J. Microbiol. Biotechnol. (2004), **14**(2), 268–275

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Detection of DNA Damage in Carp Using Single-Cell Gel Electrophoresis Assay for Genotoxicity Monitoring

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Received: January 29, 2003
Accepted: April 2, 2003

Abstract To investigate the potential application of the single-cell gel electrophoresis (SCGE) assay to carp as an aquatic pollution monitoring technique, gill, liver, and blood cells were isolated from carp exposed to a direct-acting mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), or indirect mutagen, benzo[α]pyrene (B[α]P), then the DNA strand breakage was analyzed using the assay. Based on testing 5 different cell isolation methods and 6 electrophoretic conditions, the optimized assay conditions were found to be cell isolation by filter pressing and electrophoresis at a lower voltage and longer running time (at 0.4 V/cm for 40 min). In preliminary experiments, gill and liver cells isolated from carp exposed to MNNG *in vitro* exhibited DNA damage signals even with 0.5 ppb exposure, which is a much higher dose than previously reported. In the gill cells isolated from carp exposed to 0.01–0.5 ppm MNNG *in vivo*, significant dose- and time-dependent increases were observed in the tail for 4 days. As such, the linear correlation between the relative damage index (RDI) values and time for each dose based on the initial 48-h exposure appeared to provide effective criteria for the genotoxicity monitoring of direct-acting mutagenic pollution. In contrast, the *in vivo* exposure of carp to 0.25–1.0 ppm of B[α]P for 7 days resulted in dose- and time-dependent responses in the liver cells, in which 24-h delayed responses for metabolizing activation and gradual repair after 48 h were also observed. Thus, the negative-sloped linear correlation between the RDI and time at each dose based on the initial 48 h appeared to provide more effective criteria for the genotoxicity monitoring of indirect mutagenic pollution.

Key words: Single-cell gel electrophoresis, genotoxicity, pollution monitoring, carp, direct mutagen, indirect mutagen

In recent years, scientific interest in genotoxic pollution related to inland and coastal waters has led to the development of many different mutagenesis test systems [15]. To test the mutagenicity of water and sediment samples under laboratory conditions, various biological systems such as bacteria, yeasts, plants, and aquatic animals including amphibians, mollusks, and fish have been used as bioindicators [31]. However, for the *in situ* investigation of genotoxic pollution effects, fish are considered to be more suitable organisms, as they represent the direct connection of an aquatic ecosystem to humans, accumulate pollutants, respond to mutagens at low concentrations, and activate xenobiotics through cytochrome P450-dependent oxidative metabolism, similar to mammals [7, 13]. As test organisms, fresh water fish such as carp (*Cyprinus carpio*), bullheads (*Ameiurus nebulosus*), and marine flatfish (flounder, *Pleuronectes americanus*, and turbot, *Psetta maximus*) are the most suitable for monitoring purposes, because they are sediment dwellers and sediments are known to sink with pollutants [1, 26].

From the early 1990s, studies on aquatic biomonitoring have mostly concentrated on cytochrome P450 and metallothionein as biomarkers [12]. The cytochrome P450 test has provided successful results in environmental pollution monitoring using fish [8]. The micronucleus test has also been used with fish to assess exposure to genotoxic pollutants [4]. This test estimates structural or numerical chromosome damage, which are the most commonly used indicators for assessing genotoxicity [2, 15].

However, for many years, it has also been proposed and recognized that DNA damage is useful for assessing genotoxicity and that the level of DNA strand breakage is a sensitive indicator of environmental pollutants [28]. Strand breakages are common modifications resulting from a wide range of agents and mechanisms [19] and the production of strand breakage correlates well with the mutagenic and carcinogenic properties of various pollutants [29]. As such, the measurement of strand breakage has also been applied as a biomarker.

Among the many methods used to detect strand breakage, the single-cell gel electrophoresis (SCGE) assay,

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also called the Comet assay, is a rapid, sensitive, and convenient technique for measuring DNA damage in mammalian cells [24, 30]. DNA fragments of single cells embedded and lysed in an agarose gel migrate dragging tails from the nucleoid core by electrophoresis, resulting in a 'comet' formation. The extent of DNA damage can then be determined by measuring the tail lengths or tail moments. This assay exhibits several advantages over previous techniques [5, 14]. It is highly sensitive to DNA damage expressed as single-strand breaks and alkali-labile sites, requires small samples of any eukaryotic cell population, and produces data from individual cells. Therefore, the assay is now utilized in a wide range of applications in various fields such as carcinogenesis, genotoxicity, radiotherapy, and environmental biomonitoring [5, 14, 27, 33]. Although the SCGE assay is suitable for any eukaryotic cell, there are still certain practical limitations to the application of this assay. The cell isolation techniques and experimental conditions under which assays are conducted vary considerably [33]. As such, these variables influence the apparent degree of chemically induced damage in test cells, or spontaneous damage in control cells [22].

