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Original Research

Effects of Mercury on the Proliferation of Human Peripheral Lymphocytes *in vitro*

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Abstract

Our project aimed to investigate the effects of mercury on the proliferation of human peripheral lymphocytes *in vitro*. The lymphocytes were isolated from the blood collected from healthy donors at Regionalne Centrum Krwiodawstwa i Krwiolecznictwa in Poznań, Poland. For the purpose of cell culture, the lymphocyte suspension ($25 \cdot 10^4$ cells/ml) in Eagle's medium supplemented with 10% fetal calf serum was prepared. Phytohaemagglutinin-L (PHA-L) was used in a concentration of 2.5 mg/ml to stimulate cell proliferation. Mercuric chloride ($HgCl_2$) in four different concentrations (1 μM, 10 μM, 50 μM, 100 μM) and [3H]-thymidine were added after 48 hours of incubation and the cell culture was continued for the next 24 hours. The rate of lymphocyte proliferation was measured by [3H]-thymidine incorporation method with a liquid scintillation counter.

Results indicate that higher concentrations of mercury (50 μM, 100 μM) inhibit the [3H]-thymidine incorporation of human peripheral lymphocytes *in vitro*. The incorporation was lower than the control sample by 65% at a concentration of 50 μM, while at a concentration of 100 μM it fell to virtually zero. Moreover, the phase of lymphocyte proliferation cycle affected by mercuric chloride was also investigated. For this purpose $HgCl_2$ in 2 concentrations (10 μM, 50 μM) was added to the cell culture in 4 different timepoints: at the start of the cell culture and after 4, 24, and 48 hours of incubation. After 48 hours, [3H]-thymidine was added and the cell culture was continued for an additional 24 hours. The rate of cell proliferation was estimated by [3H]-thymidine incorporation using a liquid scintillation counter. The inhibition effect was observed in samples with metal added at the start of the cell culture and after 4 h of incubation, i.e. at the initial phase of the lymphocyte proliferation cycle.

Keywords: peripheral blood lymphocytes, proliferation, mercury, heavy metal

Introduction

An increase in industrialization has led to global expansion of heavy metals, including mercury [1, 2].

Mercury occurs in three different forms: elementary, organic (MeHg), and inorganic (mercury ions) [1-3]. Sources of potential Hg exposure include: inhalation of Hg vapours, ingestion of contaminated water, fish and sea food, the production of lamps and batteries, the mining industry, and use of amalgam fillings in dentistry [1, 2, 4, 5].

Mercury has neuro-, geno- and immunotoxic properties [2, 6, 7]. It can affect the cell cycle by production of free radicals and oxidation stress, affecting cariokinetic spindle

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mictotubules, disrupting of DNA repair mechanisms, and directing interactions with nucleid acid elements [2].

The mechanism responsible for immunotoxic activity of mercuric chloride (II) is not precisely known. Studies have demonstrated that mercury (through its interaction with sulfhydryl groups on membrane proteins) disrupts cellular signaling pathways [8-10].

The aim of this study was to investigate the effect of different concentrations of mercuric chloride (II) on thymidine incorporation.

The presented issues are preliminary studies intended to provide a direction for further research on the effect of mercury on the proliferation of human peripheral blood lymphocytes.

Methods

Heparinized samples of blood (8 ml) were collected from healthy donors at Regionalne Centrum Krwiodawstwa i Krwiolecznictwa in Poznań, Poland. 100 µl/100 ml of gentamycine (Gentamycine, SIGMA) was added as a preservation to Eagle's liquid (Eagle's medium, BIOMED). A medium was later used to isolate lymphocytes and culture growth.

In order to isolate lymphocytes from blood, it was mixed with Eagle's medium in 1:1 ratio and centrifuged (25 minutes, 1750 RPM), temp 5ºC over 5 ml of Gradisol-L (Gradisol-L, AQUA-MED). Cells were washed twice. The lymphocyte suspension ($25 \cdot 10^4$ cells/ml) in Eagle's medium (Eagle's medium, BIOMED) was supplemented with 10% fetal bovine serum (Fetal Bovine Serum; SIGMA).

To stimulate proliferation, phytohaemagglutinin-L (PHA-L, Roche Diagnostics) was used in a concentration of 2.5 µg/ml. Cultures were incubated with CO_2 incubator (BINDER) under controlled conditions (5% CO_2, temp. 37ºC humidity 95%).

In the first part of experiment after 48 h of incubation $HgCl_2$ (POCH SA) was added to the culture in four different concentrations (1 µM, 10 µM, 50 µM, and 100 µM $HgCl_2$). Simultaneously, [3H]-thymidine ([3H]-thymidine, Amersham) was added in 1 µCi/well concentration and incubated for the next 24 h.

The second part of the experiment investigated which phase of lymphocyte proliferation is affected by $HgCl_2$. Cultures were prepared as above, and two concentration of $HgCl_2$ were added (10 µM, 50 µM) in four time intervals: at the beginning of culture (0 h), and after 4 h, 24 h, and 48 h of incubation.

Fifteen attempts were conducted in every part of the experiment.

The following sample marks were used: K – control, 1 – 1 µM $HgCl_2$, 10 – 10 µM $HgCl_2$, 50 – 50 µM $HgCl_2$, and 100 – 100 µM $HgCl_2$.

