In vitro protection of human lymphocytes from toxic effects of chlorpyrifos by selenium-enriched medicines

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ARTICLE INFO	ABSTRACT
	Objective(s): Chlorpyrifos (CP) is a broad-spectrum organophosphorus pesticide used extensively in agricultural and domestic pest control, accounting for 50% of the global insecticidal use. In the present study, protective effects of two selenium-enriched strong antioxidative medicines IMOD and Angipars were examined in human lymphocytes treated with CP *in vitro*. *Materials and Methods:* Isolated lymphocytes were exposed to 12 μg/ml CP either alone or in combination with effective doses (ED_{50}) of IMOD (0.2 μg/ml) and Angipars (1 μg/ml). After 3 days incubation, the viability and oxidative stress markers including cellular lipid peroxidation (LPO), myeloperoxidase (MPO), total thiol molecules (TTM), and total antioxidant power (TAP) were evaluated. Also, the levels of tumor necrosis factor-α (TNF-α), as inflammatory index along with acetylcholinesterase (AChE) activity and cell apoptosis were assessed by flow cytometry. *Results:* Results indicated that effective doses of IMOD and Angipars reduced CP-exposed lymphocyte mortality rate along with oxidative stress. Both agents restored CP-induced elevation of TNF-α and protected the lymphocytes from CP-induced apoptosis and necrosis. *Conclusion:* Overall, results confirm that IMOD and Angipars reduce the toxic effects associated with CP through free radical scavenging and protection from apoptosis and necrosis.

Introduction

Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)-phosphorothioate] (CP) is a broad-spectrum organophosphorus (OP) pesticide used extensively in agricultural and domestic pest control, accounting for 50% of the global insecticidal use (1). The US Environmental Protection Agency restricted the use of CP in June 2000 due to exposure risk especially to children; however, CP still remains one of the most widely used OPs (2). Although use of this OP is widespread, it is very dangerous and harmful because of its toxic nature (3). The main toxicity of CP is neurotoxicity, which is caused by the inhibition of acetylcholinesterase (AChE) (4). Other than AChE inhibition, induction of oxidative stress and subsequent cellular toxicity has been well defined for OPs. The ability of CP to increase generation of reactive oxygen species (ROS), reduction of antioxidant defense enzymes and glutathione (GSH), increase cellular lipid peroxidation (LPO) and protein carbonyl have been previously suggested (5-7). Besides, CP and other OPs may inhibit the mitochondrial adenosine triphosphate (ATP) production through uncoupling of oxidative phosphorylation, leading to generation of more ROS (8). Therefore, three main mechanisms of toxicity of OPs include AChE inhibition, induction of oxidative stress, and disruption of mitochondrial ATP production.

The basic and the most important defense mechanism of the human body against oxidative stress is mediated through internal antioxidative enzymes or proteins (9). The antioxidant molecules are stable enough to neutralize free radicals by donating electrons. However, in situations of increased and accelerated oxidative challenge by CP as previously reported (10-14), the natural antioxidant mechanisms are overwhelmed, thereby resulting in cellular damage (15). Therefore, it seems rational to state that supplementation with exogenous antioxidants may counteract the oxidative burden and cellular damage induced by CP.

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In recent years, a novel registered medicine named IMOD has shown positive effects on reduction of oxidative stress and pro-inflammatory status in various studies (16). IMOD as a new naturally-derived immunomodulator has been patented in USA and Europe for its efficacy in human immunodeficiency virus (HIV) infection (16). IMOD is an herbal compound comprising of ethanolic *Rosa canina*, *Urtica dioica* and *Tanacetum vulgare* extracts, which has been enriched with selenium and urea. This combination of the extracts has strong free-radical scavenging potentials that is useful in many oxidant-related diseases (17) and immunoinflammatory-based diseases like type 1 diabetes (18), pancreatic Langerhans islet transplant (19), colitis (20) and severe sepsis (21).

