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Mechanistic Insights into Monocrotophos Induced Toxicity in Neuronal Cells

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Abstract

The plethora of literature has reported the vulnerability of neuronal cells to environmental toxicants. Among such toxicants, pesticides, especially organophosphorus (OPs), have been demonstrated to exhibit severe neurotoxic effects on the brain. Monocrotophos, a widely used OP has been implicated in the onset of perilous systemic effects, in particular neurotoxicity. To gain insight into the toxicity mechanism of MCP we evaluated the potential effects of the compound on NSC derived neuronal cells in culture using a battery of cytotoxicity parameters. MCP exerted concentration dependent cytotoxicity on neuronal cells manifested by reduced cell viability, increased lipid peroxidation and reduced glutathione levels. These changes were accompanied by excessive reactive oxygen species generation and membrane depolarization with parallel alterations in markers of neuronal injury and dose dependent reduction in the expression of anti apoptotic protein Bcl-2 implicating cell apoptosis. Together these findings implicate the vulnerability of neuronal cells to MCP exposure that warrants stringent use of the pesticide.

Keywords: Monocrotophos, pesticide, neuronal cells, neurotoxicity

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Introduction

Neurotoxicity has been one of the emerging fields in neuroscience but their progress has been largely hindered by reliable and reproductive screening tools. Stem cells with their inherent ability for pluripotency and unlimited proliferation serve as one of the best tools to address the aforementioned issue. Neural stem cells (NSCs) are defined by their potential to self renew, proliferate and differentiate into neurons and glial cells, a process tightly regulated by extrinsic and intrinsic factors. Found both in the adult as well as the developing brain, they are reported to be involved in normal brain functioning, including the learning and memory processes, as well as recovery from brain injuries [1]. The last few decades have witnessed tremendous application of neural stem cells for prediction and screening of neurotoxicity of various chemicals. [2, 3]

Rampant usage of pesticides, one of the prominent environmental pollutants, has been associated with several short-term and long-term abnormalities with a strong correlation with several cases of chronic diseases like Parkinson's disease[4], amyotrophic lateral sclerosis (ALS)[5], Alzheimer[6], cancer, birth defects, reproductive anomalies, asthma, atherosclerosis, nephropathies etc. in exposed population.[7] Organophosphates (OPs), one of the major group of pesticides are highly non-specific in their action primarily targeting the acetyl cholinesterase activity at nerve endings. There have been ample reports of outbreaks of accidental poisoning by OPs.[8, 9] Various studies have also demonstrated how biological interactions between brain NSCs and OPs may lead to irreparable damages to the brain aiding in the progression of neurodegeneration.[10, 11] Monocrotophos (MCP), a widely used OP, has been declared as highly hazardous. Under chronic conditions MCP is suspected to adversely affect the central nervous system causing involuntary muscle contractions, weakness, fatigue, twitching and slurred speech and in graver cases it may paralyze the body sometimes leading to coma.[12, 13]

The toxic responses of MCP at cellular and molecular

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level have been well explored in cultured cells. However, the basic mechanism(s) underlying the toxicity induced by MCP in the brain still remains elusive. Thus, to address this issue, we propose to utilize the pluripotency potential of neural stem cell to generate neuronal cells and study cellular and molecular alterations in toxicity parameters in response to MCP thus identifying probable underlying mechanisms.

Materials and Method

Reagents and consumables

All the specified chemicals, reagents, diagnostic kits were procured from Sigma Chemical Company Pvt. Ltd. St. Louis, MO, USA, unless otherwise stated. Culture medium, serum and growth factors and antibiotics, were procured from Gibco BRL, USA.

Isolation and characterization of neural stem cells (NSCs)

NSCs were isolated from the embryonic day-12 (ED-12) rat foetuses. The tissue was dissected, washed with cold HBSS (Hanks Balanced Salt Solution), minced and then gently homogenized to obtain a single suspension of cells. The cells revealed more than 95% viability as ascertained by trypan blue dye exclusion that were then seeded at a density of 0.5×10^6 viable cells/ml in 75 cm^2 flasks in serum free neurobasal medium containing B-27 supplement (2%), N-2 supplement (1%), bFGF (10 ng/ml), EGF (10 ng/ml), and 1% antibiotic-antimycotic solution and allowed to grow as neurospheres in 5% CO2 and 95% 37 °C air under high humid conditions at 37°C. Small proliferating neurospheres appeared after 1 week, which attained maturity by day 20. The neurosphere culture was passaged every 10–12 days by triturating the neurospheres gently. To visualize the proliferating activity of the undifferentiated NSCs, neurospheres were immunostained with anti-nestin monoclonal antibody (mouse, 1:200; a neural stem and progenitor cell specific marker) and anti-BrdU antibody (rabbit, 1:500, proliferating cell marker).

