In vitro effect of cadmium on the function of human lymphocytes and neutrophils

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Abstract

Cadmium (Cd) is a heavy metal which is one of the most serious environmental pollutants. Exposure pathways include contaminated water, food and air pollution. Controversial results of Cd effect on immune response have been shown and its potential immunosuppressive action is still a subject of study. We investigated Cd effect in different concentrations on human peripheral lymphocytes proliferation and reactive oxygen species (ROS) production in neutrophils. Cadmium caused significant lower lymphocytes proliferation rate and decrease of ROS production in high concentrations (100 μM). Our and other works indicate that cadmium effect on immune response cannot be excluded and might widely differ in human population.

Key words: *cadmium, peripheral blood lymphocytes, proliferation, reactive oxygen species, neutrophils.*

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Introduction

Heavy metals pose a serious threat to human health. Among many harmful effects they can affect fertility, lead to immunodeficiency and teratogenesis [1-3]. Heavy metals occur naturally in the environment with large variations in concentration. Elevated levels of these elements have been associated mainly with agricultural and industrial activities [4]. Anthropogenic human exposures to environmental cadmium (Cd) are primarily the result of fossil fuel combustion, phosphate fertilizers, iron and steel production, cement production and related activities, nonferrous metals production and municipal solid waste incineration. The most important source of Cd exposure in the general population is food and water contamination as well as air pollution [5]. Cadmium has been classified as carcinogenic, particularly affecting lungs and kidneys [6]. However contradictory data on its immunotoxicity is available with some studies indicating stimulating effect on immune response followed by reduction of susceptibility to pathogens, other demonstrating that Cd might have immunosuppressive action, while yet other finding no effect of Cd on immune system [7, 8]. Therefore potential effects of Cd on human immune system are still a subject of study and requires subsequent *in vitro* analyses.

The aim of the following study was to examine the effect of different Cd concentrations on selected innate and adaptive immune response parameters: peripheral blood lymphocytes proliferation and reactive oxygen species (ROS) production in human neutrophils *in vitro.*

Material and methods

Lymphocytes isolation and culture growth

Heparinized samples of blood (8 ml) were collected from healthy donors at Regional Center of Blood and Blood Treatment in Poznan, Poland. Lymphocytes were isolated under sterile conditions by centrifugation (30 minutes, 1750 rpm, g = 569.4) on Gradisol-L (Aqua-Med, Poland) and washed twice in Eagle's medium (Biomed, Poland). The isolated lymphocyte suspension (1×10^6 cells ml^{-1}) in Eagle's medium was supplemented with 10% fetal bovine serum (Sigma Chemicals, USA) and antibiotic (gentamycine at concentration of 50 μg ml^{-1}, Sigma Chemicals, USA). Lymphocytes cultures were established in a 96-well microplate (200 μl aliquots per well) and incubated with CO_2 incubator under controlled conditions (5% CO_2, temp. 37°C, humidity 95%). Each culture were done in triplicate.

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Stimulation of lymphocytes proliferation and measurement

To stimulate lymphocytes proliferation phytohaemagglutinin-L (PHA-L, Roche Diagnostics, Sweden) was used in a concentration of 2.5 μg ml^{-1}. After 48 h of incubation $CdCl_2$ (POCH SA, Poland) was added to the culture in four different concentrations: 1 μM, 10 μM, 50 μM and 100 μM of Cd. For each experiment the negative control (non-treated cells) was included. Simultaneously [3H]-thymidine (Amersham, UK) was added in 1 μCi concentration per well. All samples were incubated for next 24 h. Ten repetitions of experiment for each Cd concentrations were conducted.

In order to measure lymphocytes proliferation, cultures were transferred by the harvester (SKATRON Instruments, Norway) on glass fiber filters (Perkin Elmer, USA), later placed in a scintillation cocktail (Perkin Elmer, USA). Measurement of thymidine incorporation was determined using scintillation counter (Perkin Elmer, USA). Results were expressed in counts per minute (CPM).

