Original Article

On The Protection by The Combination of CeO_2 Nanoparticles and Sodium Selenite on Human Lymphocytes against Chlorpyrifos-Induced Apoptosis *In Vitro*

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Abstract

Objective: Chlorpyrifos (CP) as an organophosphorus pesticide is thought to induce oxidative stress in human cells via producing reactive oxygen species (ROS) that leads to the presence of pathologic conditions due to apoptosis along with acetylcholinesterase (AChE) inhibition.This study aimed to evaluate the apoptotic effects of CP and to assess the protective potential of CeO_2 nanoparticle (CNP) and sodium selenite (SSe) by measuring cascades of apoptosis, oxidative stress, inflammation, and AChE inhibition in human isolated lymphocytes.

Materials and Methods: In the present experimental study, we examined the anti-oxidative and AChE activating potential of CNP and SSe in CP-treated human lymphocytes. Therefore, the lymphocytes were isolated and exposed to CP, CP+CNP, CP+SSe, and CP+CNP+SSe after a three-day incubation. Then tumor necrosis factor-alpha (TNF-α) release, myeloperoxidase (MPO) activity, thiobarbituric acid-reactive substances (TBARS) levels as inflammatory/oxidative stress indices along with AChE activity were assessed. In addition, the apoptotic process was measured by flow cytometry.

Results: Results showed a significant reduction in the mortality rate, TNF-α, MPO activity, TBARS, and apoptosis rate in cells treated with CNP, SSe and their combination. Interestingly, both CNP and SSe were able to activate AChE which is inhibited by CP. The results supported the synergistic effect of CNP/SSe combination in the prevention of apoptosis along with oxidative stress and inflammatory cascade.

Conclusion: CP induces apoptosis in isolated human lymphocytes via oxidative stress and inflammatory mediators. CP firstly produces ROS, which leads to membrane phospholipid damage. The beneficial effects of CNP and SSe in reduction of CP-induced apoptosis and restoring AChE inhibition relate to their anti-oxidative potentials.

Keywords: Organophosphorus, Chlorpyrifos, Lymphocytes, Cerium Oxide Nanoparticles, Sodium Selenite

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Introduction

Pesticides are able to modify immune responses mediated through lymphocytes as found in experimental animals and human subjects poisoned (1). The activation of lymphocytes is a prerequisite for many immunological responses (2). During the last 20 years, several experimental evidences have shown that organophosphorus (OP) compounds can interfere with the immune system and possess immunotoxic effects in the laboratory animal through the lymphocytes and other immunocompetent cells (3), that in case of chronic exposure results in incidence of human diseases (4, 5).

Chlorpyrifos (CP) [0, 0- diethyl 0-(3, 5, 6-tricloro-2- pyridinol) phosphorothionate] is a broad spectrum chlorinated OP insecticide that is now extensively used in the agricultural and residential pest control around the world (6).

As reviewed recently, OPs act through oxidative stress mechanisms (7, 8) and provide major toxicity when the organism is exposed to these compounds for a long time. Unfortunately, excessive use of pesticides in the agriculture by irresponsible persons in the line of higher production has caused the problem of entrance of pesticides into the human food cycle.

Recent studies have indicated that substances with the ability to reduce oxidative stress (9-12) or those having adenosine triphosphate (ATP) donor potentials (13) through mitochondrial mechanisms (14) can reduce toxicity of OPs. Some of antioxidant nanoparticles have been found useful in this respect and are under study of some research groups like the authors of this paper. Therefore, considering the oxidative stress mechanisms of OPs, some nanoparticles such as nanomagnesium (15), nanocerium (16), and nanoselenium (17) have been examined recently. For instance, in the recent years, the efficacy of antioxidant nanoparticles in disease models of colitis (18), diabetes (19), pancreatitis (20, 21), diabetic neuropathy (15), and cardiotoxicity (22, 23), have been proved.

One of these miracle nanoparticles is cerium oxide (CeO_2, CNP) that is thought to markedly increase the antioxidant power of exposed organs or cells via its major free radical scavenging potential (20).