Neuronal differentiation of NSCs

After passage #6, dissociated neurospheres were seeded on poly-L Lysine (PLL) pre coated flasks (5×10^4 cells/cm^2) in serum free neurobasal medium containing DMEM: F12 (1:1) supplemented with N-2 (1%), B-27 (2%) and mitogenic factors (10 ng/ml EGF & bFGF). For induction of cholinergic differentiation, cells were incubated in differentiation medium containing NGF (100 ng/ml) with reduced concentration of EGF and bFGF (1 ng/mL), B-27 (0.1%) and N-2 (1%). Neuronal differentiation was further confirmed by assessing the morphological appearance and expression of cholinergic (ChAT) and neurofilament (NF-M) proteins.

MTT assay

To ascertain the toxicity of MCP on NSC derived neuronal cells MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out following the protocol of Agarwal *et al.*[14] In brief, cells (1×10^4)/well were seeded in poly-L-lysine (PLL) pre-coated 96-well culture plates and allowed to adhere for 24 h. Thereafter, the cells were exposed to variable concentration of MCP (50μM-400μM) and further incubated for a period of 24 h. 4 h prior to completion of the incubation period, MTT (5 mg/ml of stock in phosphate-buffered saline (PBS)) was added (10 **μl**/well in 100 **μl** of cell suspension) and plates were further incubated for 4 h. At the end of the incubation period, the reaction mixture was aspirated and 200 **μl** of dimethyl sulfoxide (DMSO) was added to each well to solubilise the formazan crystals. After 10 min, absorbance was taken at 550 nm, using a multi-well microplate reader (Synergy HT; Bio-Tek, USA). The experiment was run in triplicates. Untreated sets were run simultaneously under identical conditions and served as basal control.

Estimation of glutathione

Glutathione (GSH) levels were assessed following exposure of MCP (50μM-400μM) using a commercially available kit (glutathione detection kit, Cat no. APT250; Chemicon, USA). In brief, following respective exposures for 24 h, the cells were harvested by centrifugation at 7000 x *g* for 2 min at 4°C and then lysed using lysis buffer. The samples were again centrifuged at 12000 x *g* at 4°C for 10 min and the supernatant was collected. To estimate the GSH levels, the lysed samples (90 ml/well) were then transferred to 96-well black-bottom plates and mixed with a freshly prepared assay cocktail (10 ml). Following incubation for 1-2 h, the mixture was read at an excitation wavelength of 380 nm and emission wavelength of 460 nm using a multi-well microplate reader (Synergy HT; Bio-Tek). Cells exposed to H_2O_2 (100 μM) for 2 h under

identical conditions served as positive control. The experiment was run in triplicates. Untreated sets were run simultaneously under identical conditions and served as basal control

Estimation of lipid peroxidation

Lipid peroxidation (LPO) was performed using the thiobarbituric acid reactive substances (TBARS) protocol.[15] In brief, cells (10^4 per well) were plated on poly-L-lysine pre-coated 96-well, black-bottom culture plates and allowed to adhere for 24 h under a 5% CO2–95% atmosphere at 37°C. After completion of the exposure time period, the cells were harvested by centrifugation and sonicated in ice-cold potassium chloride (1.15%) and centrifuged for 10 min at 3000 x *g*. The resulting supernatant (1 ml) was added to 2 ml of thiobarbituric acid reagent (15% TCA, 0.7% TBA, and 0.25 N HCl) and heated at 100°C for 15 min in a water bath. The samples were then placed in cold and centrifuged at 1000 x *g* for 10 min. Absorbance of the supernatant was measured at 535 nm. The experiment was run in triplicates. Untreated sets were run simultaneously under identical conditions and served as basal control.

