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Effect of fluoride on micronucleus formation and sister chromatid exchanges in some edible fishes

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Abstract
Genotoxicity of fluoride (F) in fishes of Jabalpur city was evaluated using micronuclei in erythrocytes (MNEs) assays and sister chromatid exchange (SCEs). As it is positively related to the frequency of micro nucleated erythrocytes' (MNEs) and sister chromatid exchange (SCEs) per metaphase cells. Result of the present study indicates that the MNEs and SCEs in fishes can be easily used for assessment of genotoxic effect of fluoride (F) in fresh water bodies. In present study the specific difference in MNEs and sister chromatid exchange (SCEs) frequency were found to be statistically significant. In this study it is established the mean number of sister chromatid exchange in fish maintained in high quality water in the absence of toxins.

Keywords: micronucleus, sister chromatids, fluoride, genotoxicity

Introduction
Environmental genotoxic agent rising from human activity causes Malignancies; reduce growth, abnormal development, reduced survival of embryo, larva and adults ultimately affecting the economy of fish production significantly. Genotoxicity not only reduces the fitness (i.e, growth, fertility, and fecundity) in wild fish population. But also produce risk to human health via food chain which may lead the structural and functional changes in the ecosystem (Aderson *et al.*, 1994) [2].

The environmental agents are known as genotoxic agents that directly or indirectly affect genome integrity due to mutation and damage the DNA at chromosomal level. Genetic toxicology has evolved from the initial gene mutability demonstrate by Auerbach *et al.*, (1947) [4]. Genetic toxicology investigates the interaction of chemical and physical agents with genetic material in relation to subsequent adverse effects such as cancer in case of alterations in somatic cells or genetic disease in future generations (in case of alterations in germ cells) (Aurebach *et al.*, 1947) [4].

Aquatic pollutants induce formation of micronuclei in peripheral blood cells by condensation of chromosome that are not included in the main nucleus following anaphase stage of cell division (Schemid, 1976) [15]. The importance of MN formation as biological endpoints in genotoxicity is influenced by the consequence of micronucleus formation for faith of the cell. The formation if MN may be an important process in carcinogenesis (Benner *et al.*, 1993, Garewal *et al.*, 1993) [6, 7].

Recently, fluoride (F) has been reported to induce chromosomal aberrations in bone marrow cell of mice (Podders, 2008) [12]. In earlier studies, contrary findings were reported in regard to the effect of (F) on chromosomal aberration in human peripheral blood lymphocytes in fluoride (F) endemic areas (Obe G, 1973, Slacik, 1976, Khalil, 1995) [11, 14, 9]. Although, substantial studies of the genotoxic effect have been conducted in the mammalian system, yet the available information regarding such effect on fish is very limited. Hence the present study was planned to observe the genotoxic potential of fluoride (F) in fresh water edible fish.

Fluoride is the thirteenth most abundant element (0.065% by weight) in the earth's crust and is widely distributed in our environment. In spite of the approval of fluoride for caries prevention by health authorities, controversy concerning its safety and potentially adverse effects still exists, including conflicts over possible genotoxic effects. In general, there are three different viewpoints: (i) fluoride has no genotoxic effects, (ii) fluoride is a mutagenic agent and causes DNA and chromosomal damage, (iii) fluoride has synergistic or antagonistic effects with certain known mutagens (Mukherjee and Sobels, 1968, Obe and Slacik-Erben, 1973) [10, 11, 14].

In view of the conflicting evidence, the present study is aimed to clarify the controversial issues by selecting fish to study the genotoxic influence of fluoride (in the form of sodium fluoride, NaF) on micronuclei formation and sister chromatid exchanges.

Material and Methods
The present study was carried out at the Department of P.G. Studies and Research in Biological Sciences. Rani Durgavati University Jabalpur (M.P.) for the period from February 2013 to June, 2014. Jabalpur city is lying between coordinates 23° - 36′ and 24°-05′ north latitude and 80°-55′ east longitude. It is situated on a rocky basin at height of 412 m above the mean see level and has tropical climate.

Selection and Identification of fishes
Three healthy, live fresh water fishes, weighing between 100-150 grams belonging to three different species were sampled with the help of fisherman.

The identification of fish sampled was done following the classification of Lindburg (1971)[17].

Maintenance of fishes in the aquarium

Glass aquarium measuring length 25″ × width 15″ × height 20 was fabricated locally.

