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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.

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

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

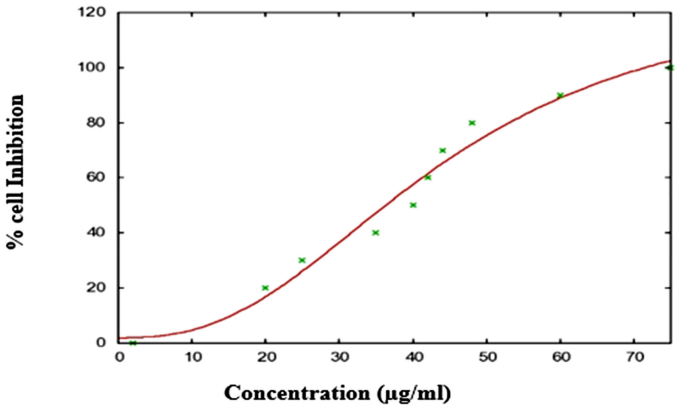

Figure 1: ICG cell mortality against different concentrations of IMI.

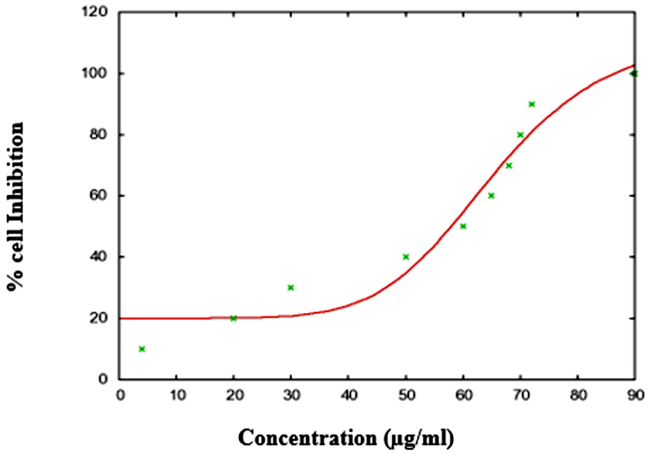

Figure 2: ICG cell mortality against different concentrations of CZ.

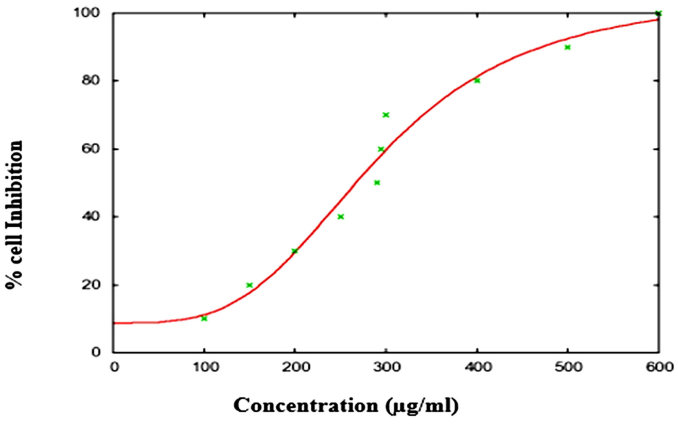

Figure 3: ICG cell mortality against different concentrations of MN.

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

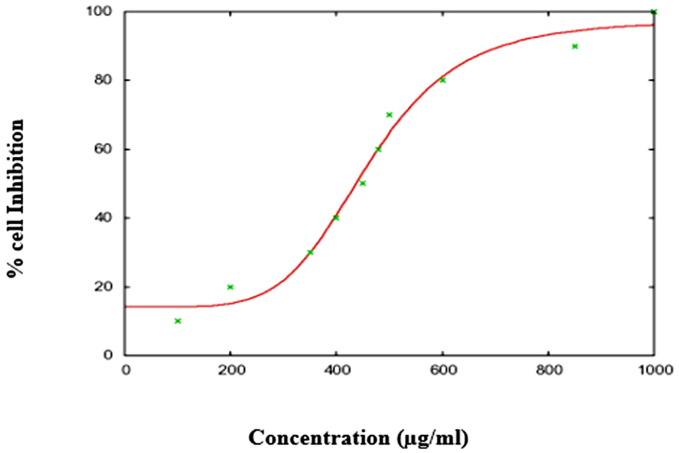

Figure 4: ICG cell mortality against different concentrations of PE.

