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In vitro effects of Bisphenol A on sperm motility characteristics in *Perca fluviatilis* L. (Percidae; Teleostei)

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Summary

In the present study, the effects of Bisphenol A (BPA) on sperm motility and velocity were investigated in perch (*Perca fluviatilis* L.). Sperm of five mature males was separately collected in a syringe. The sperm samples were diluted in an immobilizing solution containing 180 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). The sperm motility was triggered in an activation solution composed of 50 mM NaCl, 20 mM Tris, pH 8.5 containing different concentrations of BPA (2, 1.5, 1.25, 1.0, 0.5, 0.25, 0.125 and 0.0 mM) at ratio 1 : 50 (sperm : AS). At 15 s post-activation, sperm motility and velocity decreased significantly in activation medium containing 1.5 and 1 mM BPA, respectively. The motility of sperm was totally inhibited in 2 mM of BPA. Sperm motility duration decreased with increasing BPA concentrations. In the control, 25.3% of spermatozoa were still motile at 90 s post-activation, while only 1.3% was in 0.12 mM. However, sperm velocity was higher in activation medium containing 0.12 mM BPA at 15 s post-activation. There was no significant difference between the control and DMSO test group, the latter being used for dissolving BPA. The results of this study indicate that BPA decreases both motility and velocity of exposed sperm, *in vitro*. BPA may damage the flagella as 'C' shape of flagella was observed, once motility period of sperm cells are finished.

Introduction

Endocrine disrupting chemicals (EDCs) are a large group of synthetic and natural occurring agents that interfere with the normal endocrine functions (Kime, 1998). The US-Environmental Protection Agency (EPA) has defined EDC as 'an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental process' (Kavlock et al., 1996). EDCs change the endocrine system through receptor-mediated or receptor-independent mechanisms (Gregory et al., 2008), which lead to intersexes, suppress spermatogenesis, or decrease reproductive success (Sumpter and Jobling, 1995; Kime, 1998). Decrease in reproductive ability may results from altered sperm quality (such as decreased sperm production or reduced sperm motility).

Bisphenol A (BPA) is one of the highest volume chemicals produced worldwide, with over six billion pounds produced each year (Vandenberg et al., 2009). BPA is building block of polycarbonate plastic. BPA leaches from polycarbonate baby bottles, and reusable water bottles. Epoxy resins used to protect food cans are also synthesized by the condensation of BPA with epichlorhydrin to create BPA diglycidyl ether (Richter et al., 2007; Vandenberg et al., 2009). BPA act as an estrogen mimic, directly with estrogen receptor (ER) and with plasma sex-steroid binding protein (Tollefsen et al., 2004). The affinity of BPA for the ERs is approximately 10 000-fold weaker than that of estradiol (Gregory et al., 2008). In addition to estrogenic activity of BPA, there is some evidence that BPA inhibits or decrease testicular steroidogenesis at low exposure level (Sohoni and Sumpter, 1998; Akingbemi et al., 2004). It was shown that BPA alters sperm concentration in guppies (*Poecilia reticulata*) (Haubruge et al., 2000) and brown trout (*salmo trutta fario*) at the beginning of the spawning period (Lahnsteiner et al., 2005b). It can also reduce numbers of spermatogenic cysts and induce intersex in common carp (*Cyprinus carpio* L.) (Mandich et al., 2007). Increase of serum vitellogenin concentrations have been reported in male Atlantic cod (*Gadus morhua*) (Larsen et al., 2006), medaka (*Oryzias latipes*) (Tabata et al., 2001; Kang et al., 2002) and fathead minnow (*Pimephales promelas*) (Sohoni et al., 2001) exposed to BPA. Lahnsteiner et al. (2005b) studied the *in vivo* effects of BPA on sperm characteristic in brown trout. However, no information is available about *in vitro* effects of BPA on sperm motility.

In the present study, instant effects of BPA on sperm motility and velocity were studied in Eurasian perch, *Perca fluviatilis* L. (Percidae, Teleostei) using a Computer Assisted Sperm Analysis (CASA) system, *in vitro*. Spermatozoa concentration of perch was reported 29×10^9 spz ml^{-1} (Alavi et al., 2007). The spermatozoa are immotile in the seminal fluid due to osmolality and a hypo-osmotic shock is necessary for triggering of initiation of sperm motility (Alavi et al., 2007, 2010). Duration of sperm motility is very short (< 1 min in freshwater). Spermatozoa velocity, percentage of motility and beat frequency decrease rapidly after sperm activation that depends on the osmolality of the activation medium (Alavi et al., 2007, 2010). Osmolality higher than 300 mOsmol kg^{-1} totally suppress sperm activation. The means of seminal plasma osmolality (mOsmol kg^{-1}), sodium, chloride, potassium and calcium ions concentrations (mM) are measured as 298, 131, 107, 11 and 2.4 respectively (Alavi et al., 2007).

