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Research Article
Ameliorating Effects of Magnolol in Reducing the Toxicity Induced by Heavy Metal Lead in the Human Brain Cells

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Abstract
Background and Objective: Lead (Pb) is being currently used in various forms and is getting exposed to living systems via different routes. It is found to affect different metabolic functions including antioxidant defense system. The treatment procedure for the lead exposure till now is not well defined and the objective of the study was to evaluate the effects of magnolol in ameliorating the toxicity induced by Pb. **Materials and Methods:** Human SH-SY5Y cells were exposed to different concentrations (0.01-10 μM) of Pb for 48 h and the cell viability was determined and the IC_{50} was observed at 5 μM. Various markers such s glutathions, caspase-3 and PGE2 were measured. The obtained data was analyzed using the one-way analysis of variance (ANOVA) and the statistical significance was assumed at $p<0.05$. **Results:** Findings suggest that there was no change in the cell viability when the cells were pretreated with MGL with a range of concentration from 0-140 μM for 48 h. The cells were treated with 80 μM of magnolol (MGL) to demonstrate its protective effects if any and against the 5 μM Pb pretreated cells for 48 h. The results indicated that, there was a significant ($p<0.05$) increase in the cell viability at least by 23.1% when compared with Pb alone treated group. Further, to evaluate the protective effects of MGL intracellular glutathione, caspase-3 and prostaglandin E2 levels were quantified. **Conclusion:** The results clearly showed the decrease in oxidative stress, inflammation percentages and reduce the impact of apoptosis. In over all, MGL was found to be an effective antioxidant, anti-apoptotic and anti-inflammatory agent.

Key words: Lead, magnolol, neuroblastoma cells, glutathione, caspase-3, prostaglandin E_2

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Heavy metal lead (Pb) is considered to be an important environmental toxic pollutant. Literature indicates that exposure to Pb leads to several health related issues including the brain behavioral changes, mental retardation, gastro-intestinal disturbances and respiratory problems. The Pb is considered as one of the major health concern in the developed and developing nations. It is observed that brain is one of the important organ mostly affected and alters its function[1]. Children are the most affected group with cognitive dysfunction and psycho-motor problems[2]. The reduction in the ability in problem solving properties, learning and changes in the psychology is the key in the Pb exposed population[3]. Lead induced poisoning targets primarily the developing nervous system[4]. Prolonged exposure to Pb might be fatal in terms of intellectual impairment and growth retardation[5,6]. Toxic exposure leads to the alterations in the intracellular functions of the brain cells and it is considered to be a multi-factorial effect[7,8]. The chronic exposure to brain is a multi-factorial event and is executed at intra cellular level[9,10]. Neurochemical effects such as altered calcium signaling and mitochondrial permeability, mitochondrial dysfunction that is leading to the generation of oxidative stress results in damage to are the important metabolic events associated with the chronic exposure of Pb[11-13]. It is well observed that, oxidative stress is one of the eventuality by the exposure to Pb and leads to the release of free radicals in turn interfering with the functions of antioxidant defense system ultimately depleting glutathione[14]. Primarily the induction of oxidative stress together with altered calcium channel function during chronic exposure to even low-levels of Pb-exposure may ultimately result in cell death[13]. The Pb poisoning cases are very common in the developing countries and the current treatment options are very limited and there is a scope for designing alternative methods to attenuate Pb-toxicity[15]. Changes in the morphology and toxicity to the cells is a clear-cut indication of oxidative damage and during these circumstances antioxidants play an important role in the chemical toxicity being generated by Pb associated with pathological changes[12,16-18].