Water oxygenated by producing turbulence by air pump (R.S. electrode, Made in China) Voltage of the pump is 220v, 50Hz and the power of the pump is 3W used. Chambered environmental temperature and natural photoperiod utilized for acclimation of the fishes.

The physiochemical properties of holding water during acclimatization on were determined by the standard methods. (APHA 1998) [1].

Water in the aquarium were changed every alternate day after feeding. "Tokyu" was selected as fish food because of the following properties.

1. "Tokyu" is a floating type fish food
2. "Tokyu" provides carotinoid pigment in fish.
3. Presence of spirulina: spirulina is a nutritious food for fish growth.
4. Tokyu" provide necessary amount of amino acid to assist development of fish.

The ingredients of "Tokyu" is induced Shrimp meal, Cattle fish meal, Wheat flour, Thiamine, Riboflavin supplement, Soya bean meal, Sprirulinar**,** Protease**.** Minerals are also present in "Tokyu" such as Zn, Co, Fe, Mn, Cu, P, Mg, P amino benzoic acid (Paba) are present in Tokyu.

The fish were fed with 2 to 4 seed of Tokyu per fish in the morning at: 30am and evening at 6: 30pm.

Selection of fluoride (F) compound

Sodium fluoride (NaF) was selected because it is well known to cause harmful effects in both human and animals. Aquatic life can take up NaF from food and water. Fish can accumulate NaF through the food chain.

Fish treatment with sodium fluoride (NAF)

Sodium fluoride is a yellow powder with a weak musty oder and melting point of 993°C and vapor pressure of 5.43×1o¯26 mm Hg (25°C). Sodium fluoride is soluble in water at 4.10g/100 ml, at 15°C and 4.3g /100 ml at 25°C. NaF is readily soluble in many organic solvents and has a density of 2.55g/cm2

The aquarium fishes were treated with 0.35mg NaF/L for 3 day and 0.75mg/L for 3 day. The aquaria water was not change during NaF treatment and the physico chemical property of water were determine according to APHA (1998) [1].

Micro nucleus (M.N.) test

1. Collection & smearing of blood

0.01ml blood was drawn from the caudal vein at the ventral side of the fish through a sterilized syringe (2ml) and collected in a tube containing 2-3 drop of 1.7% ethylene diamine tetra acetate (EDTA solution).

A drop of blood was smeared on a clean and grease free slide and air dried over night in a dust and moisture free environment at room temperature.

2. Staining of the slides

Slides were fixed by dipping them in absolute methanol for 5 to 10 minutes and air dried for an hours, then stain the slide in to "May-Grunnwalds solution - I " for 3 minutes and wash with double distilled water (D.D.) and air dried. Again stain in "May-Grunnwalds solution - II" for 3 to 6 minutes with double distilled (D.D.) water. The slides were again stained in 6 to 8 % "Giemsa stain" solution in phosphate buffer for 30 minutes and wash with double distilled water to remove all the Giemsa particles and air dried over night.

3. Scanning and Scoring of micronucleus

Slides were made permanent with D.P.X. mount and dried over hot plate at 60˚C for overnight and observe the slide under microscope at (400X) magnification.

The frequency of micro nucleated erythrocytes (MNEs) was evaluated by examining 1000 mature erythrocytes for each species and the percentage frequency (%) was expressed as follow

$$\text{Percentage frequency of MNEs (\%)} = \frac{\text{Total no. of MNEs}}{\text{Total no. of cell examined}} \text{ X } 100$$

Only isolated nuclear fragment which fulfill the morphological criteria was counted as micronucleus (MN) i.e. rounded or ovoid shaped non-refractory particle with colour and structure similar to the principal nucleus with diameter of 1/3 to 1/50 of the main nucleus.

Sister chromatid exchange (SCEs)

1. Fish treatment

Fishes were treated with (BrdU) solution (0.5mg/100 gm) for 24 hours to get the best results. The higher concentration of BrdU increases the exchange. Than 1 ml of 0.05 % colchicines was injected (1ml/100g) intramuscularly and anesthetize the fish with ethylene glycol.

Cut small pieces of the outer region of the gill of the treated fish.

2. Preparation of the cell suspension

The small pieces of the outer region of gill were cut with the help of scissor & collect in a watch glass. Put the gill tissue in the test tube and homogenize it with glass rod in 6-8 ml hypotonic solution (0.56 % KCl) in order to prepare cell suspension.