Angipars is also a registered medicine derived from *Melilotus officinalis* under electromagnetic processes (22), which contains coumarin, flavonoids, and selenium. Clinically, it has been approved for the treatment of diabetic foot ulcers, especially through stimulation of angiogenesis (23). Coumarin which is the main component of this medicine is known for its anti-inflammatory and antioxidant activities, and for its ability to suppress superoxide and nitric oxide (NO) production in leukocytes (24, 25).

We aimed to investigate the protective effects of these two selenium-enriched medicines either alone or in combination, in alleviating the toxic effects of CP in isolated human lymphocytes.

Materials and Methods
Chemicals
All chemicals were purchased from Sigma-Aldrich Chemie (Germany) unless otherwise stated. Human specific tumor necrosis factor-α (TNF-α) ELISA kit from Bender MedSystems® (Austria) and ApoFlowEx® FITC Kit from Exbio (Czech Republic) were used. IMOD and Angipars were obtained from Rose Pharmed Biotechnology Co. (Iran).

Lymphocyte isolation and maintenance
The study was approved by the Institute 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 non-smokers and were not using medications. 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 collected, washed twice with phosphate buffer, and were counted based on the trypan blue exclusion method. After washing and counting, the cells were seeded at a density of 3×10^6 cells/well in the tissue culture medium (RPMI-1640), which consists of 10% FBS, 2 mM L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin sulfate and followed by addition of 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.

Treatment conditions
In accord with previous data (26, 27), we used 12 μg/ml of CP to induce oxidative stress in lymphocytes. In this regard, cell suspension (3×10^6 cells/well) was incubated with culture medium containing 12 μg/ml CP for 72 hrs at 37°C and 5% CO_2 humidified atmosphere. For protective treatment, optimization of dose was done by pretreating CP-induced cells with various concentrations (0, 0.1, 1, 5, 10, 20, 40, 80 and 100 μg/ml) of IMOD and (0, 0.1, 1, 25, 50, 100, 500, 1000 μg/ml) of Angipars for 72 hrs to reach the effective doses (ED_{50}). The 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay was used to calculate the concentration of IMOD and Angipars which could provide 50% of viability. After determining the ED_{50} of IMOD and Angipars, all the cells were divided into five groups including: (1) Con (negative control), lymphocytes in RPMI-1640 medium alone; (2) CP, lymphocytes in RPMI-1640 medium + CP (12 μg/ml)); (3) CP+I (lymphocytes in RPMI-1640 medium + CP (12 μg/ml) + ED_{50} of IMOD); (4) CP+A (lymphocytes in RPMI-1640 medium + CP (12 μg/ml) + ED_{50} of Angipars); (5) CP+I+A (lymphocytes in RPMI-1640 medium + CP (12 μg/ml) + ED_{50} of IMOD + ED_{50} of Angipars). Then the lymphocytes were incubated at 37°C and 5% CO_2 humidified atmosphere. After 72 hrs incubation, the cell suspension in all groups was centrifuged. The supernatant solution was removed for the biochemical assay and the deposited cells were used in viability and cell death (apoptosis vs. necrosis) assay in the next step.

Lymphocyte viability assay
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 hrs incubation. Centrifugation was done and the precipitated lymphocytes were washed twice by phosphate buffer. Then, 50 μl of MTT solution was added and it was re-incubated for 4 hrs 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 shown as the percentage of controls which assumed 100%.

Determination of TAP
The method is based on the reduction of Fe^{3+} tripyridyltriazine (TPTZ) complex (colorless) to Fe^{2+} TPTZ (blue colored) formed by the action of 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

a)

