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Environmental Contamination and Toxicology

Cadmium, Chromium, and Copper Induce Polychromatocyte Micronuclei in Carp (*Cyprinus carpio* L.)

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The dramatic bioaccumulation index of metal residues in a fish body exceeds that of all other trace substance (Weis et al. 1995; Tüzen et al. 2003). The high concentration of metal residues in a fish body interferes with the normal metabolism and causes catastrophic effects on the fishery resource (De Conto Cinier et al. 1998). For this reason, a number of studies have recently been conducted to examine the toxic mechanism of metal residues and search for the factors affecting the process of metal residues accumulation, release and distribution in fish organs (McGeer et al. 2000; Smet et al. 2001). Most of the studies, however, focused on effects of an individual metal residue (Riche et al.1995). Relatively a little information was available about synergistic toxic effects induced by combinatorial treatment of several types of metal residues.

Polychromatocyte micronucleus assay is a rapid method for monitoring the cellular genetic effect of contamination (Matter et al. 1970). Hooftman firstly applied the fish micronucleus assay to monitor fishery environment (Hooftman et al. 1982). The micronucleus assay has also been applied routinely to assess water quality and detect fish chromosome damage and DNA duplication confusion induced by pollutant (Metcalfe 1988; Hughes et al.1991; Brunetti et al.1998). In this paper, we analyzed the effects of Cd, Cr and Cu, either separately or combined on carp (*Cyprinus carpio* Linn.) with the micronucleus assay. Carp was chosen both because it is commonly cultured in China and for its ecological peculiarities. Our work objective was two-fold: 1) to assess the micronucleus test value by comparing its induction to fish as an index of environmental status of a freshwater ecosystem; 2) to express the synergistic toxic effects induced by combinatorial treatment of several types of metal residues in water by the value of the micronucleus test in fishery environment protection.

MATERIALS AND METHODS

Common carp (*Cyprinus carpio* Linn., average body weight 80±5g and body length 12±0.1cm) were obtained from a Freshwater Fisheries Research Center and divided into 13 groups. One group had 100 carp and 50 carp were maintained in a 2,000L aquatic tank (2g fish/L), filled with tap water (pH7.14, hardness8.64°dGH,

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dissolved oxygen 5.10-6.04 mgL^{-1}, no Cd, Cr and Cu content). After raised three wk, carp were treated individually and combined by different concentrations of $CdCl_2$, $K_2Cr_2O_7$ and $CuSO_4$ (the analytical grade reagents, Sigma). Water in the tanks was continuously aerated and saturated with oxygen and refreshed half every 24hr, and Cd, Cr and Cu concentrations of water were monitored and regulated daily. Carp were fed three times 1 d at a ration of 3.5% total biomass, kept at 25℃ during experiment.

Four carp were randomly sampled from each group at the exposure 2^{nd}, 4^{th}, 6^{th}, 9^{th}, 12^{th}, 17^{th}, 22^{nd}, 32^{nd} and 42^{nd} d. Blood samples were taken from the caudal vein and two slides per carp were prepared. Slides were fixed for 15 min in methyl alcohol before staining with Gimsa (from Sigma, 6:1 diluted by pH 6.4 PBS) 30min,washed several times with Millipore H_2O, imaged with a Nikon microphot-FXA303 under oil immersion lens (1000×maginification) and counted at permillage (polychromatocytes to 5,000 blood cells, see Figure1). Micronuclei frequencies were present as $\times 10^{-3}$(Hooftman et al. 1982).

Cd, Cr and Cu contents in water were monitored by a Varian AA220 atomic absorption spectrometry (with a Varian GTA-110 graphite furnace) through pre-treatment by HNO_3 (25% v/v), sample injection volume 20ul, during analyses internal argon gas flow rate through the graphite tube was 250ml/min, working condition as follow: wavelength 228.8nm Cd, 357.9nm Cr, 324.8nm Cu; slit width 0.7nm Cd, 0.7nm Cr, 0.5nm Cu; lamp current 4mA Cd, 7mA Cr, 4mA Cu. We calibrated the accuracy and precision by metal standard materials (from Varian Co.), and the recovery rates were 101.5% Cd, 99.6% Cr and 100.4% Cu. Dorsal muscle and kidney tissue samples (McGeer et al. 2000) were weighed and digested in a HNO_3-$HClO_4$-H_2SO_4 (1:8:1) at the exposure 42^{nd} d. Cd, Cr and Cu contents of digested tissues were also measured by the AAS and procedures followed as Tüzen's (Tüzen 2003). Metal residues in tissues and water were presented as mg metal kg^{-1} wet weight and mg metal L^{-1} water, respectively.

