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RESEARCH ARTICLE

Effects of heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+} on DNA damage of loach *Misgurnus anguillicaudatus*

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Abstract The effects of heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+} at 0.05, 0.5 and 5.0 mg/L level and their interactions at 0.5 mg/L level on DNA damage in hepatopancreas of loach *Misgurnus anguillicaudatus* for 1–35 days exposure were examined by single cell gel electrophoresis (SCGE). For each test group, 20 loaches with similar body size (5.17–7.99 g; 11.79–13.21 cm) were selected and kept in aquaria with dechlorinated water at (22 ± 1)°C and fed a commercial diet every 48 h. According to the percentage of damaged DNA with tail and its TL/D (tail length to diameter of nucleus) value, the relationship between DNA damage degree and heavy metal dose and exposure time was determined. Results showed that the percentage of damaged DNA and the TL/D value were increased with the prolonged exposure time. The highest percentage (84.85%) of damaged DNA was shown in 5.0 mg/L Zn^{2+} group after 28 days exposure and the biggest TL/D value (2.50) in all treated groups after 35 days exposure. During the first treated week, the damnification of DNA was mainly recognized as the first level, after that time, the third damaged level was mostly observed and the percentage of damaged DNA was beyond 80%. The joint toxic effects among Cd^{2+}, Pb^{2+} or Zn^{2+} revealed much complexity, but it generally displayed that the presence of Cd^{2+} could enhance the genotoxicity of Pb^{2+} or Zn^{2+}. In conclusion, the results suggested that there was a significant time- and dose-depended relationship between the heavy metal and DNA damage in hepatopancreas of loach, and SCGE could represent a useful means to evaluate the genotoxicity of environmental contamination on aquatic organisms.

Keywords *Misgurnus anguillicaudatus*, single cell gel electrophoresis (SCGE), heavy metals, DNA damage

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1 Introduction

In recent years, heavy metals made up 12.96% of total chemical pollutants in Lanzhou section of the Yellow River (Ma, 2002). The enrichment coefficient of heavy metal in the food chain reached as high as several ten thousand times. Once people consumed these aquatic organisms (such as fish, shrimp and shellfish) which contain large amounts of toxic heavy metals, these heavy metals would accumulate in the corresponding function organs of human body. It could cause chronic poisoning to people or even affect the normal development of human body in a serious situation. Therefore, as principal pollutants, heavy metals were attended widely by many people (Zhou et al., 2001; Sun et al., 2003; Yang et al., 2005) because of its poisoning effects on fish. At present, the content of heavy metal was considered as an important indicator of water pollution all over the world, and was seen as an important part of monitoring. Fishes were sensitive organisms in biological monitoring. Loach was a kind of freshwater fish with strong vitality, and was widespread and easy to get, all of which made it an ideal material in toxicity experiments. Large amounts of researches indicated the toxic effects on aquatic organisms when the concentrations of heavy metals critically exceeded the permitted standards in the water body. However, the previous researches mainly focused on the evaluation of acute toxicity or single metal ion toxicity. In fact, animals and plants are inevitably exposed to the mixed chemical pollutants in the nature, which was mainly the chronic or subacute toxicity of the mixed heavy metal pollution. Jia (2001) reported the effects of four kinds of heavy metals on respiratory intensity of juvenile loach *Misgurnus anguillicaudatus*, acute and subacute toxicity of cadmium on juvenile loach. Xie et al. (2003) reported the genotoxicity of four herbicides on erythrocytes of *Misgurnus anguillicaudatus*. However, the related researches on interactions of heavy metal ions and the DNA damage of

loach have not been given much attention up to now. Although single cell gel electrophoresis (SCGE) technology has been widely used for the evaluation of pollutants-induced DNA damage (Pandrangi et al., 1995; Zhao and Zhang, 1999; Gabbianelli et al., 2003; Hu et al., 2005a; Hu et al., 2005b; Li et al., 2005), there were few reports about DNA damage effects of heavy metals on fish except a few researches on the effects of heavy metals on DNA damage of lymphocytes and kidney cells in *Carassius auratus* (Zhao and Zhang, 1999; Hu et al., 2005a; Hu et al., 2005b). In this study, the DNA damage effects of single or mixed heavy metals under different concentrations and different exposure days on hepatopancreas of loach *Misgurnus anguillicaudatus* were determined by SCGE to assess the genotoxicity of heavy metals and to provide a reference for heavy metal ions discharge standards.