b)
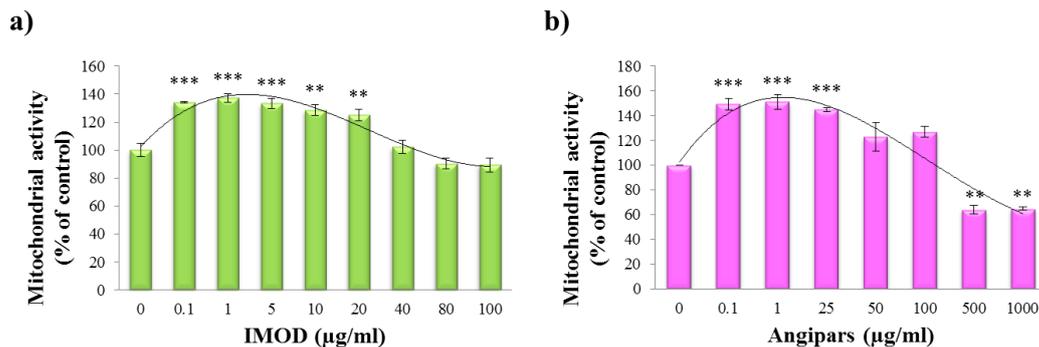
Figure 1. Effects of various concentrations of IMOD (a) and Angipars (b) on chlorpyrifos-induced toxicity in human lymphocytes *in vitro* with MTT assay
Data are expressed as mean±SEM. The cell viability by 50% as the effective dose (ED$_{50}$) of IMOD was 0.2 μg/ml and of Angipars was 1 μg/ml
**: $P<0.01$, ***: $P<0.001$ compared with control

HCl and 20 mM FeCl$_3$ in the proportion of 10:1:1 at 37°C. Ten μl of the H$_2$O 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 are shown as mmol/μg protein.

Determination of TTM

To determine TTM in the control and test groups, 0.6 ml Tris-EDTA buffer (Tris base 0.25 M, ethylene diamine tetra acetic acid 20 mM, pH 8.2) was added to 0.2 ml of supernatant, and after quick vortex mixing, 40 μl 5-5'-dithiobis-2-nitrobenzoic acid (10 mM in pure methanol) was added. The final volume of this mixture was made up to 4.0 ml by an extra addition of pure methanol. After 15 min incubation at room temperature, the samples were centrifuged at 3000 g for 10 min and ultimately the absorbance of the supernatant was measured at 412 nm. Data are shown as mM.

Determination of LPO

To measure LPO, thiobarbituric acid-reaction substances (TBARS) were measured. TBA reacts with

lipid peroxides in the samples producing a measurable pink color that has absorbance at 532 nm, as described in our previous work in detail (28). TBARS has been reported as mM.

Determination of myeloperoxidase (MPO) activity

The activity of MPO was measured spectro-photometrically 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$_2$O$_2$. The change in absorbance was recorded by spectrophotometer at 460 nm. MPO activity is defined as the absorbance change per minute at 25°C in the final reaction (29). The MPO activity is shown in Unit/ml.

Determination of AChE

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 is expressed as Unit/mg protein.

