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Benzothiazole-based ionic liquids (BIL)-induced acute toxicity attributed to damage to antioxidant enzyme system in zebrafish (*Danio rerio*)

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Ionic liquids (ILs), considered to be green solvents, are widely distributed in the environment and thus exposure to these chemicals has attracted attention due to the potential adverse consequences on organisms and ecosystems. The aim of this study was to investigate the influence of benzothiazole-based IL (BIL) on zebrafish survival and determine whether oxidation was responsible for mortality. Zebrafish (*Danio rerio*) were exposed to 5 different concentrations of 4 acidic and one neutral IL solutions for 24 h and the 50% lethal concentrations (LC50) for each benzothiazole-based IL were determined and compared. Hepatopancreas samples were taken at the 6th, 12th, 18th and 24th h after exposure. Catalase (CAT) and glutathione peroxidase (GSH-Px) activity, as well as levels of reactive oxygen species (ROS) and malondialdehyde (MDA) were measured. Data demonstrated that BIL could significantly decrease hepatopancreas CAT and GSH-Px activity accompanied by an increase in the levels of reactive oxygen species ROS and MDA. It was also found that the toxicity of acidic ionic liquids was higher than that of neutral ionic liquids.

Keywords: Ionic liquids, Oxidative stress, Reactive oxygen species, Catalase, Malondialdehyde; Glutathione peroxidase

INTRODUCTION

Ionic liquids (ILs) are organic salts consisting of organic cation and organic or inorganic anion, such as Br-, BF_4^- , PF_6^-, or -$CH_3SO_3^-$ and are considered to be green solvents [1,2]. They are extensively used in catalysis, electrochemistry, extraction separation due to specific physical and chemical properties, such as low melting points (<100^{o}C), negligible vapor pressure, high thermal stability and good conductivity [3-7]. It is found that IL are more effective than traditional solvents and are considered to be relatively "green" because of their good recyclability [7-9]. In recent years, a series of benzothiazole-based ionic liquids (BIL) were developed which demonstrated potential applications in catalysis and extraction separation [10,11]. It is an unavoidable problem that traces of IL are released in the environment during large-scale use. At present most of toxicity tests concentrated on imidazole-based IL but data regarding BIL are rare.

Zebrafish are sensitive to environmental chemical-induced effects [12-16] and have characteristics such as small size, water quality tolerance and low cost and thus are widely used as a model to test the effects of ultraviolet rays, heavy metal salts, pesticides, sewage and also to assess water quality [14-18].

Recent studies have found that some ILs are potentially toxic because of the raw organic material used in synthetic processes. The chemical stability of ILs may result in environmental accumulation and lead to certain damage to organisms and ecosystems [19,20]. The effect of IL-mediated toxicity on aquatic organisms has attracted significant attention [21-23]. Thus, this study concentrated on the acute toxicity due to exposure of fish to ILs. Moreover, it was found the SOD of zebrafish decreased after exposure to ILs, which induced oxidative stress in zebrafish [24]. This study selected five BILs including 4 acidic ILs ([HB][CH_3SO_3], [HB][ρ-TSA], [HB][BF_4], [HB][Br]) and one neutral IL ([C_4B][Br]) to examine acute toxicity in aquatic environment and to recognize the influence of different cations (or anions) on the toxicity. Finally, the underlying mechanisms of the toxicity wereexplored.

The study on the acute toxicity of pollutants to zebrafish can throw light on the possible impact of the tested chemicals on the aquatic organisms and the short-term exposure effect [25,26]. Some important bio-chemical indices such as reactive oxygen species (ROS) content, antioxidant enzyme (GSH-Px and CAT) activity and malondialdehyde (MDA) content of zebrafish could provide more detailed information on the toxicity [27-30]. Therefore, in this study, zebrafish were used as indicator organisms to study the acute toxicity and the indices above were determined. Based on the results, the toxicity of different ILs on zebrafish was evaluated and compared, which could provide the basis for the study of the effect of the change of cation or anion on the ionic liquid toxicity.

