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Comet Assay as a New DNA-Level Approach for Aquatic Ecosystem Health Assessments

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Little is known about DNA-level and physiological levels for health assessments of stream or river environments. Recently, comet assay, so called Single Cell Gel Electrophoresis (SCGE) is introduced for assessments of DNA damage in the medical science, food science and mammal toxicology. The comet assay is known as a biomarker which is one of the best barometers in assessing the DNA damage by oxidative stress. In this study, we conducted the comet assay using sentinel species, *Zacco platypus*, as one of the pre-warning alarm systems for the aquatic ecosystem health assessments and also applied it to Gap Stream as a model system. Tail extent moments in the S1 and S2 were 5.20 and 9.90 respectively and the moment was 19.89 in the S3. Statistical ANOVA in the tail moments showed a significant difference (n=75, $p<0.05$) between S1 and S3. Also, the proportions of DNA in the tail were 14.47, 23.64, and 30.04 μm in the upstream (control site), midstream, downstream sites, respectively. Our results in the downstream were accord with previous studies of individual-level, population-level, and community-level in Gap Stream. Our results suggest that the comet assay may be used as an important tool for diagnosing ecological health of aquatic ecosystems in the level of DNA.

Key words : comet assay, DNA-level, ecological health assessment, fish, pre-warning system

INTRODUCTION

Ecosystem health assessment methodologies have been widely applied to lotic systems in North America (US EPA, 2002) and Europe (Huguency *et al.*, 1996). One of the simplest methods in known as conventional chemical measurement such as BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), nutrients and toxic organic pollutants etc along with physical habitat evaluations (Kim *et al.*, 2003a). This approach, however had some limitations due to high diel variations (temporal) and site-specific variations in aquatic ecosystems. For this reason, US EPA developed a new methodology of ecosystem health assessments using three different biota of fish, macroinvertebrate, and periphyton, not by chemical approach, which is called "Rapid Bioassessment Protocol (RBP)." One of the main features in the RBP was an evaluations of ecosystem health in the community level, based on fish Index of Biological Integrity (IBI) and this approach is still widely using in the ecosystem health assessments in other developed countries (Oberdorff *et al.*, 1992; Harris, 1995; Lyons *et al.*, 1995; Koizumi

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et al., 1997) as well as USA (Karr, 1981). Also, the health assessments have been conducted in the population levels and the detailed methods are described in the book of "Munkittrick *et al.* (2000)" Also, Adams *et al.* (1993) and Barton (1994) used necropsy-based health assessment index, as one of the population level studies sentinel populations in the health assessments of aquatic systems. In addition to the studies of community and population levels, individual or organism levels were intensively studied for evaluations of aquatic ecosystem health (Anderson and Gutreuter, 1983; Goede and Barton, 1990).

In contrast, little is known about DNA-level and physiological levels for health assessments of stream or river environments (Seo and Lee, 2006). Recently, comet assay, so called Single Cell Gel Electrophoresis (SCGE) is introduced for assessment of DNA damage in the medical science (Park *et al.*, 2002), food science (Jeon *et al.*, 2003; An *et al.*, 2004) and mammal toxicology (Nehls *et al.*, 2005; Bony *et al.*, 2008). The comet assay is known as a biomarker which is one of the best barometers in assessing the DNA damage by oxidative stress (Singh *et al.*, 1988). For this reason, the comet assay has important role in ecological health/risk assessments of aquatic ecosystems (Adams, 1993) and some studies of comet assay are applied in the organic matter pollution (Li *et al.*, 2006) and heavy metal pollution (Isani *et al.*, 2008). Especially, fishes such as *Carassius auratus, Channa punctatus*, and *Danio rerio* are used for comet assay in foregin countries (Ali *et al.*, 2008; Frenzilli *et al.*, 2008) but the studies are seldom in Korea (Seo and Lee., 2006).

In this study, we introduced the comet assay using sentinel species, *Z. platypus*, as one of the pre-warning alarm systems for the aquatic ecosystem health assessments and also applied the approach to Gap Stream as a model system, which has a headwater reach as a control, and downstream reach influenced directly by domestic wastewater disposal plants (DWDPs), and midstream reach between the two.

