Environ. Eng. Res. Vol. 11, No. 5, pp. 277~284, 2006
Korean Society of Environmental Engineers

EVALUATION OF GENETIC TOXICITY FROM ENVIRONMENTAL POLLUTANTS IN *DAPHNIA MAGNA* AND *CHIRONOMUS TENTANS* FOR APPLICATION IN ECOLOGICAL RISK ASSESSMENT

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Abstract : The genetic toxicity of environmental pollutants, namely, nonylphenol (NP), bisphenol A (BPA) and chloropyriphos (CP) was investigated in aquatic sentinel species, freshwater crustacean, *Daphnia magna*, and larva of aquatic midge, *Chironomus tentans*, using Comet assay. Physiological effect of such pollutants was also investigated by studying the specimens' rates of reproduction, growth and survival. Acute toxicity results showed that, as expected, *Daphnia* was more sensitive than *Chironomus* to chemical exposure. The order of acute toxicity was CP > NP > BPA in *D. magna* and NP > CP > BPA in *C. tentans*. BPA may exert a genotoxic effect on *D. magna* and *C. tentans*, given that DNA strand breaks increased in both species exposed to this compound, whereas NP- and CP-induced DNA damage occurred only in *C. tentans*. *In vivo* genotoxic data obtained in aquatic sentinel species could provide valuable information for freshwater quality monitoring. The experiments with NP-exposed *D. magna* showed that the pollutant has long-term effects on reproduction, whereas no short-term effect on DNA integrity was found, being an example of a false-negative result from the biomarkers perspective. This result could be interpreted that other mechanism than genetic alteration might be involved in NP-induced reproduction failure in *D. magna*. False-positive results from the genotoxic biomarker obtained in BPA-exposed *D. magna* and in NP-exposed *C. tentans* make it difficult to use DNA integrity as an early warning biomarker. However, as the mere presence of genotoxic compounds, which are potentially carcinogenic, is of high concern to human and ecosystem health, it could also be important to rapidly and effectively detect genotoxic compounds in the aquatic system in ways that do not necessarily accompany a higher level of alteration. Considering the potential of *D. magna* and *C. tentans* as bioindicator species, and the importance of genotoxic biomarkers in ecotoxicity monitoring, DNA damage in these species could provide useful information for environmental risk assessment.

Key Words : *Daphnia magna, Chironomus tentans*, Nonylphenol, Bisphenol A, Chloropyriphos, Genetic toxicity, Environmental risk assessment

INTRODUCTION

The world's surface waters continue to receive large quantities of discharges including a variety of undesirable and accidental toxic compounds. Especially, the continued presence of genotoxic and potentially carcinogenic compounds in the aquatic environment is of major concern with respect to the health of aquatic media biota and humans[1-3]. To efficiently assess the presence of mutagens in the water, aside from conventional chemical analysis, genotoxicity assays should be included as additional parameters in water quality monitoring programs[4]. This is because they have been proven to be sensitive and reliable tools in the detection of mutagenic activity in the aquatic environment. Genotoxic parameters are currently the most valuable

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biomarkers for environmental risk assessment and there are many reports on the studies linking the DNA damage to subsequent molecular, cellular and tissue level alteration of aquatic organisms[2]. DNA strand breaks are potential pre-mutagenic lesions and are sensitive markers of genotoxic damage. Among the available genotoxicity indicator tests, the Comet assay has recently attracted much attention. The Comet assay, also called the single-cell gel electrophoresis (SCGE) assay, primarily measures DNA strand breakage in single cells. The Comet assay has been shown to respond quickly and accurately, and its findings are easy to measure. Thus, since the protocol was published by Singh et al[5]., it has been increasingly used in different fields of study: clinical applications, human monitoring, radiation biology, and genetic toxicology. Application of Comet assay, using many different species, has been conducted in ecotoxicology[6-10].

In this study, environmental pollutants-induced DNA damages were investigated using Comet assay in two biomonitoring species, the freshwater crustacean, *Daphnia magna,* and the larva of the aquatic midge, *Chironomus tentans,* in order to identify genotoxic biomarkers for risk assessment. *D. magna* and *C. tentas* plays a pivotal role in aquatic food webs and has been widely used as a model species for water quality biomonitoring. Taken into account of the importance of *D. magna* and *C. tentans* in the aquatic ecosystem, information concerning genotoxicity on these species can be valuable for freshwater monitoring and environmental risk assessment. As chemical stressors, nonlyphenol (NP), bisphenol A (BPA) and chloropyriphos (CP) were selected because they are widely used environmental pollutants: NP is used in the polymer industry[11], BPA is an intermediary in the production of polycarbonate and epoxyresins[12] and CP is an organophosphorous insecticide.

