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Reduction of chlorpyrifos-induced toxicity in human lymphocytes by selected phosphodiesterase inhibitors

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ABSTRACT

One of the most famous and commonly used compounds from organophosphate (OP) family is chlorpyrifos (CP) which is widespreadly used as a powerful insecticide. Previous studies have shown that OPs induce oxidative stress, inflammation and apoptosis by generating the free radicals. The protective effects of three members of phosphodiesterase inhibitor (PDEI) family, including rolipram (RLP), milrinon (MLR) and pentoxifylline (PTX) were evaluated in the human lymphocytes against CP's toxicity. In this case, the level of oxidative stress biomarkers, the viability of the cells and the rate of apoptosis by flow cytometry were investigated. The results of this study revealed that CP makes a significant increase in the level of inflammatory and oxidative stress markers such as meyloperoxidase (MPO), lipid peroxidation (LPO), total thiol molecules (TTM) and total antioxidant potential (TAP), and also makes an enhancement in the rate of apoptosis process. On the other hand, PDEIs and specifically the combination of them restored the negative effects of CP and significantly prevented the apoptosis and oxidative stress imbalance. It is concluded that these PDEIs have positive effects in attenuation, recovery, and protection of CP-induced toxicity in the human lymphocytes.

1. Introduction

The OPs have been used longtime as a group of pesticides relating to the group of molecules containing phosphorous, derived from phosphoric acid, which inhibit cholinesterase enzyme and other esterase [1]. Exposure to OPs through several routes including the food chain causes a significant number of poisonings each year [2,3]. Inhibition of AChE by OPs causes an increase in the level of acetylcholine neurotransmitter and causes continual stimulation of acetylcholine receptors, which is the main mechanism of toxicity of OPs [3]. Another mechanism, proposed for toxicity of OPs is oxidative stress. It has been shown that acute exposure to OPs causes overgeneration of reactive oxygen species (ROS) and consequently cellular LPO [4–6]. Oxidative stress is involved in the pathophysiology of several toxicants and diseases [3,4]. The oxidants have a high potency in donating oxygen to the other substances making them enormously unstable and very reactive [4,7].

One of the most famous and commonly used compounds from OP family is CP, which is widespreadly used as a powerful insecticide. The residues of CP have been detected in the air [8] which means special considerations should be taken in order to control them. CP and particularly CP-ethyl, a derivative of CP, have important toxic effects, including hepatoxicity, genotoxicity, teratogenicity, immunotoxicity as well as neurobehavioral alterations [9]. Previous studies have shown that subchronic exposure to OPs causes ACh inhibition in human [10,2]. Also, it has been shown that exposure to CP increases the level of lipid peroxides in different organs of rats, and decreases the antioxidant enzyme levels. Additionally, administration of CP in pregnant rats causes oxidative stress and consequently disturbed the antioxidant system in the liver, kidney, brain, and fetus of exposed rats [11].

Other studies show that oxidative stress makes reduction in some transcription factors, including NFAT, AP-1 and NF-kB in activated lymphocytes, causing suppression of the immune system [12]. Cytokines have an important role in the immune system operation and can increase their number, action and evolution of lymphocytes in response to OPs [13]. TNF-α as an inflammatory response indices, by activation NF-kB, causes ROS production and fat/protein peroxidation in cells which induces apoptosis [14]. Cells have different mechanisms for

Abbreviations: CP, chlorpyrifos; OP, organophosphorus; PDEI, phosphodiesterase inhibitor; RLP, rolipram; MLR, milrinon; PTX, pentoxifylline; RMP, the combination of rolipram, milrinon and pentoxifylline; AChE, acetylcholinesterase; TNF-α, tumor necrosis factor-α; MPO, myeloperoxidase; LPO, lipid peroxidation; TTM, total thiol molecules; TAP, total antioxidant potential.

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scavenging free radicals and ROS. The primary defense system is offered by antioxidants which can be in enzymatic or non-enzymatic forms. The antioxidant enzymes have been shown to be significantly affected by pesticides, including CP [15,16]. Within previous studies, it has been concluded that different treatment with antioxidants such as PDEIs also can strengthen the immune system by various pathways.

PDEIs are a group of enzymes, which have the ability of increasing a group of compounds, including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) which are responsible for signal transduction in the cells. Recent studies have shown that enhancement of cAMP and cGMP prevents the induction of oxidative stress [17]. According to this finding, PDEIs have become an important target for the treatment of OP-induced toxicity. MLR is one of the non-selective PDEI-3 inhibitors which is prescribed in cardiovascular diseases and is an inotropic agent [18]. RLP is another selective PDE-4 inhibitor which has been shown effective in different ROS-related tissue injury models, including the lung, gastric, and intestinal ischemia [19]. PTX is one of the methylxanthine derivative drug groups which has been widely used for cardiovascular and ameliorating peripheral vascular diseases. It has been indicated that PTX has the inhibitory characteristic on cytokine production, especially TNF-α. Also, according to PTX's activity as an inhibitor of xanthine oxidase, an enzyme involved in the generation of ROS, it can decrease the toxicity of OPs. In another study, it was shown that PTX has an increasing effect on bodies total antioxidant capacity and simultaneously decreases the LPO level [20].

