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Induction of chromosomal aberrations in mitotic chromosomes of fish *Boleophthalmus dussumieri* after exposure *in vivo* to antineoplastics Bleomycin, Mitomycin-C and Doxorubicin

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Summary: The mitotic chromosomes from the gills of the fish *Boleophthalmus dussumieri* were studied for the induction of chromosomal aberrations after *in vivo* treatments with three separate antineoplastic antibiotics: Bleomycin (radiomimetic drug), Mitomycin-C (bifunctional alkylating agent) and Doxorubicin (intercalating agent). Fishes were directly exposed to these agents via intramuscular injections. Two separate treatment timings, 4 hours and 24 hours were selected for all antineoplastic treatments. The concentration doses of Bleomycin (150µg and 300µg), Mitomycin-C (1 µg, 2 µg and 4 µg) and Doxorubicin (10 µg, 20 µg and 40 µg) were decided on the basis of the body weight of fishes and earlier laboratory findings. The results revealed dose and time dependent increase in the chromosomal aberrations after treatments with all antineoplastic drugs. The type of aberrations observed include chromatid breaks, acentric fragments, dicentric and ring configurations. The findings add to the fact that the fish *Boleophthalmus dussumieri* is a suitable cytogenetic model for *in vivo* detection of potential mutagens.

Keywords: *Boleophthalmus dussumieri,* chromosomes, Bleomycin, Mitomycin-C, Doxorubicin, clastogenic effects.

Introduction

Boleophthalmus dussumieri a pop-eyed edible mudskipper is a widely occurring species along the muddy coasts of back waters and creeks of western India. This fish has already been shown to be a satisfactory cytogenetic model *in vivo* for the mutagenic studies (Krishnaja & Rege, 1980). The characteristic factors of its propitious karyotype with all acrocentric 46 large chromosomes, good yield of metaphases from wide variety of tissues, convenient size, high threshold levels in experimental conditions, long maintenance in the laboratory condition and availability through out the year enhance the suitability and practical feasibility of the species to be used for mutagenic studies.

Short term cytogenetic assays have been extensively used by mammalian toxicologists; however, few aquatic toxicologists incorporate their use. The cytogenetic methodologies offer best opportunities for detecting the effects of genetically active substances on the genome of fishes. The commercial importance of many fishes that serve as food and the fact that they represent the transfer route for many of the toxic substances warrants detailed studies.

Fishes have been reported to be useful genetic models for the evaluation of pollution in the aquatic ecosystems (Mitchell & Kennedy, 1992; Park *et al*., 1993). Neoplasias in fishes have been reported on several occasions (Black *et al*., 1980; 1982). Epidemiological studies carried out by Brown *et al*. (1973) reported incidences of tumors in fishes from polluted waters as compared to non polluted waters. The detailed cytogenetic studies on two fresh water teleosts *Umbra limi* and *Umbra pygmae* have been carried out for the assessment of genotoxic agents (Klingerman, 1979; Prien *et al*., 1978; and Alink *et al*., 1980).

Krishnaja and Rege (1982) reported increase in the frequency of chromosomal aberrations from the fish *Boleophthalmus dussumieri* after *in vivo* exposure to Mitomycin-C and heavy metals mercury, selenium and chromium. Induction of SCEs by certain mutagens EMS, MMS, MNNG and MMC has been reported in transformed *Amaeca splendens* cells in culture (Barker &Rackhman, 1979). In laboratory tests involving fishes, several substances have been shown to have genotoxic potentials (Odeigah & Osaneyinpeju, 1995; Minissi *et al*., 1996).

In spite of the above referred studies there is paucity of information on *in vivo* systems where the effectiveness of antineoplastic antibiotics and their clastogenic potentials have been studied in particular on the chromosomes of fishes. This prompted us to undertake the present study. We report here the clastogenic potentials of three antineoplastic antibiotics viz Bleomycin (radiomimetic drug), Mitomycin-C (bifunctional alkylating agent) and Doxorubicin (intercalating agent) on the mitotic chromosomes of *Boleophthalmus dussumieri*.