Materials and methods

Broodfish and collection of semen

Sperm was collected from five mature males of perch (total length: 205–230 mm, and body weight: 110–162 g) in the second half of March, 2009. The males were anesthetized

before stripping in clove oil (33 mg L^{-1}). The sperm was collected by a gentle abdominal massage from the anterior portion of the testis towards the genital papilla and collected with plastic syringes to avoid sperm contamination by urine, mucus and blood cells. Samples of sperm were stored at 2–4°C and transferred to the laboratory of reproductive physiology in fish for further analysis. Spermatozoa concentration, spermatocrit and osmolality of the collected samples were between 54.3 and 68.59×10^9 spz ml^{-1}, 66–70% and 300–306 mOsmol kg^{-1}.

Sperm dilution and motility assessment

After collection of sperm, a two-step dilution was used to evaluate sperm motility. Firstly, sperm was diluted in an immobilizing solution (IS) containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). Then, the sperm was activated in activation solution (AS) containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). To test the effects of BPA, dissolved BPA in DMSO was added to AS at 2, 1.5, 1.25, 1.0, 0.5, 0.25, 0.12 mM. DMSO was < 0.1% after adding BPA in activation medium. Therefore, effect of DMSO 0.1% on sperm motility was studied as control. To avoid stickiness of sperm into the glass slide, 0.1% BSA was only added to the activation solution before sperm activation. Preliminary observations did not show any difference between activation solution containing BSA and activation solution without BSA at different concentration of BPA in terms of sperm motility (viability). In both conditions, flagellar beating was observed, but only forward motions of spermatozoa were observed in activation solution containing BSA. In activation solution without BSA, forward motions of spermatozoa inhibited due to stickiness of sperm into the glass slide.

Sperm motility was recorded using a three CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) equipped with a stroboscopic lamp at frequency adjusted to 50 Hz. A micro image analyzer (Olympus Micro Image 4.0.1 for Windows – CASA) was used to measure percentage of sperm motility (%) and sperm velocity ($\mu m\ s^{-1}$) from five successive video images, which shows positions of sperm heads. Five video frames were captured from a DVD-recorder (SONY DVO-1000 MD, Japan) and accumulated (overlapped) using CASA in real time post-activation. Figure 1 shows successive frames of sperm movement in *P. fluviatilis* at 13 s post-activation and the final output of the CASA from overlapping the five captured frames that was used for measuring the sperm parameters. The out put frame is showing positions of head of