On the other hand, CNP is able to act like superoxide dismutase (SOD) as a free radical detoxifying system (24). Besides the antioxidant effect, CNP can remain active in the living cells for an extended period of time. Selenium as sodium selenite (SSe) is an essential trace element which possesses a critical role in some protective enzymes against free radicals (25). It also inhibits the adhesive molecules induced by tumor necrosis factor-alpha (TNF-α) and deactivates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (26). In addition, SSe has been found beneficial in the rats exposed to CP by restoring the oxidative injury (27). SSe in nano or usual form or in combination with other compounds has been found a strong reducer of oxidative stress (10, 25, 27).

Given above evidences, the aim of this study was to evaluate the apoptotic effects of CP and to assess the protective potential of CNP and SSe by measuring cascades of apoptosis, oxidative stress, inflammation, and acetylcholinesterase (AChE) inhibition in human isolated lymphocytes.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemie (Germany), whereas CNP was purchased from Navarrean Nanoproducte Technology (Spain), TNF-α ELISA kit was purchased from BenderMed Systems (Austria) and ApoFlowEx® FITC Kit was purchased from Exbio (Czech Republic).

Lymphocyte isolation and culture

This experimental study was approved by the Institutional Review Board of Tehran University of Medical Sciences with code number of 90-04-151-16052 and all ethical considerations were adhered. Peripheral blood lymphocytes were isolated from heparinized venous blood, which obtained from 10 healthy male volunteers aged between 20-30 years old who were nonsmoker and taking no medications, after obtaining an informed consent from all participants. Blood was mixed with Ficoll-Paque and centrifuged at 400 g for 30 minutes. The lymphocytes were collected from the interface of plasma and Ficoll-Paque, washed three times with phosphate buffered saline (PBS), and then were counted based on the trypan blue exclusion method. After washing and counting, the cells (viability>98%) were cultured (10^5 cell/ml), in RPMI-1640 consisting of 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate that was followed by addition of 50 µl/ml lipopolysaccharide (LPS) for cell growth stimulation. Cell cultures were grown in 96-well microtiter plates and maintained at 37˚C with 5% CO_2 humidified atmosphere for 72 hours.

Chlorpyrifos, CeO_2 nanoparticle and sodium selenite dose optimization

Before performing the following tests, we determined the cytotoxicity as inhibition concentration (IC50) of CP and the effective doses (ED50) of CNP and SSe in the prevention of CP-induced oxidative stress. It was already shown that CP induces oxidative stress in human erythrocytes [red blood cells (RBCs)] at the average dose of 10 µg/ml (6). In this regard, the cell suspension was incubated with culture medium in combination with 0, 12.5, 25 and 50 µg/ml CP for 72 hours at 37˚C with 5% CO_2 humidified atmosphere. The suspension was regularly monitored for any sign of contamination or change in the pH.

According to our recent study, for determining the effective doses (ED50) of CNP and SSe in the prevention of CP-induced oxidative stress (16), we used different concentrations of CNP (0, 0.5, 1 and 2 ng/ml) and SSe (0, 0.125, 0.25 and 0.5 ng/ml) based on a pilot study, whichwere incubated at 37˚C with 5% CO_2 humidified atmosphere in the presence of CP at dose of 12 µg/ml.

Experimental groups (*in vitro*)

After determining IC50 of CP and the ED50 of CNP and SSe, all cells were divided into five groups as follows: i. control group, ii. CNP group receiving 1 ng/ml cerium oxide nanoparticles plus 12 µg/ml CP. iii. SSe group receiving 0.36 ng/ml sodium selenite plus 12 µg/ml CP. iv. CNP+SSe group as a combination group receiving 1 ng/ml cerium oxide nanoparticles plus 0.36 ng/ml sodium selenite plus 12 µg/ml CP and v. CP group receiving only 12 µg/ml CP. Then the lymphocytes were incubated at 37˚C with 5% CO_2 humidified atmosphere. After a 72-hour period, the cell suspensions in all groups were centrifuged at 250 g for 5 minutes. The supernatant solution was removed for the biochemical assays and the precipitated cells were used in methyl thiazolyl tetrazolium (MTT) reduction assay in the next step.

