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Fluoride impact as carcinogen: a case evidence in *Zaprionus indianus* (Diptera, Drosophilidae)

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ABSTRACT

Fluoride, a trace element, maintains bone and dental well-being at trace levels. However, high levels of fluoride are associated with systemic toxicity, genotoxicity, and carcinogenic effects. Drosophila melanogaster has become a widely used genetic model organism to study fluoride induced toxicity. In this study, the invasive fruit fly *Zaprionus indianus*, a robust species, was used to study the potential carcinogenic effects of fluoride. Larvae were grown on fluoride containing food media (0.0-4.0 ppm) prepared by dissolving sodium fluoride, and the mortality response was analysed using probit analysis to determine the median lethal concentration (LC… €). The LC… € value for fluoride in *Z. indianus* was estimated at 2.5 ppm, and total mortality occurred at e" 3.0 ppm. Hypertrophy of cerebral lobes and structural alterations in the ventral nerve cord were found using morphometric examination of third instar larval brains. Cytological assays using trypan blue and propidium iodide showed lowered apoptotic cell populations, suggesting that programmed cell death was disrupted. These results reinforce *Z. indianus* suitability as a viable invertebrate model for fluoride toxicity studies by providing the first evidence of fluoride induced neuromorphological and cytological changes compatible with early carcinogenic processes.

Figures : 04 References : 25 Tables : 03
KEY WORDS : Apoptosis, Carcinogenesis, Fluoride toxicity, Genotoxicity, *Zaprionus indianus*.

Introduction

Fluoride is a naturally occurring trace element that is vital for bone and dental mineralisation in trace amounts. However, chronic excessive exposure to fluoride has been associated with numerous toxicological impacts in humans and animals. Both natural and anthropogenic activities contribute to increased fluoride levels in the environment[13]. In India, excessive fluoride contamination has been observed in 23 states, with concentrations exceeding the WHO permissible limit of 1.5 mg/L[7, 17, 20, 25].

Although the manifestations of skeletal and dental fluorosis are the most obvious signs of the toxic effects of fluoride, recent reports have shown that fluoride can produce systemic toxicity[15]. Research has shown that excessive fluoride intake can lead to renal toxicity[12], reproductive toxicity[3], bone diseases[24] and alterations in the activities of critical enzymes, including phosphorylase, adenosine triphosphatase, and alkaline phosphatase, in invertebrates[16]. Mechanistic studies on fluoride toxicity have shown that it can induce oxidative stress by increasing the formation of reactive oxygen species (ROS), leading to alterations in the redox balance of cells and contributing to its genotoxic and carcinogenic effects[19].

Drosophila melanogaster is one of the fruit fly

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TABLE-1:Preparation of fluoride solutions from 100 ppm stock solution using dilution method.

S. No.	Target concentration (ppm)	Vol. of Stock Solution (mL)	Volume of Distilled water(mL)
1	1	1.00	99.00
2	2	2.00	98.00
3	2.5	2.50	97.50
4	3	3.00	97.00
5	4	4.00	98.00

species that has been used to study the genetic and toxicological effects of different environmental pollutants, including fluoride[14]. Past research has shown that sodium fluoride (NaF) exposure can lead to effects on development timing, compound eye development, and melanotic tumour formation in adult *Drosophila melanogaster*[8]. Other research has shown that there is a significant association between water fluoridation and cancer prevalence[6] in 9 fluoride prone sites in Japan[23]. Other fluoride compounds, such as fluoride pesticides containing Fipronil (FP), have shown that they can lead to mutagenic, recombinogenic, and carcinogenic effects on the somatic cells of *Drosophila melanogaster*[4,5,21]. From all the research and literature reviewed on the effects of fluoride and other fluoride compounds on organisms, there is sufficient evidence to support the proposed hypothesis that carcinogenesis can be caused in organisms through different pathways.

