



## RESEARCH ARTICLE

### EVALUATION OF TIME, DOSE AND TISSUE DEPENDENT GENOTOXIC EFFECT OF CADMIUM CHLORIDE IN A FRESH WATER FISH, CHANNA PUNCTATUS (PISCES: FAMILY - CHANNIDAE)

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#### ABSTRACT

Studies on the genotoxic effect of cadmium chloride in three tissues viz., RBCs, kidney cells and gill cells of a fresh water fish *Channa punctatus* has been carried out using the micronucleus test. The fishes were treated with four sub-lethal concentrations of cadmium chloride i.e., 1.0, 2.0, 4.0 and 8.0 mg/l. The exposed and controlled fishes were sacrificed after 24, 48, 72 and 96 hrs of exposure. Formation of micronuclei was observed to occur in RBCs, kidney cells and gill cells after heavy metal treatment. Aim of the present investigation was to determine the dose and time dependent induction of frequencies of micronuclei in different tissues examined. A comparison between micronucleus frequencies in all the tissues revealed highest micronucleus frequencies in gills followed by RBCs and least in kidney cells. Results from this study recommend the use of the micronucleus test in fish tissues as a sensitive monitor for aquatic pollution. The results also show that the assay can be employed for the evaluation and the assessment of water pollution and aquatic mutagens.

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#### INTRODUCTION

Aquatic animals have often been used in bioassays to monitor water quality of effluent and surface water (Carins *et al.*, 1975; Brugs *et al.*, 1977; Da Rocha *et al.*, 2011). Fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples since they can metabolize, concentrate and store waterborne pollutants (Al-Sabti, 1991). Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially teratogenic and carcinogenic in humans (Al-Sabti and Metcalfe, 1995).

The micronucleus test using fish tissues has been used with different fish species to monitor aquatic pollutants displaying mutagenic features (De Flora *et al.*, 1990; De Flora *et al.*, 1994; Da Rocha *et al.*, 2011; Kour *et al.*, 2013). Among the diverse genotoxicity tests, the micronucleus test has been widely applied because it is simple, reliable and sensitive, and is not strongly dependent on any karyotypic characteristics (Heddle *et al.*, 1991). Micronucleus assays which originally developed with mammalian species have been used extensively to test for the genotoxic activity of chemicals. Micronuclei are formed by condensation of chromosomal fragments or whole chromosomes that are not included in the main nucleus following anaphase. Scoring of micronuclei in the interphase is technically much easier and more rapid than the scoring of chromosomal aberrations during metaphase. The micronucleus (MN) test in fish also has potential for detecting clastogenic substances in aquatic media. Since teleost erythrocytes are nucleated, micronuclei have been scored in fish erythrocytes as a measure of clastogenic activity (Heddle *et al.*, 1991).

Cadmium (Cd) is a non essential element, with no known biological function, naturally found at low concentrations in natural waters (Viarengo, 1985). Cadmium pollution sources are diverse, but it is commonly accepted that electroplating plants are mainly responsible (Cuthbert *et al.*, 1976). Several studies have shown that Cd accumulates in specific sites in the body rather than being distributed evenly throughout. Accumulated Cd has predominantly been found in the kidney, liver and gills of exposed specimens (Mount and Stephan, 1967; Sangalang and Freeman, 1979; Kumada *et al.*, 1980). Therefore, present study was under taken to determine the tissue specific dose and time dependent frequencies of micronuclei after treatment of fish *Channa punctatus* as a test model with four sub-lethal doses of cadmium chloride.

#### MATERIALS AND METHODS

**Animals:** Experiments were performed on a fresh water fish, *Channa punctatus* (Bloch) which is relatively easy to handle and acclimate to laboratory condition because of presence of accessory air breathing organs. Adult live and apparently healthy specimens of *C. punctatus* (12-15 cms in length and 29-65 gms in weight) caught from the local lentic habitats were acclimatized to laboratory conditions in large well aerated plastic aquaria for at least for 10 days. Fishes were fed with fresh goat liver *ad libitum* and with some live food in the form of worms, insects and other small fishes. Feeding was stopped 24h prior to commencement of toxicity tests and fish were not fed during experimental period.