Materials and Methods

Live specimens of *Boleophthalmus dussumieri* were collected from 'Dumas' port near Surat and were brought to the laboratory condition at least 10

Table 1. Treatment protocol

Chemicals	Dose (µg)	Treatment duration (h)	No: of fishes
Bleomycin (BLM) (Nippon	0 - (control)	0	12
Kayaky.Co.Ltd.)	150	4	12
JAPAN	300	4	12
	0 - (control)	0	12
	150	24	12
	300	24	12
Mitomycin-C (MMC) (Kyowa, Biochem.	0 - (control)	0	12
Pharm. Inds.)	1	4	12
BOMBAY	2	4	12
	4	4	12
	0 - (control)	0	12
	1	24	12
	2	24	12
	4	24	12
Doxorubicin (DOX)	0 - (control)	0	12
(Adria Laboratories)	10	4	12
ITALY	20	4	12
	40	4	12
	0 - (control)	0	12
	10	24	12
	20	24	12
	40	24	12

days prior to experiment by maintaining in artificial sea water prepared according to Schmalz's formula (NaCl = 2815gm, KCl = 67gm, $MgCl_2$ $6H_2O$ = 551gm, $MgSO_4$ $7H_2O$ = 692gm $CaCl_2$ = 145gm and deionised water 100 Ltrs). Fishes were regularly checked for health conditions, behavioral patterns and infections, if any. Well fed fishes of 8-10 gms body weight and 7-10 cms body length were selected for the experiments. Weight difference in the test specimens was kept to the lowest possible extent. The fishes were directly exposed to the agents via intramuscular injections. Table 1 represents treatment protocols. The concentration doses of Bleomycin (150µg and 300µg), Mitomycin-C (1 µg, 2 µg and 4 µg) and Doxorubicin (10 µg, 20 µg and 40 µg) were fixed as per 10 gm body weight of fishes. The selection of these doses was based on the preliminary experiments carried out in our laboratory. The amount of solution injected was kept constant at 1 ml/100 gm body weight.

Once the treatment was over, controls as well as exposed group of fishes were given an intraperitoneal injection of 0.01 % colchicine at the rate of 0.1 ml/10 gm body weight. After 4 hours of colchicine treatment fishes were sacrificed. Gills were removed carefully and placed immediately in 0.075 M KCl hypotonic solution. The gill tissues were minced into small pieces by scissors and left in the same hypotonic for about 40-50 minutes at room temperature. Next the gill tissues were fixed in 3:1 Methanol- Acetic acid. Chromosome preparations were done following the procedure of Ojima *et al*. (1972) with some modifications (Gadhia *et al*., 1990). Air dried method was followed for slide preparations however, preheating of slides to 50^0 C (as in contemporary methods) was not preferred due to changes in the gross morphology of the chromosomes. The slides were coded blind to avoid observer's bias. Slides were stained with 2% Giemsa for 15 minutes. Metaphase plates were examined microscopically using 100-X oil immersion lens. About 35 well spread metaphases per fish were scored to detect chromosomal aberrations. Metaphase plates containing less than 40 chromosomes were not included in the scoring. Gaps were not included in the calculation of aberration frequency, but were recorded separately.

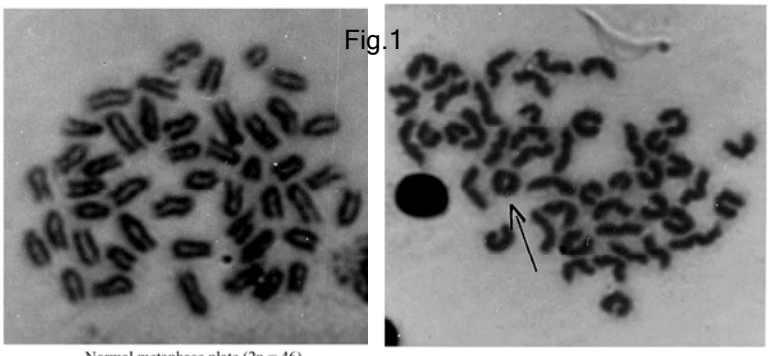
Statistical analysis

Data were expressed as mean aberrations per metaphase cell. The Students't' test was employed to compare the significance of the observed differences between the means of experimental and control groups.

