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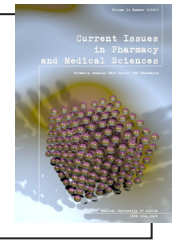

Influence of chromium (III), cobalt (II) and their mixtures on cell metabolic activity

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ABSTRACT

Chromium (III) and cobalt (II) are necessary elements required for the proper functioning of the organism, but their excess can cause toxic effects. They are the basic components of implants and are also commonly used in medicine as components of dietary supplements, vitamin and mineral products and energy drinks. The aim of this study was to investigate the effect of cobalt (II) and chromium (III) and their combination on BJ cells. In the study, BJ cells were exposed to $CoCl_2$ or $CrCl_3$ at concentrations ranging from 100 to 1400 μM, and the cytotoxicity of chromium (III) and cobalt (II) and their mixtures was assessed by MTT reduction, LDH release and NRU assays. The outcome of this work reveals the cytotoxic effects of chromium (III) and cobalt (II) and their mixtures on BJ cells. In the cytotoxicity assays, at low concentrations of $CoCl_2$ and $CrCl_3$, stimulation of cell proliferation was observed. In higher concentrations, the cell viability decreased for the tested line in all the assays. During the simultaneous incubation of fibroblasts with 200 μM of $CrCl_3$ and 1000 μM of $CoCl_2$, antagonism was observed: chromium (III) at the concentration of 200 μM induced protection from cobalt (II) toxicity; in the case of interaction of chromium chloride at 1000 μm and cobalt chloride at 200 μM, the protective effect of $CrCl_3$ on $CoCl_2$ was not observed. In the latter case, synergism between these elements was noted. Our work indicates that cobalt (II) and chromium (III) show cytotoxic properties. These metals have a destructive effect on the cell membrane, lysosomes and mitochondria, which leads to disorders of cell metabolism.

INTRODUCTION

Cobalt (II) and chromium (III) play crucial roles in human and animal body homeostasis [1]. Cobalt (II) is a microelement which is widespread in the environment. This element is very important due to its function in the biological system. This micronutrient is required in the form of vitamin B_{12} (hydroxycobalamin) in human diets. This vitamin plays a very important role in forming amino acids and some proteins in nerve cells and creating neurotransmitters which are indispensable for correct functioning of the organism [2-5]. Cobalt occurs in various oxidation states, i.e. 0, +2, +3, +4, but the most common are +2 and +3 [6,7]. Moreover, cobalt (II) is present in bacterial metalloenzymes as methionine aminopeptidase, and its functioning depends on cobalt [8].

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Epidemiological and laboratory studies report that cobalt and its compounds are carcinogenic to humans and are included in group 2 by International Agency for Research on Cancer [6,9-10]. Exposure to cobalt metal, its salts or heavy metal causes genotoxic and cytotoxic effects in many cells. The cytotoxicity of cobalt ions has been reported in many cell types and it can induce cell death by apoptosis and necrosis [2]. Cobalt (II) is capable of inducing oxidation and nitrification of proteins which lead to damage of cellular compartments in cells [11]. What is more, some studies report that cobalt induces chromosome aberrations, sister chromatide exchanges and single and double strand breaks [11,12]. This metal is able to generate active oxygen species (ROS) that are very destructive in the DNA and other biomolecules [11,13]. Moreover, cobalt (II) destabilizes the proper functioning of enzymes belonging to the antioxidant system, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (GPx). Additionally, cobalt (II)

can bind to zinc and copper sites in SOD, thus lowering the enzyme activity [14]. In addition, the element may interfere with the proper functioning of the zinc finger domain of the XPA protein, which is active in the process of nucleotide excision during the repair of mutated DNA [11].

The replacement of zinc with cobalt (II) in the XPA protein promotes the formation of ROS near the DNA, which affects its destruction [15]. Cobalt (II) disrupts the proper functioning of topoisomerase II (which requires divalent cations to carry out DNA cleavage) by forming complexes with this enzyme [11]. In addition, literature data show that cobalt (II) competes with zinc ions, which may affect the efficiency of the p53 protein binding mechanism to DNA, which is dependent on the presence of these ions [16,17].

