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Effect of butylated hydroxytoluene (BHT) on the cryopreservation of common carp (*Cyprinus carpio*) spermatozoa

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ABSTRACT

The aim of the present study was to test the effects of butylated hydroxytoluene (BHT) on the cryopreservation of common carp spermatozoa. BHT is widely used in the cryopreservation of the spermatozoa of different animal species and successfully sustains the characteristics of spermatozoa during freezing and thawing, but it has not previously been used with fish. After sampling, common carp spermatozoa were diluted with an extender composed of modified Kurokura's extender, 10% DMSO, and 10% egg yolk containing 0.0001, 0.001, 0.01, 0.1, 1, 2.5, 5, or 10 mM BHT and subsequently frozen in liquid nitrogen. The post-thaw spermatozoa characteristics (i.e., progressive motility percentage (%), duration of progressive motility (s), fertilization rate (%), and eyed-eggs rate (%)) were evaluated and compared with those of the control group. There were significant increases in the percentage of progressive motility and the duration of progressive motility at the concentrations of 0.1 and 0.001 mM BHT ($P < 0.05$). The duration of post-thawed spermatozoa progressive motility at 0.001 mM BHT was significantly greater than that of the other groups (39.6 ± 0.4 s, $P < 0.05$), and the fertilization rates and eyed-eggs rates were also higher following the 0.1 and 1 mM BHT treatments. BHT at concentrations of more than 1 mM caused sperm immobility during the preparatory stages of the sperm freezing. We concluded that 0.001–0.1 mM BHT can be beneficial for the cryopreservation of common spermatozoa.

1. Introduction

Cryopreservation techniques are widely applied to the spermatozoa of many commercial animal species and are used with cultured and endangered aquatic species. Although cryopreservation offers many advantages, including effective breeding programmes, transportation of sperm, and genetic conservation, it also causes freeze–thaw or cryoprotectant-induced damage to the DNA, protein, and membrane lipids that result in decreases in spermatozoa motility and eventually fertilization (Zilli et al., 2008; Billard et al., 1993; Munkittrick and Moccia, 1984). In fish, scientific efforts to prevent the negative effects of cryopreservation have been made since the first use of cryopreservation (Blaxter, 1953). In particular, the determination of an optimum freezing solution for a studied species is one of the critical issues for successful post-thaw sperm motility. Freezing solutions are primarily composed of the following: (*i*) a milt extender that does not induce sperm activation, (*ii*) a cryoprotective agent that is required for survival during the freezing and thawing (e.g., dimethyl sulfoxide or methanol), and

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(*iii*) a membrane stabilizer, such as avian egg yolk, that can stabilize the extra-cellular surface of the spermatozoa during freezing (Cloud and Patton, 2009). Some other additives have also been used to optimize or improve cryopreservation procedures, such as milk (Chao et al., 1987; Foote et al., 2002), honey (Chao and Liao, 2001) and propolis (Castilho et al., 2009; Ogretmen et al., 2014). In addition these components, the effects of some antioxidant vitamins (e.g., ascorbic acid and α-tocopherol (Hu et al., 2010; Martínez-Páramo et al., 2012)) and amino acids (e.g., taurine, hypotaurine, l-glutamine (Cabrita et al., 2011; Mercado et al., 2009)) on cryopreservation have been studied in recent years. Basically, these additives are thought to inhibit the creation reactive oxygen species by the spermatozoa during freeze-thaw cycles (Alvarez and Storey, 1992). Butylated hydroxytoluene (BHT) has also been used as such an additive in many studies in recent decades.