Magnolia officinalis belongs to family Magnoliaceae and is distributed over China, Japan and South Korea. *Magnolia officinalis* is used as a folk remedy for gastrointestinal disorders, cough, acute pain, anxiety and allergic diseases[19-26]. Magnolol (Mag, 5,5-diallyl-2,2-dihydroxybiphenyl), a hydroxylated biphenyl compound isolated from the root and stem bark of *M. officinalis*, is shown to have muscle relaxant, anti-oxidative, anti-atherosclerosis, anti-inflammatory and anti-microbial effects. Mag also induces differentiation and calcium mobilization[27,28]. Magnolol has showed anti-inflmmatory activity and earlier reports indicated MGL can inhibit inflammatory enzyme/cytokine production, nuclear factor (NF)-kB activation and leukocyte activation[29]. Magnolol exhibits leukocyte suppression, anti-inflammation and analgesic effects via decreased myeloperoxidase activity[20], decreased eicosanoid mediator activity[30-32] and decreased leukotriene formation[33-38]. Histamine release[36], as well as nitric oxide (NO), Tumour Necrosis Factor-α (TNF-α), basic fibroblast growth factor, matrix metalloproteinase-1 and interleukin (IL)-4 production are also decreased[39,40]. Magnolol can successfully induce apoptosis in tumour cells, including, cultured human hepatoma and colon[41] cancer cells, lung squamous[42] and thyroid carcinoma, melanoma, fibrosarcoma, monocytic leukaemia[43] and prostate cancer cells[44]. Therefore, the present report was focused to elucidate the mitigative properties of MGL against Pb-induced toxicity in human neuroblastoma cells.

MATERIALS AND METHODS

Materials: The RPMI-1640 medium, OPI were obtained from GIBCO life technologies. Fetal bovine serum, penicillin and streptomycin and phosphate buffered saline were procured from CELLGRO, Mediatech. The kits were used for the assays of MTT cell proliferation (ATCC, Manassas, VA, USA), glutathione (Dojindo Molecular Technologies, Gaithersburg, MA,, USA), prostaglandin E_2 (Amersham Biosciences Piscataway, NJ, USA) and caspase-3 (Biovision, Mountain View, CA, USA). All other chemicals were of analytical grade and procured from Sigma including Koenidine (3,11-Dihydro-8,9-dimethoxy-3,3,5-trimethylpyrano[3,2-*a*]carbazole). The study was conducted at National institute of Nutrition, Biochemistry laboratory at Hyderabad during the month of March, 2017.

Cell culture: Human SH-SY5Y neuroblastoma cells were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 $\mu g\ mL^{-1}$ penicillin-streptomycin and OPI (150 $\mu g\ mL^{-1}$ oxaloacetate, 50 $\mu g\ mL^{-1}$ pyruvate and 0.2 U mL^{-1} insulin) in a humidified air/5% CO_2 chamber at 37°C. Medium was changed every 3 days and were passed once they reached approximately 80% confluence.

Cell viability: Cell viability among the attached cell population was assessed by using trypan blue method. Trypan blue isotonic solution was added to the culture and number of deeply stained cells, representing dead cells was counted as

described by Syu *et al.*[45]. The number of stained cells was subtracted from the total count in order to determine the percentage of viable cells.

Exposure of cells to Pb acetate and magnolol (MGL): Human SH-SY5Y neuroblastoma cells were seeded at 2×10^4 cells per well in a 96 well plate. The cells were allowed to attach and grow for two days prior to the treatment. The cells were treated with varying concentrations of lead acetate (0.01-10 μM) in order to determine IC_{50} value. The cells were pretreated with MGL (0-100 μM) to determine the effect on cell growth. Further, the cells were exposed to 5 μM (IC_{50}) of Pb in the presence or absence of MGL for 48 h and the cell viability was determined by MTT reduction assay.

MTT reduction assay: Cell viability was also assessed by MTT [3-(4,5 dimethyl-2-thazolyl)-2,5 diphenyl-2H tetrazolium bromide] reduction assay as per the manufacturer's instructions. In brief, the cells 2×10^5 per well were seeded into 96 well culture plates and allowed to attach. The medium containing varying concentrations of Pb in the presence or absence of MGL was added to the cells and incubated for 48 h. Then, 10 μL of MTT reagent was added to the culture and incubated in the dark for 4 h at 37°C followed by cell lysis by the addition of 100 μL detergent reagent provided with the kit. Then plate was left at room temperature in the dark for 2 h and the relative amount of MTT reduction was determined based on the absorbance measured at 570 nm using a plate reader.

Determination of glutathione: In brief, 5×10^5 cells were centrifuged and the suspension was washed with the phosphate buffered saline. Suspended cells were lysed with 10 mM HCl and treated with 5% sulphosalycilic acid. The cells were centrifuged at 8,000×g for 10 min and to the supernatant 20 μL of enzyme working solution, 140 μL of coenzyme working solution and 20 μL of either one of the standard solution or the sample solution (cells treated with Pb in the presence or absence of MGL) were added. Then the plate was incubated at 37°C for 10 min and 20 μL of substrate, 5,5'-Dithiobis(2-nitrobenzoic acid) [DTNB] working solution was added. The plate was incubated at room temperature for 10 min and the optical density was measured at 415 nm using a micro plate reader and the concentration of GSH was calculated using a standard calibration curve. The reagents used throughout the experiment were prepared according to the manufacturer's instructions.