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EXPERIMENTAL

Materials and methods

Chemicals and reagents

The ILs were prepared as previously described [31] and purities were more than 98% as measured by high performance liquid chromatography (HPLC) following recrystallization. Analytical grade chemicals for IL synthesis were purchased from Kelong (Chengdu, China). BCA protein assay kit, reactive oxygen species (ROS) assay kit, glutathione peroxidase (GSH-Px) assay kit, catalase (CAT) assay kit and malondialdehyde (MDA) assay kit were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The IL solutions were made fresh by dissolving the IL in distilled water and diluting to the desired concentrations. The UV–Vis spectra were recorded on a UV-2800 spectrophotometer (Hengping Scientific Instrument, China) in methanol. The fluorescence intensity was recorded on a microplate reader (Molecular Devices, USA). The concentrations of the products were determined by HPLC using an LC-20AT HPLC instrument (SHIMADZU international trade, China) with a C18 column (3.9 mm×150 mm, 5 μm) with an internal standard.

Experimental fish

Zebrafish, purchased from Chengdu Aquarium Fishery (China), displayed body length of 30 ± 5 mm and weighed 0.3 ± 0.1 g with normal appearance, without visible deformity and were considered healthy. Each batch was raised in a 20 L fish tank for 1 week and mortality rate was below 10% under the lab conditions of pH = 7.4 ± 0.2 and temperature of 28 ± 1℃. The water was maintained in aerated state and was replaced with fresh water once a day to ensure clean conditions for the fish. During this period, fish were fed every two days, on a 12 h light/dark cycle [32]. Finally, fish were fasted for 24 h before test.

Preliminary acute toxicity test

Preliminary experiments were conducted according to procedure [33] with modification [34] to acquire 50% lethal concentration (LC50) after 24 hour exposure.

Index determination

All index determinations were carried out according to standard procedures. For example, ROS was measured by the DCFH-DA incineration method [35] and protein content was determined with BSA as the standard protein [36]. CAT activity was measured by the rate of ultraviolet absorption decrease (Ultraviolet spectrophotometer instrument TU - 1810，Beijing Purkinje General Instrument，Beijing China) [37]. Glutathione peroxidise (GSH-Px) was determined according to the reported method [28]. The content of MDA in zebrafish was determined by the method of thiobarbituric acid (TBA) colorimetry [38]

RESULTS AND DISCUSSION

Effect of ILs in acute toxicity test

According to the test method of Fish Acute Toxicity for Dangerous Chemical Products (GB/T 21281-2007) and International Organization for Standardization (ISO), a substance is considered to be super virulent when the LC50 of sample solution < 50 $mg \cdot L^{-1}$ and hypotoxic when LC50 of sample solution was beyond 100 $mg \cdot L^{-1}$. Therefore. it was necessary for the LC50 test to consider zebrafish's lethal data in the range from 10 $mg \cdot L^{-1}$ to 300 $mg \cdot L^{-1}$ of 5 kinds of BILs. The acute toxicity was measured in ILs solutions of pH ≈ 7. The test results are shown in Table 1. The results showed that the acid ILs had a more significant impact on the growth behavior of zebrafish than the neutral ILs.

Table 1 LC50 of 5 types of BILs on acute toxicity of zebrafish

ILs	LC_{50}/24 h	Toxicity grade
Deionized water	2L	none
[HBth][CH_3SO_3]	20.6±1.2 $mg \cdot L^{-1}$	high
[HBth][ρ-TSA]	26.4±0.4 $mg \cdot L^{-1}$	high
[HBth][BF_4]	18.21±0.8 $mg \cdot L^{-1}$	high
[HBth][Br]	31.3±1.2 $mg \cdot L^{-1}$	medium
[C_4Bth][Br]	166.7±17.8$mg \cdot L^{-1}$	low

The results of Table 1 suggested that the toxicity of acidic BILs was higher than that of neutral BILs. BILs with the same cation [HB] all caused corrosion to zebrafish. The toxicity grade of the acidic ILs ranged from highly to medium, while that of the neutral [C_4B][Br] was of low toxicity grade. This indicated that the safety of neutral ILs [C_4B][Br] was much better than that of some reported acid ILs [39].

ROS index

ROS detection could be used in toxicology studies to evaluate the toxic effect of contaminants and to explore to a certain extent the mechanism of toxic effects on organisms. The ROS level of the five groups (four tested groups and one control group) was measured for various time periods of each IL.

All ILs increased the ROS content of liver cells in zebrafish with time. In general, the ROS content of zebrafish liver cells treated with the 5 kinds of ionic liquids was significantly higher than that of the control group.

The ROS content of zebrafish liver cells treated with acidic ionic liquids was significantly higher than that of neutral ionic liquids. The analysis results are shown in Fig.1.

