# The proliferation rate and phase growth of human peripheral lymphocytes *in vitro* under zinc influence

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***Abstract***

*The effect of zinc chloride ($ZnCl_2$) upon the proliferation of human peripheral lymphocytes in vitro was investigated. Zinc chloride in four concentrations (1 μM, 10 μM, 50 μM, 100 μM) was added to the lymphocytes cultures. Results indicate that high concentrations of $ZnCl_2$ (100 μM) slightly inhibit the proliferation of human peripheral lymphocytes in vitro. The phase of lymphocyte proliferation cycle affected by $ZnCl_2$ was also investigated. For this purpose $ZnCl_2$ in two concentrations (50 μM, 100 μM) was added to the cell culture in 4 different time points: at the start of the cell culture, after 4 hours, 24 hours and 48 hours of incubation. The sooner $ZnCl_2$ was added the stronger inhibition of lymphocytes proliferation was observed; the greatest change was given by 100 μM concentration of $ZnCl_2$.*

***Key words:*** *peripheral blood lymphocytes, proliferation, zinc.*

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## Introduction

Zinc (Zn) is a trace element essential to normal growth of all living organisms. Human body contains around 2-4 g of Zn, but its physiological concentration in plasma is low and tightly regulate in 12-16 μM/L range. In human, serum zinc is protein bound: albumin (60%), transferrin (10%) and $\alpha_2$-macroglobulin (30%). Zinc is cofactor of over 300 enzymes, participates in cell processes such as signal transduction, transcription and replication [1-6].

Zinc is also necessary to proper immune system functioning. Zinc deficiency can lead to lower activity of immune system cells including T-lymphocytes, NK cells, macrophages and neutrophiles; it also decrease production of cytokines and affects apoptosis [1, 2, 5, 7, 8]. It has been shown that zinc deficiency decreases proliferation of lymphocytes after mitogen stimulation [4, 9, 10].

However, it was observed that zinc excess can be harmful causing similar symptoms to its deficiency – including inhibition of lymphocytes proliferation [1-3].

The aim of the following study was to examine the effect of different zinc chloride ($ZnCl_2$) concentrations on peripheral blood lymphocytes proliferation *in vitro*. It was also verified which phase of lymphocytes proliferation is affected by $ZnCl_2$.

## Material and methods

Heparinized samples of blood (8 ml) were collected from healthy donors at Regionalne Centrum Krwiodawstwa i Krwiolecznictwa in Poznań, Poland. Gentamycine (Sigma Chemicals, St. Louis, USA) was added as a preservation to Eagle's liquid (Biomed, Poland). Medium was later used to isolate lymphocytes and culture growing.

In order to isolate lymphocytes from blood it was mixed with Eagle's medium in 1 : 1 ratio and centrifugated (25 minutes, 1750 RPM, temp. 5°C) over 5 ml of Gradisol-L (Aqua-Med, Poland). Cells were washed twice. The lymphocyte suspension ($25 \times 10^4$ cells/ml) in Eagle's medium was supplemented with 10% fetal bovine serum (Sigma Chemicals, St. Louis, USA). To stimulate proliferation phytohaemagglutinin-L (PHA-L, Roche Diagnostics, Sweden) was used in a concentration of 2.5 μg/ml. Cultures were incubated with CO2 incubator

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under controlled conditions (5% $CO_2$, temp. 37°C, humidity 95%).

In the first part of experiment after 48 h of incubation $ZnCl_2$ (POCH SA) was added to the culture in four different concentrations (1 μM, 10 μM, 50 μM and 100 μM). Simultaneously [3H]-thymidine (Amersham, UK) was added in 1 μCi/well concentration and incubated for next 24 h.

Second part of experiment investigated which phase of lymphocytes proliferation is affected by $ZnCl_2$. Cultures were prepared as above, two concentration of $ZnCl_2$ were added (50 μM, 100 μM) in four time intervals: at the beginning of culture (0 h), after 4 h, 24 h and 48 h of incubation.

Twenty attempts were conducted in every part of the experiment.

Following samples marks were used: C – control; 1 – 1 μM $ZnCl_2$; 10 – 10 μM $ZnCl_2$; 50 – 50 μM $ZnCl_2$; 100 – 100 μM $ZnCl_2$.

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

Statistic analysis were determined by Statistica 8.0 software (StatSoft, USA). Wilcoxon signed-rank test was used to compare control and $ZnCl_2$ affected samples. *P* value < 0.05 was considered as statistically significant.

## Results

Results indicates that effect of zinc chloride on lymphocyte proliferation after 48 h is determined by its concentration. Mean thymidine incorporation in cultures affected by $ZnCl_2$ was lower than mean thymidine incorporation in the control cultures (free of $ZnCl_2$) only for 100 μM $ZnCl_2$ concentration (Fig. 1). Difference was statistically significant (Wilcoxon test, $p < 0.05$).

Effect of 1 μM, 10 μM and 50 μM zinc chloride concentrations on investigated process were inconsiderable and statistically insignificant.