Lymphocytes viability

The assay is based on the reduction of MTT, a yellow tetrazole, to purple insoluble formazan by mitochondrial respiration in viable cells. MTT assay was performed on human lymphocytes cultured after 72 hours incubation. Centrifugation was done and the precipitated lymphocytes were washed twice with PBS. Then, 30 µl of MTT (5 mg/ml PBS) was added and it was re-incubated for 4 hours at 37˚C with 5% CO_2 humidified atmosphere. Next, cells were treated with 150 µl of dimethyl sulfoxide (DMSO) and the absorbance was read at 570 nm by enzyme-linked immunosorbent assay (ELISA) reader. For subtracting the MTT background, the absorbance was read at 690 nm in order to reduce artifacts. The viability of the treatment groups was shown as the percentage of controls, assumed to be 100% (16).

Measurement of thiobarbituric acid-reactive substances as marker of lipid peroxidation

To measure lipid peroxidation, we used TBARS. TBA reactivity of lipid peroxides in the samples produces a measurable pink color that has an absorbance at 532 nm using ultraviolet (UV) spectrophotometer, described in our previous work (28, 29). The activity was shown as µM.

Measurement 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 coloring agent (30). The activity was expressed as U/ml.

Measurement of myeloperoxidase activity

To assay MPO activity, we measured it spectrophotometrically as follows: 0.1 ml of supernatant was added to 2.9 ml of 50 mM PBS 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 was defined as the absorbance change per minute at 25˚C in the final reaction (29). The MPO activity was shown in U/ml.

Measurement of tumor necrosis factor-alpha

A human specific ELISA kit was used to quantify TNF-α in the supernatant of lymphocyte culture. To assess the amount of TNF-α, the absorbance of the sample was measured at 450 nm as the primary wavelength and 620 nm as the reference wavelength by ELISA reader, as described in the manufacturer's instructions. Data are shown as µg/ml.

Measurement of apoptosis by flow cytometer

Apoptosis (a programmed cell death) is a well described phenomenon occurring in many cellular systems. Annexin-V staining was assessed by flow cytometer to investigate CP-induced apoptosis (31) pattern. Annexin V binding as an indicator of phosphatidyl serine surface exposure in early apoptotic cells and propidium iodide (PI) staining as necrosis indicator were used. Currently, the most widely used analytical assays are based on monitoring of translocation of phosphatidylserine (PS) from inner phospholipid layer to the cell surface by use of a fluorochrome-labelled Annexin V in combination with appropriate vital dyes. ApoFlowEx® FITC Kit is based on standard setup that employs Annexin V-FITC conjugate and PI. The flow cytometry test can discriminate intact cells (annexin V-/PI-), early apoptotic cells (annexin V+/PI-), late apoptosis cells (annexin V+/PI+) and necrotic cells (annexin V-/PI+). The precipitated lymphocytes were washed twice with PBS. Then, the cells were suspended in binding buffer at 3×10^5 cells/100 µl, supplemented with 5 µl of FITC-Annexin-V and 5 µl of PI, and incubated for 15 minutes at room temperature in the dark. Flow cytometric analysis (Apogee, UK) was performed.

Statistical analysis

Each experiment was carried out at least three times. Data are presented as mean ± standard error of mean (SEM). One-way ANOVA and Tukey's multiple-comparison tests were carried out by Stats-Direct 3.0.107 to determine the statistical differences, while the level of significance was set at $P<0.05$.

Results

Chlorpyrifos, CeO_2 nanoparticle and sodium selenite dose optimization

As shown in figure 1A, the MTT reduction assay was used to calculate the median IC50 for CP after 72 hours of exposure (IC50=12 µg/ml).

After 72 hours, the concentration of CNP that was able to induce the cell viability by 50% was determined by the effective dose of CNP based on MTT reduction assay, as depicted in figure 1B and C (ED50=1 ng/ml). Also, we found the effective dose of SSe that was able to increase the viability to 50% using the MTT reduction assay (ED50=0.36 ng/ml).

Lymphocytes viability

The results of MTT assay on the cultured lymphocyte after 72 hours of different treatments are shown in figure 2A. There is a significant different viability between control and CP (P=0.001) groups. The groups which were pretreated solely with CNP or SSe (P=0.001) remained more viable as compared with the CP group (P=0.001), but didn't show any different viability compared to the control group. The more improvement in the lymphocyte viability was observed when the cells were pretreated with the combination of CNP+SSe compared to the CP group (P=0.001). Also, the combination group showed no significant decrease in viability and no synergistic effects as compared to CNP (P=0.098) and SSe (P=0.086) groups.