- Pour the cell suspension in 15 ml centrifuge tube incubate it for 20-25 minutes at room temperature for swelling the cells.
- Stop hypotonic action by adding 1 ml freshly prepared chilled Carnoy`s fixative (Methanol: Acetic acid 3:1 ratio) slowly and mix it gently with pasture pipette.
- To get cell pallet at the bottom Centrifuge cell suspension at 1200-1500 rpm for 10 minutes at the room temperature.
- Remove the suspension with a pipette and slowly overlay 6-8 ml freshly prepared chilled fixative.
- Keep the tube in refrigerator for half an hour for throwing fixation.
- Mixed the content and centrifuge cell suspension at 1200-1500 rpm for 10 minutes at room temperature
- Remove the suspension without disturbing cell pellet at the bottom and add fresh fixative.
- Repeat above two steps three times till clear transparent

cell suspension is obtained.

3. Slide treatment

Take small quantity of cell suspension in pasture pipette and drop it on to grease free, pre cleaned glass slide.

a. Allow the slide to air / flam dry.
b. Treat the slide with Hoechst 33258 dye (5µ / ml) in phosphate buffer saline for 20 minutes (in dark).
c. Expose the slide to U.V. black bulb (125 W) for 10 minutes.
d. Rinse the slide with double distilled (D.D.) water and dry in air.
e. Treat the slides with 5N HCl for 10-15 minutes. Rins the slide thoroughly with double distilled.

4. Staining

- Stain the slide with 4-5 % Giemsa in phosphate buffer (pH 6.8) for 15-20 minutes.
- Wash the slide with double distilled water thoroughly.
- Keep it for air drying.
- Observe dual stain metaphase spread in bright field microscope to ascertain the quality of staining.

Scanning and screening of SCEs

- Slide were made permanent by mounting the synthetic mount e.g. D.P.X.
- Slide were observed at 1000 X magnification and screened the slide and good metaphase spreads showing sister chromatid exchange under oil immersion.
- Score the SCEs for each individual chromosome.
- Tabulate the SCEs in four categories for final analysis (based on number of exchanges per chromosomes / metaphase).

Preparation of the reagents for MN test

1. Giemsa staining solution

Composition

Giemsa powder - 0.5gm,Glycerol -33ml
Methanol - 33ml

For stock solution 0.5gm Giemsa stain powder was dissolve in 33 ml of glycerol and incubated overnight at 60°C in water both, cooled to room temperature and filtered after adding 33 ml methanol. Working solution (5%) was prepared by adding 5 ml of stock solution in 95 ml 0f phosphate buffer.

2. May-Grunnwald's solution

Composition

May- Grunnwald's - 12mg
Methanol - 100ml

May- Grunwald's staining solution-I was prepared by dissolving 125mg in 1000 ml of methanol and filtered. Similarly, May-Grunwald's staining solution-II was prepared by adding 50 ml of double distilled water to the 50 ml May-Grunwald's staining solution-I.

3. Phosphate buffer (pH 0.8)

Composition

KH_2PO_4 - 6.8045g / 100ml distilled water
Na_2HPO_4 - 8.8945g / 100ml distilled water

6.8045mg KH_2PO4 powder and 8.894mg Na_2HPO_4 powder dissolved in 100 ml distilled water to prepare separate solution of KH_2PO_4 and Na_2HPO4 and stored in umber collected bottle. Working solution was prepared taking 31.3 KH_2PO_4ml solution and 22.8ml of Na_2HPO_4 solution in conical flask (500ml) and diluted up to 50ml with distilled water.

4. Ethylene Diamine tetra Acetate (EDTA) solution

EDTA solution was prepared by dissolving 0.25mg EDTA powder in 250 ml distilled water.

Preparation of the reagents for SCEs

1. Preparation of ethylene glycol for anesthesia

Composition

Ethylene glycol - 50mg,
Water - 1000ml

Ethylene glycol for anesthesia was prepared by dissolving 50mg Ethylene glycol in 1ml in distilled water.

2. BrdU Solution

Composition

BrdU Powder - 2mg,
Double distilled water - 1ml

BrdU solution was prepared by dissolving 2mg of BrdU powder in 1ml of double distilled water.

3. Hoechest solution

Composition

Hoechst powder - 5.0mg,
PBS solution - 50ml

Hoechst solution was prepared by dissolving 5.0mg of Hoechst-33258 in 5.0ml phosphate buffer saline make aliquots of 250µl in micro centrifuge tube and store in refrigerator at concentration of 5µl / ml of PBS.