Despite extensive studies on *D. melanogaster*, little is known about fluoride toxicity in other members of the family Drosophilidae. Extension of such studies is essential to confirm species-specific effects and to find alternative model organisms for comparative carcinogenicity studies. With this aim, *Z. indianus*, a species widely distributed and easily adapted to laboratory conditions within the family Drosophilidae, has been used to assess the carcinogenic effects of fluoride exposure, especially regarding morphometric changes in brain ganglion larvae[9] and cytological features of apoptosis. The results would give us basic information about this new model organism for studying fluoride induced carcinogenesis.

Materials and Methods

Preparation of Fluoride Solutions

Anhydrous sodium fluoride (NaF; analytical grade) was used to prepare the stock solution following the protocol described[18]. Briefly, 0.015 g of NaF was dissolved in 500 mL of distilled water in a volumetric flask to obtain a 100 ppm (100 mg/L) stock solution. Working solutions of desired concentrations were prepared by serial dilution from the stock using the standard formula M V = M, V, (Table 1). All fluoride solutions were freshly prepared to ensure chemical stability and prevent precipitation.

TABLE-2 : Probit analysis for LC_{50}

Concentration (ppm)	log 10 (concentration)	% mortality	Probit of kill	SUMMARY OUTPUT	
0.5	-0.301029996	13	3.87	*Regression Statistics*	
1	0	18	4.08	Multiple R	0.904904
1.5	0.176091259	30	4.48	R Square	0.818852
2	0.301029996	37	4.67	Adjusted R Square	0.782623
2.5	0.397940009	48	4.95	Standard Error	0.423454
3	0.477121255	77	5.74	Observations	7
4	0.602059991	92	6.41		

Rearing of *Zaprionus indianus*

Z. indianus (Family: Drosophilidae) is morphologically characterised by two pairs of silvery white, zebra-like stripes on the thorax and abdomen (Fig. 1). Collected and identified species of *Z. idianus* were reared on standard *Drosophila* food medium in the genetics lab. of the department. Cultures were maintained at 25 ± 1°C, 60 ± 5% relative humidity.For experimental exposure, food media were prepared in fluorinated water with different concentrations of fluoride solutions (0.00–4.00 ppm). Six replicate culture vials were maintained for each concentration, resulting in a total of 36 replicates (6 × 6 = 36).

Determination of LC

A single mated female was allowed to oviposit overnight in a Petri dish containing standard food medium. The bifilamentous eggs were observed under a stereomicroscope, and 10 eggs were carefully transferred into each vial containing freshly prepared fluoride-supplemented food at concentrations ranging from 0.0 to 4.0 ppm, along with a control (untreated Group). All the vials were incubated in a BOD chamber at 25°C and 60% relative humidity. The parental female was removed after a week, and 14 days later, the number of adult flies that successfully emerged was recorded. The median lethal concentration (LC… €) value was determined as the fluoride concentration that inhibited the development of 50% of the eggs (5 out of 10). Percentage mortality at each concentration was recorded, followed by probit analysis. Probit analysis was conducted to determine LC_{50} statistically as described[2]. using MS –EXCEL.

Neoplasia assessment

Tumour formation (neoplasia) and tissue hypertrophy are hallmark indicators of carcinogenic transformation. To assess these endpoints, a culture of *Z. indianus* was established by rearing a naturally inseminated wild female on food medium containing fluoride at the LC… € concentration (2.5 ppm). Third instar larvae were dissected in Poels' salt solution (pH 6.8) under a stereoscopic binocular microscope. Brain ganglia were isolated, and the size of the right & left cerebral lobes and the ventral nerve cord was measured using an ocular micrometrecalibrated with a stage micrometre.Morphometric alterations were statistically compared with those of control larvae.