2 Materials and methods

2.1 Materials

Healthy loaches (*Misgurnus anguillicaudatus*) with similar body size (body weight: 5.17–7.99 g; body length: 11.79–13.21 cm) were purchased from the aquatic product market (Yellow River cultivated). They were taken into the laboratory and maintained in the dechlorinated water according to drinking water standards for 15 days, fed with market fish food every 24 h before exposure. Loaches of quick reaction were chosen as experimental materials and fed market fish food every 48 h in the same water during the experimental period. They were not fed 24 h before the samples were collected. The temperature was controlled at (22 ± 1)°C during both keeping and experimental period.

2.2 Chemicals

$CdCl_2 \cdot 2.5H_2O$ was purchased from Shanghai Tingxin chemical plant; $Pb(NO_3)_2$ was the product of Shanghai Jinshan chemical plant; $ZnSO_4 \cdot 7H_2O$ was produced by Xi'an chemical reagent plant. All reagents were analytical grade.

2.3 Experimental design

According to heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+} concentrations (0.1, 1.0, 5.0 mg/L, respectively) of national standards for wastewater discharge in China, three different concentrations of 0.05 mg/L Cd^{2+}, 0.5 mg/L Pb^{2+} and 0.5 mg/L Zn^{2+} were designed and they were lower than those of national standards for wastewater discharge in this experiment; 0.0 mg/L was the concentration of the control group (ck). Twenty loaches were put in each tank with 20 L water, and with four parallels, a total of 80 fish in each group (Table 1); all groups were maintained in natural light and background so that differences between groups were avoided and there was no dead fish during the experiment.

Table 1 Design of heavy metal concentrations in the experiment (mg/L)

Heavy metal ions \ Concentration(mg/L)	0.0 N = 80	0.05 N = 80	0.5 N = 560	5.0 N = 240
Cd^{2+}		+	+	+
Pb^{2+}		−	+	+
Zn^{2+}		−	+	+
$Cd^{2+} + Pb^{2+}$	+	−	+	−
$Cd^{2+} + Zn^{2+}$		−	+	−
$Pb^{2+} + Zn^{2+}$		−	+	−
$Cd^{2+} + Pb^{2+} + Zn^{2+}$		−	+	−

2.4 Single cell gel electrophoresis (SCGE) (Yang et al, 2002; Feng et al, 2004)

The hepatopancreas samples of each group were collected on the first, seventh, 14th, 21st, 28th and 35th days respectively. In addition, three fishes from the same concentration of each group were mixed for one sample, the total samples were 12 groups × 6 samples / group × 4 parallels = 288 samples. The degree of DNA damage was detected by single cell gel electrophoresis.

The percentage of DNA with tail in 100 nuclei per sample randomly assessed, about 400 nuclei were covered in each concentration group. The head diameters and comet tail lengths of 20% damaged DNA were measured using the fluorescence microscope ($n = 8$–64).

The degrees of DNA damage were classified based on the value of tail length and head diameter (TL/D), the value smaller than 0.3 was classified into the first damaged level or the minor damage, the value between 0.3 and 0.6 was sorted into the second damaged level or the intermediate damage, the value above 0.6 was categorized into the third damaged level or severe damage.

2.5 Data analysis

The significant differences among the groups were performed by *t*-test, moreover, the influence of treating time and ion concentrations on the measured index was analyzed by analysis of variance (ANOVA). $p < 0.05$ was accepted as statistically significant and $p < 0.01$ as greatly significant.

3 Results

3.1 Statistical analysis of DNA damage

The degrees of DNA damage in hepatopancreas of loach *Misgurnus anguillicaudatus* were statistically significant ($p < 0.05$) after the loaches were treated by different concentrations (0.05, 0.5 and 5.0 mg/L) of single heavy metal ion (Cd^{2+}, Pb^{2+} and Zn^{2+}) and the same concentration (0.5 mg/L) of mixed heavy metal ions ($Cd^{2+} + Pb^{2+}$, $Cd^{2+} + Zn^{2+}$, $Pb^{2+} + Zn^{2+}$, $Cd^{2+} + Pb^{2+} + Zn^{2+}$) for a short period (1–35 days). The specific analysis was as follows.