4. 5N HCl solution (for 50 ml solution)

Composition

Concentrated HCl - 22.25ml,
Double distilled water - 27.75ml

5NHCl solution was prepared by dissolving 22.25ml concentrated HCl in17.75ml distilled water to make 50.0ml solution.

5. Colchicine (0.05 %) solution

Composition

Colchicine powder - 0.05gm,
D.D. water - 100ml

0.05% colchicine solution was prepared by dissolving 0.05gm colchicine powder in 100ml double distilled water.

6. Hypotonic KCl solution

Composition

KCl powder - 0.56gm
D. D. water - 100ml

Hypotonic solution was prepared by dissolving 0.56gm of KCl powder in 100ml in double distilled water.

Statistical analysis

The result was performed by mean of statistical analysis of variance (ANOVA) and the variation in frequency of micro nucleated erythrocytes (MNEs) with in different species were

analyzed. The Skelton of analysis of variance table along with formula is given as under.

Table 1

Source of Variance (due to)	Degree of freedom (d.f.)	Some of square (S.S.)	mean some of squire (M.S.)	F-value
Between the sample	K-1	SSB	Sb^2 ═SSB K-1	$F=Sb^2 / Se^2$
Within the sample	N – k	SSE	Se^2 ═SSE / N -1	
Total	N -1	TSS		

1. Sum of squares between Sample (SSB)

$$SSB = \sum_{i=1}^{k} \frac{Xi^2}{ni} - C.F.$$

$$or = \sum_{i=1}^{k} \frac{Xi^2}{ni} + \frac{X2^2}{n2} + \ldots\ldots \frac{Xn^2}{ni} - C.F.$$

Where Xi = Sum of observation of individual column
Ni = Number of observation in individual column

2. Correction factor (C. F.)

$$C.F. = \frac{G^2}{N}$$

Where, G = Grand total
N = Total number of observation

3. Total sum of square (TSS)

$$TSS = \sum_{i=1}^{k} \sum_{j=1}^{Ni} xij^2 - C.F.$$

$$Or = \sum_{i=1}^{k} \sum_{j=1}^{ni} Xi^2 - X2^2 \ldots\ldots\ldots + Xn^2 - C.F.$$

Where, Xij = Individual observation

4. Sum of square within sample (SSE)

$$SSE = TSS - SSB$$

Results and Discussion

The results of the present study are broadly divided in to four section :- (I). Identification of the collected fishes (II). Physico chemical para meters of aquarium water (III). scanning of micro nucleated erythrocytes (MNes) and (IV) scanning of SCEs.

Physico chemical Parameters of aquarium

The results of the physico-chemical analysis of water are depicted in Table-2. The pH of the aquarium water range from 7.0 to 7.5, dissolved oxygen water from 8.5 to 4.2, hardness $CaCO_3$ from 224 to 991 and temperature 36 to 38, was found during acclimation. The pH, total hardness, and temperature of water were increased during acclimation but the dissolved oxygen of water was decrease during acclimation for 15th day.

Identification of fishes

The fish species collected from Hanumantal were identified by observing the morphological characteristics. They were classified as under -

1. *Labeo rohita*, Hamilton (Rohu)
2. *Heteropneustes fossilis* (Singhi)
3. *Channa punctatus* Hamilton (Karra)

Scanning of micro-nucleus erythrocytes (MNEs)

The scanning results of the micro-nucleated erythrocytes are presented in Table 3 and figure (1-3)

Table indicate that control group of all the fish species also had micro-nucleated erythrocytes, which mean that the collected fishes were already having some sort of genotoxicity. There was no significance different in the average percentage frequency of MNEs of control group and 0.35NaF/L treated group of fishes. The fish treated with 0.75NaF/L differed significantly from the control group of $\alpha = 0.05$ it can be calculated this concentration had damaging effect on chromosomes.

Scanning of sister chromatid exchange

The scanning results of sister chromate exchanges are presented in table –. 4 and figure (3-6). The highest numbers of SCEs / metaphase were observed in *Labeo rohit*a, (1.57) *Heteropneustes fossilis*, (02) *Channa punctatus*, from (2.08).

BrdU induction in the doses 0.5mg/g body weight with a exposure of 24 to 48 hours are significant for two rounds of DNA replication at 28˚+2˚C and the doses is adequate in maintaining the required BrdU level in tissues. The higher doses of BrdU or longer exposure do not improve the rate of SCE. In the present study the average temperature of water was 35°C and it is also gave a good results on SCEs rate.