Thiobarbituric acid-reactive substances levels

As shown in figure 2B, TBARS level was significantly higher in CP group compared to control group (P=0.001). The groups which were pretreated with solely CNP and SSe (P=0.009) showed an apparent reduction in TBARS when compared to CP group (P=0.008), but no differences in comparison to control group. There was a significant decrease in TBARS level of the CNP+SSe group as compared to CP group (P=0.001). Also, the combination group possessed a significant decrease in TBARS showing synergic or additive effects in comparison to CNP (P=0.018) and SSe (P=0.033) groups.

Acetylcholinesterase activity

As shown in figure 2C, AChE activity was significantly lower in the CP group compared to control group (P=0.001). The groups which were pretreated with solely CNP and SSe showed an apparent increase in AChE activity as compared to CP group (P=0.007 and P=0.001, respectively), but no differences as compared to control group. There was a significant increase in AChE activity in the CNP+SSe group as compared to CP group (P=0.001). The AChE activity improved with both CNP and SSe, especially their combination, but no synergistic or additive effects were observed.

Myeloperoxidase activity

As depicted in figure 2D, MPO activity increased in the CP group as compared to the control group (P=0.001). The CNP- and SSe-pretreated cells showed a significant decrease in MPO activity in comparison to CP group (P=0.004 and P=0.007, respectively). The CNP+SSe group reduced the MPO activity as compared with the CP group (P=0.001). This combination showed synergistic or additive effects in comparison to CNP (P=0.002) and SSe (P=0.004) alone in lowering the MPO activity.

Tumor necrosis factor-alpha release

As seen in figure 2E, TNF-α production significantly elevated in the CP group when compared to the control group (P=0.001). A significant decrease in TNF-α levels was seen in the CNP and SSe treatment groups as compared to CP group (P=0.001 and P=0.001, respectively). The combination group showed more reduction in TNF-α protein production, compared to CP group (P=0.001). Also, CNP+SSe group showed a significant decrease in TNF-α levels as well as a synergistic or additive effect in comparison to CNP (P=0.024) and SSe (P=0.039) groups.

Annexin V staining and flow cytometry analysis

As shown in figure 3, all treated groups, plus-control group appeared to contain a significantly lower percentage of apoptotic cells in comparison to the CP group.

We found that in CP-treated cells, 2% were annexin V+/PI-, 10% were annexin V+/PI+ and 30% were annexin V-/PI+ (Fig.3A).

Among the control cells, only 6% were annexin V+/PI-, 5% were annexin V+/PI+ and 12% were annexin V-/PI+ (Fig.3B).

In comparison to CP-treated cells, annexin V+/PI- cells increased to 5% and 10.3% after being treated with CNP and SSe, respectively (Fig.3C, D), although it decreased to 0.5% after being treated with their combination (Fig.3E).

Also, as compared with CP-treated cells, the annexin V+/PI+ cells decreased to 1, 1 and 0.5% after being treated with CNP, SSe and their combination, respectively (Fig.3C-E).

In addition, in comparison with CP-treated cells, annexin V-/PI+ cells decreased to 1, 1 and 1% after being treated with CNP, SSe and their combination, respectively (Fig.3C-E).

Interestingly, this combination showed synergistic or additive effects in comparison to CNP and SSe alone in reduction of early apoptosis or annexin V+/PI- cells. Also, the results of late apoptosis or annexin V+/PI+ cells confirmed synergistic or additive effects of combination as compared to CNP and SSe alone.

