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Apoptotic Response of Human Mesenchymal Stem Cells against Dose Dependent Exposure to Organophosphate Pesticide-Monocrotophos

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Abstract

The severe health implications associated with the exposure of organophosphate pesticides are highly pronounced owing to their indiscriminate use. Monocrotophos, a predominantly used organophosphate pesticide, is a hazardous chemical that predisposes the exposed population towards multiple health disorders. We investigated the toxic effects of MCP on human mesenchymal stem cells to ascertain its overall detrimental effects on the normal physiology. There was a remarkable reduction in the cell viability accompanied with increased oxidative stress and mitochondrial dysfunctions with the increasing exposure concentrations. The decreased cell viability is probably an outcome of monocrotophos induced apoptotic cell death indicated by elevated expression of Bax and caspase-3 and reduced BCl_2 expression and as such calls for a stringent abatement in its haphazard use to prevent the incidence of many life threatening ailments.

Keywords: Apoptosis, Cytotoxicity, Monocrotophos, Stem Cells

1. Introduction

All the natural or synthetic products present in the environment that can potentially be a source of stress that hinders the normal metabolic state of living system are termed as environmental stressors (Gomez-Mejiba *et al.*, 2009). These can include biotic as well as abiotic factors like toxicants (Armario *et al.*, 2012; Campbell & Ehlert, 2012). Organisms usually generate a stress response under conditions of physical or mental stress that can be a complex set of behavioral and physiological changes at multiple levels of biological organization. However the degree of response depends upon the intensity or severity of the stressor along with the duration of exposure that actually makes it stressful or non-stressful (Schulte, 2014).

The chemicals, natural or man-made, that specifically target or destroy pests (weeds, insects, rodents, etc.) are termed as pesticides. However most of them act non-specifically and might as such affect the non-target organisms as well including humans. This has raised concern amongst the toxicologists due to the reversible and irreversible harm they cause to exposed individuals. Therefore their use and exposure must be optimally controlled (Costa, 2008; Ibrahim, 2016). There is a strong correlation between pesticide usage and disease incidence in the exposed population with several cases of chronic diseases like cancer, Parkinson's disease (Dardiotis *et al.*, 2013), Amyotrophic Lateral Sclerosis (ALS) (Violi *et al.*, 2015), Alzheimer's disease (Hayden *et al.*, 2014), birth defects, reproductive anomalies, asthma, atherosclerosis, nephropathies etc. in

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exposed individuals (Mostafalou & Abdollahi, 2013). Based on their chemical structure pesticides can be clubbed into different families belonging to organochlorine and organophosphorus group of compounds including the inorganic compounds (Garcia *et al.*, 2012).

Organophosphates are highly non-specific in their action that inhibits acetylcholinesterase activity at nerve endings. Monocrotophos (MCP), an organophosphate pesticide, falls in the category of highly hazardous chemicals and primarily targets acetylcholinesterase activity. Due to this muscarinic and nicotinic cholinergic receptors in brain are hyper activated resulting in cholinergic syndrome or intermediate syndrome that is marked by excessive salivation and sweating, bronchosecretion and bronchoconstriction, miosis, diarrhoea, tremors, muscular twitching and other CNS effects (Krause *et al.*, 2013; Morris *et al.*, 2014). Its continuous use, despite the ban imposed on it, has elevated the probability of human exposure and the risks associated with it. Under chronic conditions monocrotophos might adversely affect the central nervous system causing involuntary muscle contractions, fatigue, weakness, slurred speech and twitching (Chambers & Oppenheimer, 2004; Costa *et al.*, 2005; Venkatesh *et al.*, 2009) and in graver cases it may paralyze the body sometimes leading to coma (Costa, 2008; Cannon and Greenamyre, 2011).

Based on such a rich literature associated with MCP and its toxic effects in the exposed population that results in onset of perilous health disorders we investigated the adverse effects of MCP and the probable mechanisms behind them in the exposed human mesenchymal stem cells (MSCs) that could be differentiated into any cell type upon receiving the desired stimulus and hence represent an optimal *in-vitro* model to study MCP mediated toxicity in general.