Determination of TNF-α

A human specific ELISA kit (BenderMed System) was used to quantify TNF-α in the supernatant of lymphocyte culture. To assess the amount of TNF-α,
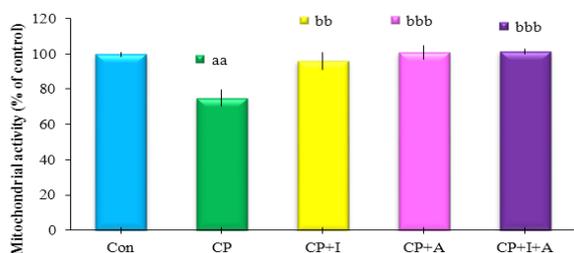
Figure 2. Effects of ED$_{50}$ of IMOD (I), Angipars (A) and their combination (I+A) on the mitochondrial activity of isolated human lymphocytes in the presence of chlorpyrifos (CP). Data are expressed as mean±SEM
aa: $P<0.01$ compared with control; bb: $P<0.01$, bbb: $P<0.001$ compared with CP
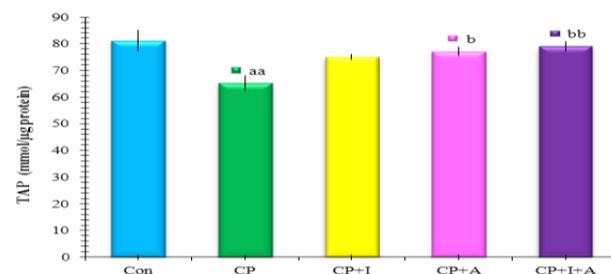
Figure 3. Effects of ED$_{50}$ of IMOD (I), Angipars (A) and their combination (I+A) on total antioxidant potential (TAP) of isolated human lymphocytes in the presence of chlorpyrifos (CP)
Data are expressed as mean±SEM
aa: $P<0.01$ compared with control; b: $P<0.05$, bb: $P<0.01$ compared with CP
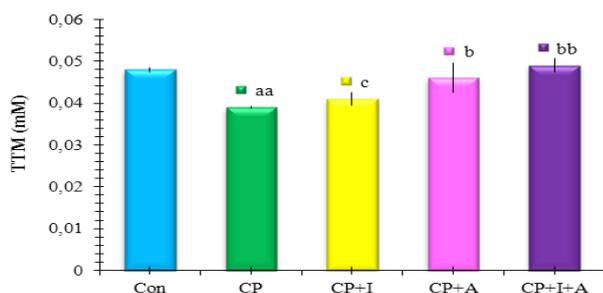
Figure 4. Effects of ED_{50} of IMOD (I), Angipars (A) and their combination (I+A) on total thiol molecules (TTM) of isolated human lymphocytes in the presence of chlorpyrifos (CP) Data are expressed as mean±SEM
aa: *P*<0.01 compared with control; b: *P*<0.05, bb: *P*<0.01 compared with CP; c: *P*<0.05 compared with CP+I+A

Figure 5. Effects of ED_{50} of IMOD (I), Angipars (A) and their combination (I+A) on lipid peroxidation (LPO) levels of isolated human lymphocytes in the presence of chlorpyrifos (CP) Data are expressed as mean±SEM
aaa: *P*<0.001 compared with control; b: *P*<0.05, bbb: *P*<0.001 compared with CP; c: *P*<0.05 compared with CP+I+A

the absorbance of sample was measured in 450 nm as the primary wavelength and 620 nm as the reference wavelength by ELIZA reader as described in the kit brochure. Data are shown as ng/ml.

Protein assay

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

Determination of cell death (apoptosis vs. necrosis)

To find out mode of lymphocyte cell death induced by CP in the presence and absence of IMOD, Angipars, and their mixture, the Annexin V-FITC/propidium iodide (PI) staining was carried out. The staining of Annexin V-FITC and PI indicates the type of death induced by the test compound i.e. apoptosis or necrosis. The cells (1×10^6) were treated with CP, alone or in combination for 72 hrs, washed and stained with Annexin V-FITC antibody and PI as per instructions given by the manufacturer. The cells were scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels. The fraction of cell population 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 post apoptotic necrosis (31).

Statistical analysis

At least three independent experiments in repetitions were carried out. Data are presented as mean±SEM. One-way ANOVA and Tukey's multi-comparison tests were carried out by Stats-Direct 3.0.117 software to determine the statistical differences while the level of significance had been set at *P*<0.05. Also, dose-effect curves and the ED_{50} for antitoxic activity of each individual drug were estimated by probit regression along with the 95% confidence intervals for each drug treatment.

Results
Effect of IMOD and Angipars on CP-induced toxicity

The ED_{50} of IMOD and Angipars in elimination of CP-induced toxicity are shown in Figure 1. As seen, the viability of cells increased with reduction of doses and the cell viability increased significantly. The estimated ED_{50} of IMOD and Angipars were 0.2 µg/ml and 1 µg/ml, respectively.