3.1.1 Statistical analysis of DNA damages of single ion in different concentrations

The results showed that the percentage of DNA with tail was increased continuously with the prolonged exposure time while in 0.05 mg/L Cd^{2+}, 0.5 mg/L Cd^{2+} and 5.0 mg/L Zn^{2+} treated groups it reached their maximum values after a treatment of 28 days. Among the single heavy metal groups, the 5.0 mg/L Zn^{2+} treated group had the highest percentage (84.85%) of the damaged DNA after exposure of 28 days, followed by 5.0 mg/L Cd^{2+} and 5.0 mg/L Pb^{2+} treated groups (Table 2).

The damnification of DNA was mainly recognized as the first level during the first treated week with the prolonged exposure time, after that time, the third damaged level was mostly observed and the percentage of the damaged DNA was beyond 80%. Except for 5.0 mg/L Zn^{2+} treated group, the DNA damage degrees in all the other groups showed a trend of going up firstly and then decreasing. Furthermore, DNA damage in high concentration groups was significantly higher than that in low concentration groups for the same heavy metal ion before 14 days ($p < 0.05$), but insignificantly after 21 days ($p > 0.05$) (Table 3).

3.1.2 DNA damage of mixed ions in the concentration of 0.5 mg/L

The mixed heavy metal groups (0.5 mg/L) were compared and the results are as follows. The percentage of DNA with tail was showed changing growth tendency with the prolonged exposure time, and switched into moderate growth after 14 days' treatment (Fig. 1). The TL/D values were fluctuant: increased significant during the first seven days, and then rose mildly during 7–14 days, while dramatically dropped during 14–21 days and followed by steady decrease after 21 days treatment (Fig. 2). In general, $Cd^{2+} + Pb^{2+} + Zn^{2+}$ treated group presented the severest damage. Other mixed-ion treated groups showed various degrees of DNA damages with the prolonged exposure time. It is concluded that the joint toxic effects among Cd^{2+}, Pb^{2+} and Zn^{2+} were definitely complex.

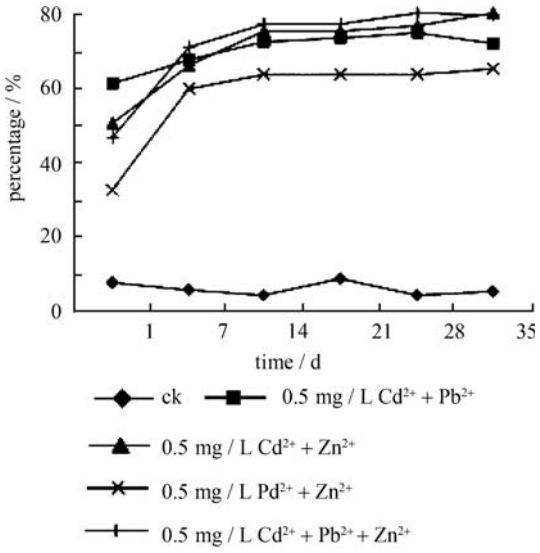

Fig. 1 Percentage of damaged DNA with tail treated by mixed heavy metals at the 0.5 mg/L

Table 2 Percentage of the damaged DNA with tail treated by single heavy metal ion

Conce-ntration (mg/L) \ Days		One day	Seven days	14 days	21 days	28 days	35 days
ck		8.00 ± 0.63	6.00 ± 0.86	4.30 ± 0.72	9.00 ± 1.24	4.17 ± 0.51	5.50 ± 0.62
Cd^{2+}	0.05	28.33 ± 2.62	45.95 ± 1.23	52.00 ± 1.28	56.00 ± 1.25	57.89 ± 2.21	53.33 ± 1.24
	0.5	30.16 ± 1.44	54.17 ± 2.66	56.00 ± 2.24	60.00 ± 1.42	62.00 ± 1.06	60.71 ± 1.11
	5.0	32.00 ± 2.91	66.67 ± 3.19	67.92 ± 2.06	74.00 ± 2.35	77.78 ± 1.19	78.13 ± 2.07
Pb^{2+}	0.5	35.45 ± 1.48	52.94 ± 1.72	58.00 ± 3.32	62.00 ± 1.31	64.86 ± 2.26	66.67 ± 3.52
	5.0	38.00 ± 2.25	57.14 ± 2.24	61.14 ± 1.46	66.00 ± 2.29	69.23 ± 2.47	71.05 ± 2.23
Zn^{2+}	0.5	35.71 ± 2.71	60.00 ± 3.26	66.00 ± 2.92	72.00 ± 1.52	72.00 ± 1.21	73.81 ± 2.94
	5.0	40.91 ± 2.59	76.19 ± 2.52	80.00 ± 3.34	82.00 ± 2.47	84.85 ± 2.27	81.25 ± 1.90