A number of approaches have also been developed for estimating DNA damage in aquatic organisms using the SCGE assay [17, 33]. Yet, despite many published studies, the SCGE assay is not the recommended test in aquatic biomonitoring due to the lack of standardization [1]. Most previous research has focused on the possible application of the SCGE assay to genotoxic pollution assessment and the effects of certain environmental factors, such as the sex of the fish, seasonality, temperature, and so on. However, for practical application, the standardization of several parameters related to the SCGE assay, including tissue dissociation, cell suspension preparation, and electrophoresis conditions, is necessary. The tissue type in a certain organism should also be standardized because the genotoxic effects of contaminants are often tissue-specific [20]. Recently, *in vivo* genotoxicity monitoring using the SCGE assay has been conducted with carp by a few groups [10, 26, 32]. In these studies, although carp was used as the test organism for detecting genotoxic pollution, standardization of the monitoring protocol, such as determining the appropriate test tissue (cell) or establishing a quantification method, was not attempted.

Accordingly, the current study was concerned with adapting and optimizing the SCGE assay for the genotoxicity monitoring of a fresh water environment using carp. As such, gill tissue (continuously in contact with the water), liver (the main metabolizing organ), and blood (easy for nondestructive sampling) were used to test the sensitivity of the SCGE assay when using a direct mutagen, MNNG, and indirect mutagen, B[α]P.

MATERIALS AND METHODS

Chemicals

The *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), benzo[α]pyrene (B[α]P), dimethyl sulfoxide (DMSO), and *N*-*t*-butyl-a-phenylnitrone (BPN) were obtained from Sigma (St. Louis, MO, U.S.A.). Phosphate buffered saline (PBS, calcium- and magnesium-free), low-melting-point agarose (LMA), and normal-melting-point agarose (NMA) were from GibcoBRL (Grand Island, NY, U.S.A.), while general chemicals were from Merck (Darmstadt, Germany) and Sigma.

Fish

Carps, aged 10–15 months with an average length of 12.0±2.3 cm, were obtained from a fish hatchery near Pohang, Kyungbuk, Korea. The fish were kept in 100-l glass tanks containing 50-l underground water that was filtered through a cloth filter. The fish were fed a commercial pellet diet and gentle aeration was also provided. Two-thirds of the water in the tanks was replaced every 3 days.

Time-Dose Exposure

Carps were received from the hatchery at least 2 weeks prior to being used in the study and 8–12 randomly selected fish were allocated per tank and kept for adaptation. The fish were exposed to a concentrated solution of MNNG or B[α]P dissolved in DMSO, and 10-l of water was replaced with fresh mutagen-containing water every 24-h, so that the final concentration remained constant. In addition, the fish were exposed to the same concentration as other aquatic organisms in related reports [17, 20], and these concentrations were determined as 0.01, 0.05, 0.1, 0.5, and 1.0 ppm for MNNG and 0.1, 0.25, 0.5, and 1.0 ppm for B[α]P. Three fish samples were taken every 24-h after exposure and compared with the control fish kept under the same conditions, except for exposure to a mutagen.

Alkaline SCGE Assay

To prepare cell suspensions, gill tissue, liver, and blood were selected. Blood samples were collected from the fish by a cardiac puncture using 5-ml heparinized syringes, and about 100 μl of blood was added to a 10% Hank's balanced salt solution (HBSS, 10 mM PBS(pH 7.4)+20 mM HEPES+0.4% (w/v) KCl+1% (w/v) glucose). Serial dilutions were prepared so that 3–4 cells would be seen in a single field of the counting chamber at a 400× magnification. The fish were then killed by decapitation, dissected, and the gills and livers were taken out. For these tissues, 5 different methods of cell dissociation were tested as described by Belpaeme *et al.* [1] with certain modifications: (i) Mincing in PBS with 100 mM EDTA for 30 or 60 sec, (ii) filter

pressing through a 100-μm mesh filter in PBS with 100 mM EDTA at room temperature or in an ice bath, (iii) homogenizing with 5 ml of Potter-Elvehjem homogenizer in PBS with 1 mM β-mercaptoethanol at room temperature or in an ice bath, (iv) incubating in PBS containing 200 mM BPN for 30 or 60 min, and (v) incubating in RPMI 1640 medium containing 0.125% collagenase for 30 or 60 min. Cell pellets were obtained by spinning down the prepared cell suspensions at 3,000 ×*g* for 10 min and resuspending in 10% HBSS. The viability of the cells was estimated with a small amount of the cell suspensions using the trypan blue dye exclusion test, which confirmed that the cell viability was above 95%.

