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PRELIMINARY STUDIES ON THE EFFECT OF ZINC AND SELENIUM ON VANADIUM-INDUCED CYTOTOXICITY *IN VITRO*

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In the present work, we investigated the cytotoxicity of vanadium and the influence of zinc and selenium on vanadium-dependent cell damage in the BALB/c 3T3 cell culture. Treatment of cells for 24 hours with medium containing 50, 100 and 200 μM $NaVO_3$ caused a significant decrease in the cell viability as measured by MTT test. Furthermore, the assays for reactive oxygen species (NBT reduction and phenol red oxidation) demonstrated the increase in superoxide and hydrogen peroxide production. In the cotreatment studies, the cells were exposed to $NaVO_3$ (50, 100 and 200 μM) in the presence of nontoxic concentrations of $ZnCl_2$ (5 μM) or Na_2SeO_3 (0.5 μM). Following 24 h incubation, the cell viability (assessed in MTT assay) and reactive oxygen species generation were evaluated. Our data suggest that zinc and selenium, in the concentrations mentioned above, provide no protection against adverse actions induced by sodium metavanadate at concentration levels of 50, 100 and 200 μM. To our knowledge, this is the first report from *in vitro* studies on interaction between pentavalent vanadium and trace elements that function as antioxidants: zinc and selenium.

Keywords: Cell culture – vanadium – zinc – selenium – reactive oxygen species

INTRODUCTION

Vanadium, zinc and selenium are trace elements, essential for normal growth and development of animals and humans. Zinc and selenium are often described as antioxidants. The precise physiological function of vanadium remains to be elucidated. Nonetheless, a lot of scientific attention is paid to pharmacological actions of this metal. The vanadium compounds were suggested to be used as therapeutic agents in obesity [19], insuline substitutes in the treatment of diabetes mellitus [27] or antitumor agents [3].

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However, the cytotoxic actions of vanadium excess are well documented both in occupationally exposed groups and in experiments involving *in vivo* and *in vitro* studies. Inhalation of vanadium dust by workers engaged in the industrial production and the use of vanadium (e.g. steel, chemistry industry) causes symptoms of respiratory tract irritation [32]. In rats, which consumed vanadium in drinking water, the number of circulating erythrocytes, hemoglobin content [35, 36] and phagocytic activity of neutrophils [34] were decreased. In the cell culture systems, vanadium compounds at high micromolar concentrations were shown to cause alternations in cell morphology [1], inhibit cell proliferation [6] and induce lipid peroxidation [5]. According to some authors, vanadium-dependent damage of mitochondria is the main mechanism of cytotoxicity induced by this metal [8, 15].

Zinc is a vital component of the oxidant defence system. Zinc-dependent antioxidant mechanisms involve: protection of sulfhydryl groups of proteins and enzymes against oxidation, stabilization of biological membranes through iron and copper antagonism as well as synthesis induction of some antioxidants, e.g. metallothioneins [29, 31]. The other element, selenium, as selenocysteine is an essential constituent of glutathione peroxidase. The primary function of this enzyme is to counteract oxidative attack. The antioxidant effects of selenium is often brought into connection with the increased activity of glutathione peroxidase [7, 37].

One of the reasons for the wide interest in vanadium toxicity is the fact that adverse actions of this metal still constitute a hindrance to its use as a drug in medicine. In contrast, zinc and selenium have been suggested to exert protective effects against cytotoxicity induced by heavy metals such as lead, copper, cadmium or arsenic [9, 10, 12, 14, 21, 25, 33]. What is more, zinc and selenium are frequently used as antioxidants in dietary supplements together with vanadium. However, zinc-vanadium and selenium-vanadium interactions are poorly examined. Therefore, this paper demonstrates a preliminary investigation of the effects of zinc and selenium, used at selected concentrations, on vanadium-induced cell damage *in vitro*.