2. Materials and Method

2.1 Reagents and Chemicals

The culture media along with their components were procured from Gibco, Thermo Scientific. These included α-Minimum Essential Medium (α-MEM, Cat No. 11900-073), RPMI-1640 (Cat No. 31800-022), MSC qualified Fetal Bovine Serum (MSC-FBS, Cat No. 12662029), 7.5% Sodium bicarbonate (Cat No. 25080094), Glutamax (Cat No. 25080094), antibiotic-antimycotic 100X (Cat No. 25080094). The culture-ware was obtained from Nunc Labware. The reagents for western blotting and culture-grade chemicals were procured from Sigma-Aldrich, India. Most of the primary antibodies used in the study were obtained from Millipore. These were anti-cytochrome-c (Cat No. 04-1043), anti-BCl2 (Cat No. 2872), anti-caspase3 (Cat No. 04-1090), anti-p53 (Cat No. 04-241), anti-apaf1 (Cat No. AB16503). The antibody for β-actin (Cat No. A1978) was bought from Sigma-Aldrich while that for Bax (Cat No. ab32503) was from Abcam. The secondary antibodies for chemiluminiscence, i.e., HRP conjugated goat anti-Mouse IgG (Cat No. AP181P) and goat anti-rabbit IgG (Cat No. AP187P) antibodies were also purchased from Millipore.

2.2 Cell Culture

The Wharton-jelly derived mesenchymal stem cells (WJ-MSCs, Cat No. CL001), procured from Himedia, were cultivated in α-MEM supplemented with 12% MSC FBS, 1% antibiotic-antimycotic, 0.2% sodium bicarbonate and 1% glutamax at 37°C and 5% CO_2 in a humidified chamber and sub-cultured at 70%-80% confluence.

2.3 MTT Assay

The first step towards assessment of neuronal secretome mediated effects on the growth and development of stem cells was to estimate the most appropriate and biologically safe concentration of MCP for treatment of Mesenchymal Stem Cells. This was achieved through the most commonly employed cytotoxicity assay, the MTT assay developed by (Mosmann, 1983). Briefly, MSCs were seeded in 96-well culture plates at a density of 1X104 per well and incubated overnight at 37°C and 5% CO_2 in a humidified chamber. The following day cells were exposed to varying concentrations (50μM-500μM) of MCP. The CM induced cytotoxicity was assessed at different time-points, i.e., 24h, 48h, 72h and 96h. 4h prior to treatment completion 10 μl of MTT (5mg/ml) was added to each well. At the end of treatment the MTT containing medium was aspirated and the resulting formazan crystals were dissolved in 200μl DMSO that led to development of deep purple color, the intensity or absorbance of which was recorded at 550nm using a spectrophotometer (Synergy HT, BioTek, USA). The untreated control sets were also run in parallel.

2.4 Trypan Blue Dye Exclusion Method

The dye exclusion test works on the principle of membrane integrity of cells under viable conditions and is used as a measure of the number of viable cells present in a given suspension. The viable cells normally possess proper and intact cell membranes and hence exclude dyes like trypan blue, Eosin, or propidium, while the dead cells due to their irregular and ruptured membranes readily take up these dyes. The viable cells possess a clear cytoplasm while the dead ones will have a blue cytoplasm (Strober, 2001). Briefly, the cells are seeded at a density of $4X10^4$ cells per well in 24-well culture plate with each sample in triplicate and incubated overnight. Post incubation cells were exposed to MCP at different concentrations for 24h and then harvested following the procedure similar to sub-culturing. The pellet obtained was dissolved in fresh culture medium and 10µl of this cell suspension was mixed thoroughly with 10µl of trypan blue dye. Further 10µl of this mixture was placed on the Neubauer haemocytometer, spread and covered with a glass cover slip and observed under the microscope for counting individual cells in all the four grids of heamocytometer. The formula used for counting the number of cells per ml was,

