Bull. Environ. Contam. Toxicol. (2004) 73:628–634

DOI: 10.1007/s00128-004-0474-1

Bulletin of Environmental Contamination and Toxicology

Toxic Effects of Aminophenols on Aquatic Life Using the Zebrafish Embryo Test and the Comet Assay

L. W. Sun,[1] M. M. Qu,[1] Y. Q. Li,[1] Y. L. Wu,[1] Y. G. Chen,[1] Z. M. Kong,[1] Z. T. Liu[2]

[1] National Key Laboratory of Pollution Control and Resource Reuse, Department of Environmental Sciences, Nanjing University, Nanjing, 210093, People's Republic of China
[2] Chinese Research Academy of Environmental Sciences, Beijing, 100012, People's Republic of China

Received: 8 December 2003/Accepted: 16 July 2004

For a wide range of environmental pollutants, the aquatic ecosystem is the final sink, where nontarget aquatic organisms can be affected. On a worldwide scale, the emission of anthropogenic chemicals has resulted in long-term ecotoxicological effects. Aminophenols, which have three isomeric forms, including ortho-aminophenol (OAP), meta-aminophenol (MAP) and para-aminophenol (PAP), are commonly used in industrial chemical syntheses such as a dye intermediate, in dyeing hair, fur, and textiles, and as a photographic developer. Massive amounts of their discharges have had detrimental effects on the environment. Take PAP for example. As a parent material for the production of paracetamol, one of the most produced pharmaceuticals worldwide, PAP is applied most widely among three isomers. The amount of PAP increases 5% per year throughout the world and the yield is about 20kt annually in China (Sun and Wang 2001). In addition, these compounds are the metabolites of related xenobiotics such as nitrobenzene, aniline, phenacetin and acetaminophen. According to the results of studies, it is well known that PAP is a possible mutagen and teratogen (Yoshida et al. 1998). However, as far as we are aware, aquatic toxicity data for these three compounds are very limited or not available.

Previous studies have shown that the embryo test of Zebrafish (*Danio rerio*) is an attractive test system that may be a possibility to replace the conventional acute fish test (Lange et al. 1995). Worldwide, the embryo test of zebrafish has been applied successfully in the last two decades (Herrmann 1995).

Since its introduction by Östling and Johanson (1984), the Single Cell Gel Electropherosis (SCGE) test or comet assay has been accepted as a rapid, simple, and sensitive visual technique for measuring DNA damage and repair, biomonitoring, and determination of genotoxicity (Fairbairn et al. 1995). Because of the broad ecological implications associated with genotoxicity in an aquatic system, it is very import that DNA damage, which acts as a biomarker of genetic toxicity in fish and other aquatic species, should be measured (Mitchelmore and Chipman 1998). Since the kidneys are common targets of xenobiotic-induced

Correspondence to: Z. M. Kong

injury and PAP is toxic to the kidney (Yoshida et al. 1998), the kidney cells of carp were employed in the comet assay.

The primary purpose of the present study was to investigate the toxic effects of three aminophenols on aquatic life, including the effects on embryo development of the zebrafish and genotoxic effects on kidney cells of carp. The possible contribution of the relative positions of the hydroxyl and amidogen substituents on the benzene ring to *in vivo* toxicity was also evaluated with the comparison among the three isomers.

MATERIALS AND METHODS

OAP (CAS No. 95-55-6), MAP (CAS No.591-27-5) and PAP (CAS No.123-30-8) were provided by the Chinese Research Academy of Environmental Sciences and the purity of these chemicals was 99%.

Zebrafish (*Danio rerio*) were obtained from a local market. A suitable number of healthy fish (ratio of females to males was 1:2) were kept in a tank containing 100L of dilution water, which was prepared according to ISO (1996) (294 mg $CaCl{\cdot}2H_2O$, 123 mg $MgSO_4{\cdot}7H_2O$, 65 mg $NaHCO_3$, 6 mg KCl, add 1 L H_2O), at 26±1°C, DO>7 mg/L, with a light/ dark cycle of 14/10 hr. They were fed daily with frozen *Artemia* sp. and commercial flake food *ad libitum*.