MATERIALS AND METHODS

1. Descriptions of sampling sites and seasons

This study was conducted in Gap Stream, which is one of tributaries of Geum River and located in the middle of Daejeon City. Three sampling sites were chosen from the stream, based on point-sources within the watershed. Sites 1 is a upstream site, mainly surrounded by pristine forest (>70%) and rural area (20%) and the mean depth is 0.2 m, which is a typical shallow stream. Site 2 is located near city households (>60%) and partially agricultural activities (rice paddy; 20%) and the mean depth is 0.5 m during the season. Site 3 is directly influenced by two point sources of industrial wastewater treatment plants (IWTP) and domestic disposal plants (DDP), which had an average effluent discharge rate of 0.6 million ton per day and the mean depth was 0.5 m. The surveys at these sites were done twice during May~September 2000. In this study, sampling periods, based on precipitation distribution in the watershed, were categorized as low-flow (May) and high-flow season (October) in order to evaluate seasonal hydrological effect on stream conditions. The sampling sites are as follows;

S1 : Bongoek 2nd bridge, Bongoek-dong, Seo-gu, Daejeon city
(Upstream, 3rd order stream)
S2 : Manyeon bridge, Wolpyeong-dong, Seo-gu, Daejeon city
(Midstream, 4th order stream)
S3 : Gapcheon bridge, Jeonmin-dong, Daedeok-gu, Daejeon city
(Downstream, 5th order stream)

2. Fish sampling methods

At the three sites, fishes were collected from all types of the habitats including riffle, run and pool according to the method of the catch per unit of effort (CPUE) (Ohio EPA, 1989). At each sampling location, stream distance sampled was 200 m and the sampling time elapsed was 60 minutes. The sampling gears such as casting net (mesh size: 5×5 mm) and hand net (mesh size: 4×4 mm) was used to three sampling sites and the samplings were conducted toward the upstream direction. *Z. platypus* among fish species collected were maintained in the potabe fish tanks and then bloods were sampled from the species for comet assay. Total length and weight of *Z. platypus* among fish species collected were measured and examined for external abnormalies of DELT such as deformities (D), erosion (E; skin, barbells), lesion (L; open sores, ulcerations) and tumors (T), based on the criteria of Sanders *et al.* (1999).

3. Sentinel species and blood sample preparation

Three individuals of sentinel species, *Z. platypus*, with a similar range of length and weight (mean total length=10.8 cm, mean weight=9.3 g) sampled from the upstream (Us), midstream (Ms) and downstream sites (Ds) of Gap Stream were used for comet assay. Fish bloods were obtained from the caudal artery through the incision of caudal peduncle using a capillary tube and collected 50 μL bloods from each individual. Fish blood samples collected were diluted with 450 μL Hank's balanced salt solution (HBSS) and then, centrifuged for 5 minutes in 3,000 rpm. After the upper solution was removed from the centrifuged samples, blood corpuscles were obtained for the experimental analysis.

4. Commet assay and slide preparation

Alkaline single-cell gel eletrophoresis, which is known as a comet assay, was employed in this experiment to evaluate whether DNA strands in the sentinel fish species had a damage or not. Experimental procedures for sample preparations were slightly modified after the approach of Singh *et al.* (1988). Low melting-point agarose gel (LMA) of 0.75% and normal melting-point agarose gel (NMA) of 0.5% were mixed with 25 mL PBS solutions (combined solutions of NaCl, KCl, KH_2PO_4, Na_2 HPO_4 and $2H_2O$) and then boiled to melt.

The NMA of 50 μL was spreaded on glass slides (size=3″ × 1″; thick=1 mm, Fisher finest microscope slide) in the first and then covered with cover glasses (24 × 50 mm). The slides were kept under 4°C for 10 minutes to allow solidification of agarose. After that, secondary spread was conducted at the same conditions of temperature. After gently removing the cover glasses, 1 μL preprocessed blood pellet of *Z. platypus* was mixed 75 μL LMA and then spread on the slides and covered with cover glasses and the slides were kept under the conditions of 4°C for 20 minutes. After the cover glass was removed from the slide, pretreated pellet of 1 μL was mixed with 75 μL LMA by voltexing, third spread was conducted, and then cool-dried for 20 minutes after the covering of cover glass. At the same time, for the last spreading, we removed the cover glass. And then LMA of 75 μL was spreaded the slides, covered with cover glasses, and the slides were kept under the conditions of 4°C for 20 minutes. These procedures were conducted in the dark room to prevent artificial DNA damage by light and also to prevent agarose gel dried easily. The room kept the conditions of 40% humidity and 20°C temperature for the sample preparations.

