

Induction of chromosomal aberrations in fish *Boleophthalmus dussumieri* after exposure in vivo to mitomycin C and heavy metals mercury, selenium and chromium

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Summary

The possibilities were explored of using fish as a cytogenetic model in vivo for the detection of potential mutagens. *Boleophthalmus dussumieri* ($2n = 46$, fairly large acrocentric chromosomes), an edible mud-skipper and a widely occurring Goby along the Bombay coast, was chosen as the test species after screening 20 species of fish locally available. I.m. injections of mitomycin C in the dose range of 0.5-2.0 mg/kg body weight resulted in a significant increase in the frequency of aberrations per metaphase compared with the control. A dose-response effect was also evident. The types of aberration observed included chromatid and isochromatid breaks, fragments, rings, exchanges and unclassified markers. A significant increase in the number of gaps was also observed. Clastogenic effects of metals such as Hg, Se and Cr in the form of phenyl mercuric acetate, selenium dioxide and sodium dichromate following direct (i.m. injections) and indirect (dissolved in the aquarial water) exposures were studied. A marked enhancement was noticed in the aberration frequency at most of the dose levels tested. Spontaneous chromosomal aberrations in this species were rather rare and occurred at a rate close to zero. If developed along proper lines, fish could be a useful biological model for studying the teratogenic, carcinogenic and mutagenic effects of environmental chemicals.

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Abbreviations: Cr, chromium; Hg, mercury; MMC, mitomycin C; $\text{Na}_2\text{Cr}_2\text{O}_7$, sodium dichromate; PMA, phenyl mercuric acetate; Se, selenium; SeO_2 , selenium dioxide.

Current awareness of the potential hazards of pollutants in the aquatic environment has stimulated much interest in the use of fishes as indicators for the monitoring of environmental carcinogens, teratogens and mutagens. This is mainly because aquatic environments serve as convenient repositories for man's biological and technological wastes. Epidemiological investigations have brought to light a higher incidence of tumours in fishes from polluted water systems compared with their counterparts from relatively non-polluted waters (Brown et al., 1973). Experimental studies have shown that fishes are very sensitive to the induction of tumours by such diverse chemical carcinogens as dimethyl nitrosamine, 2-acetylaminofluorine and aflatoxin B₁, and have indicated the advantages of using fishes for bioassays of environmental carcinogens (Sato et al., 1973; Matsushima and Sugimura, 1976; Kraybill and Helmes, 1978). The utility of the guppy *Poecilia reticulata* as a model test system in which water-borne chemical mutagens could be assayed for dominant lethal effects has been amply demonstrated by Mathews et al. (1978). Studies on the clastogenic effects of acute irradiation in cultured embryonic cells of *Ameioba splendens* have indicated that the spectrum of aberrations found in these cells is similar to that obtained in irradiated mammalian cells in culture (Woodhead, 1976). Induction of SCEs by certain mutagens such as EMS, MMS, MNNG and MMC has also been reported in transformed *Ameioba splendens* cells in culture (Barker and Rackham, 1979). A cytogenetic model system, in vivo, making use of 2 fresh-water teleosts, *Umbra limi* and *Umbra pygmaea*, for the assessment of genetic damage that might occur on exposure to genotoxic agents has been developed in recent years (Kligerman et al., 1975; Kligerman, 1979; Prein et al., 1978; Alink et al., 1980). However, studies in vivo on the clastogenic effects of chemical mutagens in fishes have been rather limited.

In the present work, the possibilities of using fish species available in this part of the country as a cytogenetic model in vivo for the detection of potential mutagens were explored. Accordingly, 20 species of fish were screened for the selection of a satisfactory karyotype. Of these, *Boleophthalmus dussumieri* with a favourable karyotype consisting of 46 acrocentric chromosomes of fairly large size was chosen as the test species (Krishnaja, 1980). Other factors, such as easy maintenance in the laboratory, relatively small size, ability to withstand experimental conditions, availability in large numbers throughout the year and good yield of a number of metaphases from a wide variety of tissues, were also taken into consideration in the selection of the test species. *Boleophthalmus dussumieri* (Cuv. and Val.), an edible mudskipper, is a widely occurring goby along the Bombay coast and is a semi-terrestrial fish spending its time in tidal shallows and wet muddy flats of Bombay backwaters and creeks. The present report pertains to (1) studies with mitomycin C which is often used as a reference mutagen, (2) clastogenic effects of metal compounds such as phenyl mercuric acetate, selenium dioxide and sodium dichromate, in *B. dussumieri* after direct and indirect exposures.

