ORIGINAL ARTICLE

Biochemical and molecular evidences on the protection by magnesium oxide nanoparticles of chlorpyrifos-induced apoptosis in human lymphocytes

Vida Heydary[1,2,3,*], Mona Navaei-Nigjeh[1,2,4,*], Mahban Rahimifard[1,2], Azadeh Mohammadirad[1,2], Maryam Baeeri[1,2], Mohammad Abdollahi[1,2,3,4,5]

[1]Toxicology and Poisoning Research Center, [2]Pharmaceutical Sciences Research Center, [3]Islamic Azad University, Ahar Branch, [4]Department of Tissue Engineering, School of Advanced Technologies in Medicine, [5]Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

*These two authors contributed equally.

Background: Chlorpyrifos (CP) is one of the most widely used organophosphate (OP) insecticides in agricultural and residential pest control with its attendant adverse health effect. In the present study, it is proposed to investigate the possible modulatory role of magnesium oxide nanoparticles (MgO NPs) against CP-induced toxicity in human lymphocytes and determine the mechanisms lying behind this protection by viability and biochemical assays. **Materials and Methods:** Isolated lymphocytes were exposed to 12 μg/mL CP either alone or in combination with different concentrations of MgO NPs (0.1 μg/mL, 1 μg/mL, 10 μg/mL, and 100 μg/mL). After a 3-day incubation, the viability and oxidative stress markers including cellular mitochondrial activity, caspase-3 and -9 activities, total antioxidant power, lipid peroxidation, and myeloperoxidase (MPO) activity were measured. Also, the levels of tumor necrosis factor-α (TNF-α) as inflammatory index, along with acetylcholinesterase (AChE) activity were measured. Statistical differences were determined using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. **Results:** It is indicated that CP-exposed lymphocytes treated with MgO NPs resulted in a substantial reduction in the pace of mortality as well as the stages of oxidative stress in a dose-dependent manner. Also, MgO NPs (100 μg/mL) meaningfully restored CP-induced increase of TNF-α ($P < 0.001$) and decrease of AChE activity ($P < 0.001$) and were capable of preventing CP-treated human lymphocytes from apoptosis ($P < 0.001$). **Conclusion:** Our results demonstrate that MgO NPs in approximate 100 nm diameter not only make cells resistant to the toxic properties of CP but also attenuate toxic effects of CP, which is demonstrating the potential of MgO NPs to be applied in future immune deficiency therapeutic strategies.

Key words: Acetylcholinesterase (AChE), antiapoptotic, antioxidant, chlorpyrifos (CP), human lymphocytes, magnesium oxide nanoparticle (MgO NP), organophosphate (OP), oxidative stress

How to cite this article: Heydary V, Navaei-Nigjeh M, Rahimifard M, Mohammadirad A, Baeeri M, Abdollahi M. Biochemical and molecular evidences on the protection by magnesium oxide nanoparticles of chlorpyrifos-induced apoptosis in human lymphocytes. J Res Med Sci 2015;20:1021-31.

INTRODUCTION

Chlorpyrifos (CP) is one of the most widely used organophosphate (OP) insecticides in agricultural and residential pest control with its attendant adverse health effect. The primary toxicity of CP is attributed to the irreversible inhibition of the enzyme acetylcholinesterase (AChE).[1] Likewise, it has been postulated that CP has multiple effects on the cells including the production of reactive oxygen species (ROS) and initiation of intracellular oxidative stress, thereby disrupting normal cellular development and differentiation.[2] In summation, it has been reported that CP, like other OP compounds, causes immunological abnormalities in animals and man through lymphocytes and other

Access this article online	
Quick Response Code:	Website: www.jmsjournal.net
	DOI: ****

Address for correspondence: Prof. Mohammad Abdollahi, Department of Toxicology and Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran.
E-mail: Moahammad@TUMS.Ac.Ir

Received: 29-12-2015; **Revised:** 15-04-2015; **Accepted:** 09-11-2015

immunocompetent cells,[3] which in the event of chronic exposures result in the incidence of human diseases.[4,5] Consequently, since the role of CP is on the lift, especially in agriculture, the need to identify factors that have abated the adverse health consequence posed by long- and short-term exposures to the insecticide becomes worthy.

It is always a demand for creating and exploring novel materials for biological applications, particularly in the area of medicine owing to their immense impact on wellness maintenance. Nanomaterials offer promising opportunities for improved and tailored properties for applications in various fields due to their unique physicochemical characterization apparent at the nanoscale such as large surface area, surface reactivity, surface derivatization, and altered electronic properties.[6,7] Nanomaterials are of special interest not only for basic research but also for their interesting applications in various fields such as pharmaceutical cosmetics, drug delivery, additives to drugs, and food products.[8] In this respect, in the recent years, the efficacy of some antioxidant nanoparticles (NPs) such as nanomagnesium, nanocerium, nanoselenium, and nanoyttrium in disease models of colitis,[9] diabetes,[10] pancreatitis,[11-13] diabetic neuropathy,[14] cardiotoxicity,[15,16] and spermatotoxicity,[17] have been proven and it has been found that they are useful in reducing oxidative stress.

Magnesium oxide nanoparticle (MgO NP) is one of the metal oxide NPs, which is extensively used in several industries such as pharmaceuticals, cosmetics, food packaging, electrical insulation, desiccant, and medicine.[18] It has the advantage of nontoxic, high thermal stability, biocompatible, low cost, and has considerable potential as an antibacterial agent.[19] A number of studies have also been performed on MgO NPs and have evaluated different mechanistic endpoints. Stoimenov *et al*. (2002) reported that the nanoscale MgO powder showed a pronounced bacterial action against vegetative cells and spores of bacteria.[20] This antibacterial activity of MgO NPs was reported by other studies.[19,21] According to the findings of these studies, the toxicity of NPs toward microorganisms is due to either physical disruption or oxidative stress, which increased as the concentration of MgO increased.[19-21] In contrast, Ge *et al*. (2011) reported low concentration (below 200 μg/mL) of MgO. NPs not only have no cytotoxicity but also promote the human umbilical vein endothelial cells (HUVECs) proliferation.[22] Accordingly, their findings provide elementary cytocompatibility investigation about MgO NPs. This noncytotoxicity of MgO NPs in low concentration was confirmed by other researchers. Lai *et al*. (2008) showed that the treatment of U87 cells with MgO NPs for 48 h did not significantly reduce their survival until concentrations were higher than 50 μg/mL.[23] In addition, Hasbullah *et al*. (2013) indicated that MgO NPs have no toxicity on human neuroblastoma SH-SY5Y cell lines.[18] All these researches mentioned above indicated that the beneficial or detrimental biological effects of MgO NPs were dependent on particle composition, particle concentration, particle size, exposure time, and the nature of the biological target.

Given the above evidences, the present endeavor was undertaken to develop a cell culture model using lymphocytes and the immunocompetent cells of the body, and to evaluate compatibility of MgO NPs in low concentration. It is also aimed to investigate the possible modulatory role of MgO NPs against CP-induced oxidative stress and determine the mechanisms lying behind this protection by viability and biochemical assays.

