Cell viability in normal fibroblasts and liver cancer cells after treatment with iron (III), nickel (II), and their mixture

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Abstract

Introduction: Nickel and iron are very commonly occurring metals. Nickel is used in industry, but nowadays it is also used in medical biomaterials. Iron is an element necessary for cell metabolism and is used in diet supplements and biomaterials, whence it may be released along with nickel. **Material and Methods:** BALB/3T3 and HepG2 cells were incubated with iron chloride or nickel chloride at concentrations ranging from 100 to 1,400 μM. The following mixtures were used: iron chloride 200 μM plus nickel chloride 1,000 μM, or iron chloride 1,000 μM plus nickel chloride 200 μM. The cell viability was determined with MTT, LHD, and NRU tests. **Results:** A decrease in cell viability was observed after incubating the BALB/3T3 and HepG2 cells with iron chloride or nickel chloride. A synergistic effect was observed after iron chloride 1,000 μM plus nickel chloride 200 μM treatment in all assays. Moreover, the same effect was observed in the pair iron chloride 200 μM plus nickel chloride 1,000 μM in the LDH and NRU assays. **Conclusions:** Iron (III) and nickel (II) decrease cell viability. Iron chloride at a concentration of 200 μM protects mitochondria from nickel chloride toxicity.

Keywords: nickel (II), iron (III), cytotoxicity, interactions.

Introduction

Nickel and iron are very commonly occurring metals all over the Earth where iron's abundancy is as large as 5% of the planet's crust. Nickel is used in electroplating and in the manufacture of different alloys, batteries, and electronic devices. Moreover, nowadays nickel is used in medical biomaterials (11, 16). Nevertheless, nickel and its compounds are considered industrial health hazards. It has been proved that reactive oxygen species (ROS) may play a major role in the toxicity of nickel and its compounds. Nickel is involved in the Fenton reaction, in which hydroxyl radicals are generated. These radicals cause lipid peroxidation and DNA strand breakage (5). In the scientific literature, there are many reports on the effect of nickel compounds on DNA, among them an account of nickel compounds inducing regional decondensation, deletions, and formation aberrations in the Chinese hamster X chromosome. Sister chromatid exchanges and mutations were also observed (3). In mitochondria, nickel and its compounds inhibit mitochondrial succinate dehydrogenase activity and cause the loss of mitochondria membrane potential. This may lead to accumulation of cells in G2/M phase of cell cycle and apoptosis (25).

Iron exists in oxidation states ranging from −2 to 6. For biological systems the most important are Fe^{2+} and Fe^{3+}. The crucial function of iron is to form haeme, which is a prosthetic group of haemoglobin and myoglobin. It is also crucial for formation of iron-sulphur clusters of cytochromes, ferredoxins, and dehydrogenases, which are involved in redox and electron transfer reactions (6). Iron is required for many

cellular functions such as the cell cycle, growth, and DNA replication, and is involved in intracellular respiration (21). However, an excess of iron concentration results in formation of ROS. Iron ions can react *via* the Fenton and Haber–Weiss reactions: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^\bullet + OH^-$ and $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^\bullet + H^+$. Superoxides and hydroxyl radicals formed *via* these reactions can react with cell biomolecules such as DNA, with proteins, and with cell organelles such as mitochondria or cell membranes, leading to cytotoxic and genotoxic effects (6). There are many reports of iron genotoxicity. Iron compounds induce DNA damage, mutations in *Salmonella* Typhimurium, and formation of micronuclei (2).

Cytotoxicity studies are often used in *in vitro* investigations. MTT, NRU, and LDH assays are most commonly applied to detect cell viability after exposure to various substances. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a water-soluble tetrazolium salt, which is converted to an insoluble formazan by succinate dehydrogenase within mitochondria. This formazan accumulates in metabolically active cells (9), and so the MTT assay measures mitochondrial activity in cells. Another assay used is the neutral red uptake (NRU) assay. As in the case of the MTT assay, the NRU assay also measures cell viability. Neutral red can be accumulated in lysosomes of living cells. Therefore, the NRU assay measures lysosomal activity. Another frequently used assay is the LDH leakage assay based on measurement of lactate dehydrogenase released to the cell culture medium. This assay measures membrane integrity (20).