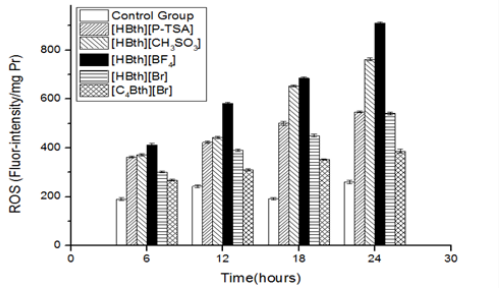

Fig. 1. Reactive oxygen species (ROS) in the hepatopancreas of zebrafish at different kinds of ionic liquids. Each bar is the mean of three replicates. The error bars represent standard deviation (SD).

The liver is an important organ that regulates the mechanism of redox reaction and is also the main detoxification organ. Some key enzymes such as Most of the antioxidant enzymes are present in the liver and these enzymes can be used to remove excess ROS. Under normal circumstances, the metabolism of living oxygen free radicals is in equilibrium, however, when the body is destroyed after contamination, ROS free radical metabolism in the liver cells may be abnormal, resulting in excessive production of ROS, which induced cell oxidative stress. In this experiment, the ROS content of all the treated groups was higher than that of the control group and the ROS content in the zebrafish treated by the acidic ionic liquid was significantly higher than that of the neutral ionic liquid. The ionic liquid induces the ROS content of zebrafish and increases its accumulation over time. With the accumulation of time, the ROS content of each concentration group exceeded the control group, indicating that time accumulation is also a reason for the increase of ROS content. The exposure time has a certain effect on the toxicity accumulation of ionic liquids.

Enzyme index

The CAT and GSH-Px levels of the five groups (four test groups and a control group) were measured at various time intervals for each IL. The analysis results are shown in Figs.2 and 3. It is seen that after exposure to the five BILs for different time intervals, the CAT and GSH-Px activities were remarkably reduced. In particular, the CAT and GSH-Px levels of the acidic ILs were lower than that of the neutral IL with time.

CAT belongs to the antioxidative system which plays the role of indirectly clearing hydroxide radicals. It follows from Fig. 2 that after exposure to the five BILs for different times, the CAT activities were remarkably reduced. The CAT activity significantly dropped after 6 h for all tested acidic ILs [HB][p-TSA], [HB][CH_3SO_3], [HB][BF_4] and [HB][Br], while it gradually decreased for the neutral IL [C4B][Br]. However, after 12-h exposure, the activity of acid ILs apparently increased. This phenomenon might occur through an internal regulation mechanism which is similar to the feedback regulation of fish. CAT activity rapidly decreased during the first 6 h. Due to the zebrafish suffering from the ILs solution, the internal system of fish would produce corresponding hormones or steroids to steadily adjust internal environment, and it was raised during the second 6 h [40,41]. When exposed longer than 12 h, the CAT activity decreased because the internal system was out of balance and could not take any available adjustment reaction. These results proved that the acidic ILs could significantly impact on the enzyme regulation mechanism of zebrafish but the monotonously decrease of CAT activity demonstrates that the neutral ILs [C_4B][Br] has a weak effect on the regulating ability.

GSH-Px belongs to antioxidant enzymes which present in the liver. GSH-Px can remove viable intracellular peroxides and play a key role in protecting cells from free radical damage. Intracellular lipids easily react with free radicals to produce lipid peroxides. GSH-Px can use GSH to reduce lipid peroxides, thereby eliminating the toxic effects of free radicals.

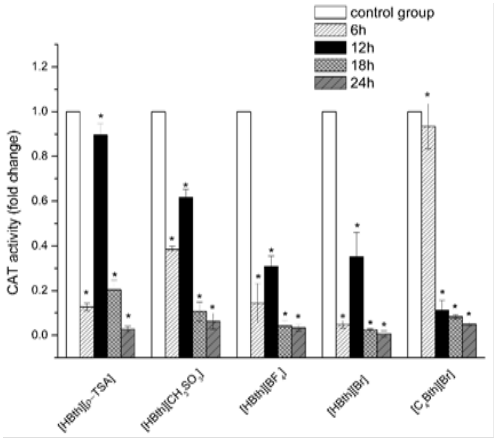

Fig. 2. Catalase (CAT) activity (multiple numerals) in hepatopancreas of zebrafish at different time intervals. Each bar is the mean of three replicates. The error bars represent standard deviation (SD). The asterisk denotes a response that is significantly different from the control (*$p<0.05$)

Fig. 3 demonstrates decrease in the antioxidant enzyme activity. In particular, the GSH-Px level of acidic ILs was lower than that of neutral ILs and with time, GSH-Px activity in the body gradually decreased. This is due to the accumulation of ionic liquids in the body to destroy the antioxidant activity of the organism.