**Test chemical:** Cadmium as CdCl<sub>2</sub>.1/2H<sub>2</sub>O was dissolved in dechlorinated tap water at a concentration of four sub-lethal doses i.e., 1.0 mg/l, 2.0 mg/l, 4.0 mg/l and 8.0 mg/l (LC<sub>50</sub> value at 96h as Finney (1980) was 12.0 mg/l).

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**Experimentation:** The fish were made to float in the solution of cadmium chloride so as to match the way in which they are exposed in nature. As the test chemical is added into water, it reaches the target organ via a longer route i.e., along blood circulation and absorption through operculum, gill epithelium and body surface. After natural modification and change in the course of circulation, only a small fraction of the chemical hits the target organ. Thus, the results obtained by this method would be very close to the expected actual values in the natural environment.

Toxicity tests were conducted in accordance with standard methods in dechlorinated tap water having hardness of 140-185 mg as  $\text{CaCO}_3/\text{l}$  and alkalinity of 98-126 mg/l as  $\text{CaCO}_3$ . The exposure system consisted of plastic containers of 50L capacity. Acclimatized and apparently healthy and uninfected fish specimens were released in each well aerated container in semi-static bioassays. Test concentrations were renewed every day. The fishes of control group were maintained on the normal food i.e., fresh goat liver and these were kept in similar experimental conditions, without the heavy metal treatment. The fish of exposed groups were treated with four sub-lethal concentrations of each heavy metal salt mentioned above for 24, 48, 72 and 96h of duration.

Four fish specimens were kept for treatment with each heavy metal salt concentration for 24, 48, 72 and 96h. Separate batch of same number of fish was kept as control for each duration of exposure i.e., 24, 48, 72 and 96h. Presently, peripheral erythrocytes (RBCs), gill epithelium and kidney cells were used for performing MNT (Sadhukhan and Manna, 1989).

#### **Preparation of erythrocyte micronuclei slides**

The MN test was performed according to published protocols (Al-Sabti and Metcalfe, 1995). After each duration of exposure peripheral blood samples were collected individually from caudal veins of fishes in each group. Blood smears were prepared on clean glass slides, air dried and fixed in methanol for 5 minutes. Slides were then stained for 10 min with a mixture of 10 ml Giemsa and 90 ml phosphate buffer (pH 6.8). The slides were then rinsed with distilled water, air dried and examined using optical microscopy under 100x objective for the presence of micronuclei.

#### **Preparation of micronuclei slides from kidney and gills**

Control as well as exposed fish specimens were dissected from the ventral side so as to remove the kidneys and gills (Manna and Sadhukhan, 1986; Rishi and Grewal, 1995). The micronuclei slides were made following the air-drying Giemsa staining technique (modified after Tijo and Whang, 1965). The tissues were cut into small pieces and homogenized in 0.56% KCl solution (hypotonic) and kept as such for 20 to 25 minutes. It was then centrifuged at 1000 to 1500 rpm for 10 minutes. Supernatant was discarded and the pellet was suspended in the fixative acetic- methanol in the ratio of 1:3. Several changes of fixative were given but the last change was in a small amount of fixative. This suspension was dropped on clean and dry slides and then air dried. Air dried slides were stained in 2-4% Giemsa solution at a pH 7-8 for 30-40 minutes and slides were differentiated in distilled water and then air dried.

#### **Scoring of MN slides**

Only micronuclei not exceeding  $1/3^{\text{rd}}$  of the main nucleus diameter, clearly separable from the main nucleus and with

distinct borders and of the same colour as the nucleus were scored. The frequency of micronuclei in each tissue was established by estimating the number of micronuclei in at least 1500 interphase cells/specimen (total of 6000 interphase cells from four specimens used per concentration and duration) and was expressed as mean $\pm$ standard deviation (SD) per 1000 cells. The micronucleated interphase cells from different tissues were photomicrographed at 1000x magnification.

#### **Statistical analysis**

Data from the micronucleus test were expressed as mean $\pm$ SD. Data were then compared by the non-parametric Kruskal-Wallis test. Statistical analysis was performed using the computer software called 'PRIMERS-4.0'.  $p < 0.05$  was considered to be the level of significance. Statistical significance in the frequencies of micronuclei between exposed and control groups after each dose and duration of exposure were evaluated.