All values were given as means±SD. Mean values and standard deviations of the samples in this study were within 10% ranges. Statistical analyses were preformed with Matlab 6.0 program, using one way analysis of variance (ANOVA), and data were plotted on graphs by Origin 6.1 version. Significance levels of tests were indicated by English letters according to the following probability ranges: $^{a}p>0.05$, $0.05>^{b}p>0.025$, $0.025>^{c}p>0.01$, $0.01>^{d}p>0.001$ and $^{h}p<0.001$.

RESULTS AND DISCUSSION

The relationship between metal concentrations and micronuclei frequencies: carp were treated with different concentrations of Cd, Cr and Cu individually or combined, and the data of micronuclei frequencies were averaged from 4 carp (2 slides per carp), presented as means±SD ($\times 10^{-3}$) and plotted in Table1. For Cu-exposed carp, micronuclei frequencies didn't show higher than those of control carp ($p>0.05$). With respect to Cd-exposed carp, micronuclei frequencies were not also significant difference between treatment by $0.001 mgL^{-1}$ Cd^{2+} and

Table1. Carp (*Cyprinus carpio* Linn.) polychromatocyte micronucleus frequencies induced individually and combined by different concentrations of Cd, Cr, Cu ($\times 10^{-3}$) (means±SD).

Element	Concentration (mgL^{-1})	Carp micronuclei frequencies data during exposure time ($\times 10^{-3}$) (n=8,means ± SD)								
		2	4	6	9	12	17	22	32	42
Cd^{2+}	0.001	0.27	0.24	0.23	0.28	0.29	0.27	0.28	0.22	0.24
		$\pm 0.024^{a}$	$\pm 0.082^{a}$	$\pm 0.016^{a}$	$\pm 0.086^{a}$	$\pm 0.024^{a}$	$\pm 0.021^{a}$	$\pm 0.017^{a}$	$\pm 0.062^{a}$	$\pm 0.073^{a}$
Cd^{2+}	0.01	1.02	2.12	2.73	3.33	3.64	4.07	4.34	4.74	4.62
		$\pm 0.15^{b}$	$\pm 0.25^{c}$	$\pm 0.37^{c}$	$\pm 0.11^{c}$	$\pm 0.22^{c}$	$\pm 0.52^{d}$	$\pm 0.73^{d}$	$\pm 0.43^{d}$	$\pm 0.21^{d}$
Cd^{2+}	0.1	4.29	6.72	5.58	5.29	5.15	5.28	4.82	4.78	4.83
		$\pm 0.65^{d}$	$\pm 0.78^{h}$	$\pm 0.23^{d}$	$\pm 0.45^{d}$	$\pm 0.73^{d}$	$\pm 0.24^{d}$	$\pm 0.15^{d}$	$\pm 0.11^{d}$	$\pm 0.32^{d}$
Cr^{6+}	0.001	0.83	0.98	1.32	1.39	1.43	1.74	1.80	1.92	1.97
		$\pm 0.17^{b}$	$\pm 0.36^{b}$	$\pm 0.39^{b}$	$\pm 0.37^{b}$	$\pm 0.32^{b}$	$\pm 0.62^{b}$	$\pm 0.20^{b}$	$\pm 0.31^{b}$	$\pm 0.27^{b}$
Cr^{6+}	0.01	3.18	3.27	3.30	5.46	5.23	5.13	5.24	4.80	4.78
		$\pm 0.35^{c}$	$\pm 0.17^{c}$	$\pm 0.33^{c}$	$\pm 0.29^{d}$	$\pm 0.21^{d}$	$\pm 0.17^{d}$	$\pm 0.19^{d}$	$\pm 0.11^{d}$	$\pm 0.21^{d}$
Cr^{6+}	0.1	6.02	6.02	5.95	5.87	6.04	5.90	5.95	6.02	6.03
		$\pm 0.86^{h}$	$\pm 0.92^{h}$	$\pm 0.37^{h}$	$\pm 0.39^{h}$	$\pm 0.41^{h}$	$\pm 0.35^{h}$	$\pm 0.43^{h}$	$\pm 0.87^{h}$	$\pm 0.31^{h}$
Cu^{2+}	0.01	0.31	0.29	0.31	0.28	0.27	0.22	0.27	0.24	0.29
		$\pm 0.087^{a}$	$\pm 0.037^{a}$	$\pm 0.021^{a}$	$\pm 0.020^{a}$	$\pm 0.089^{a}$	$\pm 0.062^{a}$	$\pm 0.067^{a}$	$\pm 0.073^{a}$	$\pm 0.043^{a}$