Cytological Assessment of Apoptosis

To evaluate fluoride-induced alterations in apoptosis, cytological assays were performed using the Trypan Blue exclusion assay[22] and the propidium iodide (PI) fluorescencedye exclusion method[11]. Larval brain ganglia were incubated in 0.4% Trypan Blue for 5 min, washed in phosphate-buffered saline (PBS, pH 7.4), and examined under a compound microscope. For PI staining, tissues were incubated in 10 µg/mL PI for 5 min, rinsed with PBS and visualised under a fluorescence microscope. The proportion of stained

TABLE-3:Comparative brain lobe size in control and fluoride-exposed (2.5 ppm) larvae of *Zaprionus indianus*.

Replica	Control (0.0 ppm) Right lobe (µm)	Control (0.0 ppm) Left lobe (µm)	Treated (2.5ppm) Right lobe (µm)	Treated (2.5ppm) Left lobe (µm)	% Increase (Right lobe)	% Increase (Left lobe)
1	26.5	26.5	30.8	35.42	16.2	33.6
2	26.5	26.5	33.24	35.30	25.43	33.20
3	26.5	26.42	28.2	29.00	6.41	9.76
4	25.8	26.25	30.8	35.42	19.37	34.93
5	26.43	26.52	33.24	35.30	25.76	33.10
6	26.8	26.5	33.5	35.20	25.00	32.83
Mean ± SD	**26.42 ± 0.33**	**26.44 ± 0.05**	**31.63 ± 2.09**	**34.27 ± 2.58**	**19.71 ± 7.57**	**29.57 ± 9.73**

Fig. 1 : Experimental model: *Zaprionus indianus* showing characteristic zebra-like silvery white thoracic and abdominal stripes

(non-viable) and unstained (viable) cells was visualised (Fig. 4) under a fluorescence microscope and an Olympus light microscope.

Results

Mortality of *Zaprionus indianus* at different fluoride concentrations used for LC... € determination, which indicated that 50% of flies died at the fluoride concentration of 2.5 ppm, indicating LC_{50}. (Table-2).Further probit analysis was performed to estimate the LC_{50}, which revealed a value of 1.91 ppm.

Neoplasia Assessment after measuring the size of the enlargement of the brain and metastasis revealed significant size variation at 2.5 ppm of fluoride (Fig. 3)

Apoptotic (programmed cell death)

Apoptotic (programmed cell death) assessment was performed to visualise the cell's fate. Dye exclusion with both dyes, PI (3-1) and trypan blue (3-2), clearly

