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Developmental and acute toxicity of cetylpyridinium chloride in *Bombina orientalis* (Amphibia: Anura)

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ABSTRACT

In an effort to evaluate the toxicity of cetylpyridinium chloride (CPC), a cationic surfactant in amphibians, we examined the developmental and acute toxicity of CPC in *Bombina orientalis* embryos and tadpoles. Embryonic exposure to 2.0 μM (0.72 mg/l) CPC for 7 days significantly decreased the survival rates and increased DNA damage in the intestine of developed tadpoles. Exposure to 1.5 μM (0.54 mg/l) CPC significantly decreased the growth of embryos and increased developmental abnormalities. The 168-h LC_{50} and EC_{50} values of CPC were 1.95 μM (0.697 mg/l) and 1.48 μM (0.531 mg/l) in embryos, respectively. In an extended acute toxicity test using tadpoles, the 168-h LC_{50} value of CPC was 5.07 μM (1.82 mg/l). In terms of survival and growth rates, the lowest observed effective concentration of CPC was 1.5 μM. At sub-lethal concentrations (1.0 and 2.0 μM) CPC treatment to embryos increased lipid peroxidation in the intestine and gills of developed tadpoles, indicating that CPC can impose oxidative stress. At 2.0 μM CPC, pro-apoptotic Bax and Bak mRNA levels were significantly increased together with DNA fragmentation, indicative of apoptotic cell death. CPC in freshwater system may threaten the normal development of amphibian embryos.

1. Introduction

Since the initial use of quaternary ammonium compounds (QACs) in the late 1930s, cationic surfactants have been increasingly used for various industrial, pharmaceutical, and consumer products during the past decades (Walker, 2003). Among the QACs, cetylpyridinium chloride (CPC) is used in oral hygiene products, skin lotions, and antiperspirant deodorants (Scientific Committee on Consumer Safety, 2012). In particular, CPC is a well-known antiseptic used mainly in mouthwash and toothpaste for preventing dental plaques and reducing gingivitis (reviewed in McDonnell and Russell, 1999; Pardini et al., 2005; Costa et al., 2013). Currently, CPC has also been used to remove reactive dyes, phenols, and other organic solutes from sewage or treated wastewater (Ahmad and Puasa, 2007; Luo et al., 2010; Buffet-Bataillon et al., 2012).

In general, the acute toxicity of cationic surfactants is higher than those of anionic, amphoteric, and nonionic surfactants in various prokaryotes, invertebrates, and vertebrates (Ivanković and Hrenović, 2010). Although CPC has been commonly used in ionic surfactants in a wide range of applications, the aquatic toxicity of CPC has previously been studied only in limited species such as planarians and fish. In the freshwater planarian (*Dugesia japonica*), the 96-h LC_{50} of CPC was 0.04 mg/l, which was lower than those of seven other surfactants including benzalkonium chloride (0.21 mg/l), saponin (0.44 mg/l), 4-*tert*-octylphenol (0.78 mg/l), ammonium lauryl sulfate (1.04 mg/l), sodium lauroylsarcosinate (2.99 mg/l), dioctyl sulfosuccinate (10.03 mg/l), and dodecyl trimethyl ammonium bromide (43.52 mg/l) (Li, 2012). To date, however, the acute toxicity of CPC in aquatic vertebrates has been studied in only one fish species, *Cyprinus carpio* (Reagentworld MSDS; https://www.reagentworld.com/products/msds2.asp?proid_2=20178). In the *C. carpio*, the 96-h LC_{50} of CPC was 0.01 mg/l according to MSDS.

Recently, fresh water amphibian populations have rapidly declined in Korea, mostly in the urban freshwater system (Park et al., 2014). The chemical contamination of freshwater systems is an important factor responsible for amphibian decline (Collins and Storfer, 2003). Recently, CPC was measured at 0.15 μM (0.052 mg/l) in river water in Kaohsiung, Taiwan (Shrivas and Wu, 2007). However, effects of CPC at environmental relevant concentration on development and survival of amphibian embryos were not studied. In the present study, we examined the developmental and acute toxicity of CPC in *Bombina orientalis* embryos and tadpoles using a method adopted from the Frog Embryo Teratogenesis Assay-

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Xenopus (FETAX; ASTM, 2004). This method was previously used to test the toxicity of pesticides, endocrine disruptors, and environmental samples (Kang et al., 2009, 2010; Park et al., 2010, 2014). Specifically, we examined oxidative stress in the embryonic organs. In addition, DNA fragmentation and expression of apoptotic pathway genes mRNA were examined in the intestine which was the most frequently affected by CPC exposure during early development of *B. orientalis*.

2. Materials and methods

2.1. Test organisms

Adult *B. orientalis* were bred in the Hanyang University Aquarium and maintained on a diurnal 14-h light and 10-h dark cycle at 20–22 °C. They were fed crickets three times a week. The experiments conducted on amphibian embryos followed the "Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research" (ASIH, 2004) (http://www.asih.org/files/hacc-final.pdf). Adult frogs, 3 years of age, were bred by artificial fertilization from wild parent pairs sampled from Gapyeong, Gyeonggi-do, Korea. To obtain embryos, superovulation, mating, and fertilization were performed by gonadotropin stimulation, as described previously (Kang et al., 2010). Briefly, adult males and females, bred in the laboratory, were given a single injection of 200 IU and 300 IU human chorionic gonadotropin (Sigma-Aldrich, St Louis, MO), respectively, in the dorsal lymph sac. Fertilized eggs were obtained from 5 different male/female pairs for each experiment. Unfertilized and necrotic eggs were removed, and healthy fertilized eggs with first cleavage furrows were selected 2 h post-fertilization. When the fertilized eggs developed to the blastula stage, the embryos were de-jellied with a 2% L-cysteine solution (pH 8.1) for 2 min. Normal embryos were selected according to the FETAX (ASTM, 2004).

