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Oxidative stress, genotoxicity and cytotoxicity of 1-methyl-3-octylimidazolium chloride on *Paramisgurnus dabryanus*

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ABSTRACT

This study evaluated the toxicity of 1-methyl-3-octylimidazolium chloride ([C_8mim]Cl) on *Paramisgurnus dabryanus* by enzyme analysis, comet assay, and apoptosis analysis. The study showed that [C_8mim]Cl had an obvious toxic effect inducing oxidative stress, genotoxicity, and cytotoxicity to fish liver cells. [C_8mim]Cl also induced changes in the activities of superoxide dismutase and catalase, and the glutathione content and malondialdehyde level in fish exposed at 20–80 mg L^{-1}. With increased exposure concentration and time, the four antioxidant enzyme activities, three different comet parameters and apoptosis rates of tested cells were significantly increased, with significant differences ($P < 0.05$ or $P < 0.01$) observed between control group and each treatment group. This study shows that [C_8mim]Cl could be a threat to aquatic organism health when accidentally released into aquatic ecosystems.

1. Introduction

With economic development and industrialization, most of the volatile organic compounds commonly used in industrial applications cause environmental pollution problems (Schmid et al., 1998). Thus, many researchers have focused on the development of "green engineering" which aims at finding environmental friendly alternatives to harmful chemicals. Among the reputable "green engineering" solvents, ionic liquids (ILs) have garnered increasing attention over others such as supercritical CO_2 or aqueous biphasic systems (Pham et al., 2010).

ILs are organic salts formed by cations and anions with melting points below 100 °C. The main cations of ILs are imidazolium, pyridinium, ammonium, piperidinium, and pyrrolidinium, while the anions are chloride (Cl^-), bromide (Br^-), tetrafluoroborate (BF_4^-), and hexafluorophosphate (PF_6^-) (Ranke et al., 2007). Recently, ILs have attracted considerable attention mainly because they have high thermal stability, excellent solvation ability, and negligible vapor pressure (Jungnickel et al., 2008), which reduce the risk of air pollution. ILs are claimed to be "environmental friendly" chemicals because of their negligible vapor pressure (Sheldon, 2005). At present, ILs are used as reaction media for organic synthesis, catalysis, or biocatalysis in commercial applications and by individual consumers (Jungnickel et al., 2008). Although ILs can lessen the risk of air pollution due to their insignificant vapor pressure, most are water soluble and might enter the aquatic environment by accidental leakage or with effluents (McFarlane et al., 2005). Many reports have indicated that most ILs are poorly decomposed by microorganisms and many ILs are toxic to aquatic organisms (Ventura et al., 2010; Li et al., 2012). Therefore, ILs may pose environmental risks to aquatic ecosystems.

In this study, the biochemical methods were used to evaluate oxidative stress, genotoxicity and cytotoxicity of 1-methyl-3-octylimidazolium chloride ([C_8mim]Cl) on *Paramisgurnus dabryanus* by analyzing liver antioxidant enzyme activities, comet assay and apoptosis in liver cells. *P. dabryanus* is a common loach, which is a freshwater fish commonly found in Chinese water bodies. With its short life cycle, ease of capture, and quick response to environmental disturbances, loach is commonly used in toxicity tests (Seok-Ki et al., 2010; Nan et al., 2015). The aim of this study was to evaluate the toxicological risk for fish exposed to [C_8mim]Cl by using liver antioxidant enzyme activities, comet assay, and apoptosis analysis as indicators of biological toxicity. This study contributes significantly to the knowledge of the ecological consequences of [C_8mim]Cl occurrence in the aquatic environment.

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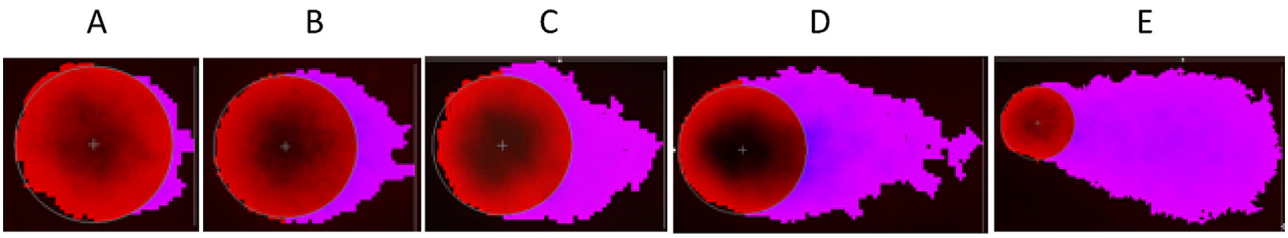

Fig. 1. The Comet scale of *P. dabryanus* liver cells induced by [C_8mim]Cl (×400), a Five-class classification based on tail DNA% (TD). Comet classes: (A) no or minimal damage (class 1, TD < 5); (B) Low damage cells (class 2, TD: 5–20); (C) Mid damage cells (class 3, TD: 20–40); (D) High damage cells (class 4, TD: 40–75); (E) Extreme damage cells (class 5, TD > 75).