MATERIALS AND METHODS

Chemicals

All the chemicals were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany) unless otherwise expressed. The MgO NPs suspension was purchased from NanoZino (Tehran, Tehran Province, Iran). The human-specific tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kit from Bender MedSystems (Vienna, Austria) and ApoFlowEx® fluorescein isothiocyanate (FITC) kit from Exbio (Vestec, Czech Republic) were obtained.

Characterization of magnesium oxide nanoparticle

Ultraviolet visible (UV-Vis) spectra of MgO NPs used in the experiment were recorded (apparatus UV-VIS CECIL BioAquarius 7250, Cambridge, England). The morphology of the NPs was determined using transmission electron microscopic (TEM) technique.

Lymphocyte isolation and care

The study was approved by the institute's review board with code number of 90-04-151-16052. Peripheral blood lymphocytes were isolated from heparinized venous blood, which was obtained from healthy volunteers who were nonsmokers and were not using medicines. Blood was mixed with Ficoll-Paque and centrifuged at 400 g for 30 min. The lymphocytes, from the interface of plasma and Ficoll-Paque, were picked up and washed twice with phosphate buffer, and were then counted based on the trypan blue exclusion method. After washing and weighing, the cells were seeded at a density of 3×10^6 cells/well in the tissue culture medium (RPMI-1640), which consisted of 10% FBS, 2 mM L-glutamine, 100 u/mL penicillin, and 100 μg/mL streptomycin sulfate, and 50 μl/ml LPS for cell growth stimulation. The lymphocyte cultures were grown in a humidified incubator with 5% CO_2 at 37°C in 96 microtiter plates.

Experimentation 1: Cytotoxic effect of magnesium oxide nanoparticles on human lymphocytes

Effect of magnesium oxide nanoparticles on human lymphocytes

Before undergoing the following test and seeing the effect of MgO NPs on the prevention of CP-induced toxicity, the human lymphocytes were exposed to various concentrations of MgO NPs. In a former study, Ge *et al*. (2011) have shown that low concentration (below 200 μg/mL) of MgO NPs showed no cytotoxicity by 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay on HUVECs *in vitro*.[22] Consequently, in our study human lymphocytes (3×10^6 cells/well) were incubated with the culture medium in combination with different concentrations of MgO NPs (0 μg/mL, 0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 200 μg/mL) for 72 h at 37°C and 5% CO_2 humidified atmosphere. After treatment, the lymphocytes were processed for cytotoxicity by MTT assay.

Lymphocyte viability assay

The assay is based on the reduction of MTT, a yellow tetrazole, to purple insoluble formazon by mitochondrial respiration in viable cells. MTT assay was performed on human lymphocytes cultured after 72 h incubation. Centrifugation was performed and the precipitated lymphocytes were washed twice by phosphate buffer. Then, 50 μl of MTT solution was added and it was reincubated for 4 h at 37°C and 5% CO_2 humidified atmosphere. At the end, 150 μl of DMSO solution was added and the absorbance was determined at 570 nm by ELISA reader. The viability of the treatment groups was expressed as the percentage of controls, which assumed 100%.

Experimentation 2: Protective effects of magnesium oxide nanoparticles against chlorpyrifos-induced toxicity

Treatment conditions and experimental groups

According to previous data,[24,25] we used 12 μg/mL as the concentration of CP that induced oxidative stress in the lymphocytes. In this regard, cell suspension (3×10^6 cells/well) was incubated with the culture medium in combination with 12 μg/mL CP for 72 h at 37°C and 5% CO_2 humidified atmosphere. For protective treatment, optimization of dose was performed by pretreating CP-induced cells with logarithmic concentrations (0.1 μg/mL, 1 μg/mL, 10 μg/mL, and 100 μg/mL) of MgO NPs for 72 h to ascertain the most efficacious dosage. To fulfill this purpose, all the cells were split into half a dozen groups of four per each interval ($n = 4$). Treatment conditions of experimental groups included:

(1) Con (negative control), lymphocytes in RPMI-1640 medium alone;
(2) CP, lymphocytes in RPMI-1640 medium + CP (12 μg/mL);
(3) MgO NPs I [lymphocytes in RPMI-1640 medium + CP (12 μg/mL) + MgO NPs (0.1 μg/mL)];
(4) MgO NPs II [lymphocytes in RPMI-1640 medium + CP (12 μg/mL) + MgO NPs (1 μg/mL)];
(5) MgO NPs III (lymphocytes in RPMI-1640 medium + CP (12 μg/mL) + MgO NPs (10 μg/mL));
(6) MgO NPs IV [lymphocytes in RPMI-1640 medium + CP (12 μg/mL) + MgO NPs (100 μg/mL)].

Then the lymphocytes were incubated at 37°C and 5% CO_2 humidified atmosphere. After a 72-h period, the cell suspensions in all groups were centrifuged. The supernatant solutions were removed for the biochemical assays and the deposited cells were used for viability assays in the succeeding measure.

Viability assays

Mitochondrial activity assay

We used this assay for investigation of the viability of treated lymphocytes as described in the previous section.

Caspase-3 and -9 activities assays

Caspase-3 and -9 activities were measured by colorimetric assays based on the identity of specific amino acid sequences by these caspases. The tetrapeptide substrates were labeled with the chromophore r-nitroaniline (ϱNA). ϱNA is released from the substrate upon cleavage by caspase and produces a yellow color that is monitored by an ELISA reader at 405 nm. The amount of caspase activity present in the sample is proportional to the quantity of yellow color produced upon cleavage.[26] Briefly, the pretreated lymphocytes were lyzed in the supplied lysis buffer and were incubated on ice for 10 min. The whole cell lysates were incubated in caspase buffer (100 mM HEPES, pH 7.4, 20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol) containing 100 mM of caspase-3 and -9-specific substrate [N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-ϱNA:), N-acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-ϱNA)] for 4 h at 37°C. Then, absorbance was measured at 405 nm. The caspase-3 and -9 activities of the treatment groups were expressed as the percentage of controls, which assumed 100%.

Determination of cell death (apoptosis vs necrosis)

To find out the mode of lymphocyte cell death induced by CP in the presence and absence of MgO NPs, the annexin V-FITC/propidium iodide (PI) staining was performed. The staining of annexin V-FITC and PI indicates the character of death caused by the test compound, i.e., apoptosis or necrosis. The cells (1×10^6) were treated with CP, alone or in combination with several concentrations of MgO NPs for 72 h, washed and stained with annexin V-FITC antibody and PI as per the instructions presented by the producer. The cells were scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels. The fraction of cell populations in

different quadrants was analyzed using quadrant statistics. Cells in the lower right quadrant represented apoptosis and those in the upper right quadrant represented necrosis or postapoptosis necrosis.[27]

Biochemical assays

Determination of total antioxidant power (TAP)

The method is based on the reduction of Fe^{3+} tripyridyltriazine (TPTZ) complex (colorless complex) to Fe^{2+} TPTZ (blue colored complex) formed by the action of the electron donating antioxidants at low pH. The ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM acetate buffer, 10 mL TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in the ratio of 10:1:1 at 37°C. Ten µl of H_2O diluted sample was then added to 300 mL freshly prepared reagent warmed at 37°C. An intense blue color complex was formed when Fe^{3+} TPTZ complex was reduced to Fe^{2+} form and the absorbance at 593 nm was recorded against a reagent blank after 30 min incubation at 37°C. Data were shown as mmol/µg protein.

