially the average of progressive motility duration determined in this treatment was higher than the value in the control ($P \leq 0.05$).

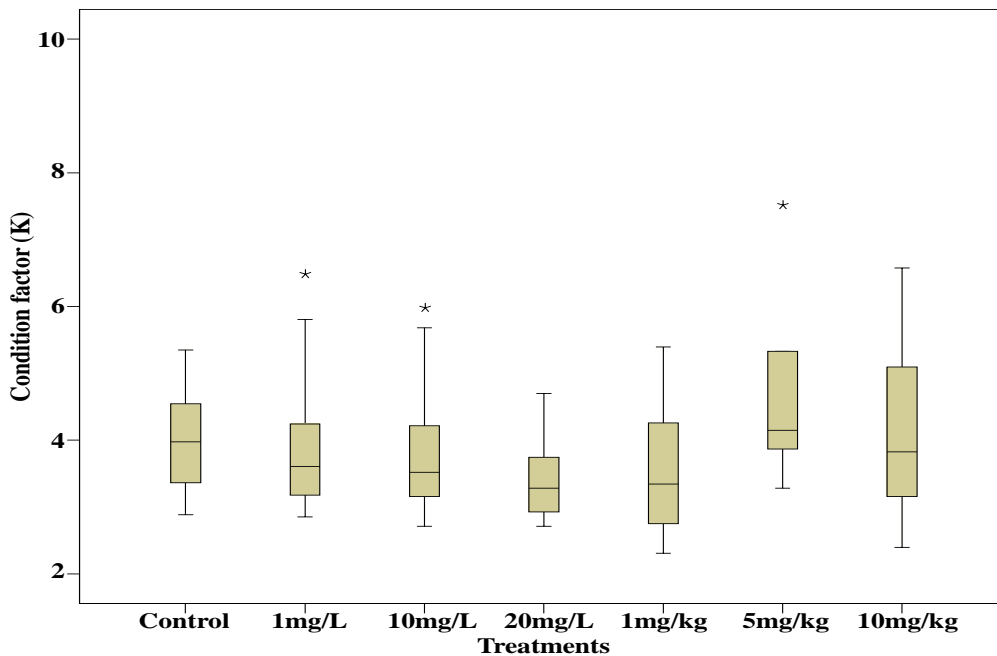


Figure 1. The condition factor (K) of the male fish in the control and the treatments (water-borne; 1, 10 and 20 mg/L of B in water and food-borne; 1, 5 and 10 mg/kg of B in pellets) at the end of the experiment. No significant differences were found among the groups ($n=36$ for each group, $P > 0.05$). The box plot represents the median and 25/75th percentile, whereas asterisks indicate outliers.

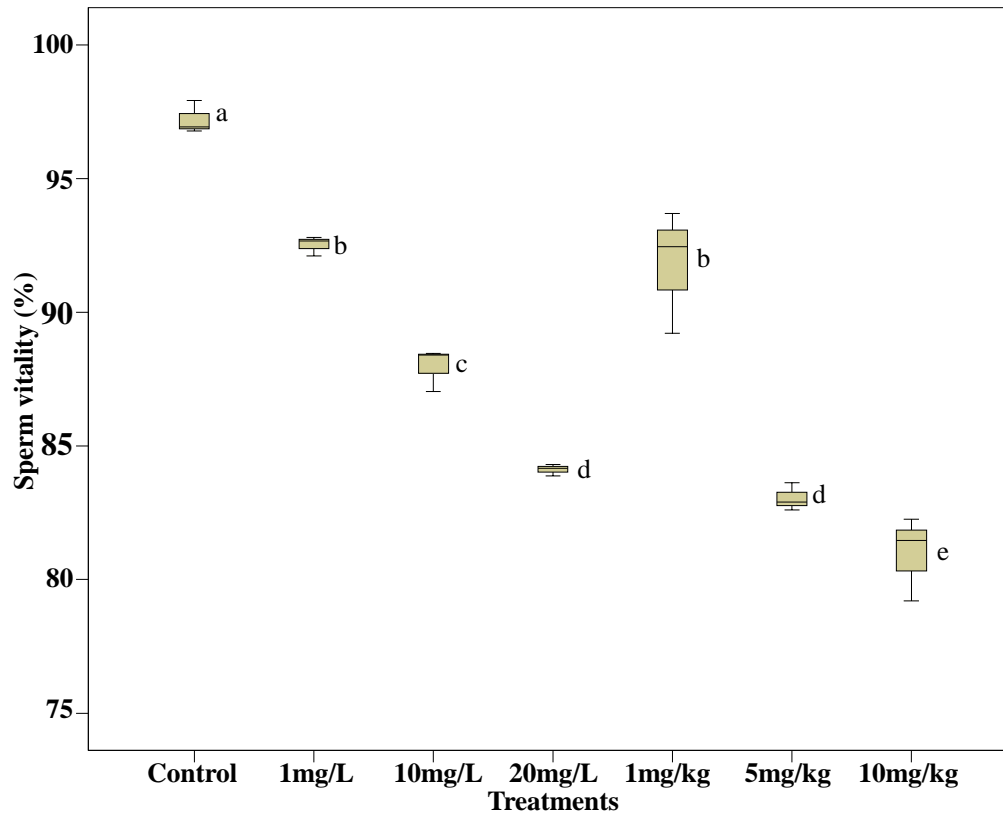


Figure 2. The percentages of sperm vitality (%) of the male fish in the control and the treatments (water-borne; 1, 10 and 20 mg/L of B in water and food-borne; 1, 5 and 10 mg/kg of B in pellets) at the end of the experiment. The same superscript letters did not differ from each other (n=36 for each group, P ≤ 0.05). The box plot represents the median and 25/75th percentile.