MATERIALS AND METHODS

Chemicals and materials

Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), antibiotic-antimycotic solution (10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 μg/ml amphotericin B), sodium metavanadate ($NaVO_3$), zinc chloride ($ZnCl_2$), sodium selenite (Na_2SeO_3), phenol red, Hanks' Balanced Salt Solution (HBSS) and horseradish peroxidase were all obtained from Sigma-Aldrich (St. Louis, MO, USA). TACS^{TM}MTT Assay was from R&D Systems Europe (UK). Nitroblue tetrazolium (NBT) and sodium peroxide were purchased from Polish Chemical Reagent (Gliwice, Poland). Trypsin (0.25% solution) and phosphate buffered saline were obtained from BIOMED (Lublin, Poland). Tissue culture dishes (plastic flasks and 96-well plates) were from NUNC (Germany).

The individual metal compounds of vanadium ($NaVO_3$), zinc ($ZnCl_2$) and selenium (Na_2SeO_3) were dissolved in deionized water to prepare 10 mM stock solutions. The solutions were kept in refrigerator. On the day of experiments, concentrated stock solutions were diluted in DMEM to 1 mM working solutions before the addition to culture medium.

Cell culture

The mouse BALB/c 3T3 embryo fibroblasts (hereafter named BALB/3T3) were a generous gift from Dr. D. Śladowski (Department of Transplantology & Central Tissue Bank, Center of Biostructure Medical University of Warsaw). The cells were grown in 75 cm^2 plastic flasks at 37 °C in DMEM supplemented with 5% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution in a humidified atmosphere of 95% air and 5% CO_2. The cells were subcultured three times a week using 0.25% trypsin solution.

Treatment of cells with $NaVO_3$ *alone and in combination with* $ZnCl_2$ *or* Na_2SeO_3

For the experiments, newly confluent cell layers were trypsinised, resuspended in culture medium containing 4% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution and seeded in sterile 96-well plates at a density of 5×10^4/ml (100 µl/well). The cells were cultured for 24 hours at 37°C and in 5% CO_2. Next, the medium was replaced with the fresh medium (supplemented with 3% v/v FBS and 1% v/v antibiotic-antimycotic solution) without $NaVO_3$ addition (control) or with the addition of $NaVO_3$ at 20, 50, 100 or 200 µM. After 24 h incubation, the cell viability (measured by MTT assay), superoxide and hydrogen peroxide production were determined as described further under "Materials and Methods".

For the cotreatment studies the cells were seeded into 96-well plates at a density of 5000 cells per well in 100 µl of culture medium supplemented with 4% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution and allowed to grow for 24 hours at 37 °C in an atmosphere of 95% air and 5% CO_2. Next, the medium was replaced with the fresh medium containing 3% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution without metal compounds addition (control) or with the addition of:

(1) 50 µM $NaVO_3$
(2) 100 µM $NaVO_3$
(3) 200 µM $NaVO_3$
(4) 5 µM $ZnCl_2$
(5) 0.5 µM Na_2SeO_3
(6) 50 µM $NaVO_3$ + 5 µM $ZnCl_2$
(7) 100 µM $NaVO_3$ + 5 µM $ZnCl_2$
(8) 200 µM $NaVO_3$ + 5 µM $ZnCl_2$

(9) 50 μM $NaVO_3$ + 0.5 μM Na_2SeO_3
(10) 100 μM $NaVO_3$ + 0.5 μM Na_2SeO_3
(11) 200 μM $NaVO_3$ + 0.5 μM Na_2SeO_3

and cultivated for another 24 h. The non-toxic concentrations of $ZnCl_2$ (5 μM) and Na_2SeO_3 (0.5 μM) were established on the basis of our preliminary experiments (Table 1). Following incubation period, the cells were submitted to the MTT, superoxide and hydrogen peroxide assays as described below.

Cell viability

After experimental treatments, the cell viability was determined using the MTT assay. This test monitors the conversion of soluble yellow tetrazolium compound MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to insoluble purple formazan by mitochondrial dehydrogenases in viable cells.

The MTT assay was performed according to the manufacturer's procedure. For the assay, 10 μl of MTT reagent was added to each well. The plate was returned to the incubator (37 °C, 5% CO_2) for 3 h. Detergent reagent was added at 100 μl per well to dissolve the coloured formazan crystals. After 2 h incubation at 37 °C the absorbance of the wells was measured at 570 nm with a reference setting of 630 nm on a microplate spectrophotometer. Blank values containing only culture medium (without cells), MTT and detergent were subtracted from all samples.