Materials and methods

Boleophthalmus dussumieri, collected from Thane near Bombay, were kept in the laboratory in artificial sea water at pH 7–7.2, temperature 27–30°C. This synthetic medium was prepared according to Schmalz's formula for making large quantities of artificial sea water (NaCl 2815 g, KCl 67 g, MgCl₂ · 6H₂O 551 g, MgSO₄ · 7H₂O 692 g, CaCl₂ 145 g, deionized water 100 l). A 1:1 dilution of the above medium with deionized water was found suitable for maintaining *B. dussumieri* in the laboratory.

Well-fed healthy fishes (5–10 g body wt.), in groups of 6, were given a single dose, by intramuscular injection, of mitomycin C in the doses of 0.5, 1, 1.5 and 2 mg/kg. Mitomycin C–Kyowa (2 mg potency of crystalline mitomycin C and 48 mg of NaCl) was dissolved in distilled water. The amount of solution injected was kept constant at 1 ml/100 g body wt. The control group of 6 fishes was given the same amount of NaCl in dilution, as for the 2 mg/kg group. The doses were selected on the basis of preliminary data. A comparative study on different tissues such as gill, kidney, intestine, scale, fin, and stomach tissues in *B. dussumieri* revealed that gill tissue yielded 3 times as many usable metaphases as did the other tissues; hence this tissue was used in subsequent chromosomal aberration scorings (Krishnaja, 1980). At 72 h after treatment, an i.m. injection of 0.02% colchicine (1 ml/100 g body wt.) was given and fishes were killed 4 h later. The gills were removed and placed in 0.56% KCl hypotonic solution for 25–30 min. The gill tissues were then fixed in 3:1 methanol–acetic acid, and chromosome preparations were made according to the method already described (Krishnaja and Rege, 1980).

The following metal compounds were tested. (1) Phenyl mercuric acetate, C₆H₅HgOCOCH₃ (Excel India Ltd., Bombay). PMA is an organomercurial compound used as a fungicide in agriculture and as a slimicide in the paper and pulp manufacturing industry. (2) Selenium dioxide, SeO₂ (E. Merck), (3) sodium dichromate, Na₂Cr₂O₇ (Golden Chemicals Ltd., Bombay). Of these, PMA is acetone soluble, the other two are water soluble.

Direct exposure series

Fishes in groups of 6 were given single doses by i.m. injection in the following dose ranges. PMA, 2 and 1 mg/kg (in terms of PMA; in terms of Hg, these correspond to 1.19 and 0.59 mg/kg, respectively). SeO₂, 0.25 and 0.10 mg/kg (in terms of Se). Na₂Cr₂O₇, 5 and 1 mg/kg (in terms of Cr). Appropriate control groups were also maintained. The PMA control group was injected with the same amount of acetone as for the 2-mg group.

Indirect exposure series

This was carried out with a view to simulating the natural way of exposure, as well as to find out whether, with such a mode of exposure, the system could be used to detect mutagens present in the aquatic environment. The logarithmic series of concentrations given in Table No. 801(vi) from the American Public Health Association (1975) was used, and the following concentrations were selected: PMA, 0.56, 0.10, 0.056 and 0.010 ppm; SeO₂, 3.2, 2.1 and 1.0 ppm; Na₂Cr₂O₇, 30.5 and 24 ppm

TABLE 1

ANALYSIS OF CHROMOSOMAL ABERRATIONS INDUCED IN *B. dussumieri* AFTER TREATMENT IN VIVO WITH VARIOUS DOSES OF MITOMYCIN C

Dose (mg/kg)	Number of metaphases analysed (number of fishes)	Number of metaphases with at least 1 aberration ^a	Total number of aberrations	Aberration per metaphase ^b \pm S.D.	Total number of gaps	Gaps per metaphase ^c
0.0 (control)	350 (6)	1	1	0.0017 \pm 0.0040	0	0
0.5	343 (6)	8	8	0.0270 * \pm 0.0244	23	0.0734 ** \pm 0.0548
1.0	405 (6)	25	32	0.0794 ** \pm 0.0496	39	0.0897 ** \pm 0.0566
1.5	391 (6)	27	36	0.1010 *** \pm 0.0520	36	0.1019 ** \pm 0.0690
2.0	132 (5)	10	15	0.1186 ** \pm 0.0801	16	0.1062 ** \pm 0.0823
^d	1037 (10)	1	1	0.0004 \pm 0.0014	2	0.0022 \pm 0.0046

^a χ^2 test; $P < 0.001$.^{b,c} 2-sample t test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.^d Chromosomal aberrations in fish not deliberately exposed to any mutagens (spontaneous aberrations).