2.2. Developmental toxicity test

CPC (CAS. 6004-24-6, Sigma-Aldrich) was dissolved in distilled water has adequate grade (maximum electrical conductivity, 20 μS/cm at 25 °C) for FETAX. The concentration of stock solution was 25 mM. In total, 168 de-jellied blastulae were subjected to the bioassay. Embryos from the same female were randomly placed in six-well dishes and exposed to various concentrations of CPC (0, 0.5, 1.0, 1.5, 2.0, and 3.0 μM; 0, 0.18, 0.36, 0.54, 0.72, and 1.07 mg/l) in 7 ml of 0.1 X modified Ringer's solution (MR; Godsave et al., 1988). Experiments were replicated four times. In each well, 7 embryos exposed to 7 ml medium. The embryos were cultured in an incubator (MIR550, Sanyo, Osaka, Japan) at 22 °C for 7 days. The test solution was replaced daily with fresh solution, and dead embryos and larvae were removed. At the end of the treatment, surviving embryos were euthanized by tricaine methanesulfonate (MS-222) and examined for malformations under a stereomicroscope. All embryos were fixed with Bouin's fixative. Staging and patterning of abnormal development were conducted as described by Nieuwkoop and Faber (1994) and Rugh (1962). Dead embryos were not counted (ASTM, 2004).

2.3. Extended acute toxicity test

For comparison of the acute toxicity and developmental toxicity of CPC, 7-day extended acute toxicity test was performed. In total, 192 tadpoles were included in the bioassay. Operculum complete stage tadpoles corresponding to Nieuwkoop and Faber (NF) stage 45 from the same female were randomly placed in 6-well dishes and exposed to various concentrations of CPC (0, 3.0, 5.0, 5.5, 6.0 and 6.5 μM; 0, 1.07, 1.79, 1.97, 2.15, and 2.33 mg/l) in 0.1 X MR. Whether hatched tadpoles reached operculum complete stage was determined through the internalization of external gills. Experiments were replicated eight times. In each well, 4 tadpoles exposed to 4 ml medium. The tadpoles were cultured in an incubator (MIR550, Sanyo) at 22 °C for 7 days. The test solution was replaced daily with fresh solution, and dead tadpoles were removed. At the end of the treatment, surviving tadpoles were euthanized and fixed with Bouin's fixative.

2.4. RT-PCR analysis

Tadpoles that were exposed to CPC from blastula to operculum complete stage for 7 days were euthanized with 0.02% buffered 3-aminobenzoic acid ethyl ester (MS-222; CAS. 886-86-2, Sigma-Aldrich) to minimize pain, and their intestine was removed. Total RNA was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. The concentrations of all RNA preparations were determined prior to storage at −85 °C until use. For cDNA synthesis, RNA (0.5 μg) was reverse transcribed for 60 min at 42 °C in a 20 μl reaction with 50 units of MuLV reverse transcriptase and 2.5 μM oligod(T)16 primer, according to the manufacturer's standard protocol (Applied Biosystems, Foster City, CA). PCR was performed with degenerate primers for anti-apoptotic Bcl-2 (DGP Bcl-2), pro-apoptotic Bax (DGP Bax) and Bak (DGP Bak) cDNA sequences, which were based on multiple vertebrate mRNA sequences (Table 1). PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI), and the resultant constructs were transformed into DH5α competent cells. More than 10 positive colonies were selected by blue/white screening. DNA sequencing was performed using M13 primers. From the sequencing of the insert, partial sequences of *B. orientalis* Bcl-2 (213 bp), Bax (201 bp), and Bak (273 bp) were defined. The deduced amino acid sequence of the corresponding *B. orientalis* Bcl-2, Bax, and Bak ORF was 92, 80, and 72% identical with the anuran *X. laevis* sequences (GenBank accession no. NP_001139565.1, NP_001079104.1, NP_001089587.1), respectively (Fig. S1 in the online version at DOI: 10.1016/j.aquatox.2016.06.022). RT-PCR was performed according to the method outlined by Gye and Kim (2005) and Park et al. (2014). Briefly, PCR reactions contained 0.5 μl intestine cDNA, 1.25 units of Ex Taq™ polymerase (Takara, Japan), 1 × Ex PCR™ Buffer II (Mg^{2+}-free), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.2 μM of each primer in a total volume of 20 μl. β-actin mRNA was amplified as a reference gene (Table 1). Real-time PCR was carried out using the same primers and iQ SYBR Green Super mix reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. PCR was performed in a MyiQ i Cycler (Bio-Rad) using the following thermo-cycling conditions: 1 cycle for 3 min at 95 °C followed by 40 cycles at 95 °C for 30 s, the appropriate annealing temperature for 30 s, and 72 °C for 30 s. Bcl-2, Bax and Bak mRNA levels are expressed as arbitrary units relative to β-actin mRNA, and the relative quantification was performed using the comparative CT method according to the manufacturer's protocols (Bio-Rad).

2.5. Apoptosis assays—DNA fragmentation

Since the abnormality was mostly found in the intestine, apoptosis assay was performed using intestinal tissues. De-jellied blastulae from the same female were randomly placed in six-well dishes and exposed to CPC (0, 1.5, and 2.0 μ*M*) in 5 ml of 0.1 X MR solution. The test solution was replaced daily with fresh solution. When the embryos have reached the operculum complete stage by day 7, DNA was extracted from the intestine of control or CPC-exposed tadpoles for apoptosis assay. Briefly, intestinal tissues of NF stage 45 tadpoles were homogenized with 0.5 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 20 mM