The mean absorbance of the control wells, which received no metal compounds, was regarded as 100% and the viability of exposed cells was calculated as follows:

Cell viability (% of control) = (mean absorbance of exposed cells/mean absorbance of control cells) × 100

The experiments were independently repeated three times with 3 wells per control and exposed cells ($n = 9$).

Measurement of superoxide anion production

The superoxide anion content was determined by nitroblue teterazolium (NBT) reduction microassay according to the method by Pick [20]. The assay is based on an intracellular reduction of NBT by superoxide-dependent reactions leading to the formation of insoluble formazan.

After experimental treatments the cells were covered with 100 μl/well of a 1 mg/ml solution of NBT in HBSS. The plate was placed in a humidified incubator at 37 °C in 95% air – 5% CO_2 for 90 minutes. The generation of insoluble formazan was verified by microscopic examination. The amount of reduced NBT (precipitated formazan) accumulating in the cells was measured in the spectrophotometer at 550 nm absorbance after performing the blanking procedure on the wells containing only NBT solution (1 mg/ml) without cells.

Finally, the results were expressed as absorbance of formazan formed per milligram protein during 90 minutes. Four independent sets of experiments were done with 3 wells per control and exposed cells ($n = 12$).

Hydrogen peroxide assay

The level of H_2O_2 was measured according to the method by Pick [20]. The principle of the assay is based on horseradish peroxidase dependent oxidation of phenol red by hydrogen peroxide leading to the formation of a compound that, at alkaline pH exhibits increased absorbance at 600 nm.

After experimental treatments, the cultures were rinsed with the Hanks' Balanced Salt Solution (HBSS). Next, the cells were covered with 100 µl/well of HBSS containing phenol red (0.2 g/L) and horseradish peroxidase (20 U/ml) and incubated for 90 minutes at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The reaction was stopped by adding 10 µl/well of 1N NaOH. The absorbance of the experimental wells was read at 600 nm against the blank wells on a microplate spectrophotometer. Blank wells contained only phenol red – horseradish peroxidase solution (100 µl) + 10 µl of 1N NaOH (without cells).

The concentration of H_2O_2 (nanomoles H_2O_2/wells) was calculated using the following formula:

Nanomoles H_2O_2 per well = absorbance at 600 nm × 16.7 (based on the phenol red extinction coefficient: $\Delta E_{600\ nm} = 19.8 \times 10^3\ M^{-1}\ cm^{-1}$)

Results were finally expressed as nanomoles H_2O_2 produced per miligram of protein during 90 minutes. Four independent sets of experiments were done with 3 wells per control and exposed cells ($n = 12$).

Protein determination

Cell protein concentration was determined in parallel with the NBT and H_2O_2 assay by bicinchoninic acid (BCA) assay (Sigma – Aldrich).

The cells were washed once with phosphate-buffered saline, suspended in deionized water and subjected to two freeze-thaw cycles. Each freeze-thaw cycle consisted of freezing (–80 °C) for 10 minutes followed by thawing at room temperature. Next, cell debris was removed by centrifugation and protein content in cell supernatants was determined using 96 well plates according to the manufacturer's instructions with bovine serum albumine (BSA) as a standard.

For protein assay, cell samples (75 µl of cell supernatant + 75 µl deionized water/well) were prepared on microplates. Next, 150 µl of QuantiPro Working Reagent was added to each well and plates were incubated at 37 °C for two hours. The absorbance at 562 nm of each sample was found by subtracting the absorbance of the blank (150 µl of dH_2O + 150 µl QuantiPro Working Reagent) from the absorbance for the cell samples. The protein content was calculated from a standard curve prepared with known BSA standard concentrations.

Statistics

All results in the text are expressed as means ± S.D. Statistical analyses were performed with the SPSS 14.0 PL for Windows. The concentration-effect data were evaluated for statistical significance using one-way ANOVA with Dunnett test to compare control means with each of the concentration means. Data showing the influence of $ZnCl_2$ or Na_2SeO_3 on $NaVO_3$-induced cytotoxicity (cotreatment studies) were analysed using one-way ANOVA followed by the Tukey post-test ($p < 0.05$ was considered statistically significant).

RESULTS

The viability of the cells and reactive oxygen species production after treatment with $NaVO_3$

A dose-dependent decrease in the viability of BALB/3T3 fibroblasts was observed after 24 h exposure to 20, 50, 100 and 200 μM $NaVO_3$, as measured by MTT assay (Fig. 1).