Caspase-3 activity assay: Briefly, the assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. After the Pb-treatment of the cells in the presence or absence of MGL, $3\text{-}5\times10^6$ cells were collected, resuspened in the 50 μL of chilled cell lysis buffer included in the kit and incubated for 10 min on ice and then centrifuged at 10,000×g for 1 min. To the supernatant, 50 μL of the reaction buffer containing 10 mM dithiothreitol and 5 μL of the 4 mM DEVD-pNA substrate was added and incubated at 37°C and the optical density was measured at 405 nm after 2 h. The relative fold increase in caspase-3 activity was determined by comparing with the levels of the untreated control.

Determination of prostaglandin E_2: The reagents used in the procedure were included in the Prostaglandin E_2 kit (Amersham Biosciences Piscataway, NJ, USA) contents. The SH-SY5Y neuroblastoma cells 10^4 to 10^5 cells per well were placed in a standard 96 well microplate. The plate was incubated in a humidified air/5% CO_2 chamber at 37°C and allowed the cells to attach. The cells were treated with Pb in the presence or absence of MGL and continued the incubation for 48 h. Then, 20 μL of buffer A (2.5% dodecyl trimethylammonium Bromide in 0.1 M Phosphate buffer pH 7.5). The cells were lysed by simple agitation and 50 μL of lysate was transferred to goat anti-mouse IgG coated plate (included in the kit) and 50 μL of lysis reagent containing dodecyltrimethyl ammonium bromide (prepared as described with 0.1 M phosphate buffer pH 7.5 containing 0.9% bovine serum albumin and 0.5% Kathon). Further, 50 μL of diluted PGE_2 antibody and 50 μL of diluted conjugate (PGE_2 conjugated to Horseradish Peroxidase) were added to the plate and incubated at room temperature for 1 h. The cells were washed and added 150 μL of enzyme substrate (3,3',5,5' tetramethylbenzidine) readily provided with the kit. The contents were mixed thoroughly exactly for 30 min and the reaction was stopped by the addition of 100 μL of 1 M sulfuric acid. The optical density was measured at 450 nm within 30 min and concentration of total cellular PGE_2 was calculated using appropriate standards as provided in the kit.

Data analysis: Data were expressed as Mean±SD of at least four determinations from each group, repeated at least three times in different occasions. Statistical analysis was performed using one-way analysis of variance (ANOVA) using SPSS (version 15.0) software and the statistical significance was assumed at $p<0.05$[46].

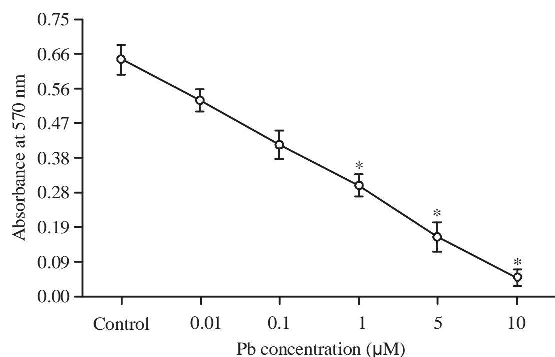

Fig. 1: Effect of Pb exposure on human SH-SY5Y neuroblastoma cells

The lead was exposed in increasing concentrations (0.01-10 μM) for 48 h The cell viability was determined by MTT reduction assay. Data are presented as Mean+SD from four samples from each group. *Significantly different from control at $p<0.05$

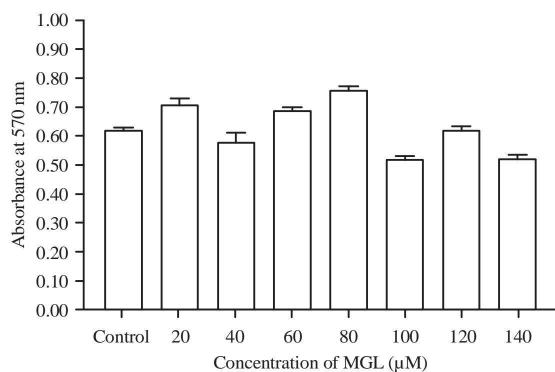

Fig. 2: Effect of MGL treatment (0-140 μM) on human SH-SY5Y neuroblastoma cells for 48 h