(all in terms of the respective metal ions). These concentrations were selected on the basis of earlier pilot experiments.

No mortality occurred during the 96-h exposure tests at these concentrations, except at Cr 30.5 ppm in which 50% mortality occurred by 96 h. Fishes in groups of 6 (12–15 g body wt.) were exposed to the above-mentioned concentrations, and 96-h exposure tests were carried out following, as far as possible, the standard methods prescribed by the American Public Health Association (1975) for toxicity bioassays. Aliquots of the salt solutions were added to the aquarial water to provide the necessary toxic concentrations. Appropriate control groups were also maintained. For the PMA control group, acetone was added to the aquarial water in an amount equal to the aliquot of the stock solution used for dilution for 0.56 ppm, the highest concentration tested here.

In the direct and indirect exposure series, fishes were given an injection of colchicine after 72 and 92 h, respectively, and the gill tissue was processed 4 h later for chromosome analysis as mentioned earlier. A minimum of 50 metaphase spreads per fish were examined where ever possible. Well-spread metaphases were selected, and aberration scoring was done under oil immersion according to generally accepted conventions (Kilian et al., 1977). Scoring was limited to metaphase plates that contained 40–46 chromosomes. Gaps were not included in the calculation of aberration frequency, but were scored separately. Data are expressed as aberrations per metaphase cell.

The mitotic index was established by estimating the number of metaphases in 2000 cells counted. Only those cells obviously still capable of entering mitosis were included. In fact only a rough estimation of the frequency of mitosis was possible from these preparations from small fragments of tissues in which the cells were not mixed at random. 10 slides from each group were examined for this purpose. The percentage of usable metaphases (in which 40–46 chromosomes were clearly visible) was calculated from 100 metaphase spreads counted at random. The data obtained were analysed statistically.

Results

Treatment *in vivo* with mitomycin C in the dose range of 0.5–2.0 mg/kg body wt. resulted in a significant increase in the frequency of aberrations per metaphase compared with the control (Table 1). A dose–response effect was also evident. Isochromatid breaks constituted more than 50% of the total aberrations produced at 0.5 and 1 mg dose levels. Ring chromosomes, fragments, exchanges and unclassified markers were observed more frequently at 1.5 and 2 mg dose levels. Metaphase cells with 2 or more aberrations were more frequent in the higher dose groups. The maximal number of severely damaged cells was observed in the 2-mg dose group. An increase in the frequency of gaps per metaphase was noticed in all experimental groups. The number of usable metaphases decreased with increased MMC dosage (control, 80%; 0.5 mg, 70%; 1 mg, 60%; 1.5 mg, 57%; 2 mg, 44%) although an increased mitotic index was evident compared with the control (0.5 mg, 3.96%; 1 mg,

TABLE 2

ANALYSIS OF CHROMOSOMAL ABERRATIONS INDUCED IN *B. dussumieri* AFTER DIRECT EXPOSURE (I.M. INJECTION) TO VARIOUS DOSES OF PMA, SeO₂ AND Na₂Cr₂O₇

Metal compound tested	Dose (mg/kg) ^a	Number of metaphases analysed (number of fishes)	Number of metaphases with at least 1 aberration	Total number of aberrations	Aberrations per metaphase ^b ± S.D.	Total number of gaps	Gaps per metaphase ^c ± S.D.	Poly-ploid cells
PMA	0.0 (control)	300 (6)	1	1	0.0033 ±0.0081	0	0	0
	2	321 (6)	16	23	0.0704 *	9	0.0288 * ±0.0108	3
	1	300 (6)	9	9	0.0300 ** ±0.0161	2	0.0066 ±0.0103	0
SeO ₂	0.0 (control)	300 (6)	0	0	0.00	1	0.0033 ±0.0081	0
	0.25	350 (5)	31	43	0.1200 * ±0.0313	32	0.0892 * ±0.0181	4
	0.10	300 (6)	8	10	0.0333 ** ±0.0204	8	0.0266 *** ±0.0118	2
Na ₂ Cr ₂ O ₇	0.0 (control)	300 (6)	1	1	0.0033 ±0.0081	2	0.0066 ±0.0103	0
	5	310 (6)	16	22	0.0705 * ±0.0115	11	0.0350 ** ±0.0150	4
	1	300 (6)	6	6	0.0192 ** ±0.0063	7	0.0211 *** ±0.0104	0

^a PMA, dose in terms of PMA; SeO₂ and Na₂Cr₂O₇, dose in terms of the respective metal ions.