Number of cells/ml = average of four grids X dilution factor X 10^4

2.5 Neutral Red Uptake Assay

The neutral red uptake assay is a quantitative assessment of the number of live cells in a culture. The neutral red dye accumulates in the lysosomes according to the pH gradient present in them which further depends upon a proper maintenance of pH gradient by the live cells. In absence of a pH gradient this dye cannot accumulate and hence the amount of dye retained by the cells is a direct measure of the number of viable cells (Repetto *et al.*, 2008). Briefly, the cells were seeded at a density of $1X10^4$ per well in 96-well culture plates and incubated overnight. The cells were then exposed to varying concentrations of MCP for 24h. Post treatment the medium was aspirated and cells were washed with 1X PBS. The cells were then further incubated with the normal growth medium supplemented with neutral red dye (50µg/ml) was added for 3h at 37°C. After incubation cells were washed with 0.5% formaldehyde and 1% calcium chloride and then fixed with 1% acetic acid and 50% ethanol for 20 minutes at 37°C. The absorbance was recorded at 540nm using a spectrophotometer (Synergy HT, BioTek, USA).

2.6 Oxidative Stress Generation

ROS estimation involves the use of 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescent probe that usually reacts with several Reactive Oxygen Species (ROS), the marker of oxidative stress. Dead or dying cells produce ROS. Briefly the cells were seeded in 6-well culture plates at a density of $1\text{-}1.5X10^5$ cells/well and incubated. The cells were then exposed to varying concentrations of MCP for 24h. H_2O_2 (500 µM) exposed cells served as positive control for ROS generation. After the treatment duration was completed cells were harvested and incubated in 10µM DCFDA dye (Cat No. D6883, Sigma Aldrich) for 20 minutes in dark, followed by another wash. Finally the pellet obtained was resuspended in 1XPBS and analyzed through flow cytometry (BD influx, USA).

2.7 Mitochondrial Membrane Potential (MMP)

Changes in the mitochondrial membrane potential can be measured to assess the normal mitochondrial activity of cells. Briefly $1x10^5$ cells were seeded per well in 6-well culture plate and incubated overnight. Following this cells were exposed to different concentrations of MCP. Staurosporine (100nM) served as the positive control group. After 24h of treatment the cells were trypsinized and harvested and then washed with 1XPBS. The pellet obtained was again resuspended in PBS and incubated with JC-1 dye (5µg/ml) in dark for 15 minutes followed by another wash with PBS. Finally the pellet obtained was resuspended in 1XPBS and analyzed using flow cytometer (BD influx, USA).

2.8 Changes in Expression of Markers of Apoptosis

Changes in protein expression level in response to MCP exposure were determined by immunoblotting. Equal amount (40µg) of proteins was loaded on 10-12% SDS gel and blotted on polyvinylidene fluoride membrane. After blocking for 1hr at room temperature, the membranes were incubated overnight at 4°C with anti-protein primary antibodies specific for apoptosis, viz., BCl_2, caspase-3 and Bax. β-actin was used as an internal control. The membranes were then incubated for 2h at room temperature with secondary anti-primary immunoglobulin G (IgG)-conjugated with horseradish peroxidase. The blots were developed using luminal, and densitometry for protein

specific bands was done with the help of AlphaEaseTM FC StandAlone V. 4.0.0 software.

3. Results and Discussion

Environmental contaminants and their associated adverse effects on human physiology are far and many. World Health Organization's report suggested approximately one million deaths across the globe occurring through unintentional poisoning of pesticides while two million suicidal deaths occur through poisoning annually (Jokanović & Kosanović, 2010). The exposure to these contaminants can be acute (single or multiple exposures within a short time span, say 24h), developmental (prenatal, in utero or early postnatal exposures), subchronic (exposures extending up to as long as <90 days), and chronic (longer than sub-chronic) (Voorhees *et al.*, 2016). Pesticides like OPs are also genotoxic under chronic and acute exposure (Ojha *et al.*, 2013). Monocrotophos is a commonly used pesticide to control pests that hamper the crops like cotton, rice and sugarcane (IPCS, 1993). Based on WHO report, MCP has been declared as highly hazardous.