Superoxide and hydrogen peroxide production in BALB/3T3 cells was elevated following 24 h incubation in medium with 20–200 μM $NaVO_3$. The increase in superoxide content was substantial after exposure to all tested concentrations of $NaVO_3$ (Fig. 2), while hydrogen peroxide production rose significantly in the concentration range of 50–200 μM $NaVO_3$ (Fig. 3).

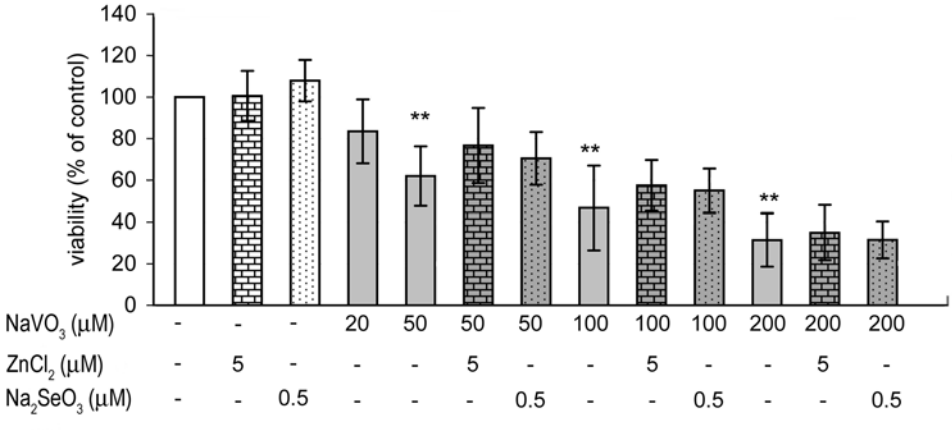

Fig. 1. The viability of BALB/3T3 cells following 24 h incubation in medium supplemented with $NaVO_3$ alone and $NaVO_3$ in the presence of 5 μM $ZnCl_2$ or 0.5 μM Na_2SeO_3 measured by MTT assay. Data are expressed as a percentage of the control cultures (= 100%). Each bar represents the mean (± S.D.) from three independent experiments carried out in triplicates ($n = 9$). Significance is indicated by asterisks: ***P*-value < 0.001 in comparison to control cells incubated in medium without the metal compounds addition (Tukey test)

The viability of the cells incubated in medium supplemented with $NaVO_3$ + $ZnCl_2$ *or* $NaVO_3$ + Na_2SeO_3

For cotreatment experiments, we selected three doses of $NaVO_3$ (50, 100 and 200 μM), which considerably decreased the viability of cells (MTT assay). Neither $ZnCl_2$ at 5 μM nor 0.5 μM Na_2SeO_3 were cytotoxic in our culture conditions. The concentrations of $ZnCl_2$ and Na_2SeO_3 were established on the basis of our preliminary experiments (mitotic index, Table 1). The preliminary studies showed that incubation of cells with $ZnCl_2$ at concentration less than 20 μM (5 and 10 μM) had no effect on mitotic divisions of BALB/3T3 fibroblasts (we even observed a minimal increase in mitotic index for 5 μM $ZnCl_2$ in comparison with control, Table 1). Sodium selenite, starting from 2.5 μM, significantly inhibited mitosis and mitotic index in BALB/3T3 cells. Thus, we selected 5 μM $ZnCl_2$ and 0.5 μM Na_2SeO_3 as non-toxic concentrations of zinc and selenium in our experimental conditions. Accordingly, for concurrent incubations, $NaVO_3$ was added to the medium together with 5 μM $ZnCl_2$ or 0.5 μM Na_2SeO_3.

Table 1
Mitotic index in culture of BALB/3T3 cells following 24 h incubation in medium supplemented with 5–20 μM $ZnCl_2$ or 1–5 μM Na_2SeO_3 (two independent experiments were done in triplicate)

Analysis	Control	$ZnCl_2$			Na_2SeO_3		
		5 μM	10 μM	20 μM	1 μM	2.5 μM	5 μM
The number of cells examined	29,240	14,040	10,059	13,945	9037	14,093	13,917
The number of mitotic cells	2666	1432	916	954	800	830	30
Mitotic index (‰)	91.2	102	91.1	68.4*	88.5	58.9*	2.2*

Statistically significant in comparison to the control *$p < 0.001$.