BHT is a substituted toluene and phenolic antioxidant that is used in the manufacture of plastics, elastomers, oils, lubricants, vitamins and fragrances. BHT is also found in cosmetic product formulations and is used for prolonging the shelf life of foodstuffs at defined levels (Grillo and Dulout, 1995; Lanigan and Yamarik, 2002; Sonnenschein and Soto, 1998). The application of BHT to animal spermatozoa was initially studied in 1970s (Kamra, 1973; Hammerstedt et al., 1976). Pursel (1979) reported that BHT protects boar sperm membranes against cold shock damage (at 0 °C for 10 min). Subsequently, the progressive motilities and acrosomal integrities of boar spermatozoa that were treated with different levels of BHT were shown. The results indicated that the BHT-treated spermatozoa managed exposure to cold shock better than the untreated (control) spermatozoa (Bamba and Cran, 1992). The addition of BHT to the freezing solution clearly improves the survival and fertility of cryopreserved boar spermatozoa (Roca et al., 2004). The applications of BFT to the freezing solution for bull and goat spermatozoa have also been discussed in many studies. In bulls, extenders containing BHT have been found to result in 10% greater sperm motility than samples without BHT after thawing (Killian et al., 1989). It has been determined that both BHT and its analogues have different effects on cold-induced membrane stress in bull sperm (Graham and Hammerstedt, 1992). The positive effects of BHT with different extenders have not only been reported for bull spermatozoa (Anderson et al., 1994; Shoae and Zamiri, 2008; Asadpour and Nasrabadi, 2012; Suttiyotin et al., 2011) but also for the spermatozoa of Nili-Ravi buffalo (Ijaz et al., 2009) and Sahiwal bulls (Ansari et al., 2011). Moreover, BHT is useful for the cold storage (4 °C) of bovine oocytes and provides protection against direct chilling injury (Zeron and Arav, 1996). The effectiveness of BHT in cryopreservation has been observed for goat spermatozoa using different extenders, and better motility parameters have been observed in BHT-treated groups compared to control groups (Khalifa and El-Saidy, 2006; Memon et al., 2012; Naijian et al., 2013). Furthermore, it has been reported that the inclusion of specific levels of BHT to extenders enhances the post-thaw quality of canine (Ziaullah et al., 2012), ram (Farshad et al., 2010) and turkey sperm (Donoghue and Donoghue, 1997). Despite increasing interest in the use of BHT in the cryopreservation of spermatozoa, no studies have yet examined its effectiveness in fish.

Cyrinus carpio (the common carp) is an important commercial cyprinid species that is found from Europe to Asia. According to FAO statistics, more than 3 million tonnes of *C. carpio* have been produced each year since 2007. The common carp is also the subject of many studies due to its use as a model organism in various fields and the cryopreservation of its spermatozoa. The objective of the present work was to determine the effects of BHT on the post-thaw spermatozoa motility characteristics in terms of fertilization and the eyed-eggs rates of common carp eggs following insemination with frozen-thawed and fresh sperm.

2. Materials and methods

2.1. Collection of the gametes

Gametes were obtained from 3- to 4-year-old breeders at the Fish Production Farm of General Directorate of State Hydraulic Works (Izmir, Turkey) in May. The breeders were held in sand ponds under a natural photoperiod regime in running water at a temperature of 22 ± 1 °C. The first injections of carp pituitary extract (1 mg kg^{-1}) were administered to both male and females, and 12 h later, the second injections were performed (3 mg kg^{-1}) for only the females before. This procedure was followed 12 h later with stripping. The fish were anaesthetized in a 1:3000 aqueous solution of 2-phenoxyethanol (Sigma–Aldrich, Germany). Gamete samples were collected by manual abdominal stripping while avoiding any contamination from water, blood, urine, or faeces. The motility characteristics of the collected samples of spermatozoa (expressed as durations and percentages) were estimated by mixing an activation solution (45 mM NaCl, 5 mM KCl, and 30 mM Tris–HCl, pH 8.2, Horváth et al., 2003) under a light microscope at 200× magnification at a semen:activation solution ratio of 1:100 on a microscope slide as described below. Samples from seven males that exhibited greater than 90% motility were used for cryopreservation. After examination of the motility characteristics of the spermatozoa, the samples (1 ml from each of the males) were pooled for the cryopreservation process. Following the stripping and pooling of the semen samples, they were placed in 12-ml glass test tubes cooled to 4 °C immediately under aerobic conditions. The eggs were maintained in dry plastic bowls at room temperature and used for fertilization within 30 min of stripping.

