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Elevated DNA-Protein Cross-Links in Red Blood Cells of German Carp, *Cyprinus carpio*, from the Lower Passaic River, New Jersey, USA

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Recent interest in biomonitoring for exposure to environmental genotoxins has resulted in a need for detection systems in lower organisms. Fish erythrocytes are nucleated and represent a potentially advantageous model for detection of chemically-induced DNA damage. A simple, non-lethal method has been applied to the study of DNA-protein crosslinks (DPXs) in fish. The SDS/KCl precipitation technique for determination of DPXs has been demonstrated to be reliable and sensitive in several primary mammalian cell cultures and immortalized cell lines (Costa et al. 1993; 1997a; 1997b; Kuykendall et al. 1993; 1995). Several studies have demonstrated an inherent thermal instability of some chemically-induced DPXs in human and rodent cells (Kuykendall and Bogdanffy 1992; 1993; Costa et al. 1997b; Quievryn and Zhitkovich 2000). Since the ambient temperature of aquatic environments can vary greatly due to geographic, seasonal and climatic differences, an investigation of the effects of temperature on DPX formation in vitro was conducted. Formaldehyde has been previously shown to serve as an effective crosslink inducer at low doses in this assay system (Kuykendall and Bogdannfy 1995; Costa et al. 1997b; Quievryn and Zhitkovich 2000) and was used for method validation in fish RBCs isolated from hatchery rainbow trout (*Oncorhynuchus mykiss*) in this study.

Blood samples from 12 German carp (*Cyprinus carpio*) caught from an industrial, polluted river in northern New Jersey were compared to those of cohorts caught in a nearby non-industrial lake. Statistically significant increases in DPXs were detected in RBCs from river carp above those from lake carp. The role in DPXs in health is under intense investigation in various workplace situations, but has not been investigated for environmental impact. This method may serve as a reliable, rapid indicator of chemical damage to genetic material of fish in various aquatic habitats. To our knowledge, this is the only report of the use of DPXs in fish RBCs as a model system to monitor genotoxicity in an environmental study.

MATERIALS AND METHODS

Rainbow trout (0.25-0.75 kg) were collected by dip netting from the Clearspring Trout Hatchery, Sterling, Idaho. Blood was collected immediately by gill incision and drainage of 1-2 ml of blood into 15 ml Falcon polypropylene tubes containing

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approximately equal volumes of cell harvesting buffer [containing phosphate-buffered saline (PBS) with 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 50 units of heparin/ml]. To insure that clot formation did not occur, the gills were sprayed with a solution containing PBS with 1 mM EDTA (pH 7.4) just prior to incision. After blood collection, tubes were gently inverted several times to mix contents and inspect for clot formation. Those containing visible clots were discarded. Blood samples were transported back to the lab on ice and RBCs were collected by centrifugation for 5 min at 500 x g. After the plasma and buffy coat were removed, RBCs were rinsed with PBS and pelleted by centrifugation. Aliquots of 10 µl of the packed RBCs were incubated for a 23 hour acclimation period at temperatures of 5, 10, 15 and 20°C in a 15 ml volume (25 ml Falcon culture flasks). Increasing concentrations of formaldehyde (0, 0.1, 1, 5 and 10 µM) were added at 23 hrs and incubated with the cells for an additional 1 hr. After treatment, cell viability greater than 95% was assured using trypan blue exclusion microscopy. Cultured cells were harvested, rinsed once with 1 ml PBS, and resuspended to 900 µl with PBS in 1.5 ml Eppendorf tubes. RBCs were immediately lysed by the addition of 100 µl of 1 % sodium dodecyl sulfate and gentle inversion of the tube five times. The samples may be frozen at this point and stored at -70°C, if necessary. The determination of DPXs was carried out as previously described (Kuykendall and Bogdannfy 1992).

Triplicate analyses of DNA concentrations of both free and bound DNA were used to determine the percent DNA in protein-crosslinked fractions of each sample (mean value). The average values of triplicate samples ($\pm$ standard error) were used to compute a value for each crosslink level at each data point. Statistical significance was determined by analysis of variance (ANOVA) with a level set at $p<0.05$.

Carp (n=12) were electro-shocked and dip-netting on separate days in mid-December at two locations in northern New Jersey, one in a (non-industrial) lake on the Upper Passaic River basin and another on the (industrial) Lower Passaic River in Newark City, NJ. Fish were kept in a live well and blood samples were collected at the end of the day. All fish were then released unharmed. Blood was collected by gill incision and RBCs were collected, lysed and frozen at -70°C until further sample preparation could be carried out at our laboratory. Triplicate DPX determinations were carried out on 12 carp (2-5 kg live weight) from each location in a single-blind study. Data were expressed by scatter plots and analyzed by two-way ANOVA with level set a $p<0.05$.