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.

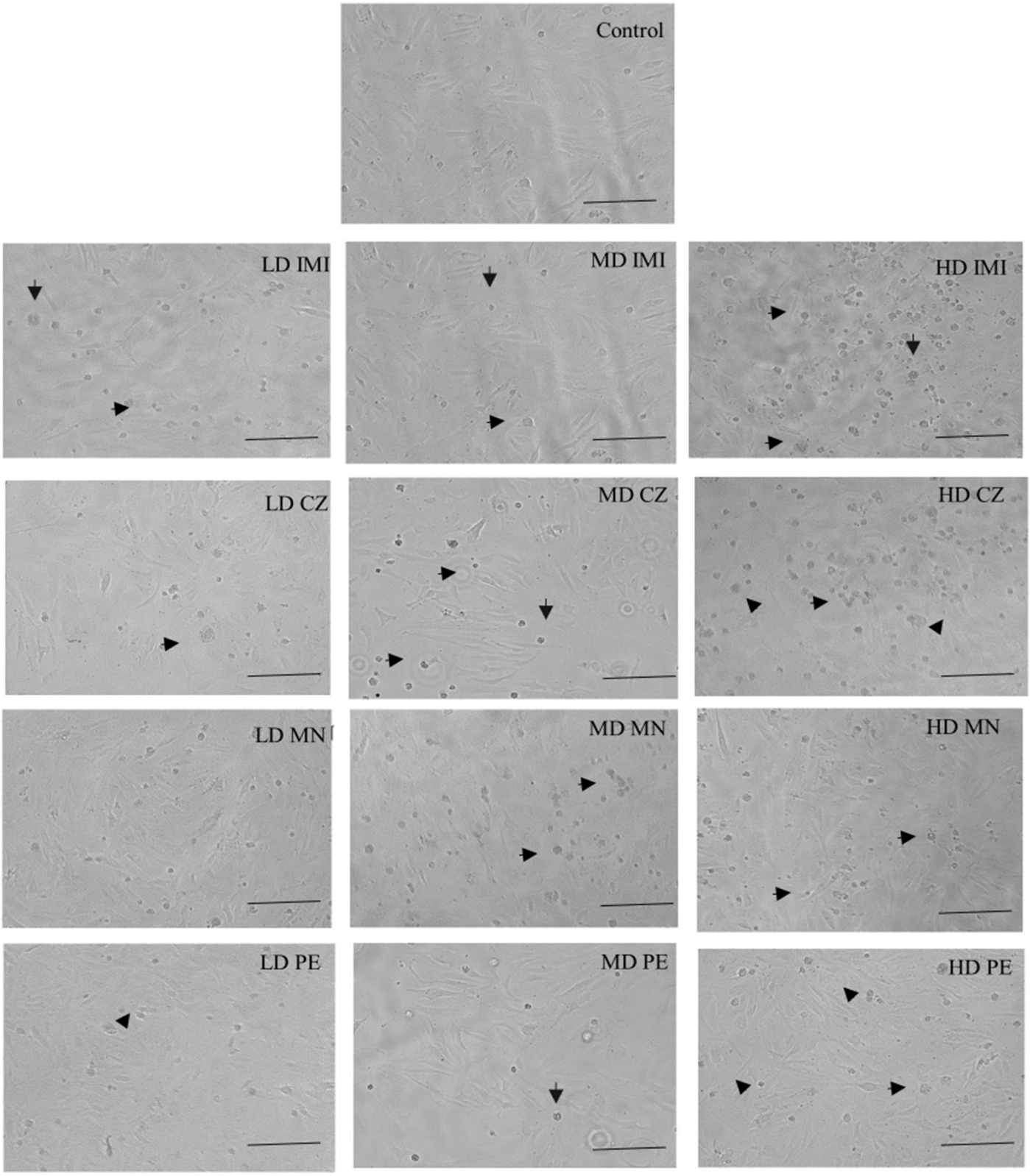

Figure 5: Alterations in the cell morphology of ICG cells exposured to agrochemicals.