The aim of this study was to evaluate sensitivity of the BALB/3T3 and HepG2 cells after their challenge with iron chloride, nickel chloride, and the mixture of the chlorides. This sensitivity was measured by three cytotoxicity assays.

Material and Methods

Cell culture and treatment. Mouse embryo fibroblasts BALB/3T3 clone A31 cells (ATCC CCL-163) were obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured as adherent monolayers in plastic tissue culture dishes in complete growth medium: Dulbecco's Modified Eagle Medium (ATCC, USA) supplemented with 10% (v/v) heat-inactivated calf bovine serum (ATCC, USA) and antibiotic antimycotic solution (1 mL per 100 mL of cell culture medium) (Sigma-Aldrich, USA). The cells were maintained at 37ºC in a humidified incubator in an atmosphere with 5% CO_2 (24).

Liver cancer HepG2 cells (ATTC HB-8065) were also obtained from ATCC (USA). These cells were cultured as adherent monolayers in plastic tissue culture dishes, in a different complete growth medium: Eagle's Minimum Essential Medium (ATCC, USA) supplemented with 10% (v/v) heat-inactivated foetal

bovine serum (ATCC) and antibiotic antimycotic solution (1 mL per 100 mL of cell culture medium) (Sigma-Aldrich, USA). The cell cultures were maintained at 37ºC in a humidified incubator in an atmosphere with 5% CO_2 (24).

$FeCl_3 \times 6H_2O$ and $NiCl_2$ (Acros Organics, Belgium) were dissolved in PBS at the concentration of 10 mM. The final concentration was obtained by dilution in complete culture medium (24).

Cytotoxicity assays. To perform the MTT reduction, LDH release, and neutral red uptake assays, the cells were cultured on 96-well plates (2×10^5 cells/mL) in 100 μL of complete growth medium. After 24 h, the medium was exchanged for fresh medium supplemented with iron chloride or nickel chloride within a range of concentrations from 100 to 1,400 μM. The cells exposed to the mixture of trace elements were similarly plated at a density of 2×10^5 cells/mL and incubated for 24 h. Next, the medium was exchanged for fresh medium supplemented with 200 μM of iron chloride and 1,000 μM of nickel chloride and, in the other case, supplemented with 1,000 μM of iron chloride and 200 μM nickel chloride. After 24 h of incubation, MTT reduction, LDH release, and neutral red uptake assays were performed (24).

MTT reduction assay. The MTT reduction colorimetric assay was performed using the commercial TOX-1 kit (TOX-1) from Sigma-Aldrich (USA) according to the manufacturer's instructions. On the basis of spectrophotometric measurements, the cell viability was calculated compared to the control cells (the absorbance of the control cells as 100% viability). The 50% inhibitory concentration (IC_{50}) value was calculated from the graph of the dose-response curve. Six independent experiments were performed with three wells per treatment preparation (24).

LDH release assay. The LDH release assay was performed using the commercial TOX-7 kit also from Sigma-Aldrich (USA) according to the manufacturer's instructions. The percentage of released LDH was calculated according to the formula: % of released LDH = (absorbance of R LDH/absorbance of T LDH) × 100%.

On the basis of the results, a curve was plotted. The IC_{50} value was calculated from the graph of the dose-response curve. Again, six independent experiments were performed with three wells per each treatment preparation (24).

Neutral red uptake assay. The NRU assay was performed using the commercial TOX-4 kit from the same supplier, Sigma-Aldrich (USA) according to the manufacturer's instructions. On the basis of spectrophotometric measurements, the cell viability was calculated compared to the control cells. A curve was plotted from on the results. The IC_{50} value was calculated from the graph of the dose-response curve. Consistent with the MTT and LDH protocols, six independent experiments were performed with three wells per each treatment preparation (24).