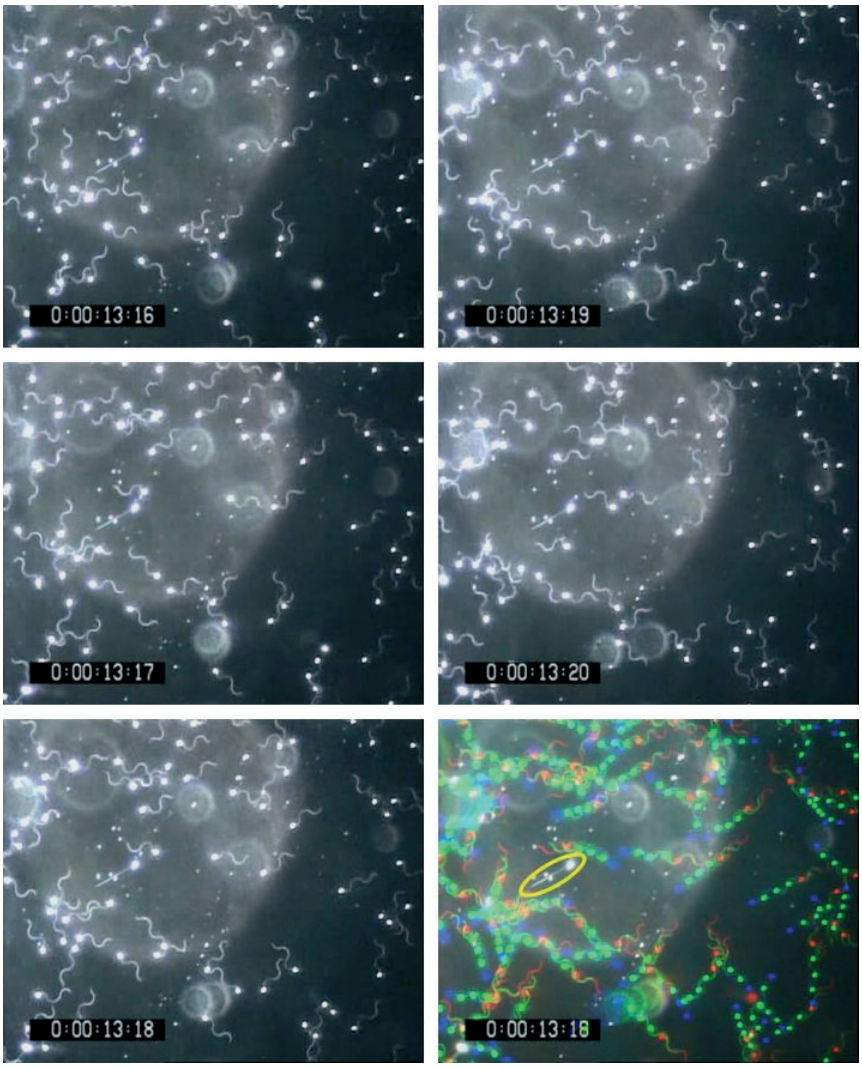

Fig. 1. Successive frames of spermatozoa movement in *Perca fluviatils* identified by the time (bottom left, in this case at 13 s post-activation). The out put frame from overlapping of five captured frames is showing five positions of heads of motile spermatozoa in red-green-green-green-blue, while the immotile sperm cell(s) is in white color (in this case the spermatozoon in yellow circle). The percentage of motile spermatozoa was calculated by counting of red or blue spots versus the number of white spots. To measure the sperm velocity, distance of five head positions (distance between red and blue spots) were measured and divided into time spent for moving such distance

motile spermatozoa in five spots (red-green-green-green-blue), while the immotile spermatozoa are shown in white color. Therefore, the percentage of motile spermatozoa was calculated by counting of red or blue spots versus the number of white spots. To measure the sperm velocity, distance of five head positions (distance between red and blue spots) were measured and divided into time spent for moving such distance. In this study, the sperm velocity shows data of only motile spermatozoa (approximately 50–60 spermatozoa per each treatment). In each treatment, sperm motility was assessed three times per each male.

Data analysis

Data presented are mean ± standard error of mean (SE). Mean of data from three analyses per each treatment were measured and used for statistical analysis. Before analysis of data by ANOVA, Kolmogorov–Smirnov 's and Levene 's tests were used for normality of data distribution and homogeneity of variances. Then, statistical comparisons were made based on sperm motility and velocity as dependent variables and BPA concentration as independent variable at each time post sperm activation following Duncan's test (SPSS 10.0).

Results

Significant effect of BPA on sperm motility was observed at each time post-activation (df = 4, $P < 0.001$, F = 66.87, 67.04, 49.40, 33.17 and 60.11 at 15, 30, 45, 60 and 90 s post-activation, respectively). The motility of sperm was totally inhibited in 2 mM BPA (Fig. 2). The duration of sperm motility decreased with increasing BPA concentrations. In control, 25.3% of spermatozoa were motile at 90 s post-activation, while only 1.3% was motile in 0.12 mM. At 15 s post-activation, the percentage of sperm motility significantly decreased after activation in 1.5 mM BPA. The percentage of motile spermatozoa did not differ significantly between control and sperm exposed to lower than 1.25 mM BPA at 15 s post-activation, but decreased significantly in 1.0 mM BPA at 30 and in 0.5 mM BPA at 45 s post-activation. Closer to the end of the motility period, sperm motility was significantly low in very low concentrations of BPA (0.25 and 0.12 mM at 60 and 90 s post-activation, respectively). There was no significant difference between control and activation solution containing 0.1% DMS in terms of sperm motility (Fig. 2).