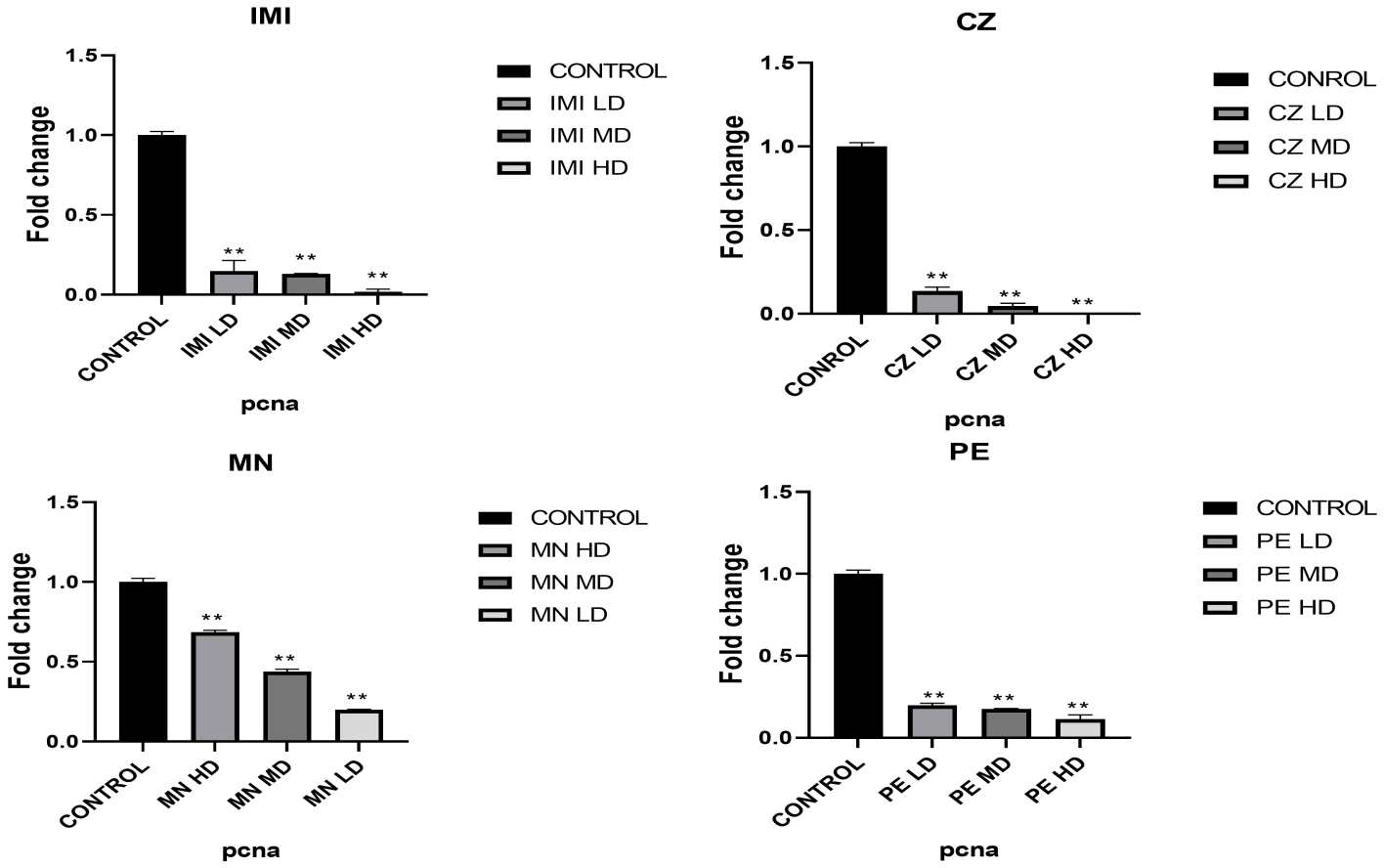

Figure 6: The level of PCNA (in folds) in ICG cells treated with sublethal doses of IMI, CZ, MN, and PE. Each value represents the mean ± SEM ($n = 3$). The significant level is indicated by * $p < 0.05$ and ** $p < 0.01$.

