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Evaluating the Genotoxicity of Surface Water of Yangzhong City Using the *Vicia Faba* Micronucleus Test and the Comet Assay

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Yangzhong City,which is located on an island in the middle of the Changjiang River, has an area of 332 square kilometers and it is a relatively closed ecosystem. The mortality of a malignant upper digestive tract tumor in Yangzhong City is among the highest in China, especially the malignant tumor of the oesophagus. Many studies have demonstrated that pollution of water sources has relationships with endemic cancers in humans (Zhu 1987;Zhu and Jiang 1984,). The detection of genotoxicity in potable water and its source has aroused more and more attention. The detection can be established using short-term genotoxicity tests such as the Ames test, sister-chromatid exchange assay and micronucleus test (Ohe et al. 1993;Sujburt et al. 1993;Gauthier et al. 1994). Among the different genotoxicity methods, the micronucleus test using *Vicia faba* root tips is of particular interest because it is sensitive to both mitoclastic and clastogenic agents. Its results are reliable, it is simple and requires minimal laboratory facilities (Degrassi and Rizzoni 1982; Ma 1982; Marco et al. 1990). In this study, the micronucleus test using *V. faba* root tips was utilised to make a preliminary screen of the mutagenic pollution of surface water in Yangzhong.

Based on the screening for mutageuicity, organic extracts from four representative water samples examined by the single cell gel electrophoresis (comet assay) to detect the DNA damage induced by water samples in human lymphocytes. The comet assay is a novel, rapid, simple, sensitive and inexpensive technique for measuring and analyzing DNA breakage in individual cells (Singh et al. 1988;Olive et al. 1990;Tice et al. 1990). Most studies performed to date have utilized the comet assay to evaluate DNA damage induced by a certain compound, and only a few studies have been performed to consider the potential value of the comet assay for environmental monitoring (Mitchelmore and Chipman 1998;Verschaeve and Gilles 1995). In this study, the comet assay was conducted to assess the DNA damage in human lymphocytes induced by surface water samples in Yangzhong. Using both the micronucleus test and the comet assay, the objective was to obtain a more comprehensive assessment of the genetic toxicity of the surface water in YangZhong.

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MATERIALS AND METHODS

Fourteen representative sites for sampling were chosen and one thousand liter water samples were collected for each site in July 1998 (Fig. 1). The samples were immediately preserved in polythylene bottles at 4^0C in darkness

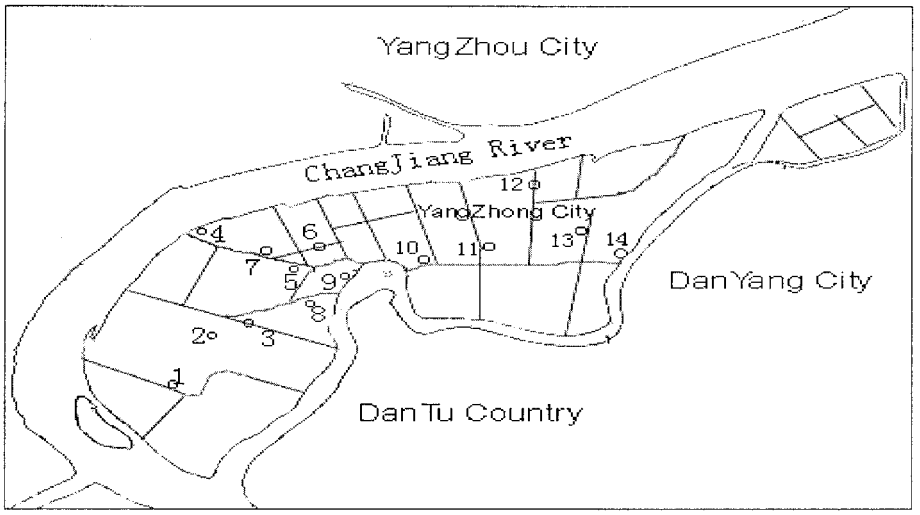

Figure 1. Map of YangZhong City with sampling sites
Number 1-14:sampling sites; Lines in Yangzhong City: ditches.

The test material was *Vicia faba* (songzi green-skin) seeds, provided by Middle-China Normal University. The test was performed according to the method reported by Chen et al. (1985). Five beans were set in a group and five root tips were examined by microscope for each site and 1000 cells were counted per slide. The MNC‰ and the pollution index (PI) were calculated as blow:

$$\text{MCN‰} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 1000‰$$

$$\text{PI} = \frac{\text{MCN‰ of sample}}{\text{MCN‰ of the negative control}} \qquad (1)$$

The student's *t*-test was used to determine significant differences between each experimental point and the control.