Bagdonas *et al.*, (2003) [5] also show that under some treatment of fluoride different fish species had variability in the frequency of MNEs and sister chromate exchange (SCEs) depending upon metabolic states. The present study also shows similar variation amongst *Labeo rohita*, *Heteropneustes fossilis*, *Channa punctatus*.

The frequency of the micro-nucleated erythrocytes and sister chromate exchanges are positively related to the level of genotoxicity. (Alink *et al.*, 1980) MN test and sister chromatid exchange are very sensitive, rapid and popular methods for detecting the chemically induced chromosome damage / genotoxicity.

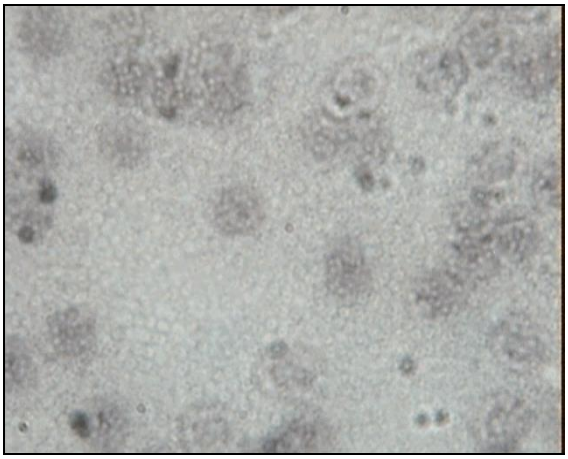

Fig 1: photograph showing that micro-nucleated erythrocytes' fluoride treatment for control group.

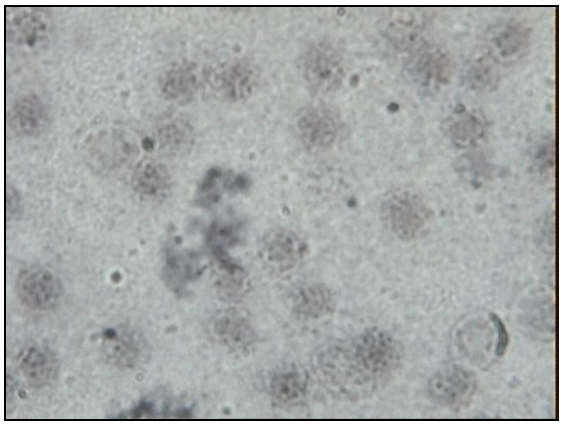

Fig 2: Overview showing that micro nucleated erythrocytes' fish treated with 0.35mgF/L.

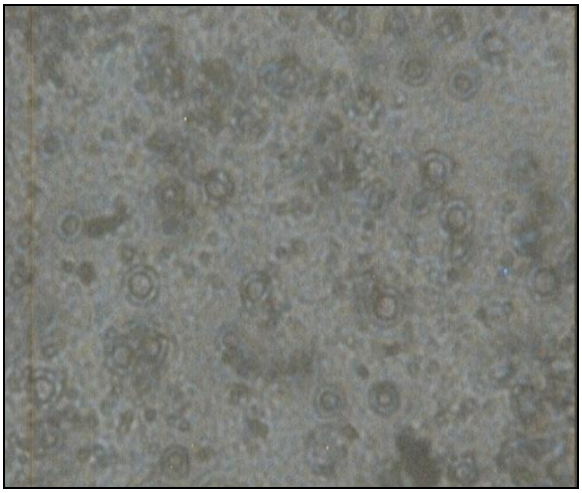

Fig 3: Overview showing that micro nucleated erythrocytes' fish treated with 0.75mgF/L.

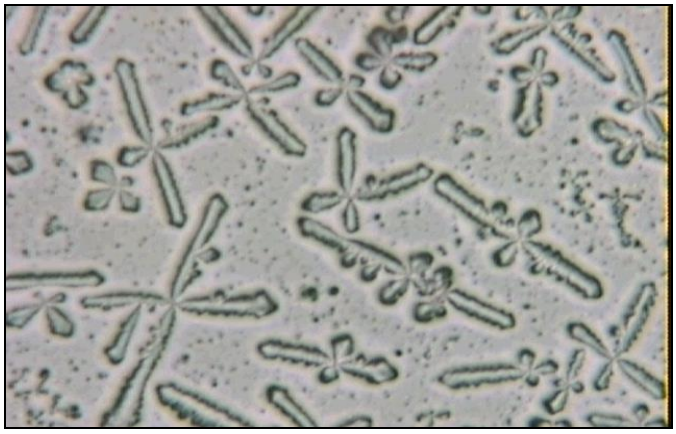

Fig 4: Microphotographs showing that sister chromatid exchange (SCEs) in gill cells of fishes showing that sister chromatid exchange for Control group (without fluoride)