Statistical analysis. The results were expressed as mean ±SD, and the data were analysed using one-way analysis of variance (ANOVA) with Tukey's multi-comparison post-test using the Statistica programme (Tibco Software, USA). In all cases, $P < 0.05$ was considered significant.

Results

In the MTT and NRU assays, the HepG2cells were more sensitive than the BALB/3T3 cells. The IC_{50} values obtained by three assays are presented in Table 1.

Cell viability assessed in the MTT assay after iron chloride and nickel chloride treatment is shown in Fig. 1. Cell viability in both cell lines was significantly decreased when compared to control cells. The viability assessed by the MTT reduction assay in both cell lines significantly decreased in a concentration-dependent manner. However, at low doses (100 and 200 µM) of both trace elements, cell viability increased. In this assay, the IC_{50} for iron chloride in the HepG2 cells was 1,300 µM. The IC_{50} for nickel chloride in the BALB/3T3 cells was 700 µM, while in the HepG2 cells it was 400 µM (Table 1).

The toxic effects of iron chloride or nickel chloride on membrane integrity of the BALB/3T3 and HepG2 cells were examined with the use of the LDH release assay. As shown in Fig. 2, both cell lines displayed increasingly significant LDH leakage after exposure to the elements. In this assay, the IC_{50} for iron chloride in the BALB/3T3 cells was 550 µM, while in the HepG2 cells it was 900 µM. The IC_{50} for nickel chloride in the BALB/3T3 cells was 300 µM, while in the HepG2 cells it was 800 µM (Table 1).

The concentration-response profile of cell viability assessed in the NRU assay is shown in Fig. 3. The cell viability of both cell lines significantly decreased in a concentration-dependent manner. However, at low doses (100, 200, and 400 µM) of both trace elements, cell viability increased. In this assay, the IC_{50} for iron chloride in the HepG2 cells was 1,200 µM, and for nickel chloride in the BALB/3T3 cells was 1,100 µM, while in the HepG2 cells it was 1,200 µM (Table 1).

Figs 4–6 show the changes in cell viability (measured by MTT reduction, LDH leakage, and NRU assays) by mixing Fe (III) at concentration of 200 µM with Ni (II) at concentration of 1,000 µM, and in the other case by mixing Fe (III) at concentration of 1,000 µM with Ni (II) at concentration of 200 µM. Addition of Fe (III) at 1,000 µM plus Ni (II) at 200 µM showed a synergistic effect in a decrease in cell viability assessed by MTT, LDH, and NRU assays in both cell lines. In cells treated with Fe (III) at 200 µM plus Ni (II) at 1,000 µM, a synergistic effect was also evident, bringing about a decrease in cell viability assessed by LDH and NRU assays in both cell lines. However, in the MTT assay Fe (III) at concentration of 200 µM protected cells from Ni (II) toxicity at a concentration of 1,000 µM.
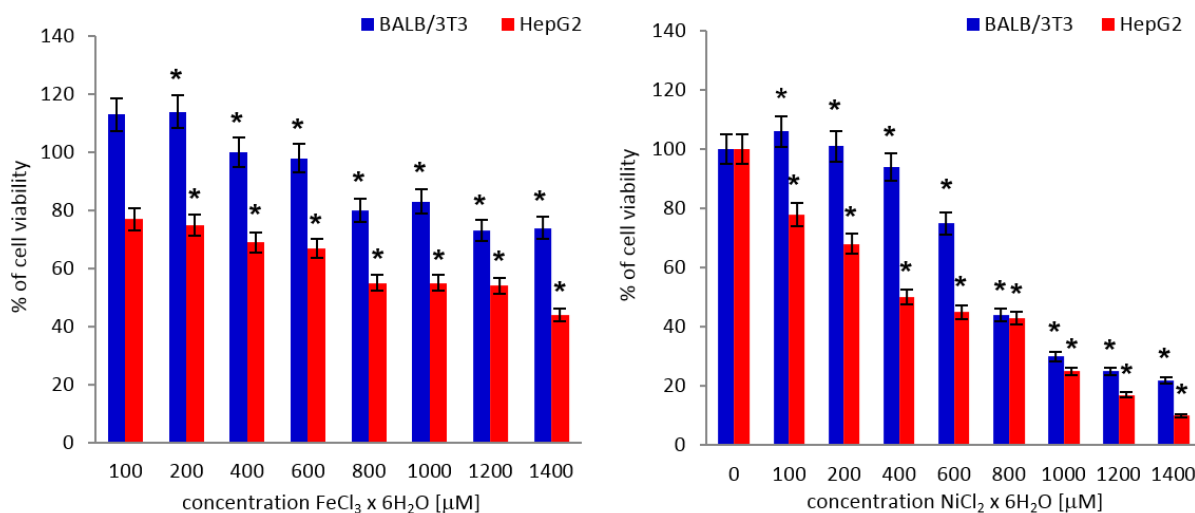