Cu^{2+}	0.1	0.29	0.31	0.30	0.24	0.22	0.25	0.26	0.30	0.25
		$\pm0.013^{a}$	$\pm0.042^{a}$	$\pm0.027^{a}$	$\pm0.031^{a}$	$\pm0.017^{a}$	$\pm0.019^{a}$	$\pm0.092^{a}$	$\pm0.084^{a}$	$\pm0.012^{a}$
Cu^{2+}	1.0	0.28	0.30	0.29	0.31	0.31	0.25	0.29	0.29	0.30
		$\pm0.083^{a}$	$\pm0.017^{a}$	$\pm0.029^{a}$	$\pm0.014^{a}$	$\pm0.039^{a}$	$\pm0.017^{a}$	$\pm0.083^{a}$	$\pm0.091^{a}$	$\pm0.024^{a}$
Cd^{2+}+ Cr^{6+}	0.001+0.001	4.12	6.72	6.05	6.19	5.89	6.21	6.17	6.02	6.12
		$\pm0.20^{d}$	$\pm0.21^{h}$	$\pm0.12^{h}$	$\pm0.73^{h}$	$\pm0.97^{h}$	$\pm0.74^{h}$	$\pm0.89^{h}$	$\pm0.21^{h}$	$\pm0.14^{h}$
Cd^{2+}+ Cu^{2+}	0.001+0.01	0.37	0.65	0.93	1.25	1.74	2.16	2.26	2.34	2.27
		$\pm0.020^{a}$	$\pm0.012^{b}$	$\pm0.016^{b}$	$\pm0.15^{c}$	$\pm0.13^{c}$	$\pm0.72^{c}$	$\pm0.87^{c}$	$\pm0.75^{c}$	$\pm0.19^{c}$
Cd^{2+}+Cr^{6+}	0.001+0.001	7.67	7.75	7.43	7.86	7.52	7.68	7.54	7.71	7.56
+Cu^{2+}	+0.01	$\pm0.25^{h}$	$\pm0.16^{h}$	$\pm0.11^{h}$	$\pm0.43^{h}$	$\pm0.97^{h}$	$\pm0.35^{h}$	$\pm0.95^{h}$	$\pm0.87^{h}$	$\pm0.17^{h}$
Control						0.20±0.017				

The micronuclei frequencies data were averaged from 4 carp (2 slides per carp) treated separately and combined by different Cd, Cr and Cu concentrations and statistics analysis *t*-test showed non-significantly different ($^{a}p>0.05$), significantly different ($^{b}p<0.05$, $^{c}p<0.025$, $^{d}p<0.01$ and $^{h}p<0.001$, respectively) from control during exposure time. From the synergistic effects, carp exposed to $0.001mgL^{-1}Cd^{2+}$+$0.01mgL^{-1}Cu^{2+}$, $0.001mgL^{-1}Cd^{2+}$+$0.001mgL^{-1}Cr^{6+}$ and $0.001mgL^{-1}Cd^{2+}$+ $0.001mgL^{-1}Cr^{6+}$+$0.01mgL^{-1}Cu^{2+}$, and micronuclei frequencies were higher than those of control ($^{c}p<0.025$, $^{h}p<0.001$ and $^{h}p<0.001$, respectively).

