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Induction of Micronuclei and Nuclear Abnormalities in the Erythrocytes of Mudminnows (*Umbra limi*) and Brown Bullheads (*Ictalurus nebulosus*)

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The discovery of high incidences of tumors among fish populations has stimulated concern over the possible association between these diseases and exposure to carcinogens/mutagens. Cytogenetic studies using fish have shown that chromosome aberrations and sister chromatid exchanges (SCE's) are induced by exposure to genotoxic agents in water (Kligerman 1982; Prein et al 1978). However, there are disadvantages to using metaphase assays such as the SCE and chromosome aberration tests. Most fish karyotypes consist of large numbers of small, irregular chromosomes.

Hooftman and de Raat (1982) described an in vivo micronucleus (MN) assay using the peripheral erythrocytes of the eastern mudminnow (Umbra pygmaea). The mature erythrocytes of this species showed well-defined MN after aqueous exposure to ethyl methanesulphonate (EMS). However, other nuclear abnormalities were reported, which have not been described for assays using mammalian species. In addition, the incidence of MN induced in peripheral erythrocytes was low (less than four per thousand) in comparison to mammalian in vivo assays.

We are interested in determining whether the MN technique can be developed as an in situ assay for genotoxicity in fish species; and in particular, the brown bullhead (Ictalurus nebulosus). In this study, central mudminnows (Umbra limi) and brown bullheads were exposed to EMS and benzo(a)pyrene (BaP) by intraperitoneal injection. The various experiments were designed to: i) Compare the sensitivity of the MN and SCE assays using the mudminnow model; ii) Determine whether MN are induced in the erythrocytes of the brown bullhead by exposure to genotoxic chemicals (EMS and BaP); iii) Determine whether erythrocytes harvested from the erythropoietic tissues of fish (pronephros) have a greater incidence of MN than peripheral erythrocytes; iv) Examine the association between the induction of MN and the induction of nuclear abnormalities in erythrocytes after exposure to genotoxic agents.

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The brown bullhead was selected as a candidate species for in situ monitoring of MN because several populations of these fish in the Great Lakes have high incidences of hepatic and epithelial carcinomas (Baumann et al 1987; Black 1983). They may be a suitable organism for examining the possible relationship between the development of fish tumors and in situ exposure to genotoxic/carcinogenic chemicals.

MATERIALS AND METHODS

Mudminnows (Umbra limi) weighing 7-11 g were collected by seine, and brown bullheads (Ictalurus nebulosus) weighing 120-180 g were collected by trap-net from uncontaminated lakes in Ontario. Mudminnows and bullheads were exposed to EMS (Sigma, St. Louis, Mo.) and BaP (Sigma) by intraperitoneal injection. For EMS, the carrier and control solutions were sterile phosphate-buffered saline (PBS). For BaP, the carrier and control solutions were corn oil. All solutions of EMS and BaP were made up just prior to injection at concentrations which would deliver doses in approximately 1 ml of solution for mudminnows, and in approximately 5 ml of solution for bullheads. Fish were treated with either a single injection of chemical, or by four injections at four-day intervals. There were three fish tested per treatment.

Mudminnows were prepared for SCE analysis according to the method of Kligerman (1979). Total time between injection of EMS solution and sacrifice of fish was 96 h. Kidney tissue was removed and placed in hypotonic solution (0.4% KCl), then fixed in 3:1 methanol/acetic acid. Slides of kidney metaphases were prepared and stained in Hoechst 33258 (Sigma). After exposure to black light in Sorenson's buffer, slides were stained with Geimsa and mounted with DPX mountant (BDH Canada, Toronto).

SCE's were scored blind for 25 metaphases per fish (n=3) from each treatment, using a magnification of x 1000. The mean number of SCE's in controls and experimentals were compared using a one-sided t-test.

Erythrocytes from both peripheral blood and the pronephros (head kidney) were prepared from mudminnows and bullheads for analysis of MN frequency. Peripheral blood was taken with heparinized capillary tubes after caudal severance, and blood was smeared on pre-washed slides. With bullheads, kidney smears were also prepared from pronephros tissue. With mudminnows, the pronephros was aspirated into a solution of 0.25% trypsin in PBS. The tissue was agitated for 15 min and the resultant cell suspension decanted into a 30-mL centrifuge tube. The suspension was centrifuged at 3,000 rpm for 5 min and the pellet resuspended in 0.5 mL PBS. The cell suspension was then smeared on slides. Smears were fixed overnight in methanol.