The alkaline SCGE assay was performed essentially as described by Singh *et al.* [30] with certain modifications. Briefly, 35 μl of 0.5% NMA solution was coated on a glass slide, solidified in an ice bath, and dried over the flame of an alcohol lamp. The appropriate amount of the cell suspensions (10 μl for the blood and gill cells and 30 μl for the liver cells) was mixed with 80 μl of 0.75% LMA at 40°C and layered on top of the precoated slide. After this layer was solidified in an ice bath, a third layer of 75 μl 0.75% LMA was added and solidified again in an ice bath. The slides were immersed in an alkali lysis buffer (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 M Tris HCl, pH 10) for 1 h at 4°C in the dark. The slides were then placed into a horizontal electrophoresis apparatus (Hoefer, San Francisco, U.S.A.), covered with electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH), and allowed to stand for 20 min, followed by electrophoresis for 40 min at 300 mA (0.4 V/cm). Thereafter, the slides were gently rinsed with distilled water for 5 min and air-dried, followed by staining with 100 μl of a fluorescent dye, YOYO-1 (10 μg/ml, Molecular Probes, Eugene, U.S.A.), mounting under a cover glass, and air-drying for 20 min. The ensuing fluorescent microscopy was carried out as previously described [25]. For one sample (slide), 100 cells were randomly selected and measured. The images were analyzed using the Comet Assay II image analysis system (Perceptive Instruments, Suffolk, U.K.) in which the tail length and tail moment were measured. All data sets were statistically analyzed to express the results as the mean tail lengths (or mean tail moments)±SD (n=100) and compared using Student's *t*-test. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Estimation of Genotoxic Sensitivity in Isolated Cells

To optimize the cell isolation from carp gill and liver tissue and the conditions of the SCGE assay, 5 different isolation methods (mincing, filter pressing, homogenizing, BPN treatment, and collagenase treatment) and several electrophoresis conditions were tested, among which the filter pressing method and a lower voltage-longer running time were selected for the best resolution (data not shown). In addition, based on results from a preliminary experiment, the blood cells were ruled out as their sensitivity was found to be lower than that of gill and liver cells (data not shown). Consequently, gill and liver cells were isolated by filter pressing, and the SCGE assay was performed at 0.4 V/cm for 40 min in all further experiments.

To examine the relevancy of the established experimental conditions for genotoxicity monitoring and to estimate the genotoxic sensitivity, the cells isolated from gill and liver tissue were treated with various concentrations of MNNG and assayed. Both types of cells revealed a dose-dependent and near-linear response from 0.5 ppb to 0.1 ppm (Fig. 1), plus there was no loss of cell viability even at a 0.1 ppm concentration. However, at a concentration higher than 0.1 ppm, there was no further increase in the response and a loss of viability was observed. Therefore, based on the results that a response could be detected at an extremely low concentration of MNNG (0.5 ppb), the SCGE assay would appear to be sensitive and appropriate for detecting DNA damage in carp. The dose-dependent response within a wide range of concentrations also indicated a good accuracy for the assay. Furthermore, the sensitivity exhibited by carp cells was higher than any previous reports on genotoxic studies of MNNG with other animal cells using the SCGE assay [17, 18, 20, 23, 35].

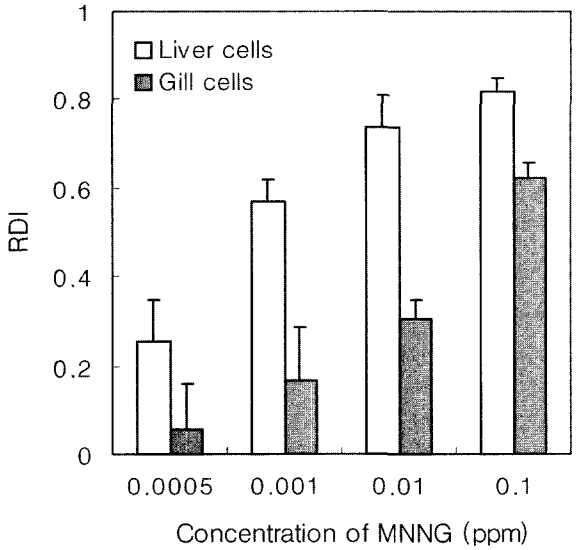

Fig. 1. *In vitro* genotoxic effects of MNNG on carp liver and gill cells.
Cell suspensions were exposed to various concentrations of MNNG at room temperature for 30 min. RDIs were then calculated from the mean tail lengths of the controls and MNNG-treated cells. Error bars represent the SD obtained from 3 independent experiments (n=300). RDIs were calculated as follows: RDI=(mean tail length of cells obtained from MNNG-treated carp- that of cells obtained from control carp)/(that of cells obtained from control carp).

Table 1. Genotoxic effect of 0.5 ppm MNNG in gill and liver cells from carp.