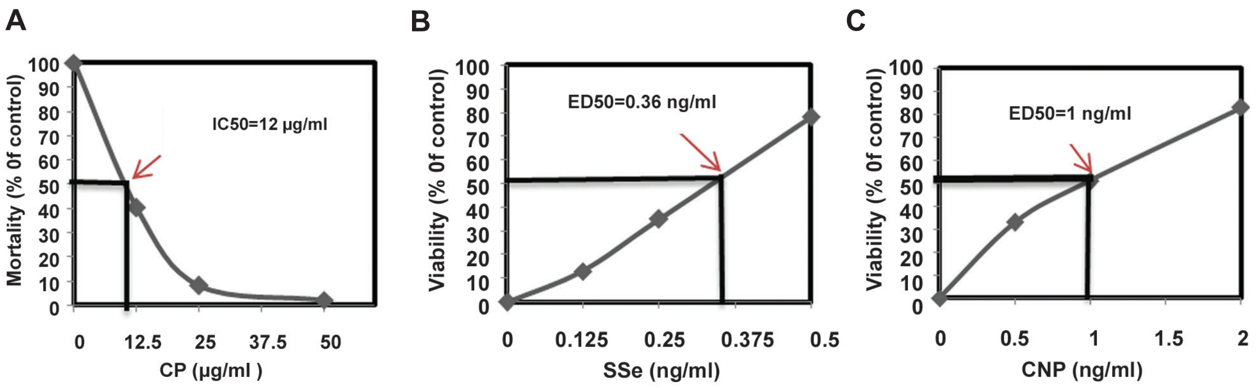

Fig.1: Effects of **A**. CP, **B**. SSe and **C**. CNP on mortality of isolated human lymphocytes.
The median inhibitory concentration (IC50) of CP was 12 µg/ml. The cell viability by 50% as the effective dose (ED50) of CNP was 1 ng/ml and of SSe was 0.36 ng/ml.
CP; Chlorpyrifos, SSe; Sodium selenite and CNP; Cerium oxide nanoparticle.