Figure 6. Effects of ED_{50} of IMOD (I), Angipars (A) and their combination (I+A) on meyloperoxidase (MPO) activity of isolated human lymphocytes in the presence of chlorpyrifos (CP) Data are expressed as mean±SEM
a: *P*<0.05, a'a': *P*<0.01 compared with control; bb: *P*<0.01, bbb: *P*<0.001 compared with CP; c: *P*<0.05 compared with CP+I+A
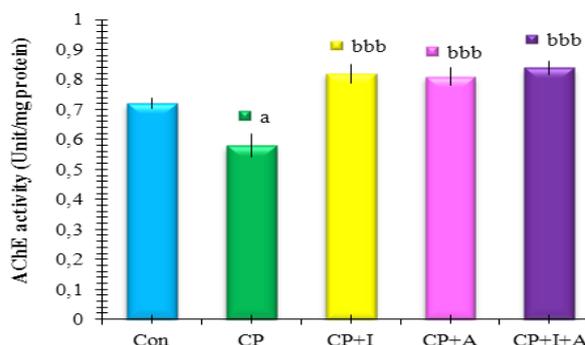
Figure 7. Effects of ED_{50} of IMOD (I), Angipars (A) and their combination (I+A) on acetylcholinesterase (AChE) activity of isolated human lymphocytes in the presence of chlorpyrifos (CP) Data are expressed as mean±SEM
a: *P*<0.05 compared with control; bbb: *P*<0.001 compared with CP
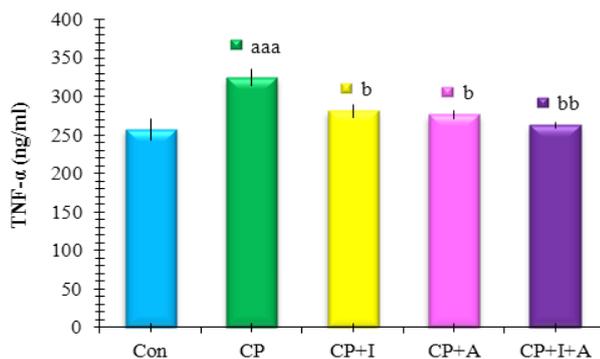
Figure 8. Effects of ED$_{50}$ of IMOD (I), Angipars (A) and their combination (I+A) on TNF-α release of isolated human lymphocytes in the presence of chlorpyrifos (CP)
Data are expressed as mean±SEM
aaa: *P*<0.001 compared with control; b: *P*<0.05, bb: *P*<0.01 compared with CP

Cell viability

The results of MTT assay on the cultured lymphocytes, 72 hrs post incubation, are shown in Figure 2. There is a significant decrease in viability in the CP group as compared with control group (*P*<0.01). The groups which were pretreated with IMOD, Angipars, and their combination remained more viable in comparison with CP (*P*<0.01, *P*<0.001, and *P*<0.001 respectively), but did not show different viability when compared to the control. No significant improvement in the lymphocyte viability was observed when the cells were pretreated with the combination of IMOD and Angipars in comparison with each of them alone.

TAP

As shown in Figure 3, there is a significant decrease in control TAP by administration of CP (*P*<0.01). The groups which were pretreated with IMOD, Angipars, and their combination increased

TAP of lymphocytes when compared with CP which are significant in Angipars and combination groups (*P*<0.05 and *P*<0.01, respectively).

TTM

As shown in Figure 4, TTM considerably decreased in CP group when compared to controls (*P*<0.01). No significant improvement in the lymphocyte TTM was observed when the cells were pretreated with IMOD as compared with CP group. However, treatment with Angipars and its combination with IMOD significantly increased TTM (*P*<0.05 and *P*<0.01, respectively). Noticeably, the combination group of IMOD and Angipars showed additive effects in comparison with IMOD group (*P*<0.05).

LPO

There was a significant elevation in LPO (*P*<0.001) in CP group in comparison with controls (Figure 5). The groups which were pretreated with IMOD or Angipars showed apparent reductions in LPO when compared with CP group (*P*<0.05 and *P*<0.001, respectively), but no difference was observed when compared with control group. There was a significant decrease in LPO of combination group in comparison with CP (*P*<0.001). Also, the combination group induced a significant decrease in LPO showing incremental trend in comparison with IMOD group.