Chromium (III) occurs naturally in the environment and is the most common and stable form found in living organisms. This element is characterized by low reactivity, which is extremely important in biological systems [18-21]. Chromium (III) is involved in carbohydrate, lipid and protein metabolism. It is very important for normal functioning of the insulin receptor, which is an integral component of glucose tolerance factor [20,22]. This element occurs in the environment in various degrees of oxidation – ranging from -2 to +6. However, in chemical compounds it is found at +1, +2, +3 and +6 degrees, and exceptionally at +4 and +5. Chromium with a zero oxidation state does not occur naturally in the Earth's crust and is biologically inert [18,23].

The hexavalent form is associated with the toxic effect on organism and is classified as a human carcinogen and mutagen [20]. Chromium(VI) in the cell is reduced by the redox system, which leads to the formation of various forms, including the trivalent one [26]. Chromium (III) has a destructive effect on the antioxidant system, which is unable to remove harmful oxygen species under conditions of disturbed cellular equilibrium. The activity of the superoxide dismutase enzymes, glutathione peroxidase, gradually decreases under the influence of ROS, which leads to irreversible destructive changes, i.e. lipid peroxidation and the formation of malondialdehyde [24-26]. As reported in the literature, chromium(III) and its compounds are toxic and cause formation of DNA-DNA crosslink and single-strand breaks. Some disturbance in metabolism of oxygen and depletion of ATP level has also been noted [26].

Living organisms are constantly exposed to chromium and cobalt because these elements are widely used in the metallurgical, aerospace, electronic and tool industries [27,28]. Moreover, they are the fundamental components of implants commonly used in medicine. Nowadays, modern medicine offers dietary supplements and vitamins that are widely consumed globally [29-33]. Cobalt (II) is a component of many dietary supplements; manufacturers recommend its consumption in the amount of 1 mg Co per day, in order to ensure proper metabolism of fats and sugars, protein synthesis and the production of red blood cells. In addition, some energy drinks also contain cobalt, which is found in the form of cyanocobalamin. This metal is present in significant amounts, about 41.677%, corresponding to about 100 µg of cobalt per serving at the FDA (Food and Drug Administration) recommended dosage of only 6 µg [34].

Chromium (III) is also a component of many dietary supplements, most often in the form of chromium picolinate – which reduces the fatty acid content [35-38]. Moreover, cocktails and supplements for athletes are often spiked with chromium (III) and are very often self-administered to reduce body fat, increase the body›s aerobic capacity and to achieve better training results [34].

Thus, these metals can enter the body in a variety of ways. In high concentrations, they can be potentially toxic to cells and can cause a number of changes at the cellular level. Despite many reports, the metabolism of chromium (III) and cobalt (II) is not well-known and the interactions between these elements are unknown. Bearing in mind the importance of both elements, the aim of the present work was to determine the influence of chromium (III) and cobalt (II) and their mixtures on cell organelles. Additionally, it aimed at determining the relationships between these elements.

MATERIALS AND METHODS

The research was carried out on human fibroblast BJ cell lines, obtained from American Type Culture Collection (ATCC). The cells were grown as adherent monolayers in Eagle's Minimum Essentials Medium (EMEM), supplemented with 10% fetal bovine serum (FBS) obtained from ATCC, and antibiotic antimycotic solution (10.000 U/ml of penicillin, 10 mg/mL of streptomycin, 25 µg/mL of amphotericin B), at 37°C and 5% CO_2. Hexahydrate chromium chloride ($CrCl_3 \times 6H_2O$) and hexahydrate cobalt chloride ($CoCl_2\ 6H_2O$) were dissolved in Phosphate Buffered Saline (PBS) to concentration of 1mM. Solutions of chromium chloride or cobalt chloride at the concentration range from 100 to 1400 µM were prepared by diluting them in culture medium EMEM supplemented with FBS and antibiotics.

Cytotoxic assays

In vitro cytotoxicity assays are commonly used to indicate cytotoxic effects induced by chemicals in different compartments of cells. In our work, these methods allow determining cell viability assessment by measuring the number of cells, dead or alive, after exposure to chromium chloride or cobalt chloride [40]. All cytotoxic assays detect very specific changes in different cell organelles that occur after exposure to toxic substances. In this work, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction, the neutral red uptake (NRU) and lactate dehydrogenase (LDH) cytotoxicity assays were used. All the assays were performed according to the original manufacturer's instructions – In vitro Toxicology Assay Kit, Sigma Aldrich (MTT – TOX-1; LDH – TOX-7; NR – TOX-4).

