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Assessment of sublethal toxicity using proliferation markers in fish cell line-ICG exposed to agrochemicals

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ABSTRACT

The purpose of this study was to determine the cytotoxic impact of four agrochemicals on *Catla catla* Hamilton 1822 Indian Catla catla gill cell line (ICG): insecticide [imidacloprid (IMI)], fungicide [curzate (CZ)], herbicide [pyrazosulfuron-ethyl (PE)], and fertilizer micronutrients (MN). The cytotoxic study was carried out by following the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method for 96 hours and inhibition concentration (IC_{50}) values were determined. For further subacute studies, sublethal concentrations (1/20th of IC_{50} as low dose, 1/10th of IC_{50} as medium dose, and 1/5th of IC_{50} as high dose) were selected. The ICG cells were exposed to all agrochemicals for 7 days and toxicity was analyzed with respect to untreated control. The morphological changes were observed and Trypan blue assay was used to understand the effect of agrochemicals on the ICG cells viability. The study reported a dose-dependent alteration in morphology and viability in ICG cells when exposed to agrochemicals. Furthermore, the expression of proliferative markers like proliferating cell nuclear antigen and cyclin genes (cyclin E and A) were analyzed through quantitative polymerase chain reaction. There was a significant decrease observed in gene expression of proliferating cell nuclear antigen, cyclin A, and cyclin E, which indicates the toxicity of agrochemicals IMI, CZ, PE, and MN, resulting in alterations in the cell cycle of the ICG cell line.

1. INTRODUCTION

Pesticide residues have been detected in many ecosystems of the environment, generating serious concerns about their uncontrolled use, which has outweighed the benefits gained [1,2] The scientific community is concerned about the possibility of pesticide management having a negative influence on numerous natural environment components [3,4]. Pesticides have been used in India alone in excess of 100,000 tons, mostly for agricultural pest control, because of their low cost and broad-spectrum toxicity [5–8]. Agricultural fertilizers and pesticides are applied worldwide in excess of 140 billion kilos per year, creating a major source of pollution through agricultural runoff [6]. Agricultural pollution refers to the biotic and abiotic waste products of agriculture that pollute, degrade, and/or harm humans, their economic interests, as well as the environment and ecosystems surrounding them [9,10]. Agrochemicals have the potencies to pollute food and water, putting human health at risk [11,12]. Due to their increasing toxicity, persistence, and potencies to accumulate in organisms, the use of such agrochemicals poses a significant risk to human health and has become a serious issue for the aquatic environment [13].

Human health has been posed with a huge risk when it comes to pesticides and their usage. However, initially, pesticides were synthesized to control pest population, but their usage has led to posed prospective risks to human health and nontarget environmental species [14–18]. Traditional toxicity testing highly depends on *in-vivo* single constituent studies, which have been thoroughly investigated at all levels of the system, including producer and consumer levels. However, *in-vivo* testing is time-consuming and expensive, and it necessitates a lot of upkeep and a large number of animals, which raises ethical concerns [19]. Thus, for economic, practical, and ethical reasons, *in-vitro* techniques have risen tremendously, and the use of cell lines as an alternative to *in-vivo* testing is being seriously examined [14,20,21].

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In-vitro analysis of fish cells is gaining a promising alternative for mechanistic evaluation for toxicological assessment, with the potential to replace or reduce the usage of fish as a whole organism [22], which is also supported by the idea where maintenance of fish cells in culture conditions is easy and cost-effective. There have been a lot of studies carried out on hazardous substances to correlate the toxicity of xenobiotic in *in-vitro* and *in-vivo* experimentation, which has resulted in the usefulness of mitigating the usage of it [23,24]. Research is now intended toward assessing the toxicity of agrochemicals on different cell lines derived from fish organs. For instance, several cell lines have been developed from India, such as the Indian Catla catla Heart cell line from the heart of *Catla catla*; RE and Indian Catla catla Brain Cell line from the eye of *L. rohita* and brain of *C. catla*, respectively [25]; rohita eye cell line, rohita Fin cell line, and Cell line from L. rohita swim bladder from fin, heart and swim bladder of *L. rohita*, respectively [26]; from the fin tissue of *Tor tor* [27]; two cell lines from the fin and eye tissues of *Tor chelynoides* [28,29]; and heart and gill cell lines from *C. catla* [30,31].

Previous *in-vivo* studies have well established the toxic potential of all the classes of agrochemicals, viz. imidacloprid (IMI), curzate (CZ), micronutrients (MN), and pyrazosulfuron-ethyl (PE), which elucidated the alteration in hematological, histological, biochemical parameters, behavior, and neuroendocrine response as well [4,12,23,24,32–35]. However, there is a gap in our understanding with regards to the molecular mechanism. Thus to understand the mechanism of action, the present study was undertaken to unravel the alteration in cell cycle on exposure to agrochemicals (PE, CZ, MN and IMI) in fish cells Indian Catla catla gill cell line (ICG). More precisely, the loss of normal cell orchestration and cell proliferation was addressed by studying the cell cycle regulation and key proliferation markers.

Proliferating cell nuclear antigen (PCNA) is a vital component in replication in which it acts as a progression factor and DNA clamp for DNA polymerase δ, and additionally, it also plays a pivotal role in DNA repair, chromatin remodeling, and epigenetics. It is considered as a universal marker for cell proliferation [36,37]. The control of DNA replication is a key element in the proper functioning of a cell, and it influences genome stability [38]. Duplication of the genetic material that occurs in S phase of the cell cycle has to be coordinated with other cellular processes like mitosis. DNA replication is regulated mainly at the initiation step as a result of cooperation between different signaling pathways controlling the cell cycle [39,40].

In addition to PCNA, cyclin and cyclin-dependent kinases (CDKs) are yet other universal markers which are known to control cell cycle transitions. Several classes of cyclins have been described, of which cyclin E binds to G1 phase Cdk2, which is required for the transition from G1 to S phase of the cell cycle that determines initiation of DNA duplication [40]. During the S phase of the cell cycle, cyclin A is found in the nucleus and is involved in the initiation and completion of DNA replication [39,41]. Quantification of proliferative markers (PCNA and cyclin genes) can thus be crucial in understanding its role of xenobiotics in the cell cycle. The present inventory aims to understand the alterations in the expressions of the proliferative markers in fish cell line-ICG due to the exposure of different classes of agrochemicals (IMI, CZ, MN, and PE). The selection of the agrochemicals was based on the routine usage in the agricultural field and its *in-vivo* assessment [4,12,23,24]. Moreover, the gill cell line was taken as it is the first organ of the fish that is acquainted with any toxicant in the natural habitat.

2. MATERIALS AND METHODS

2.1. Chemicals

Agrochemicals insecticide IMI (TATAMIDA), fungicide CZ (DuPont™ Curzate M8), herbicide PE (Saathi, UPL), and MN (Librel™, Ciba) were purchased from the local vendors and they were dissolved (individually) in water for the further experimentation.

2.2. Culturing of ICG Cells

The ICG gill cell line of *C. catla* was procured from the National Repository of Fish Cell Line (NRFC), Indian Council of Agricultural Research, National Bureau of Fish Genetic Resources (ICAR-NBFGR), Lucknow. The cell line was cultured in Leibovitz's L-15 (AL0011A, HiMedia, India) supplemented with 10% FBS (RM9955, HiMedia, India) [11]. The flasks were incubated at 28°C in a biological incubator (LabTech) and the medium was changed every fourth day. Upon reaching 80%–85% confluence, the cells were subcultured in the ratio of 1:2 by using trypsin–EDTA solution (TC007, HiMedia, India).

2.3. MTT Assay

ICG cells were seeded in the density of 2×10^4 cells per well in 96-well tissue culture plates (TPC96, HiMedia, India) and were incubated overnight at 28°C. The medium was removed after incubation and the cells were treated with a medium containing agrochemicals (CZ, IMI, PE, and MN) for 96 hours. After a 96-hour exposure period, the test medium was replaced by 10 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (TC191, HiMedia, India) in phosphate buffered saline (PBS) (M1866, pH 7.4, HiMedia, India). After incubation for 4 hours at room temperature in the dark, the solution was removed carefully and dimethyl sulfoxide (6644, SRL, India) was added per well to solubilize the purple formazan crystals produced. The absorbance of each well was measured at 570 nm and cell inhibitions were obtained using the following formula:

$$\% \text{ Cell inhibition} = 100 - \frac{\text{Average OD of test}}{\text{Average OD of control}} \times 100$$

After obtaining the inhibition concentration (IC50), sublethal (1/20th, 1/10th, and 1/5th doses of IC50) concentrations were selected for further subacute studies as low dose (LD), medium dose (MD), and high dose (HD), respectively. Moreover, the ICG cells were exposed to all agrochemicals, i.e., IMI, CZ, MN, and PE, for 7 days and toxicity was analyzed with respect to untreated control ($n = 3$).

