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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