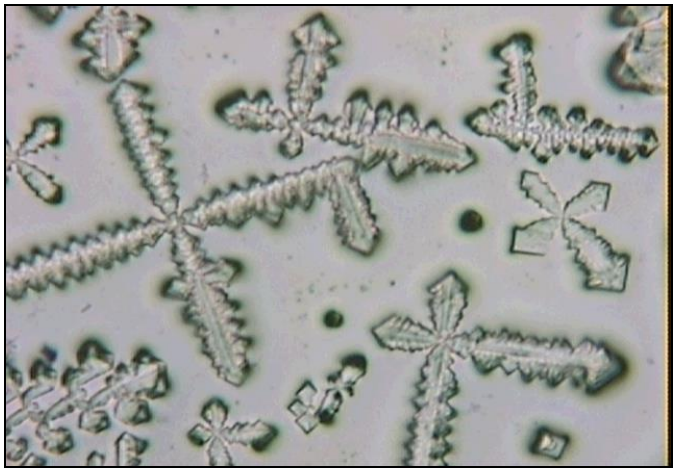

Fig 5: Treated with 0.35mgNaF/L

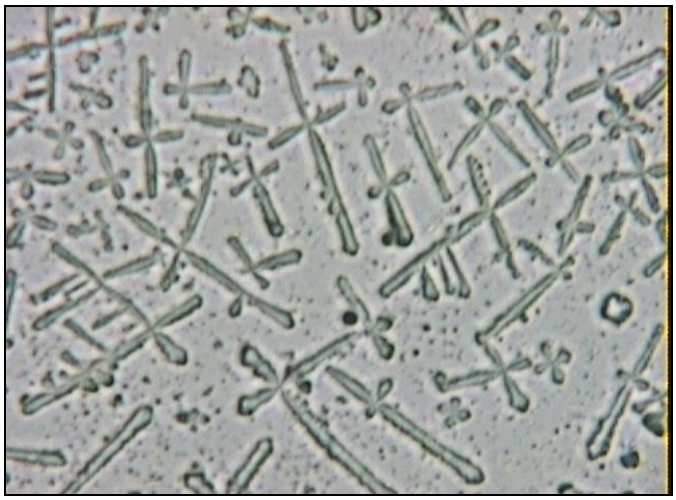

Fig 6: Treated with 0.75mgNaF/L)

Table 2: Physico chemical parameters of aquarium waters, during sodium fluoride (NaF) treatment.

	NaF 0.35mg/L			NaF 0.75mg/L		
Para-meters	**Ist day**	**IInd day**	**IIIrd day**	**Ist day**	**IInd day**	**IIIrd day**
Ph	7.0	7.2	7.2	6.65	7.0	6.9
Dissolved-(O_2 mg/L)	8.5	8.9	8.2	9.3	9.1	8.8

Hardness ($Caco_3$) (mg / L)	115	112	125	113	119	115
Temperature (°C)	36	33	35	32	36	39

Table 3: Number of micro-nucleated erythrocytes (MNEs) and their average percentage frequency in red blood cells.

S. No.	Dosage	Number of MNEs per 1000 cells examined			
		Heteropneustes fossilis	***Labeo - rohita***	***Channa punctatus***	**Average (%) frequency of MNEs)**
1	Control				
2	Ist day	2	1	1	0.133
3	IInd day	1	1	1	0.00
4	IIIrd day	0.6	0.5	1	0.07
5	0.35 mg F / L				
6	I st day	3	2	2	0.233
7	II nd day	3	3	2	0.266
8	III rd day	1	3	4	0.333
9	0.75 mg F / L				
10	Ist day	2	3	3	0.266
11	IInd day	3	3	3	0.3
12	IIIrd day	4	2	4	0.4
Total	09 days	19.6	18.5	21	2.001

Table 4: Number of sister chromatid exchange in Meta phase stage.

Species	Tested metaphase	Control	Group - I	Group- II
Heteropneustes fossilis	220	60	240	30
Labeo rohita	119	40	240	20
Channa punctatus	301	40	199	26

Group – I Control group
Group – II Fish treated with 0.35mgF/L
Group – III Fish treated with 0.75mgF/L

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