## **RESULTS**

Result of MNT in Kidney (Fig 1 and 2): Frequency of micronuclei determined in different treatments is summarized in table 1 and fig 7. Fish exposed to different concentrations and durations of  $\text{CdCl}_2$  showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at 24h, 48h and 72h,  $p < 0.001$ ; at 96h,  $p < 0.01$  versus respective controls in all treatment groups). In fish treated *in vivo* with 1.0 mg/l of  $\text{CdCl}_2$ , frequency of MN was recorded to be  $3.82 \pm 0.35$  (24h),  $7.47 \pm 0.670$  (48h),  $13.97 \pm 1.103$  (72h) and  $13.47 \pm 1.118$  (96h) wherein the incidence of MN was slightly decreased after 96h compared to 72h. An elevated response was observed upto 72h of exposure in the frequency of MN ( $9.8 \pm 0.627$  after 24h,  $12.15 \pm 0.834$  after 48h,  $18.47 \pm 1.147$  after 72h and  $13.8 \pm 1.749$  after 96h) after treatment with 2.0 mg/l which was decreased after 96h of exposure. Specimens exposed to 4.0 mg/l showed significantly elevated incidence of MN at 24h ( $12.12 \pm 1.5$ ), at 48h ( $13.12 \pm 1.477$ ), at 72h ( $19.62 \pm 1.576$ ) and at 96h exposure ( $20.62 \pm 1.103$ ). Treatment with 8.0 mg/l showed a highly significant increase of micronuclei in relation to respective controls at all the exposure periods. The values recorded were  $17.47 \pm 1.47$  (24h),  $20.3 \pm 1.288$  (48h),  $27.45 \pm 1.35$  (72h) and  $28.95 \pm 1.377$  (96h).

Result of MNT in Gills (Fig 3 and 4): Frequencies of MN recorded in gill tissue after different treatments and exposure periods have been shown in the table 1 and fig 8. MN frequencies observed in fish treated *in vivo* with 1.0 mg/l increased from  $6.95 \pm 0.404$  at 24h to  $17.12 \pm 1.118$  at 72h and decreased afterwards to  $15.95 \pm 1.559$  at 96h. Increased levels of MN were observed after all the exposure periods in experimental fish treated with 2.0 mg/l. Maximum frequency of MN was obtained after exposure period of 96h ( $28.97 \pm 2.085$ ) whereas minimum frequency was obtained after exposure period of 24h ( $12.27 \pm 1.164$ ). Significant difference (at 24, 48, 72 and 96h,  $p < 0.001$  versus respective controls) in the incidence of MN between the experimental and control values was also observed after all the exposure periods in treatment with 4.0 mg/l ( $21.12 \pm 1.765$  at 24h,  $24.62 \pm 0.943$  at 48h,  $25.97 \pm 1.42$  at 72h and  $32.82 \pm 0.994$  at 96h) and 8.0 mg/l ( $19.45 \pm 1.35$  at 24h,  $26.82 \pm 0.994$  at 48h,  $34.45 \pm 1.399$  at 72h and  $40.62 \pm 1.466$  at 96h).

Result of MNT in RBCs (Fig 5 and 6): Values of MN in the peripheral erythrocytes after different treatments and exposure periods have been shown in the table 37 and fig 9. Fish treated *in vivo* with 1.0 mg/l of CdCl<sub>2</sub> showed a significant increase (at 24, 48, 72 and 96h, p<0.001 versus control values) in the incidence of MN versus respective controls at all the exposure periods. The values recorded were 7.12±0.35, 8.3±0.346, 13.3±1.206 and 16.62±0.943 after 24h, 48h, 72h and 96h respectively. An elevated response was also observed during treatment with 2.0 mg/l wherein the values recorded were 11.8±1.458, 13.8±0.627, 19.8±0.627 and 29.12±1.118 after 24, 48, 72 and 96h of exposure respectively. Fish exposed to sub lethal concentration of 4.0 mg/l revealed significant (p<0.001 at all the exposure periods versus respective control values) induction of MN frequency after all the exposure periods in comparison to controls. Maximum frequency (26.47±1.5) was recorded after 72h after which the value was decreased. Exposure to 8.0 mg/l induced 15.47±0.670, 30.47±0.994, 29.8±1.458 and 39.65±1.377 MN frequencies after 24, 48, 72 and 96h.