3.1 Cell Viability of MCP Exposed Stem Cells

There have been earlier reports of MCP induced cytotoxicity under both acute and chronic conditions that increases in a dose and time-dependent manner (Tripathi et al., 2014). The results obtained from cytotoxicity assessment based on different parameters and assays in this study too depict a concentration dependent reduction in the cell viability of exposed MSCs (figure 1A-C). Based on the data obtained from MTT assay it was observed that there was a severe loss in cell viability of MCP exposed cells as compared to the untreated control group. The cell viability declined at 50µM dose followed by 100µM and a drastic reduction at 200µM and 500µM respectively relative to the control group. A similar concentration dependent decrease in the cell viability was also observed in other cytotoxicity assays performed indicating the direct relationship between increasing MCP concentration and cytotoxicity in stem cells. According to the results obtained 100µM dose seemed biologically safe but toxic enough to be treated as cytotoxic for carrying out further experiments for cytotoxicity. The % cell viability of hMSCs treated with different concentrations of MCP was determined by (a) MTT assay, (b) trypan blue dye exclusion method, (c) NRU assay, at 24h, 48h, 72h and 96h. The values obtained for control samples for each day was considered basal and compared with the values of corresponding treated samples. Values are mean±SE of the data obtained from three independent experiments. * = p< 0.05, * * = p< 0.01, * * * = p< 0.001.