The addition of 5 μM $ZnCl_2$ to cultures incubated with 50, 100 and 200 μM $NaVO_3$ had no effect on $NaVO_3$-induced cytotoxicity. Similarly, the employed selenite concentration (0.5 μM Na_2SeO_3) did not influence the viability of cells treated with $NaVO_3$ (Fig. 1).

The effect of $ZnCl_2$ *on* $NaVO_3$*-induced superoxide and hydrogen peroxide production*

The addition of 5 μM $ZnCl_2$ to cultures incubated with 50, 100 and 200 μM $NaVO_3$ had no effect on $NaVO_3$-induced superoxide anion generation (Fig. 2).

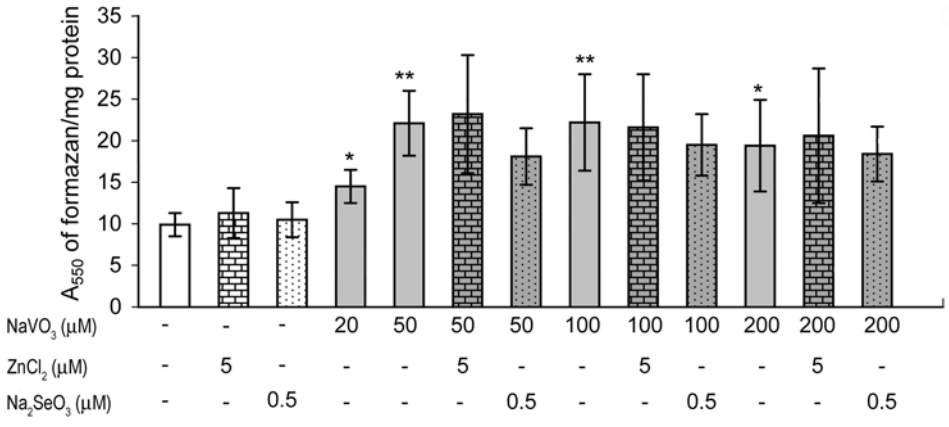

Fig. 2. Level of superoxide anion (measured by NBT reduction assay) in BALB/3T3 cells following 24 h incubation in medium supplemented with $NaVO_3$ alone and $NaVO_3$ in the presence of 5 μM $ZnCl_2$ or 0.5 μM Na_2SeO_3. Each bar represents the mean (± S.D.) from four independent experiments carried out in triplicates ($n = 12$). Significance is indicated by asterisks: *P-value < 0.01, **P-value < 0.001 in comparison to control cells incubated in medium without the metal compounds addition (Tukey test)

Vanadium-dependent hydrogen peroxide production by cells incubated with 50 or 100 μM $NaVO_3$ was minimally altered when 5 μM $ZnCl_2$ was added to the medium (Fig. 3). In cells treated with 50 and 100 μM $NaVO_3$ alone, the H_2O_2 contents were 536 and 650 nM, whereas in cultures treated with 50 and 100 mM $NaVO_3$ in the presence of 5 μM $ZnCl_2$ the H_2O_2 levels were 478 and 520 nM, respectively but the differences were far from significance ($p = 0.958$ and 0.394). The addition of 5 μM $ZnCl_2$ to cultures exposed to 200 μM $NaVO_3$ did not have any effect on $NaVO_3$-induced H_2O_2 production.

The effect of Na_2SeO_3 *on* $NaVO_3$*-induced superoxide and hydrogen peroxide production*

Selenite (0.5 μM) minimally reduced the vanadate-enhanced superoxide generation in cultures treated with 50 and 100 μM $NaVO_3$ (p-values: 0.236 and 0.666, respectively, Fig. 2). However, the addition of selenite to the medium of cells treated with 200 μM $NaVO_3$ had no effect on $NaVO_3$-dependent rise in superoxide production compared to respective vanadium control (200 μM $NaVO_3$).