A comparison between micronucleus frequencies in all the tissues revealed highest MN frequencies in gills followed by RBCs and least in kidney cells. Therefore, cadmium chloride was found to be potentially genotoxic as was evident from the extent of frequencies of MN in fish *C. punctatus* exposed even to sub-lethal concentrations.

## DISCUSSION

An attempt was made during the present work to assess the time-dose-dependent, tissue specific peculiarities of cadmium genotoxicity in fish, *Channa punctatus*, as aquatic model test organism. During the present studies, it is reported that cadmium induced the micronuclei formation in *C. punctatus*. Sub-lethal concentrations of cadmium clearly increased the micronuclei frequencies in all the tissues which were dose and time dependent, compared to respective controls. A comparison between the micronucleus frequencies in all the tissues revealed highest micronuclei frequencies in the gill cells followed by RBCs and least in kidney cells (Table 1; Fig. 7, 8 and 9). The observation that cadmium caused greater increase in micronuclei frequencies in cells of gills followed by RBCs and least in kidneys is most likely due to the fact that the gills were in direct contact with the test chemical in solution and therefore, were exposed to the highest *in situ* concentration of the compound. Furthermore, since cadmium is highly reactive, its effects could be attenuated as it travels in the blood to the kidney and so on. However, differences in metabolic activation and deactivation properties of these tissues or different sensitivities of the tissues to the mutagens cannot be ruled out as alternative explanations.

The *in vivo* induction of micronuclei by cadmium exposure in present investigation gets confirmed from the results obtained by Sadhukhan and Manna (1989) who reported a dose and time dependent relationship between micronuclei in peripheral erythrocytes, gill cells and kidney cells on exposure to rogor-30 in *O. mossambicus*. They found an increased number of micronuclei by increasing the concentration and duration of exposure of rogor-30 in all tissues. Positive relationship between metal concentrations and micronuclei frequencies observed in the present study was confirmed by the results of Zhu *et al.*, (2004). They treated carp with different

concentrations of cadmium (as CdCl<sub>2</sub>; doses of 0.001 mg/l, 0.01 mg/l and 0.1 mg/l) for exposure periods of 2, 4, 6, 9, 12, 17, 22, 32 and 42 day. They found that micronuclei frequencies increased with the rise of cadmium concentrations during exposure time. Hughes and Hebert (1991) found that micronuclei frequencies of fish exposed to high concentration pollutant could elevate many times with the rise of exposure time. Similarly, Zhu *et al.*, (2004) found that micronuclei frequencies would increase to a smooth level after a peak value with the rise of cadmium concentrations. Carp exposed to 0.1 mg/l cadmium, micronuclei frequencies increased after first few days of exposure, peaked on day 4 and day 9, respectively and then smoothly changed. But in present investigations, a gradual increase in frequencies of micronuclei was recorded at all exposure periods in all the sub-lethal concentrations tested (Table 1; Fig. 7, 8 and 9).

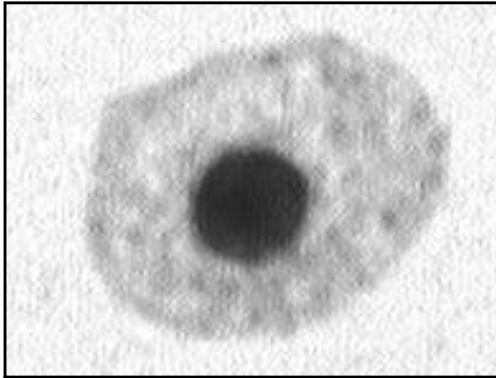
Jiraungkoorskul *et al.*, (2007) investigated the effects of cadmium and ascorbic acid on the red tailed foil barb (*Puntius altus*) using the micronucleus and nuclear abnormality tests for the period of 24, 48, 72 and 96h. The micronuclei frequencies in erythrocytes, gill, liver and fin cells were analyzed comparatively to evaluate the sensitivity and suitability of these different cell types. Nuclear abnormalities (NA) shapes in erythrocytes were scored into blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) and binuclei (BN). In general, the highest value of both micronuclei and NA cells were significantly increased in the cadmium treated group followed by the combination of cadmium and AA (ascorbic acid) treated group. On the other hand, micronuclei and NA frequencies in erythrocytes were most sensitive to the treatment and could provide valuable information than those in gill, liver and fin cells. In contrast, micronuclei frequencies in gill cells were reported to be highest among various tissues during the present work. Jiraungkoorskul *et al.*, (2007) also revealed highest number of micronuclei and NA after 48h treatment in all cases and decreased within the longer time exposure while in the present study, frequencies of micronuclei were found to increase from 24 to 96h being highest at 96h of exposure (Table 1; Fig. 7, 8 and 9).