RESULTS AND DISCUSSION

Trout RBCs were incubated for 24 hours in RPMI medium containing 5% trout serum at temperatures from 5, 10, 15 and 20°C with increasing concentrations of 0, 0.1, 1, 5 and 10 µM formaldehyde during the final hr of a 24 hr incubation

period. Control cultures (no formaldehyde) exhibited background levels near 1.26% of the DNA in the crosslinked fraction, and were not statistically different between various incubation temperatures. Statistically significant ($p<0.05$), dose-dependent increases in DPX formation were found at 1 μM formaldehyde (and above) in cells cultured at 5, 10, 15 and 20°C (Table 1). DPX increases were from 137 % to 353% above control levels in cultures containing 10 μM formaldehyde incubated at 5°C and 20°C, respectively. These data provide evidence of the usefulness of the DPX procedure in fish RBCs cultured with a known crosslinking agent and suggest that evironmental exposure of fish of other species to crosslinking agents may be demonstrated by this assay.

Table 1. DNA-protein crosslink formation in trout red blood cells cultured with increasing concentrations of formaldehyde at various temperatures

Conc.	5 °C	10 °C	15 °C	20 °C
0	1.16+0.12	1.32±0.12	1.36±0.48	1.20±0.46
0.1 μM	1.80 ± 0.30* (55.2%)	1.40±0.54 (6.1%)	2.02±0.28* (50.5%)	2.34±0.54* (95.0%)
1 μM	2.30±0.12* (98.3%)	1.88±0.14* (42.4%)	2.74±0.24* (101.5%)	3.30±1.78* (175.0%)
5 μM	2.40±0.18* (106.9%)	2.90±0.24* (119.7%)	3.36±0.58* (147.1%)	4.04±1.40* (236.7%)
10 μM	2.76±0.34* (137.9%)	4.06±0.34* (207.8%)	4.54±0.26* (233.8%)	5.44±0.88* (353.3%)

Data are expressed as % DNA in DPX fraction ± S.E. of triplicate assays. Percent change above control (in parentheses), was calculated as [(experimental-control value)/control] x100. *Significantly different from corresponding control, $p<0.05$.

This DPX procedure was then used to determine crosslink levels in carp caught from two environmental sources (Figure 1). There was a significant difference ($p<0.006$, two-way ANOVA) between DPX levels in RBCs from carp caught in a lake (1.276 ± 0.04 %, mean ± S.D.) in the Upper Passaic River basin, compared to carp caught in Newark City, Essex County, NJ on the Lower Passaic River (1.450 ± 0.067 %). The lake is a source of potable water (US Geological Survey, Water Sources Investigation Report 02-4272), while the Lower Passaic River in Newark City is home to 11 of the 18 critical sites mentioned in the Passaic River Directive of the New Jersey's State Natural Resource Damage program (Willner et al. 2003). This particular area of the Lower Passaic River contains multiple superfund sites and is known to contain high concentrations of dioxins, mercury, lead, polychlorinated biphenyls, as well as industrial dyes, heavy metals and numerous other chemicals (Wolfskill and McNutt 1998). Of particular importance is the sediment found in the Lower Passaic River, which is contaminated with high levels of dioxins, pesticides, divalent metals, chlordane, volatile organic compounds and many other hazardous substances (EPA 823-R-97-006, 1997). Contractual obligations prevent the disclosure of the exact locations of the water sources surveyed in this study.

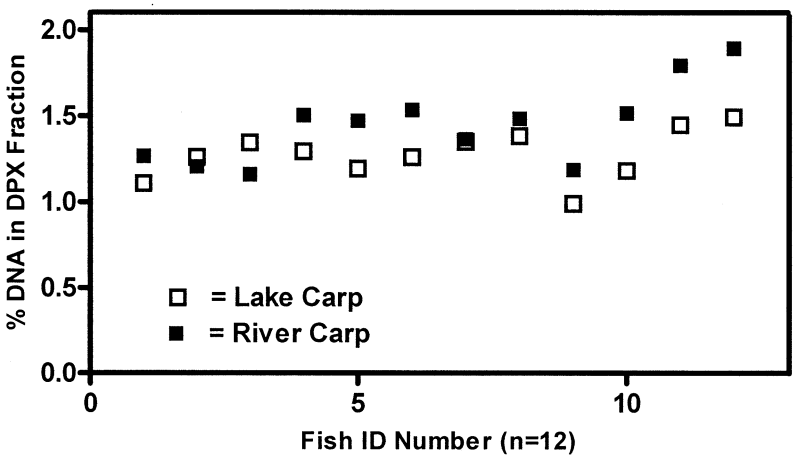

Figure 1. Comparison of DPX level in RBCs from carp taken from a polluted, industrial river and a nearby, non-industrial lake. Data represents the percent DNA in the DNA-protein crosslinked fraction of RBCs isolated from twelve carp from each location. Each data point represents the mean of triplicate samples taken (along with triplicate analyses of each sample) from each animal.

These data suggest that the assay may be useful to monitor DPX formation in fish living in environments known to have multiple, high level chemical contaminants. The use of carp as an indicator species may be advantageous due to their bottom feeding habits, an area of the biosystem where industrial sludge may form and concentrate natural toxicants. An immediately apparent limitation of this method in environmental studies is an inability to determine which type of chemical may have caused the DPXs. However, this assay would be useful to detect recent or ongoing exposure to DPX agents, as erythrocyte lifespan is on the order of several months. Studies are ongoing to determine DPX formation in RBCs of fish following controlled exposure to several known carcinogens found as aquatic pollutants, including chromium and aliphatic aldehydes. Target sites for environmental assessment of DPXs in RBCs of native fish populations would likely include industrial sites with high levels of heavy metals in the sediment.

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