At 15 s post-activation, sperm velocity was significantly influenced by BPA concentrations and decreased significantly in 1 mM BPA (df = 4, F = 151.10, $P < 0.001$). Sperm velocity was higher in 0.12 mM than in control and DMSO groups (Fig. 3). BPA concentrations also affected sperm velocity at 30 and 45 s post-activation (df = 4, F = 29.06 and 17.17, $P < 0.001$). At 30 s post-activation, the highest sperm velocity was observed in activation solution containing 0.12 mM BPA. Sperm velocity in BPA 0.25–1.25 mM were higher than control and DMSO (Fig. 3). BPA concentration showed significant effect on sperm velocity at 45 s post-activation (df = 4, F = 15.17, $P < 0.01$). The highest sperm velocity was observed in activation solution containing 0.12–1.25 mM BPA. Sperm velocity was also influenced by BPA concentrations at 60 (df = 4, F = 1.58, $P < 0.05$) and 90 s post-activation (df = 4, F = 1.16, $P < 0.05$). Significant differences were not observed between the control, DMSO and BPA concentrations (0.12–0.25 mM), in which the spermatozoa were motile. There was no significant difference in terms of sperm velocity between control and DMSO, which was used for dissolving BPA (Fig. 3).

Figure 4 shows sperm motility of *P. fluviatilis* after activation in control or different BPA concentrations at different time post-activation. Interestingly, a 'C' shape of flagella was observed at 1.25 mM BPA (at 60 s post-activation) and at 1.5 mM BPA (at 30 s post-activation), when the motility period is finished. In the control, flagellum of sperm is in straight position once the motility period is finished at 90 s post-activation.

Discussion

The present study showed that BPA affect on sperm motility and velocity of perch, *in vitro*. LC50 was estimated at 1.37 mM BPA (Fig. 2). BPA may damage the flagella as different form

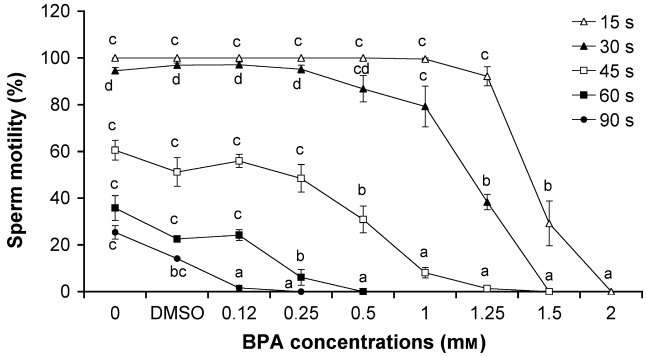

Fig. 2. *In vitro* effects of bisphenol A (BPA) on sperm motility in *Perca fluviatilis*. Sperm of five males were separately diluted in an immobilizing solution containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). Immediately after dilution, the sperm motility was activated in activation solution containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). BPA was only added into activation solution (DMSO concentration was lower than 0.1% after adding BPA in activation solution). Effect of DMSO 0.1% on sperm motility was also studied. At the same time post-activation, values (mean ± standard error of mean) with the same letters are not significantly different ($P > 0.05$)

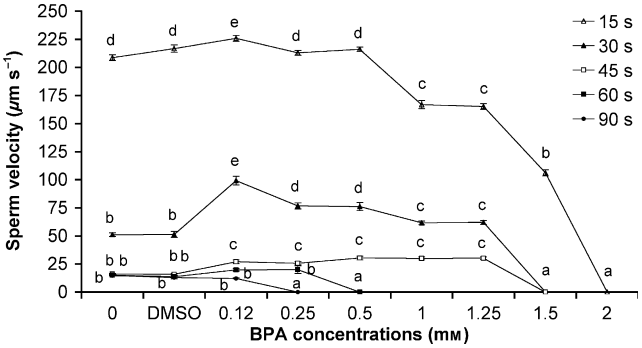

Fig. 3. *In vitro* effects of bisphenol A (BPA) on sperm velocity in *Perca fluviatilis*. Sperm of five males were separately diluted in an immobilizing solution containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). Immediately after dilution, the sperm motility was activated in activation solution containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). BPA was only added into activation solution (DMSO concentration was lower than 0.1% after adding BPA in activation solution). Effect of DMSO 0.1% on sperm motility was also studied. At the same time post-activation, values (mean ± standard error of mean) with the same letters are not significantly different ($P > 0.05$)