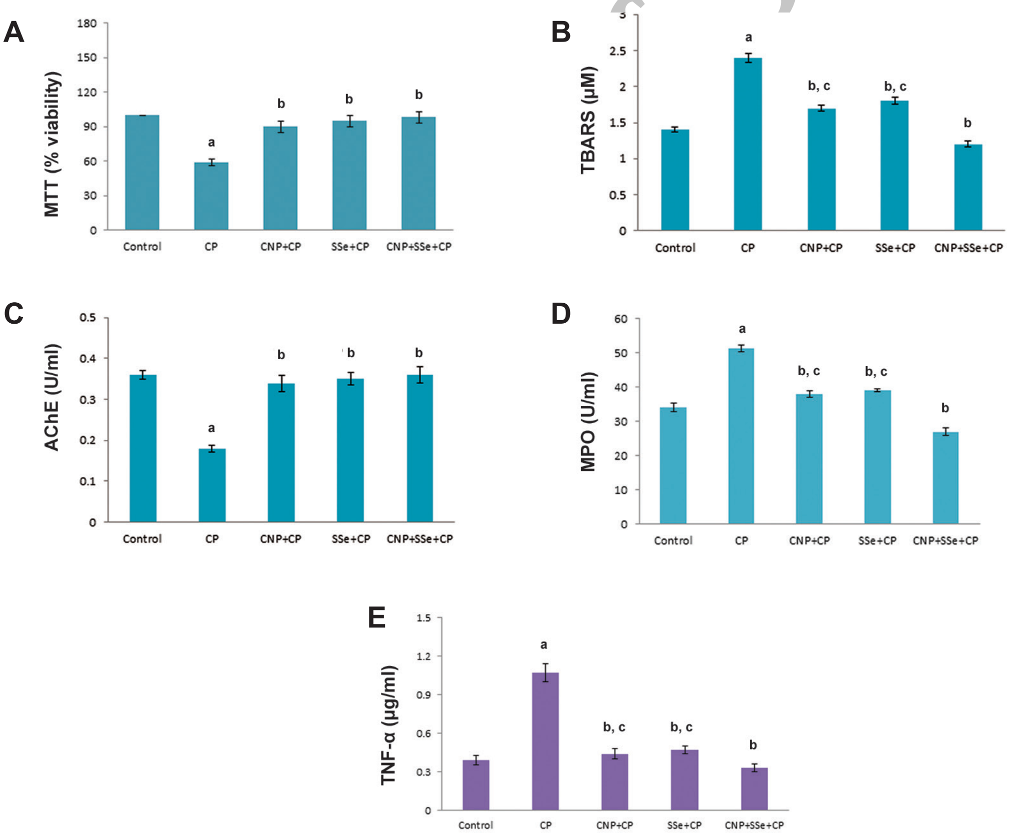

Fig.2: A. Effects of CP, CNP+CP, SSe+CP and CNP+SSe+CP in MTT assay, **B**. TBARS levels, **C**. AChE activity, **D**. MPO activity and **E**.TNF-α release of isolated human lymphocytes. Data are expressed as mean ± SEM. a; Significant difference between control and other groups, b; Significant difference between CP and other groups and c; Significant difference between CNP+SSe+CP and other groups.
CP; Chlorpyrifos , CNP; Cerium oxide nanoparticle, SSe; Sodium selenite, MTT; Methyl thiazolyl tetrazolium, TBARS; Thiobarbituric acid-reactive substances, AChE; Acetylcholinesterase, MPO; Myeloperoxidase, TNF-α; Tumor necrosis factor-alpha and SEM; Standard error of mean.

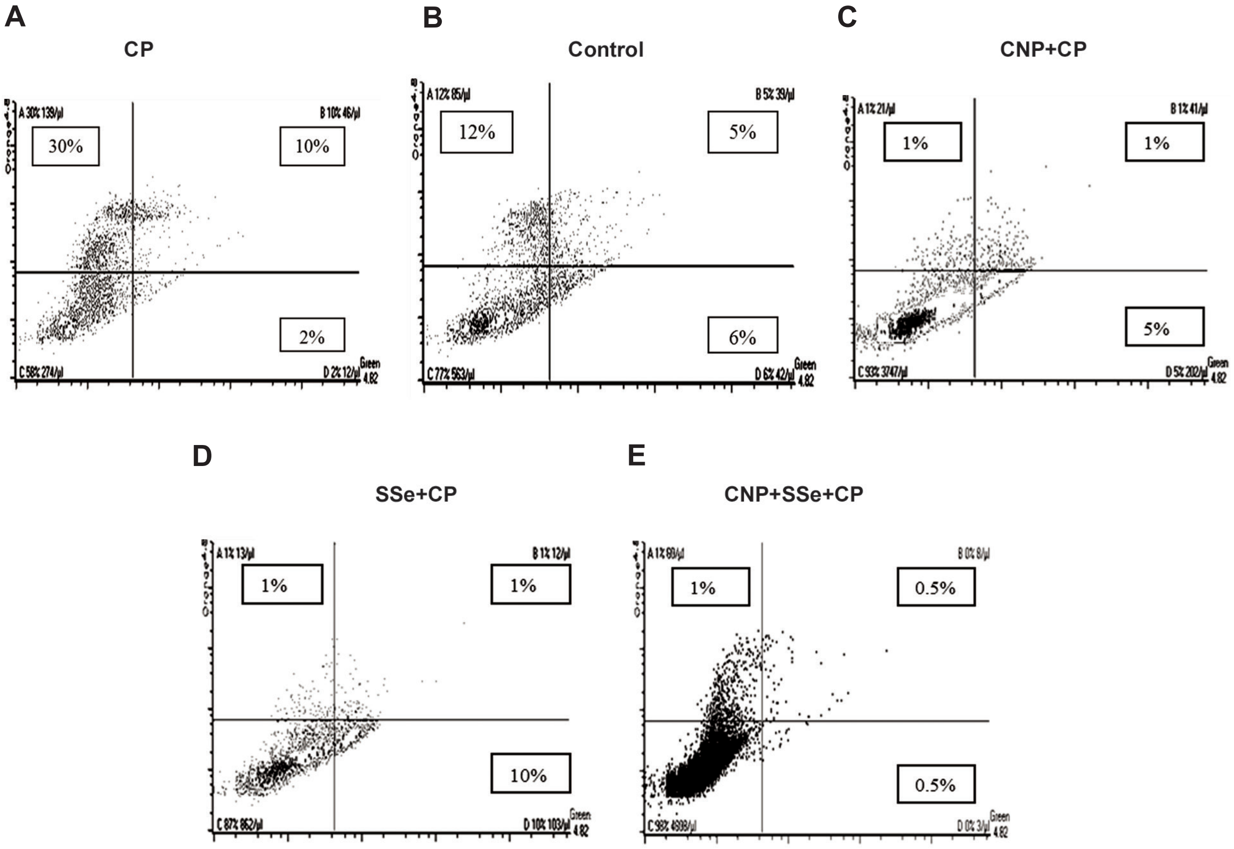

Fig 3: Effects of CP, CNP+CP, SSe+CP and CNP+SSe+CP in apoptosis of isolated human lymphocytes. Data are expressed as mean ± SEM. CP; Chlorpyrifos, SSe; Sodium selenite, CNP; Cerium oxide nanoparticle and SEM; Standard error of mean.