Treatment time	Gill/control		Gill/MNNG treated		Liver/control		Liver/MNNG treated	
	TL[a]	TM[b]	TL	TM	TL	TM	TL	TM
0 h	15.00±4.76[c]	0.29±0.42	-	-	17.51±7.75	1.23±2.97	-	-
	15.22±3.0	0.29±0.35	-	-	19.37±9.91	1.91±4.23	-	-
	16.19±3.68	0.45±1.28	-	-	18.67±7.84	1.43±3.30	-	-
24 h	14.39±4.39	0.47±1.38	24.64±10.1	2.10±3.06	17.41±7.92	1.25±3.24	23.65±11.1	2.42±3.39
	16.11±6.73	1.02±2.86	22.37±8.78	1.70±2.79	17.99±6.60	1.01±2.09	24.97±10.9	2.83±4.19
	15.55±7.90	0.57±1.91	23.75±9.35	2.08±3.09	19.96±9.73	1.60±2.92	25.88±12.6	3.59±5.09
48 h	19.54±8.33	1.33±2.80	32.20±8.12	4.88±4.51	22.90±11.3	2.65±4.26	29.45±8.58	3.60±3.54
	16.53±5.42	0.63±1.71	29.39±7.92	3.37±3.32	20.39±9.71	1.56±3.27	32.33±9.86	4.96±4.50
	17.02±4.97	0.54±0.98	32.63±10.3	5.16±3.79	19.52±8.50	1.09±1.86	31.33±13.1	3.56±2.94
72 h	18.70±7.60	0.93±1.55	31.75±6.61	2.10±3.06	19.71±10.1	1.14±2.24	25.54±9.23	1.97±2.01
	16.59±5.18	0.49±0.72	30.49±6.71	1.70±2.79	22.93±9.83	1.59±2.38	28.36±9.65	2.68±2.56
	17.94±6.09	0.65±0.92	31.94±8.11	2.08±3.09	22.01±9.41	1.39±2.21	27.01±9.41	2.63±2.11
96 h	18.14±6.01	0.78±0.52	33.71±8.11	4.20±2.70	19.08±7.42	1.39±2.21	40.13±11.8	5.88±4.29
	17.89±5.43	0.67±0.82	34.94±9.23	4.08±3.09	24.71±10.3	1.73±2.48	35.08±9.14	4.36±2.45
	19.86±6.89	1.05±1.32	35.12±7.87	5.30±4.01	20.36±8.33	1.39±2.21	34.01±8.66	5.17±3.31

[a]TL; tail length.

[b]TM; tail moment.

[c]Values represent mean tail lengths and tail moments±SD of 100 cells isolated from each carp (3 carps per condition).

***In Vivo* Dose- and Time-Dependent Response to MNNG**

MNNG, an alkylating agent (direct acting mutagen), is commonly used as a positive control compound for the production of DNA strand breaks in cells from aquatic species treated *in vitro* or *in vivo* [27]. Carps were exposed to 0.5 ppm MNNG for 4 days with the random sampling of 2 or 3 carp/condition every 24 h. Gill and liver cells isolated from carps showed significant increases ($p< 0.01$) in the DNA tail lengths and tail moments at 24 and 48 h after exposure (Table 1). After 48 h, the DNA damage seemed to be saturated in gill cells, while in liver cells, the damage decreased after 72 h, then increased again after 96 h. The coefficient of the variation [CV=(standard deviation/mean)×100] in the comet data of the tail length from the individual carp in each condition varied from 2.2% (MNNG-treated gill cells) to 13.8% (control liver cells). This substantial inter-animal variation was previously discussed by Mitchelmore and Chipman [18], where the CV between different hepatocyte preparations from different fish was found to be a mean of 29.5% for direct-acting compounds and 50.6% for compounds that require metabolic activation. Belpaeme *et al.* [1] also reported a CV for the tail length ranging from 0.18 to 39.96% for controls and from 0.13 to 61.92% for exposed fish among marine flatfish. Therefore, when compared with these reports, the variability of the data in the current study was considerably low, indicating a good stability for the SCGE assay with carp. Gill cells also exhibited a more stable response than liver cells possibly because gill tissues were in more direct and continuous contact with MNNG. It is also possible that DNA damage repair occurred in liver cells between 48 h and 72 h, as there have been various previous reports on DNA damage repair observed in the SCGE assay, yet more studies are required to investigate this further [27].

Carps were exposed to concentrations of 0.01, 0.05, 0.1, and 0.5 ppm of MNNG for 1, 2, 3, or 4 days, then gill and liver cells were isolated from 3 carps exposed to each concentration, respectively, everyday. The data points in Figs. 2 and 3 represent the RDI values calculated from the mean tail lengths of the control and exposed carps at each exposure time. The DNA damage observed in gill cells increased with the exposure time with concentrations of 0.1 and 0.5 ppm, while the concentrations of 0.01 and 0.05 ppm produced no correlation between the RDI and the exposure time (Fig. 2A). A correlation between a high concentration and a high RDI was obtained with a concentration of 0.5 ppm at all exposure times and with 0.1 ppm after 24 h of exposure, yet 0.01 and 0.05 ppm produced no significant difference. Based on the data showing that the main DNA damage occurred within 48 h, the linearity of the RDI and exposure time correlation at each concentration was analyzed. Figure 2B shows that the slopes of the linear regression increased with the exposure concentration, thereby suggesting that the linear regression of a dose- and time-dependent response curve within a certain initial period of exposure could be used as a criterion for genotoxicity monitoring. However, conversely, the variability of the DNA damage data obtained from liver cells was so high that any regression analysis using such data would provide no useful information on the dose and time dependencies of damage resulting from MNNG exposure (Fig. 3).