^{b,c} 2-sample *t* test; * *P* < 0.001; ** *P* < 0.01; *** *P* < 0.05.

TABLE 3

ANALYSIS OF CHROMOSOMAL ABERRATIONS INDUCED IN *B. dussumieri* AFTER INDIRECT EXPOSURE (DISSOLVED IN AQUARIAL WATERS) TO VARIOUS CONCENTRATIONS OF PMA, SeO₂ AND Na₂Cr₂O₇

Metal compound tested	Dose (ppm) ^a	Number of metaphases analysed (number of fishes)	Number of metaphases with at least 1 aberration	Total number of aberrations	Aberrations per metaphase ^b ± S.D.	Total number of gaps	Gaps per metaphase ^c ± S.D.	Poly-ploid cells
PMA	0.0 (control)	300 (6)	0	0	0	0	0	0
	0.56	320 (6)	8	11	0.0333 * ±0.0127	12	0.0372 * ±0.0096	5
	0.10	300 (6)	5	5	0.0167 * ±0.0081	5	0.0166 ** ±0.0106	0
	0.056	300 (6)	3	3	0.0100 *** ±0.0100	2	0.0066 ±0.0103	0
	0.01	300 (6)	2	2	0.0066 ±0.0103	4	0.0133 ±0.0162	0
SeO ₂	0.0	300 (6)	0	0	0	0	0	0
	3.2	260 (6)	5	5	0.0186 * ±0.0094	4	0.0153 ** ±0.0120	1
	2.1	300 (6)	2	2	0.0066 ±0.0103	6	0.0200 *** ±0.0400	0
	1.0	350 (6)	2	2	0.0033 ±0.0074	2	0.0066 ±0.0140	0
Na ₂ Cr ₂ O ₇	0.0	300 (6)	0	0	0	0	0	0
	30.5	108 (5)	6	7	0.0637 ±0.0112	10	0.0937 * ±0.0285	0
	24.0	300 (6)	5	7	0.0300 * ±0.0109	8	0.0333 * ±0.0142	2

^a Dose in terms of the respective metal ions.^{b,c} 2-sample *t* test; * *P* < 0.001; ** *P* < 0.01; *** *P* < 0.05.

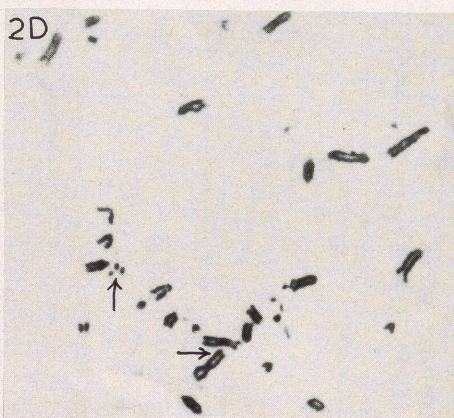
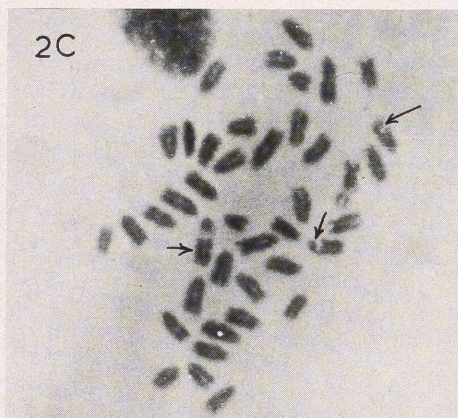
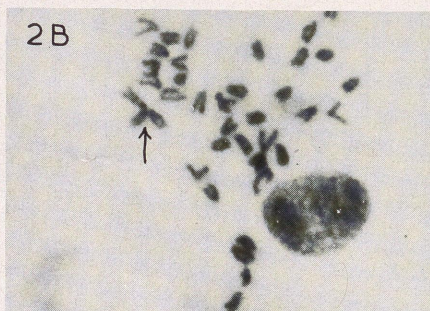
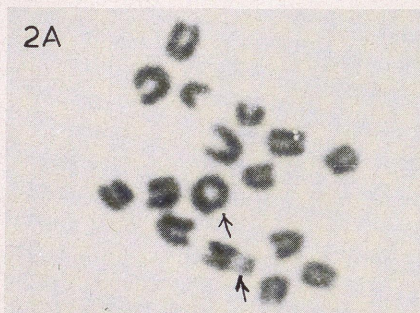
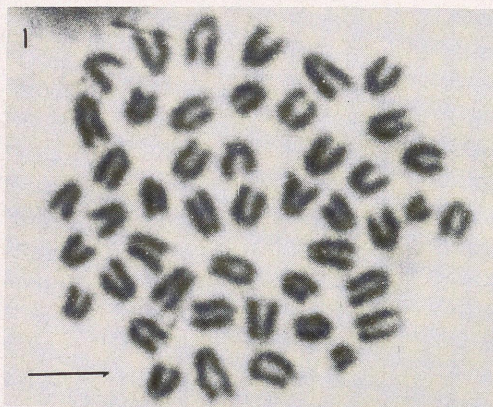


