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Genotoxicity evaluation and a primary risk assessment of organic pollutants in the drinking water sources of Nanjing, China

LI Yi-qiang[1,2], WU Yu-lin[1], CHEN Yuan-gao[1], KONG Zhi-ming[1,*]

(1. The State Key Laboratory of Pollution Control and Resource Reuse, School of Environmental, Nanjing University, Nanjing 210093, China. E-mail: zmkong@nju.edu.cn; 2. National Accredited Laboratory, Environmental Monitoring Center of Jiangsu Province, Nanjing 210036, China)

Abstract: An increasing number of industrial, agricultural, and commercial chemicals in the aquatic environment leads to various deleterious effects on organisms, which is becoming an increasingly serious problem in China. In this study, the comet assay was conducted to investigate the genotoxicity to human body caused by organic concentrates in the drinking water sources of Nanjing City from Yangtze River of China, and health and ecology risk due to expose to these organic pollutants were evaluated with the multimedia environmental assessment system (MEAS). For all the water samples, they were collected from four different locations in the drinking water source samples, es of Nanjing City. The results of the comet assay showed that all the organic concentrates from the water samples could induce different levels DNA damages on human peripheral blood lymphocytes, and a statistically significant difference ($p<0.01$) was observed compared with the solvent control, which demonstrated the genotoxicity was in existence. According to the ambient severity (AS) of individual compound, we had sorted out the main organic pollutants in the drinking water source of the four waterworks, and the results showed that there was some potential hazard to human body for all the source water, namely the total ambient severity (TAS) of health for each water source was more than 1. However, the TAS of ecology for each water source was less than 1, which indicated that it was safe to ecology. The results of this investigation demonstrate the application of the comet assay and the MEAS in aquatic environmental monitoring studies, and the comet assay found to be fast, sensitive, and suitable for genotoxicity monitoring programs of drinking water source.

Keywords: genotoxicity; comet assay; risk assessment; organic pollutants; drinking water sources; Nanjing City

Introduction

Human health is closely bound up with the quality of drinking water, however, more and more pollutants with genotoxic substances are released into the aquatic environment, which is threatening the safety of drinking water (Turgeon *et al.*, 2004; Rajaguru *et al.*, 2002). Since early in 1970s when Environmental Protection Agency (EPA) discovered the derivatives of chlorine in the drinking water for the first time, an increasing concern of human health was showed for the organic chemicals in drinking water (Zhu, 1995). Many organic matters have genotoxicity, they also can induce tumor even cause death under the condition of low dose and long-term exposure. Therefore, it should be accepted as the main index to evaluate the water quality for the human health and population bio-monitoring to see whether there are genotoxic agents in the drinking water source (Feng *et al.*, 1990). Generally speaking, the genotoxicity evaluation of organic matters in the water body is evaluated *in vitro* by different short-term bioassays such as the Ames test, micronucleus test and the mutagenicity test in V79 cells etc.(Zhang *et al.*, 1997). Comparatively the comet assay (single cell gel electrophoresis) is a more rapid, simple and sensitive visual technique, and which has extensively been used as a basic tool in environmental monitoring programs in the recent years (Rajagura *et al.*, 2002; Avishai, 2002).

The chief drinking water source of Nanjing City is Yangtze River, including Qinhuai River, Chu River etc. Because of the effect of environmental pollution of industry and agriculture, all the water sources have the environment safe hidden trouble of different degrees, which are threatening the drinking safety to more than hundreds million people (Xie *et al.*, 1998). For evaluating the pollution level and genotoxicity damage to human health resulted from the organic pollutants in the drinking water sources of Nanjing City, from 2002 to 2004, a project was sponsored and founded by scientific and technological drawing room of Jiangsu Province, China. In this study, we investigated the DNA damage to human peripheral blood lymphocytes (PBL) caused by organic concentrates in the water samples from Yangtze River with the comet assay, and evaluated the health risk and the ecology risk due to expose these organic pollutants with the MEAS suggested by USEPA, which is a procedure that can determine quantitatively whether one pollutant or some compounds are potentially dangerous to man and the environment (Baasel, 1985). MEAS was a scientific and effective method for investigating the health risks of organic pollutants in the drinking water (Liu *et al.*, 1994). The purpose of this investigation was to introduce the comet assay and the MEAS as the monitoring and assessment tool for the detection and assessment of organic pollutants in the drinking water sources, and preserve a safe drinking water and protecting human

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health.