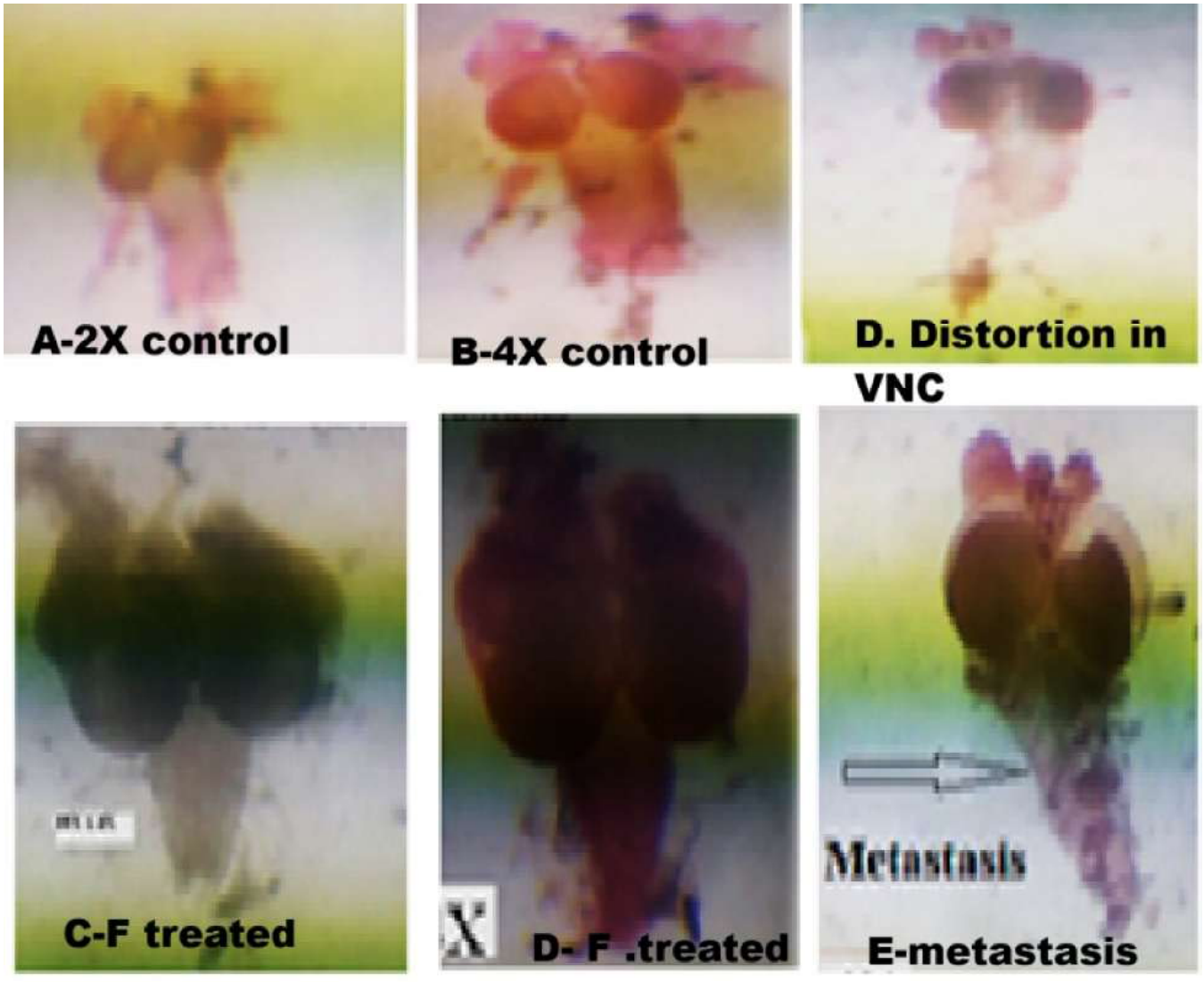

Fig. 2 :Neoplasia in the cerebral ganglion. (A-B) Control, (B-C) Neoplasia, (D) Distortion in Ventral Nerve Chord (VNC),(E)Metastasis in VNC