Zebrafish eggs were obtained according to the method described by OECD (1998). The eggs were collected in 30 min and washed with tap water. As soon as possible, a suitable number of eggs were transferred into prepared chemical concentrations without regard for fertilization. Then the eggs were observed and the non-fertilized eggs were discarded. After differentiation, the eggs were transferred into wells of a 24-well multiplate (1 egg per well). 20 wells were prepared with 2 mL test solution each, 4 wells were filled with dilution water and served as internal controls. The concentrations were identified in a preliminary experiment and acetone (0.1mL/L) was used as solvent control. The tests were replicated at least three times (60 eggs per concentration, and 7 concentrations tested of each chemical) consecutively, and only considered as valid when at least 90% of the embryos in the control (dilution water) were viable after 72hr. The eggs were incubated at 26±1°C and the embryonic development was observed with an inverted microscope (TMD-EF, Nikon, Japan) and recorded daily. The toxicological endpoint was embryo mortality. The values of 72hr-LC_{50} were determined by TOXCALC.

Carp (*Cyprinus caprio*) are an edible fish, commercially valuable and distributed

all over China. Live fish weighing 13.5-17.1g were obtained from a local market. Fish were kept in tanks with aerated water at 18°C with a natural light-dark cycle. Fish were fed on fish food, and acclimatized to laboratory conditions for two weeks.

Exposure experiments were performed in tanks containing 80L of solution. The carp were exposed to the test solution (5 fish/tank) for 72hr. The concentrations were identified in a preliminary experiment and acetone (0.1mL/L) was used as the solvent control. One quarter of the volume was renewed every 24 hr and gentle aeration was provided.

After exposure, fish were killed by a blow to the head and dissected. The kidneys were rapidly removed, put into cold phosphate buffered saline (PBS) and washed twice. The tissue was then dissected into very small pieces, pressed through a 100-μm mesh filter and digested in PBS containing 100mM EDTA at 4°C for 1hr. The cell suspensions were centrifuged at 3000 rpm for 5 min and the supernatant was removed. The final concentration of the cells was adjusted to $1\text{-}3\times10^5$cells/mL. The viability of the individual cells was determined by the trypan blue exclusion technique and found to be more than 90%.

The comet assay was performed as described by Singh et al. (1988). The DNA was stained with ethidium bromide and the slides were examined with a fluorescent microscope (BX41，Olympus, Japan). One observer scored the slides to minimize variability. Images were analyzed according to the method of Collins et al. (1995). 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). The "arbitrary units (AU)" was used to express the extent of DNA damage and was calculated as follows:

$$\text{Arbitrary units} = \sum_{i=0}^{4} i \times Ni,$$

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

At the same time, the electrophoretic patterns were also analyzed by the Comet Assay Software Project (CASP), which is a free software downloaded from SourceForge.net. At least 50 cells/slide were analyzed and three parameters, Tail DNA % (TDNA, percent of DNA in the comet tail)，Tail moment（TM, percent of DNA in the tail × tail length)，and Olive Tail Moment（OTM, percent of DNA in the tail × distance between the center of gravity of DNA in the tail and the center of gravity of DNA in the head）were measured. The data were analyzed using a one-way analysis of variance (ANOVA) test followed by Student Newman-Keuls analysis.

RESULTS AND DISCUSSION

The mortality for developing zebrafish embryos after exposure to each test solution varied greatly. In the acetone control, 10% of the embryos were dead within the first 12hr after exposure and remained constant thereafter for the duration of the experiment. All the groups showed a dose- and time-related increase in mortality. The 72hr-LC_{50} values of three aminophenols were 0.033 mg/L (ortho-, 95% confidence interval was 0.033-0.034 mg/L), 22.34 mg/L (meta-, 95% confidence interval was 22.13-22.55 mg/L), and 1.43 mg/L (para-, 95% confidence interval was 1.42-1.44 mg/L), respectively. Therefore, the order of the acute toxicity on embryo development of zebrafish was determined as: OAP> PAP>MAP.