To determine the sublethal exposure condition, acute toxicity tests were performed using 24-hr EC50 and 24-hr LC50 for *D. magna* and *C. tentans,* respectively. Genotoxicity was then measured upon sublethal exposure condition. In order to identify higher level consequences of genotoxic effects, reproduction failure, growth retardation and survival rate were investigated as physiological level descriptors by chemical exposure in *D. magna* and *C. tentans.*

MATERIALS AND METHODS

Organism Culture

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), we obtained *D. magna* and *C. tentans* larvae from adults reared in our laboratory. *D. magna* were individually placed in glass beakers containing a culture medium, aerated M4 media, for 2 days. Cultured daphnids were fed on the green alga *Chlorella sp.* at concentrations of 1×10^6-10^9cells/mL every 2 days; the larvae of *C. tentans*, which were fed with fish flake food (Tetramin, Tetrawerke, Melle, Germany), were reared in a 2 L glass chamber containing dechlorinated tap water and acid-washed and aerated sand. Culture of *D. magna* and *C. tentans* were maintained at 20°C ± 1°C, 16hr light and 8 hr dark cycle photoperiod regime.

Exposure Condition

We conducted the experiment at a constant temperature of 20 ± 1 ℃ under light conditions of 16 hr to 8 hr of light and darkness. For the *Daphnia* experiment, neonates less then 24 hr old were used for acute toxicity, reproduction and survival tests, and 7-day-old adults were used for the Comet assay. The fourth instar larvae collected in rearing aquaria were used for all *Chironomus* experiments. Based on the results of the acute toxicity test (data not shown), three concentrations corresponding to 1/1000, 1/100 and 1/10 of the 24-hr L(E)C50 were selected for sublethal exposure conditions. *D. magna* were exposed to 0.3, 3, and 30 μg/L for NP and BPA, and 0.001, 0.01 and 0.1 μg/L for CP for Comet assay and reproduction and survival tests, whereas, *C. tentans* were exposed to 1, 10 and 100 μg/L for NP and 5, 50 and

500 μg/L for BPA and 2, 20 and 200 μg/L for CP for the Comet assay and growth and survival tests. We transferred organisms into 200 mL beakers containing 100 mL of culture medium (for *D. magna*) or of dechlorinated tap water (for *C. tentans*), and treated them with chemicals for sublethal exposure. For each experiment, we added 0.1 mL of the test solution into the experimental beakers before introducing the larvae. Acetone was used as solvent. Three concentrations of each test chemical, solvent control (Acetone) and control were prepared for each experiment. Three replicates were prepared for each concentration.

Comet Assay

A total of 15 *Daphnia* and 10 larvae of *C. tentans* were collected 24 hr after treatment from the control and experimental tanks and were pooled for a Comet assay. Treated organisms were placed in 1 mL of phosphate -buffered saline (PBS) containing 20 mM ethylene diaminetetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and disintegrated mechanically by mincing. Cell suspension was precipitated by vortexing, and then immediately mixed with 100 μL 1% low-melting-point (LMP) agarose for use in the Comet assay. An alkaline comet assay was performed, as described by Singh et al[5]. Briefly, 100 μL 1% LMP agarose was spread on a normal agarose pre-coated microscope slide and placed at 4°C for 5 min to allow for solidification. The cells were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 NaCl, 10% DMSO, 10% Triton x 100, pH 10), and subsequently exposed to alkali (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4°C to allow for DNA unwinding and expression of alkali -liable sites. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min. After the electrophoresis, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place until image analysis. Before analysis, the slides were stained with 50-μL ethidium bromide (5 μg/mL), then analyzed at 400 × magnification using a fluorescence microscope (Nikon, kanagawa, Japan) equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm. Approximately, 25 cells per slide (3 slides per treatment) were examined. DNA damage was expressed as the tail moment (tail length × tail % DNA/100) using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

Daphnia Reproduction Test

The experiments were performed in accordance to the standard protocol for *D. magna* reproduction tests[13]. Briefly, daphnids less than 24 hr old were used at the start of each experiment. They were exposed to various concentrations of test chemicals and observed and fed daily for 21 days. Three replicates were prepared for each concentration, each consisting of 10 daphnids in 100 mL of test media. Each group was provided with *Chlorella* as food at a concentration of 5×10^5 cells/mL daily. Test animals were transferred to new medium every 2 days. Neonates were removed daily, and the numbers of neonates were counted. Survival rate was also evaluated at the end of the experiment.