The hydrogen peroxide concentrations in cells incubated with 0.5 μM Na_2SeO_3 and 50 or 100 μM $NaVO_3$ were comparable with H_2O_2 contents in respective vanadium controls (50 or 100 μM $NaVO_3$ alone, Fig. 3). However, the presence of 0.5 μM Na_2SeO_3 in cultures treated with 200 μM $NaVO_3$ minimally increased the vanadium-dependent H_2O_2 production, but the increase was not statistically significant.

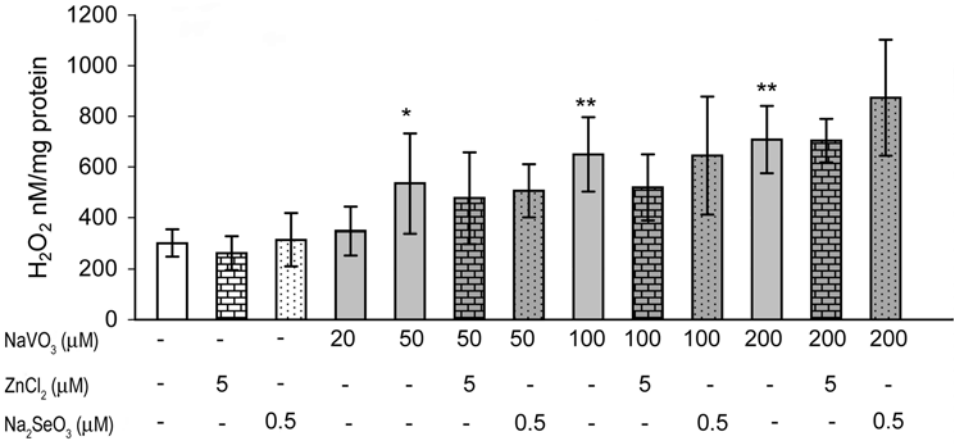

Fig. 3. Level of hydrogen peroxide (measured by oxidation of phenol red) in BALB/3T3 cells following 24 h incubation in medium supplemented with $NaVO_3$ alone and $NaVO_3$ in the presence of 5 μM $ZnCl_2$ or 0.5 μM Na_2SeO_3. Each bar represents the mean (±S.D.) from four independent experiments carried out in triplicates ($n = 12$). Significance is indicated by asterisks: **P*-value < 0.01, ***P*-value < 0.001 in comparison to control cells incubated in medium without the metal compounds addition (Tukey test)

DISCUSSION

The main goal of this work was to find out whether zinc or selenium, which are often described as antioxidants, affect vanadium-induced cytotoxicity in cell culture. We conducted our experiments using the mouse BALB/c 3T3 embryo fibroblasts, since this cell line turned out to be a reliable experimental model in studying the cytotoxicity of metal compounds *in vitro* [16, 17]. The MTT assay was used to measure the cell viability. The marker of viability, which is determined in this test, is the metabolic activity of mitochondria [22]. We also investigated the intracellular level of superoxide and hydrogen peroxide, as an oxidative stress marker.

In the first part of the study, we observed that $NaVO_3$ at 50–200 μM caused a dose-dependent loss of cell viability in parallel with an enhancement of superoxide and hydrogen peroxide production. Oxidative stress thus appears to be connected with the cytotoxicity induced by this metal compound in BALB/3T3 fibroblasts. This finding concurs with other reports. Zhang et al. [39] suggested that once inside the cell, vanadate (5+) is reduced to vanadyl (4+) generating reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical, which mediated vanadium-dependent damage of A549 cells. Hydrogen peroxide has been found to play a primary role in the toxic effects of vanadate in mouse epidermal JB6 cells [15], lung epithelial cell line [38] and epithelial cell line Ma 104 [2].