Estimation of reactive oxygen species generation

Estimation of MCP-induced reactive oxygen species (ROS) generation was carried out using the standard protocol Srivastava *et al.*[16] Briefly the cells were plated on PLL pre coated 6-well culture plates at a density of 1 –1.5X10^5 cells/well and incubated overnight. The cells were then exposed to varying concentrations of MCP (50μM-400μM) for 24 h. H_2O_2 (500 μM) exposed cells (for 2 h) served as positive control for ROS generation. On completion of incubation period, the cells were collected and incubated in 10μM DCFDA dye (Cat No. D6883, Sigma Aldrich) for 30 minutes in dark at 37°C, followed by wash with 1X PBS. Finally the pellet obtained was resuspended in 1X PBS and analyzed through flow cytometry (BD influx, USA) at an excitation wavelength of 485 nm and emission wavelength of 528 nm. Untreated sets were run simultaneously under identical conditions and served as basal control.

Assessment of alterations in mitochondrial membrane potential

Briefly 1-1.5X10^5 cells were seeded per well in PLL pre coated 6-well culture plate and incubated overnight. Following this cells were exposed to different concentrations of MCP (50μM-400μM) for 24 h. Staurosporine (100nM) served as the positive control group. After 24 h of exposure the cells were harvested and washed with 1X PBS. The pellet obtained was again resuspended in 1X PBS and incubated with JC-1 dye (5μg/ml) in dark for 20 minutes at 37°C followed by another wash with 1X PBS. Finally the pellet obtained was resuspended in 1X PBS and analyzed through flow cytometry (BD influx, USA) at an excitation and emission wavelength of 535 nm and 590 nm respectively. Untreated sets were run simultaneously under identical conditions and served as basal control.

Alterations in expression of apoptosis markers

Translational changes in the selected genes (Bcl-2 and caspase-3 and Bax) involved in the apoptosis pathway were assessed in neuronal cells exposed to various concentrations of MCP for 24 h. In brief, cells were lysed using the CelLytic™ M Cell Lysis Reagent (catalog no. C2978; Sigma) in the presence of a protease inhibitor cocktail (catalog no. P8340; Sigma). Protein estimation was carried out using a BCA Protein Assay kit (catalog no. G1002; Lamda Biotech Inc., St Louis, MO, USA). Denatured proteins (40 μg/well) were loaded and electrophoresed using 10% Tricine–SDS (sodium dodecyl sulfate) gel. The separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, USA; catalog no. IPVH00010) by the wet transfer method under 250 mA current for 2 h. Nonspecific sites was blocked with 5% non-fat dry milk powder in TBST (20 mM Tris–HCl (pH 7.4), 137 mM NaCl and 0.1% Tween-20] for 1 h at RT. After blocking, the membranes were incubated overnight at 4°C using primary antibodies specific for Bcl-2 and caspase-3 and Bax (1:1000; Chemicon International, USA), in blocking buffer (pH 7.5). After incubation for 2 h at RT with a secondary anti-primary antibody conjugated with horseradish peroxidase (Chemicon International), the blots were developed using the Super Signal West Femto Chemiluminescent Substrate™ (Thermo Fisher Scientific, USA) and Bio-Rad Versa Doc™ Imaging System 4000 (Bio-Rad, PA, USA). Densitometry for protein-specific bands was performed using a gel documentation system (Alpha Innotech, USA) with the help of the Alpha Ease™ FC Stand Alone V.4.0 software. β-Actin was used as an internal control to the normalize data. Untreated sets

were run simultaneously under identical conditions and served as basal control.

Statistical analysis

Results are expressed as mean and standard error of means (mean ±SE) for at least three experiments. One-way analysis of variance followed by *post hoc* Dunnett's test was employed to detect differences between the exposed and control groups.

Results

Isolation, characterization and differentiation of NSCs

NSCs isolated from embryonic day-12 (ED-12) rat foetuses expressed over 95% viability (Figure 1A). The proliferative cells developed into small neurospheres by day 7 (Figure 1B) that attained maturity by day 20 (Figure 1C). Cells in the neurospheres showed expression of both progenitor cell marker-nestin (green) and proliferating cell marker-BrdU (red) (Figure 1D-F) counterstained with DAPI (Figure 1C). Morphologically differentiated neuronal cells showed expression for neural marker NF-M (green color) (Figure 1G), for cholinergic specific marker ChAT (red color) (Figure 1H) and DAPI (Figure 1I)

Cell viability of MCP exposed neuronal cells

We performed a concentration response experiment to determine whether MCP exerted toxicity on the neuronal cells. The data obtained from cytotoxicity assessment based on MTT assay revealed a concentration dependent reduction in the cell viability of exposed cells as compared to the control group (Figure 2A-C). The cell viability reduced to around 86% at 50μM dose followed by 81% viability in 100μM and a drastic reduction to 74% and 66% at 200μM and 400μM respectively in comparison to the control group.