Reactive oxygen species production measurement

Heparinized samples of blood (1.5 ml) were collected from healthy donors at Regional Center of Blood and Blood Treatment in Poznan, Poland. 50 μl of blood samples were incubated in room temperature with $CdCl_2$ (POCH SA, Poland) in 2 ml tubes for 1 h with 1 μM, 10 μM and 100 μM of Cd concentrations. Simultaneously control samples without $CdCl_2$ were prepared and incubated under the same conditions. Two trials of control and Cd affected samples were conducted.

To evaluate the intensity of ROS production dihydrorhodamine (123 DHR, Sigma, USA) at the concentration of 0.1 mg ml^{-1} was added to all samples and incubated in CO_2 incubator under controlled conditions (5% CO_2, temp. 37°C, humidity 95%) for 5 minutes.

Afterwards the strong stimulator of the respiratory burst – phorbol 12-myristate 13-acetate (PMA, Sigma, USA) at the concentration of 40 μg ml^{-1} was added to one trial of samples which were further incubated for 15 minutes in darkness and room temperature. Second trial of samples was not stimulated with PMA and incubated under the same conditions. Following incubation, 1 ml of red blood cell lysis solution (prepared by ourselves) was added to both trials of samples. The process of red blood cell lysis was performed at room temperature for 10 minutes, in darkness.

The measurement of samples was conducted using FACScan flow cytometer (Becton Dickinson, USA). Based on forward and side scatters parameters (FSC and SSC), cells were fractioned by virtue of their size, shape and internal granularity, and in such a manner the population of neutrophils was identified, gated on a 2D-graph with a linear scale and taken for further analysis. Within a gate, 5,000 events were acquired. Reactive oxygen species production was assessed by the intracellular oxidative transformation of 123 DHR (dihydrorhodamine) to the fluorescent R 123 (rhodamine 123). Fluorescence of R 123 was measured using FL3 channel at 515-548 nm, within the green band of the spectrum, with logarithmic amplification of the signal. The mean fluorescence channel was used as measure of ROS production. Ten repetitions of experiment for each Cd concentrations were conducted.

Statistical methods

Statistic analysis were determined by Statistica 8.0 software (StatSoft, USA). Wilcoxon signed-rank test was used to compare control and Cd affected samples. P value < 0.05 was considered as statistically significant.

Results

Effect on lymphocytes proliferation

Results indicate that effect of cadmium chloride on lymphocyte proliferation after 48 h incubation is determined by its concentration. There were no significant differences between control cultures (free of $CdCl_2$) and affected by cadmium in 1 μM and 10 μM concentrations.

However thymidine incorporation was strongly inhibited by 50 μM and 100 μM, resulting in respectively 71% and 90% lower proliferation rate in comparison to control samples. Differences were statistically significant (Wilcoxon test, $p < 0.001$) (Fig. 1).

Effect on reactive oxygen species production

Experiments performed in the present study demonstrated that exposure to cadmium may affect ROS production in human peripheral blood neutrophils *in vitro*. In rest-

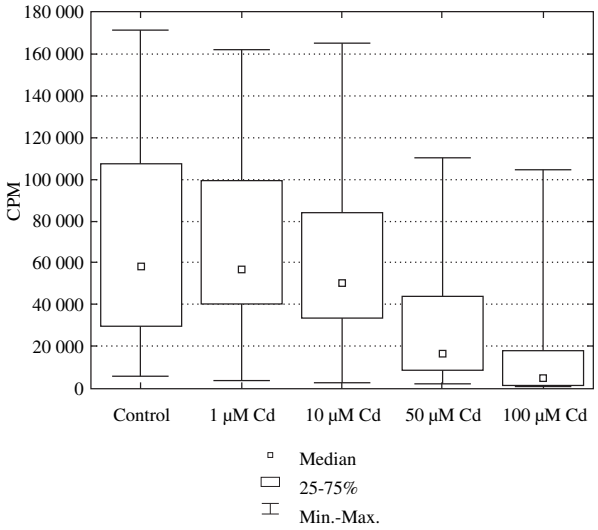

Fig. 1. Effect of different cadmium (Cd) concentrations on lymphocytes proliferation (CPM – counts per minute)