2.4. Cell Viability Assay

Trypan blue assay was used to understand the effect of agrochemicals on the viability of ICG cells. The cells were seeded at a density of 1×10^5 cells/ ml in a complete L-15 medium. Following 24 hrs of cell growth, different concentrations of agrochemicals (LD,

MD, and HD) were added to the cells. After 7 days, cells were trypsinized, washed, and resuspended in PBS containing 0.4% trypan blue (TCL046, HiMedia, India). The number of viable cells was counted using hemocytometer (GW088, HiMedia, India) as per the standard protocol. Each experiment was carried out with three replicates ($n = 3$) for each group for statistical analysis.

$$\%\,\text{Cell viability} = \frac{\text{No of viable cells}}{\text{Total no of cells}} \times 100$$

2.5. Cell Morphology Analysis

Cells were plated into a 6-well culture plate (9.5 cm^2, TPC6, HiMedia, India) at a density of 2×10^5 cells (in 2 ml complete medium). After overnight growth, the supernatants from the culture plates were aspirated and fresh aliquots of growth medium containing various concentrations (LD, MD, and HD) of agrochemicals were added. Upon incubation for 7 days, cells were washed with PBS (M1866, HiMedia, India, pH 7.4) and morphological changes were observed under an inverted phase-contrast microscope at 100× magnification.

2.6. Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from ICG cells from control and treated cells for all agrochemicals using TRIzol reagent (15596-026, Invitrogen, USA) with standard protocol. The pellet was resuspended by adding 40 µl of diethyl pyrocarbonate in water (DBOS009, SRL, India), which was quantified spectrophotometrically using NanodropC and was stored at −20°C. The cDNA was synthesized from each sample using the standard kit protocol of Thermo Scientific Verso cDNA Synthesis Kit (AB-1453/A), for which 1 µg RNA was used as a template per reaction for single-strand cDNA synthesis using oligo dT primers.

2.7. Quantitative PCR Amplification

Quantitative RT-polymerase chain reaction (PCR) was carried out using the method where PowerUp SYBR Green Master Mix (A25741, Applied Biosystems, USA) was used and the amplification was carried out in Quant Studio 12K (Life technology) FAST real-time PCR machine with primers of PCNA, cyclin A, and cyclin E (Table 1). The melting curve of each sample was measured to ensure the specificity of the products. glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the variability in the expression levels and data were analyzed using the $2^{\Delta\Delta CT}$ method [42].

2.8. Statistical Analysis

Experiments were carried out in triplicate ($n = 3$) for each exposure concentration. Data were analyzed with GraphPad Prism 9 (GraphPad Software) and one-way analysis of variance ($p \leq 0.05$) was carried out. The *post-hoc* test was carried out by Dunnett's multiple comparison test to further understand the level of significance ($p \leq 0.05$; $p \leq 0.01$).

3. RESULTS

Table 2 and Figures 1–4 show the IC_{50} values and sublethal concentrations of different classes of agrochemicals. IMI was determined to be the most harmful of all the agrochemicals, followed by CZ and MN, with PE being the least toxic. ICG cells were treated with sublethal concentrations [LD (1/20th), MD (1/10th), and HD (1/5th)] of all agrochemicals (IMI, CZ, MN, and PE) for 7 days. Cell viability assay carried out by Trypan blue (Table 3) showed that cell proliferation was significantly ($p < 0.05$) affected upon treatment with agrochemicals in a dose-dependent manner. At all exposures, cell viability was found to be highest in

Table 1: Real-time PCR primer sequences.

	Gene name	Primer type	Sequence	Tm
1	GAPDH	Forward	CTCACACCAAGTGTCAGGACGAACAG	66.38
		Reverse	GTCAAGAAAGCAGCACGGGTCACC	66.13
5	PCNA	Forward	GCACGTCTGGTTCAGGGATCTATCC	66.26
		Reverse	TGCAGAGAAATGCCCGACGAGC	63.98
7	Cyclin A	Forward	CTCAAGCCCGGCCAAAGAGTTG	63.98
		Reverse	GCATCCATCTGAACGAGTCCAGGATC	66.38
8	Cyclin E	Forward	CGTGAAACCAAAGGGTGAAGACACTG	64.80
		Reverse	GCATCCATCTGAACGAGTCCAGGATC	66.38

Table 2: IC_{50} values and their sublethal doses for IMI, CZ, MN, and PE for ICG cell line.

Agrochemical	IC_{50} value	LD (1/20th IC_{50})	MD (1/10th IC_{50})	HD (1/5th IC_{50})
IMI	43.95 µg/ml	2.19 µg/ml	4.39 µg/ml	8.7 µg/ml
CZ	65.34 µg/ml	3. 26 µg/ml	6.53 µg/ml	13.06 µg/ml
MN	290.8 µg/ml	14.54 µg/ml	29.08 µg/ml	58.16 µg/ml
PE	460.85 µg/ml	23.04 µg/ml	46.08 µg/ml	92.17 µg/ml

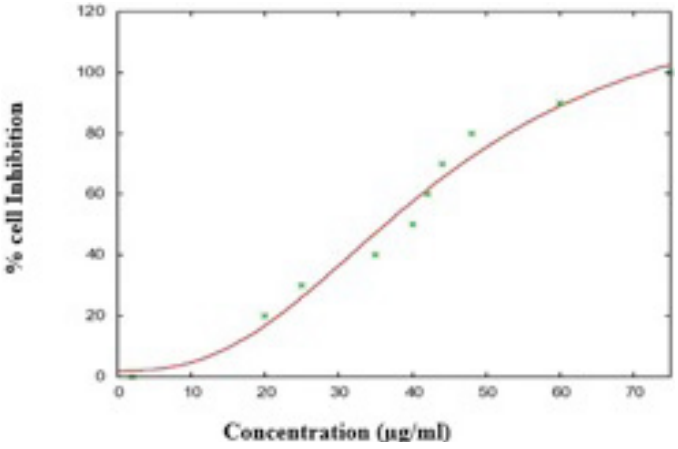

Figure 1: ICG cell mortality against different concentrations of IMI.

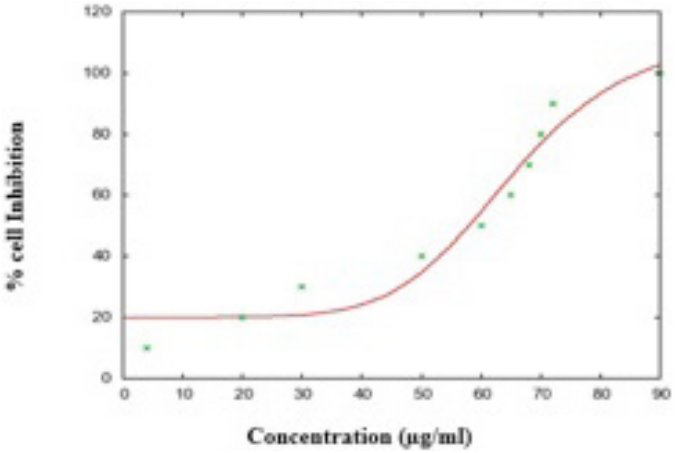

Figure 2: ICG cell mortality against different concentrations of CZ.