Determination of lipid peroxidation (LPO)

To measure LPO, thiobarbituric acid reactive substances (TBARS) were evaluated. TBA reacts with lipid peroxides in the samples producing a measurable pink color that has an optical density at 532 nm read by the ELIZA reader as described in our previous study.[28] The activity was shown as mM.

Determination of myeloperoxidase activity

To assay myeloperoxidase (MPO) activity, we measured it spectrophotometrically as follows: 0.1 mL of supernatant was added to 2.9 mL of 50 mM phosphate buffer containing 0.167 mg/mL O-dianisidine hydrochloride and 0.0005% H_2O_2. The change in absorbance was recorded by spectrophotometer at 460 nm. MPO activity is set as the absorbance change per minute at 25°C in the final reaction.[29] The MPO activity was shown in unit/mL.

Determination of acetylcholinesterase

AChE activity in lymphocytes was assayed according to the modified Ellman method using acetylthiocholine iodide as the substrate and 5-5-bis dithionitrobenzoic acid (DTNB) as a coloring agent.[30] The action was expressed as unit/mg protein.

Determination of tumor necrosis factor-α

A human-specific ELISA kit (Bender MedSystem, Vienna, Austria) was used to quantify TNF-α in the supernatant of lymphocyte culture. To measure the amount of TNF-α, the absorbance of the sample was quantified at 450 nm as the primary wavelength and 620 names as the reference wavelength by the ELIZA reader as described in the kit brochure. Data were shown as ng/mL.

Protein assay

To see the total protein concentration of cells, Bradford reagent was added to dilute samples and the absorbance was measured by the spectrophotometer at 595 nm after 5 min. The bovine serum albumin was used as the criterion.

Statistical analysis of data

The data were first tested for normality (Shapiro-Wilk test) and as this assumption was met, one-way ANOVA and Dunnett's multiple comparison tests were held out by Stats-Direct 3.0.117 to determine the statistical differences while the degree of significance was placed at $P < 0.05$.[29] Data were presented as mean ± standard error of mean (SEM) of four different experiments, each read in duplicate.

RESULTS

Characterization of magnesium oxide nanoparticles

The optical properties of the MgO NPs were studied in particular by means of the UV-Vis absorption spectra in the wavelength range of 200-700 nm at room temperature. Figure 1 shows the UV-Vis absorption spectrum of the MgO NPs depicting the enhanced absorbance in the low ultraviolet (UV) region. The maximum absorption band of MgO NPs was found at 246.2 nm. The particle size was also examined using transmission electron microscopy (TEM). Figure 2 displays TEM micrographs of MgO NPs, revealing that the particle size is approximately 100 nm.

Lymphocytes viability in the presence of magnesium oxide nanoparticles

As shown in Figure 3, the MTT assay was applied for investigation of the viability of treated lymphocytes after 72 h of exposure. It illustrated that the mitochondrial activity of lymphocytes was about 100% when the MgO NPs suspension concentration was lower than 200 µg/mL. Thus, not only was the range of 0.1-200 µg/mL of MgO NPs safe but also at 100 µg/ml, the viability improved to 25% of the control ($P < 0.05$).

Protective effects of magnesium oxide nanoparticles against chlorpyrifos-induced toxicity with viability assays

Mitochondrial activity

The results of MTT assay on the cultured lymphocytes post 72 h of different treatments are shown in Table 1. There is a significant decrease in the viability of the CP group as compared with the control ($P < 0.01$). The groups, which were pretreated with MgO NPs at doses 1 µg/ml, 10 µg/ml, and 100 µg/ml remained more viable in comparison with CP ($P < 0.05$) but did not prove any different viability when compared with the control. No significant improvement in the lymphocyte viability was observed when the cells

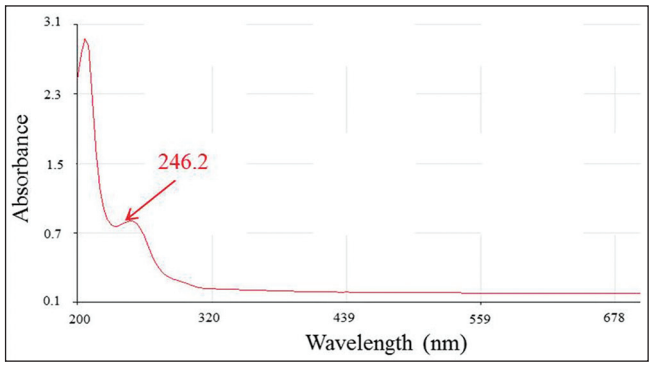

Figure 1: UV-Vis spectra of MgO NPs used in this study

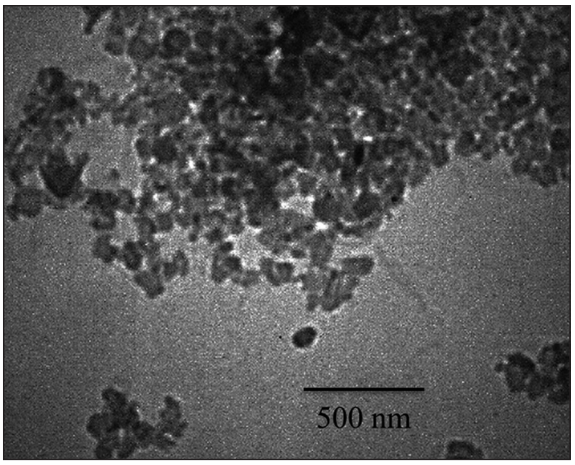

Figure 2: TEM micrograph of MgO NPs

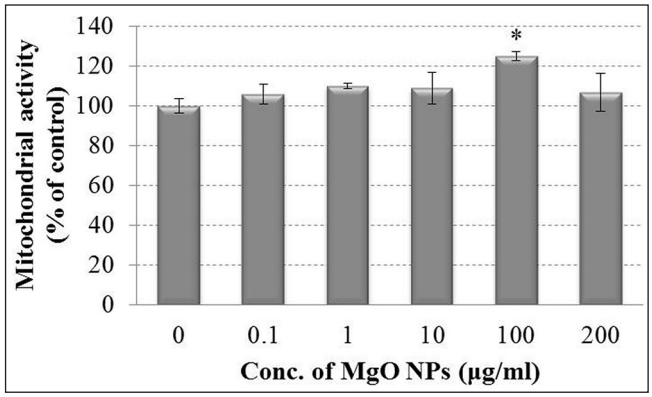

Figure 3: Effects of various concentrations of MgO NPs in the mitochondrial activity of isolated human lymphocytes; Data are expressed as mean ± SEM; Significantly different from control at $^{*}P < 0.05$

pretreated with the group of dose 0.1 µg/mL MgO NPs as compared with CP.