In the second part of experiment relation between time of $ZnCl_2$ addition and lymphocyte proliferation was demonstrated. The sooner zinc chloride was added, the stronger inhibition of thymidine incorporation was observed; the greatest change was given by 100 μM concentration of $ZnCl_2$ (Fig. 2). Statistically significant differences between control and zinc-affected samples (50 μM and 100 μM) were observed when zinc was added at the beginning (0 h) and after 4 hours of the cell culture. Addition of $ZnCl_2$ after 48 h of cell culturing resulted in statistically significant difference with control sample only for 100 μM concentration (Wilcoxon test, $p < 0.01$).

Samples with $ZnCl_2$ added at the start of the cell culture have shown a decrease of mean thymidine incorporation to 84% (50 μM) and 59% (100 μM) in comparison to control. In subsequent time intervals following values were observed: 4 h: 90% (50 μM) and 72% (100 μM); 24 h: 102% (50 μM) and 93% (100 μM) and 48 h: 102% (50 μM) and 81% (100 μM).

## Discussion

In our research the effect of zinc on lymphocytes proliferation was determined by its concentration in cell culture. Only 100 μM $ZnCl_2$ concentration significantly inhibited tested process.

As mentioned before, zinc excess, as well as its deficiency, can negatively affect immune system, including lymphocytes proliferation. Moreover, zinc can stimulate or inhibit the same process depending on its concentration [1]. Ryu *et al.* who studied thymidine incorporation in mouse embryonic stem cells observed that low (40 μM)

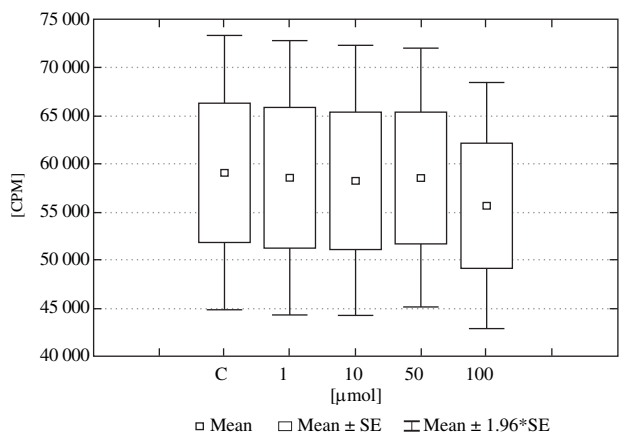


**Fig. 1.** Effect of different $ZnCl_2$ concentrations on lymphocyte proliferation (CPM – counts per minute, samples marked as in methodology)

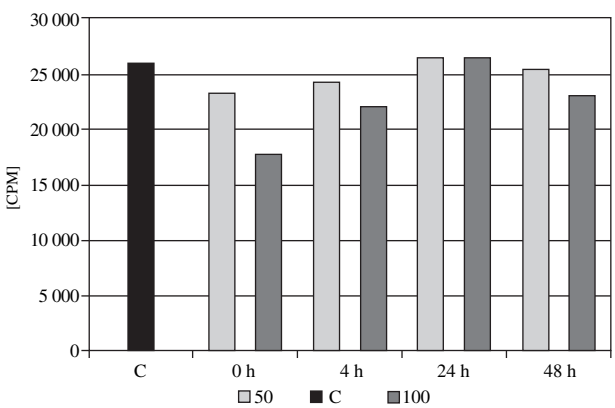


**Fig. 2.** Time-dependent effect of $ZnCl_2$ on lymphocyte proliferation (CPM – counts per minute, samples marked as in methodology)

concentrations of $ZnCl_2$ stimulates the process, while higher doses (≥ 200 μM) blocks the proliferative effect [11].

High zinc concentrations, exceeding physiological concentration 7-8 times (circa 100 μM) can inhibit lymphocytes functions and its proliferation [1-3].

Inhibition of lymphocytes proliferation by high concentrations of Zn was also observed by others [12-14]. Chang *et al.* demonstrated that physiologic concentrations of zinc has no effect on human peripheral blood mononuclear cells, higher doses (≥100 μM) decreased cell proliferation and induced apoptosis [12]. Wellinghausen *et al*. [13] suggests that observed inhibition effect is connected with inhibition of type-1 receptor for IL-1 associated with protein kinase (IRAK) and decrease of signal transduction at very early stage. It was also observed that 100 μM concentration stimulates monocytes, but inhibits T-lymphocytes activation due to its lower zinc content in cells, resulting in higher sensitivity. Concentration higher 3-4 times (60 μM) than physiological doesn't lead to T-lymphocytes suppression but inhibits alloreactivity of mixed lymphocytes cultures MLC [14].

Viability of normal human lymphocytes and human myelogenous leukemia K562 cells exposed to different zinc concentrations was also studies [15]. Zinc evoked a concentration-dependent decrease in the viability of both types of cells, but normal lymphocytes much better survived zinc treatment than the cancer cells. Also some of the latest studies indicates that high concentrations of zinc can inhibit proliferation of some cancer cells such as Eca 109 [16].

In our research inhibition of lymphocytes proliferation was most significant for samples affected by $ZnCl_2$ at the beginning of *in vitro* cultures (0 h). It means that high concentrations of zinc have negative effect on early phases of signal transduction after mitogen stimulation, what corresponds with Wellinghausen *et al.* [14] research.

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