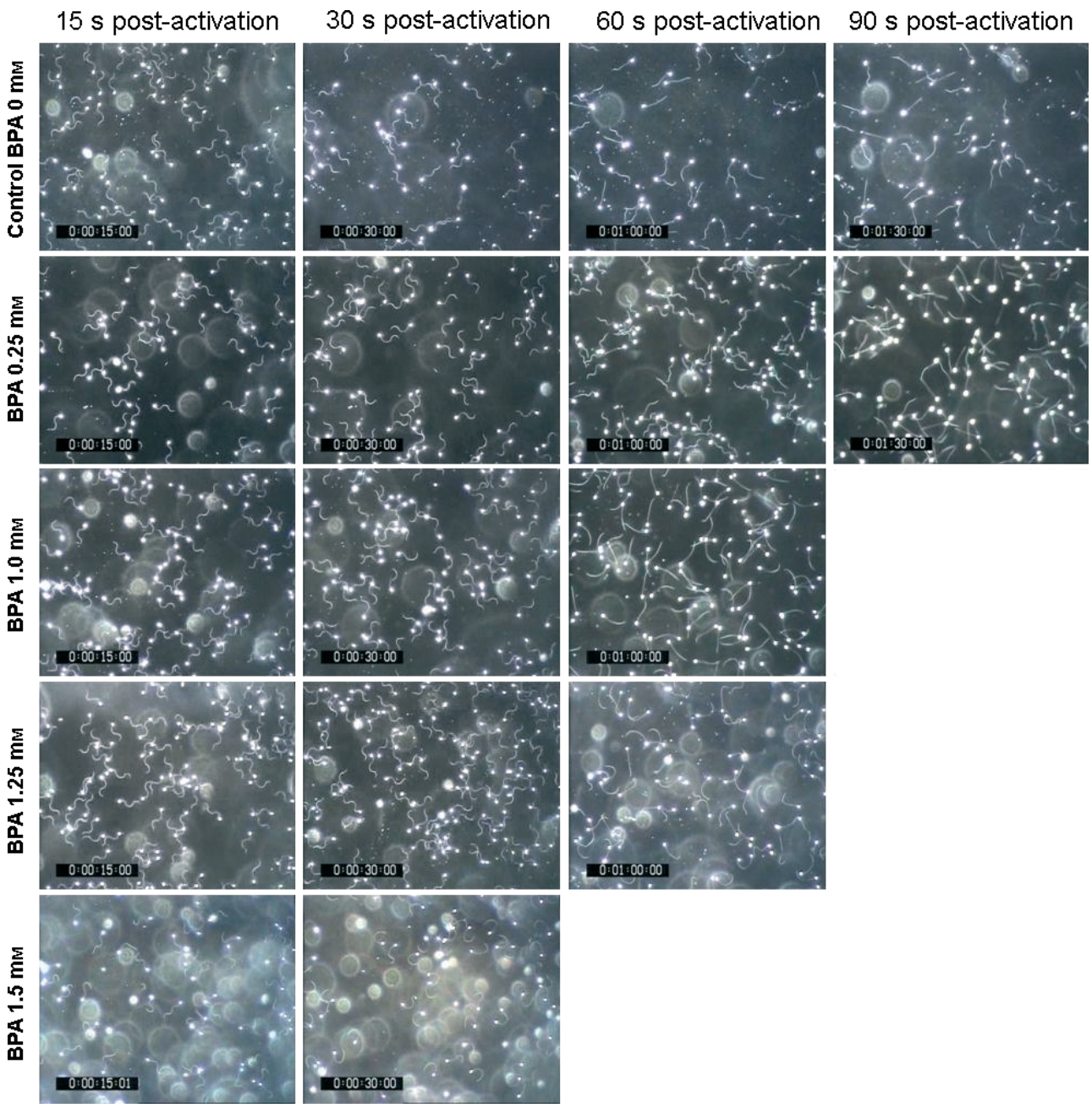

Fig. 4. *In vitro* effects of BPA on sperm flagella in *Perca fluviatilis*. Once the motility period of sperm is finished after activation, structure of flagella become 'C' shape in sperm that has been exposed to high concentration of BPA (> 1.25 mM) compared to straight structure of sperm in control. In all cased, sperm was firstly diluted in an immobilizing solution containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). The sperm motility was activated in activation solution containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). BPA was only added to activation solution