In a study performed by Rosa *et al.*, (2003), it was indicated that the micronuclei induction may be interpreted as a consequence of oxidative stress, upholding the view that cadmium induced DNA damage is via generation of (intermediate) reactive oxygen species in *V. faba*. A study by Unyayar *et al.*, (2006) demonstrated high lipid peroxidation in *V. faba* root cells treated with different concentrations of cadmium and proposed that cadmium might delay mitosis by damaging the transport mechanism due to the attack of free radicals on fatty acid component of membrane lipids. Reactive oxygen species overproduction coupled with deficiency of antioxidant defense mechanisms may be an important factor contributing to the increase in micronuclei. In some studies, it has been implied that there is a relationship between micronuclei formation and lipid peroxidation under several stress conditions such as dietary habits (Mayer *et al.*, 2000). Mayer *et al.*, (2000) also demonstrated a positive correlation between lipid peroxidation status and genotoxicity as reflected by increased micronuclei formation in lymphocytes.

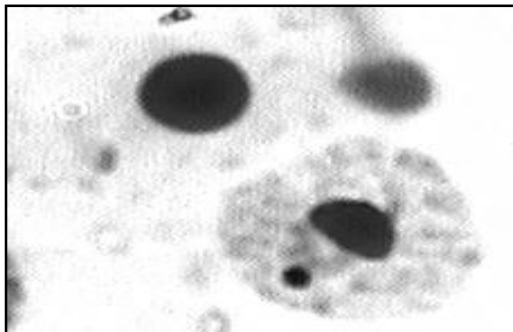
Present investigation suggested that individual tissues differ in their rates of accumulation of toxicants and this was evident from the highest MN frequencies in gills followed by RBCs

and least in kidney cells. For this reason, it is preferable to assess genetic damage in a variety of tissues whenever possible.

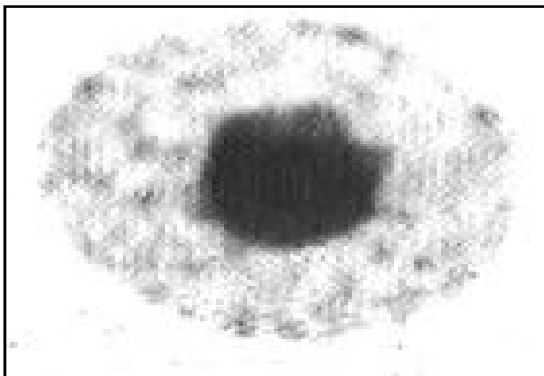
**Appendices**



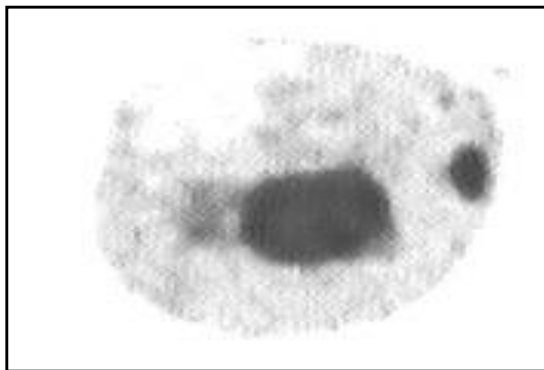
**Fig. 1** Photomicrograph of kidney cell of *Channa punctatus* with normal nucleus from control specimen.