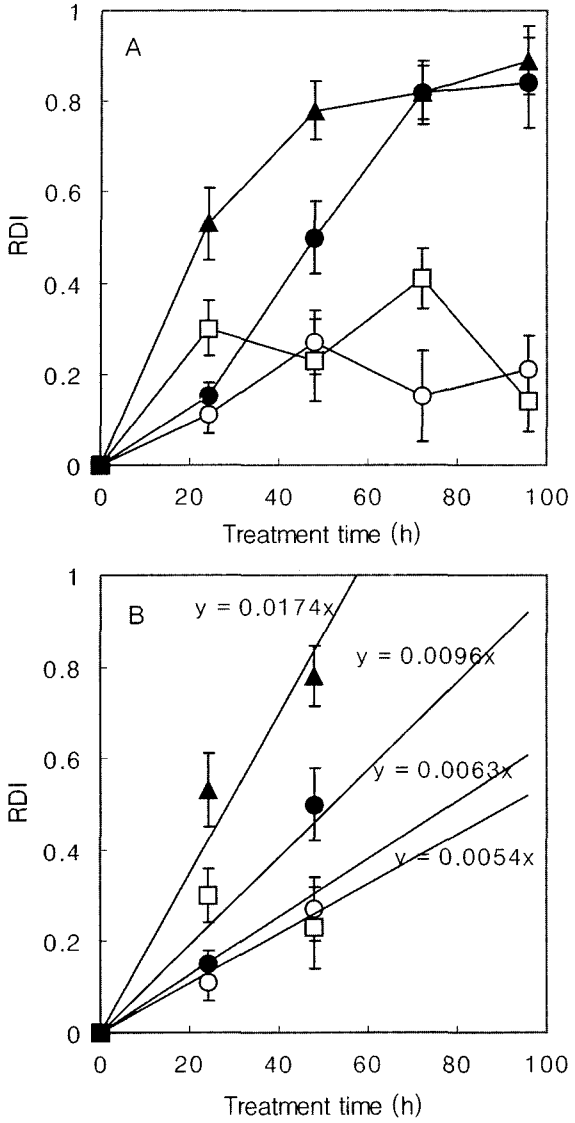

Fig. 2. Genotoxic effect of MNNG in gill cells from carp with increased exposure time (A) and correlation between RDI and exposure time with each MNNG concentration (B).
Each point represents the average value of the mean tail lengths obtained from 3 carp samples (300 tail length values). The concentrations of MNNG were 0.01 (○), 0.05 (□), 0.1 (●), and 0.5 ppm (▲). The RDIs were calculated as follows: RDI=(mean tail length of cells obtained from MNNG-treated carp- that of cells obtained from control carp)/(that of cells obtained from control carp).

In Vivo Dose- and Time-Dependent Response to B[α]P

Carps were exposed to concentrations of 0.25, 0.5, and 1.0 ppm of B[α]P for 7 days, and 3 carps were sampled each day to perform the same observations as conducted forthe MNNG exposure. Contrary to the MNNG exposure, liver cells showed dose- and time-dependent responses to B[α]P exposure (Fig. 4A). Even though the damage during the initial 24 h was slight, it was increased markedly in the 48-h exposed carp, then gradually decreased thereafter until day 7. B[α]P has already been extensively studied as an

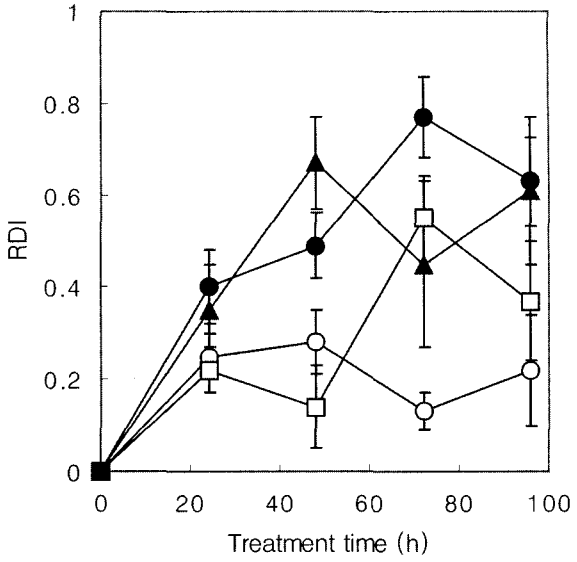

Fig. 3. Genotoxic effect of MNNG in liver cells from carp with increased exposure time.
Each point represents the average value of the mean tail lengths obtained from 3 carp samples (300 tail length values). The concentrations of MNNG were 0.01 (○), 0.05 (□), 0.1 (●), and 0.5 ppm (▲). The RDIs were calculated as follows: RDI=(mean tail length of cells obtained from MNNG-treated carp- that of cells obtained from control carp)/(that of cells obtained from control carp).