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

Statistical analysis was determined by Statistica 8.0 software (StatSoft, U.S.A.). Lilliefors test was used in order to examine normality of distribution. In the first part of the experiment, the Wilcoxon signed-rank test was used to compare control and $HgCl_2$-affected samples. In the second part of the experiment Kruskal-Wallis one-way analysis of variance was used with post-hoc Tukey test. P value <0.05 was considered statistically significant.

Results and Discussion

Mean thymidine incorporation in cultures affected by mercuric chloride (II) was lower than mean incorporation in control cultures, free of mercuric chloride (II) (Fig. 1).

Statistically significant changes of thymidine incorporation was observed within cell cultures under the effect of mercury salt in concentrations of 50 µM and 100 µM $HgCl_2$ ($p<0.01$).

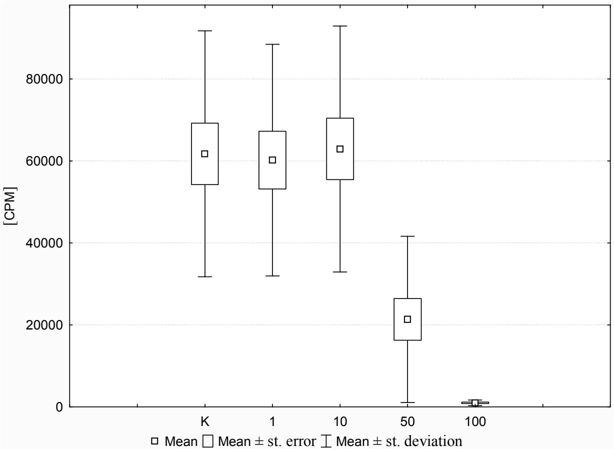

Fig. 1. Effect of mercuric chloride (II) on thymidine incorporation (samples marked as in methodology).

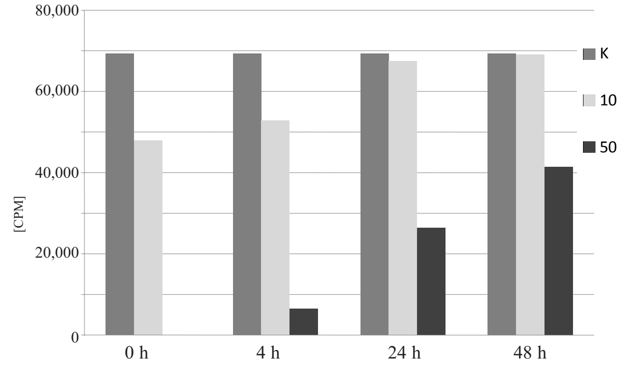

Fig. 2. Effect of mercuric chloride (II) thymidine incorporation (samples marked as in methodology).

Table. 1. Comparison of thymidine incorporation in control and in researched samples dependent on time of $HgCl_2$ addition.

Time of metal addition [h]	Compared samples	Kruskal-Wallis test
0	Kvs10	ns
	Kvs50	**p<0.05**
4	Kvs10	ns
	Kvs50	**p<0.05**
24	Kvs10	ns
	Kvs50	**p<0.05**
48	Kvs10	ns
	Kvs50	**p<0.05**

Statistically significant results were bolded, ns – statistically insignificant.

The second experiment reported a decrease of mean thymidine incorporation depending on time of mercury addition. The sooner mercuric chloride (II) was added, the stronger thymidine incorporation inhibition was observed (Fig. 2).

Samples with metal added at the start of the cell culture (0 h) have shown a decrease of mean thymidine incorporation to 69% (10 µM) and 0.1% (50 µM). Values of incorporation in next-time intervals were shaped as follows: 4 h: 76% (10 µM) 9% (50 µM); 24 h: 97% (10 µM) 38% (50 µM); and 48 h: 99% (10 µM) 60% (50 µM).

Also, it was demonstrated that only 50 µM concentrations of $HgCl_2$ affected thymidine incorporation statistically significantly (Table 1).

Mercuric chloride (II) has a considerable effect on thymidine incorporation. It differs depending on metal concentration and time of its addition to cell culture. In the presented investigation decrease of mean thymidine incorporation under mercuric chloride (II), activity was demonstrated. 50 µM and 100 µM concentrations inhibit incorporation most significantly. We also demonstrated that the effect of mercury is strongest at initial phases of the cell cycle – between G1 and S phases.

Based on statistical analysis, cytotoxic impact of high mercury concentrations can be concluded.

The negative effect of mercury salt on a cell's immune system has been widely described, although origins of a disruption mechanism were not clearly demonstrated. Ben-Ozer observations showed inhibitory effect of high mercury concentrations on proliferation of human immune system cells. The following concentrations of $HgCl_2$ were investigated: 0.1, 0.5, 1, 5, 10, 50, 100 µM, 50 µM, and 100 µM concentrations decreased proliferation to 5% level. In lower concentrations a decreasing effect was not observed [11]. Our research confirms these conclusions. Ben-Ozer investigation also demonstrated that DNA damages emerge under mercury doses acknowledged as nontoxic for cells (concentrations below 10 µM). It was proposed that a cell's DNA damage can be caused by factors independent of apoptosis – the production of free oxygen radicals (ROS) or reactive oxygen metabolites (ROM) [11]. It can lead to an increase of chromosome aberrations [12-14] and the formation of oxidation stress that causes an increase of 8-hydroxydeoxyguanosine [14].

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