1 Materials and methods

1.1 Sample collection

Water samples were collected in the source water from 4 different waterworks (Beihekou waterworks (S1), Shangyuanmen waterworks (S2), Chengnan waterworks (S3) and Chengbei waterworks (S4) in October 2003. At each sampling site, 25 L water was collected at 0.5 m beneath the water-surface. The four waterworks all lie in the south bank of Yangtze River and all chose the river as their river intake, and almost all the drinking water of Nanjing City is supplied by them.

1.2 Comet assay

1.2.1 Sample preparation and cell exposure

Twenty liter water samples were calmed down and placed about 24 h, and then each water sample was fist filtered by gauze and filter paper, so as to remove suspended material or sediment, then it was passed through a column with non-polar neutral resin (XAD-2) to adsorb organic pollutants. The velocity of flow was controlled at 30—40 ml/min. Finally these constituents were first eluted with carbinol, acetone and dichloromethane, then dried by blowing with nitrogen at 50℃ and re-dissolved in 2.0 ml dimethysulphoxide (DMSO) (Greenberg *et al.*, 1992; Bian *et al.*, 1994).

PBL samples (4 healthy, non-smoking, adult male volunteers) subsided unaffectedly for 30 min in 3% glutin solution at 37℃ , and then the layer containing lymphocytes (0.2 ml) were transferred into a test tube with 0.8 ml phosphate-buffered saline (PBS). The water layer in the tube was removed carefully after being centrifuged at 3000 r/min for 3 min, and then the pellet containing lymphocytes was incubated in 1 ml different concentration of organic concentrate solutions (1 ml equivalent to 20, 100, and 500 ml of water respectively) for 1 h at 37℃. For the organic concentrates, they were diluted with PBS. The survival rate of lymphocytes after the exposure was more than 90% as examined by trypan blue exclusion test.

1.2.2 Procedure

The procedure used was basically the same as that described by Singh *et al.* (1988). Modifications due to the equipment available were relatively minor (Zhong *et al.*, 2001). The modified procedure was described in detail by Zhong *et al.* (2001).

Samples were immediately placed on ice for comet assay. The essential steps of comet assay involve at least 1 h lysis of cells by detergent at high salt concentration and electrophoresis under alkaline conditions (300 mmol/L NaOH and 1 mmol/L Na_2EDTA, pH 13; 30 min unwinding; 60 min electrophoresis at 100 mA and 25 V). Nucleoids were stained with ethidium bromide and examined with a "BX41" fluorescent microscope (Olympus, Japan). One hundred cells per slide were scored visually and given scores 0 (undamaged), 1, 2, 3 or 4 (maximally damaged) according to tail intensity (size and shape) (Maluf and Erktmann, 2000; Zang *et al.*, 2000; Zhong *et al.*, 2001; Kamerand Rinkevich, 2002). Thus, the total score for 100 comets ranges from 0 (all undamaged) to 400 (all maximally damage). The arbitrary unit (AU) was used to express the extent of DNA damage and was calculated as follows:

$$AU = \sum_{i=0}^{4} i \times N_i$$

where N_i is the number of cells in i degree; i is the degree of damage (0, 1, 2, 3, 4).

1.2.3 Statistical analysis

Comparisons of DNA damage induced by all the control and treated groups were analyzed using a one-way analysis of variance (ANOVA) test by the statistical product and service solutions (SPSS). In addition, differences among all the groups were analyzed applying with the Duncan test, and significance level was 0.05.

1.3 Risk assessment

1.3.1 Sample preparation

In this investigation, solid-phase extraction (SPE) technique was applied to collect organic pollutants in water samples, the variety and constitute of organic compounds of the drinking water sources of the four waterworks were analyzed and identified by gas chromatography-mass spectrometry (GC/MS).