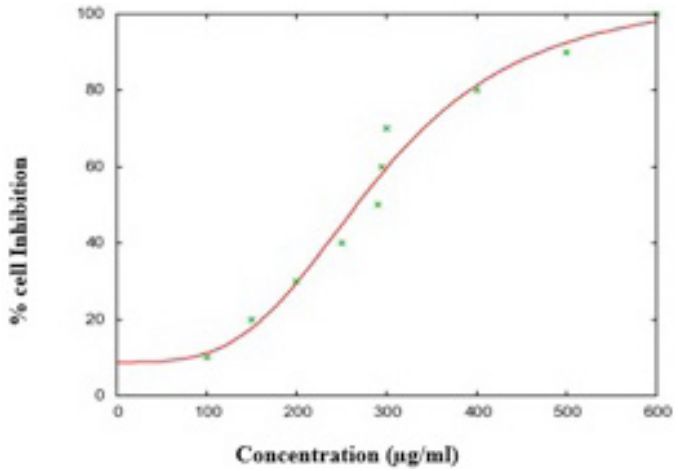

Figure 3: ICG cell mortality against different concentrations of MN.

PE and MN, low in CZ, and lowest in IMI. Among all the groups, HD of IMI, CZ, MN, and PE showed a significant decrease in viability compared to control.

Morphological alterations were also observed, such as loss of integrity of membrane, membrane blebbing, detachment of

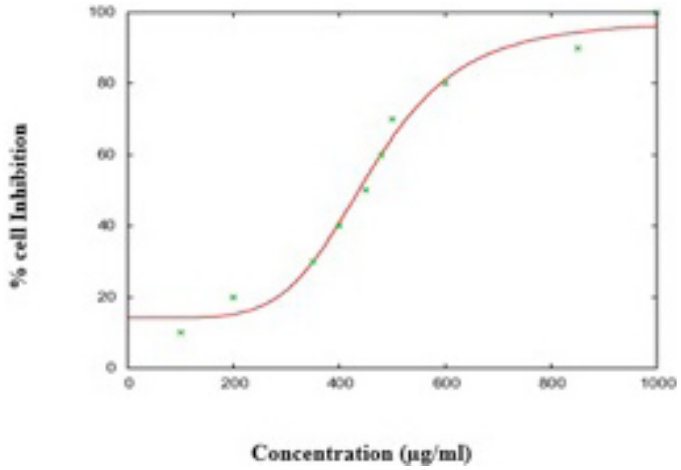

Figure 4: ICG cell mortality against different concentrations of PE.

cells, and formation of apoptotic bodies compared to the control cells which showed healthy cell morphology. Dose-dependent morphological changes were observed in cells exposed to agrochemicals, where MN- and PE-treated cells exhibited fewer alterations, whereas IMI- and CZ-treated cells exhibited the highest alterations in comparison to control. The observed alterations in morphology of ICG cells are shown in Figure 5.

Subacute exposure of agrochemicals for 7 days resulted in differential expressions of the proliferative markers. Expression of the proliferative marker genes, such as PCNA and cyclin A, showed different expressions. A significant dose-dependent decrease ($p < 0.01$) was seen in PCNA expression (Fig. 6) in all the treated groups for all the doses compared to control, while cyclin A was found to be significantly decreasing only at MD and HD of IMI ($p < 0.05$; $p < 0.01$), CZ ($p < 0.01$), and MN ($p < 0.01$) exposure compared to the control. PE exposure resulted in a significant ($p < 0.01$) decrease only at HD (Fig. 7). Cyclin E expression resulted in a dose-dependent significant ($p < 0.01$) decrease in exposure to IMI, CZ, and PE. However, MN exposure was found to be significantly decreased ($p < 0.01$) only at HD compared to the control (Fig. 8).

4. DISCUSSION

Under the Green Revolution, agrochemicals and chemical fertilizers were widely employed to protect crops from pests and increase yield, resulting in increased productivity and economic benefit of agricultural output to satisfy the rising demand for food due to the fast-growing population [43]. Runoff and groundwater leaching from a range of chemicals used in agricultural activities have a significant potential of contaminating aquatic habitats that flow through the agricultural regions. Fish is the most economically important nontarget species that is adversely affected by severe agrochemical pollution [43–45].

To evaluate the toxic potential of agrochemicals many scientists have worked on the toxic effect on fish in *in-vivo* and *in-vitro* systems. ICG cells have been found to be good candidates for assessing *in-vitro* acute cytotoxicity of hazardous compounds and heavy metals [29]. We employed ICG cells to assess the *in-vitro* toxicity of agrochemicals such as IMI, CZ, MN, and PE.

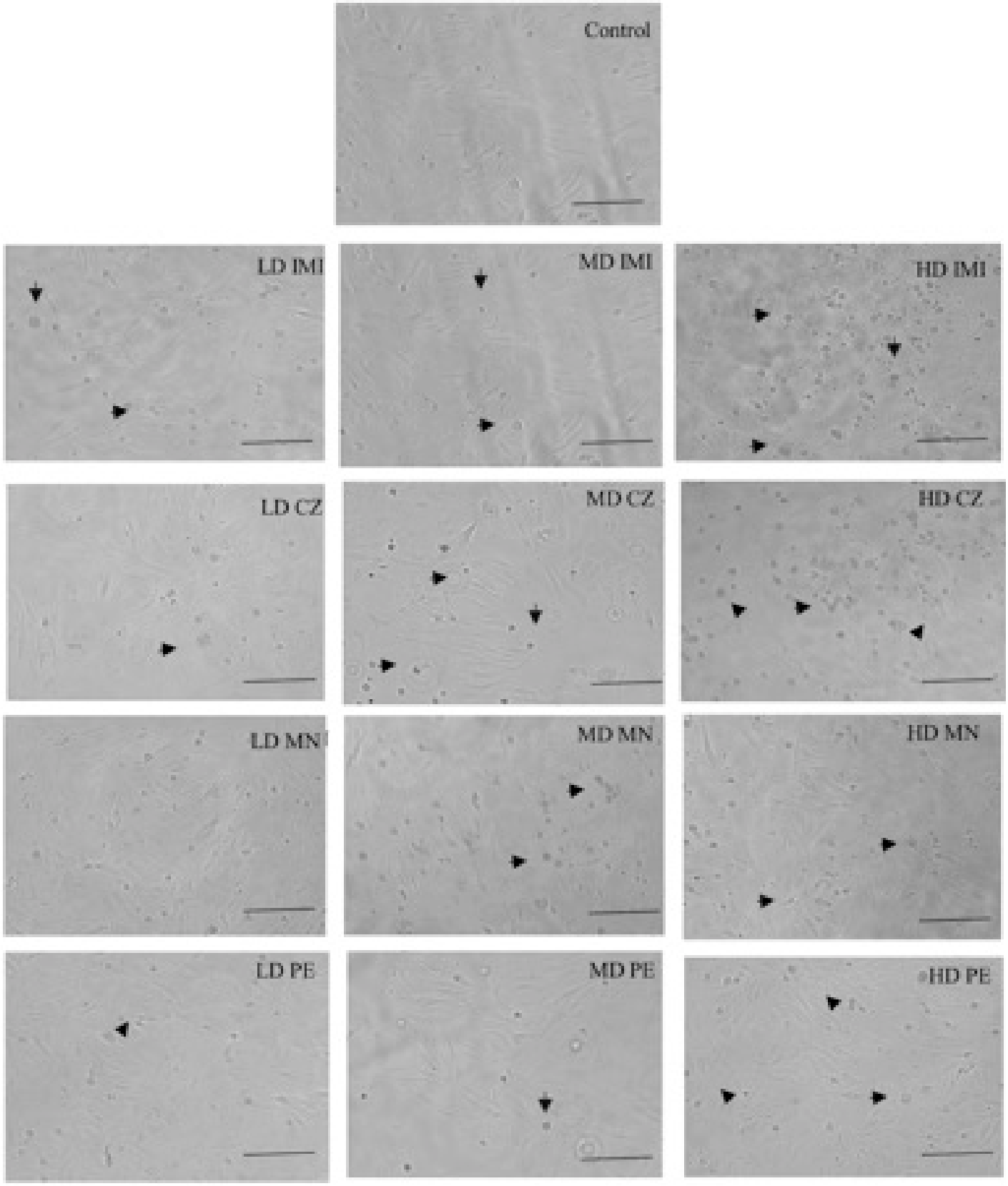

Figure 5: Alterations in the cell morphology of ICG cells exposured to agrochemicals.