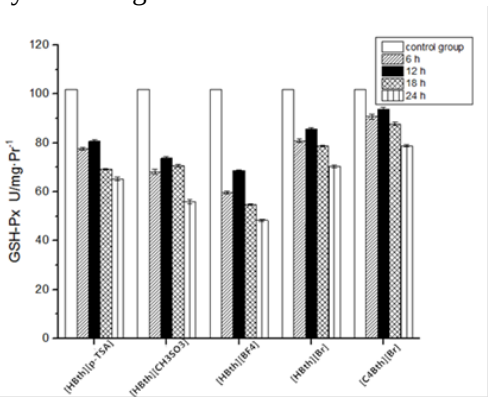

Fig.3. Glutathione peroxidase (GSH-Px) activity (multiple numerals) of zebrafish at different time intervals. Each bar is the mean of three replicates. The error bars represent standard deviation (SD).

The test on SOD [24] showed that the zebrafish took in ionic liquids into the body, and the liver metabolism had an impact on the reduction of SOD activity which on its turn reduced the ability to eliminate oxygen free radicals producing toxic effects on zebrafish.

The MDA levels of the five groups (four tested groups and one control group) were measured in various time periods for each IL. The results are shown in Fig.4 which demonstrates that the MDA contents of all test groups notably increased.

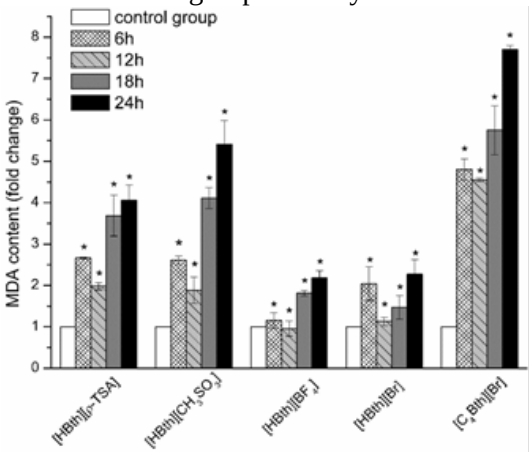

Fig.4. MDA activity (multiple numerals) of zebra fish at different time intervals. Each bar is the mean of three replicates. The error bars represent standard deviation (SD). The asterisk denotes a response that is significantly different from the control (*p<0.05)

Lipid peroxidation is a series of reactions caused by free radicals, which have an important relationship with the active oxygen content in the organism. MDA is the product of lipid peroxidation. The change in MDA content reflects the degree of lipid peroxidation and indirectly reflects the degree of oxidative damage. The MDA contents of all test groups notably increased. The acidic ILs groups decreased a little at 12 h, which could be attributed to feedback regulation. The MDA contents of all ILs tended to rise up during the whole experiment, which proved that the damage of the cells by the ILs solutions was gradually accumulated.

CONCLUSIONS

The ROS in zebrafish increased and inhibited the activity of antioxidant enzymes under the experimental conditions. It was deduced that if the experimental time was prolonged, with the reactive oxygen species accumulation and damage of the anti-oxidation system, the reactive oxygen species caused tissue damage - at high concentrations of the treatment group zebrafish sick or even death phenomenon was likely to appear. The benzothiazole-based ILs, especially those with acidic anion, exhibited toxicity to zebrafish at a certain lethal concentration. The toxicity test indicates that the acidic ILs are more corrosive than the neutral ILs to superficial and internal organs of aquatic life. The CAT, GSH-Px and SOD activity of fish exposed to acidic or neutral ILs solution decreased. The MDA level of fish increased significantly for all ILs and exhibited a higher level for the neutral ILs. These results suggest that acidic ILs have higher lethal toxicity while neutral ILs have a more serious impact on internal enzyme activity. This research could provide basic toxicological data and reference indicators of enzyme activity for benzothiazole-based ILs and be supposed to be beneficial for further study of the toxicity of ILs.

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