MPO activity

As depicted in Figure 6, MPO activity increased in CP group as compared with the control group (*P*<0.05). The IMOD- and Angipars-pretreated cells showed a significant decrease in MPO activity in comparison with CP group (*P*<0.01). The combination group reduced the MPO activity when compared with the CP and control
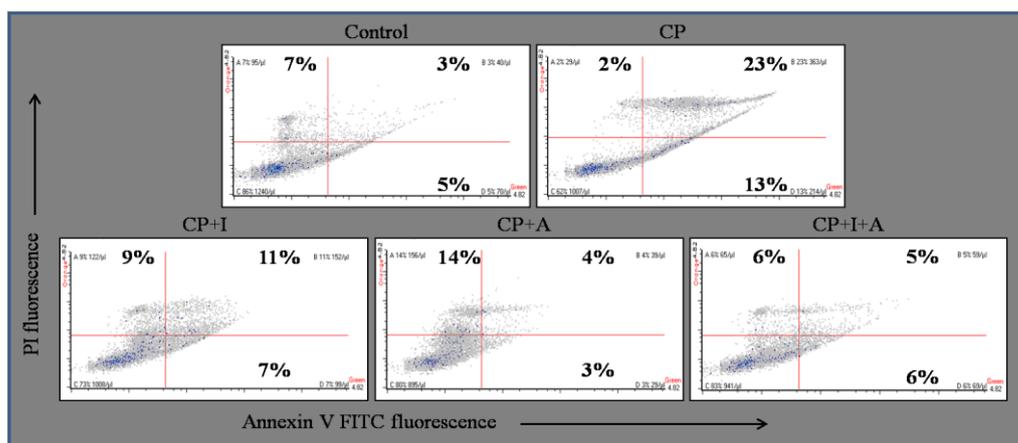
Figure 9. Flow cytometric analysis of apoptosis and necrosis in human lymphocytes induce by chlorpyrifos (CP), alone or in combination with IMOD (I), Angipars (A) and their mixture (I+A), using Annexin V-FITC and PI double staining. Human lymphocytes (1×10^6/200 μl) were incubated with indicated concentrations of antioxidants and CP for 72 hrs and stained with Annexin V-FITC/PI. Quadrant analysis of fluorescence intensity of non-gated 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 (post-apoptotic necrotic) cells, respectively. The percentages of positive cells are indicated in each panel

groups ($P<0.001$ and $P<0.01$, respectively). This combination showed significant effects in comparison to IMOD alone in lowering MPO activity ($P<0.05$).

AChE activity

As shown in Figure 7, AChE activity was significantly lower in CP when compared with control group ($P<0.05$). The groups which were pretreated with IMOD, Angipars, and their combinations showed an apparent increase in AChE activity when compared with CP group ($P<0.001$), but no synergistic or additive effects were observed in combination group.

TNF-α release

As seen in Figure 8, TNF-α production was significantly elevated in the CP group when compared with controls ($P<0.001$). A significant decrease in TNF-α was seen in IMOD or Angipars treatment groups when compared with CP ($P<0.05$). The combination group showed more reduction in TNF-α when compared with CP ($P<0.01$), but no significant effect was shown in comparison with each of them alone.

Lymphocyte death (apoptosis vs. necrosis)

Figure 9 represents typical flow cytometry plots of Annexin V and PI staining of different treatments of lymphocyte. After 72 hrs incubation with CP, there was an increase in the number of lymphocytes exposing phosphatidylserine as indicated by an increase in the Annexin V-positive and PI-negative cell population as well as 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. The groups which were pretreated with IMOD and Angipars remained more viable and the number of apoptotic and necrotic cells decreased when compared with CP (% of apoptotic cells: 7 and 3 vs. 13, % of necrotic cells: 11 and 4 vs. 23). The combination group showed more reduction in the number of lymphocytes that were apoptotic and necrotic (6% and 5%, respectively) in comparison with CP. This combination showed additive effects, in comparison with IMOD alone, in reduction of apoptosis and necrosis. Besides, there was no significant difference in number of viable cells in comparison with control.