Caspase-3 and -9 activities

As shown in Table 1, CP-induced apoptosis significantly increased the activities of caspase-3 and-9 when compared with the controls ($P < 0.05$ and $P < 0.001$, respectively), whereas pretreatment with different concentrations of MgO NPs in these conditions reduced the activities of caspase-3 and 9 in a dose-dependent fashion. Nevertheless, no obvious change in the activities of caspase-3 and -9 was caused by MgO NPs at 0.1 µg/mL when compared with CP after 3 days but the highest concentration caused a significant ($P < 0.001$) decrease in caspase-3 and -9 activities in this condition.

Lymphocyte death (apoptosis vs necrosis)

Figure 4 represents typical flow cytometry plots of annexin V and PI staining of different treatments of lymphocyte. After 72 h incubation with CP, there was an increase in the number of lymphocytes that were apoptotic as indicated by an increase in the annexin V-positive and PI-negative cell population as well as an increase in the number of lymphocytes that were necrotic or in the later stages of apoptosis as indicated by the increase in the annexin V-positive and PI-positive cell population as compared with the control group (% of apoptotic cells: 13 vs 2, % of necrotic cells: 27 vs 3). In addition, the treated cells with MgO NPs at dose of 0.1 µg/mL in comparison with CP-treated cells showed no obvious changes in apoptotic and necrotic cell populations (% of apoptotic cells: 13 vs 13, % of necrotic cells: 23 vs 27). In contrast, the groups, which were pretreated with MgO NPs at doses of 1 µg/mL, 10 µg/mL, and 100 µg/mL remained more viable and the number of apoptotic and necrotic cells decreased as compared with the CP (% of apoptotic cells: 9, 5, and 3 vs 13, % of necrotic cells: 6, 3, and 3 vs 23). Interestingly, there were no significant differences in the viable cells in comparison with the control group.

Protection effects of magnesium oxide nanoparticles against chlorpyrifos-induced toxicity with biochemical assays

Total antioxidant power

As shown in Figure 5a, there is a decrease in control TAP by administration of CP. In contrast, the groups,

Table 1: Effects of various concentrations of magnesium oxide nanoparticles (MgO NPs) on mitochondrial and caspase-3 and -9 activities of isolated human lymphocytes in the presence of chlorpyrifos (CP)

Experimental groups	Control Mean ± SEM	CP Mean ± SEM□	CP + MgO NPs I Mean ± SEM	CP + MgO NPs II Mean ± SEM	CP + MgO NPs III Mean ± SEM	CP + MgO NPs IV Mean ± SEM
Mitochondrial activity (% of control)	100±2.3	73±5**	90±5.2	96±2.6$^{\#}$	99±1$^{\#}$	95±8.4$^{\#}$
Caspase-3 activity (% of the control)	100±1	117±2.9*	114±4	106±0.3	97±3.7$^{\#}$	85±6.7$^{*,\#\#\#}$
Caspase-9 activity (% of the control)	100±2.4	112±0.77***	110±0.26***	104±1.53$^{\#\#\#}$	102±0.07$^{\#\#\#}$	99±1.28$^{\#\#\#}$

Significantly different from the control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Significantly different from CP at $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, □SEM: Standard error of mean