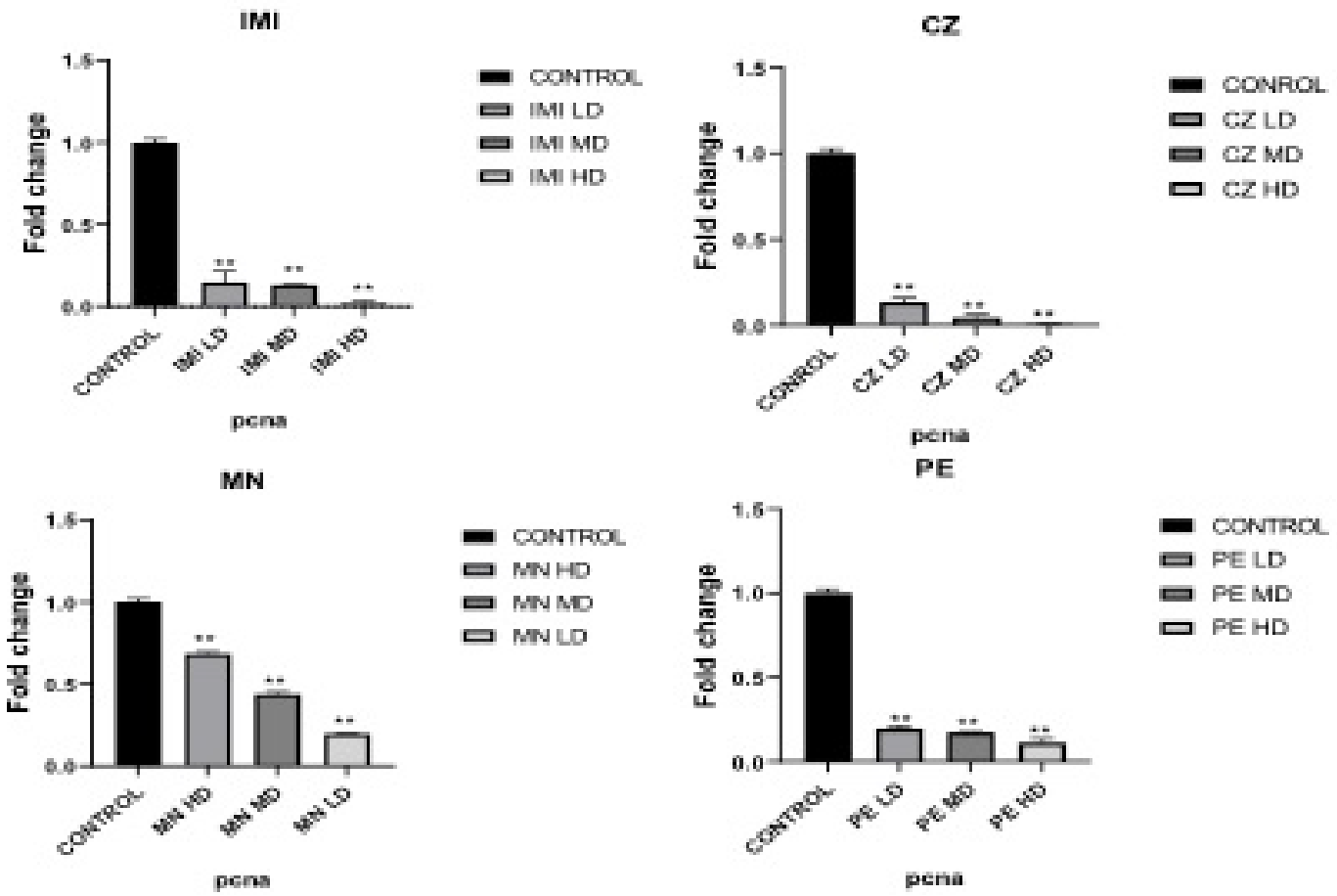

Figure 6: The level of PCNA (in folds) in ICG cells treated with sublethal doses of IMI, CZ, MN, and PE. Each value represents the mean ± SEM ($n = 3$). The significant level is indicated by * $p < 0.05$ and ** $p < 0.01$.

The half-maximal inhibitory concentration (IC_{50}) is a measure of the potency of a chemical in inhibiting a specific biological or biochemical function [45–47].

The effect of agrochemicals on the ICG cells of *C. catla* was assessed by the uptake of MTT and its following reduction in the mitochondria of living cells to MTT formazan [48]. This is the first time *in-vitro* studies are reported in which we found the IC_{50} of four different agrochemicals in fish gill cells ICG. According to previous studies, IMI proved to be toxic to many nontarget organisms [19,49–51]. Earlier studies have reported that LC_{50} values have proved that neonicotinoids IMI is the most toxic to the nontarget organisms in *in-vivo* conditions, followed by CZ, MN, and PE [4,12,23,24,32,35]. Furthermore, *in-vitro* studies have also suggested that the neonicotinoids are more toxic compared to other agrochemicals. The IC_{50} of IMI is 0.023 mM, which was reported previously in the prostate epithelial WPM-Y.1 cell line [52]. The IC_{50} values of neutral red, MTT, and total cell protein were 41.86, 38.46, and 39.08 g/ml, respectively, in an *in-vitro* study of the pesticide IMI in the gill cell line of *Flounder* (FG) [53].

Microscopic observation also revealed the presence of many abnormal cells; some cells had lost their normal cell morphology: loss of cell shape and sphericity and increase in cell granularity. Moreover, the cells were seen to get detached, float, and die. The morphological alterations were observed to be in the proportion of concentrations of the agrochemicals. Our studies are in agreement with previous reports on the assessment of cytotoxicity of the organophosphorus pesticide parathion on FG-9307 cells *in-vitro* system. They concluded that with the increase in the parathion concentration, the degree of damage to the cellular structures was more serious [54]. Moreover, morphological changes were observed in two fish cell lines, RTG-2 cells and PLHC-1 cells, on exposure to sodium fluoroacetate during previous cytotoxic studies [55]. Cytotoxic effects of benzonitrile herbicides using two human cell lines, Hep G2 and HEK293T, were studied where they have reported the alteration in morphology in a dose-dependent and time-dependent manner [56]. Apart from these assessment studies on cytotoxicity of imidazolium in the ovarian fish cell line CCO, the human cell line HeLa also revealed the same results [57]. Our results support the previously reported changes in cell shape, granularity, and alter morphology observed on exposure to toxicants. Of all the agrochemicals, the morphological changes in the IMI and CZ-treated groups were much more significant. The observed toxicity in ICG cells could be ranked in the