The cells were cultured in 96-well plates (2×10^5 cells/mL) in 100 µL in complete growth medium (EMEM supplemented with 10% FBS and mixture of antibiotics). After 24-hour incubation of fibroblast, the culture fluid was exchanged into a new one in the control case or supplemented with chromium chloride or cobalt chloride at concentrations ranging from 100 to 1400 µM. In order to determine the interactions between the above-mentioned microelements, the cells were similarly plated and incubated for 24 hours. In the next phase, the culture fluid was changed into a

fresh one and supplemented with mixture compounds with the following combinations: 200 µM chromium chloride and 1000 µM cobalt chloride or 1000 µM chromium chloride and 200 µM cobalt chloride. Subsequently, MTT, LDH and NRU assays were performed. The cultures were then subjected to spectrophotometric analysis.

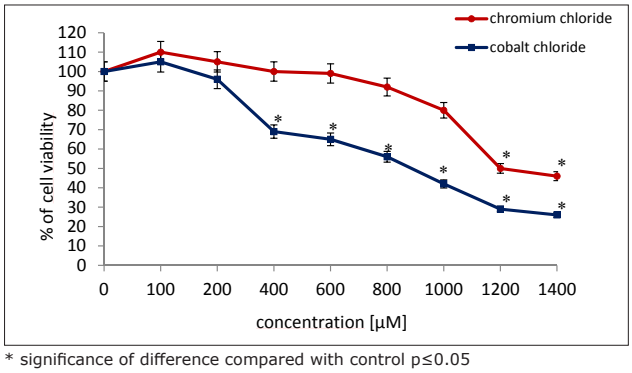
The percentage of cell viability was calculated by relating the absorbance values obtained for each concentration of the tested compounds to the absorbance value of the control cells, taking the absorbance of the solution for the control cells as 100% (in the case of the MTT and NRU assays). In the LDH assay, the percentage of LDH release was calculated (% release LDH = amount of spontaneously released LDH from the cell into the culture fluid/amount of total LDH spontaneously released and remaining in the cell ×100%). A dose-response curve was then prepared for each of the compounds tested, and the IC_{50} value was determined.

Statistics

The results were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons, using Statistica version 4.0.

RESULTS

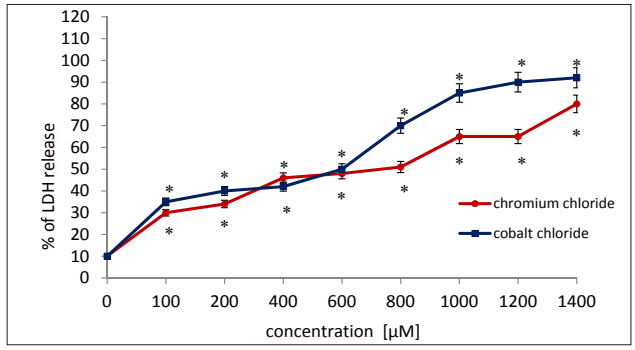
In the cytotoxic assays at low concentrations of both tested compounds, stimulation of cell proliferation was observed. However, in higher concentrations, the cell viability decreased. Additionally, an increase in release of lactate dehydrogenase in the LDH assay in the case of both tested compounds was observed. Cytotoxic effects of chromium chloride and cobalt chloride are shown in Figures 1, 2 and 3. After incubation of the BJ cells with chromium chloride in the concentration range of 100 and 200 µM, an increase in cell viability, which was approximately 10% at the concentration of 100 µM, was noted. In the concentrations ranging from 1000 to 1400 µM, a statistically significant decrease in cell viability as compared to the control cells was observed. Incubation of cells with cobalt chloride caused a statistically significant decrease in cell viability at concentrations of 400 µM and more, along with the increasing concentrations, as compared to the control system. An increase in cell proliferations was observed only at the lowest concentration. The above-mentioned cytotoxic effects were observed in the MTT assay.