Discussion

OPs have been known immunotoxics since early 1970s (32). *In vivo*, CP has been shown to cause immunologic abnormalities in both animals (33) and humans (34), through toxicity on human T lymphocytes, either in the presence or absence of oxidative stress condition (35). In the present *in vitro* study, results show that incubation of lymphocytes with CP induces oxidative stress and inflammatory factors in human lymphocytes along with inhibition of AChE activity. Moreover, lymphocytes pretreated with IMOD and Angipars ameliorated CP-induced LPO as well as protein oxidation. These results indicate that IMOD and Angipars may have a beneficial role in lowering CP toxicity but no meaningful additive effect was observed by use of combination therapy.

Previous studies have shown that OPs such as CP cause an *in vitro* increase in LPO in human cells (36), which has been suggested as one of the molecular mechanisms involved in OP-induced toxicity (37, 38). CP is lipophilic and may enhance LPO by direct interaction with cellular plasma membrane (39). Therefore, the results of present study strongly support the suggestion that oxidative stress may play a critical role in the human cell damage caused by CP exposure. Furthermore induction of MPO activity as a heme protein that generates ROS and bioactive LPO products (40) can be taken into account. Interestingly, our study showed that CP-induced increases in LPO and MPO were restored by pretreatment with IMOD and Angipars. In agreement with us, previous studies demonstrated that IMOD and its main component *Urtica dioica* have antioxidant activity and remarkably reduced LPO and MPO in colitis (20) and diabetes (18, 41). Meanwhile, other studies showed that the antioxidative potential of Angipars is exerted mainly through enhancing TAP and reduction of LPO (22).

In addition, several studies have shown the antioxidative activity of the main components of IMOD and other strong antioxidants with the same components such as *Urtica dioica*, *Teucrium* and selenium through reduction of free radicals and inhibition of inflammatory mediators (41-44). Also, it has been proven that the immunomodulatory effect of IMOD results in the attenuation of TNF-α, IL-1β, and MPO in many inflammatory conditions (45). *Melilotus officinalis*, the main constituent of Angipars, has been proven helpful, in previous studies, in reducing inflammation and regulating the immune system which is thought to be due to inhibitory effect of natural coumarins on LPO and oxidative DNA damage (22). Coumarin has also been found to reduce synthesis of NO in the phagocytes in anti-inflammatory action (24).

The findings of Annexin V staining and MTT assay indicated that CP-induced lymphocyte death is due to apoptosis. This is the first *in vitro* report on CP-induced apoptosis in human lymphocytes, although it has been reported that CP induces apoptosis in human monocyte cell line (46), rat primary cortical neurons (27) and murine preimplantation embryos (47). Previous studies have shown that CP exposure induces immunotoxicity via induction of apoptosis

partly mediated through the activation of caspase 3 (46). Also, it was demonstrated that chronic CP exposure may cause abnormality in the immune system including depression of T lymphocytes (48). Besides, free radical-induced oxidative damage, which has been widely implicated in the molecular mechanism of CP cytotoxicity, is initiators of apoptosis (49), which may have been involved in the depression of T lymphocytes in the group exposed to CP. Present results of MTT, suggest that CP disrupts mitochondrial function. Many reports have identified two potential cellular targets for CP, cell signaling cascades from one side and the expression and function of gene transcription factors from the other side (50-52). ROS interact with receptors, second messengers and transcription factors, and alter gene expression and influence cell growth and survival (53) as supported by present positive protective effects of IMOD and Angipars on cell viability. Moreover, antioxidant potentials of IMOD and Angipars explain inhibition of apoptosis.

Conclusion

IMOD and Angipars reduce the toxic effects associated with CP through free radical scavenging, restoring AChE inhibition and protecting from apoptosis and necrosis. Strong anti-oxidative stress activity and enrichment with selenium, which has shared properties, bring us to the conclusion that toxic effects of this organophosphorus insecticide are avoidable with these medicines.

Acknowledgment

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