Results

The results of cytogenetic analysis in *Boleophthalmus dussumieri* after direct exposure to various doses of Bleomycin, Mitomycin-C and

Fig.1



Normal metaphase plate (2n = 46) Metaphase plate showing ring chromosome

Table 2. Analysis of chromosomal aberrations induced in B. dussumieri after treatment in vivo with Bleomycin (data pooled from twelve fishes per treatment)

Dose/Concentration µg/10 mg body weight	Treatment Timings (Hours)	Total No. of Metaphases Analysed	Aberration %	Type of Aberrations		
				Chromatid Fragments	Chromosome Fragments	Ring Chromosomes
0 - (control)	0	405	0	0	0	0
150	4	415	1.92	3	2	3
300	4	398	3.26*	5	4	4
0 - (control)	0	400	0.25	1	0	0
150	24	380	3.94*	8	4	3
300	24	412	5.58**	10	7	6

** Significantly different at (P < 0.05) from the respective control*
*** Significantly different at (P < 0.01) from the respective control*

Doxorubicin are summarized in Tables 2, 3 & 4 respectively. Significant increase in the enhancement of chromosomal aberrations per metaphase cell was noticed particularly after 24 hours exposure for each of the agents tested. With whole, time and dose dependent increase in the aberrations were recorded after treatment of each agents.

Both chromosome and chromatid type aberrations were noticed. The chromosome type

Table.3. Analysis of chromosomal aberrations induced in B. dussumieri after treatment in vivo with Mitomycin-C (data pooled from twelve fishes per treatment)

Dose/Concentration µg/10 mg body weight	Treatment Timings (Hours)	Total No. of Metaphases Analysed	Total No. of Aberration	Aberrations per metaphase ± S.D.	Total No. of Gaps	Gaps per metaphase ± S.D.
0 - (control)		430	0	0	0	0
1	4	380	3	0.0078 ± 0.003	7	0.0184 ± 0.002
2	4	410	11	0.0268 ± 0.002*	9	0.0219 ± 0.001*
4	4	390	18	0.0461 ± 0.007**	15	0.0384 ± 0.004
0 - (control)		410	1	0.0024 ± 0.0001	0	0
1	24	390	9	0.0230 ± 0.007	23	0.0589 ± 0.004
2	24	410	23	0.0560 ± 0.005*	37	0.0902 ± 0.007*
4	24	400	34	0.0857 ± 0.008**	44	0.1100 ± 0.009**

** Significantly different at (P < 0.05) from the respective control*
*** Significantly different at (P < 0.01) from the respective control*

4 hours treatment timing only higher doses of the agents seemed to induce aberrations. Thus as a aberrations (mainly observed after 4 hours treatment timing) included acentric fragments,

Table 4. Analysis of chromosomal aberrations induced in B. dussumieri after treatment in vivo with Doxorubicin (data pooled from twelve fishes per treatment)

Dose/Concentration µg/10 mg body weight	Treatment Timings (Hours)	Total No. of metaphases analysed	Total No. of Aberration	Aberrations per metaphase ± S.D.	Total No. of Gaps	Gaps per metaphase ± S.D.
0 - (control)		380	0	0	0	0
1	4	415	3	0.0072 ± 0.003	7	0.0240 ± 0.003
2	4	395	7	0.0177 ± 0.006*	9	0.0430 ± 0.006*
4	4	440	10	0.0227 ± 0.005**	15	0.0477 ± 0.007*
0 - (control)		450	0	0	0	0
1	24	385	8	0.0207 ± 0.006*	23	0.0415 ± 0.003
2	24	390	13	0.0333 ± 0.003**	37	0.0538 ± 0.007*
4	24	420	17	0.0404 ± 0.007**	44	0.0809 ± 0.008**

* *Significantly different at (P < 0.05) from the respective control*

** *Significantly different at (P < 0.01) from the respective control*

dicentric and ring configurations (Fig.1) as well as translocations; while chromatid type of aberrations (mainly observed after 24 hours treatment timing) included chromatid breaks and interchanges.

Since gaps were not included in calculating aberration frequency and so were recorded separately, their quantification is also given separately in the Tables 3 & 4. A similar trend of dose and time dependent increase in the gaps was observed after treatments of Mitomycin-C and Doxorubicin.