2.2. Application of BHT to the cryomedia, freezing–thawing methods, and fertilization

The semen samples were mixed in a ratio of 1:9 (v/v) with an extender composed of modified Kurokura (MK) solution (62 mMol NaCl, 134 mMol KCl, 2 mMol $CaCl_2$, 1 mMol $MgCl_2$, and 2 mMol $NaHCO_3$, pH 8.2, 378 mOsm), 10% DMSO, and 10% egg yolk (Magyary et al., 1996). Due to the solubility properties of BHT, a stock solution was prepared in DMSO and diluted with DMSO for the experimental doses. Thus, BHT added to the extenders through

the cryoprotectant (DMSO) to calculate the final doses in the extender. Nine experimental extenders were prepared at concentrations of 0 (control group), 0.0001, 0.001, 0.01, 0.1, 1, 2.5, 5, and 10 mM BHT. The results from the use of these extenders also compared those from fresh semen.

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 20 °C water bath for 30 s.

For the fertilization assay, 1.2 g of eggs (approximately 1000 eggs) were fertilized at a ratio of 10^5:1 sperm/egg with the activation solutions described above using both fresh and thawed sperm in 500 ml round-bottomed plastic bowls (Linhart et al., 2000). The fertilization tests were performed in triplicate. Following 2 min of stirring after the addition of the activation solution, the solution was replaced with a solution of 0.4% NaCl and 0.3% urea to eliminate the stickiness of eggs. The eggs were stirred in this solution for 1 h and then rinsed twice in a 0.5% solution of tannic acid for 20 s, and the bowls were then gently transferred to an experimental stand that was supplied with 22 ± 1 °C hatchery water at a flow rate of 10 l h^{-1}. 3 h after insemination, the fertilization ratios were calculated using a stereomicroscope at 20× magnification, and the dead eggs were removed from each bowl during incubation. The eyed embryos were counted after three days of incubation.

2.3. Evaluation of the post-thaw motility of the spermatozoa

The activation solution described above was used to evaluate the motility characteristics of freshly collected and cryopreserved samples. A 1 μl drop of sperm 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 spermatozoa 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 (20 °C) 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). Three aliquots of each sample were determined, and the average motility characteristics were then calculated.

2.4. Statistical analyses

The freezing experiments were replicated seven times, and the fertilization procedures were replicated three times. The results are shown as the means ± the standard errors of the mean (SEMs). Non-parametric Mann–Whitney U tests followed by Kruskall–Wallis test were used, and $\alpha < 0.05$ were taken to indicate a significant difference between the treatments in terms of post-thaw motility percentages, durations, and fertilization and eyed-egg rates.

3. Results

The post-thaw progressive motility percentages, durations, and fertilization and eyed-egg rates of the carp spermatozoa that were cryopreserved in BHT concentrations in MK extender, the control and those of fresh samples are shown in Table 1. The average motility percentages and durations of the fresh sperm were 91.4 ± 1.4% and 59.1 ± 2.2 s ($n = 7$), respectively. In contrast, the average motility percentages and durations of the cryopreserved semen ranged from 41.4 ± 2.6 to 81.4 ± 1.4% and 23.6 ± 0.2 to 39.6 ± 0.4 s, respectively. The highest post-thaw motility values were achieved using the extenders that contained 0.01 mM BHT (81.4 ± 1.4%) and 0.001 mM BHT (72.9 ± 3.6%). Significant differences in the motility durations of all groups were found ($P < 0.05$); the control group exhibited the lowest spermatozoa post-thaw motility duration (23.6 ± 0.2 s).

Among the cryopreserved samples, the highest average fertilization ratio (87.3 ± 3.7%) was obtained using the extender that contained 0.1 mM BHT. No significant difference in the fertilization rates was found between the fresh group and that stored with extender containing

Table 1
Post-thaw motility percentages, durations ($n = 7$), and fertilization and eyed-egg rates ($n = 3$) of the carp spermatozoa that were cryopreserved with different BHT ratios in the MK extender, the controls (MK extender) and the fresh samples (presented as the means ± the SEMs).