Fig. 1. Cytotoxicity expressed as percentage of cell viability ±SD induced by iron chloride or nickel chloride in the BALB/3T3 and HepG2 cell lines as assessed by MTT assay, *P < 0.05, compared to control
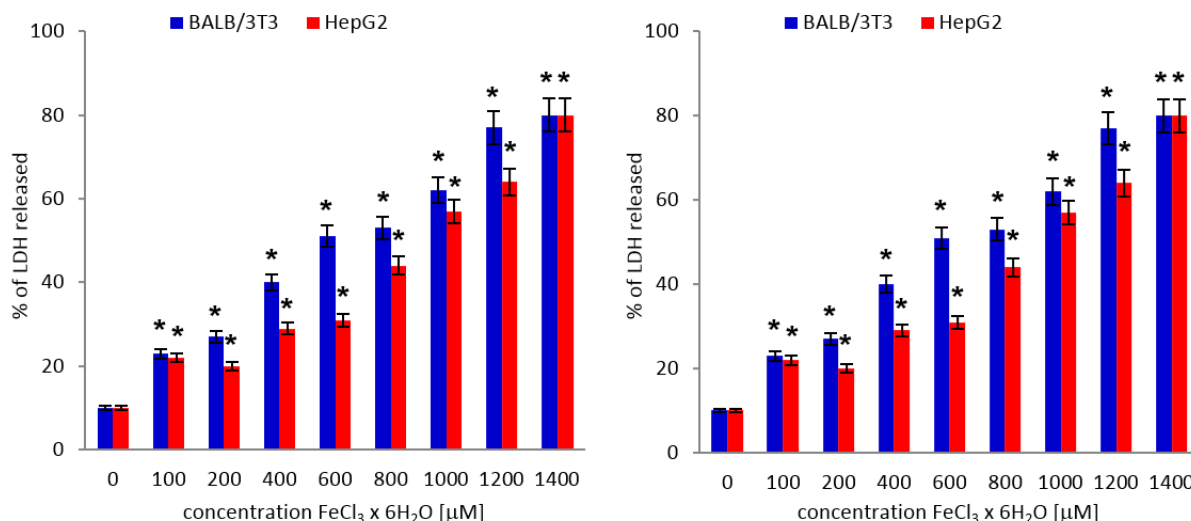
Fig. 2. Cytotoxicity expressed as percentage LDH released ±SD induced by iron chloride or nickel chloride in the BALB/3T3 and HepG2 cell lines as assessed by LDH assay, *P < 0.05, compared to control
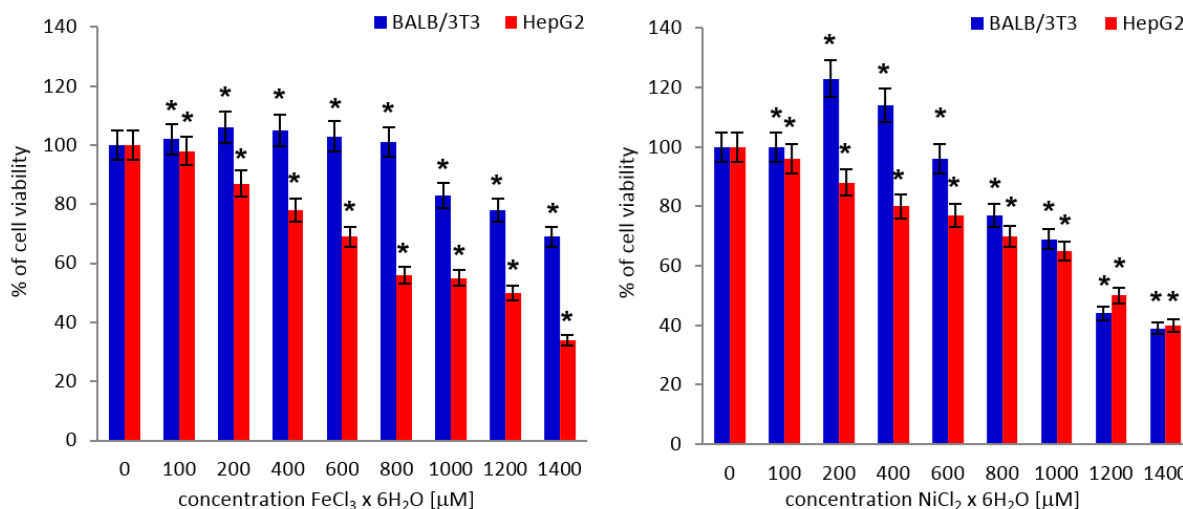