Glutathione level

The data of MCP-induced alterations in the levels of GSH are presented in Figure 3. A dose dependent decrease in GSH level was observed at 24 h on exposure to MCP relative to unexposed control cells.

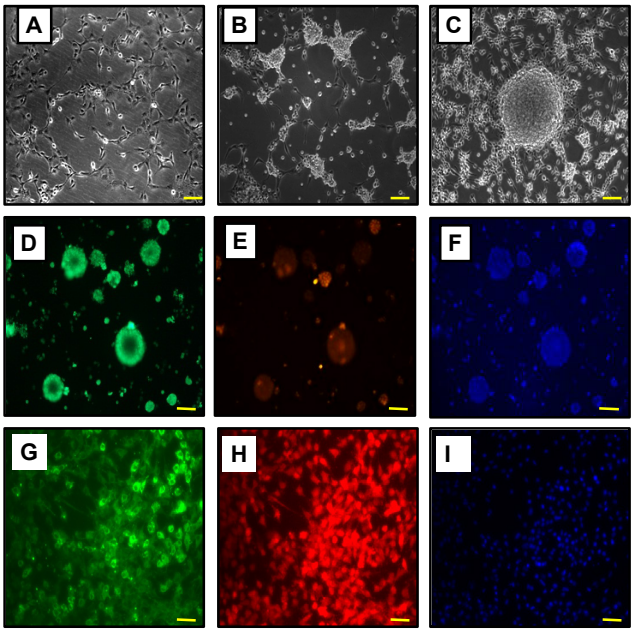


Figure 1. **(A)** Rat brain NSCs isolated from embryonic day-12 (ED-12) rat foetuses **(B)** Proliferative cells seen as small neurospheres by day 7 **(C)** The neurospheres attained maturity by day 20 **(D-F)** NSCs showing immunoreactivity for both progenitor cell marker-nestin (green) and proliferating cell marker-BrdU (red) counterstained with DAPI **(G-I)** Differentiated NSCs showed neuronal morphology and stained positive for neural marker NF-M (green) and cholinergic specific marker ChAT (red) counter stained with DAPI.