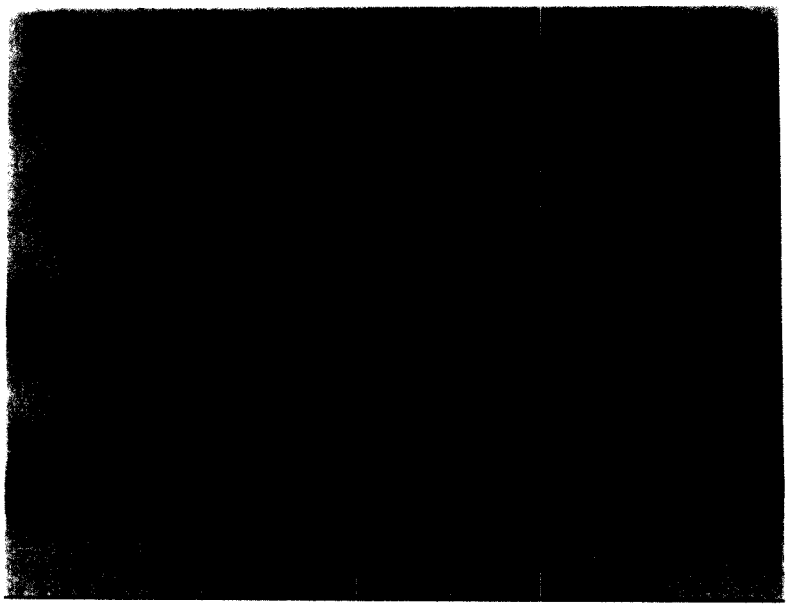

Figure 1. Microscopic picture of carp polychromatocyte micronucleus. The picture was imaged with a Nikon microphot-FXA303 under oil immersion lens (1000×maginification). Arrow indicated carp (*Cyprinus carpio* Linn.) polychromatocyte micronucleus.

control groups ($p>0.05$). However, we found that micronuclei frequencies increased with the rise of Cd and Cr concentrations during exposure time. Carp treated by $0.01mgL^{-1}$ and $0.1mgL^{-1}$ Cd^{2+}, micronuclei frequencies were higher than those of control group($p<0.025$ and $p<0.01$, respectively)(Figure2).Carp exposed to $0.001mgL^{-1}$, $0.01mgL^{-1}$ and $0.1mgL^{-1}$ Cr^{6+}, micronuclei frequencies were induced significantly higher than those of control group (*t*-test, $p<0.05$, $p<0.025$, $p<0.01$and $p<0.001$, respectively). Hughes also found that micronuclei frequencies of fish exposed to high concentration pollutant could elevate many times with the rise of exposure time (Hughes et al. 1991).

The relationship between exposure time and micronuclei frequencies: carp exposed to low Cd and Cr concentrations, micronuclei frequencies increased with exposure time. However, we interestingly found that micronuclei frequencies would increase to a smooth level after a peak value with the rise of Cd and Cr concentrations. Carp exposed to $0.1mgL^{-1}$ Cd^{2+} and $0.01mgL^{-1}$ Cr^{6+}, micronuclei frequencies increased after the first few days of exposure, peaked on d 4 and d 9, respectively, and then smoothly changed. Carp exposed to $0.1mgL^{-1}$ Cr^{6+}, micronuclei frequencies increased toward a relatively smooth level, might peak within exposure 24hrs (Figure 3). Brunetti thought that the higher pollutant

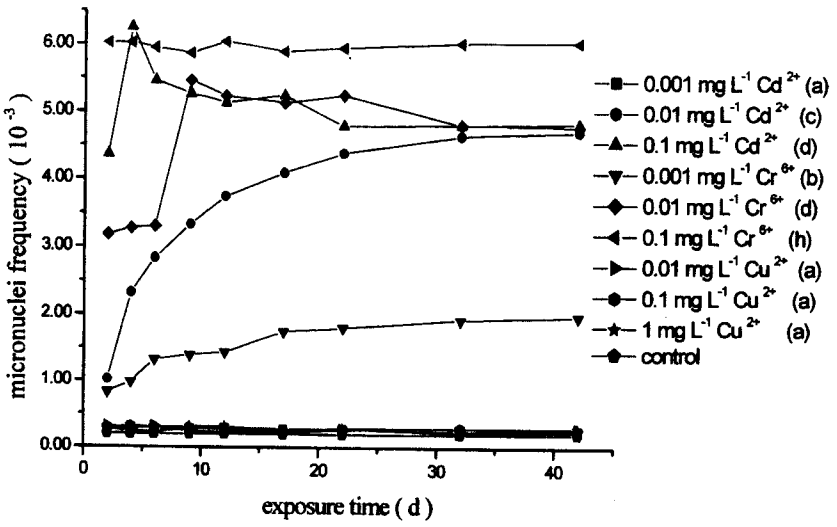

Figure 2. The relationship between metal concentrations and micronuclei frequencies.

Micronuclei frequencies data were averaged from 4 carp (2 slides per carp) in different Cd, Cr and Cu concentrations. Statistics analysis *t*-test showed non-significantly different ($^{a}p>0.05$), significantly different ($^{b}p<0.05$, $^{c}p<0.025$, $^{d}p<0.01$ and $^{h}p<0.001$, respectively) from control during exposure time.

concentration might inhibit normal cell division, damage erythrocyte chromosome and interdict DNA duplication, thus micronuclei frequencies more or less declined. Then micronuclei frequencies trended to the smooth change and fish might promote some defensive mechanism to reduce some of residues in body, so as to stabilize the micronuclei frequencies relatively (Brunetti et al.1998). Likewise Nepomuceno also held a similar view (Nepomuceno et al.1997).