Chironomus Growth Test

Body fresh weight and water content were measured on 10 larvae collected 48 hr after commencing exposure. Fresh weight was immediately measured. Larval dry weight was evaluated after placing the larvae at 105°C for 24 hr and water content was calculated from the respective dry and fresh weights. The larvae, which have been used for dry weight measurement, were subsequently placed at 540°C for 4 hr for the evaluation of ash-free dry weight. Weighing was performed to the nearest 0.1 mg. At the end of exposure, survival rate was also evaluated by counting dead larvae.

Data Analysis

Log-probit transformation of the data was

used in order to estimate 24 hr L(E)C50 values and the corresponding 95% confidence intervals. The sublethal exposure results were tested for significance using analysis of variance (ANOVA) test with Dunnett's multiple comparison test. All statistic tests were performed using spss® 12.0 KO (SPSS Incorporated, Chicago, IL, USA).

RESULTS & DISCUSSION

In this study, genetic toxicity of NP, BPA and CP was investigated in aquatic sentinel species, *D. magna* and *C. tentans*, as well as physiological level effects of such toxicity. Evaluation of genetic toxicity using Comet assay was performed on numerous wildlife organisms, including plants[14-16], worms, mollusks[9], fish[17,18], amphibians[19], and mammalians[20]. However, only few studies have been conducted on chironomid and daphnid[21].

DNA damage, particularly DNA strand breaks, was measured by Comet assay in order to evaluate whether these compounds induce any genetic toxicity in *D. magna* and in *C. tentans* (Table 1). As shown in Table 1, tail moment increased significantly in cells isolated from D. magna that have been exposed to BPA (3 and 30 μg/L). NP and CP did not seem to induce DNA strand breaks in this species. Increased tail moment by chemical exposure occurred at all concentrations tested by BPA exposure (5, 50, 500 μg/L), whereas at two highest concentrations by NP exposure (10 and 100 μg/L), and at the highest concentration by CP exposure (200 μg/L) in *C. tentans*.

As shown in Table 1, BPA may exert a genotoxic effect on *D. magna* and *C. tentans*, given that DNA strand breaks increased in both species exposed to this compound, whereas NP- and CP-induced DNA damage occurred only in *C. tentans*. For screening of genotoxic activities in aquatic environment, most genotoxic tests using Comet assays have been performed *in vitro* system from aquatic species, mostly using fish-driven cell lines[3,6] ; in this study, however, *D. magna* and *C. tentans* were exposed to each pollutant *in vivo* and DNA damage was assessed in cells subsequently isolated from them. *In vivo* genotoxic biomarker obtained in aquatic sentinel species, as in our study, could be a powerful tool in environmental monitoring. Indeed, according to Ohe *et al.*[2] and Chen and White[22], DNA damage in wildlife species measured by Comet assay could provide a sensitive and rapid genotoxic biomarker in environmental monitoring.

Table 1. DNA damage measured in NP-, BPA-, and CP-exposed *D. magna* and *C. tentans*. The results were expressed as tail moment obtained by comet assay (n=3, mean ± standard error of mean, * p<0.05)

Species	Chemicals	Concentration (μg/L)	Tail moment
D. magna	NP	0 (control)	2.914±0.833
		0.3	3.128±0.291
		3	3.951±0.144
		30	4.123±0.296
	BPA	0 (control)	3.061±0.599
		0.3	3.887±0.172
		3	4.907±0.423*
		30	5.274±0.935*
	CP	0 (control)	5.571±0.288
		0.001	6.086±1.007
		0.01	6.595±0.746
		0.1	8.090±0.326
C. tentans	NP	0 (control)	5.123±0.137
		1	7.264±1.113
		10	10.357±0.588*
		100	12.007±0.650*
	BPA	0 (control)	4.470±0.276
		5	7.080±0.452*
		50	8.8400±0.518*
		500	9.0400±0.297*
	CP	0 (control)	5.384±0.369
		2	7.155±1.776
		20	7.890±0.656
		200	12.370±2.415*

The 21-day reproduction test was performed on *D. magna* upon exposure to sublethal concentrations of each chemical (Figure 1). The number of neonates per female that had been exposed to NP decreased significantly in *D.*