Fig. 3. Cytotoxicity expressed as percentage of cell viability ±SD induced by iron chloride or nickel chloride in the BALB/3T3 and HepG2 cell lines as assessed by NRU assay, *P < 0.05, compared to control

Table 1. IC_{50} values for the BALB/3T3 and HepG2 cell lines following exposure to iron chloride and nickel chloride derived from MTT reduction, LDH leakage, and NRU assays

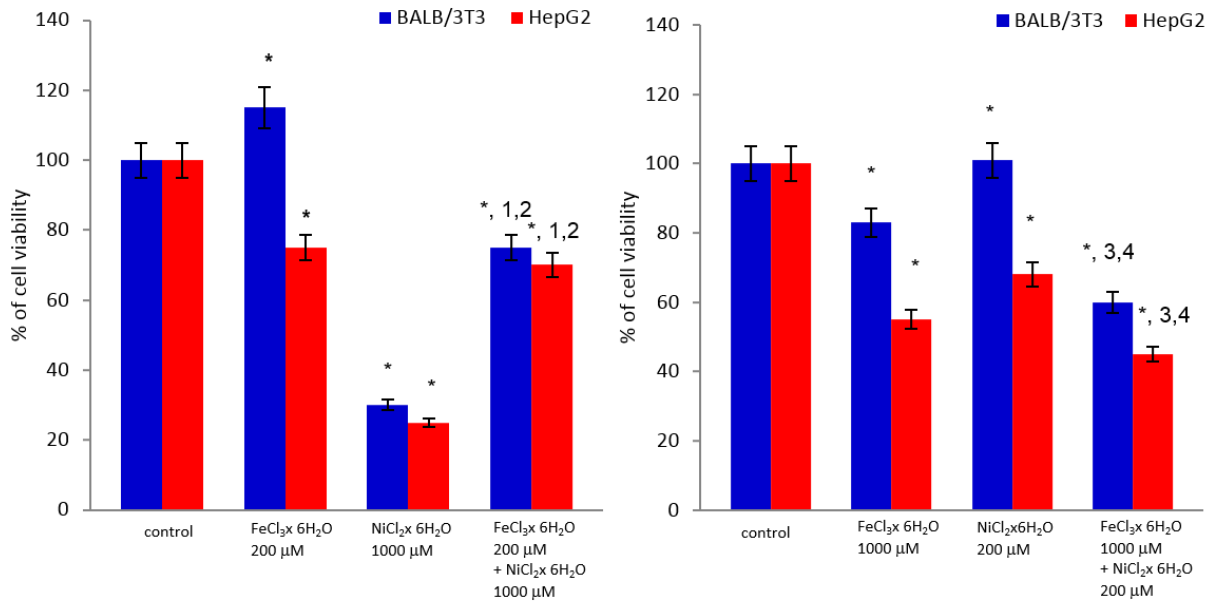| Metal compounds	Assay	BALB/3T3 (µM)	HepG2 (µM)
Iron chloride			
	MTT	-	1,300
	LDH	550	900
	NRU	-	1,200
Nickel chloride			
	MTT	700	400
	LDH	300	800
	NRU	1,100	1,200