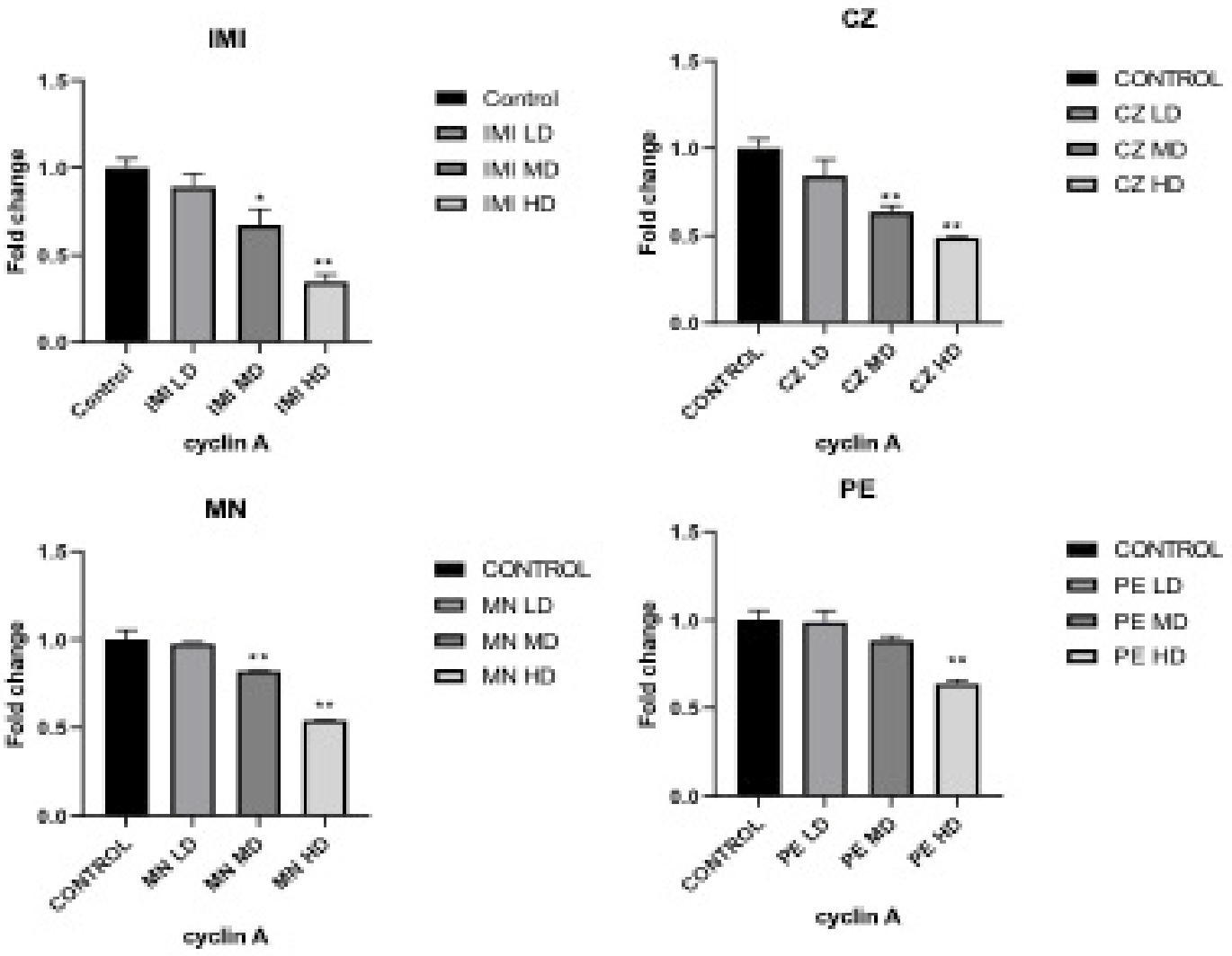

Figure 7: The level of cyclin A (in folds) in ICG cells treated with sublethal doses of IMI, CZ, MN, and PE. Each value represents the mean ± SEM ($n = 3$). The significant level is indicated by * $p < 0.05$ and ** $p < 0.01$.

following decreasing order: IMI > CZ > MN > PE on exposure to agrochemicals.

PCNA is a well-conserved protein present in all eukaryotic species, as well as Archaea. PCNA was initially discovered to function as a processivity factor for DNA polymerase, which plays a role in DNA replication [57]. Moreover, PCNA activities are involved with other critical cellular processes such as chromatin remodeling, DNA repair, sister-chromatid cohesion, and cell cycle control [37,57]. Because cells spend more time in the G1 to S phase transition, PCNA expression is considered an indicator of cell proliferation. Furthermore, as part of the DNA replication and repair mechanism, this plays an important function in nucleic acid metabolism [58].

PCNA has been found in a variety of cell types in mammalian tissues, as well as in a variety of fish organs [21]. The effect of Mirex pesticide on the expression of PCNA levels has been reported [59]. It has been stated that organophosphate insecticides cause a substantial decrease in cell proliferation in liver cells [60]. Our results are in agreement with earlier reported studies. There was a significant dose-dependent decrease observed on the exposure of all the agrochemicals, suggesting that the decrease in the PCNA mRNA has probably lead to an impaired repair mechanism leading to a decreased replication process in the S-phase of the cells. Furthermore, the results also indicate that cells may have undergone stress conditions leading them to cell death [61].

The control of cell cycle progression is central to not only maintaining homeostasis but its alteration may also lead to imbalances in proliferation; cell death is governed by cyclins and CDKs. Normal cell proliferation is regulated by checkpoints that are situated at different stages of the cell cycle. Deregulation of these checkpoint events and the chemicals linked to them may cause cell cycle progression to halt. Cyclin D and E govern the transition from G1 to S phase; cyclin A regulates the development from G2

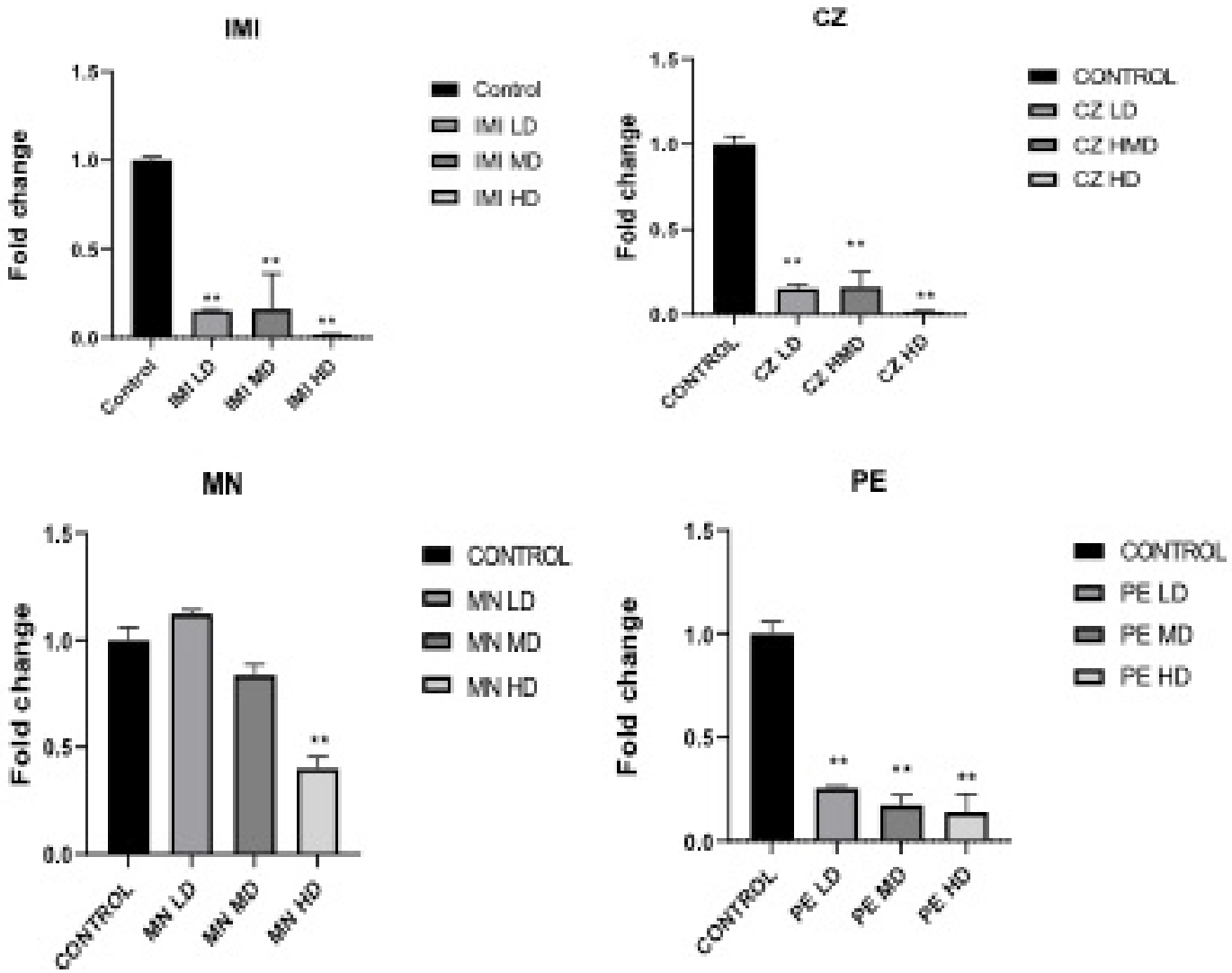

Figure 8: The level of cyclin E (in folds) in ICG cells treated with sublethal doses of IMI, CZ, MN, and PE. Each value represents the mean ± SEM (n = 3). The significant level is indicated by * $p < 0.05$ and ** $p < 0.01$.

Table 3: Cell viability at sublethal doses for IMI, CZ, MN, and PE for ICG cell line.