of flagella are observed once motility period of sperm cells are finished (Fig. 4). However, it is not well know if BPA damage the plasma membrane or axoneme. Plasma membrane plays an important role in the initiation of sperm motility due to hypo-osmotic signals (Alavi et al., 2007). Thomas and Doughty (2004) observed that nonestrogenic as well as estrogenic organic compounds could interfere with a rapid nongenomic progestin action to upregulate sperm motility in Atlantic croaker. Most of these chemicals (such as BPA, estradiol, *p,p′*-DDT, *o,p′*-DDE, naphthalane, zearalenone) completely blocked the hormonal response to 17,20β,21 – trihydroxy-4-pregnen-3-one (20β-S) at 0.1 μM, but none of them caused a decrease in percentage of motile sperm below control levels. Some of these chemicals can bind to the sperm membrane progestin receptor such as *o,p′*-DDE and zearalenone (Thomas et al., 1998) suggesting that their mechanisms of disruption involve binding to the 20β-S receptor. In addition, the effect of BPA on sperm energetics (ATP content and consumption during sperm activation) is another option that could be considered in further studies. ATP is the most energetic source required for axonemal beating in fish sperm (Perchec-Poupard et al., 1998; Rurangwa et al., 2002). Lahnsteiner et al. (2005b) studied the *in vivo* effects of BPA on sperm characteristics in brown trout during reproductive season. They found that sperm motility and velocity were lower in males exposed to 1.75, 2.40 and 5.0 μg L^{-1} BPA for 3 weeks at the beginning

and the middle of the reproductive season, but no effects was observed at the end of reproductive season. Our recent study on goldfish (*Carassius carassius* L.) showed decrease of sperm motility after exposing fish to BPA higher than 1 μg L^{-1} for 1 month that might be related to anti-androgenic activity of BPA (Hatef, A., Alavi, S.M.H., Abdalfatah, A., Fontaine, P., Linhart, O., unpubl. data). There are many studies showing estrogenic activity of BPA through direct ER binding or indirect actions of endogenous E2 (Crain et al., 2007). BPA enhances VTG mRNA expression and increase the VTG concentration in serum (Tabata et al., 2001; Yamaguchi et al., 2005; Huang et al., 2010). BPA can also act through sex steroid binding proteins (Tollefsen et al., 2004). This may answer study carried out by Lahnsteiner et al. (2005b). They observed no change of sperm production in *S. trutta fario* exposed to BPA (1.75–5 g L^{-1}) during the spawning period, but sperm concentration significantly decreased in the beginning of the spawning period. These findings suggest that *in vivo* or *in vitro* effects of BPA on sperm characteristics are different and might be varied at different time during the reproductive season. The different effects of BPA might be correspond to dose of exposure, duration of exposure and maturity stage in fish (see reviews by Richter et al., 2007; Gregory et al., 2008; Vandenberg et al., 2009).

In literature, we have found effects of the other EDCs on sperm motility characteristics. In sewage effluents, there are different compounds of Alkylphenols such as 4- Nonylphenol. Lahnsteiner et al. (2005a) did not observe *in vivo* effects of 4-nonylphenol on sperm motility in rainbow trout at concentration up to 750 ng L^{-1}. Recently, Hara et al. (2007) published decrease of motility and velocity of medaka sperm incubated for 60 s in 100μM L^{-1} Nonylphenol. The 2,4-Dichlorophenol (a chlorinated derivative of phenol) also significantly decreased both sperm motility and velocity in African catfish (*Clarias gariepinus*) at 1 mg L^{-1}, *in vitro* (Lahnsteiner et al., 2004). In brown trou and burbot (*Lota lota* L.), the 2,4-Dichlorophenol significantly decreased sperm motility at concentrations 0.01 and 10 mg L^{-1}, respectively. But it had no effect on sperm velocity. In contrast, 2,4-Dichlorophenol significantly increased the sperm velocity in *Luciscus cephalus*, but it did not affect percentage of motility of sperm at concentration 100 mg L^{-1}. Reduction of fertility was also reported in medaka exposed to 100 μg L^{-1} 4-nonylphenol (Kang et al., 2002).

In addition, there are some other EDCs such as heavy metals, pesticide and fungicide and paper and mill effluents, which have negative effects on sperm motility. Van Look and Kime (2003) and Kime et al. (1996) observed decrease of sperm motility and velocity in goldfish and African catfish exposed to mercury or zinc, *in vitro*. Among fungicides and pesticides, tributyltin and DDT decreased sperm motility in *Orechromis mossambicus* and *Clarias gariepinus* (Rurangwa et al., 2002; Marchand et al., 2008). McMaster et al. (1992) observed inhibition of sperm motility white sucker (*Catostomus commersoni*) in exposed to paper and mill effluents.

In conclusion, the present study showed that BPA decreases both sperm motility and velocity, *in vitro*. Its mechanisms of action on spermatozoa are not known. They may act through damage of plasma membrane, axonemal apparatus, or depletion of ATP contents in the spermatozoa. The BPA can also reduce the sperm production in fish, which might be through negative impact on production of sex steroids. Alternations of sex steroid production subsequently modify process of sperm maturation that is essential for acquisition of potentiality for sperm motility.

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