* significance of difference compared with control p≤0.05

Figure 1. Cytotoxic effect of $CrCl_3 \times 6H_20$ or $CoCl_2 \times 6H_20$ in the BJ line – as detected via the MTT assay

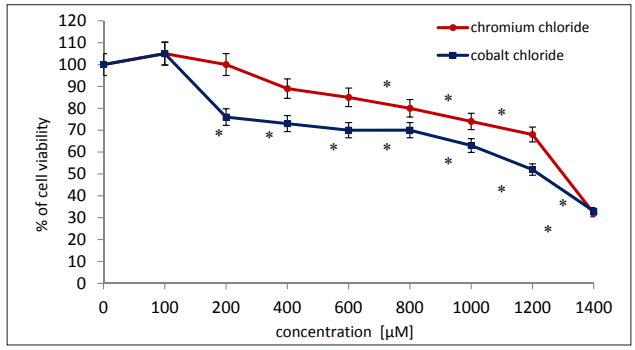
In the LDH assay, a statistically significant increase in release of enzyme (decreased cell viability), along with an increase in the concentration of the tested compound, compared to the control was observed.



* significance of difference compared with control p≤0.05

Figure 2. Cytotoxic effect of $CrCl_3 \times 6H_20$ or $CoCl_2 \times 6H_20$ in the BJ line – as detected via the LDH release assay

An analysis of the graph in the NRU assay reveals that after incubating the BJ cells with chromium chloride at concentrations of 100 and 200 µM, a slight increase in cell viability, as compared to the control, was observed.



* significance of difference compared with control p≤ 0,05

Figure 3. Cytotoxic effect of $CrCl_3 \times 6H_20$ or $CoCl_2 \times 6H_20$ in the BJ line – as detected via the NRU assay

A statistically significant decrease in cell viability at the concentration of 800 µM for the human fibroblasts was also noted. The same results were received after incubation of human fibroblast cell lines with cobalt chloride. For each test, the value IC_{50} was determined (Table 1).

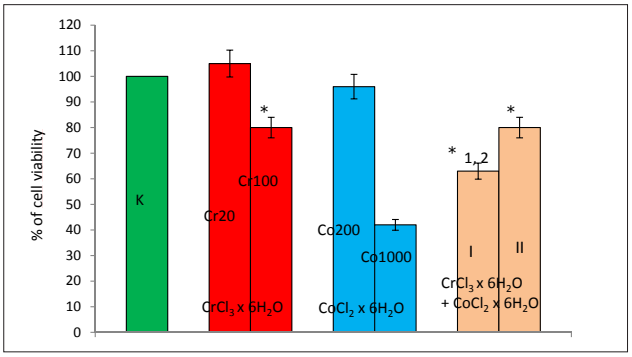
Table 1. IC_{50} values for the BJ line following exposure to $CrCl_3 \times 6H_20$ and $CoCl_2 \times 6H_20$ – as determined in cytotoxicity assays

Tested compounds	MTT	LDH	NRU
$CrCl_3 \times 6H_2O$	1200 µM	800 µM	1250 µM
$CoCl_2 \times 6H_2O$	900 µM	600 µM	1200 µM

MTT – the MTT reduction assay
LDH – the LDH release assay
NRU – the neutral red uptake assay

On the basis of the results obtained at the first stage of the research, the concentrations of the elements were selected in order to determine the interactions between them. The BJ cells were treated with the mixtures of the following compounds: 200 µM chromium chloride and 1000 µM cobalt chloride or 1000 µM chromium chloride and 200 µM cobalt chloride. During the simultaneous incubation with chromium chloride at the concentration of 200 µM and cobalt chloride at the concentration of 1000 µM of the BJ cells, a statistically significant decrease in cell viability

was noted when compared to the control cells and those incubated with chromium chloride at the concentration of 200 μM, while as to the cells incubated only with cobalt chloride at the concentration of 1000 μM, a statistically significant increase in cell viability was observed. In the case of simultaneous incubation of fibroblast with chromium chloride at the concentration of 1000 μM and cobalt chloride at the concentration of 200 μM, a decrease in cell viability was noted, but not statistically significant when compared to the cells incubated only with cobalt chloride at the concentration of 200 μM. Compared to the cells incubated only with chromium chloride at the concentration of 1000 μM, no changes in viability of human fibroblasts were noted. The effect of mixtures of chromium chloride and cobalt chloride on viability of cells in the MTT assay is shown in Figure 4. In the next assay, simultaneous incubation of cells with 200 μM chromium chloride and 1000 μM cobalt chloride decreased lactate dehydrogenase release compared to cells incubated only with 200 μM chromium chloride or 1000 μM cobalt chloride.