Agrochemicals	% Cell viability		
	LD	MD	HD
IMI	76.16 ± 0.67	68.13 ± 0.67	52.17 ± 0.70
CZ	83.00 ± 0.62	64.57 ± 0.81	57.77 ± 1.06
MN	94.87 ± 0.74	90.77 ± 0.98	86.83 ± 1.03
PE	93.77 ± 1.01	88.40 ± 0.84	71.62 ± 0.84

to M phase; and cyclin B regulates the transition from G2 to M phase [39]. By connecting with and activating its catalytic partner Cdk2, cyclin E is required for advancement through the G1 phase of the cell cycle and activation of DNA replication. The targets of cyclin E/Cdk2 phosphorylation are Rb, which is the critical component of cell proliferation, and Cdc6 and nucleophosmin, which are important for DNA replication [62]. The results of the present study show a decrease in the dose-dependent manner in the expression of cyclin A on exposure to IMI, CZ, and MN; however, with reference to PE, the pattern was not the same and a significant decrease was noted only at a high dose.

There was a dose-dependent significant reduction observed in cyclin e expression in cells exposed to IMI, CZ, and PE, whereas cells exposed to MN showed decreased expression in HD only. A decrease in cyclin A and E is suggestive of a decrease in the

transition from G1 to S phase and an arrest happening at S phase through which the cell cycle regulation is getting hampered. Most likely, pesticide exposure changed this process by preventing cell cycle progression from G1 to DNA synthetic S phase, where certain endogenous anti-mitogenic signals might have been working through CDK inhibitors to decrease the cyclin–CDK complex activity and impede G1/S transition [63–65].

5. CONCLUSION

According to the results of the study on alterations in proliferation in ICG cells exposed to pesticides, IMI is the most toxic of all the agrochemicals studied, followed by CZ, MN, and PE. The study also suggests that dose-dependent morphological alterations were observed in ICG cells exposed to all agrochemicals compared to the control which showed healthy cell morphology. There was a significant decrease in proliferation markers like PCNA and cyclin genes in ICG cells when exposed to all agrochemicals. The study suggests that agrochemicals possess multimodal actions, i.e., it does not alter a single gene, instead works on multiple pathways.

6. ACKNOWLEDGMENT

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7. AUTHORS' CONTRIBUTION

All authors contributed significantly to the conception, design, data analysis, and interpretation; participated in the drafting of the manuscript and critically revised it for its content; and have approved the final draft submitted for publication to the current journal. All the writers are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

9. FUNDING

The authors received no direct funding for this research.

10. ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

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Behavioral alterations and neurotoxicity of Imidacloprid on freshwater Teleost *Oreochromis mossambicus*

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Abstract

Agrochemicals are a significant cause of concern for the aquatic environment due to their toxicity, persistence and propensity to build up in the organisms. Imidacloprid (IMI) is a neonicotinoid insecticide widely used across the world. Improper and widespread usage and accidental exposure through agriculture runoff, IMI poses a major threat to non-target aquatic organisms. Although fish being a major source of protein-rich food for the human being, concerns are raised against the health status and vulnerability of fish, leading to the entry of toxicants into the food chain. In this context, the present study is a replicating condition of insecticide exposure in the laboratory, where the toxic potential of IMI with their two sub lethal concentration ($LC_{50/10th}$ and $LC_{50/20th}$) was tested (0.074 ppm and 0.04 ppm). Furthermore, the acute toxicity effect of IMI was validated by the behavioral response, histological alteration and biochemical estimation of Acetylcholine esterase (AchE) in the brain of *Oreochromis mossambicus*. Our result demonstrates that IMI has resulted in neuronal injury in the brain, resulting in severe abnormalities and suggests that alteration in AchE levels can be a good bio-indicator for monitoring neuro-toxicity caused by insecticides.

Keywords: *Imidacloprid, sub-lethal, Oreochromis mossambicus, brain.*

Introduction

The potentially deleterious effect on various natural environment components has elevated a great deal of concern in scientific circles for pesticide management [1-2].

Due to low cost and broad-spectrum toxicity, it is estimated that more than 100,000 tons of pesticides have been applied in India, primarily agricultural pest control [3]. The increase in agricultural production, crop protection, and yield have increased due to the use of chemical pesticides. The abundant use of these chemicals, under the adage, "If little is good, a lot more will be better" has played an essential role in increasing the consumption [4-5]. The annual application of agricultural fertilizers and pesticides is over 140 billion kilograms, a vast source of pollutants through agricultural runoff [6-9]. Application of such agrochemicals shows potential health hazards and has become a major concern for aquatic habitat due to their toxicity, persistency and tendency to accumulate in the organisms. [10-12]. The application of pesticides has increased several folds in India and is likely to increase in the coming years. The applied insecticides into agriculture fields easily get washed away, enter into the aquatic system in vast quantities and imbalance the ecosystem and induce physiological and biochemical effects on aquatic organisms. Fishes are most essential and highest interacted species of the aquatic ecosystem. They become a bridge between aquatic and terrestrial ecosystems as they are getting used by birds and mammals as a food source. [13-16].

Over the last period, a new class of insecticides, the neonicotinoids, has developed the most important and fastest emerging pesticides on the global market [17-19]. When used as plant protection products, neonicotinoids function by becoming distributed systemically throughout the growing plant following seed or soil application. IMI is potential groundwater and surface water contaminant because it can leach and runoff from soil and crops [20-22]. It may also enter water bodies from spray drift or accidental spills, leading to local point-source contamination. There are reports which suggest that the minimum concentration of 10 µg/L IMI in the aquatic environment may have adverse effects on the embryonic and larval stages of common carp. [23] and in *L. variegatus* by decreasing survival, inhibiting behavior, interfering with the growth process, and shortening life span [24]. Imidacloprid is a neonicotinoid insecticide that causes paralysis of the central nervous system in insects. Moreover, it may also affect the central nervous system of humans and animals [25]. Previous work of our lab has also reported physiological, biochemical, and histopathological alteration in *O. mossambicus* in the exposure of IMI in liver, kidney, and gills. [9,26] However, there was a lacuna for the evaluation of histoarchitectural alteration and analysis of Acetylcholine esterase activity from the brain; hence the present work is an attempt to assess the neurotoxic potential of IMI regarding Acetylcholine Esterase activity and histological change in the brain of *O. mossambicus* on the exposure of IMI.

Methodology

Animal maintenance

Mature Tilapia *Oreochromis mossambicus* (15± 2 cm, 25 ± 1.9 g) of similar size in length and weight were acquired from the pure brooders of the Vadodara district and transferred to the laboratory. The acclimation period was 15 days at 27 ± 40°C, pH 7.4 ± 0.5, dissolved oxygen 8 ± 0.3 mg/L, total hardness 188 mg/L CaCO3 with a 12:12 light: dark photoperiod. Fish were supplied daily with commercial fish food during acclimation. The maintenance of animal and experimental protocols were in accordance with the guideline of APHA-AWWA-WEF [27].

Experimental protocol

Based on the LC_{50} value [9] of IMI sub-lethal study, where the toxic potential of IMI with their two sub-lethal concentrations $LC_{50/10th}$ High Dose (HD) and $LC_{50/20th}$ Low Dose (LD) was tested (0.074 ppm and 0.04 ppm). The experimental regime was maintained in the laboratory for 14 days, with a control group having three replicates in each group. The experiment was performed semi-statically with a group of 10 fishes (5 males and 5 females) in experimental aquaria. All the groups were kept under continuous observation during the experimental period for behavioural study like hyperactivity, restlessness, jerky movement, loss of equilibrium, fin movement and mucus secretion. After the completion of the exposure of 14 days, fishes were caught very gently using a small dip net, one at a time

with the least disturbance. They were slowly released in the tough containing 1% clove oil to make it immobile and afterwards allowed to dissect out the brain and was analysed by HE staining for its cellular architectural changes and histological alterations in fish exposed to low dose (LD) and high dose (HD) of IMI as compare to control. After measuring the weight, fresh tissues were fixed in 4% paraformaldehyde for 2 hrs, degraded and embedded in paraffin wax and sectioned at 10 -12 um then stained with haematoxylin and eosin and examined microscopically and photographed using a digital camera. After 14 days of exposure, the brains of Tilapia were homogenized in 0.1 M PBS. The homogenate was centrifuged at 10000 g at 4 °C for 15 min to obtain the supernatant. The supernatant was found to determine AChE activity. Triplicate samples from each treatment group were collected and measured the AChE activity by the Elman method 1961[28].