Next, we evaluated the impact of two antioxidant elements: zinc and selenium on vanadium-induced cytotoxicity. We observed that 5 μM $ZnCl_2$ had no effect on the viability of BALB/3T3 cells, when added to cultures treated with $NaVO_3$. The inves-

tigation carried out by Riley et al. [24] showed that $ZnCl_2$ at 25 μM markedly reduced cytotoxicity (assessed with the MTT assay) induced by 600 μM VCl_4 in RLE-6TN cells. In our work, we used BALB/3T3 cells and pentavalent vanadium compound, which is more toxic than vanadium with an oxidation state +4 used in Riley et al. [24] investigations. Therefore, the comparison of our results with Riley's may be inadequate. Moreover, in the present work, we tested fivefold lower concentration of $ZnCl_2$ (5 μM) in combination with vanadium. The reason for this was that $ZnCl_2$ at concentration of 20 μM or higher was found to be toxic to BALB/3T3 cells in our preliminary investigations (Table 1). Still, no protection was observed under our experimental conditions in contrast to Riley's work. Nevertheless, such a comparison indicates complexities in zinc effects on vanadium adverse actions that remain to be resolved. In available literature, the investigation of Riley et al. [24] is the only report, in which the interaction between zinc and vanadium *in vitro* was described.

The assays for reactive oxygen species production revealed that 5 μM $ZnCl_2$ had no effect on vanadium-dependent superoxide generation. Merely a minimal reduction of hydrogen peroxide level was observed in cells cotreated with 5 μM $ZnCl_2$ and 50 or 100 μM $NaVO_3$. However, the observed inhibition of hydrogen peroxide production by $ZnCl_2$ was far from reaching statistical significance. Thus, taking into account results from viability and reactive oxygen species assays we conclude that $ZnCl_2$, in the concentration used by us, was not an effective protectant against vanadium toxicity in BALB/3T3 cells.

Zinc for its antioxidant role is often mentioned in studies as a protective agent against the toxicity induced by a variety of metals. The results, however, are conflicting. For example, Shimizu et al. [28] observed that zinc acetate (125 μM) although inducing metallothionein synthesis, did not have any effect on arsenite-induced cytotoxicity (as measured by MTT test) in rat myoblast cells. Rudolf et al. [26], on one hand, noted that 12 hour incubation of hepatocytes (Hep-2 cell line) in medium with 10 μM K_2CrO_4 and 100 μM $ZnSO_4$ caused a decrease in superoxide and hydrogen peroxide level and increased cell viability in comparison with control cells treated with 10 μM K_2CrO_4 alone. On the other hand, they also demonstrated that zinc enhanced the cytotoxicity of chromium salt when K_2CrO_4 was given at a higher dose. In other observations [30] zinc chloride (10 μM) inhibited apoptosis in HeLa cells and bovine aorta endothelial cells induced by 10 μM $CdCl_2$.

Our studies showed that the addition of 0.5 μM Na_2SeO_3 to cultures treated with 50, 100 and 200 μM $NaVO_3$ induced no protection against vanadium toxicity, as measured by MTT and superoxide assay. In the literature, the interaction between selenium and vanadium has not been studied extensively. Haider et al. [13] observed effective reduction of vanadium adverse actions by selenium in *in vivo* experimental model. *In vivo* conditions, however, differ from those *in vitro*. Moreover, Haider et al. [13] focused their investigations on differentiated cells (neurons), which are less sensitive to toxins than proliferating BALB/3T3 fibroblasts. So far, we could not find reports on the effect of selenium on vanadium-induced adverse actions in cell lines.

Many authors suggest that selenium-dependent protection often involves lowering of H_2O_2 level, which is mediated by Se-enzyme glutathione peroxidase [4, 7, 11]. However, in our studies H_2O_2 concentration produced in the presence of vanadium was not decreased by selenium.

A possible explanation of this rather unexpected result can be related with peroxovanadium compounds. They are formed in the reaction of vanadyl or vanadate with hydrogen peroxide. Some authors suggest that peroxovanadium species are more toxic than vanadyl or vanadate alone [2, 18]. On top of that, Rao et al. [23] showed that hydrogen peroxide attached to vanadate was more stable and thus less sensitive to degradation in comparison with hydrogen peroxide alone.

To sum up, sodium metavanadate at used concentrations (50, 100 and 200 μM) greatly increased superoxide and hydrogen peroxide production in BALB/3T3 fibroblasts. Considering statistics, neither zinc nor selenium efficiently inhibited vanadium-dependent cell damage in our experimental conditions. Our data only indicate a trend towards a reduction of vanadium-induced toxicity when zinc or selenium was added to the culture medium, but which was not significant. This calls for future experiments, which should be aimed at finding out if there are more effective concentrations of zinc and selenium for reducing vanadium cytotoxicity *in vitro*.

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