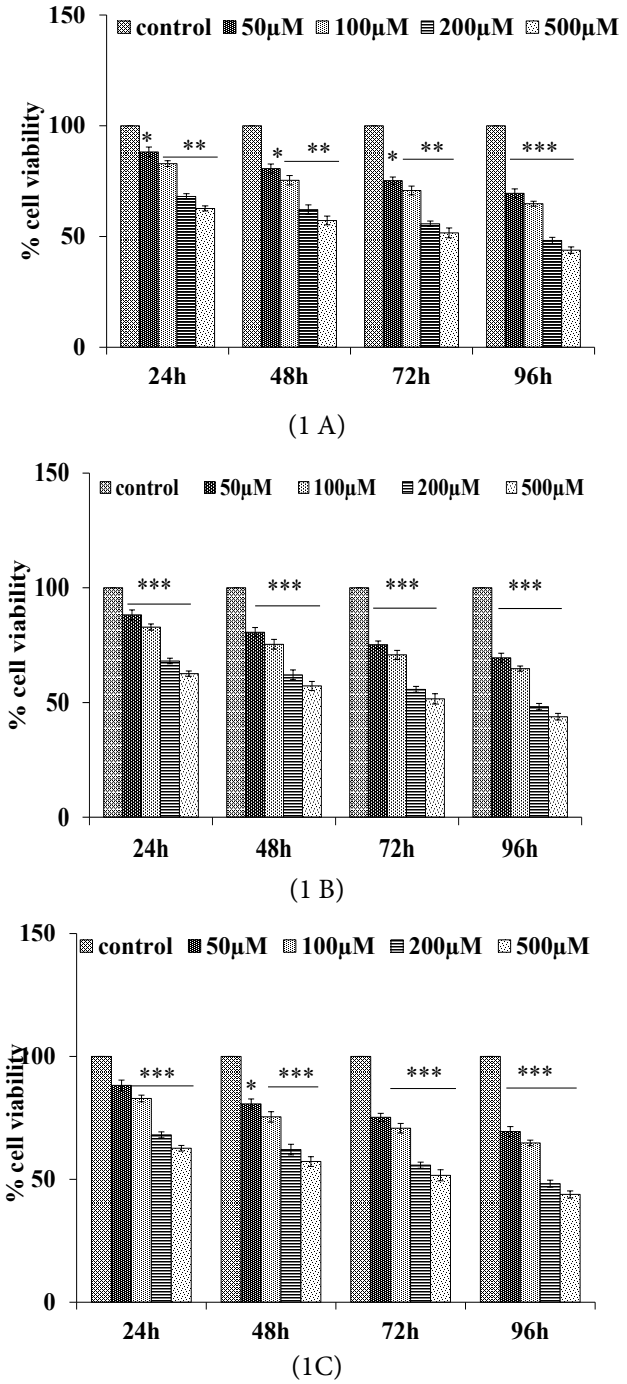

Fig. 1 Cell viability estimation through (A) MTT assay, (B) Trypan blue dye exclusion method, (C) Neutral Red Uptake assay. Values are mean±SE of the data obtained from three independent experiments. * =p< 0.05, * * = p< 0.01, * * * = p< 0.001.

3.2 MCP Induced Oxidative Stress

A significant elevation in Reactive Oxygen Species (ROS), Lipid Peroxide (LPO), and the ratio of Glutathione Disulfide (GSSG)/reduced glutathione (GSH) has also been reported in cells exposed to selected doses of MCP that can possibly be linked with mitochondrial dysfunctions (Kashyap *et al.*, 2010; Karami-Mohajeri & Abdollahi, 2013). Experimental analysis reveals the disturbed balance between prooxidants and antioxidants in the body accompanied with membrane damage due 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 toxicity (Yaduvanshi *et al.*, 2010). The oxidative stress generated as a result of formation of superoxide radicals or free radicals due to MCP exposure was assessed through estimation of ROS using flow cytometry. Higher ROS suggests higher cell death that can be observed on a flow cytometer on the basis of red to green shift in the fluorescence. As per our expectation and the earlier studies the increasing concentrations of MCP produced oxidative stress in a direct proportion, i.e., increasing concentrations of MCP led to increased ROS production in the exposed mesenchymal stem cells.

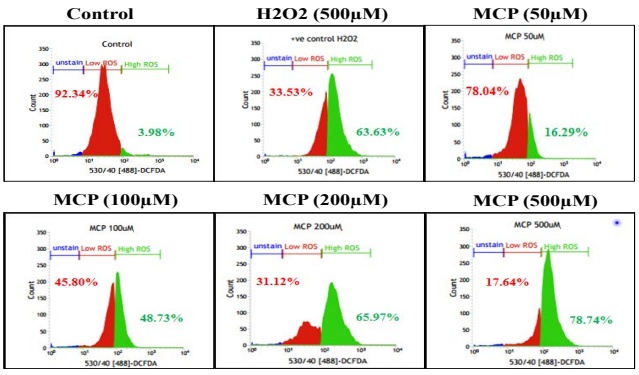

Fig. 2 ROS generation in hMSCs exposed to varying concentrations of MCP was analyzed through flow cytometry. The red to green shift indicates cell death. The numbers in red and green indicate percentage of live and dead cells respectively. Cells exposed to H_2O_2 served as positive control for ROS generation.

3.3 Alterations in MMP in Response to MCP

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. Earlier studies based on estimation of MCP toxicity reported a significant reduction the mitochondrial membrane potential (Kashyap *et al.*, 2013). 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.

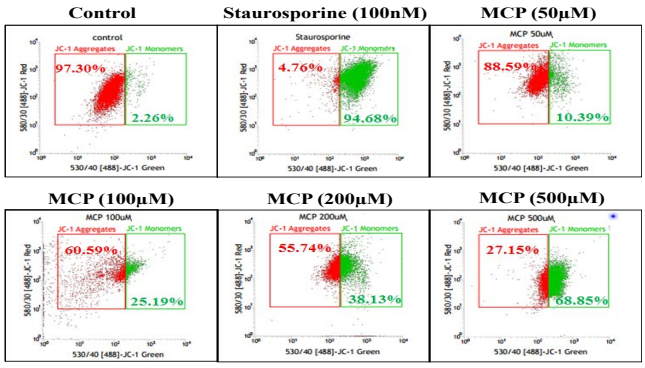

Fig. 3 The alterations in MMP induced in hMSCs in response to different concentrations of MCP were recorded through flow cytometry using JC-1 dye. Staurosporine (100nM) served as the positive control.