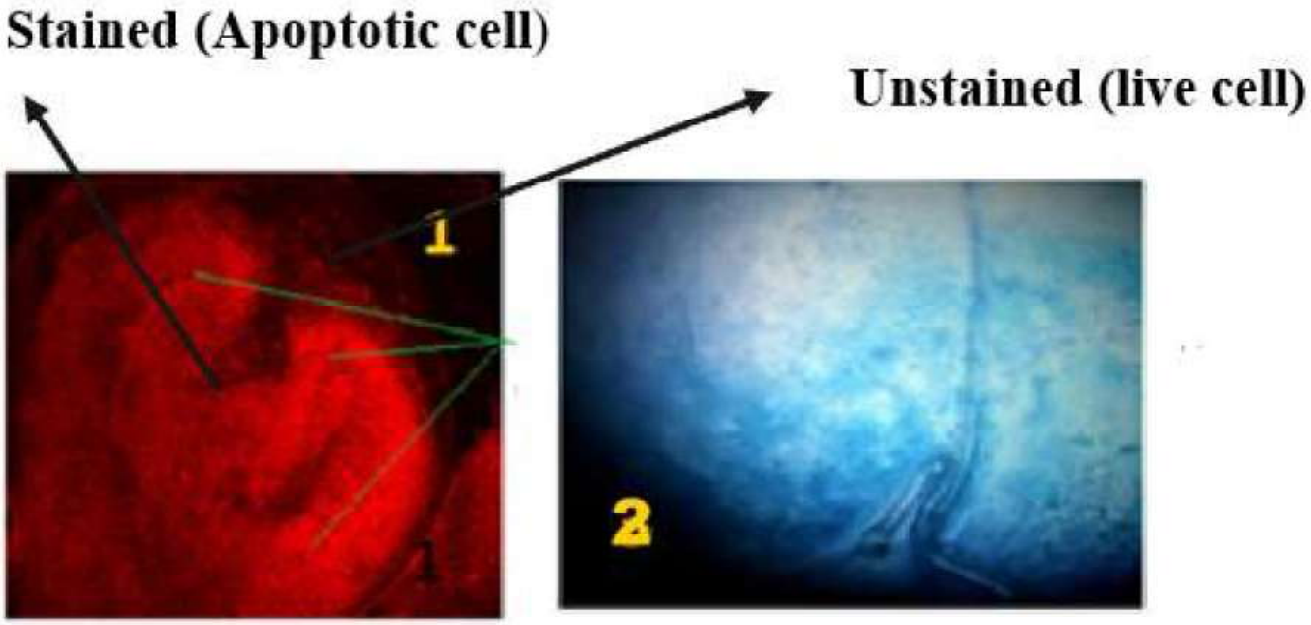

Fig. 3 :Dye exclusion for apoptosis (1) with Propidium iodide, (2) with trypan blue (stained dead cell and unstained region live cell)

showed stained regions corresponding to dead cells and unstained regions indicating live cells.

Discussion

The current work highlights *Z. indianus*'s high susceptibility to fluoride stress by showing that fluoride exposure causes notable toxic, morphological, and cytological changes in the organism. Strong toxicity even at relatively low concentrations is indicated by the observed concentration-dependent increase in mortality and the LC_{50} value of 2.5 ppm (Figure 4). Complete mortality (100%) was reported at 3.0 and 4.0 ppm, whereas approximately 51.7% mortality was observed at 2.5 ppm, which corresponds to the LC_{50}. Fluoride also exhibits developmental toxicity and promotes mortality.The log-transformed concentration and the probit of mortality showed a strong positive linear association, with a correlation coefficient value of 0.905.Similar results have been documented in other biological systems, where high fluoride levels cause damage to vital organelles such as the nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus, as well as alter cellular homeostasis by blocking important enzyme processes[10]. Increased cellular stress and compromised metabolic processes are frequently linked to these disturbances.

Severe neuro developmental abnormalities are suggested by the significant enlargement of brain lobes and distortion of the ventral nerve cord seen in larvae treated with fluoride. Chemically induced carcinogenic-like alterations in *Drosophila* species have previously been linked to such structural defects[1]. The underlying dysregulation of cell proliferation and differentiation processes, which are crucial steps in the start of carcinogenesis, may be reflected in these morphological alterations.

Apoptosis is vital for maintaining cell stability because it eliminates defective cells. Apoptosis is considered fundamental for maintaining cell balance and is vital for removing defective cells. The blockage of apoptosis can lead to the preservation of defective cells. Results from cytological analysis showed that there was a reduction in the number of apoptotic cells within the brain tissue of larvae exposed to fluoride. This was shown through a reduction in Trypan Blue and propidium iodide stain uptake, implying that there was an inhibition of apoptosis due to fluoride treatment.

Conclusion

The results of the study suggest that fluoride exposure has the capacity to induce carcinogenic changes in *Z. indianus* at both the cellular and organismal stages. The increased mortality rate, structural deformation of the brain tissue, and suppression of apoptosis are all suggestive of the possibility of fluoride poisoning inducing the early stages of carcinogenesis. *Z. indianus* has thus been found to be a viable and cost-effective substitute for the study of fluoride-induced carcinogenic changes and the development of treatment possibilities.

Declarations

Conflict of interest

The authors declare no conflict of interest.