All 0.8 L water samples were fist filtered through a 0.45-μm PTFE membrane to remove suspended material or sediment before being applied to the cartridges. SPE was carried out in the positive pressure mode and nitrogen was used as the source of pressure. Before sample application, the C-18 SPE cartridges were preconditioned by 5 ml methanol and distilled water. The flow-rate of the sample through the cartridge was controlled at 2 ml/min by adjusting the nitrogen pressure. After all the samples had percolated through the cartridge, the cartridge was centrifuged at 4000 r/min for 5 min, and nitrogen was allowed to continue to flow through the apparatus to remove the residential water in the adsorbent bed. Analytes were first eluted from the SPE by 5 ml dichloromethane, and then eluted by rinses of 1 :1 dichloromethane-hexane (v/v). After sufficient anhydrous sodium sulfate was added, the eluate was mixed with a vortex-mixer and concentrated at 45℃ with a nitrogen stream to 0.1 ml (note: for the quantitative analysis of volatile organic compounds (VOCs), the water sample should first adjust pH to 2.3—3.0 with 1 mol/L HNO_3 before preparation).

1.3.2 GC-MS conditions

Analysis of final extracts was conducted using an Agilent 6890 GC/ECD (HP, USA) and a capillary column (30.0 m × 250 μm × 0.25 μm, J&W 122-1032). The GC-MS parameters are as follows: carrier gas was 99.9% ultrahigh helium, and the flow rate was 2 ml/min. The temperature programs were as the following: 60℃ (0 min)→(25℃/min)→170℃→(4℃/min)→220℃→(2℃/min)→240℃→(20℃/min)→300℃ (5 min) for GC/ECD, 60℃ (15 min)→(4℃/min)→250℃ (5 min) for GC/FID and GC/MS.

1.3.3 Procedure

In this study, the MEAS, namely the Multimedia Environmental Goal (MEG) was used to evaluate health and ecology risk due to exposure organic pollutants from the drinking water sources. The MEG is the highest concentration of a compound in a medium that will not cause significant harm (Baasel *et al.*, 1980).

The ratio of the actual concentration to the MEG is called the ambient severity (AS) when it is applied to ambient conditions, $AS= C_{ij}/AMEG_{ij}$. If the harmful effects caused by the two or more were not synergistic or antagonistic, the AS of a medium may be approximated by the sum of AS of its components. This is known as the total ambient severity (TAS):

$$TAS_{ij}= \sum AS_{ij}$$

where, C_{ij} is the concentration of compound i in medium j; $AMEG_{ij}$ is ambient MEG of compound i in medium j; AS_{ij} is ambient severity of compound i in medium j; TAS_{ij} is total ambient severity in medium j.

The consequences of this addition are that although no individual compounds in the water (rivers, streams or lakes) may be considered dangerous (AS<1.0), yet the water itself may be considered dangerous. This can occur if there are three compounds present in the water, each with an AS of 0.5. The TAS for the three would be 1.5, i.e. TAS>1, so the water is considered potentially harmful.

2 Results

2.1 Comet assay

The results of DNA damages in human peripheral blood lymphocytes induced by organic concentrates from source water of all waterworks are presented in Table 1, and the parameter AU was used for compa, similar results were obtained. From Table 1 we can see all the water sample concentrates could induce DNA damage diversely, and a statistically significant difference (p<0.01) was observed comparing with the solvent control. Table 1 also demonstrates that the DNA damage score increased with the increase of concentrations. But the trypan blue staining showed the cell livability of different dose group was more than 90% before and after exposure, so we could confirm that the main cause of DNA damage was genotoxicity, rather than cytotoxicity. Multiple comparisons at the dose of 100 ml of each tube (amount equivalent to 100 ml of the original water sample) were made among S1, S2, S3 and S4, the results revealed that it was very different for the possible contribution of compounds from different water sources (the significant level was 0.05), and the degree of DNA damages to lymphocytes for all the four water sources was S3> S1> S2≈S4.