Table 1. Motility percentages (%) and durations (s) of fresh pooled and post-thaw sperm samples of goldfish (*C. auratus*) treated by boron (B) with water-borne (1, 10 and 20 mg/L of B in water) and food-borne (1, 5 and 10 mg/kg of B in pellets).

Treatments	Fresh Pooled Sperm Samples		Post-thaw Sperm Samples	
	Progressive motility (%)	Duration of progressive motility (s)	Progressive motility (%)	Duration of progressive motility (s)
Control	91.7±2.9 ^a	58.3±2.9 ^a	48.3±2.9 ^a	33.0±2.6 ^a
1mg/L	83.3±2.9 ^{ad}	62.7±1.2 ^b	25.0±5.0 ^b	18.7±1.5 ^b
10mg/L	86.7±2.9 ^a	54.7±1.5 ^a	36.7±5.8 ^b	34.0±6.6 ^{ad}
20mg/L	66.7±5.8 ^{bc}	44.3±2.5 ^c	31.7±2.9 ^b	23.7±2.1 ^c
1mg/kg	85.0±5.0 ^{ae}	51.3±1.2 ^d	48.3±2.9 ^a	32.3±2.1 ^a
5mg/kg	73.3±5.8 ^{bde}	56.0±1.0 ^a	53.3±5.8 ^a	40.3±2.5 ^d
10mg/kg	58.3±2.9 ^c	49.3±1.2 ^d	33.3±2.9 ^b	33.3±1.2 ^a

It has been shown that B supplemented diets improve testis weight in Frog (Fort *et al.*, 1999). In general, percentages of sperm vitality significantly decreased with increasing B levels ($P \leq 0.05$). The lowest percentage of sperm vitality was observed in 10 mg/kg of B in food-borne treatments. The negative effects of B levels on progressive motility percentages were recorded in fresh sperm samples ($P \leq 0.05$). Like sperm vitality, the lowest percentage of sperm motility was found in 10 mg/kg of B treatment. Similarly, a decrease in motility percentages was also reported in frog and some mammalian species (Fort *et al.*, 1999; Scialli *et al.*, 2010). In humans, no adverse effects of B on male reproduction can be demonstrated (Liu *et al.*, 2005; Duydu *et al.*, 2012). However, it was emphasized that the possibility of a bias in the selection of subjects for semen evaluation may have been emerged (Scialli *et al.*, 2010). Also, B exposures of selected subjects could be under the conditions of human handling and use (Bolt *et al.*, 2012).

Additionally, results of our study showed that durations of progressive motility were also decreased with the exception of the highest motility durations in 1 mg/kg of B food-borne group, even higher than the control slightly but significantly ($P \leq 0.05$). In cryopreserved sperm samples, while percentages of progressive motility were close, duration of progressive motility recorded in 5 mg/kg of B food-borne group higher than those values in control significantly. B functions, particularly at cell level, could induce this positive effect on motility duration. Cellular membrane functions of B and its role in anti-oxidative defense systems have been suggested clearly in plants (Goldbach *et al.*, 2001; Cakmak and Römheld, 1997). Whereas B functions in plants are well documented, its functions in animals have been started to study lately. For instance, it was shown that a boron transporter, called NaBC1, as mammalian homologue of the AtBOR1 borate transporter, occurring ubiquitously in animal cell membranes (Park *et al.*, 2004), This NaBC1 transporter conducts Na^+ which one of the major cations in semen (Alavi and Cosson, 2006) and in the absence of borate. Also, B has effects on energy metabolism and Ca^{++} and Mg^{++} metabolisms which are substantial for sperm motility (Alavi and Cosson, 2006; Kabu and Akosman, 2013).

Conclusion

In essence, results of our study showed that the motility characteristics of sperm of *C. auratus* were inhibited by exposing to water-borne and food-borne B. On the other hand, after cryopreservation of the samples, the motility characteristics in the 5 mg/kg of B from the food-borne groups were improved, comparing with the control group signifi-

cantly. Regarding the observable effects of B on sperm quality, using of B in studies on reproductive biology and fish as a model species are to encourage for further studies. Our study suggest the need for further studies of *in vitro* effect of B on quality of sperm, particularly adding B to cryomedium at low doses. Furthermore, analysis of oxidative stress of semen samples treated by B and also determining the accumulation of B in gonadal tissue and semen are needed to be studied.

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