5. Cell lysis and unwinding

The slides removed cover glasses were treated with lysis buffer solution (2.5 M NaCl plus 100 mM Na_2EDTA plus 10 mM Tris) containing Triton X-100, 10% DMSO (dimethyl sulfoxide) in coplin jar to remove erythrocyte. The slides were remained during 60 minutes at the dark refrigerated container to prevent artificial DNA damages by the light. The remaining blood cells were taken to analysis the DNA damage. After the lysis, the slides were immersed in unwinding buffer solutions (300 mM NaOH plus 10 mM Na_2EDTA) of another coplin jar for 40 minutes. The buffer allowed double-strand DNA to separate or be made single strand.

6. Electrophoresis and neutralization

After the lysis, the slides were placed in electrophoresis chamber and allowed the slides to be submersed in electrophoresis buffer solutions (300 mM NaOH plus 10 mM Na_2EDTA). Electrophoresis was then carried out for 20 minutes under the condition of 25 volt and 300 mA. During the electrophoresis, the chamber is surrounded by ice-pack to maintain temperature and keep steady voltage regulating the volume of buffer. Slide after electrophoresis was neutralized 3 times for 5 minutes each in 0.4 M Tris buffer and fixed in ethanol (95%) for 5 minutes. After this step, slide was dried over 30 minutes in the room temperature. The dried slides were refrigerated untill the analysis and the next day, stained to observe the DNA damage.

7. Estimation of DNA damage

The DNA in the slides were stained with ethidium bromide (ETBR) and then, covered with the cover glasses, observed from the point of 1/3 slides changing direction in zigzags. Twenty five blood cells randomly selected were counted in each slide using a fluorescence microscope (FM, Leica, Germany). Komet 4.0 comet image analyzing system (Kinetic Imaging, UK) was applied for the analysis. To determine DNA damages, we

analyzed the length of DNA fragment from the nucleus (tail length, μm, TL) and the proportion of DNA in the tail (%). Also, it was estimated as tail extent moment (TEM) × percentage of DNA in the tail (%) / 100 as other way of assessments for the DNA damage in the *Z. platypus*.

8. Statistical analysis

Statistical tests are performed using Version 12.0 of SPSS/Windows, and the results were expressed as a mean. The statistical significance tests ($p < 0.05$) between the sampling sites were determined by one way analysis of variance (one-way ANOVA).

RESULTS AND DISCUSSION

Analysis of Single Cell Gell Electrophoresis (SCGE) showed distinct differences in nucleus shapes in the three sampling sites, so the magnitude of DNA damage in the blood cell were easily identified in Gap Stream (Fig. 1a). The shape of nucleus in site 1 (S1) did not show any damage of DNA in the comet assay. In contrast in the site 3 (S3) the shape of nucleus was oval form and small tiny parts broken from the nucleus were scattered longitudinally in the image analysis (Fig. 1c). This result in the S3 indicates toward the positive (+) polar by the principles of electrophoresis. In the site 2 (S2) the shape of nucleus was similar to that in the site 1 (S1), but the tiny parcles suggested that the magnitude of DNA damage is closely associated with concentrations of various pollutants, and that when the DNA damage is severe, the head part of nucleus is shorter and the tail part is longer (Kim *et al.*, 2003b). Our image analysis of comet assay indicated downstream increases of DNA damages in Gap Stream, and the damage is most pronounced in the S3, which is directly influenced by effluents from domestic wastewater disposal plants. Our results in the S3 are accord with previous studies of individual-level (frequency of fish abnormalities) population-level (HAI values) (An *et al.*, 2006), and community-level (IBI values) (An *et al.*, 2004) in Gap Stream. The main causes of DNA damage in the S3 are not identifiable in this study but could regard that chemical measurements related with organic pollutant was the highest in S3 by previous report (An *et al.*, 2004).

We determined the magnitude of DNA damage by analyzing tail extent moment (tail length × tail% DNA/100) percentage of DNA in the tail (%) and tail length (μm) previous studies showed that in case of high damage in single DNA stand, values of the tail extent moment tail DNA (%) and tail length are getting increase in the comet assay (Cotelle and Ferard, 1999).