Fig. 1. Metaphase spread from *B. dussumieri*, $2n=46$. Tissue, gill. Bar indicates $5\ \mu\text{m}$.

Fig. 2. Various types of chromosomal aberrations obtained after treatment. (A) ring chromosome, isochromatid gap, MMC 1.5 mg/kg; (B) exchange figure, MMC 1.5 mg/kg; (C) isochromatid break, chromatid break, chromatid gap, Cr 24 ppm; (D) part of a severely damaged cell, fragments and other unusual chromosome morphology, MMC 2 mg/kg.

4.17%; 1.5 mg, 4.6%; 2 mg, 4.6%; control, 1.5%). An analysis of spontaneous chromosomal aberrations in *B. dussumieri* showed that these were rather rare and occurred at a rate close to zero (Table 1).

The results of cytogenetic analysis after direct and indirect exposure to PMA, SeO_2 and $\text{Na}_2\text{Cr}_2\text{O}_7$ are summarized in Tables 2 and 3. Out of a total of 1800 metaphases examined in the control series, only 2 chromatid breaks and 3 isochromatid gaps were noticed. Direct as well as indirect exposure to PMA, SeO_2 and $\text{Na}_2\text{Cr}_2\text{O}_7$ increased the frequency of chromosomal aberrations at all dose levels except for Hg at 0.01 ppm and Se at 2.1 and 1.0 ppm. Aberrations noticed included chromatid and isochromatid breaks, rings, fragments, unclassified markers and exchanges. Exchange figures were seen frequently only in the selenium-exposed series. A significant number of gaps was also induced by exposure to these metal compounds except for PMA 1 mg/kg, Hg 0.056 and 0.01 ppm and Se 1.0 ppm. At all exposure levels, the numbers of usable metaphases showed a decreasing trend, although increased mitotic activity was noticed. A metaphase spread with 46 acrocentric chromosomes from the gill epithelial cells of *B. dussumieri* as well as representative aberration patterns obtained after treatment with MMC and the metal compounds are shown in Figs. 1 and 2.

Gill and muscle tissue from the control group analysed for determining the background concentration of mercury revealed no detectable concentration of mercury (flameless atomic absorption spectrophotometric method). The background concentrations of selenium and chromium in these samples were not determined.

Discussion

The present study has shown that a significant number of chromosomal aberrations was induced in *B. dussumieri* by mitomycin C and also by Hg, Se and Cr compounds, after direct as well as indirect exposures. Almost all the mitomycin-induced isochromatid breaks and exchanges were located near the centromere regions. Earlier cytogenetic studies showed that MMC preferentially affects centromeric heterochromatin (Natarajan and Raposa, 1975). Studies in vivo in mice had also shown that the maximally affected parts of chromosomes are the late-replicating constitutive heterochromatin zones, as identified by C banding (Adler, 1974). An overall comparison of the chromosome-breaking efficiency of MMC in the present study with mammalian systems in vivo showed that MMC induced a smaller number of aberrations in *B. dussumieri*. Barker and Rackham (1979) also reported that fish cells (transformed *Ameioba splendens* cells in culture) are less sensitive to MMC than higher vertebrate cells in the induction of SCEs. Inherent inter- and intra-species differences in drug metabolic pathways might also play a role in such findings.