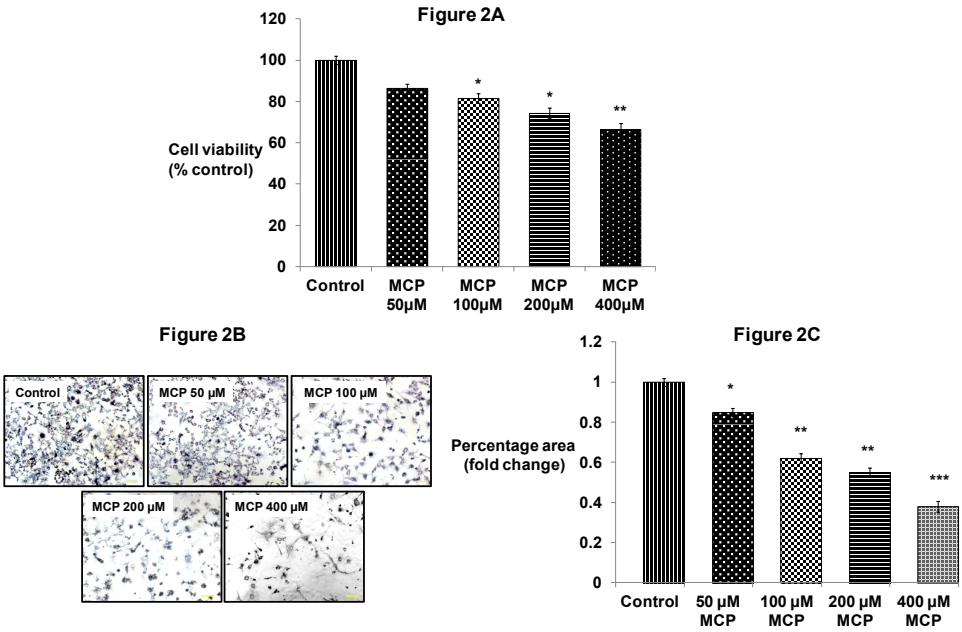


Figure 2. **(A)** Cell viability assay of neuronal cells at 24 h following the exposure of different concentrations of MCP by MTT assay (**B**) Representative microphotographs of formazan crystals indicative of concentration dependent cytotoxicity of MCP (**C**) Image quantification was done using ImageJ image analysis software and expressed in fold change. Values are mean ± SE of the data obtained from three independent experiments. * = p< 0.05, * * = p< 0.01, * * * = p< 0.001.

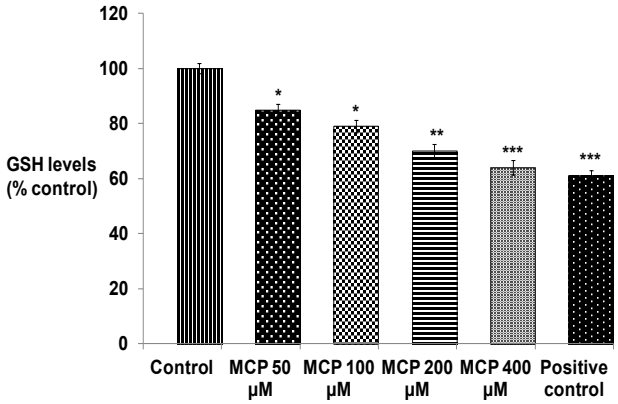


Figure 3. Glutathione (GSH) levels in neuronal cells exposed to different concentrations of MCP for 24 h assessed by a fluorescence-based glutathione detection kit. The data are expressed as percentage intracellular concentration of GSH ± SEM. Values are mean ± SE of the data obtained from three independent experiments. * = p< 0.05, * * = p< 0.01, * * * = p< 0.001.

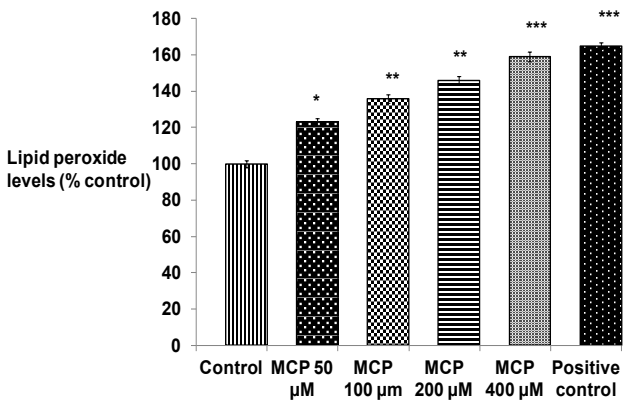


Figure 4. LPO levels in neuronal cells exposed to various concentration of MCP for 24 h was assessed by the thiobarbituric acid reactive substances (TBARS) protocol. The data are expressed as percentage intracellular concentration of LPO ± SEM. Values are mean ± SE of the data obtained from three independent experiments. * = p< 0.05, * * = p< 0.01, * * * = p< 0.001.

Lipid peroxidation level

A statistically significant increase in the LPO levels were recorded with respect to the unexposed control after 24 h exposure period to MCP (Figure 4).

ROS generation

Significant induction in ROS production was recorded on exposure to increasing concentrations of MCP when compared with the controls (Figure 5).