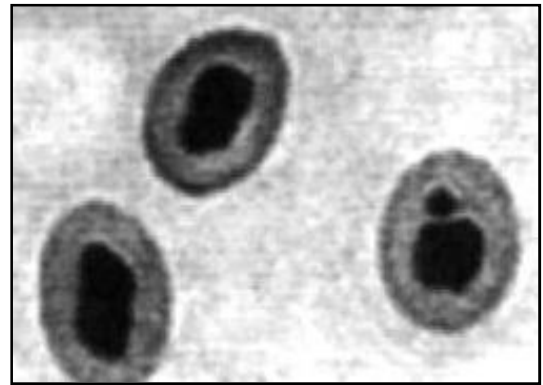
**Fig. 2** Photomicrograph of kidney cell of *Channa punctatus* with micronuclei from treated specimens.



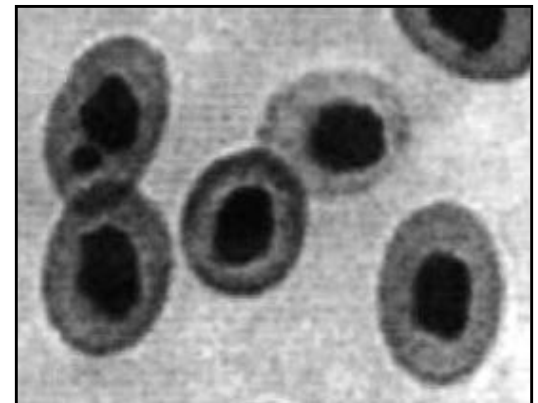
**Fig 3** Photomicrograph of a gill cell of *Channa punctatus* with normal nucleus from control specimen.



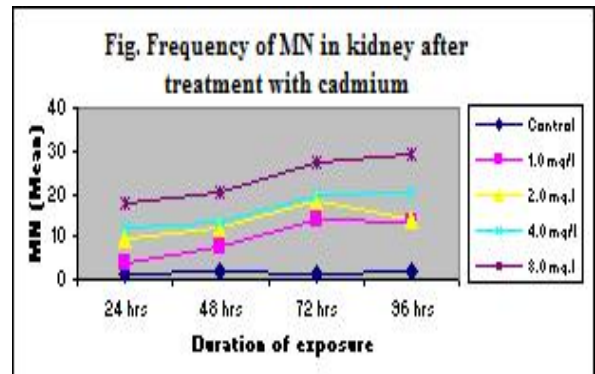
**Fig. 4** Photomicrograph of a gill cell of *Channa punctatus* with micronuclei from treated specimen.



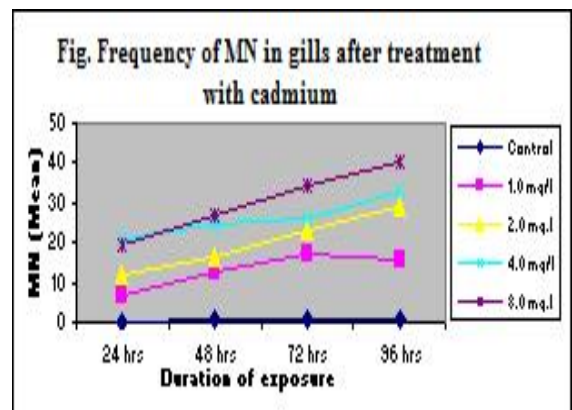
**Fig 5** Photomicrograph of red blood cell of *Channa punctatus* with normal nucleus from control specimen.