Fig. 4. Cytotoxicity expressed as percentage of cell viability ±SD induced by iron chloride or nickel chloride in the BALB/3T3 and HepG2 cell lines as assessed by MTT assay
*$P < 0.05$, compared to control
[1]$P < 0.05$, compared to iron chloride at concentration of 200 μM
[2]$P < 0.05$, compared to nickel chloride at concentration of 1,000 μM
[3]$P < 0.05$, compared to iron chloride at concentration of 1,000 μM
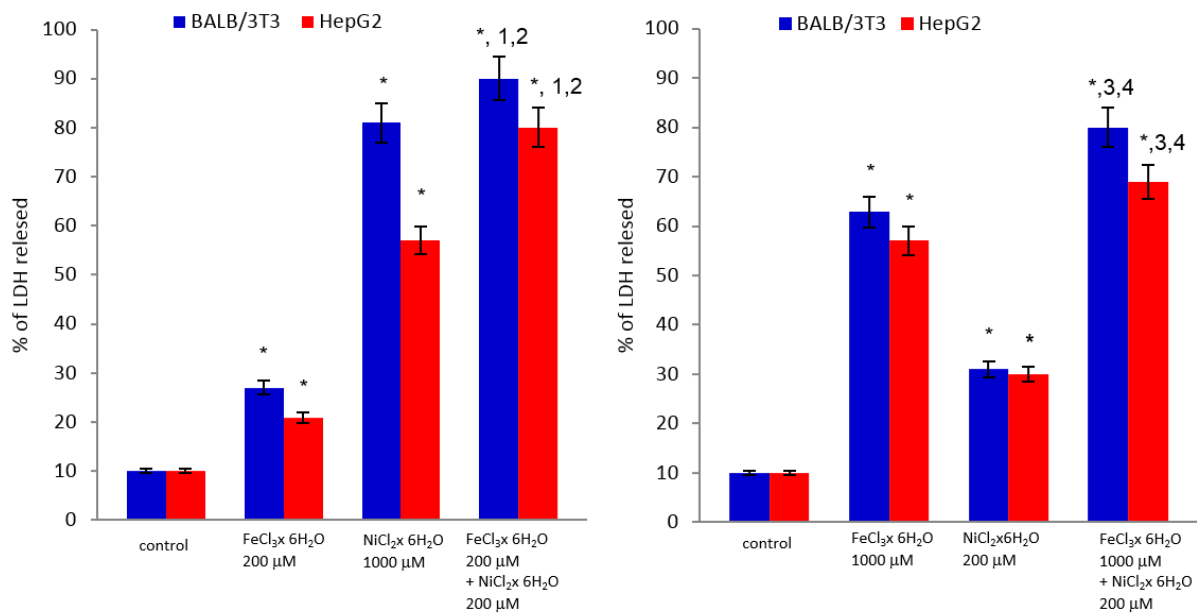[4]$P < 0.05$, compared to nickel chloride at concentration of 200 μM