2. Materials and methods

2.1. Chemicals

[C_8mim]Cl with a purity of more than 99% was purchased from the Shanghai Jiecheng Bioengineering Institute (Shanghai, China). Diagnostic reagent kits for determination of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), malondialdehyde (MDA), and total protein content were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animals and treatments

Adult *P. dabryanus* (16.4 ± 3.2 g) were collected from wetlands in the old course of the Yellow River (Yanjin, Henan, China) and handled according to the China ethics law [ethics approval no. SCXK (YU) 2005-0001]. Each group of tested fish were raised in room temperature water under laboratory conditions in a glass jar (6 L in volume), and no food was provided during the tests. Acute toxicity was assessed by exposure for 96 h to 126–360 mg L^{-1} [C_8mim]Cl with 1.3-fold serial dilutions (126, 164, 213, 278, and 360 mg L^{-1}) by using the Spearman–Kärber methods (Nan et al., 2015). There were three parallels in each tested group.

2.3. Liver antioxidant system and lipid peroxidation assays

According to the result of an acute toxicity test, 60 adult *P. dabryanus* were randomly divided into four groups (15 fish in each group), out of which three served as treatment groups that were exposed to [C_8mim]Cl solutions at concentrations of 20, 40, and 80 mg L^{-1} for 12 days, and the other was a control group. After 4, 8, and 12 days exposure to [C_8mimCl], three fish from each group were anaesthetized with 100 mg L^{-1} tricaine methanesulfonate (Guangzhou Chongjun Chemical Co., Ltd., Guangzhou, China) and dissected. Samples of fish liver were collected to determine the activities of the liver antioxidant enzymes SOD, CAT, and GSH, and of MDA using diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Results are given in enzymatic activity units per milligram protein (U mg^{-1} protein), with 1 U of SOD being defined as the amount of enzyme producing 50% inhibition of SOD, and 1 U of CAT as the amount of enzyme decomposing 1 μmol L^{-1} H_2O_2 per second. GSH was expressed in mg per gram protein, and the concentration of MDA was expressed as nmol per milligram protein. Total protein contents were measured by a Coomassie Blue diagnostic reagent kit (Nanjing Jiancheng Bioengineering Institute, China).

2.4. Comet assay

The experimental design and treatment process of the comet assay were similar to the above antioxidant enzyme activity and lipid peroxidation assays test. After 2, 4, 8, and 12 days of exposure, three fish from each group were anaesthetized with 100 mg L^{-1} MS-222 (tricaine methanesulfonate, China Langchem Inc., Ltd., Beijing, China). Then, the livers were quickly dissected from the tested fish, placed in phosphate buffer solution (pH 7.5), kept on ice and gently cut up with scissors. After hepatocytes were isolated and released as a suspension, the cell number and cell viability were measured as described by Mitchelmore and Chipman (1998) with some modifications, i.e. cell number and cell viability were measured automatically (Vi-Cell Viability Automatic Analyzer, Beckman Coulter Commercial Enterprise (China) Co., Ltd., Shanghai, China). Cells were typically more than 80% viable, with 1×10^6–1×10^8 cells isolated per liver.

Single cell gel electrophoresis was performed as described by Singh et al. (1988) with minor modifications (Nan et al., 2015). After cell lysis in 2.5 mol L^{-1} NaCl, 10 mmol L^{-1} Tris, 100 mmol L^{-1} Edetate disodium, 1% Triton X-100, 10% Dimethyl sulfoxide, pH 10.0, for 1.5 h, the microgels were placed in an alkaline electrophoresis buffer (300 mmol L^{-1} NaOH, 1 mmol L^{-1} Edetate disodium; pH 13, 4 °C) for 10 min to allow the DNA to unwind, followed by electrophoresis in the same buffer for 20 min at 25 V, 200 mA at 4 °C. Samples were stained with ethidium bromide (20 μg mL^{-1}, 10 min) and examined under a fluorescent microscope (OLYMPUS BX60, Guangzhou Special Inspect Instrument Co., Ltd., Guangzhou, China). All slides were coded and were scanned randomly. Two slides per specimen were prepared and fifty random cells per slide were analyzed and scored by using an image analysis system CytoVision NT (Beijing Biolaunching Technologies Co., Ltd., Beijing, China). Cells with damaged DNA appeared as comets (Fig. 1), whose tail length, tail DNA (%) and olive tail moment (OTM) were assessed by using the CytoVision NT automatic (Beijing Biolaunching Technologies Co., Ltd., Beijing, China) image analysis system. The cells were then categorized into five grades of damage (using tail DNA%) as adopted from Mitchelmore and Chipman (1998) with minor modifications [grade of damage: Class 1, no or minimal damage (<5%); Class 2, low damage (5%–20%); Class 3, mid damage (20%–40%); Class 4, high damage (40%–75%); Class 5, extreme damage (>75%)].

2.5. Apoptosis analysis

The experimental design and treatment process of apoptosis analysis were similar to the liver antioxidant system and lipid peroxidation assay tests above. After 4, 8, and 12 days of exposure, three fish of each group were anaesthetized with 100 mg L^{-1} MS-222 tricaine methanesulfonate. Then, the livers were dissected from the fish, and quickly made into frozen tissue sections (8 μm). Apoptosis in each frozen tissue section was detected by using a TUNEL FITC apoptosis detection kit (Vazyme Biotech Co., Ltd., Nanjing, China). All sections were coded and scanned randomly. Two