Each water sample (20 liters) was initially filtered by gauze and filter paper to remove suspended material or sediment, then passed through a column with non-polar neutral resin (XAD-2) to absorb organic pollutants. These constituents were eluted by 50mL acetone and CH_2Cl_2. The eluates were concentrated under

reduced pressure and dried with nitrogen gas, then were dissolved by 20mL DMSO (dimethylsulphoxide) and were kept at -18^{0}C. Four sites were chosen: (1) Xinba Bridge, (2) Lianhe Pond, (3) Yangzi River, and (4) Hongxin River(Table 1).

Whole-blood samples (2mL) were mixed with 5mL gelatin (3%) in centrifuge tube and were vertically rinsed in water bath at 37^0**C** for 30 min. The mixture was divided into two layers and the lymphocytes were suspended in the upper layer. 200μL of lymphocyte layer was mixed with 800μL of PBS (phosphate-buffered saline) and was centrifuged at 3000×g for 5 min. The cells were then suspended in 900μL of phosphate-buffered saline. Water samples (100μL) of various concentrations were then added into the microcentrifuge tube to make the final exposure dose were 2mL, 10mL, 50mL water sample per tube. Dimethylsulphoxide (100μL) was added as the negative control and 10mM H_2O_2 was used as positive control. The contact periods were 60 min in the dark at 4^0**C**. After the treatment period, cells were sedimented at 3000×g for 5 min and were put on ice before performing the comet assay.

The comet assay was performed as described by Singh et al.(1988). Essential steps involve 1-hr lysis of the cells by detergent at high salt concentrations and electrophoresis under alkline conditions (20 min unwinding followed by 45 min electrophoresis at 150 mA and 25V). EB (ethidium bromide) stained nucleoids were examined with a "TMD-EF" fluorescent microscope (Nikon, Japan). Images were analysed according to the method of Collins et al.(1989). One hundred comets on each slide were scored visually as belonging to one of five classes according to tail intensity and given a value of 0, 1, 2, 3 or 4 (from undamaged 0, to maximally damaged, 4). Thus, the total score for 100 comets could range from 0 (all undamaged) to 400 (all maximally damaged). The percentage (%) of damaged cells was calculated and the results were analyzed with the x-square test. The 'arbitrary units' was used to express the extent of DNA damage and was calculated as shown below.

$$\text{Arbitrary units} = \sum_{i=0}^{4} N_i \times i \qquad (2)$$

Ni= number of cells in i degree.
i=damage degree (0, 1, 2, 3, 4)

Statistical comparisons between the grade of DNA damage in control/exposed group were analysed by Kruskal-Wallis test.

RESULTS AND DISCUSSION

Table 1 lists the MCN‰ and PI value for each sampling site. There are five sites (Lianhe Pond, Fengshou River, Yanzi River, Hongxing River, Yongsheng Ditch) that have significant higher MCN‰ than that of the control. According to the PI values, the sampling site can be divided into three types. The Yangzi River and the Hongxing River were heavily polluted (PI>3.5). The Lianhe Pond, the Qidong

Pond and the Yongsheng Yangzi River flows through Sanmao town, and the domestic wastewater flows into this river and then into the Changjiang River. The high MCN‰ of this site indicated that the domestic sewage of the city may have

Table 1. The PI value and MCN permillage of *Vicia faba* of each sampling site

Site no.		MCN‰±S.E	PI	Site no.	MCN‰ ±S.E	PI
1	Xinba Bridge	3.25±1.55	1.29	9 Shajia floodgate	2.22±0.55	0.88
2	Lianhe Pond	4.73±0.91*	1.88	10 Xinglong Pond	2.46±0.61	0.98
3	Lianfeng River	2.86±0.92	1.13	11 Hongxing River	9.06±4.01*	3.60
4	Dongfanghong floodgate	2.44±1.23	0.97	12Yongsheng Ditch	3.91±1.07*	1.55
5	Sanmao River	2.46±0.55	0.98	13 Hongqi River	3.29±1.34	1.31
6	Fengshou River	3.43±0.65*	1.36	14Yingfeng floodgate	2.94±1.72	1.17
7	Yangzi River	10.69±2.42*	4.24	Positive Control	16.9±4.49	
8	Qidong Pond	3.88±2.13	1.54	Negative Control	2.52±0.64	

*P< 0.05

Table 2. Number of cells in each damage degree and the DNA damage scoring in control and treated group