Fig. 5. Cytotoxicity expressed as percentage LDH released ±SD induced by iron chloride or nickel chloride in the BALB/3T3 and HepG2 cell lines as assessed by LDH assay
*$P < 0.05$, compared with control
[1]$P < 0.05$, compared to iron chloride at concentration of 200 μM
[2]$P < 0.05$, compared to nickel chloride at concentration of 1,000 μM
[3]$P < 0.05$, compared to iron chloride at concentration of 1,000 μM
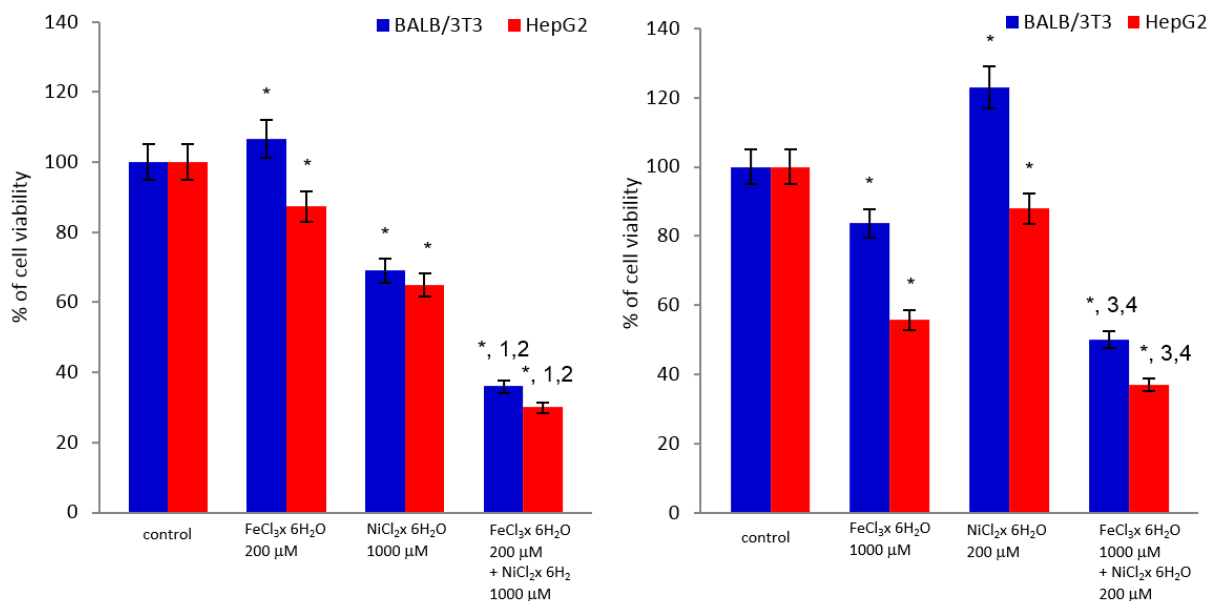[4]$P < 0.05$, compared to nickel chloride at concentration of 200 μM

Fig. 6. Cytotoxicity expressed as percentage of cell viability ±SD induced by iron chloride or nickel chloride in the BALB/3T3 and HepG2 cell lines as assessed by NRU assay
*$P < 0.05$, compared to control
[1]$P < 0.05$, compared to iron chloride at concentration of 200 μM
[2]$P < 0.05$, compared to nickel chloride at concentration of 1,000 μM
[3]$P < 0.05$, compared to iron chloride at concentration of 1,000 μM
[4]$P < 0.05$, compared to nickel chloride at concentration of 200 μM

Discussion

The aim of this study was to evaluate the toxicity of iron chloride, nickel chloride, and their mixture. For this purpose, three cytotoxicity assays were used. The MTT assay detects perturbations in mitochondria and their functions. The NRU assay detects loss of lysosomal activity on cells. The third, the LDH leakage assay, detects loss of cell membrane integrity (9).