indirect mutagenic contaminant in aquatic organisms [3, 16, 21], and is principally metabolized by cytochrome P4501A (CYP1A) to form certain genotoxic metabolites, such as 7,8-diol-9,10-epoxide-benzo[α]pyrene (BPDE), and reactive oxygen species (ROS) that can react with DNA to cause DNA lesions such as DNA adducts, and oxidative DNA damage [3]. If the lesions are not correctly repaired, they can lead to the formation of DNA strand breaks [6]. The results of the delayed damage signal in the current study demonstrated that for a certain period of time liver cells metabolized B[α]P to active mutagenic compounds yet the cell DNA was eventually dramatically damaged by the activated compound(s). Subsequently, the damaged cells may have also activated detoxification process(es) based on the xenobiotic metabolizing enzymes usually involved in both metabolic activation and detoxification [9, 36]. In contrast to the case of MNNG exposure, the correlation between RDI and exposure time of more than 24 h at each concentration exhibited a negative-slope linearity (Fig. 4B). As such, if further investigations and observations are conducted and the results are statistically confirmed, RDI value at 48 h and/or the correlating absolute value of the slope could be used as criteria for the qualitative and quantitative measuring of B[α]P pollution.

In addition, as with liver cells exposed to MNNG, B[α]P-exposed gill cells exhibited a poor correlation among RDI, exposure time, and concentration (Fig. 5). Although the induction of CYP1A has previously been

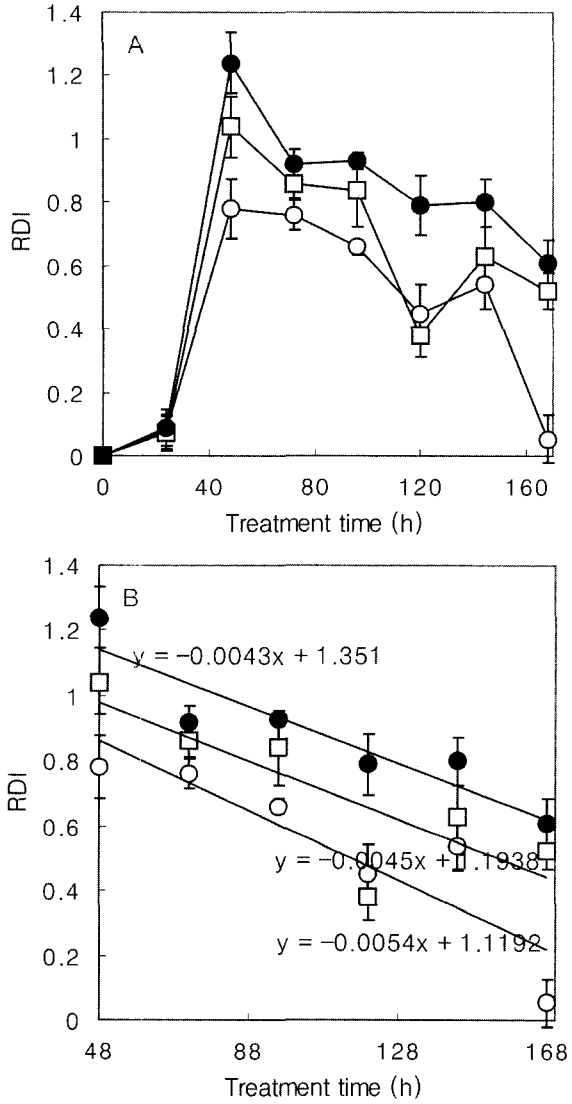

Fig. 4. Genotoxic effect of B[α]P in liver cells from carp with increased exposure time (A) and correlation between RDI and exposure time with each B[α]P concentration (B).
Each point represents the average value of the mean tail lengths obtained from 3 carp samples (300 tail length values). The concentrations of B[α]P were 0.25 (○), 0.5 (□), and 1 ppm (●). The RDIs were calculated as follows: RDI=(mean tail length of cells obtained from B[α]P-treated carp-that of cells obtained from control carp)/(that of cells obtained from control carp).

observed in gill cells [34], the specific activities are lower than those in liver and unlikely to be high enough to produce genotoxic metabolites sufficient for causing DNA strand breaks [11]. When CYP1A expression in liver and gills of rainbow trout was compared by Levine and Oris [11], it was found that liver CYP1A mRNA and ethoxyresorufin-O-deethylase levels were increased with the initial B[α]P exposure, yet decreased to a basal level thereafter, while gill CYP1A mRNA and B[α]P hydroxylase levels were increased with time and remained at high levels. These response patterns are very similar to the DNA damage