3.4 Expression Changes in Apoptosis Markers

The *in-vitro* studies conducted on PC12 cells depicted a remarkable increase in the expression of apoptosis markers in MCP exposed cells that further stimulated the activity of caspases and cytochrome P450 (Kashyap *et al.*, 2010; Kashyap *et al.*, 2011). Likewise a number of *in-vitro* and *in-vivo* studies reported the active involvement of various apoptotic signaling pathways in MCP-exposed neuronal cells (Kumar *et al.*, 2013). To further analyze the biochemical changes introduced in MSCs upon MCP exposure the expression pattern of some of the key apoptosis markers was observed. In sync with the previous findings it was observed that the increasing concentrations of MCP promoted the expression of pro-apoptotic proteins Bax and caspase-3 while reducing the expression of anti-apoptotic BCl_2 in comparison to the untreated control group of cells. Such an expression profile is indic-

ative of apoptosis induction in response to increasing MCP concentrations.

Changes in expression of apoptosis markers in response to MCP exposure at various concentrations (in µM) were observed. The expression of pro-apoptotic markers increased with increasing concentration of MCP while that of anti-apoptotic BCl2. The fold change in expression of each marker in each treatment group was compared with one another and with control using Newman-Keules multiple comparison test. Values are represented as mean±SE of the data obtained from three independent experiments (*** - p<0.001)

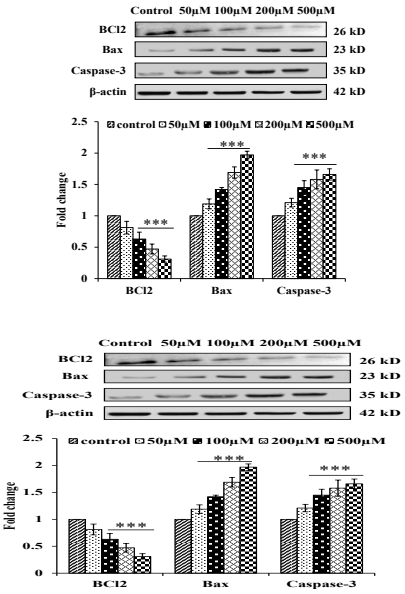

Fig. 4 Expression of apoptosis markers in response to various concentrations of MCP. The expression of pro-apoptotic proteins Bax abd Caspase-3 increased while that of anti-apoptotic BCl2 decreased with increasing MCP concentrations. Values are represented as mean±SE of the data obtained from three independent experiments (*** - p<0.001)

MCP additionally manifests its neurotoxic potential by hampering the dopaminergic neurotransmission in mice (Ali & Rajini, 2016). Besides neuronal anomalies MCP exposure also generates cardiovascular stress. The cardiotoxicity as a result of prolonged usage of MCP leads to mild-hyperglycemia and dyslipidemia in the blood of exposed animals along with oxidative stress. The markers of cardiac damage cTn-I, CK-MB and LDH additionally had an elevated expression in blood plasma (Velmurugan *et al.*, 2013). It also causes growth inhibition in diatoms at low concentrations with complete seizure at higher doses (Karthikeyan *et al.*, 2015).

In sync with the above mentioned alterations induced by MCP in cellular physiology our results too suggested apoptotic cell death in the MCP exposed Mesenchymal stem cells. The exposure to MCP led to induction of hugely magnified oxidative stress with the increase in reactive oxygen species and depolarization of mitochondrial membrane thus reducing the cell viability. This was also accompanied with the increased activity of pro-apoptotic protein markers and a reduction in the anti-apoptotic ones thus highlighting the major key features of an apoptotic cell. The cytotoxic potential of MCP in exposed mesenchymal stem cells thus presents a considerably clear picture of a general overview of pesticide induced toxicity. Since stem cells can be converted into any desired cell type this pilot study to some extent proves the overall toxic potential of MCP in the exposed population that can manifest its toxic effects in any of the organ system along the entire life span of an individual.

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