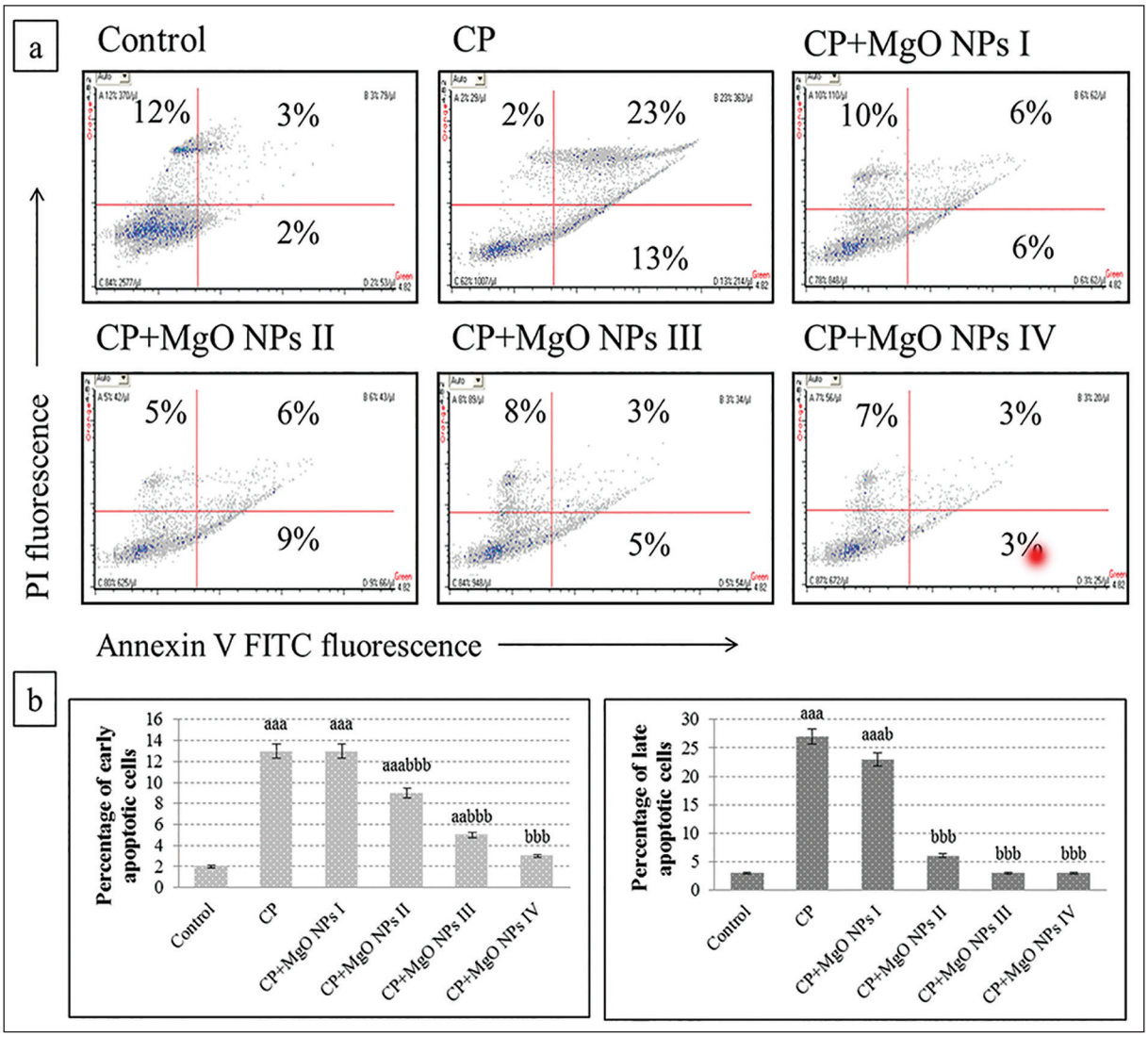

Figure 4: Flow cytometric analyses of apoptosis and necrosis in human lymphocytes induced by CP, alone, or in combination with various concentrations of MgO NPs using Annexin V-FITC and PI double staining; Human lymphocytes (1 × 10^6/200 µl) were incubated with indicated concentrations of MgO NPs and CP for 72 h and stained with annexin V-FITC/PI as described previously. Quadrant analysis of fluorescence intensity of nongated cells in FL1 (annexin V) vs. FL2 (PI) channels was from 5,000 events. The values shown in the lower left, lower right, upper left, and upper right quadrants of each panel represent the percentage of viable, apoptotic, necrotic, and late apoptotic (postapoptotic necrotic) cells, respectively. The percentages of positive cells were indicated in each panel

which were pretreated with MgO NPs at doses of 0.1 µg/mL, 1 µg/mL, 10 µg/mL, and 100 µg/mL, showed an apparent increase in TAP when compared with the CP group in a dose-dependent manner ($P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively), which was also significant in comparison with the control group at doses of 10 µg/mL and 100 µg/mL ($P < 0.05$ and $P < 0.001$, respectively).

Lipid peroxidation

There was a significant elevation in LPO ($P < 0.01$) in CP compared with the control group [shown in Figure 5b]. In addition, the groups which were pretreated with low concentrations of MgO NPs (0.1, 1 and 10 µg/mL) showed no significant reduction in LPO as compared with the CP group and LPO levels of these groups still higher than control group ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively). In contrast, high concentration of MgO NPs (100 µg/mL) could decrease LPO as compared with CP, which was significant at dose of 100 µg/mL ($P < 0.01$).

Myeloperoxidase activity

As depicted in Figure 5c, MPO activity considerably increased in the CP group as compared with the control group ($P < 0.05$). No significant improvement in the lymphocyte MPO activity was observed when the cells were pretreated with MgO NPs at dose 0.1 µg/mL in comparison with CP. However, treatment with the other concentrations of MgO NPs (1 µg/mL, 10 µg/mL, and 100 µg/mL) decreased MPO activity in a dose-dependent manner. Among these concentrations, 100 µg/mL MgO NPs showed significant effects in comparison

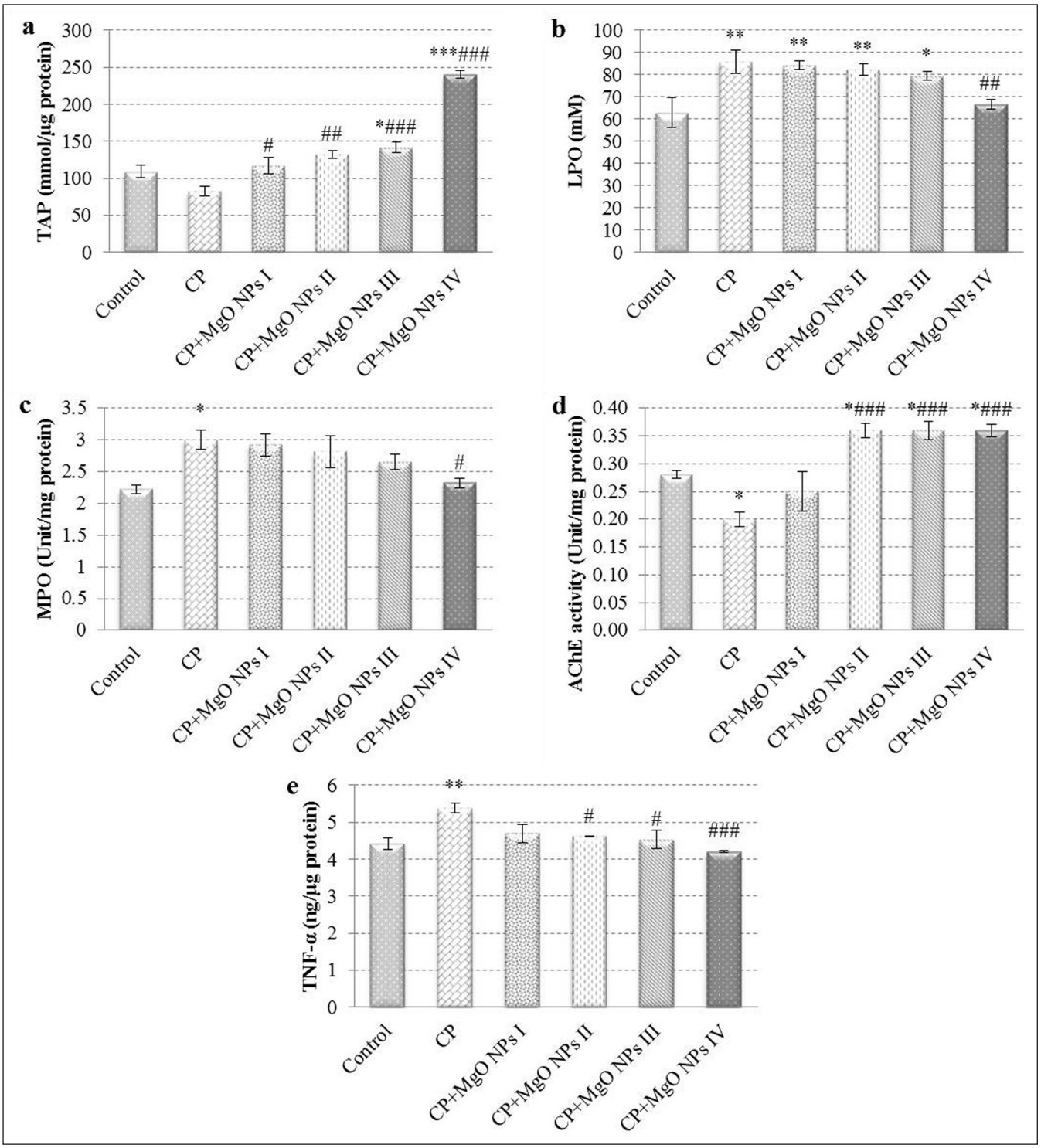

Figure 5: Effects of various concentrations of MgO NPs in TAP values (a), LPO levels (b), MPO activity (c), AChE activity (d), and TNF-α release (e) of isolated human lymphocytes in the presence of CP; Data are expressed as mean ± SEM ; Significantly different from control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Significantly different from CP at $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$

with CP ($P < 0.05$) and reached close to the control values.