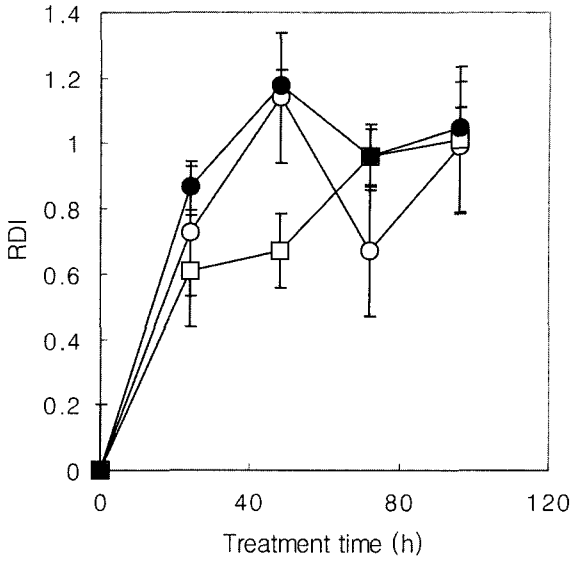

Fig. 5. Genotoxic effect of B[α]P in gill cells from carp with increased exposure time.
Each point represents the average value of the mean tail lengths obtained from 3 carp samples (300 tail length values). The concentrations of B[α]P were 0.25 (○), 0.5 (□), and 1 ppm (●). RDIs were calculated as follows: RDI=(mean tail length of cells obtained from B[α]P-treated carp-that of cells obtained from control carp)/(that of cells obtained from control carp).

responses of liver and gills found in the current study (Figs. 4 and 5). Accordingly, for the genotoxicity monitoring of mutagens causing DNA strand breakage, it would seem that gill cells are effective as the testing cells for direct mutagens, whereas liver cells are more appropriate for indirect mutagens.

Acknowledgment

This work was supported by grant (No. R01-2001-000-00222-0) from the Korea Science & Engineering Foundation.

REFERENCES

1. Belpaeme, K., K. Cooreman, and M. Kirsch-Volders. 1998. Development and validation of the *in vivo* alkaline comet assay for detecting genomic damage in marine fish. *Mutat. Res.* **415:** 167-184.
2. Campana, M. A., A. M. Panzeri, V. J. Moreno, and F. N. Dulout. 1999. Genotoxic evaluation of the pyrethroid lambda-cyhalothrin using the micronucleus test in erythrocytes of the fish *Cheirodon interruptus interruptus*. *Mutat. Res.* **438:** 155-161.
3. Canova, S., P. Degan, L. D. Peters, D. R. Livingstone, R. Voltan, and P. Venier. 1998. Tissue dose, DNA adducts, oxidative DNA damage and CYP1A-immunopositive proteins in mussels exposed to waterborne benzo[α]pyrene. *Mutat. Res.* **399:** 17-30.