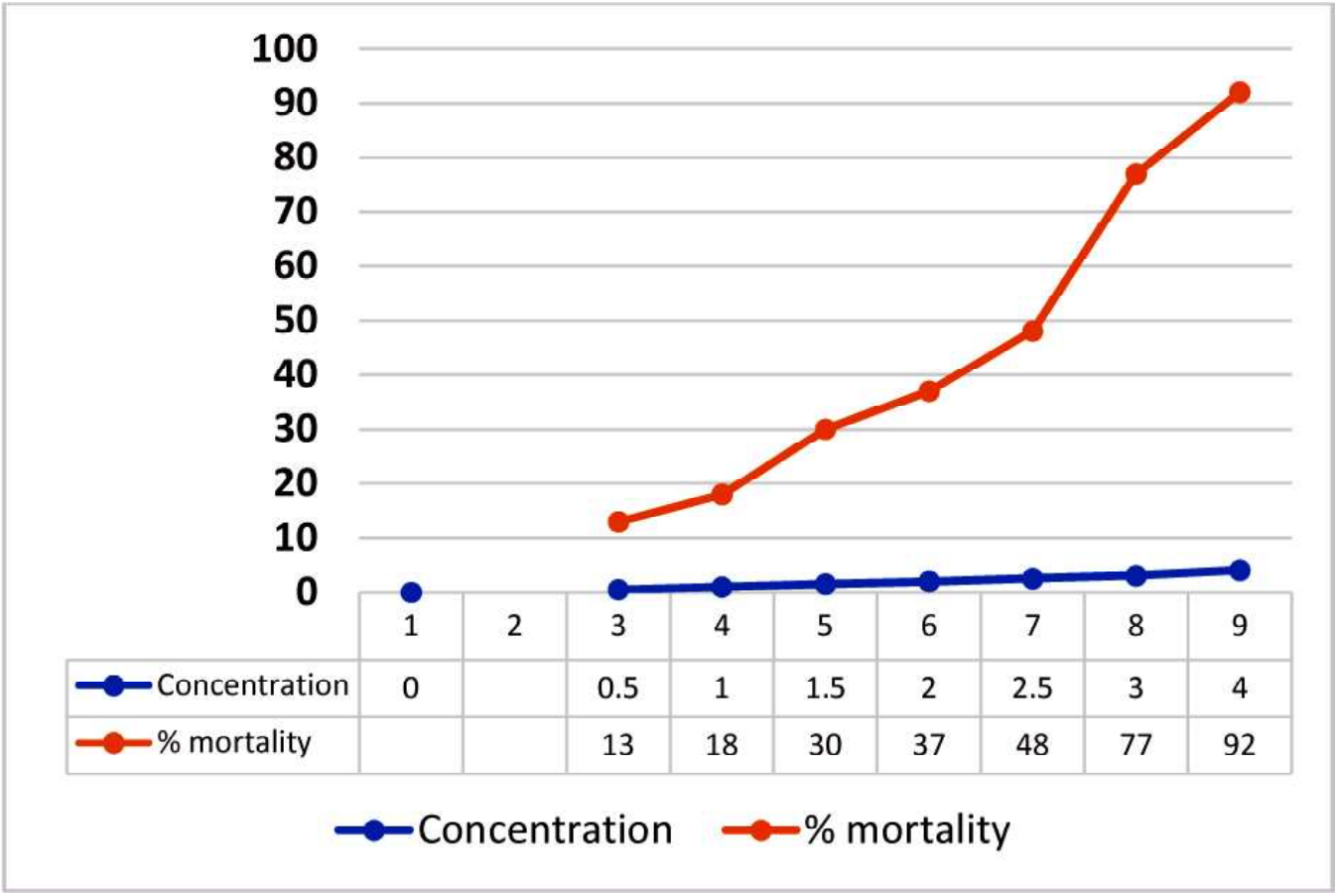

Fig. 4 :Positive Correlation between Fluoride & mortality percentage

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