Acetylcholinesterase activity

As shown in Figure 5d, AChE activity in the CP group was significantly lower compared with the control group ($P < 0.05$). The groups, which were pretreated with MgO NPs at doses of 1 µg/mL, 10 µg/mL, and 100 µg/mL, showed an apparent increase in AChE activity as compared with the control and CP groups ($P < 0.05$ and $P < 0.001$, respectively). But the group of dose 0.1 µg/mL, MgO NPs showed no difference in AChE activity as compared with CP.

Tumor necrosis factor-α release

As seen in Figure 5e, TNF-α production significantly elevated in the CP group when compared with the control group ($P < 0.01$). Conversely, administration of MgO NPs in different concentrations decreased TNF-α release in dose-dependent manner as compared with CP, which was significant at doses 1 μg/mL, 10 μg/mL, and 100 μg/mL ($P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively).

DISCUSSION

Previous works, either *in vivo* or *in vitro*, have shown that NPs have a wide range of effects on humans. As we know, size, condition, and surface area of NPs,[31] duration of the exposure time[32] and a compactness of them[33] are important in rendering them as toxic property or safety feature. About MgO NPs, in that respect are some articles, which have indicated toxic properties[22,34] and also other articles, which have exhibited the positive effects of MgO NPs on different organisms by changing the concentration and size of MgO NPs.[18,23,35] Hence, in this work we prospected increasing maintenance of the human lymphocytes by testing different concentrations of MgO NPs.

Before beginning the task, MgO NPs were investigated by spectroscopic methods for determining the peak concentration. As shown in the Figure 1, peaking absorption of them was approximately 250 nm, which was confirmed range of pure MgO NPs by another study.[36] After that, since the cytotoxic effect of MgO NPs on human lymphocytes *in vitro* has not been read yet, their toxicity investigated precisely. As shown in the Figure 3, the viability of lymphocytes in exposure to MgO NPs with a scope of 0.1–200 μg/mL for 72 h, based on MTT assay was tested and it showed that MgO NPs are safe and can even increase the viability of the cells. There is a study indicating that concentrations up to 200 μg/mL of MgO NPs have no toxicity on Human umbilical vein endothelial cell (HUVE) cells.[22] The study in which an exposure time of MgO NPs was similar to our approach (72 h) showed similar results with the MTT assay.

Regarding such a straightforward response from the viability test of human lymphocytes, the efficacy of MgO NPs was tested against CP-induced toxicity on lymphocytes.[37,38] Interestingly, in the first portion of the study, all results that are channeled out for viability tests of lymphocytes, confirmed each other and showed that MgO NPs could lessen the harmful effects of CP in human lymphocytes. As determined in Table 1, CP significantly decreased the viability of the lymphocytes ($P < 0.01$) and MgO NPs compensated for that reduction effect. A previous report has indicated that CP has effects on mortality of the cells such as neurons[39] by different mechanisms such as cell signaling and starting cascades, and also changing gene transcription factors, which are activated by reactive oxygen species (ROS).[40] Toxic effect of CP is supported by results of flow cytometry in the present study. As shown in the Figure 4, in the control group, which was not exhibited to any dose of MgO NPs, lymphocytes were distributed mostly in quarter C (83%) and the rate of apoptotic and necrotic cells was really depressed. As we expected, the rates of early and late apoptosis increased significantly by CP (up to 13% and 27%). In contrast, these rates decreased upon treatment with MgO NPs. The best result in the flow cytometric assay was got by a dose of 100 μg/mL MgO NPs, which decreased the percentages of early and late stages of apoptotic cells down to 3% (from 13% and 27% of the CP group). A previous study has reported that magnesium sulfate could decrease apoptosis in nerve cells after cerebral ischemia-reperfusion injury, perhaps via decreasing expression of Bax and caspase-3.[41] In general, there are two mechanisms for justifying the apoptosis; one of the mechanisms for declined apoptotic cells is linked to suppression of caspase enzyme activity,[42] which is depicted in this study by measuring caspase-3 and -9 activities. Given the effects of caspase in Figure 4, we learned that MgO NPs can alleviate caspase activity in CP-induced human lymphocytes. In both caspase-3 and -9, concentration of 100 μg/mL MgO NPs more than other concentrations made a reduction in enzyme activity and was the best effective dose, which correlated well with the flow cytometry results. The other mechanism, which justifies apoptosis and causes internal signal in apoptosis, relates to increasing levels of cytochrome C and apoptosis-inducing factor (AIF) by depressing the mitochondrial function.[43] So because of the positive effects of MgO NPs on the viability of mitochondria, such as decrease in apoptosis, is not surprising.

In the second section of our study, oxidative stress factors were assessed. As shown in the Figure 5, MgO NPs were in a concentration of 100 μg/mL in comparison with the CP group's alleviated levels of LPO and MPO ($P < 0.05$). Also, the level of TAP, which was decreased by CP upon treatment with MgO NPs increased significantly ($P < 0.001$). A previous study has shown that CP makes a significant increase in LPO and thus, induces cytotoxicity and apoptosis in the follicular cells.[44] Also, MPO produced a more toxic oxon group by replacing sulfur atom with oxygen in CP; thus, it made more cytotoxicity.[45] Other studies have reported that the metal form of Mg reduces LPO level in the plasma of the rat. The researchers concluded that Mg had an effect on glutathione peroxidase enzyme, which has a central role in LPO.[46] As indicated in another study, Mg in metal form has neuroprotective effect and reduces LPO.[47] Thus, nanoscale and oxide forms of magnesium may have similar properties to the metal form. The present study also showed a substantial elevation in the degree of AChE by MgO NPs compared with the CP group ($P < 0.001$). It was reported that CP in different tissues such as

the kidney, liver, spleen, and brain caused inhibition of AChE activity.[48] Likewise, another study on the blood samples of workers who were in contact with OP pesticide, recommended that measuring AChE activity could be useful for detecting OP poisoning.[49] Because of the freshness of this approach, this is the first time that MgO NPs has been tried out at the level of AChE in lymphocytes but as we know, the reduction of AChE activity is accompanied by oxidative stress;[50,51] so increasing of AChE by MgO NPs has a good correlation with the increasing of TAP level.

At the end, due to the important role of lymphocytes in the immune system, the level of TNF-α was investigated. The cytokine TNF-α is one of the best indexes inflammatory processes, which is discharged because of immunologic responses from mononuclear cells in human peripheral blood. TNF-α stimulates production of ROS by inhibiting the action of NF-kB, and by creating oxidative stress resulting in cellular lipid and protein peroxidation, and apoptosis.[52] As shown in Figure 5d, the concentration of 100 µg/mL MgO NPs, which was the best dose in decreasing oxidative stress and apoptosis, lessened the CP-induced increase in TNF-α. This confirms that it can markedly protect lymphocytes against CP toxicity.