2.2 Risk assessment

The results of health and ecology risk of organic

Table 1 DNA damages in human peripheral blood lymphocytes exposed to organic concentrates

Station	Concentrates, ml/tube	Number of cells in each damage degree, %					Arbitrary units (AU)
		0	1	2	3	4	
S1	20	25.19±7.89	72.49±5.83	2.31±1.60	0.00±0.00	0.00±0.00	77.12±10.20**
	100	1.32±0.93	70.36±10.67	26.99±8.17	1.33±1.89	0.00±0.00	128.33±11.20**b
	500	0.00±0.00	22.65±2.15	54.68±2.22	22.35±3.49	0.32±0.46	200.34±4.39**
S2	20	31.33±1.73	61.77±1.43	6.91±0.29	0.00±0.00	0.00±0.00	75.58±2.02**
	100	20.45±1.40	54.67±1.60	19.67±0.14	5.21±1.10	0.00±0.00	109.64±2.97**a
	500	13.57±1.81	45.14±1.02	32.05±1.28	8.27±1.54	0.97±0.01	137.92±6.19**
S3	20	15.26±2.81	73.71±2.54	10.37±0.68	0.66±0.72	0.00±0.00	96.42±4.03**
	100	0.00±0.00	53.52±3.82	39.44±2.93	7.04±1.45	0.00±0.00	153.52±4.97**c
	500	0.00±0.00	15.04±1.18	38.27±4.00	42.76±4.32	3.93±1.66	235.59±5.51**
S4	20	32.27±1.51	56.18±1.11	10.17±1.20	1.37±0.38	0.00±0.00	80.64±2.46**
	100	15.92±2.71	52.43±1.49	29.73±4.24	1.92±0.04	0.00±0.00	117.66±6.87**a
	500	8.77±1.30	39.08±1.96	43.36±4.72	7.52±2.11	1.26±0.89	153.43±1.78**
Solvent control	0	51.74±5.25	47.53±5.02	0.74±1.27	0.00±0.00	0.00±0.00	49.00±5.76

Notes: Data is expressed as mean ±*SD*; ** P<0.01; the same letter indicated no difference, significance level is 0.05

compounds in the drinking water sources of the two waterworks are shown in Table 2. The results indicated that the TAS of health of S1, S2, S3 and S4 was 7.789, 13.749, 3.281 and 3.933 respectively, and the TAS of health for each water source was more than 1, therefore there was some potential damage to human health for organic pollutants in the drinking water source of the four waterworks. However, all the TAS of ecology for each water source was less than 1, and with that of 0.544, 0.556, 0.354 and 0.342, they were considered safe to ecology.

Table 2 TAS of health and ecology of organic pollutants from different water samples