The cell viability was determined by MTT reduction assay. Data are represented as Mean+SD of four different samples from each group

RESULTS

The Pb was exposed to cells at different ranges from 0.01-10 μM for 48 h resulted with significant ($p<0.05$) decrease in the cell viability from 94.2-21.6% (Fig. 1). A 50% decrease in cell viability (IC_{50}) was observed at 5 μM Pb. When 0-140 μM concentrations of MGL were exposed to cells for 48 h, the cell viability was unaffected and there were no significant alterations in the growth of the cells (Fig. 2). The MGL was treated (60 μM) to the cells to verify its protective effects against the pretreatment of Pb at a concentration of 5 μM 48 h, has resulted in the significant ($p<0.05$) increase in the cell viability at least by 26.1% from 10.2% when compared with Pb alone treated group (Fig. 3). These results indicated that, MGL can act as effective protective agent in enhancing the cell viability that is been affected by Pb exposure. Further, intra cellular GSH levels indicated that, there was a significant ($p<0.05$) decrease in the levels in the cells exposed to Pb for about 48 h in a concentration dependent manner (Fig. 4). About 55.4% of decrease in GSH levels were observed in the cells at 5 μM of Pb exposure, where as cells treated with MGL alone (1-140 μM) for 48 h the GSH levels were unaltered. When pre-treated with MGL (60 μM) along with Pb (5 μM) has

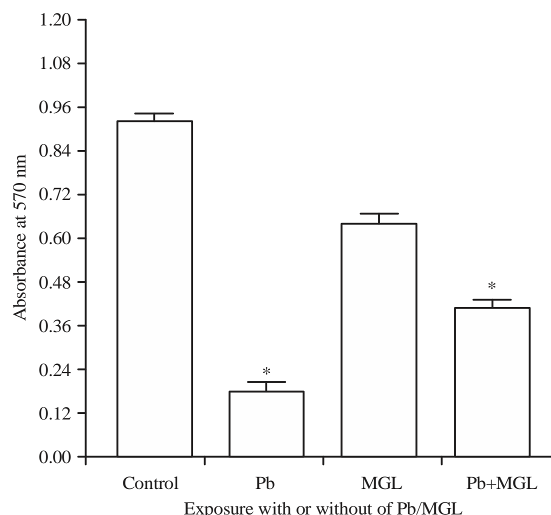

Fig. 3: Effect of Pb (5 μM) exposure for 48 h in the presence or absence of MGL (60 μM)

The cell viability was determined by MTT reduction assay. Data are represented as Mean+SD of four different samples from each group. *Significantly different from the control at $p<0.05$

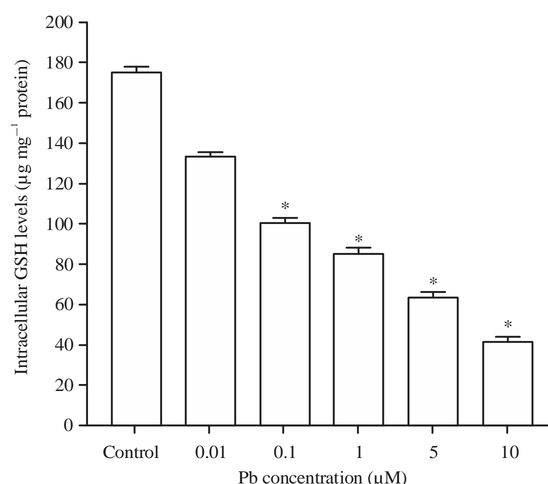

Fig. 4: Changes in the percentage of intra-cellular GSH levels after 48 h incubation of the cells with increased concentrations of Pb (0.01-10 μM)

The values are Mean+SD of five different values from each group. *Significantly different from control at $p<0.05$