Taken together, the present results from MgO NPs on human lymphocytes in terms of increasing the viability and decreasing apoptosis, oxidative stress, and also inflammation show the potential of MgO NPs to be applied in future immune deficiency therapeutic strategies.

CONCLUSION

By this study, the toxic effects of CP as an OP compound, on human lymphocytes was investigated and it was proved that it could increase caspase-3 and-9 activities, which lead to increased apoptosis and decreased viability of the cells. Likewise, it can decrease the antioxidant capacity and make inflammation by increasing TNF-α. Interestingly, our results together with the previous studies, demonstrate that MgO NPs in approximately 100-nm diameter not only make cells resistant to the toxic properties of CP but also attenuate bad effects of CP, which is reported for the first time. Accordingly, the use of MgO NPs in low concentration (100 µg/mL) can play antiapoptotic, antioxidative, and anti-inflammatory roles against toxic properties caused by CP in human lymphocytes.

Acknowledgments

The authors thank the Toxicology and Poisoning Research Center and the Tehran University of Medical Sciences for financial support of the study as a part of a major project.

Financial support and sponsorship

Authors wish the support of Iran National Science Foundation.

Conflicts of interest

There are no conflicts of interest.

AUTHOR'S CONTRIBUTIONS

VH provided assistance in the design of the study, performed some experiments, and participated in manuscript preparation. MNN contributed in the conception and design of the work, provided assistance for all experiments, and participated in manuscript preparation. MR and AM participated in the design and manuscript preparation. MB provided assistance for all experiments. MA conceived the whole project. All the authors have read and approved the content of the manuscript.

REFERENCES

1. Moser VC. Dose-response and time-course of neurobehavioral changes following oral chlorpyrifos in rats of different ages. Neurotoxicol Teratol 2000;22:713-23.
2. Bebe FN, Panemangalore M. Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissues of rats. J Environ Sci Health B 2003;38:349-63.
3. Corsini E, Liesivuori J, Vergieva T, Van Loveren H, Colosio C. Effects of pesticide exposure on the human immune system. Hum Exp Toxicol 2008;27:671-80.
4. Mostafalou S, Abdollahi M. Pesticides and human chronic diseases: Evidences, mechanisms, and perspectives. Toxicol Appl Pharmacol 2013;268:157-77.
5. Abdollahi M, Karami-Mohajeri S. A comprehensive review on experimental and clinical findings in intermediate syndrome caused by organophosphate poisoning. Toxicol Appl Pharmacol 2010;258:309-14.
6. Pourmand A, Pourmand MR, Wang J, Shesser R. Application of nanomedicines in emergency medicine; Point-of-care testing and drug delivery in twenty - first century. Daru 2012;20:26.
7. Gwinn MR, Vallyathan V. Nanoparticles: Health effects — pros and cons. Environ Health Perspect 2006;114:1818-25.
8. Pourmand A, Abdollahi M. Current opinion on nanotoxicology. Daru 2012;20:95.
9. Saadatzadeh A, Atyabi F, Fazeli MR, Dinarvand R, Jamalifar H, Abdolghaffari AH, *et al*. Biochemical and pathological evidences on the benefit of a new biodegradable nanoparticles of probiotic extract in murine colitis. Fundam Clin Pharmacol 2012;26:589-98.
10. Navaei-Nigjeh M, Rahimifard M, Pourkhalili N, Nili-Ahmadabadi A, Pakzad M, Baeeri M, *et al*. Multi-organ protective effects of cerium oxide nanoparticle/selenium in diabetic rats: Evidence for more efficiency of nanocerium in comparison to metal form of cerium. Asian J Anim Vet Adv 2012;7:605-12.
11. Pourkhalili N, Hosseini A, Nili-AhmadabadiA, Rahimifard M, Navaei-Nigjeh M, Hassani S, *et al*. Improvement of isolated rat pancreatic islets function by combination of cerium oxide nanoparticles/sodium selenite through reduction of oxidative stress. Toxicol Mech Meth 2012;22:476-82.
12. Hosseini A, Abdollahi M. Through a mechanism-based approach, nanoparticles of cerium and yttrium may improve the outcome of pancreatic islet isolation. J Med Hypotheses Ideas 2012;6:4-6.