Table 1: Effects of CP, CNP+CP, SSe+CP and CNP+SSe+CP on MTT assay, TBARS levels, AChE activity, MPO activity, and TNF-α release of isolated human lymphocytes

	MTT (%viability)	P value	TBARS (µM)	P value	AChE (U/ml)	P value	MPO (U/ml)	P value	TNF-alpha (pg/ml)	P value
Control	100 ± 1	-	1.4 ± 0.034	-	0.36 ± 0.034	-	34 ± 1.2		0.39 ± 0.04	
CP	59+2.8[a]	0.001	2.4 ± 0.06[a]	0.001	0.18 ± 0.06[a]	0.001	51.2 ± 0.99[a]	0.001	1.07 ± 0.07[a]	0.001
CNP+CP	90 ± 4.8[b]	0.001	1.7 ± 0.04[b,c]	0.009, 0.018	0.35 ± 0.043[b]	0.007	38 ± 0.97[b,c]	0.004, 0.002	0.44 ± 0.04[b,c]	0.001, 0.024
SSe+CP	95 ± 4.8[b]	0.001	1.8 ± 0.05[b,c]	0.008, 0.033	0.34 ± 0.038[b]	0.001	39 ± 0.42[b,c]	0.007, 0.004	0.47 ± 0.03[b,c]	0.001, 0.039
CNP+SSe+CP	98 ± 5.2[b]	0.001	1.2 ± 0.04[b,c]	0.001	0.36 ± 0.048[b]	0.001	27 ± 1.03[b]	0.001	0.33 ± 0.03[b,c]	0.001

Data are expressed as mean±SEM. [a]; Significant difference between control and other groups. [b]; Significant difference between CP and other groups and [c]; Significantly different from CNP+SSe+CP and other groups.
CP; Chlorpyrifos, CNP; Cerium oxide nanoparticle, SSe; Sodium selenite, MTT; Methyl thiazolyl tetrazolium, TBARS; Thiobarbituric acid-reactive substances, AChE; Acetylcholineesterase, MPO; Myeloperoxidase, TNF-α; Tumor necrosis factor-alpha and SEM; Standard error of mean.

Discussion

The goal of the present study was to investigate one of the widely used pesticides CP for its potential to induce apoptosis and oxidative stress in the isolated human lymphocytes and also to evaluate the protective effects of CNP and SSe, especially their combination (Table 1). The results of this study showed that incubation with CP significantly increased levels of oxidative stress and key inflammatory biomarkers such as TBARS, MPO, and TNF-α, whereas their activities were prevented when CNP, SSe or their combination were used. Although, the AChE activity improved with both CNP and SSe, especially their combination, no synergistic or additive effects were observed.

Moreover, lymphocytes treated with CNP, SSe, and their combination, resulted in a significant decrease in the percent of mortality and apoptotic cells, inflammatory as well as oxidative markers.

It has been reported that Annexin-V staining is able to detect apoptosis in the early stage based on alterations of the cell membrane (31). In this regard, our findings of Annexin-V staining assay indicated that CP-induced cell death consisted of apoptosis in human lymphocytes. In support of this result, it has been reported that CP induces apoptosis in human natural killer (NK) cells (32), human monocyte cell line U937 (33), and a murine EL4 T-lymphocytic leukemia cell line (34).

It has been well established that OP causes mitochondrial damage and dysfunction due to increased generation of ROS, induction of proteolytic enzymes, and apoptotic death (35). In addition, the phospholipid component of cell membranes is suggested as a site of toxic action of OP compound (14). Exposure of phospholipids on the external surface of the cell membrane has been reported for activated apoptosis process. On the other hand, apoptosis is a cell death process characterized by specific features occurring at different stages. At a stage of early apoptosis (annexin V+/PI-), translocation of phospholipid phosphatidylserine and reduction of apoptosis occurs, whereas on stage of late apoptosis cells (annexin V+/PI+), the cells are already dead and phospholipid translocation has already occurred; therefore, use of CNP and SSe combination is better than that of CNP or SSe alone. So, the results are in favor of synergism or additive effects of combination.