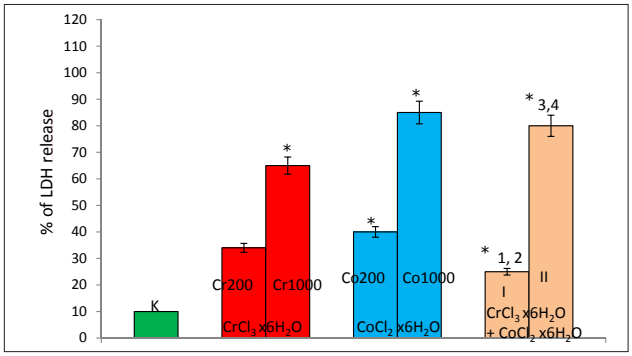


K – Control, I: simultaneous incubation with $CrCl_3 \times 6H_2O$ at concentration of 200 μM + $CoCl_2 \times 6H_2O$ at concentration of 1000 μM. II: $CrCl_3 \times 6H_2O$ at concentration of 1000 μM + $CoCl_2 \times 6H_2O$ at concentration of 200 μM, * – significance of difference compared with control p≤ 0.05, 1 – significance of difference compared with chromium chloride at concentration of 200 μM, 2 – significance of difference compared with cobalt chloride at concentration of 1000 μM

Figure 4. Effect of mixtures of chromium chloride and cobalt chloride on cell viability of the BJ lines in the MTT reduction assay

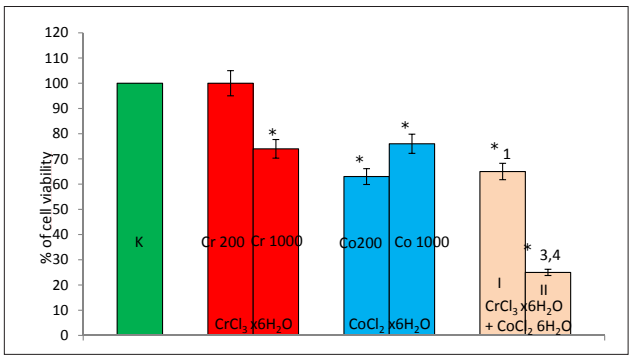
However, in the case of simultaneous incubation of chromium chloride at the concentration of 1000 μM and cobalt chloride at the concentration of 200 μM of fibroblast cell line, a statistically significant increase in the release of enzyme lactate dehydrogenase was noted when compared to control cells and incubated only with chromium chloride at the concentration of 1000 μM or only with cobalt chloride at the concentration of 200 μM (Fig. 5).

Figure 6 shows the results obtained in the NRU assay. During simultaneous incubation with chromium chloride at the concentration of 200 μM and cobalt chloride at the concentration of 1000 μM of the BJ cells, there was a statistically significant decrease in cell viability when compared to the control cells and incubated only with chromium chloride at the concentration of 200 μM, while a statistically significant increase in cell viability compared to cells incubated only with 1000 μM cobalt chloride, and an increase in cell viability were noted. However, in the case of simultaneous incubation of chromium chloride at the concentration of 1000 μM and cobalt chloride at the concentration of 200 μM of fibroblast lines, a statistically significant decrease in cell viability was observed when compared to control cells and incubated only with chromium chloride at the concentration of 1000 μM or cobalt chloride at the concentration of 200 μM.