13. Pourkhalili N, Hosseini A, Nili-Ahmadabadi A, Hassani S, Pakzad M, Baeeri M, *et al*. Biochemical and cellular evidence of the benefit of a combination of cerium oxide nanoparticles and selenium to diabetic rats. World J Diabetes 2011;2:204-10.
14. Hosseini A, Abdollahi M, Hassanzadeh G, Rezayat M, Hassani S, Pourkhalili N, *et al*. Protective effect of magnesium-25 carrying porphyrin-fullerene nanoparticles on degeneration of dorsal root ganglion neurons and motor function in experimental diabetic neuropathy. Basic Clin Pharmacol Toxicol 2011;109:381-6.
15. Mohammadi H, Karimi G, Seyed Mahdi Rezayat, Ahmad Reza Dehpour, Shafiee H, Nikfar S, *et al*. Benefit of nanocarrier of magnetic magnesium in rat malathion-induced toxicity and cardiac failure using non-invasive monitoring of electrocardiogram and blood pressure. Toxicol Ind Health 2011;27:417-29.
16. Shafiee H, Mohammadi H, Rezayat SM, Hosseini A, Baeeri M, Hassani S, *et al*. Prevention of malathion-induced depletion of cardiac cells mitochondrial energy and free radical damage by a magnetic magnesium-carrying nanoparticle. Toxicol Mech Meth 2010;20:538-43.
17. Rezvanfar MA, Rezvanfar MA, Shahverdi AR, Ahmadi A, Baeeri M, Mohammadirad A, *et al*. Protection of cisplatin-induced spermatotoxicity, DNA damage and chromatin abnormality by selenium nano-particles. Toxicol Appl Pharmacol 2013;266:356-65.
18. Hasbullah NI, Mazatulikhma MZ, Kamarulzaman N. Nanotoxicity of magnesium oxide on human neuroblastoma SH-SY5Y cell lines. Adv Mat Res 2013;667:160-4.
19. Krishnamoorthy K, Manivannan G, Kim SJ, Jeyasubramanian K, Premanathan M. Antibacterial activity of MgO nanoparticles based on lipid peroxidation by oxygen vacancy. J Nanopart Res 2012;14:1-10.
20. Stoimenov PK, Klinger RL, Marchin GL, Klabunde KJ. Metal oxide nanoparticles as bactericidal agents. Langmuir 2002;18:6679-86.
21. Jin T, He Y. Antibacterial activities of magnesium oxide (MgO) nanoparticles against foodborne pathogens. J Nanopart Res 2011;13:6877-85.
22. Ge S, Wang G, Shen Y, Zhang Q, Jia D, Wang H, *et al*. Cytotoxic effects of MgO nanoparticles onhuman umbilical vein endothelial cells *in vitro*. IET Nanotechnol 2011;5:36.
23. Lai JC, Lai MB, Jandhyam S, Dukhande VV, Bhushan A, Daniels CK, *et al*. Exposure to titaniumdioxide and other metallic oxide nanoparticles induces cytotoxicityon human neural cells and fibroblasts. Int J Nanomedicine 2008;3:533-45.
24. Shankarjit S, Aruna B. Biotherapeutic potential of Ziziphusmauritiana (Lamk.) extract against chlorpyrifos induced oxidative stress (an *in-vitro* study). Biochem Mol Biol 2013;1:58-62.
25. Caughlan A, Newhouse K, Namgung U, Xia Z. Chlorpyrifos induces apoptosis in rat cortical neurons that is regulated by a balance between p38 and ERK/JNK MAP kinases. Toxicol Sci 2004;78:125-34.
26. Kim JY, Lim DM, Moon CI, Jo KJ, Lee SK, Baik HW, *et al*. Exendin-4 protects oxidative stress-induced b-cell apoptosis through reduced JNK and GSK3b activity. J Korean Med Sci 2010;25:1626-32.
27. Kumar A, Malik F, Bhushan S, Sethi VK, Shahi AK, Kaur J, *et al*. An essential oil and its major constituent isointermedeol induce apoptosis by increased expression of mitochondrial cytochrome c and apical death receptors in human leukaemiaHL-60 cells. Chem Biol Interact 2008;171:332-47.
28. Astaneie F, Afshari M, Mojtahedi A, Mostafalou S, Zamani MJ, Larijani B, *et al*. Total antioxidant capacity and levels of epidermal growth factor and nitric oxide in blood and saliva of insulin-dependent diabetic patients. Arch Med Res 2005;36:376-81.
29. Navaei-Nigjeh M, Asadi H, Baeeri M, Pedram S, Rezvanfar MA, Mohammadirad A, *et al*. *In vitro* protection of human lymphocytes from toxic effects of chlorpyrifos by selenium-enriched medicines. Iran J Basic Med Sci 2015;18:284-91.
30. Ellman GL, Courtney KD, Anders V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
31. Arora S, Rajwade JM, Paknikar KM. Nanotoxicology and *in vitro* studies: The need of the hour. Toxicol Appl Pharmacol 2012;258:151-65.
32. Fukui H, Horie M, Endoh S, Kato H, Fujita K, Nishio K, *et al*. Association of zinc ion release and oxidative stress induced by intratracheal instillation of ZnO nanoparticles to rat lung. Chem Biol Interact 2012;198:29-37.
33. Chalkidou A, Simeonidis K, Angelakeris M, Samaras T, Martinez-Boubeta C, Balcells L, *et al*. *In vitro* application of Fe/MgO nanoparticles as magnetically mediated hyperthermia agents for cancer treatment. J Magn Magn Mater 2011;323:775-80.
34. Jebali A, Hekmatimoghaddam S, Kazemi B, Allaveisie A, Masoudi A, Daliri K, *et al*. Lectin coated MgO nanoparticle: Its toxicity, antileishmanial activity, and macrophage activation. Drug Chem Toxicol 2014;37:400-9.
35. Krishnamoorthy K, Moon JY, Hyun HB, Cho SK, Kim SJ. Mechanistic investigation on the toxicity of MgO nanoparticles toward cancer cells. J Mater Chem 2012;22:24610-7.
36. Sadeghi M, Hosseini M. Nucleophilic chemistry of the synthesized magnesium oxide (magnesia) nanoparticles via microwave@sol-gel process for removal of sulfurous pollutant. Int J Bio-Inorg Hybd Nanomat 2012;1:175-82.
37. Das GP, Shaik AP, Jamil K. Estimation of apoptosis and necrosis caused by pesticides in vitro on human lymphocytes using DNA diffusion assay. Drug Chem Toxicol 2006;29:147-56.
38. Li Q, Kobayashi M, Kawada T. Chlorpyrifos induces apoptosis in human T cells. Toxicology 2009;255:53-7.
39. Karami-Mohajeri S, Nikfar S, Abdollahi M. A systematic review on the nerve-muscle electrophysiology in human organophosphorus pesticide exposure. Hum Exp Toxicol 2014;33:92-102.
40. Elelaimy IA, Ibrahim HM, Abdel Ghaffar FR, Alawthan YS. Evaluation of sub-chronic chlorpyrifos poisoning on immunological and biochemical changes in rats and protective effect of eugenol. J Appl Pharm Sci 2012;2:51-61.
41. Zhou H, Ma Y, Zhou Y, Liu Z, Wang K, Chen G. Effects of magnesium sulfate on neuron apoptosis and expression of caspase-3, bax and bcl-2 after cerebral ischemia-reperfusion injury. Chin Med J (Engl) 2003;116:1532-4.
42. Leist M, Jäättelä M. Four deaths and a funeral: From caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2001;2:589-98.
43. Kaufmann SH, Hengartner MO. Programmed cell death: Alive and well in the new millennium. Trends Cell Biol 2011;11:526-34.
44. Shady AM, Noor El-Deen FI. Effect of chlorpyrifos on thyroid gland of adult male albino rats. Egypt J Histol 2010;33:441-50.
45. Lazarević-Pašti T, Nastasijević B, Vasić V. Oxidation of chlorpyrifos, azinphos-methyl and phorate by myeloperoxidase. Pestic Biochem Physiol 2011;101:220-6.
46. Buha A, Bulat Z, Dukić-Ćosić D, Matović V. Effects of oral and intraperitoneal magnesium treatment against cadmium-induced oxidative stress in plasma of rats. Arh Hig Rada Toksikol 2012;63:247-54.
47. Süzer T, Coskun E, Islekel H, Tahta K. Neuroprotective effect of magnesium on lipid peroxidation and axonal function after experimental spinal cord injury. Spinal Cord 1999;37:480-4.
48. Ojha A, Yaduvanshi SK, Srivastava N. Effect of combined exposure of commonly used organophosphate pesticides on

lipid peroxidation and antioxidant enzymes in rat tissues. Pestic Biochem Physiol 2011;99:148-56.
49. Yu F, Wang Z, Ju B, Wang Y, Wang J, Bai D. Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina *in vivo via* oxidative stress and protection of combination of vitamins C and E. Exp Toxicol Pathol 2008;59:415-23.
50. Ranjbar A, Pasalar P, Abdollahi M. Induction of oxidative stress and acetylcholinesterase inhibition in organophosphorous pesticide manufacturing workers. Hum Exp Toxicol 2002;21:179-82.
51. Teimouri F, Amirkabirian N, Esmaily H, Mohammadirad A, Aliahmadi A, Abdollahi M. Alteration of hepatic cells glucose metabolism as a non-cholinergic detoxication mechanism in counteracting diazinon-induced oxidative stress. Hum Exp Toxicol 2006;25:697-703.
52. Djavaheri-Mergny M, Javelaud D, Wietzerbin J, Besançon F. NF-kappaB activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNFα-treated Ewing sarcoma cells. FEBS Lett 2004;578:111-5.