Discussion

The mitotic chromosomes from the gills of the fish *B. dussumieri* were studied with an initiative to gain information about the nature and extent of the damage that may be produced by *in vivo* treatments of three mutagens Bleomycin, Mitomycin-C and Doxorubicin. Our observations showed dose and time dependent genotoxicity of all the three agents.

As far as we are aware, no attempts have been made to study the clastogenic effects of Bleomycin on the chromosomes of *B. dussumieri*, despite of the various reports on cytogenetic effects of bleomycin in mammalian cell systems (Paul & Gonzward, 1980; Sognier *et al*., 1982) as well as human lymphocytes (Dresp *et al*., 1978). The results of the present study may be used to draw a preliminary conclusion that *B. dussumieri* cells are sensitive to radiomimetic drug Bleomycin.

The ability of Mitomycin-C to induce chromosomal aberrations has been reported in *Vicia faba* (Shah *et al*., 1972); in human lymphocytes (Latt, 1974); in Chinese hamster ovarian cells (Natrajan & Reposa, 1975) and in fish (Krishnaja & Rege, 1982). In the present study chromosome type aberrations that included acentric fragments, dicentric and ring configurations as well as translocations were observed after 4 hours treatment timing of Mitomycin-C; while long duration treatment timing (24 hours) mainly resulted in chromatid type of aberrations that included gaps breaks and interchanges. The reason for this is precisely inconclusive as we have not studied the cytokinetics of the gill cells.

Unlike clastogens which produce aberrations by interfering with condensation mechanisms, Mitomycin-C induced aberrations are due to direct cleavage of DNA strands. In the present study chromatid gaps and breaks were mainly observed in the centromeric regions. This may be due to the selective action of MMC in centromeric heterochromatin, as explained earlier (Natrajan & Ahmstorm, 1969; Natrajan & Raposa, 1975). Further Shah (1975) reported that MMC

concentration as high as 1mg/ml had almost no deleterious effects on frog lymphocyte chromosomes that lack considerable amount of heterochromatin.

The identification and conformation of the mutagenic activity of Doxorubicin upon fish chromosomes is much lacking in the literature. Therefore, the experiments of the present study further included treatment of Doxorubicin - an antibiotic isolated from the mutant strain of *Streptomyces pencetius*. The genotoxicity of Doxorubicin has been established only in some test systems (Benedict et al., 1977; Lee & Dixon 1978).

Biochemically Doxorubicin is capable of intercalating with DNA, RNA and proteins and also inhibits their synthesis. Interaction of Doxorubicin with DNA results in inhibition of topoisomerase-II activity, the formation of DNA single and double strand breaks, mutations and chromosomal aberrations (Singh & Gupta, 1983; Cebula, 1986; Chen & Liu, 1994; Yu. et al., 1994). The ability of Doxorubicin to induce chromosomal aberrations in the germ cells of mice has been shown in various occasions (Alder, 1980; Alder & Bruden, 1982). Further, Ola *et al*., (2000) showed that intraperitoneal injection of Doxorubicin at 3mg/ml when given to rats induced significantly the incidence of chromosomal aberrations in the bone marrow cells and inhibited mitosis to about 50% of its normal rate. The results of our study also indicated significant increase in the frequency of chromosomal aberrations from the gill tissues of *B. dussumieri* after treatment of Doxorubicin. Where in treatment of longer duration (24 hours) mainly resulted in chromatid-type aberrations (gaps, breaks and interchanges) while shorter duration (4 Hours) treatment induced chromosome-type aberrations (ring, dicentrics and translocations). In some cases total destruction of chromosome complement with complex abnormality was noticed particularly after 40µg/10gm body weight treatment of Doxorubicin for 24 hours.

The overall results of the present study revealed that compounds Bleomycin, Mitomycin-C and Doxorubicin induced clastogenic effects in the cells of *B. dussumieri*. Further, this study made use of the whole organism, therefore the *in vitro* problems of accounting for metabolic activation, detoxification, dilution, excretion and different tissue sensitivities were greatly reduced. The findings of the present study, in general, add to the fact that fish such as *Boleophthalmus dussumieri* is a promising model *in vivo* for the cytogenetic studies on mutagenecity.

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