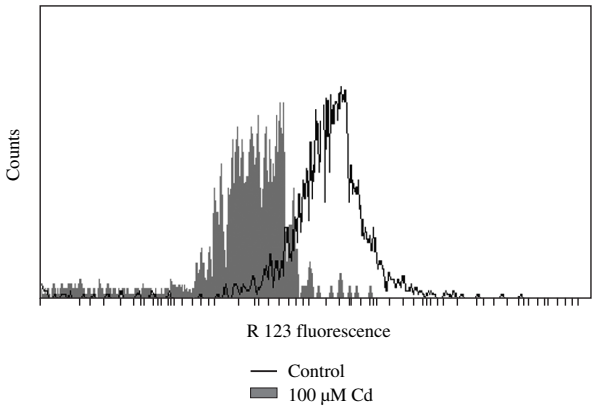

Fig. 2. Typical image of difference in rhodamine 123 fluorescence between control and 100 μM cadmium (Cd) affected samples for PMA-stimulated neutrophils

ing not stimulated cells, incubation with $CdCl_2$ caused a decrease in cell activity, i.e. ROS production. The effect was observed for every Cd concentration, but only 100 μM affected ROS production with statistic significance (Wilcoxon test, $p < 0.05$). However observed differences were small and resulted in 14% difference with control sample. The ROS production of PMA-stimulated cells also decreased with higher Cd concentrations. Statistically significant difference was observed for 100 μM concentration of Cd (Wilcoxon test, $p < 0.05$) and resulted in 25% difference in 123 DHR fluroescence with control sample (Fig. 2).

Discussion

Obtained results indicate that Cd might affect immune response in human. However significant impact on both, lymphocytes proliferation and ROS production in neutrophils was found only for higher concentrations, especially 100 μM of Cd. As described by Marth *et al.* [9] heavy metals may influence cells in two major ways: by penetration of the cell through calcium channels of the L-type and by reaction with surface structures of the cell.

The effect of Cd on human lymphocytes proliferation has been described so far only in the few publications [10]. Marth *et al.* [11] who studied the effect of $CdCl_2$ in 5-100 μM Cd concentrations obtained results similar to our observations. The most significant inhibition of proliferation was also observed for 100 μM concentrations. Interesting research was conducted by Jung *et al.* [12] who analyzed cadmium concentrations in the air and lymphocytes proliferation *in vivo* of exposed individuals and found strong negative correlation. Studies of Cd impact on immune system were also conducted in animal model i.e. rats, fishes and sheep [13-15]. However low doses of Cd were observed to slightly stimulate the lymphocytes proliferation, higher concentrations (10-100 μM) induced significant inhibition.

The inhibition of ROS production under heavy metals such as lead, mercury or copper has been already observed [16, 17]. As we already mentioned in introduction, contradictory effects of Cd on respiratory burst has been demonstrated. Zhong *et al.* [18] found that neutrophils incubated with Cd did not generate hydrogen peroxide (H_2O_2). On the other side, Freitas *et al.* [19] demonstrated that Cd induces neutrophils respiratory burst only in high concentrations (> 100 μM), mainly via activation of protein kinase C, precluding a significant contribution of other cellular pathways for ROS generation mediated by this metal. It should be noted that sustained overproduction of ROS may result in oxidative stress which is associated with detrimental effects, namely alterations on the normal function of lipids, proteins or DNA and as well as with aging, arteriosclerosis and cancer [20, 21]. Our study shows that Cd at higher concentrations reduces the ROS production in human neutrophils, both stimulated and not stimulated with PMA. This in turn leads to conclusion that innate immune response is disrupted and the protection against the pathogens might be lowered [22].

Our and other works indicate that exact Cd effect on immune responses, both innate and adaptive, is controversial although can not be excluded. Obtained results confirm previous studies of Cd impact on lymphocytes proliferation. It should be noted that levels of Cd contamination varies in the different places in the world [23]. Therefore individual and inter-population variability in Cd resistance and tolerance is possible and might be responsible for different observations of the impact on the ROS production as well as on the other parameters of immune response.

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