Inhibition of AChE as a main mechanism of toxicity of CP is related to the cell membrane of lymphocytes and monocytes (36) that may lead to structural or functional alterations in immunocyte populations. Treatment of CNP, SSe and their combination alleviated AChE inhibitory activity in the CP-induced human lymphocytes, demonstrating that alteration of AChE activity might be the down stream effect of oxidative stress. Although, this is the first study targeting the effects of CNP in increasing AChE activity, there are several evidences about potential of SSe in augmentation of AChE activity (37). This element is capable of improving the toxicity of cadmium, mercury and lead which induced AChE inhibition in fish brain (38). On the other hand, supplementation with SSe (0.05 mg/kg/day) produced a beneficial effect on the buffalo calves intoxicated with CP (39).

In our study, CP caused lipid damage as indicated by the rise of TBARS, the marker of lipid peroxidation, resulting from the direct interaction of ROS and unsaturated fatty acids (40). This increase was associated with the protection provided by CNP, SSe and their combination. In support of the present findings, available reports have exhibited that CP increases TBARS in the erythrocytes (*in vivo* and *in vitro*) as well as in the brain, lung, testes, kidney and the liver (41-43). MPO, a heme protein, performs as an oxidant enzyme in the process of inflammation and generates reactive intermediates that progress lipid peroxidation *in vitro* (44). Interestingly, in this study, the results confirmed an increase in the TBARS which was associated with an enhancement of MPO activity in the lymphocytes treated with CP. In addition, this result is supported by our previous reports of *in vitro* effects of CNP, SSe and their combination, as anti-oxidative agents on isolated rat islets (16). Moreover, CNP has been reported to diminish oxidative signaling and cell mortality induced by cigarette smoke, diesel exhaust, and hydrogen peroxide (45-47). In addition, SSe has been found beneficial in the rats exposed to CP by restoring the oxidative injury (27).

Cytokines, regulators of immuneresponses play an important role in activation, proliferation and differentiation of lymphocytes in response to pesticide exposure (48). Release of TNF-α from human blood mononuclear cells, following an immunologic response, is an index of the inflammatory processes which may result in the peroxidation of cell proteins, lipids and cell apoptosis (49). Our data supported previous studies showing that TNF-α levels increase in animals exposed to CP (50), while interestingly showed the protective effects of CNP, SSe and their combination in reduction of TNF-α in CP-treated lymphocytes. The anti-inflammatory effects of CNP in macrophages showed its effect by reduction of inducible nitric oxide expression (46). Also, inflammatory factors were reduced by CNP in a murine cardiomyopathy model (51). SSe, as an essential trace element, possesses a critical role in some protective enzymes against free radicals (25), inhibits the adhesive molecules induced by TNF-α, and deactivates NF-κB (52).

The results of MTT assay suggest that CP disrupts mitochondrial function, showing involvement of the mitochondrial pathway (33). In addition, our result of the protective effect of CNP, SSe and their combination, is supported by our previous reports of *in vitro* effects of these elements, as antioxidant agents on isolated rat islets (19). It has been reported that CP induces apoptosis in rat neurons via a balanced mechanism regulated by p38 mitogen-activated protein (MAP) kinases, extracellular signal-regulated protein kinase (ERK), and c-Jun NH_2-terminal protein kinase (JNK) (53). Further studies are necessary to explore the detailed effect of CP on the mitochondrial pathway.

Conclusion

Our results demonstrated that CP induces apoptosis in isolated human lymphocytes via oxidative stress and inflammatory mediators. This kind of apoptosis in lymphocytes would without doubt affect its function and can be named immunotoxicity, although it is not new for OP compounds. It seems that CP firstly produces ROS, which leads to membrane phospholipid damage. The beneficial effects of CNP and SSe in reduction of CP-induced apoptosis and restoring AChE inhibition are related to their antioxidant potentials. Therefore, application of the CNP and SSe combination is reasonable in protection of toxic effects of CP. Of course, this remains to be further examined *in vivo* and in the clinic.

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