K – Control, I: simultaneous incubation with $CrCl_3 \times 6H_2O$ at concentration of 200 μM + $CoCl_2 \times 6H_2O$ at concentration of 1000 μM. II: $CrCl_3 \times 6H_2O$ at concentration of 1000 μM + $CoCl_2 \times 6H_2O$ at concentration of 200 μM, * – significance of difference compared with control p ≤0.05, 1 – significance of difference compared with chromium chloride at concentration of 200 μM, 2 – significance of difference compared with cobalt chloride at concentration of 1000 μM, 3 – significance of difference compared with chromium chloride at concentration of 1000 μM, 4 – significance of difference compared with cobalt chloride at concentration of 200 μM

Figure 5. Effect of mixtures of chromium chloride and cobalt chloride on cell viability of the BJ lines in the LDH release assay.



K – control, I: simultaneous incubation with $CrCl_3 \times 6H_2O$ at concentration of 200 μM + $CoCl_2 \times 6H_2O$ at concentration of 1000 μM. II: $CrCl_3 \times 6H_2O$ at concentration of 1000 μM + $CoCl_2 \times 6H_2O$ at concentration of 200 μM, * – significance of difference compared with control p≤0.05; 1 – significance of difference compared with chromium chloride at concentration of 200 μM, 2 – significance of difference compared with cobalt chloride at concentration of 1000 μM, 3 – significance of difference compared with chromium chloride at concentration of 1000 μM, 4 – significance of difference compared with cobalt chloride at concentration of 200 μM

Figure 6. Effect of mixtures of chromium chloride and cobalt chloride on cell viability of the BJ lines in the NR uptake assay.

DISCUSSION

The cytotoxic activity of the elements can be assessed by direct or indirect measurement of changes in the cells that are exposed to some chemicals when compared to the control system. By using properly selected assays, it is possible to determine the efficiency of individual cell organelles. The MTT assay allows determining the efficiency of the key cell organelle, which is the mitochondria, it also detects perturbations and their functions. The NRU assay detects loss of lysosomal activity on cells and the LDH release assay detects the loss of cell membrane integrity. After analyzing the IC_{50} values obtained in the above-mentioned tests, it can be seen that the examined compounds damaged the cell organelles in the following order: cell membrane, then mitochondrion and, finally, lysosomes.

The carried out studies showed that cobalt chloride and chromium chloride caused a concentration-dependent decrease in cell viability in the MTT and NRU assays, with the exception of 100 and 200 μM concentrations in which cell proliferation was observed. In the LDH assay, an increase in enzyme release (decreased cell viability) with an increase in the tested compound concentration was noted. The results were confirmed by other previous experiments in which WTHBF-6 cell lines were exposed to cobalt chloride, and a decrease of cell viability had been observed. Other studies reported that cobalt chloride caused a decrease in membrane potential and depletion of ATP in astrocytes [12]. The toxic effect of cobalt chloride was confirmed in studies conducted on the astrocyte line, where a decrease in cell viability was noted [41]. Toxicity of this element is probably due to its ability to generate reactive oxygen species.

Literature data clearly indicate that cobalt and chromium are cytotoxic metals. Cobalt generates reactive oxygen species that are highly destructive to molecules. Cobalt ions in the presence of hydrogen peroxide can increase the level of damage by activating the caspase system by reacting with hydrogen peroxide in the Fenton reaction [11,12,41-43]. One of the most dangerous effects of cobalt is the reduction of the membrane potential of the mitochondria and the peroxidation of the membrane lipids of these organelles [40,44,45]. Toxicity of cobalt may result from two mechanisms: from direct destruction by free radicals of biomolecules or from the destruction of the antioxidant system. Previous studies have shown changes in the activity of enzymes of antioxidant system in the liver cells of mice after exposure to cobalt chloride. An increase of GPx and a decrease of ACT and SOD were observed [42]. The decrease of activity of SOD enzyme is related to the binding of cobalt to the sites of zinc and copper in SOD, thus reducing the activity of the enzyme [36]. Moreover, the toxicity of cobalt could be related to its high affinity for sulfhydryl groups. Cobalt has influence upon the mitochondrion function because it causes inhibition of crucial enzymes in mitochondrial respiration [18].

The experiments carried out in this study also confirmed the toxic effect of chromium chloride. In our work, decrease of cell viability along with increased concentrations was noted; the exception was at 100 and 200 μM, when stimulation of cell proliferation was noted. This investigation was confirmed by earlier research carried out on the keratinocytes line, where decreased cell viability in the MTT assay was observed [26]. Moreover, other research was carried out on ovarian Chinese Hamster cells exposed to chromium chloride at the concentration of 1000 μM or chromium picolinate (at a dose of 80 $\mu g/cm^2$) in a colony formation test.