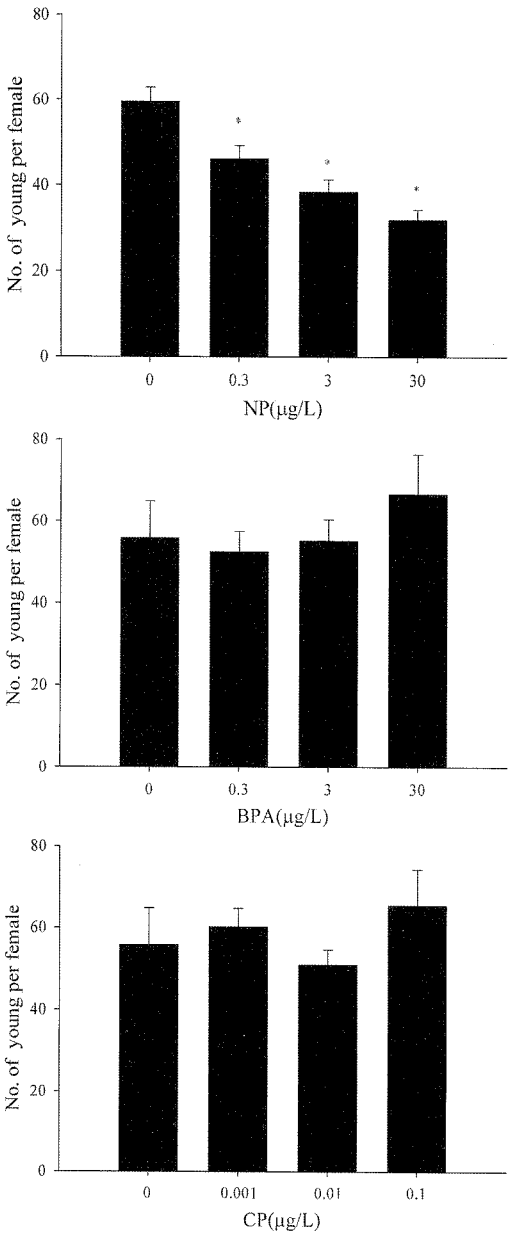

Figure 1. Number of young per female counted 21 day after exposure of NP-, BPA-, and CP in *D. magna* (n=3, mean ± standard error of mean, * $p<0.05$).

magna, which was dependent on exposure concentration. BPA and CP did not provoke any alteration on this parameter in *D. magna.*

As a growth indicator, body fresh weight and ash-free body dry weight were examined 48 hr after chemical exposure (Table 2). Larval water content was also examined as osmoregulation parameter. Body fresh weight and water content decreased at highest concentration of BPA (500 μg/L). At two highest concentrations of BPA (50 and 500 μg/L) and CP (20 and 200 μg/L), ash free body dry weight decreased in statistically significant way. No significant change was observed by NP exposure.

The survival rate was also evaluated at the end of the reproduction and growth experiments in *D. magna* and *C. tentans,* respectively (Table 3). Mortality was not observed at *Daphnia* control whereas, *Chironomus* control tests had less than 15% mortality. In both species no clear trend among treatments was apparent, except 30 μg/L of NP exposed Daphnia, where statistically significant decrease in survival rate was observed.

Biomarkers can be used to assess changes at individual and/or population levels. However, it has been widely recognized that the implementation of biomarkers, including genotoxic biomarkers in environmental monitoring is hampered by the lack of knowledge of how biomarker responses are related to population dynamics of the species in which the biomarker is applied[23,24]. Indeed, although pollutants may influence the genetic constitution of populations by causing direct damage to DNA molecules within the individual cell nucleus, the ecological relevance of changes in single cells within some tissues of some individual organisms is extremely difficult to assess[25]. Nonetheless, sensitive detection of DNA damage in wildlife species is necessary, as pollutant-induced DNA damage might influence the genetic constitution of populations. Therefore, in this study, to provide insight into the relative sensitivity and higher biological level consequences of DNA damage observed in Table 1, reproduction (*D. magna*), growth (*C. tentans*) and survival parameters (*D. magna and C. tentans*) were investigated. The experiments with NP-exposed *D. magna* show there are long-term effects on reproduction and survival, while no short-term effect on DNA integrity was found, being an example of a false-negative result from the

Table 2. Body fresh weight, water content and ash free body dry weight measured in NP-, BPA-, and CP-exposed 4th instar larvae of *C. tentans* (n=3, mean ± standard error of mean, * p<0.05)