4. De Flora, S., L. Vigano, F. DAgostini, A. Camoirano, M. Bagnasco, C. Bennicelli, F. Melodia, and A. Arillo. 1993. Multiple genotoxicity biomarkers in fish exposed *in situ* to polluted river water. *Mutat. Res.* **319:** 167-177.
5. Fairbairn, D. W., P. L. Olive, and K. L. ONeill. 1995. The comet assay: A comprehensive review. *Mutat. Res.* **339:** 37-59.
6. Garry, S., F. Nesslany, E. Aliouat, J.-M. Haguenoer, and D. Marzin. 2003. Assessment of genotoxic effect of benzo[α]pyrene in endotracheally treated rat using the comet assay. *Mutat. Res.* **534:** 33-43.
7. Goksøyr, A., T. Andersson, D. R. Buhler, J. J. Stegeman, D. E. Williams, and L. Forlin. 1991. Immunochemical cross-reactivity of beta-naphthoflavone-inducible cytochrome P450 (P450 IA1) in liver microsomes from different fish species and rat. *Fish Physiol. Chem.* **9:** 1-13.
8. Goksøyr, A. 1995. Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Arch. Toxicol.* **17(suppl.): 17:** 80-95.
9. Huuskonen, S., T. Rasanen, K. Koponen, and P. Lindstrom-Seppa. 1995. Time-course studies of the biotransformation enzymes in control rainbow trout when adjusting to new habitats. *Mar. Environ. Res.* **39:** 79-83.
10. Kammann, U., J. C. Riggers, N. Theobald, and H. Steinhart. 2000. Genotoxic potential of marine sediments from the North Sea. *Mutat. Res.* **467:** 161-168.
11. Levine, S. L. and J. T. Oris. 1999. CYP1A expression in liver and gill of rainbow trout following waterborne exposure: Implications for biomarker determination. *Aquat. Toxicol.* **46:** 279-287.
12. Livingstone, D. R. 1993. Biotechnology and pollution monitoring: Use of molecular biomarkers in the aquatic environment. *J. Chem. Technol. Biotechnol.* **57:** 195-211.
13. Livingstone, D. R. 1996. Cytochrome P450 in pollution monitoring: Use of cytochrome P4501A as a biomarker of organic pollution in aquatic and other organisms, pp. 143-160. *In* M. Richardson (ed.), *Environmental Xenobiotics.* Taylor & Francis, London.
14. McKelvey-Martin, V. J., M. H. L. Green, P. Schmezer, B. L. Pool-Zobel, M. P. De Meo, and A. Collins. 1993. The single cell gel electrophoresis assay(comet assay): A European review. *Mutat. Res.* **288:** 47-63.
15. Minissi, S., E. Ciccotti, and M. Rizzoni. 1996. Micronucleus test in erythrocytes of *Barbus plebejus* (Teleostei, Pisces) from two natural environments: A bioassay for the *in situ* detection of mutagens in freshwater. *Mutat. Res.* **367:** 245-251.
16. Mitchelmore, C. L., C. Birmelin, D. R. Livingstone, and J. K. Chipman, 1998. Detection of DNA strand breaks in isolated mussel (*Mytilus edulis* L.) digestive gland cells using the "Comet" assay. *Ecotoxicol. Environ. Safety* **41:** 51-58.
17. Mitchelmore, C. L. and J. K. Chipman. 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutat. Res.* **399:** 135-147.
18. Mitchelmore, C. L. and J. K. Chipman. 1998. Detection of DNA strand breaks in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell eletrophoresis (comet) assay. *Aquat. Toxicol.* **41:** 161-182.
19. Nacci, D., S. Nelson, W. Nelson, and E. Jackim. 1992. Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves. *Mar. Environ. Res.* **33:** 83-100.
20. Nacci, D. E., S. Cayula, and E. Jackim. 1996. Detection of DNA damage in individual cells from marine organisms using the single cell gel assay. *Aquat. Toxicol.* **35:** 197-210.
21. Nunn, J. W., D. R. Livingstone, and J. K. Chipman. 1996. Effect of genetic toxicants in aquatic organisms, pp. 225-251. *In* E. W. Taylor (ed.), *Toxicology and Aquatic Pollution: Physiological, Molecular and Cellular Approaches.* Society for Experimental Biology Seminar Series, Vol. **57,** Cambridge Univ. Press, Cambridge.
22. Olive, P. L., D. Wlodek, R.E. Durand, and J. P. Banath. 1992. Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp. Cell Res.* **198:** 259-267.
23. Olive P. L., J. P. Banath, and R. E. Durand. 1997. Detection of subpopulations resistant to DNA-damaging agents in spheroids and murine tumors. *Mutat. Res.* **375:** 157-165.
24. Östling, D. and K. J. Johanson. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.* **123:** 291-298.
25. Park, K. J. and C. K. Hyun. 2002. Antigenotoxic effect of the peptides derived from bovine blood plasma proteins. *Enzyme Microb. Technol.* **30:** 633-638.
26. Pandrangi, R., M. Petras, S. Ralph, and M. Vrzoc. 1995. Alkaline single cell gel (Comet) assay and genotoxicity monitoring using bullheads and carp. *Environ. Mol. Mutagen.* **26:** 345-356.
27. Rojas, E., M. C. Lopez, and M. Valverde. 1999. Single cell gel electrophoresis assay: Methodology and applications. *J. Chromatogr. B* **722:** 225-254.
28. Shugart, L. R., J. Bickham, G. Jackim, G. McMahon, W. Ridley, and J. Stein. 1992. DNA alterations, pp. 125-153. *In* R. J. Huggett, R. A. Kimerle, P. M. Mehrle, and H. L. Bergman (eds.), *Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress.* Lewis, Boca Raton.
29. Sina, J. F., C. L. Bean, G. R. Dysart, V. I. Taylor, and M. O. Bradley. 1983. Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. *Mutat. Res.* **113:** 357-391.
30. Singh, N. P., M. T. McCoy, R. R. Tice, and E. L. Schneider. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175:** 184-191.
31. Stahl, R. Jr. 1991. The genetic toxicology of organic compounds in natural waters and wastewaters. *Ecotoxicol. Environ. Safety* **22:** 94-125.
32. Sumathi, M., K. Kalaiselvi, M. Palanivel, and P. Rajaguru. 2001. Genotoxicity of textile dye effluent on fish (*Cyprinus*

carpio) measured using the Comet assay. *Bull. Environ. Contam. Toxicol.* **66:** 407–414.

33. Tice, R. 1995. The single cell gel/comet assay: A microgel electrophoretic technique for the detection of DNA damage and repair in individual cells, pp. 315–339. *In* D. H. Philips and S. Venitt (eds.), *Environmental Mutagenesis*. BIOS Scientific Publishers, Oxford.
34. Van Veld, P. A., W. K. Vogelbein, M. K. Cochran, A. Goksøyr, and J. J. Stegeman. 1997. Route-specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. *Toxicol. Appl. Pharmacol.* **142:** 348–359.
35. Yendle, J. E., H. Tinwell, B. M. Elliott, and J. Ashby. 1997. The genetic toxicity of time: Importance of DNA-unwinding time to the outcome of single-cell gel electrophoresis assays. *Mutat. Res.* **375:** 125–136.
36. Zaleski, J., R. A. Steward, and H. C. Sikka. 1991. Metabolism of benzo[a]pyrene and (-)-trans-benzo[α]pyrene-7,8-dihydrodiol by freshly isolated hepatocytes from mirror carp. *Carcinogenesis* **12:** 167–174.