Considering above mentioned facts, this study is aimed to examine the protective effects of RLP, PTX and MLR on CP-induced oxidative stress in the human lymphocytes.

2. Materials and methods

2.1. Chemicals

Human specific tumor necrosis factor-α (TNF-α) ELISA kit was obtained from Bender MedSystems (Vienna, Austria; Cat. No. BMS2034) and ApoFlowEx® FITC Kit were purchased from Exbio (Vestec, Czech Republic; Cat. No. ED7044). PTX, RLP, MLR and other chemicals, including RPMI 1640 medium, bovine serum albumin (BSA), 3–4,5-dimethyl thiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-ρNA), N-acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-ρNA), ethylenediaminetetraacetic acid (EDTA), HEPES sodium salt, dimethyl sulfoxide (DMSO) and thiobarbituric acid (TBA) were used from Sigma-Aldrich (Munich, Germany).

2.2. Preparing lymphocytes

2.2.1. Human volunteers

Vein bloods were obtained in sterile conditions from three different 25 years old male volunteers, which were not addicted to cigarettes and opioids and have not used any drug. The blood was immediately heparinized in order to prevent coagulation. This part of the study was approved by applying all ethical aspects according to institute review board (IRB) with code number of 90-01-45-13671.

2.2.2. Separating and counting lymphocytes

In the first step, under the laminar hood and sterile condition, the heparinized blood was diluted in order to make the separation easier. This was done by mixing 1:1 ratio of heparinized blood and RPMI 1640 medium cautiously by the means of a micropipette. In the next step, in order to separate lymphocytes from different blood components, Ficoll-Paque with a density of 1077 g/ml was mixed slowly with an equal volume of blood in order to prevent the combination of two compounds. After a few minutes, red blood cells got precipitated. Samples were centrifuged at 400 *g* for 30 min. The lymphocytes, from the interface of plasma and Ficoll-Paque, were collected and washed twice with phosphate buffer, then were counted based on the trypan blue exclusion method under a microscope. Live cells displayed completely bright and clear with a uniformed cell wall. However, the dead cells appear dark blue with a disordered cell wall.

2.3. Study design

After counting, 3×10^6 lymphocytes/well were exposed to 12 μg/ml CP, which can induce oxidative stress in lymphocytes according to previous studies [21,22], and different concentrations of PTX, MLR and RLP, for 72 h at 37 °C, to find effective dose of drugs for 50% of the group (ED_{50}) against CP. ED_{50} of the drugs was determined by measuring the viability of the cells based on MTT assay. After determining the ED_{50} of PTX, MLR and RLP, all the cells were divided into six groups, including: cell suspension alone (Control), cell suspension with CP toxicant (CP), cell suspension with CP toxicant containing ED_{50} of RLP (CP + R), cell suspension with CP toxicant containing ED_{50} of MLR (CP + M), cell suspension with CP toxicant containing ED_{50} of PTX (CP + P) and cell suspension with CP toxicant containing ED_{50} of PTX, MLR and RLP (CP + RMP). According to these groups, lymphocytes were incubated in RPMI 1640 medium for 72 h at 37 °C and 5% CO_2 humidified atmosphere, and different experiments, including viability assays (MTT test and apoptosis vs. necrosis assay) were done; also oxidative stress markers and inflammatory levels were determined.

2.4. Investigating viability and mortality of the lymphocytes

2.4.1. MTT assay

MTT assay was performed on human lymphocytes which were cultured and incubated for 72 h. In the first step, defined amount of yellow MTT powder was dissolved in phosphate buffer and was shacked thoroughly until completely dissolved. The cell precipitants were washed with phosphate buffer carefully and then 0.5 μl MTT solution (0.5 mg/ml) was added. In the next step, microtubes were incubated in 37 °C and 5% CO_2 pressure. After incubation, 150 μl DMSO was added to the suspension and shaken for 15 min on a shaker [23]. Finally, 100 μl of each samples was placed on a plate, the absorption was read at 570 nm by ELISA reader three times and the viability was reported as percent of the control group.

2.4.2. Flow cytometry assay

Flow cytometry is assumed as a high quality technique to study physicochemical specifications of the cells. This experiment was intended to differentiate the necrotic, late apoptotic, early apoptotic and viable cells. To find out the mode of lymphocyte cell death induced by CP in the presence of MLR, RLP and PTX, 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), which were treated for 72 h were washed and stained with 5 μl of Annexin V-FITC antibody and 5 μl of PI for 15 min at room temperature. The cells were scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels by a flow cytometer (Apogee Flow Systems, UK). The fraction of cell populations in different quadrants was analyzed using quadrant statistics [24].

2.5. Investigating inflammatory marker

A human specific ELISA kit, obtained from Bender MedSystems (Vienna, Austria; Cat. No. BMS2034), was used to quantify TNF-α in the supernatant of lymphocyte culture. To assess the amount of TNF-α, the absorbance of the samples was measured at 450 nm as the primary wavelength and 620 nm as the reference wavelength by an ELISA reader as described in the kit brochure. Data were shown as ng/ml.