Chemical	Concentration (μg/L)	Body fresh weight (mg/larva)	Water contents (mg/larva)	Ash free body dry weight (mg/larva)
NP	0 (control)	7.608±0.330	6.591±0.279	0.174±0.038
	1	6.501±0.413	5.820±0.295	0.136±0.049
	10	7.100±0.500	6.307±0.474	0.107±0.033
	100	7.092±0.022	6.260±0.074	0.123±0.029
BPA	0 (control)	7.650±0.288	6.680±0.191	0.205±0.033
	5	6.769±0.419	5.906±0.355	0.117±0.019
	50	6.613±0.599	5.800±0.537	0.087±0.024*
	500	6.614±0.225*	5.747±0.212*	0.114±0.027*
CP	0 (control)	7.608±0.330	6.591±0.279	0.174±0.038
	2	6.797±0.227	6.041±0.222	0.082±0.010
	20	6.801±0.646	6.016±0.552	0.064±0.010*
	200	6.552±0.308	5.763±0.260	0.069±0.006*

Table 3. Survival rate measured in NP-, BPA- and CP-exposed *D. magna* and *C. tentans* (n=3, mean ± standard error of mean, * p<0.05) Results are expressed as percentage of the total number of animal introduced at the beginning of the experiment

Species	Chemicals	Concentration (μg/L)	Survival rate (%)
D. magna	NP	0 (control)	100±0.0
		0.3	79.2±20.8
		3	71.3±23.4
		30	85.5±2.8*
	BPA	0 (control)	100±0.0
		0.3	95.8±4.2
		3	100±0.0
		30	100±0.0
	CP	0 (control)	100±0.0
		0.001	91.7±8.3
		0.01	89.2±5.8
		0.1	79.6±9.8
C. tentans	NP	0 (control)	94.4±5.6
		1	94.4±5.6
		10	83.3±0.0
		100	77.8±5.5
	BPA	0 (control)	87.8±6.2
		5	100±0.0
		50	94.4±5.6
		500	93.3±6.7
	CP	0 (control)	94.4±5.6
		2	88.9±5.6
		20	94.4±5.6
		200	88.9±5.6

biomarkers' perspective. It is clear that this type of error can occur; however, this result could be interpreted that mechanism other than genetic alteration might be involved in NP-induced reproduction failure in *D. magna*. Decrease of ash free dry weight, an indicator of *Chironomus* growth, at the high concentrations of BPA- and CP-exposure suggests that alteration of this parameter might be considered a consequence of a serious progression of the toxic effect. The fact that DNA damage occurred concomitantly with decrease in growth parameter suggests DNA alteration by these compounds might provoke higher level consequences. Moreover, in BPA exposure, *Chironomus* water content decreased at the highest concentration, it may be hypothesized that larval osmoregulation is related to alteration in DNA integrity, and thus, severe perturbation of this process may lead to growth retardation. However, our data are not sufficient to provide a clear explanation for this phenomenon. If more subcellular parameters had been tested with longer exposure period, involvement of observed DNA damage in growth and/or osmoregulation pathway could probably be better evaluated and explained. On the other hand, effects on DNA integrity in NP -exposed *C. tentans* were not related to a degree of impairment of growth or survival of the organism. False-positive results from geno-

toxic biomarker obtained in BPA-exposed *D. magna* and NP-exposed *C. tentans* make it more difficult to use DNA damage as an early warning biomarker.

The relationships between genotoxic biomarker responses and physiological/individual/population effects are complicated because of compensatory mechanisms that regulate physiological/individual fitness and population dynamics in a natural system. Some biomarkers, such as, NP- (*C. tentans*) and BPA- induced DNA damage (*D. magna*), do not appear to have a direct relationship to a higher level of biological organization. In this case, the use of biomarker will not give a reliable prediction of toxic effects upon organisms and is, therefore, only ever likely to indicate exposure to chemicals. In using such biomarkers of exposure, it is difficult to predict effects at the population level from biomarker changes measured in a sample of individuals[24,26]. However, as the mere presence of genotoxic compounds, which are potentially carcinogenic, is a major concern in human and ecosystem health, sensitive and rapid detection of genotoxic property in aquatic system itself is considered important, although it does not necessarily include alteration at a higher level of biological organization. Considering the potential of *D. magna* and *C. tentans* as bioindicator species, and the importance of genotoxic biomarkers in ecotoxicity monitoring, measurement of DNA damage in these species could provide useful information for freshwater monitoring and risk assessment.

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