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

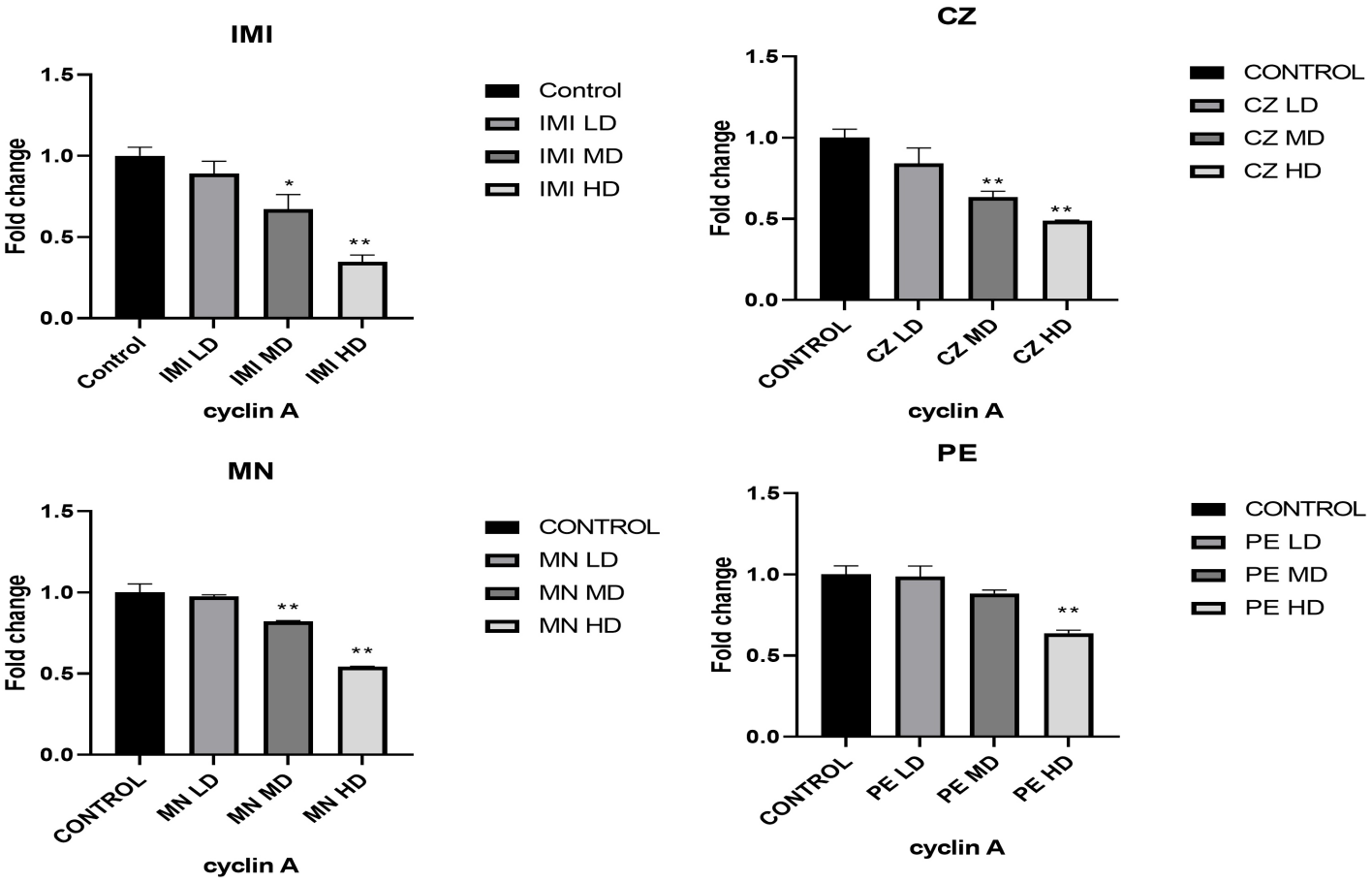

Figure 7: The level of cyclin A (in folds) in ICG cells treated with sublethal doses of IMI, CZ, MN, and PE. Each value represents the mean ± SEM ($n = 3$). The significant level is indicated by * $p < 0.05$ and ** $p < 0.01$.