Table 3 TL/D value of damaged DNA treated by single heavy metal ion

Conce-ntration (mg/L) \ Days		One day	Seven days	14 days	21 days	28 days	35 days
ck		0.10 ± 0.06	0.36 ± 0.12	0.42 ± 0.14	0.36 ± 0.10	0.36 ± 0.09	0.20 ± 0.09
Cd^{2+}	0.05	0.11 ± 0.07	1.31 ± 0.74	1.37 ± 0.68	1.49 ± 0.71	1.79 ± 0.57	1.25 ± 0.49
	0.5	0.13 ± 0.08	1.73 ± 0.89	1.79 ± 0.74	1.85 ± 0.99	1.78 ± 0.67	1.55 ± 0.64
	5.0	0.18 ± 0.04	1.90 ± 0.76	2.02 ± 0.66	1.96 ± 0.98	1.90 ± 0.79	1.90 ± 0.82
Pb^{2+}	0.5	0.21 ± 0.08	1.96 ± 0.43	2.08 ± 0.85	1.55 ± 0.72	1.43 ± 0.87	1.07 ± 0.65
	5.0	0.23 ± 0.10	2.14 ± 0.88	2.26 ± 0.66	1.67 ± 0.58	1.37 ± 0.74	1.43 ± 0.72
Zn^{2+}	0.5	0.14 ± 0.04	1.49 ± 0.55	1.55 ± 0.72	1.79 ± 0.78	1.85 ± 0.82	1.73 ± 0.84
	5.0	0.33 ± 0.12	1.79 ± 0.64	1.90 ± 0.49	2.14 ± 0.85	2.08 ± 0.88	2.50 ± 0.96

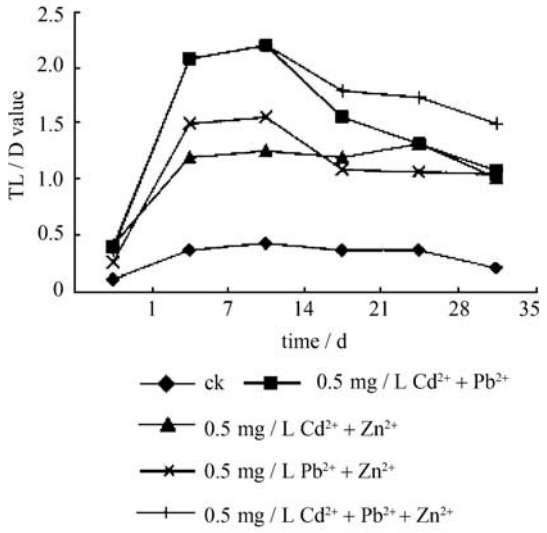

Fig. 2 TL/D vaule of damaged DNA treated by mixed heavy metals at the 0.5 mg/L

3.2 DNA damage degrees

The SCGE images of loach hepatopancreas cells showed (Fig. 3) that the normal nucleus was a round fluorescence core (Fig. 3(a)). The different degrees of DNA damage were caused in all the treated groups (Fig. 3(b)–(d)). The damaged nucleus had a comet tail which was tightly linked to the fluorescence forehead.

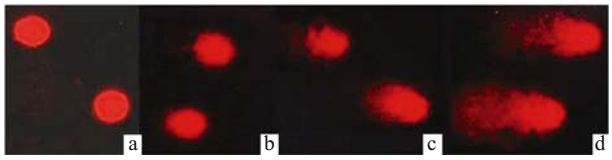

Fig. 3 SCGE images of loach hepatopancreas cells
(a) 21 days, control, no damage; (b) 21 days, 0.05 mg/L Cd^{2+}, the first damage level; (c) 35 days, 0.5 mg/L $Cd^{2+} + Zn^{2+}$ group, the second damage level; (d) 28 days, 5.0 mg/L Zn^{2+} group, the third damage level.