Test samples	Dose (mL water sample tube^{-1})	Number of cells in each damage grade (Mean±SD)					Damage percentage(%)	DNA damage scores#
		0	1	2	3	4		
No.1	2	75.7	10.3	9.3	4.7	0	24.3**	43.0
Xinba	10	66.0	11.7	10.7	11.7	0	34.0**	68.0
Bridge	50	24.0	10.0	36.0	22.0	8.0	76.0**	180.0
No.2	2	52.5	39.5	7.0	1.0	0	47.5**	56.5
Lianhe Pond	10	16.0	30.0	42.3	10.7	1.0	84.0**	150.7
	50	1.3	18.0	40.7	24.3	15.7	98.7**	235.0
No.7	2	11.8	66.8	19.5	2.0	0	88.2**	111.8
Yanzi River	10	1.0	30.0	48.7	15.3	5.0	99.0**	193.3
	50	0	2.0	36.0	46.0	16.0	100.0**	276.0
No.11	2	31.5	37.5	27.0	4	0	68.5**	103.5
Hongxing	10	1	33.0	48.7	13.7	4.0	99.0**	187.3
River	50	0	5.0	42.5	40.0	12.5	100.0**	260.0
DMSO	0.1mL tube^{-1}	89.5	10.5	0	0	0	10.5	10.5
H_2O_2	10mM	0	0	0	8.9	91.1	100.0**	391.1

** P<0.01 (with control: χ -square test).

DNA damage score was expressed as arbitrary units

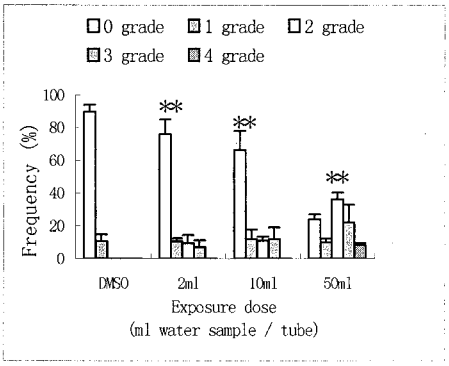

Figure 2. The frequency histogram of the grade of damage in the control group and groups exposed to different doses of water sample from Xinba Bridge (Nos 1). Bars indicate standard errors of means of four or more experiments. **: $p<0.01$ (with control: Kruskal-Wallis test)

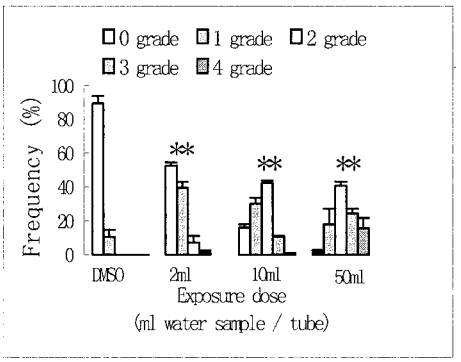

Figure 3. The frequency histogram of the grade of damage in the control group and groups exposed to different doses of water sample in Lianhe Pond (Nos 2). Bars indicate standard errors of means of four or more experiments. **: $p<0.01$ (with control: Kruskal- Wallis test)

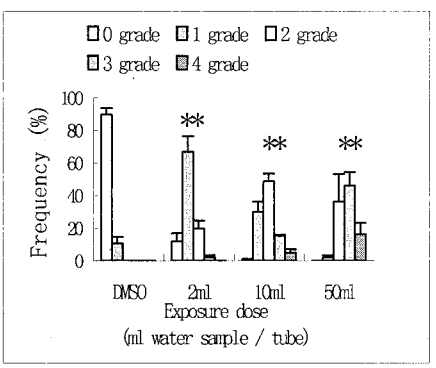

Figure 4. The frequency histogram of the grade of damage in the control group and groups exposed to different doses of water sample from Yangzi River (Nos 7). Bars indicate standard errors of means of four or more experiments. **: $p<0.01$ (with control: Kruskal-Wallis test)

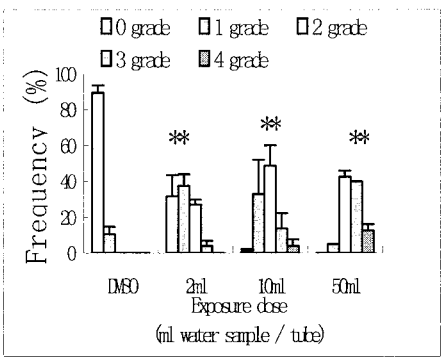

Figure 5. The frequency histogram of the grade of damage in the control group and groups exposed to different doses of water sample from Hongxing River (Nos 11). Bars indicate standard errors of means of four or more experiments. **: $p<0.01$ (with control: Kruskal- Wallis test)

potential mutagenic properties. The result of the micronucleus test demonstrated that the water quality in the main streams and lock entrances to the Changjiang River is higher than that of the smaller rivers, stagnant water pond and ditch. The stagnant ponds and ditches in rural area were shown to be slightly polluted. Although tap water is provided in these areas, some people still maintain their habits of using water of the ponds and ditches to wash rice and vegetables. It might be expected that the water quality in these ponds and ditches is closely related to the health of people in these areas and the report of the "Effect of water resource pollution on public health" Program has demonstrated that the mortality due to cancer is higher in people who drink pond water than in people who drink surface flowing water (Collins et al. 1989). Therefore, the authors suggest that should be improved the water quality in these water bodies, and the health education about the potable water is necessary.