We also saw a decrease of cell viability and destruction in the mitochondrion structure. They were swollen with destruction of mitochondrial combs after exposure to chromium toxicity [46]. Studies by other authors indicated that chromium (III) compounds had the ability to generate reactive oxygen species, with a destructive effect on mitochondria and DNA, leading to apoptosis [47]. What is more, chromium (III) has a destructive effect on lipids, causing their peroxidation in liver and kidney cells [25]. As reported in the literature, chromium (III) has a destructive effect on the antioxidant system, which is unable to remove harmful forms during cell imbalance. A gradual increase in activity of CAT and GPx when compared to the control system was observed [48].

The interaction of the elements may be of different nature. In the case of reducing the effects of the examined elements, antagonism can be observed, and in the case of their intensification – synergism. During the simultaneous incubation with chromium chloride at the concentration of 200 μM and cobalt chloride at the concentration of 1000 μM of the BJ cells, antagonism was observed – chromium (III) at the concentration of 200 μM served as protective function against the toxic concentration of cobalt (II) at the concentration of 1000 μM. These observations were made in the MTT and the NRU, where a statistically significant increase in viability of fibroblast cell was noted in comparison to the cells incubated with chromium chloride at the concentration of 1000 μM, and in the case of the LDH test, a decrease in enzyme release was observed. However, in the case of the interaction of chromium chloride with the concentration of 1000 μM and cobalt chloride with the concentration of 200 μM, no protective effect of chromium chloride against cobalt chloride was observed. In this case, synergism was observed between the examined elements. Such observations were made in the case of cytotoxicity assays, where there was a decrease in cell viability in the MTT and NRU tests, and an increase in the release of the enzyme lactate dehydrogenase in the LDH test was noticed. The lack of protective effect of chromium chloride with the concentration of 1000 μM is associated with a decrease in the efficiency of the antioxidant system [49]. These results are confirmed by another study conducted by Chen *et al.,* in which enzymes of the antioxidant system (in a non-cellular system) were treated with chromium (III) in the range of 0 to 5.0×10^{-4} mol l^{-1}. In low concentrations, *i.e.* 2.0×10^{-4} mol, chromium chloride was observed to increase catalase activity, which decreased at higher concentrations. The destructive effect was due to the destruction of the active center of this enzyme by chromium (III) [49].

The ability of low concentrations of chromium (III) to stimulate catalase activity results in its protective effect against the post-oxidative effect of cobalt (II) used in high concentrations. In the case of interaction of chromium chloride at the concentration of 1000 μM and cobalt chloride at the concentration of 200 μM, no protective effect of chromium chloride against cobalt chloride was observed. In this case, synergism was observed between the studied elements. Such observations were made in the case of cytotoxicity tests, where a decrease in cell viability was noted in the MTT and NRU, while there was an increase in the release of the enzyme lactate dehydrogenase in the LDH test. The lack of protective effect of chromium chloride at the concentration of 1000 μM is associated with the generation of oxygen free radicals and decreased efficiency of the antioxidant system [49]. In the case of interaction of chromium (III) at 1000 μM concentration with cobalt chloride at 200 μM concentration, the first element enhances the proapoptotic effect of cobalt (II).

CONCLUSION

Chromium (III) and cobalt (II) are trace elements required for good health, but their excess can cause toxic effects. These metals are commonly widespread. They are components in dietary supplements and energy drinks. In addition, they are components of biomaterials. According to several studies, the toxicity of the elements is dose-dependent. Cobalt and chromium have a destructive effect on the cell membrane, lysosomes and mitochondria, which leads to disorders of cell metabolism. In addition, the elements have the ability to generate reactive oxygen species that are destructive to cells. In our work, during the first interaction (200 μM of chromium chloride and 1000 μM of cobalt chloride), antagonism was observed. However, in the case of the other interaction (chromium chloride at 1000 μM and cobalt chloride at 200 μM), the protective effect of chromium chloride on cobalt chloride was not observed. In this case, synergism between these elements was noted.

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