Final BHT concentration (mM)	Progressive motility (%)	Duration of progressive motility (s)	Fertilization rate (%)	Eyed-eggs rate (%)
1	41.4 ± 2.6[a]	28.4 ± 0.4[a]	85.7 ± 1.2[a]	79.7 ± 0.9[a]
0.1	51.4 ± 3.4[b]	35.6 ± 0.3[b]	87.3 ± 3.7[abf]	64.3 ± 2.3[b]
0.01	81.4 ± 1.4[c]	36.1 ± 0.3[b]	79.0 ± 2.6[bcd]	35.3 ± 2.4[c]
0.001	72.9 ± 3.6[d]	39.6 ± 0.4[c]	76.0 ± 2.5[cd]	38.3 ± 1.2[c]
0.0001	54.3 ± 3.0[b]	24.4 ± 0.2[d]	75.0 ± 1.5[d]	35.0 ± 2.5[c]
Control	45.7 ± 3.0[ab]	23.6 ± 0.2[e]	69.3 ± 0.7[e]	13.3 ± 0.9[d]
Fresh[a]	91.4 ± 1.4[e]	59.1 ± 2.2[f]	91.7 ± 0.9[f]	87.3 ± 1.2[e]

The values with the same superscript letters in the same column did not differ from each other ($P \leq 0.05$).
[a] Percentage and duration of motility values were taken post-thaw with the exception of the fresh samples.

0.1 mM BHT. Moreover, the average fertilization rate obtained with the extender that contained 1 mM BHT was not significantly different from that of the 0.1 mM BHT group. Regarding the eyed-eggs rates, the highest rate ($79.7 \pm 0.9\%$) was achieved with 1 mM BHT in the extender. No differences between the extenders containing 0.01, 0.001, and 0.0001 mM BHT were found. The fertilization and eyed-eggs rates in all BHT groups were higher than those of the control group ($P < 0.05$). The spermatozoa samples that were treated with the extenders containing 2.5, 5, 10 mM BHT could not be activated.

4. Discussion

To preserve sperm motility after thawing, cryopreservation protocols have been improved via not only the use of extenders with varied ionic and non-ionic compositions and different cryoprotectants but also via evaluations of the appropriate ratios of these components in the cryomedium and alterations in the elapsed freezing and thawing times. Sperm damage that results in low post-thaw sperm motility is induced by distinct mechanisms at each of the phase of cryopreservation (Medeiros et al., 2002). The primary causes of such damage, also called as cryoinjury, are heat removal, pH fluctuations, cold shock, ice crystal formation, osmolarity effects, and cryoprotectant toxicity (Chao and Liao, 2001). There have been scientific efforts to prevent such damage that has resulted in the development of extenders that contain various ionic contents, have different osmolality and pH values and are generally supported by sugar (e.g., glucose) and protein (e.g., albumin). Different cryoprotectants (e.g., dimethyl sulfoxide, methanol, ethylene glycol, and glycerol) have also been added to the extenders to prevent intracellular ice formation (Lim and Le, 2013; Orfão et al., 2011; Chao and Liao, 2001). In addition to these factors, the cryoprotective roles of some additives have been demonstrated to improve post-thaw sperm motility. For example, an extender composed of milk combined with methanol has been applied to red tilapia (*Tilapia niloticus*) sperm, and the fertilization rate was to be found 93.4% compared the control rate of 90% (Chao et al., 1987). Moreover, the addition of 0.5% honey to Ringer's solution has been found to be effective in the cryopreservation milkfish (*Chanos chanos*) and black porgy (*Acanthopagrus schlegeli*) (Chao and Liao, 2001) sperm. Additionally, the cryoprotective effects of the inclusion of 0.8 and 1 mg ml^{-1} propolis in the extender have been demonstrated in terms of the post-thaw motilities and hatching abilities of common carp spermatozoa (Ogretmen et al., 2014).