No.	MEG No.	Pollutants	MEG, μg/L		S1			S2			S3			S4	
						TAS			TAS			TAS			TAS	
			Health	Eco-logy	Conc., μg/L	Health	Eco-logy	Conc., μg/L	Health	Eco-logy	Conc., μg/L	Health	Eco-logy	Conc., μg/L	Health	Eco-logy
1	02A100	Chloroform	140	500	2	0.014	0.004	0.9	0.006	0.002	1.8	0.013	0.004	1.3	0.009	0.003
2	02A100	Carbon tetrachloride	170	500	0.46	0.003	0.001	1.16	0.007	0.002	0.62	0.004	0.001	1.08	0.006	0.002
3	02A240	β-BHC	4	4000	0.00038	0.000	0.000	0.000185	0.000	0.000	0.000525	0.000	0.000	0.000455	0.000	0.000
4	02A380	Lindane	4	4000	0.000585	0.000	0.000	0.00078	0.000	0.000	0.000975	0.000	0.000	0.001225	0.000	0.000
5	02A380	Frichloroethylene	7400	5000	ND			ND			ND			ND		
6	02B070	Tetrachloroethene	9200	500	ND			ND			ND			ND		
7	02B080	2,2-Dichlorodiiso-propyl ether	96		0.55	0.006		ND			ND			0.24	0.003	
8	04B100	Isophorone	350		ND			ND			1.41	0.004		ND		
9	07B080	Di-2-ethylhexyl phthalate	70		0.63	0.009		ND			0.85	0.012		ND		
10	08D300	Butyl benzyl phthalate	1300	50000	ND			ND			ND			ND		
11	08D320	Bezene	110	500	1	0.009	0.002	1	0.009	0.002	1	0.009	0.002	1	0.009	0.002
12	15A020	1,2-Dichlorobenzene	4100	50	1.49	0.000	0.03	0.75	0.000	0.015	0.91	0.000	0.018	0.11	0.000	0.002
13	16A100	1,3-Dichlorobenzene	4100	50	ND			ND			ND			ND		
14	16A120	1,4-Dichlorobenzene	6200	50	ND			ND			ND			ND		
15	16A140	Polychlorinated benzenes	420	50	1.57	0.004	0.031	1.15	0.003	0.023	4.58	0.011	0.092	0.91	0.002	0.018
16	16A160	1,2,4-Trichloro benzene	420	50	0.86	0.002	0.017	ND			ND			ND		
17	16A161	2,6-Dinitrotoluene	21	500	0.36	0.017	0.001	1.43	0.068	0.003	0.4	0.019	0.001	1	0.048	0.002
18	17A084	2,4-Dinitrotoluene	21	500	1.74	0.083	0.003	2.62	0.125	0.005	0.48	0.023	0.001	1.54	0.073	0.003
19	18A142	2,4-Xylenol	1	100	2.28	2.280	0.023	0.55	0.550	0.006	ND			ND		
20	19A020	2-Chlorophenol	1	10	ND			ND			ND			ND		
21	19A040	2,4-Dichlorophenol	1	40	ND	20		ND			ND			ND		
22	19A050	2,4,6-Trichlorophenol	1	10	1.36	1.360	0.136	0.9	0.90	0.09	0.93	0.93	0.093	1.19	1.19	0.119
23	19A060	Pentachlorophenol	1	12.5	1.86	1.860	0.149	0.96	0.960	0.077	ND			0.66	0.66	0.053
24	20A020	2-Nitrophenol	1	100	ND			ND			ND			ND		
25	20A060	4-Nitrophenol	10	100	14.6	1.460	0.146	12.7	1.270	0.127	13	1.300	0.130	13.5	1.350	0.135
26	20B020	4,6-Dinitro-*o*-cresol	1	50	0.7	0.700	0.014	9.86	9.860	0.197	0.98	0.980	0.020	0.6	0.600	0.012
27	21A020	Naphthalene	690	50	ND			0.83	0.001	0.017	ND			ND		
28	21A140	Anthracene	2000		9.92	0.005		20.4	0.01		2.92	0.001		10	0.005	
29	21A180	Phenanthrene	57		0.17	0.003		0.08	0.001		ND			0.1	0.002	
30	21B040	Benz[a]anthracene	1.7		ND			ND			ND			ND		
31	21B120	Chrysene	79		ND			ND			ND			ND		
32	21B180	Pyrene	8300		0.06	0.000		ND			ND			ND		
33	22C020	Benzo[k]fluoranthene	58		ND			ND			ND			ND		
34	22D020	Indeno[1,2,3-cd] pyrene	59		ND			ND			ND			ND		
		Sum				7.789	0.544		13.749	0.556		3.281	0.354		3.933	0.342

3 Discussion

S1 and S3 are located in Dashengguan water source conservation district of Nanjing City, of which there are 42 and 17 pollutant sources distributed in the range from the 5000 m upper course to the 2000 m lower reaches respectively, the industry waste water and the sewage of the urban living were contributed to pollute the drinking water sources. S2 lies in the bottomland of Yangtze River, where there are so many docks and wastewater pumping stations, of which there are about 30 pollutant sources distributed in the range from the 5000 m upper course to the 2000 m lower reaches. S4 is located in between Ertaidong and Santaidong of Swllow Rock along the Yangtze River, in the range from the 5000 m upper course to the 2000 m lower reaches, there are about 16 pollutant sources. The characters of S2 and S4 were where there were many docks and pumping stations, so they affected the water quality less and show less toxic impact on Lymphocytes. All these drinking water sources potentially damaged the quality of the drinking water of the four waterworks (Nanjing Municipal Environmental Monitoring Center, 2003).