Early life stages of fish are usually regarded to be the most sensitive stages in fish development, if compared to juveniles and adults (Strmac et al. 2000). In another test, we studied the acute toxicity of three aminophenols on adult zebrafish (10 fish per concentration, and 5 concentrations tested of each chemical) according to Chinese National Environmental Protection Agency Guidelines (1990), and found the 96hr-LC_{50} values for OAP and PAP were 1.92 mg/L (95% confidence interval was 1.69-2.05 mg/L) and 1.44 mg/L (95% confidence interval was 1.32-1.65 mg/L) respectively, and the 96hr-LC_{50} for MAP was ranged from 60 to150 mg/L (data not published). The comparison of embryo data with that of adult zebrafish showed that the embryo test had greater or comparable sensitivity versus the adult fish test. It was surprising that the toxicity of OAP on the embryo was much greater (about 58 fold) than the toxicity on adult fish. A possible explanation is that the metabolic system of the embryos may not be developed sufficiently so that the chemical cannot interact with an enzymatic system (i.e., detoxication). Moreover, the complex properties of the chorion should be taken into account as well.

DNA damage induced by the three aminophenols is shown in Table 1. Four different parameters, AU, TDNA, TM and OTM, were used for comparison and similar results were obtained. The results indicated that all three compounds could induce different levels of DNA damage and the damage showed a dose-related increase. Almost all concentrations of the three tested substances produced statistically significant increases (P at least <0.05) in all comet parameters when compared to the control (apart from TDNA measurements following OAP and MAP treatment at 0.008mg/L, the lowest concentration).

To assess the possible contribution of the relative positions of the hydroxyl and amidogen on the benzene ring to *in vivo* toxicity, multiple comparisons were made

among three isomers (AU measurement was analyzed only). The results revealed that OAP and PAP could induce more serious DNA damage than MAP, and significant differences ($P<0.05$) could be observed at all concentrations tested, except for the OAP and MAP treatment at 0.008mg/L. It also indicated that PAP might be a more potent genotoxin than OAP since the values of AU were higher at 0.008, 0.2 and 1 mg/L, but no statistical differences were found ($P>0.05$) at 0.008, 0.04 and 0.2 mg/L.

Table 1. DNA damage in *Cyprinus caprio* kidney cells induced by aminophenols

Concentration (mg/L)	AU	TDNA (%)	TM	OTM
OAP				
0.008	55.60±6.8**	11.17±1.47	3.11±0.85*	2.02±0.46**
0.04	114.01±3.33**	18.18±2.95**	6.05±1.82**	4.30±0.96**
0.2	139.56±4.12**	23.25±1.53**	7.39±0.95**	4.88±0.53**
1	174.24±1.34**	27.46±0.69**	9.56±0.11**	5.54±0.09**
MAP				
0.008	46.86±1.69**	10.12±1.16	2.52±0.24*	1.95±0.20**
0.04	77.01±4.13**	14.74±1.97**	4.19±1.11**	2.97±0.61**
0.2	107.50±3.32**	19.37±1.20**	5.75±0.68**	3.83±0.34**
1	120.64±2.78**	20.96±1.45**	6.55±0.40**	4.39±0.30**
PAP				
0.008	70.47±4.60**	12.95±0.95*	3.58±0.37**	2.67±0.22**
0.04	112.22±6.64**	19.56±1.64**	5.97±0.69**	3.81±0.32**
0.2	142.33±3.39**	22.57±1.54**	7.98±0.58**	4.96±0.34**
1	191.07±3.66**	29.12±2.65**	9.90±0.59**	6.57±0.69**
Solvent control	21.03±3.24	8.02±1.03	1.23±0.17	0.912±0.10

Data are expressed as mean±S.E.M

*: $P< 0.05$;**: $P< 0.01$ (significantly different from control (ANOVA test)).