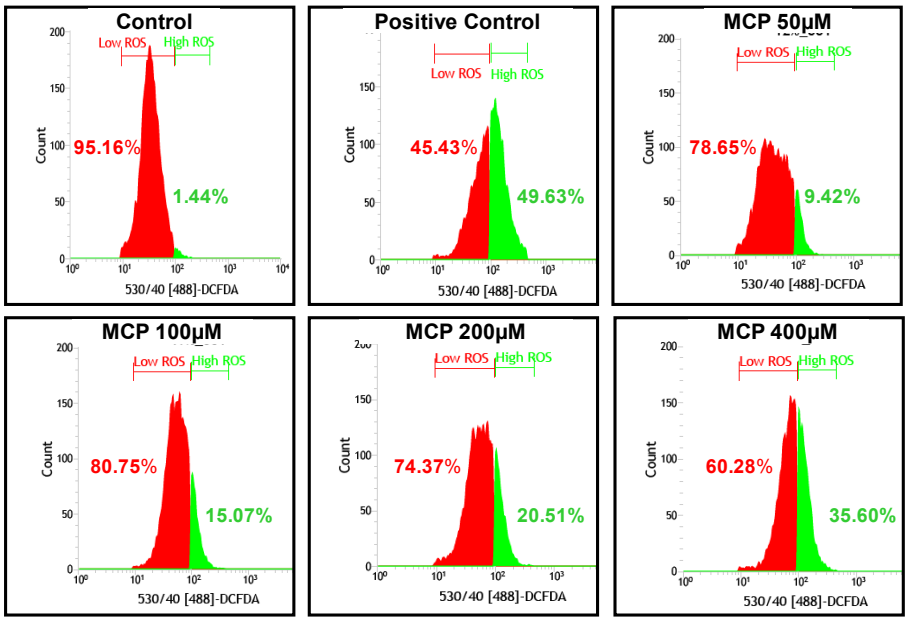


Figure 5. ROS generation in neuronal exposed to varying concentrations of MCP was analyzed through flow cytometry. The red to green shift indicates cell death. The numbers in red and green indicate percentage of live and dead cells respectively. Cells exposed to H_2O_2 served as positive control for ROS generation.

Mitochondrial membrane potential (MMP)

The mitochondrial membrane potential and the changes induced in it can be associated with cell death due to apoptosis characterized by release of cytochrome c due to formation of membrane transition pore resulting from a perturbed MMP. The dye JC-1 accumulates in mitochondria in a potential dependent manner and emits fluorescence. The red to green shift in the fluorescence is representative of increasing membrane depolarization suggesting increasing cell death. In the present study this shift increased with increasing concentrations of MCP that signifies increased depolarization of mitochondrial membrane leading to increased apoptosis in the MCP exposed cells.

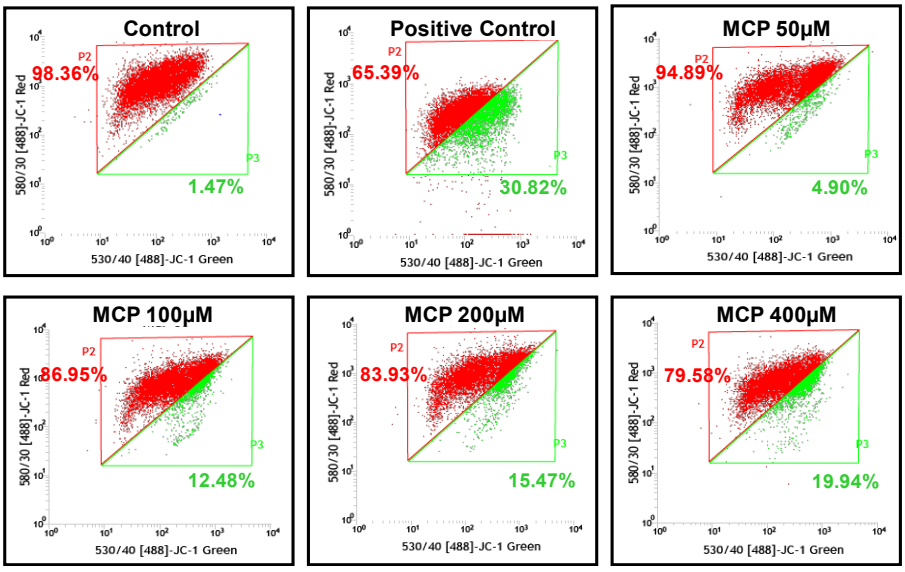


Figure 6. The alterations in MMP induced in neuronal cells in response to different concentrations of MCP for 24 h were recorded through flow cytometry using JC-1 dye. Staurosporine (100nM) served as the positive control. P2: JC1 aggregates, P3: JC1 monomers.

Translational changes

To further analyze the biochemical changes introduced in neuronal cells upon MCP exposure the expression pattern of some of the key apoptosis markers was studied. A dose dependent decrease in expression of Bcl-2 was observed with a parallel induction in expression of caspase -3 and Bax that are a hallmark of apoptosis. Such an expression profile was indicative of apoptosis induction in response to increasing MCP concentrations.