The relationship between micronuclei frequencies and Cd, Cr residues in tissues: We selected dorsal muscle and kidney tissue as samples: 1) the lowest metal residues are always found in fish muscle tissue, while kidney is the main targeting tissue for metal accumulation (Metcalfe 1988; McGeer et al. 2000); 2) test micronucleus assay sensitivity (Metcalfe 1988; Brunetti et al.1998). Metcalfe thought that the fish micronucleus assay had some drawbacks, whereas the relationship between micronuclei and residues in kidney could test the fish micronucleus assay sensitivity (Metcalfe 1988). Therefore, we plotted the relationship between micronuclei frequencies and Cd, Cr residues of kidney tissue in Table2. An association between micronuclei frequencies and Cd residues in kidney tissue had been described as $r=0.8568$ ($t>t_{0.05,3}$), $y=0.5042+0.6302x$; the same as Cr residues in kidney tissue e.g. $r=0.9280$ ($t>t_{0.025,3}$), $y=1.3950+0.9007x$. (y was micronuclei frequencies, x was Cd, Cr residues in kidney tissue). However,

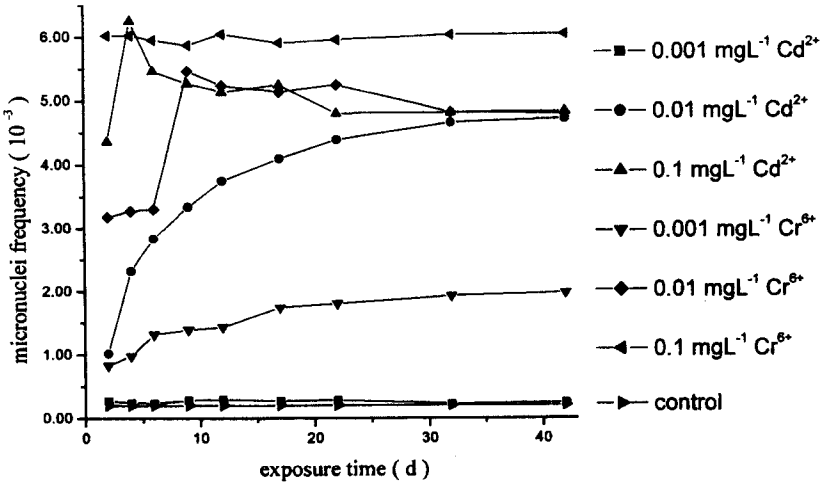

Figure 3. The relationship between micronuclei frequencies and exposure time.

Micronuclei frequencies data were averaged from 4 carp (2slides per carp).Carp exposed to 0.1mgL^{-1}Cd^{2+}, 0.01mgL^{-1}Cr^{6+} and 0.1mgL^{-1}Cr^{6+}, micronuclei increased after the first few days of exposure, peaked on d4, d9 and 24thhr of exposure, respectively, and then smoothly changed.

we interestingly found that there was not association between micronuclei frequencies and metal residues in muscle tissue, and Manna also held a similar view (Manna et al.1986).

Table 2. The relationship between Cd, Cr residues in tissues and micronuclei frequencies (means ± SD).

Element	Concentration (mgL^{-1})	Muscle (mgkg^{-1})(n=4)	Kidney (mgkg^{-1}) (n=4)	Micronuclei Frequencies (×10^{-3}) (n=8)
Cd^{2}	0.001	0.17±0.043	1.02±0.41	0.24±0.073
Cd^{2+}	0.01	0.50±0.012	3.21±0.17	4.62±0.21
Cd^{2}	0.1	0.67±0.028	8.12±0.32	4.83±0.32
Cr^{6+}	0.001	0.12±0.084	0.85±0.093	1.97±0.27
Cr^{6+}	0.01	0.39±0.029	1.20±0.087	4.78±0.21
Cr^{6+}	0.1	0.73±0.084	6.08±0.57	6.03±0.31
Control		Not detected	Cd0.093±0.0029 Cr0.054±0.0093	0.20±0.017

Micronuclei frequencies induced by synergistic toxic effects of Cd, Cr and Cu: carp exposed to both 0.001mgL^{-1}Cd^{2+} and 0.01mgL^{-1}Cu^{2+}, and micronuclei frequencies were not higher than those of control group ($p>0.05$).