BHT is the one of the most commonly used additives, particularly for the cryopreservation of mammalian sperm. The use of BHT in the cryopreservation of the sperm of different mammalian species is summarized in Table 2. Generally, 0.5–2 mM BHT is used in the extender to improve sperm motility and preserve sperm morphology, plasma membrane integrity, acrosomal integrity and viability. In contrast, concentrations of BHT above 2 mM have some negative effects on sperm quality. Favourable effects of BHT on sperm cryopreservation resulting from supplementations of extenders with suitable concentrations have been clearly observed for Nili-Ravi buffalo, Sahiwal bull, boar and canine sperm (Labrador dogs) (Ijaz et al., 2009; Ansari et al., 2011; Roca et al., 2004; Ziaullah et al., 2012). Moreover, it is well established that the presence of 1 mM BHT in the extender improves post-thaw membrane integrity of mongrel dog sperm (Neagu et al., 2010). In contrast, there are no significant differences in the post-thawed spermatozoa characteristics between treatments containing and not containing BHT for certain species (e.g., rams and Mahabadi goats). In addition to the above mentioned mammalian species, the addition of 1.25 mM BHT to the extender improves the spermatozoa survival, membrane integrity, and preserved motility of turkey semen (Donoghue and Donoghue, 1997). It is also clear that the optimum concentrations of BHT for cryoprotection are species-dependent.

In this study, higher concentrations of BHT were found to decrease the quality of the spermatozoa of the common carp. Concentrations of BHT greater than 1 mM resulted in nearly complete immobilization of the spermatozoa even before freezing. However, BHT concentrations below than 1.0 mM, particularly 0.01 and 0.001 mM, increased the percentages of progressive motility compared to the other concentrations. Specifically, at the eyed-egg stage, the eggs fertilized by thawed spermatozoa that were treated with BHT exhibited significantly better results than did the controls.

Two primary mechanisms have been proposed to explain the protective effect of BHT on sperm characteristics after thawing. The first is that BHT is penetrates into the sperm membrane and increases the flexibility and fluidity of the membrane to protect the sperm from cold-shock (Hammerstedt et al., 1976). Alternatively, BHT reduces the damaging effects of lipid peroxyl radicals by converting them to hydroperoxides, which are harmless to spermatozoa (Aitken and Clarkson, 1988). Cryopreservation induces lipid peroxidation in mammalian spermatozoa, which is negatively correlated with sperm characteristics (Alvarez and Storey, 1992; Chatterjee and Gagnon, 2001). Similarly, it is well established that increases in lipid peroxidation decrease the quality of fish sperm (Martínez-Páramo et al., 2012; Shaliutina et al., 2013). It has been shown that BHT, as a lipid-soluble chain-breaking antioxidant, suppresses lipid peroxidation at defined concentrations in thawed bull and boar sperm (Killian et al., 1989; Roca et al., 2004). Moreover, Roca et al. (2004) suggested that BHT might have an additional protective effect on cryopreserved spermatozoa that prevents the loss of DNA integrity and reported differences in developing embryos that underwent different BHT treatments. Such effects might explain the higher eyed-eggs rates observed at different BHT treatments compared to the control in our study. Consequently, one of (or both) of these mechanisms might be responsible for the protective effects of BHT on the motility of common carp sperm after thawing, which eventually improves fertilization and eyed-eggs rates.

Our results showed that the use of concentrations of BHT that are lower than those used for mammals had positive effects on the cryopreservation of fish spermatozoa. This finding might be related to the differences between the reproductive mechanisms of fish and mammalians, particularly the differences in the selectivity of the spermatozoa

Table 2
The effect of the use of BHT on the cryopreservation of the sperm of different animal species, effective doses, overdoses and tested sperm parameters.