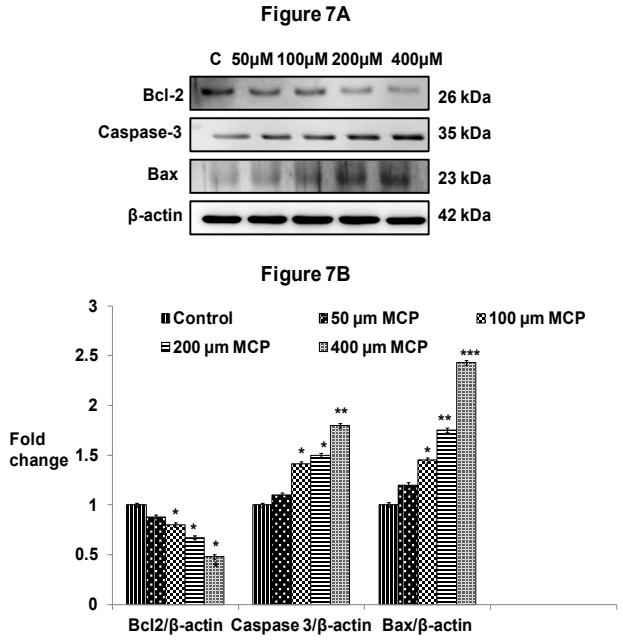


Figure 7. **(A)** Alterations in the expression of the proteins involved in the apoptosis pathway viz. bcl-2, caspase-3 and bax studied in neuronal cells exposed to MCP for a 24□h time period **(B)** Quantification was done using a gel documentation system (Alpha Innotech) with the help of the Alpha Ease™ FC Stand Alone V.4.0 software and expressed in fold change. β-actin was used as an internal control to normalize the data. * = p< 0.05, * * = p< 0.01, * * * = p< 0.001.

Discussion

Brain, both adult as well as in developmental stages, as compared to other organs is more susceptible to various environmental toxicants. OPs are primarily known to act by inhibiting acetyl cholinesterase (AChE) activity in the neuronal system.[17] However, the toxicological responses of OPs have also been linked with increased generation of ROS that been reported to cause cell injury and subsequent apoptotic cell death. Further, brain is an easy target of ROS due to inefficient anti-oxidant capacity and therefore, imbalance in the pro-oxidant and anti-oxidant ratio may cause oxidative damage.[18]

In view of involvement of free radicals in chemical induced neurotoxicity, a number of studies have been directed towards assessment of the impact of oxidative stress to understand the associated mechanism with MCP. A significant induction in reactive oxygen species (ROS), lipid peroxide (LPO), and the ratio of glutathione disulfide (GSSG)/reduced glutathione (GSH) has been demonstrated in cells exposed to particular doses of MCP that could possibly be correlated with mitochondrial deficits.[19-21] Likewise, *in-vitro* studies carried out on PC12 cells revealed a drastic increase in the expression of apoptosis markers in MCP exposed cells by changing the expression and activity of caspases and cytochrome P450. Experimental analysis also revealed the disturbed balance between pro oxidants and antioxidants accompanied by membrane damage attributed to lipid peroxidation. Organophosphates like MCP attack the cell by inducing the production of reactive oxygen species (ROS) that could be a major reason underlying MCP induced toxicity.[22]

In complete sync with the aforementioned cellular and molecular alterations induced by MCP our results too suggested apoptotic cell death in the MCP exposed neuronal cells. The exposure to MCP led to dose dependent reduction in the cell viability. Further 24 h exposure of MCP was found to increase the LPO levels and decrease the GSH level dose dependently. These changes were accompanied by alterations in parameters of oxidative stress viz increase in reactive oxygen species generation and a state of mitochondrial membrane depolarization. Paralleling these changes was an increased activity of pro-apoptotic protein markers like caspase-3 and Bax while drastically reducing the anti-apoptotic Bcl-2 thus highlighting the major key features of an apoptotic cell.

The cytotoxic potential of MCP in exposed neuronal cells thus presents a considerably clear picture of apoptotic mediated toxicity. Our studies aim to establish the neurotoxicity mechanisms of MCP in rat brain neural stem cell derived neuronal cells which would help us to delve deeper and further into the molecular and cellular insights of pesticides induced neurodegeneration.

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Conflict of Interest

The authors declare no conflict of interest.

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