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

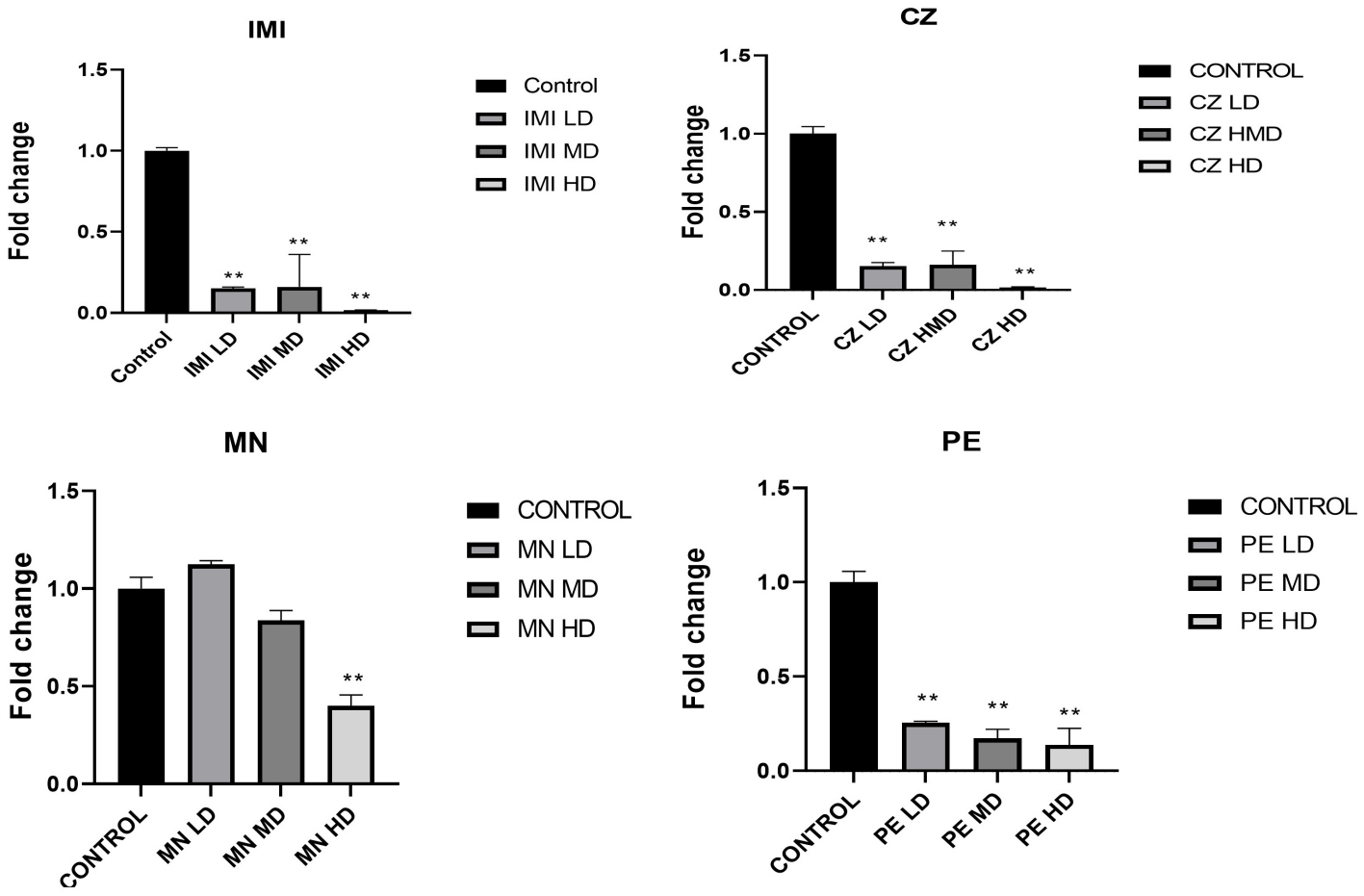

Figure 8: The level of cyclin E (in folds) in ICG cells treated with sublethal doses of IMI, CZ, MN, and PE. Each value represents the mean ± SEM ($n = 3$). The significant level is indicated by * $p < 0.05$ and ** $p < 0.01$.

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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