Erythrocytes were Feulgen stained, and counter-stained with aqueous light green according to the method of Hooftman and de

Raat (1982). In each treatment, either 7,000 or 14,000 erythrocytes were scored blind for MN and nuclear abnormalities. The scored cells were divided equally between the three individuals tested per treatment. Only cells with intact cell and nuclear membranes were scored. The frequency of MN or nuclear abnormalities in pooled control and experimental erythrocytes were compared statistically using the tables of Kastenbaum and Bowman (1970).

RESULTS AND DISCUSSION

In Experiment I, comparisons were made between the induction of SCE's and MN in mudminnows exposed to EMS. Mudminnows were exposed to 0, 25, 50, and 75 µg/g of EMS by a single i.p. injection. As indicated in Table 1, kidney cells showed elevated numbers of SCE's, with an optimum response at the 50 µg/g dose. Low frequencies of MN were observed in erythrocytes prepared from peripheral blood and the pronephros. The maximum frequency of MN (3.5 o/oo) was observed at the 25 µg/g dose. There were also low frequencies of nuclear abnormalities in erythrocytes, which were almost all gross irregularities in nucleus shape. Again, the 25 µg/g dose induced the highest number of nuclear abnormalities. There was no significant difference in the frequency of MN scored in peripheral erythrocytes in comparison to pronephros erythrocytes.

Table 1. Mean number of SCE's per kidney metaphase, number of MN per 1,000 erythrocytes (peripheral and pronephros), and number of nuclear abnormalities per 1,000 erythrocytes (peripheral and pronephros) in *Umbra limi* exposed to single i.p. injections of EMS. "Periph" refers to peripheral erythrocytes; "Proneph" refers to pronephros erythrocytes.

Treatment	SCE's/Metaphase[a] (Mean ± S.D.)	Micronuclei[b]		Abnormalities[b]	
		Periph	Proneph	Periph	Proneph
Control	4.41 (± 0.50)	0.14	0	0.14	0.71
EMS					
25 µg/g	7.73 (± 1.46)*	3.71*	3.42*	0.14	0.71*
50 µg/g	16.97 (± 2.63)*	2.00*	1.00*	1.00*	1.86*
75 µg/g	9.73 (± 1.55)*	0.86	0.71	0.86	0.29

a) 25 metaphases scored per fish in each treatment (3 fish).
b) 7,000 erythrocytes scored per treatment (3 fish pooled).
* Frequency greater than controls at $\alpha = 0.05$ level of significance.

In these experiments with the mudminnow, the *in vivo* SCE and MN assays were comparable in sensitivity. However, there were differences in the doses producing a maximum response in the two

assays (Table 1). Renault et al (1982) showed that SCE and MN induction occurs independently. MN may occur as a consequence of chromosome breakage and spindle dysfunction, while SCE's are DNA interchanges between sister chromatids at presumably homologous loci.

In Experiment II, brown bullheads were exposed to 0, 25, 50, and 100 µg/g of EMS by a single i.p. injection, or by four i.p. injections at intervals of four days. When bullheads were given single injections, there were low frequencies of MN in erythrocytes from both peripheral blood and the pronephros (Table 2a). In contrast to mudminnow data (Table 1), the only significant increase in MN frequency above controls in peripheral erythrocytes was at the 100 µg/g dose of EMS. The MN frequencies in pronephros erythrocytes were not significantly greater than controls. There were nuclear abnormalities in peripheral and pronephros erythrocytes (Table 2a) which closely resembled those observed for mudminnows.

When bullheads were exposed to multiple injections of EMS at four day intervals, the maximum genotoxic response in peripheral erythrocytes shifted to the 25 µg/g dose (Table 2b). The maximum frequencies of MN or nuclear abnormalities in these treatments were not statistically higher than the maximum frequencies in single injection treatments. In the multiple injection treatments, no erythrocytes from the pronephros were scored.