Effective dose (mM)	Overdose (mM)	Tested characteristics of spermatozoa	Post-thawed values (means% ± SEM)		Species	References
			BHT	Control		
2	>3	• Motility	58.5 ± 1.2^{a}	44.6 ± 1.1^{b}	Nili-Ravi buffalo	Ijaz et al. (2009)
		• Plasma membrane integrity	52.3 ± 1.2^{a}	44.5 ± 1.1^{b}		
		• Acrosomal integrity	28.7 ± 0.7^{a}	21.0 ± 1.1^{b}		
		• Viability	67.6 ± 1.0^{a}	59.7 ± 1.9^{b}		
0.5	>2	• Motility	32.9 ± 1.4^{ns}	33.3 ± 0.9 ^{ns}	Bull	Shoae and Zamiri (2008)
		• Acrosomal integrity	56.4 ± 0.9^{a}	50.5 ± 1.0^{b}		
		• Viability	56.9 ± 0.8^{a}	51.7 ± 0.9^{b}		
0.5	>3	• Motility	58.3 ± 1.3^{a}	41.7 ± 1.3^{b}	Sahiwal bull	Ansari et al. (2011)
		• Plasma membrane integrity	57.0 ± 1.2^{a}	39.3 ± 1.4^{b}		
		• Viability	72.7 ± 1.9^{a}	61.7 ± 0.7^{b}		
0.2	1.6	• Motility	45.4 ± 2.0^{a}	32.2 ± 2.6^{b}	Boar	Roca et al. (2004)
2	–	• Motility	66.3 ± 0.5^{a}	64.3 ± 0.5^{b}	Boar goat	Memon et al. (2012)
		• Morphology	74.2 ± 0.8^{ns}	72.3 ± 1.1^{ns}		
		• Plasma membrane integrity	68.8 ± 1.2^{a}	58.1 ± 1.5^{b}		
		• Acrosomal integrity	65.1 ± 0.6^{a}	61.2 ± 0.6^{b}		
		• Viability	73.4 ± 0.8^{a}	69.0 ± 1.1^{b}		
1	>2	• Motility	60.2 ± 5.8^{ns}	54.4 ± 5.4^{ns}	Mahabadi goat	Naijian et al. (2013)
		• Plasma membrane integrity	47.6 ± 3.9^{ns}	46.2 ± 4.2^{ns}		
		• Acrosomal integrity	26.3 ± 4.5^{ns}	22.3 ± 3.6^{ns}		
		• Viability	62.8 ± 3.8^{a}	52.8 ± 3.8^{b}		
2	3	• Motility	76.1 ± 0.9^{ns}	73.7 ± 0.8^{ns}	Ram	Farshad et al. (2010)
		• Plasma membrane integrity	81.4 ± 0.9^{ns}	79.2 ± 0.8^{ns}		
		• Acrosomal integrity	5.7 ± 0.2^{ns}	5.9 ± 0.2^{ns}		
		• Viability	84.2 ± 0.7^{ns}	82.3 ± 0.6^{ns}		
1	>1.5	• Motility	52.3 ± 2.4^{a}	38.6 ± 2.2^{b}	Canine	Ziaullah et al. (2012)
		• Plasma membrane integrity	59.5 ± 1.0^{a}	54.2 ± 1.5^{b}		
		• Acrosomal integrity	20.0 ± 1.3^{a}	23.4 ± 1.3^{b}		
		• Viability	60.0 ± 1.5^{a}	50.2 ± 2.7^{b}		

Different superscript letters within each row in the *post-thawed values column* indicate statistically significant differences at $P < 0.05$; ^{ns} no significant difference.

membranes, or the result of the interaction between egg yolk and BHT. The effectiveness of BHT in improving post-thaw spermatozoa characteristics might be related to the cooperative interaction between BHT and egg yolk (Roca et al., 2004). It has been suggested that BHT and its analogues interact synergistically with egg yolk to protect spermatozoa from cold shock (Graham and Hammerstedt, 1992). In contrast, the presence of egg yolk apparently affects the optimal concentration of BHT for use in cryopreservation. Lower concentrations of BHT are optimal when egg yolk-free extender is used in the conservation of goat semen (Khalifa et al., 2008). Thus, the egg ratio used in the extender for freezing is important. Generally, extenders containing 20% egg yolk are used for the cryopreservation of mammalian spermatozoa. However, the extender used in our study contained 10% egg yolk, which is similar to the extenders used in sperm cryopreservation for many other fish species. The lower egg yolk ratio that was for the cryopreservation of common carp sperm might explain why the lower concentrations of BHT had effects on the characteristics of the post-thaw spermatozoa.

In summary, we reported the cryoprotective role of BHT for fish sperm for the first time. The presence of BHT at concentrations ranging from 0.001 to 0.1 mM in the extender improved sperm motility and duration of motility after thawing. Additionally, the addition of BHT to the extender significantly increased fertilization and eyed-egg rates among the eggs that were inseminated with post-thawed common carp sperm.

Conflict of interest

None declared.

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