The concentrations of iron chloride and nickel chloride were chosen on the basis of other reports and our earlier investigations (17, 12, 23). At 200 μM concentrations of the two trace elements, no changes in cell viability were observed. Moreover, in some cases, the cell viability was slightly stimulated. However, at concentrations of 1,000 μM and above, a decrease in cell viability was noticed (24).

The BALB/3T3 cell line was chosen for our investigation because it was suggested as a cellular model in studying the morphological and biochemical changes induced by biometals (17). Human cell lines are sensitive tools for high-throughput toxicity screening which can potentially reduce toxicological testing in animals (18). The human hepatoblastoma HepG2 cells have been well characterised and very often used as an *in vitro* toxicity model (8, 22, 23). They have also been used to distinguish toxic and non-toxic substances.

The use of these three assays showed that there are differences between the BALB/3T3 and HepG2 cell lines concerning their sensitivity to iron chloride or nickel chloride. The HepG2cells were more sensitive than the BALB/3T3 cells after treatment with both trace elements with the use of MTT and NRU assays.

Iron is essential for cell metabolism. It is involved in haemoglobin synthesis, oxidation-reduction reactions, and cellular proliferation and differentiation (13). However, the excess of iron concentration can cause toxic effects. The investigations conducted by He *et al.* (14) showed that Fe (III) decreased cell viability when assessed in the MTT assay. Moreover, the same study showed an increase in LDH leakage. This observation was correlated with an increase in MDA production. The same results were obtained by Minaeva *et al.* (19). It may suggest that Fe (III) alters membrane stability through ROS production in the way that excess ROS production can cause lipid peroxidation and damage to cell membranes, DNA, and proteins. These observations confirm our study. LDH leakage assay was most sensitive in both cell lines after iron chloride treatment. The weakening of mitochondrial activity and loss of lysosome integrity resulted in cell membrane damage. The other observations showed that Fe (III) bound to nitrilotriacetate (at molar rate of 1:1) could decrease membrane stability of Caco-2 cells (14). These observations confirm that the cell membrane is the most sensitive organelle after Fe (III) treatment.

Nickel and its compounds are used in industry. The toxicity of nickel has been investigated by many researchers, among them Ermoli *et al.* (7) whose experiments showed that Ni (II) decreased cell viability

on the HaCaT cell line (7). The experiments performed by Wang *et al.* (25) showed that Ni (II) acetate decreased viability of HK-2 cells in a concentration-dependent manner. The cell viability of the HK-2 cells (assessed by MTT assay) was less than 50% when treated with 480 µM of Ni (II) acetate. At the same time, the level of intracellular ROS increased. In normal rat kidney cells, an increase of ROS after Ni (II) treatment was observed, and this ROS production was associated with cytotoxicity in cells (4). Similar results were obtained by Lee *et al.* (15). Ni (II) treatment resulted in a gradual decrease in cell viability in nasal epithelial cells in a dose-dependent manner. In our study, nickel chloride decreased cell viability when assessed by MTT, NRU, and LDH leakage assays. The main mechanism of Ni-induced toxicity was ROS production. ROS can directly or indirectly interact with cell membranes, mitochondria, proteins, and DNA. Moreover, it has been shown that nickel inhibits mitochondrial succinate dehydrogenase activity. These mechanisms cause cell death (1, 10).

In the cells treated with iron chloride at a concentration of 200 µM and nickel chloride at a concentration of 1,000 µM, the protective effect of iron chloride was observed in the MTT assay. Iron is critical for formation of iron-sulphur clusters in mitochondrial succinate dehydrogenase (6). Probably, at low doses, iron provides proper activity of this enzyme and protects cells from nickel toxicity. However, after incubation of both cell lines with iron chloride at concentration of 1,000 µM and nickel chloride at concentration of 200 µM, no protective effect was observed in any assay. Probably, the concentration of ROS was very high. As a cofactor, nickel inhibits mitochondrial succinate dehydrogenase activity. These two mechanisms lead to cell death.

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Animal Rights Statement: None required.

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