**Fig. 6** Photomicrograph of red blood cell of *Channa punctatus* with micronuclei from treated specimen.



**Fig. 7** Frequency of micronuclei in kidney cells after treatment with cadmium.

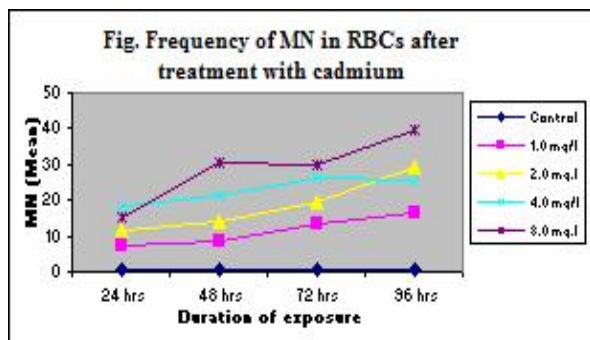


**Fig. 8** Frequency of micronuclei in gill cells after treatment with cadmium.

**Table 1** Frequencies of micronuclei (%) in different tissues of *Channa punctatus* exposed to cadmium.

Conc. (mg/l)	Duration (Hrs)	No. of specimens	Total interphase cells studied	Frequency of micronuclei (Mean±SD)		
				Kidney	Gills	RBCs
Contr.	24	4	6000	1.12±0.670	0.3±0.346	0.45±0.3
	48	4	6000	1.62±0.865	0.47±0.618	0.62±0.531
	72	4	6000	1.47±0.35	0.47±0.618	0.8±0.627
	96	4	6000	1.82±0.35	0.8±0.848	0.62±0.531
1.0	24	4	6000	3.82±0.35 <sup>c</sup>	6.95±0.404 <sup>c</sup>	7.12±0.35 <sup>c</sup>
	48	4	6000	7.47±0.670 <sup>c</sup>	12.45±0.834 <sup>c</sup>	8.3±0.346 <sup>c</sup>
	72	4	6000	13.97±1.103 <sup>c</sup>	17.12±1.118 <sup>c</sup>	13.3±1.206 <sup>c</sup>
	96	4	6000	13.47±1.118 <sup>b</sup>	15.95±1.559 <sup>c</sup>	16.62±0.943 <sup>c</sup>
2.0	24	4	6000	9.8±0.627 <sup>c</sup>	12.27±1.164 <sup>c</sup>	11.8±1.458 <sup>c</sup>
	48	4	6000	12.15±0.834 <sup>c</sup>	16.47±1.5 <sup>c</sup>	13.8±0.627 <sup>c</sup>
	72	4	6000	18.47±1.147 <sup>c</sup>	23.45±0.834 <sup>c</sup>	19.8±0.627 <sup>c</sup>
	96	4	6000	13.8±1.749 <sup>b</sup>	28.97±2.085 <sup>c</sup>	29.12±1.118 <sup>c</sup>
4.0	24	4	6000	12.12±1.5 <sup>c</sup>	21.12±1.765 <sup>c</sup>	18.3±0.852 <sup>c</sup>
	48	4	6000	13.12±1.477 <sup>c</sup>	24.62±0.943 <sup>c</sup>	21.47±0.670 <sup>c</sup>
	72	4	6000	19.62±1.576 <sup>c</sup>	25.97±1.42 <sup>c</sup>	26.47±1.5 <sup>c</sup>
	96	4	6000	20.62±1.103 <sup>b</sup>	32.82±0.994 <sup>c</sup>	25.62±0.865 <sup>c</sup>
8.0	24	4	6000	17.47±1.47 <sup>c</sup>	19.45±1.35 <sup>c</sup>	15.47±0.670 <sup>c</sup>
	48	4	6000	20.3±1.288 <sup>c</sup>	26.82±0.994 <sup>c</sup>	30.47±0.994 <sup>c</sup>
	72	4	6000	27.45±1.35 <sup>c</sup>	34.45±1.399 <sup>c</sup>	29.8±1.458 <sup>c</sup>
	96	4	6000	28.95±1.377 <sup>b</sup>	40.62±1.466 <sup>c</sup>	39.65±1.377 <sup>c</sup>

<sup>a</sup>=p<0.05, <sup>b</sup>=p<0.01, <sup>c</sup>=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control



**Fig. 9** Frequency of micronuclei in red blood cells after treatment with cadmium.

**CONCLUSION**

Fishes are an important source of human nutrition. Aquatic ecosystems polluted with heavy metals, may therefore, threaten human nutrition and health directly. To measure the effects these substances could have on fish species and subsequently on human populations, an investigation of such substances on the genetic material of aquatic organisms may produce meaningful and useful results. Ultimately, the goal of the studies such as this is to provide information that will help mediate the environmental damage resulting from pollutant releases like those of heavy metals.

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