In the direct-exposure series, the gill tissue was processed for chromosome analysis after exposure for 76 h. This was based on an earlier finding by Kligerman and Bloom (1976), who reported that chromosomes from gill tissue of *Umbra limi* displayed the highest incidence of sister chromatid differentiation 6.25 days after

injection of BrdU. Thus, it is reasonable to assume that the cell-cycle duration in gill epithelial cells of fish may be around 75 h, unless of course it varies in different fishes. The finding of an increased mitotic index after exposure to mitomycin C and the metal compounds may be attributed to a stimulation of mitotic activity to compensate for an earlier mitotic delay or cell damage that might have occurred.

Selenium induced a large number of breaks followed by mercury and chromium in the direct-exposure series. Selenium was the most toxic when given as i.m. injections, so much so that the highest dose tested could be only 0.25 mg/kg. Even at this dose, one of the experimental fishes died at 72 h. For selenium, therefore, the dose range tested had to be narrow. Lo et al. (1978) pointed out that one of the difficulties in uncovering the mutagenic action of selenium compounds is their high toxicity and the relatively narrow range of concentrations that lead to a mutagenic effect rather than a lethal one.

In the indirect-exposure series with the 96-h exposure period, mercury induced a higher number of aberrations followed by selenium and chromium. Chromium at 30.5 ppm (96 h LC₅₀ value) reduced the mitotic index drastically, resulting in a smaller number of metaphases available for scoring of chromosomal aberrations. In fact the frequency of aberrations detected at this concentration may be lower than the actual value, because many of the cells might have been severely damaged, rendered incapable of division and therefore eliminated. The lowest concentrations of mercury and selenium tested did not show any increase in aberration frequency. It appears that, during indirect-exposure tests with low dose levels, a long enough time lag is essential before the metal is absorbed and accumulated for causing mutagenic effects.

Moreover, absorption and accumulation studies with mercury, selenium and chromium in fishes have clearly brought out the fact that there is a differential absorption in various tissues, and the concentrations of these metals are comparatively low in gills (Mckim et al., 1976; Buhler et al., 1977; Hodson et al., 1980). Mckim et al. (1976) showed that, on exposure to methyl mercury chloride, blood, spleen and kidney accumulated Hg most rapidly and contained the highest residues followed by liver, brain, gonad and muscle in first and second generations of brook trout. Buhler et al. (1977) found that hexavalent chromium tends to accumulate more in opercular bone, spleen, kidney, gastro-intestinal tract and gall bladder than in gills. Further, chromium accumulation was not distributed proportionally among various subcellular fractions, and in liver and gill it was concentrated in the cell cytosol. Hodson et al. (1980) also reported that higher selenium concentrations were noticed in liver, digestive tract and kidney and these organs appeared to be better indicators of selenium exposure than other tissues. Considering the fact that the accumulation of these metals is comparatively low in gills, the frequency of chromosomal aberrations induced in the gill epithelial cells of *B. dussumieri* following exposure to these metallic compounds is noteworthy.

Valency state is important in the expression of mutagenic effects of metals, as evidenced from studies with selenium and chromium. Nakamuro et al. (1976) found tetravalent selenium to be more effective than hexavalent in inducing chromosomal aberrations and gaps. Hexavalent chromium was a more potent inducer of chro-

mosomal aberrations than the trivalent form (Nakamuro et al., 1978). The metals studied here are known to be mutagenic in most of the systems tested.

In general, the present study indicates that fish as a cytogenetic model in vivo could be a promising system in mutagenicity studies, especially in monitoring the aquatic environment, and merits further exploration. The advantages of this model are various; for example it is a system in vivo that takes into account the metabolic and detoxifying mechanisms, the possibility of direct and indirect exposures, the availability of good quality metaphase spreads from a wide variety of tissues, the consistently low incidence of spontaneous aberrations and the feasibility of testing a large number of specimens at low cost.

However, to bring this system to the level of a standardized testing protocol, much remains to be done. Some of the aspects needing to be covered are (1) a more comprehensive screening involving a larger number of fish species to detect an ideal karyotype, (2) cell-cycle kinetics studies for the tissues concerned to make the chromosomal aberration analysis more meaningful, and (3) collection of more data to establish the exposure time for indirect exposure.

Increasing interest is now being evinced in using fish as a model for studying the teratogenic, carcinogenic and mutagenic effects of environmental chemicals. If developed along suitable lines, fish as a system could be a useful biological model in such studies.

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