The comet assay has been successfully applied to aquatic species for detecting and quantifying DNA damage. Devaux et al. (1997) showed that DNA single strand-breaks were less pronounced after Benzo[a]pyrene (B[α]P) exposure of hepatocytes for 24 and 48 hr than at 4 hr. The decrease in DNA strand breaks with increasing time and higher B[α]P could be due to a high degree of fragmentation of DNA and the loss of these fragments during electrophoresis. In addition, activation of the DNA repair system and modulation of the enzyme reaction could account for a decrease in DNA strand breaks after a prolonged time. The present study showed that after 72 hr exposure *in vivo* to three aminophenols, the DNA

damage of carp kidney cells significantly increased in all the treatment groups when compared with the control. Thus, the comet assay appears to be a useful tool to screen the genotoxic potential of compounds found in an aquatic system.

It is reported that PAP is toxic to the kidney. Although the biochemical mechanism responsible for PAP nephrotoxicity is not yet established, oxidative metabolism and subsequent conjugation with glutathione (GSH) are believed to be the key steps in the nephrotoxic process (Yang and Kulkarni 2000). PAP is also genotoxic based on its induction of DNA cleavage in mouse lymphoma cells, Chinese hamster ovary (CHO) cells and human lymphoblastoid cells. The DNA cleavage and cytotoxicity induced by PAP were related to autoxidation, but the precise mechanism remains obscure (Yoshida et al. 1998). In this study, the data from the comet assay indicated that not only PAP but also OAP and MAP could induce DNA damage on carp kidney cells even at low concentrations.

It is well known that the relative position of radicals plays a very important role in chemical toxicity. The present study suggested that the relative positions of the hydroxyl and amidogen on the benzene ring appeared to modify toxicity on aquatic species. When the hydroxyl group was ortho or para to the amidogen group, the aminophenols displayed more genotoxic effects on kidney cells in the comet assay. On the other hand, in the embryo test of zebrafish, the OAP was the most toxic of three isomers. Further studies would be necessary to determine the mechanism responsible for the difference in toxicity.

Acknowledgements. This study was supported by the National Nature Sciences Fund of China (No.29977020). We are also grateful to Di Wu for providing the software and her excellent technical assistance.

REFERENCES

Collins AR, Ma AG, Duthie SJ (1995) The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res 336:69-77

Devaux A, Pesonen M, Monod G (1997) Alkaline comet assay in rainbow trout hepatocytes. Toxicol in Vitro11: 71-79

Fairbairn DW, Olive PL, O'Neill KL (1995) Comet assay, a comprehensive review. Mutat Res 339: 37-42

Herrmann K (1995) Teratogenic effects of retinoic acid and related substances on the early development of zebrafish (*Brachydanio rerio*) as assessed by a novel scoring system. Toxic in Vitro 9:267-283

International Organization for Standardization (1996) 7346/1, 2, and 3-Water quality - Determination of the acute lethal toxicity of substances to a freshwater fish

Lange M, Gebauer W, Markl J, Nagel R (1995) Comparison of testing acute toxicity on embryo of zebrafish, *Brachydanio rerio* and RTG-2 cytotoxicity as possible alternatives to the acute fish test. Chemosphere 30: 2087-2102

Mitchelmore CL, Chipman JK (1998) DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat Res 399:135–147

National Environmental Protection Agency (1990) NEPA Guidelines for testing of chemicals, Beijing

Organization for Economic Cooperation and Development (1998) Fish, short-term toxicity test on embryo and sac-fry stages. OECD Guideline for Testing of Chemicals, OECD TG212, Paris

Östling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123: 291–298

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184-191

Strmac M, Oberemm A, Braunbeck T (2002) Effects of sediment elutes and extracts from differently polluted small rivers on zebrafish embryos and larvae. J Fish Bio 61:24-38

Sun CJ, Wang YY (2001) Research state on treatment of *p*-aminophenol production wastewater. Environ Protect Chem Ind 21: 270-273

Yang X, Kulkarni AP (2000) Lipoxygenase-mediated biotransformation of *p*-aminophenol in the presence of glutathione: possible conjugate formation. Toxicol Lett 111:253-261

Yoshida R, Oikawa S, Ogawa Y, Miyakoshi Y, Ooida M, Asanuma K, Shimizu H (1998) Mutagenicity of *p*-aminophenol in *E. coli* WP2*uvrA*/pKM101 and its relevance to oxidative DNA damage. Mutat Res 415: 139–150