The above four waterworks are located between the section of Swllow Rock and Chixia of Yangtze River and all choose the Yangtze River as their drinking water source. The all-year monitoring data indicated that the index of chemical toxicity is low comparatively, and the quality of their source water can achieve the secondary category of GB3838-2002 on the whole. Nevertheless, an increasing number of industrial, agricultural, and commercial organic pollutants in the aquatic environment leads to various deleterious effects on organisms, and in lower concentrations, the organic pollutants may have no detectable acute effects on organisms, but may reduce their survival via long-term chronic effects. Such effects could be manifested by minor or major genetic damage to somatic and germ cells and by the development of disorders, e.g. cancer, that requires long, latent periods before becoming clinically visible (White and Rasmussen, 1998). In this study, our data also indicate that the source water in the study area is contaminated with substances capable of inducing DNA damage in human cells. But the methods of chemical tests and the short-term bioassays could not reflect well and truly the potential dangerous to the aquatic organisms and human health in time. So it is very important to develop a rapid, sensitive, and reliable screening methodology for the evaluation of genotoxic impact at low levels and in early exposure stages.

Since the development of the alkaline single-cell gel electrophoresis (SCGE) method by Singh *et al.* (1988), it has been extensively utilized in research ranging from environmental bio-monitoring to clinical diagnosis, to understand DNA repair processes and also in genetic toxicology (Tice *et al.*, 2000; Rojas *et al.*, 1999; Sun *et al.*, 2005). Human peripheral blood lymphocyte (PBL) is a good material for the toxicity research of organic pollutants in drinking water, the results from PBL could well demonstrate the adverse effects of pollutants on human, and could be used to predict the risk on human healthy immediately. Many tests evaluated the genotoxicity of pesticide and environment endocrine disruptors (EEDS) using the comet assay (Feng *et al.*, 2000; Zhang *et al.*, 2002; Rajaguru *et al.*, 2002; Kassie *et al.*, 2000; Zhong *et al.*, 2001). The results of the present comet assay show that all the organic extracts from all the water samples could induce different levels that DNA damages on human peripheral blood lymphocytes. However, the variety and constitute of organic compounds of the drinking water sources of the four waterworks are very different, so the levels that DNA damages on human peripheral blood lymphocytes at the same dose are S3 > S1 > S2 $\approx$ S4, by analyzing the genotoxicity of water samples at the dose of 100 ml of each tube with multiple comparisons.

According to the results of the qualitative and quantitative analysis for organic pollutants in source water, it reveal that there are many organic chemicals clarified by the Blacklist of Principal Environment Pollutants in China and EPA priority pollutants, such as 2-nitryl-4,6-dinitrophenol, 4-nitrophenol, Anthracene and di-*n*-butyl phthalate etc., and they have a great harmful contribution to the exposed aquatic organisms and human, which was confirmed by the results of health and ecology risk. Table 2 also indicates that there is some potential damage to human health, namely all TAS of health are more than 1. The degree of potential harmless for the four source water is S2 > S1 > S4 > S3. Furthermore, the organic agents from different locations vary in composition, this could be a reason for the observed difference in effect. As opposed to the comet assay of human peripheral blood lymphocytes, the results of health and ecology risk were not displayed well. The main cause could be that the locations of water samples were arranged casually and the two tests were not conducted at the same time, and the industry waste water and the sewage of the urban living could affect the water quality more seriously for their so many chemicals, such as anthracene and di-*n*-butyl phthalate etc.; the preparation procedure of water samples was different; and the analytical and assessment method of multi-medium pollution had some disadvantages inescapability, for example, an assumption was made that the impact and the concentration of a pollutant in a medium to health and ecology was a linear relationship, and all kinds of compound effect was

summed simply without considering the synergistic effect or the antagonistic effect among them. In addition, it must be pointed out that there could be other organic pollutants undetected in water samples.

4 Conclusions

In summary, our data indicate that the source water of Nanjing City is contaminated with substances capable of inducing DNA damage in human cells. Therefore, the direct and indirect exposure to this contaminated water may cause mutagenic/carcinogenic changes in exposed individuals. The study also demonstrated that the comet assay and the MEAS could be applied to the detection and assessment of geotaxis materials in the drinking water sources. Compared with the later, the comet assay is a more sensitive tool for environmental monitoring, especially for evaluating mutagenic/carcinogenic changes in exposed individuals. At the same time we also should expand SCGE further applications in practice by enhancing the methodology study of the comet assay further, and establishing a standard comet assay programs of human blood lymphocyte.

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