The number of cells in each grade for each of the sample sites, the percentage of damaged cells and DNA damage score of each group were shown in Table 2.

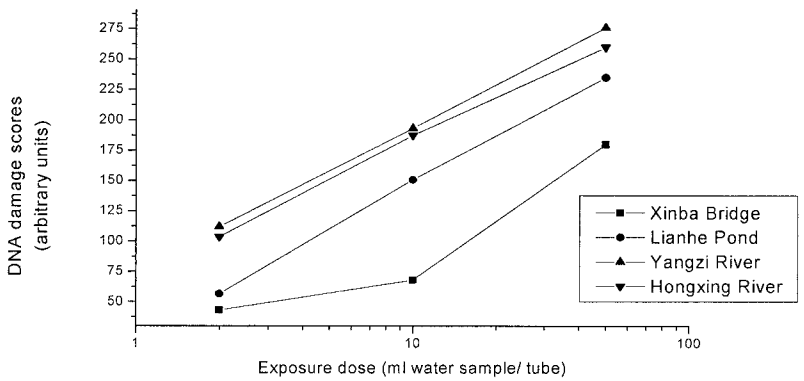

Figure 6. Dose-effect relationships of water samples to the DNA damage scores in human lymphocytes.

Significant increases ($p<0.01$) in the percentage of damaged cells were observed in all the groups treated with the water samples. Frequency histograms of the damage grade in control and treated groups are shown in Figs. 2-5. The distributions in the damage grades in all the treated groups were significantly different from the control ($p<0.01$). DNA damage scores (expressed as arbitrary units) increase as the exposure doses of the four water samples increase and a dose-effect relationship was observed (Fig. 6) for all the four water samples (r =0.938, 0.999, 0.999 and 0.999 for Xinba Bridge, Lianhe Pond, Yangzi River, Hongxing River respectively). The Yangzi River and Hongxing River have the highest mutagenicity and the Xinba Bridge has the lowest mutagenicity, which is in accord with the result of the *Vicia faba* micronucleus test. However, the results of comet assay showed that all the four water sample can induce significant DNA

damage in human lymphocytes, including the water sample of Xinba Bridge in which no mutagenicity was detected by micronucleus test. Therefore, we can conclude that both the comet assay and micronucleus test are able to detect the genotoxic potential of different water bodies, but the comet assay seemed to be more sensitive than micronucleus test.

With its introduction by Singh et al. (1988), the comet assay has become accepted as a rapid, simple, and sensitive visual technique for measuring DNA damage, and for repair studies, biomonitoring, and determination of genotoxicity. The potential value of comet assay in environmental monitoring has aroused more interest in recent years. Verschaeve and Gilles (1995) demonstrated that the comet assay in earthworms may be very valuable for the monitoring and detection of genotoxic compounds in terrestrial ecosystems. Mitchelmore and Chipman (1998) explored the possibility of using DNA strand breaks, measured by comet assay, to act as a biomarker of genetic toxicity in fish and other aquatic species. They concluded that the comet assay is a sensitive, rapid and economic technique for the detection of DNA damage, which is ideally suited as a non-specific biomarker of genotoxicity in fish and other aquatic species. Our study demonstrated that the comet assay seemed to be more sensitive than the micronucleus test to assess the DNA damage potential of the surface water in the Yangzhong city. Moreover, the possibility of evaluating *in vivo* DNA damage in human peripheral blood lymphocytes from 10-20μL blood, allows the use of this assay in the biomonitoring of people exposed to genotoxic compounds and in specific environment (Meo et al. 1991). Therefore, it is suggested that the comet assay is a very easy and convenient way to assess the DNA damage induced by chemicals and may be useful in screening chemicals, or a specific environment, for their DNA damaging properties.

Although the comet assay is a sensitive method to assess DNA damage, different test endpoints and different mechanisms are involved in different genotoxicity tests. Essentially, in this study, the micronucleus tests detects lesions which survived at least one mitotic cycle, while the comet assay identifies repairable DNA lesions or alkali-labile sites (Goethem et al. 1997). There is also a need to exploit the comet assay for more detailed information on cell-specific effects and inter-individual variability and the standard isoline of the method. Taken as a whole, there is a need to combine the comet assay with the use of other genotoxicity tests such as micronucleus test, to get a more comprehensive understanding of the pollution situation of a certain environment.

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