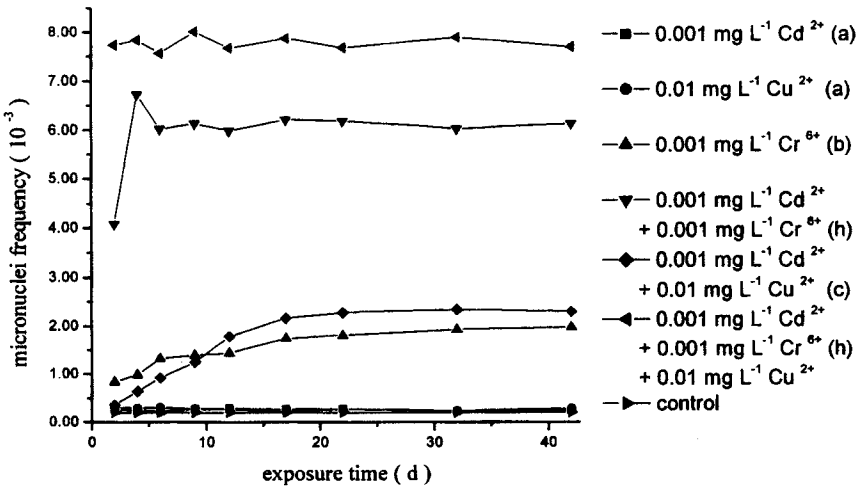

Figure 4. Micronuclei induced by combinatorial treatment of Cd, Cr and Cu. Micronuclei frequencies data were averaged for 4 carp (2 slides per carp).Carp exposed to $0.001mgL^{-1}Cd^{2+}+0.01mgL^{-1}Cu^{2+}$, $0.001mgL^{-1}Cd^{2+}+0.001mgL^{-1}Cr^{6+}$ and $0.001mgL^{-1}Cd^{2+}+0.001mgL^{-1}Cr^{6+}+0.01mgL^{-1}Cu^{2+}$, micronuclei frequencies were higher than those of control ($^{c}p<0.025$, $^{h}p<0.001$ and $^{h}p<0.001$).

However, carp exposed to $0.001mgL^{-1}Cd^{2+}+0.01mgL^{-1}Cu^{2+}$, and the micronuclei frequencies were significantly higher than both those of individual treatment by 0.001 $mgL^{-1}Cd^{2+}$ and control groups ($p<0.025$).Carp expose to $0.001mgL^{-1}Cd^{2+}+0.001mgL^{-1}Cr^{6+}and0.001mgL^{-1}Cd^{2+}+0.001mgL^{-1}Cr^{6+}+0.01mgL^{-1}Cu^{2+}$,micronuclei frequencies were significantly higher than those of control ($p<0.001$). Meanwhile, carp exposed to $0.001mgL^{-1}Cd^{2+}+0.001mgL^{-1}Cr^{6+}$, micronuclei frequencies were more significant than those of carp exposed to $0.01mgL^{-1}Cr^{6+}$ ($p<0.05$); carp exposed to $0.001mgL^{-1}Cd^{2+}+ 0.001mgL^{-1}Cr^{6+}$, and micronuclei frequencies were more significant than those of carp exposed to $0.001mgL^{-1}Cd^{2+}+0.01mgL^{-1}Cu^{2+}$ ($p<0.01$). Carp exposed to $0.001mgL^{-1}Cd^{2+}+0.001mgL^{-1}Cr^{6+}+0.01mgL^{-1}Cu^{2+}$, and micronuclei frequencies were significantly higher than those of carp exposed to both$0.001mgL^{-1}Cd^{2+}+0.001mgL^{-1}Cr^{6+}and0.001mgL^{-1}Cd^{2+}+0.01mgL^{-1}Cu^{2+}$ ($p<0.05$ and $p<0.001$, respectively).

Our studies showed that carp polychromatocyte micronuclei were not induced separately by Cu, and carp exposed to low concentrations of Cd and Cr, micronuclei frequencies increased with the exposure time. The induction of carp polychromatocytes micronuclei was enriched by synergistic toxic effects of multiple metal residues. And our data showed that the fish micronucleus assay was a valid technique to monitor toxic effects induced by heavy metals in water.

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