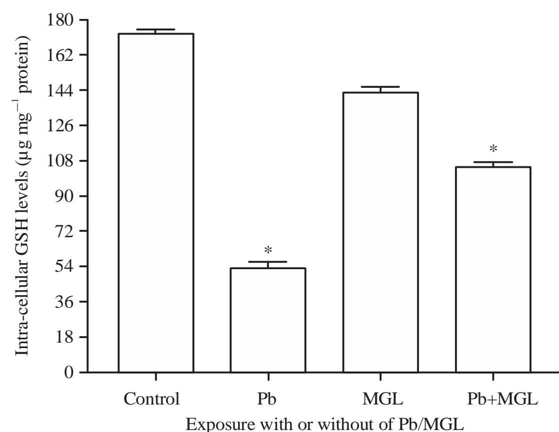

Fig. 5: Effect of Pb (5 µM) exposure on the levels of intra-cellular GSH over 48 h in the presence or absence of MGL (60 µM)

The values are Mean+SD of five different samples from each group. *Significantly different from control at $p<0.05$

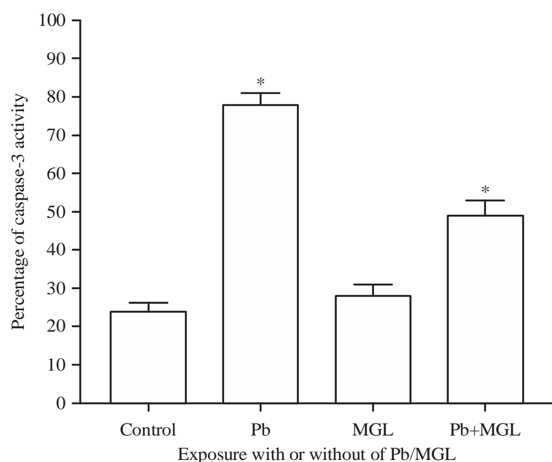

Fig. 6: Caspase-3 levels after 48 h of Pb exposure (5 µM) in the presence or absence of MGL (60 µM)

Data are represented as Mean+SD of four independent assays from each group. *Significantly different from control at $p<0.05$

shown a significant increase (26.3%) in the levels of GSH when compared with the group with Pb alone indicating the fact that, MGL has an antioxidant effect (Fig. 5). An executioner enzyme in the apoptosis pathway, caspase-3 levels were significantly increased $p<0.05$ at least by 51.3% when compared with the untreated control group. Cells treated with MGL alone has not shown any effect on the levels of the enzyme when compared with Pb alone group, where as the combination of Pb and MGL has resulted in significant $p<0.05$ decrease in the enzyme levels by 25.2% when compared with the Pb alone group (Fig. 6). Significant $p<0.05$ increase in the PGE_2 levels were observed in the Pb alone group, but not

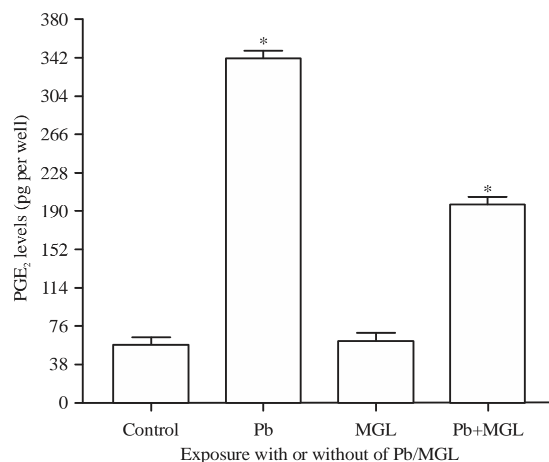

Fig. 7: Alterations in the levels of total cellular PGE_2 after 48 h of Pb/MGL exposure

Data are represented as Mean+SD of four independent assays from each group. *Significantly different from control at $p<0.05$

MGL group, Whereas, the combination of MGL (60 µM) and Pb (5 µM) exposure resulted in a significant $p<0.05$ decrease in PGE_2 by 133.4 pg per well when compared the Pb alone group (Fig. 7).