Statistical analysis-

The difference between the mean of control and the exposed fishes was determined by one-way ANOVA using Graph Pad Prism software version 6. If there was any significant difference, a post hoc test was carried out where Dunnett's multiple comparison tests were employed to recognize differences in the alterations that were found between the control and the exposed groups. The significant level of the tests was set at 5% ($p < 0.05$).

Results and Discussions

Behavioral Study

Sub-lethal exposure of IMI resulted in an overall increase in behavioral patterns alteration compared to control. Frequency in the alteration of behavior, like restlessness, jerky movement, loss of equilibrium, and hyperactivity, was found to be more increased in HD than in LD treated IMI group and control. A significant change was observed in the tendency of fin movement and mucus secretion also. Based on this study, we can predict the alteration in behavior patterns is dose and exposure time dependent. (Table 1)

Histological Study

A dose dependent alterations in the brain of *O. mossambicus* was observed on the exposure of IMI for a period of 14 days. Severity in histological changes was characterized by vacuolation (VC), necrosis of neurons (NC), with degeneration of neural cells, distended sinusoids (DS), and mild structural damages (SD) were observed in the brain of the fish exposed to a low dose of IMI. Whereas, when exposed to a high dose for a period of 14 days, significant changes were observed in the normal cytoarchitecture of the fish brain compared to control. Severe structural damage, increased necrosis, degenerative changes (DC), mononuclear infiltration (MI), congestion (C), cytoplasmic vacuolization (VC), and severe lesions (L) were also noticed in the brain. A significant haemorrhage (HE) was also noticed in some places. (Figure 1 and Table 2)

Table 1: Behaviour study in *O. mossambicus* on exposure of sub lethal dose of IMI

Exposure time	Day1			Day 5			Day 10			Day 15		
Dose	C	LD	HD	C	LD	HD	C	LD	HD	C	LD	HD
Jerky movement	+	+	+	+	+	+	+	+	++	+	+	++
Hyperactivity	-	-	-	-	+	+	-	+	++	-	+	+++
Restlessness	-	-	-	-	+	+	-	+	++	-	+	+++
Loss of Equilibrium	-	-	-	-	+	+	-	++	++	-	++	+++
Fin Movement	+	+	+	+	++	++	+	++	+++	+	++	+++
Mucus Secretion	-	-	-	-	+	+	-	+	++	-	++	++

Note: (-) → Normal, (+) → mild, (++) → moderate, and (+++) → maximum behavior pattern

Table 2: Summary of histological changes observed in the brain of *O. mossambicus* subjected to LD and HD of IMI

Characterization	Control	LD	HD
Vacuolation	-	++	+++
Necrosis of neurons	-	++	+++
Distended sinusoids	-	++	+++
Degenerative changes	-	++	+++
Mononuclear infiltration	-	+++	+++
Congestion	-	++	+++
Lesions	-	+	+++
Hemorrhage	-	++	+++

Note: (-) → none, (+) → mild occurance, (++) → moderate occurrence, and (+++) → maximum occurrence

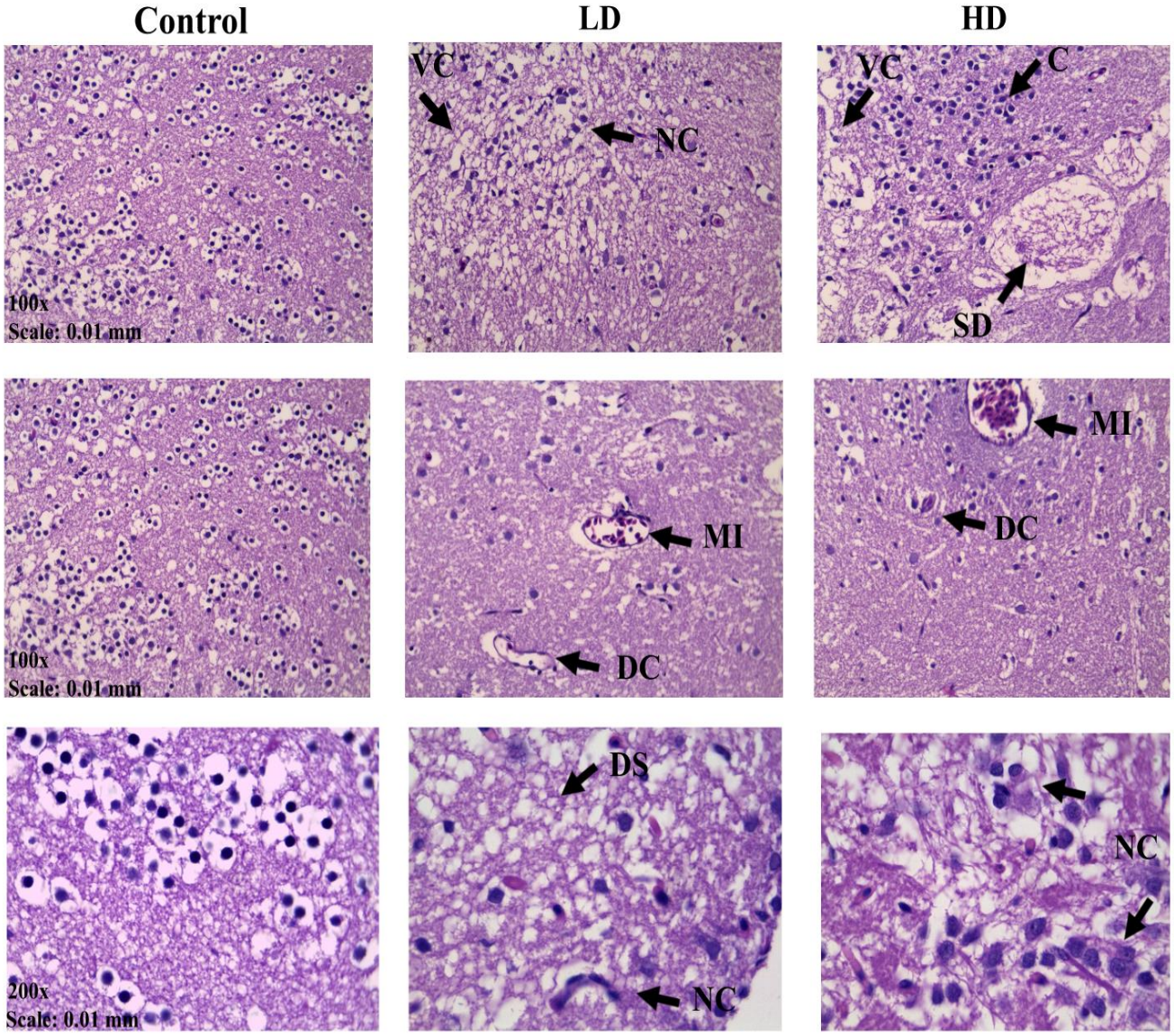

Figure 1: Alteration in histology of brain in *O. mossambicus* on exposure of sub lethal dose of IMI

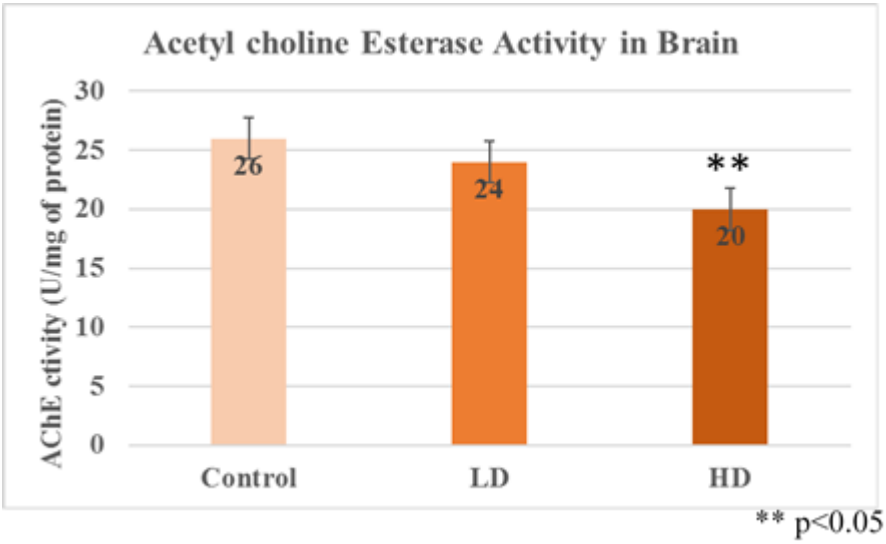

Figure 2: Acetylcholine Esterase Activity of the brain in *O. mossambicus* on exposure of sub lethal dose of IMI

Acetylcholine Esterase Activity

A Sublethal exposure of IMI resulted in a significant ($p<0.05$) decline of AChE activity, at high dose only as compared to control. (Figure 2)

Discussion

In this study, the acute toxicity effect of IMI was studied in terms of alterations in the behavioral pattern, histopathological changes, and AChE activity in the brain of tilapia (*O. mossambicus*). The major aim was to validate the neurotoxic potential of IMI; the work was focused on alteration in behavior, AChE levels, and correlate it with the histoarchitectural changes in the brain.