4 Discussion

The mixture of heavy metal ions in water could impose toxic effects on fish to different extent, such as affecting breath, immunity, enzyme activity, embryonic development and causing DNA damage and so on, by the process of physico-chemistry, physiology and detoxification (Sun et al., 2003). Moreover, these effects were closely related to the properties and the concentrations of heavy metal ions (Zhou et al., 2001). However, the reports on the effects of heavy metal mixture on the genotoxicity of fish were rare. The present paper studied the effects of essential element Zn and non-essential element Cd and Pb on DNA damages in loach hepatopancreas cells by SCGE. The results showed that the DNA damage effects exhibited certain regularity with the treatment of single heavy metal ion in different concentrations and mixed heavy metal ions ($Cd^{2+} + Pb^{2+}$, $Cd^{2+} + Zn^{2+}$, $Pb^{2+} + Zn^{2+}$ and $Cd^{2+} + Pb^{2+} + Zn^{2+}$) at the same concentration (0.5 mg/L). The loach hepatopancreas DNA was significantly damaged in every treated group, and the percentage of DNA with comet tail and the values of TL/D showed an ascending tendency with the prolonged exposure time. These results were consistent with the study on the DNA damages in *Carassius auratus* lymphocytes treated by mixed heavy metals (Hu et al., 2005a), and both researches displayed significant dose-effect relationship. The joint toxic effects of Cd^{2+}, Pb^{2+} and Zn^{2+} were rather complex in the concentration of 0.5 mg/L. The existence of Zn^{2+} could weaken the genotoxic effect of Pb^{2+}, while the presence of Cd^{2+} could strengthen the genotoxic effects of Zn^{2+} and Pb^{2+} (Fig. 2). Additionally, Zhou et al. (2001) reported that the existence of Cu^{2+} could attenuate the toxic effects of Zn^{2+}, Pb^{2+} and Cd^{2+} on cerebrum, gill and hepatopancreas of *C. auratus*, thus the interactions among heavy metal ions were quite perplexing. It was clearly undesirable to take the genotoxic effect of single heavy metal species as the toxicity of water pollution, because a variety of toxic heavy metals coexist in the aquatic environment and their combined toxic effects would be much more intricate. Therefore, not only the detection of single toxin content should be applied to the pollution evaluation of natural water, but also the biotoxicity test of the associated toxicity should be accepted to make objective and systemic assessment.

The main mechanism of DNA damage caused by heavy metal ions, which contributed to genotoxicity, is the induction of a large number of free radicals. These free radicals attack DNA double chains and make them broken. If the broken DNA strands can not be repaired timely, it will affect the function of DNA and result in genotoxicity. However, the acting mechanisms of essential elements and non-essential elements are slightly different in organisms. Zn, an essential element, is involved in a variety of enzyme-catalyzed biochemical reactions. However, once its concentration exceeds the necessary limit *in vivo*, it will induce the mass production of free radicals, which further stimulate the cell membrane lipid peroxidation. However, for Cd^{2+} and Pb^{2+}, the non-essential elements, they can induce the production of free radicals in very low amounts and affect the activities of endonuclease and polymerase, even result in DNA mutations in higher concentrations (Sun et al., 2003). In this study, there were no remarkable differences in the ability of single heavy metal ion inducing loach hepatopancreas DNA damage in the concentration of 0.5 mg/L with the extended time ($p > 0.05$), but in the 5.0 mg/L Zn^{2+} group, it showed the most noticeable inducing ability, which might be attributed to the different detoxification abilities of loach hepatopancreas to different heavy metals.

In the present paper, the effects of 0.05 mg/L Cd^{2+}, 0.5 mg/L Pb^{2+} and 0.5 mg/L Zn^{2+} were investigated. The designed concentrations were lower than those of the national standards for wastewater discharge. The results indicated that

DNA damages of loach hepatopancreas cells could be caused by Cd^{2+}, Pb^{2+} and Zn^{2+} solely both in low concentration and in short time period, whereas the interactions of mixed heavy metal ions were much more evident. Animals would be in an abnormal or a pathological state when the toxicities of pollutants exceeded zoic metabolic transformation. Therefore, both single heavy metal ion and integrated environmental factors should be taken into consideration when revise the water quality standards for fishing, so that we could establish more scientific and reasonable water quality standards for wastewater discharge.

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