DISCUSSION

In this study MGL was found to mitigate the toxic effects of Pb. Heavy metal Pb is known to induce alterations and causes damage to different pathways of metabolism in both cellular and animal models[47-50]. However, the studies related to the effects of low levels of exposure of and resultant damage to the nervous system is very scanty. The mechanism of Pb induced oxidative stress involves an imbalance between generation and removal of ROS in tissues and cellular components causing damage to membranes, DNA and proteins is one of the very important events that are been associated with the cell death[48]. In the present study, the investigations associated with the effects of exposure of Pb on human neuroblastoma cells and protective role of magnolol during toxic incursion of Pb in the cells were undertaken. Results suggested that, the MGL along with Pb exposed cells has shown a significant improvement in the cell viability. Maiti *et al*.,[51] reported the dynamics of oxidative stress in different pathologies appears to be multifactorial with the involvement of environmental and metabolic factors ultimately leading to cell death. Neuroprotective effects of MGL might be related to its anti-oxidative actions and the mechanism could be by antagonizing the toxicity induced by excitatory amino acids, this herbal compound could potentially be used to treat toxic assaults to the brain[52]. Rather

our results indicates that MGL treatment alone has not shown any effect on the cell viability but when it is pretreated with Pb has a significant effect on the cell proliferation where it showed toxic ameliorating role of MGL.

The Pb induced cognitive impairment, abnormal behavior and alterations in the motor functions are could be because of the process known as apoptosis, where the ultimate effects may result in the brain damage, this can be triggered by several of kinds of stimuli. A family of cysteine proteases known as caspases, transducer apoptotic cell death signals in a hierarchical fashion, where the initiator caspases cleave and activate the effector caspases leading to cell death[52]. One of the important executioner enzymes in the group of caspases is caspase-3 which promotes the cell dis-assembly via the cleavage of several structural proteins and repairs the enzymes essential for maintaining cellular homeostasis. The proteolytic activity of caspase-3 is a critical determinant of whether or not cell commits suicide[53]. Altered stimulation or inhibition of process of apoptosis has lead to several physiological changes. Recently, Wolf and Green[54] demonstrated antiapoptotic effects of melatonin in the multitarget hybrids during amyloid induced toxicity in the neurodegenerative situations. Results indicated that, levels of caspase-3 was significantly enhanced $p<0.05$ with the exposure of Pb. Whereas, cells that are pre-treated with MGL followed by Pb has shown a significant $p<0.05$ reduction in the caspase-3 levels. This indicates that, MGL could have played an important role in antagonizing the apoptotic effect, thus acting as anti-apoptotic agent. In fact, caspase-3, a member of the caspase family has been shown to be the key modulator of apoptotic changes[55].

The GSH is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine and the carboxyl group of cysteine is attached by normal peptide linkage to aglycine. A significant $p<0.05$ reduction in the GSH levels was also observed suggesting the fact that oxidative stress has been induced in the Pb exposed cells. Previous studies have demonstrated that, Pb can effectively stimulate oxidative hemolysis by inhibiting several antioxidative enzymes[12,16,56]. Present study results indicated that cells pretreated with MGL were resistant to the effects of Pb, suggesting the fact that, MGL acts as neuroprotecive agent in interfering the toxicity induced by Pb. Technically, MGL might have strongly bonded to Pb thus leading to increase in intracellular GSH levels could be via up-regulation of machinery of GSH-synthesizing enzymes[56,57]. It is possible that the metal-chelating effect of MGL has reduced cell damage and increased the production of intracellular GSH.

The significant release PGE_2 was observed in the cells as a result of low levels of Pb exposure. Cycloxygenase activation and production of PGE_2 is an essential key mechanism in the process neuronal loss[58,59]. An increase in neurotrophic factors could alter the levels of prostaglandins and will make them potentially valuable in the treatment of neurodegenerative disorders[60-62]. Similarly, from the results, it is evident that, Pb has played a key role in the activation of cycloxygenase pathway that has resulted in the significant release of PGE_2. Rather, MGL exposed cells and there after treatment with Pb has resulted in the reduction of PGE_2 has showed the importance of MGL in the inflammatory pathway.

CONCLUSION

It is concluded from the present study that exposure of Pb has induced apoptosis, oxidative stress and alteration in the inflammatory pathways which said to play an important role in the development of neuro-toxicity. However, the present study reported that, a natural compound such as magnolol could effectively work against the lead induced toxicity and can ameliorate the toxic effects that is been produced by Pb.

SIGNIFICANCE STATEMENTS

The study emphasizes the protective effects of magnolol against the lead induced toxicity in the human brain cells. It was well known fact that, lead toxicity was one of the major issues associated with the environmental pollution. It was the need of the hour to address the lead toxicity by natural procedures instead of drugs treatment. Thus finding alternative treatment with naturally available bioactive components such as magnolol may be of use and this particular research was aimed at finding the ameliorating effects of magnolol against the lead induced toxicity in the brain cells.

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