Tail extent moments in the S1 and S2 were 5.20 and 9.90 respectively and the moment was

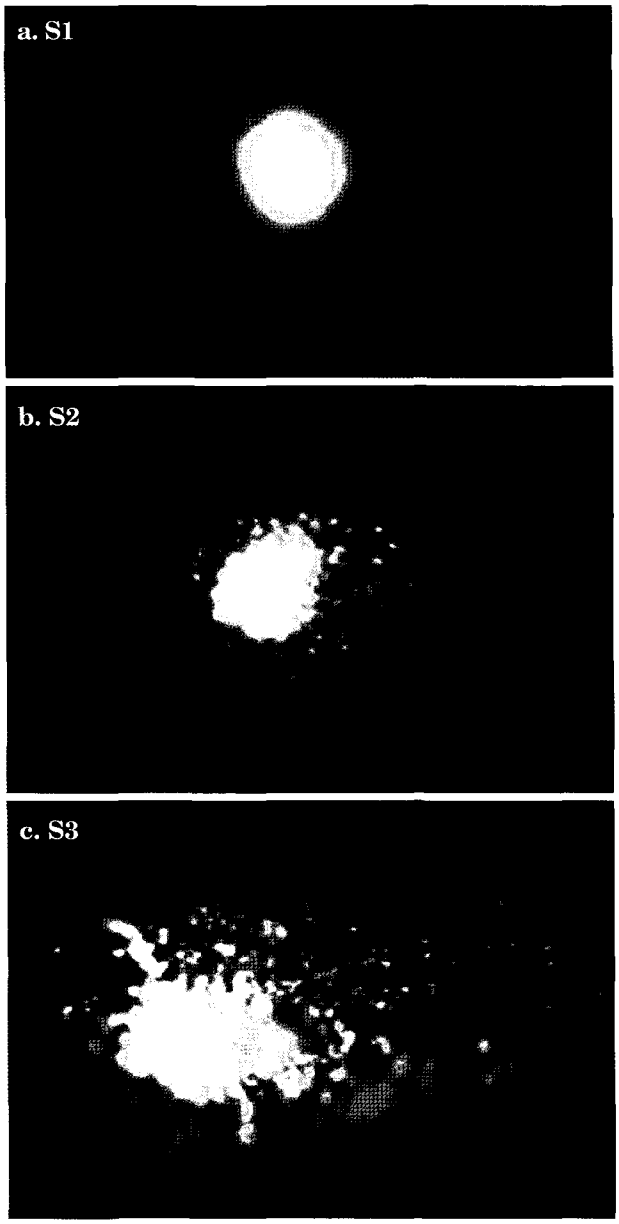

Fig. 1. Image analysis of comet assay using blood samples of *Zacco platypus* as a sentinel indicator species in three site (S1=upstream, S2=midstream and S3=downstream) of Gap Stream.

Table 1. Summary of DNA damage, assessed by comet assay (Single-Cell Gel Eletrophoresis, SCGE) from sentinel species, *Zacco platypus* in three sites of upstream (S1), midstream (S2) and downstream (S3) of Gap Stream.

Site	Sample (n=75)	Tail extent moment	Tail DNA (%)	Tail length (μm)
		Average	Average	Average
Us (S1)	1	3.94	11.73	22.54
	2	6.17	17.41	22.95
	3	5.48	14.27	22.46
	Mean	5.20	14.47	22.65
Ms (S2)	1	9.81	24.80	31.83
	2	9.67	22.20	30.54
	3	10.23	23.90	32.63
	Mean	9.90	23.64	31.67
Ds (S3)	1	22.82	29.20	59.81
	2	15.35	26.11	51.95
	3	21.50	34.81	52.39
	Mean	19.89	30.04	54.72

19.89 in the S3. Statistical t-test in the tail moments showed a significant difference (n=75, $p < 0.05$) between S1 and S3. Also, the proportions of DNA in the tail were 14.47, 23.64, and 30.04 μm in the upstream, midstream, downstream sites, respectively. Tail length also showed a longitudinal decline from the upstream to downstream sites: Tail length in the S1 and S2 were 22.65 and 31.67 while the value was 54.72. Thus, tail length was 2-fold grater in the downstream than the upstream (Table 1). Overall our results in the comet assay suggest that tail extent moment, percent of DNA in the tail and tail length were significantly (n=75, $p < 0.05$) greater in the downstream than the upstream. Thus the magnitude of DNA damage was greater in the downstream than any other sites and the greater impacts in the downstream were closely associated with degraded chemical conditions (COD > 10 mg L^{-1}, TN > 8 mg L^{-1}, TP > 1,000 μg L^{-1}) as previous studies pointed out. We believe that the comet assay as a DNA-level biomarker may be effectively used as a pre-warning monitoring tool for stream health assessments.

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