Behavior provides a unique perspective on the physiology and ecology of an organism and its environment, operating through the central and peripheral nervous system [29]. Since behavior is not a random process, instead of a highly structured and anticipated sequence that ensures fitness and survival of the species, behavioral endpoints serve as a valuable tool to distinguish and evaluate the effects of exposure to environmental stress. Alteration in the fish behavior provides relevant indices for ecosystem assessment, any change in their behavior indicates the deterioration of water quality, so they are considered a biological indicator [30]. In the present scenario, the control fish were active and alert. They had a well-synchronized movement, whereas, in the case of IMI, exposure exhibited a dose-dependent severity in hyperactivity, loss of equilibrium, restlessness, and jerky movement. These symptoms may be due to inhibition of AChE activity leading to the accumulation of Ach in cholinergic synapses ensuing hyperstimulation. Since inhibition of AchE activity is a typical characteristic of neonicotinoids [31-32]; our findings corroborate with observations made by Rossi *et al.*, 2020 in sentinel freshwater fish following sublethal exposure of neonicotinoids. The observed behavioral changes also suggest that the brain was affected by IMI exposure. To confirm these histological studies were conducted. Histology is used as a biomonitoring tool in toxicity studies as it provides early warning signs of disease. Histopathological observations have been commonly used to determine the health of the entire ecosystem population for toxicity testing of the effects of xenobiotic compounds at the sub-organismal or organismal level. [33-34]. The damage and injury in different organs are usually dependent on dose, duration, exposure, and type of pesticides. The utility of histological lesions is sensitive and reliable indicators of fish health as

reported in early studies. [20, 35]. Tissues obtained from the control group showed regular features with no apparent lesions. However, dose-dependent alterations in the brain were observed, such as Hyperplasia, edema, necrosis, vacuolation, and overall increase in brain cells. Vacuolation and increased cell size may have been due to glycolysis leading to microsomal and mitochondrial dysfunctions. [36-38]. The recent results of the present study show that the IMI causes major neurotoxicity in the brain and impairs its behavioral pattern. The problem can be more severe in the fish farms as the ponds are habitually located in or near the agricultural land, which is loaded with pesticides of all kinds. To this, if the groundwater used for domestic purposes can exacerbate the problem with simulated laboratory experimental conditions. Therefore, a scientific detoxification approach is necessary to improve the health of these economically valuable fish and reduce the losses caused by anthropogenic stress.

Conclusion

The study suggests that sub-lethal concentration of IMI leads to neurotoxicity, and fishes were found to be highly stressed, therefore it is necessary to carry out field studies and check the fish health status and IMI residues in the environment. Furthermore, monitoring AChE levels can be used as a well-supported marker for IMI neurotoxicity in fishes in general and tilapia in particular.

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Compliance with ethical standards The research carried out is compliance with ethical standards.

Ethical approval
All the procedures were in accordance with the guidelines of APHA-AWWA-WEF (1998) for which the care and use of animals were followed.

Conflicts of interest: The authors stated that no conflicts of interest.

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NEUROSCIENCE RESEARCH NOTES

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Chemical hypoxia in human pluripotent NT2 stem cell-derived neurons: Effect of hydroxamic acid and benzamide-based epigenetic drugs

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ABSTRACT: Hypoxia-induced oxidative stress contributes to neuronal damage leading to many neurodegenerative disorders. Hypoxia promotes many downstream effectors such as hypoxia-inducible factor-1α (HIF-1α) in order to restore respiratory homeostasis due to low oxygen availability and increased ROS. Use of histone deacetylase (HDAC) inhibitors may modulate hypoxia-induced neuronal cell damage. In this study, we used two chemically diverse HDAC inhibitors to investigate their effect on hypoxia-exposed neuronal cells. Human pluripotent NT-2 stem cell-derived neuronal differentiated cells were exposed to $CoCl_2$ pre-treatment for 6h to induce hypoxia, prior to supplementation of HDAC inhibitor (SAHA or MGCD0103). Treatment with HDAC inhibitor improved cell viability in hypoxia-induced neuronal cells. The increased HIF1α expression in hypoxia-induced neuronal cells was blunted by these HDAC inhibitors with a concomitant decrease in ROS production. $CoCl_2$ treatment caused an increase in IL-1β, which was significantly inhibited by these HDAC inhibitors. Furthermore, apoptosis induced in these $CoCl_2$ treated neuronal cells was mitigated by SAHA as well MGCD0103 suggesting that these HDAC inhibitors are capable of reducing cellular toxicity, inflammation and apoptosis, and thus, could be beneficial as therapeutic molecules for many neuropathological conditions.

Keywords: pluripotent; neuron; hypoxia; histone deacetylase; gene expression

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ORIGINAL ARTICLE

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Evaluating the toxic potential of agrochemicals on the hypothalamic-pituitary-thyroid axis in tilapia (*Oreochromis mossambicus*)

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Abstract

Agrochemicals are a major cause of concern for the aquatic environment because of their toxicity, persistence, and tendency to accumulate in the organisms. The impact of these chemicals on aquatic organisms is due to their movement from various diffuse or point sources, which poses a great threat to aquatic fauna especially fishes, which constitute one of the major sources of protein-rich food for mankind. The present study is a first of its kind, where the toxic potential of two sublethal concentration ($LC_{1/10th}$ and $LC_{1/25th}$) of four different classes of agrochemicals have been tested (Insecticide- Imidacloprid-0.074 ppm, 0.02 ppm, Fungicide-Curzate-4.9 ppm, 1.96 ppm, Herbicide- Pyrazosulphuron ethyl-50 ppm, 20 ppm and Fertilizer-Micronutrient mixture 500 ppm, 200 ppm) on candidate markers of hypothalamus pituitary-thyroid axis (TSH, T_3, T_4, TSHβr) in *Oreochromis mossambicus* (tilapia) by validating hormonal level and mRNA expression. The results reveal that exposure to agrochemicals resulted in a broad range of alterations with maximum damage being caused by insecticide followed by herbicide and fungicide in that order on the thyroid axis. The results of the present study highlight the need for more detailed studies on the effects of agrochemicals that accumulate in organisms and propose that there should be a check on the rampant use of agrochemicals.

KEYWORDS

agrochemicals, hypothalamus-pituitary thyroid axis, sublethal, toxic potential

1 | INTRODUCTION

The increasing use of synthetic agrochemicals is escalating worldwide pollution risks. Agrochemicals are toxic and are principally designed to kill unwanted organisms, but when applied on land, they wash down into the surface waters and adversely affect the life of aquatic organisms (Mosesso, 2012; Silva & Wheeler, 2017; Woo et al., 2010). The aquatic environment is a receptacle of several undesirable contaminants, including agrochemicals. Therefore, possible contamination of the aquatic environment by pesticides poses tremendous environmental concern worldwide (Alvarez-Moya, 2014; Guilherme, Santos, Gaivão, & Pacheco, 2014). The use of these hazardous pollutants posing potential health hazards has become a great cause of concern for the aquatic environment because of their toxicity, persistence, and tendency to bio-accumulate within organisms (Joseph & Raj, 2011). The profound impact of these agrochemicals on aquatic organisms is due to the active movement of pesticides from various diffuse or point sources that constitute a severe threat to aquatic fauna especially fishes, which typically constitute one of the major sources of protein-rich food for mankind (Sharma & Singh, 2007).

Animals in general and fishes, in particular, are known to exhibit alterations in hormone profile on exposure to the changing environment/toxic conditions. Agrochemicals have been known to interrupt