The most striking features of EMS exposures to both mudminnows and bullheads are the low frequencies of induced MN (less than four per thousand erythrocytes). Hooftman and de Raat (1982) noted similar low frequencies of MN in the peripheral erythrocytes of mudminnows after short-term, aqueous exposure to EMS. The reasons for these low frequencies are unknown, but one can speculate that the rate of erythropoiesis is low in these fish species. Walton et al (1984) reported that mudminnow heart and fin cells *in vitro* showed a greater incidence of mutagen-induced MN than did mammalian cell lines; presumably because of slower rates of DNA repair in fish cells. Thus, the low frequencies of MN noted in fish *in vivo* are not due to any cellular resistance to clastogenic activity. Therefore, it is possible that long-term, *in situ* exposure to genotoxic agents may increase the frequencies of MN in the circulating erythrocytes of bullheads.

The low frequency of MN in individual fish creates a problem for statistical analysis. If MN frequencies are to be compared between individuals, our data indicate that greater than 4,600 erythrocytes per individual should be scored. In this study, total frequencies of MN were pooled for the three fish in each treatment and tables for determining the significance of mutation frequencies (Kastenbaum and Bowman 1970) were used to test treatments against controls. Multiple injections of bullheads with high concentrations of EMS (50 and 100 µg/gm) did not increase the frequency of MN above a single treatment (Table 2).

Table 2. Number of MN and nuclear abnormalities per 1,000 cells in erythrocytes of bullheads exposed to single and multiple (4) i.p. injections of EMS.

Treatment	Micronuclei		Abnormalities	
	Periph	Proneph	Periph	Proneph
a) Single injection[a]				
Control	0	0	0.14	0
EMS				
25 µg/g	0	-	0	-
50 µg/g	0.14	0.43	2.71*	1.00*
100 µg/g	1.86*	0.14	3.14*	0.14
b) Multiple injections[b]				
Control	0.14	-	0.14	-
EMS				
25 µg/g	1.71*	-	0.71*	-
50 µg/g	0.50*	-	0.79*	-
100 µg/g	0.43	-	0.21	-

a) 7,000 erythrocytes scored per treatment (3 fished pooled).
b) 14,000 erythrocytes scored per treatment (3 fish pooled).
* Frequency greater than controls ($\alpha = 0.05$).

Table 3. Number of MN and nuclear abnormalities per 1,000 cells in peripheral erythrocytes of bullheads exposed to multiple (4) i.p. injections of BaP.

Treatment[a]	Micronuclei	Abnormalities
Control	0	0.14
BaP 5 µg/g	0.14	0.43
25 µg/g	0.71*	1.00
50 µg/g	0.86*	2.20*

a) 7,000 erythrocytes scored per treatment (3 fish pooled).
* Frequency greater than controls ($\alpha = 0.05$).

These data conform to similar multiple injection studies using mammalian species (Kliesch et al 1981).

In Experiment III, brown bullheads were exposed to 0, 5, 25, and 50 µg/g of BaP by four i.p. injections at intervals of four days. Peripheral erythrocytes showed low frequencies of MN and nuclear abnormalities after multiple exposures to BaP (Table 3). The maximum genotoxic response was at the highest chemical dose (50 µg/g). This positive response to BaP in the bullhead MN assay is significant because the polynuclear aromatic hydrocarbon class of compounds, of which BaP is a member, is an important group of environmental mutagens/carcinogens which has been implicated in the development of tumors among feral bullhead populations (Baumann et al 1987; Black 1983).

Because of low MN frequencies after short-term exposures, this genotoxicity assay does not show promise as an in vivo bioassay technique for fish. However, it may be the only suitable method for in situ monitoring of feral fish for genotoxic activity. Further tests are required to determine whether long-term in situ exposures increase MN frequency, and to determine the effects of various biotic and abiotic parameters (e.g. temperature, age, disease) upon erythropoiesis and upon MN frequency.

As in the previous study of MN in mudminnows (Hooftman and de Raat, 1982), nuclear abnormalities were noted in the erythrocytes of mudminnows and bullheads exposed to genotoxic chemicals. It is possible that these nuclear abnormalities could also be enumerated as a indicator of genotoxic activity. However, the relative frequencies of these abnormalities in the various treatments did not always conform to the dose-responses observed for MN. It is difficult to speculate on whether these abnormalities are an indicator of genotoxic activity. Further studies are required to determine their origin.

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