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Cytotoxicity of iron (III), molybdenum (III), and their mixtures in BALB/3T3 and HepG2 cells

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Abstract

Introduction: Iron and molybdenum are essential trace elements for cell metabolism. They are involved in maintaining proper functions of enzymes, cell proliferation, and metabolism of DNA. **Material and Methods:** BALB/3T3 and HepG2 cells were incubated with iron chloride or molybdenum trioxide at concentrations from 100 to 1,400 μM. The cells were also incubated in mixtures of iron chloride at 200 μM plus molybdenum trioxide at 1,000 μM or iron chloride at 1,000 μM plus molybdenum trioxide at 200 μM. Cell viability was determined with MTT reduction, LHD release, and NRU tests. **Results:** A decrease in cell viability was observed after incubating both cell lines with iron chloride or molybdenum trioxide. In cells incubated with mixtures of these trace elements, a decrease in cell viability was observed, assessed by all the used assays. **Conclusions:** Iron (III) and molybdenum (III) decrease cell viability in normal and cancer cells. A synergistic effect of the mixture of these elements was observed.

Keywords: molybdenum (III), iron (III), cytotoxicity, interactions.

Introduction

Micronutrients in human and animal organisms play a crucial role in the prevention and treatment of various diseases. They also play an important role in the optimisation of physical and mental functions. Moreover, the elements they contain are the components of biomaterials and dietary supplements. The relationship between trace elements *in vitro* and *in vivo* has attracted the attention of many investigators, among other reasons because nutrient-nutrient interactions may negatively or positively affect cell viability.

Molybdenum is an essential element for plants, animals, and microorganisms. Molybdenum belongs to the group of trace elements which are necessary to normal metabolism but is only beneficial when ingested in small amounts. In larger amounts, it can be toxic. Molybdenum is taken mainly with water and food. This element is a part of the active sites of many metalloenzymes. In eukaryotes, Mo enzymes can be subdivided into two classes: the xanthine oxidase (XO) family which is represented by xanthine dehydrogenase (XDH), aldehyde oxidase (AO), pyridoxal oxidase, and nicotinate hydroxylase and the SO class formed by sulphite oxidase (SO) and nitrate reductase (NR). These enzymes are involved in the catabolism of sulphur amino acids, purines, and pyrimidines (12, 13). However, molybdenum can induce oxidative stress and reactive oxygen species (ROS) formation. Molybdenum-induced ROS attack cell biomolecules such as cell membranes, polyunsaturated fatty acids, and DNA (19, 21). For this reason, many studies have shown that molybdenum induces genotoxic and mutagenic effects. Genotoxicity was assessed by comet and micronucleus assays, in which the percentage of DNA tail and frequency of micronuclei (MN) were observed in Syrian hamster embryonic (SHE) cells (3).

Iron is an essential metal. It is involved in oxidation-reduction reactions, the cell cycle, and

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replication and metabolism of DNA. Iron is fundamental for binding iron-sulphur cluster proteins such as dehydrogenases in mitochondria (14). However, an excessive iron concentration in cells causes ROS production (9). The ROS production induced by iron is mainly through the Fenton reaction, in which ROS react with nucleic acid bases, consequently forming 8-hydroxyguanine (8-OHG). Iron-induced ROS also react with lipids to form lipid hydroxyl-peroxide (ROOH) as the main product. The final products of this reaction are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (9). ROS may also react with DNA, causing its damage. There are many reports about iron-induced genotoxicity and mutagenicity, among which one is positing that iron compounds can induce mutations in *Salmonella* Typhimurium (1).

For toxicity evaluations, mitochondrial function (MTT reduction assay), cell membrane integrity (lactate dehydrogenase (LDH) release assay), and lisosome integrity (neutral red uptake (NRU) assay) were assessed under control conditions and when exposed to iron (III), molybdenum (III), and their mixture. The most common applications of cytotoxicity testing techniques are MTT or NRU assays. As the cytotoxicity of chemicals can be mediated by different toxic mechanisms that influence different endpoints, many reports recommend the use of multiple, mechanistically different endpoint parameters for the evaluation of xenobiotic cytotoxicity. From this standpoint, the LDH assay is very useful for examining the three different endpoints on the same cells at the same time (4).

The aim of this study was to evaluate cytotoxicity of molybdenum trioxide or iron chloride and their mixtures on BALB/3T3 and HepG2 cell lines with the use of MTT, LDH, and NRU assays.

Material and Methods

Cell culture and treatment. Mouse embryo fibroblast 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 Dulbecco's modified eagle medium (ATCC) supplemented with 10% (v/v) heat-inactivated calf bovine serum (ATCC) and antibiotic-antimycotic solution (Sigma-Aldrich, USA). The cells were maintained at 37ºC in a humidified incubator in an atmosphere with 5% CO_2 (20).

The liver cancer HepG2 cells (ATTC HB-8065) were also obtained from the ATCC. These cells were cultured as adherent monolayers in plastic tissue culture dishes, but in Eagle's minimum essential medium (ATCC) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (ATCC) and antibiotic-antimycotic solution (Sigma-Aldrich). The cell cultures were maintained at 37ºC in a humidified incubator in an atmosphere with 5% CO_2 (20).

$FeCl_3 \times 6H_2O$ and MoO_3 (Acros Organics, Belgium) were dissolved in PBS at a concentration of 10 mM. The final concentrations were obtained by dilution in culture medium supplemented with serum and antibiotics (20).

Cytotoxicity assays. In order to perform MTT reduction, LDH release, and neutral red uptake tests, 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 molybdenum trioxide within a range of concentrations from 100 to 1,400 μM. The cells now exposed to the mixture of the 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 molybdenum trioxide in one case, and in the other case with 1,000 μM of iron chloride and 200 μM molybdenum trioxide. After 24 h of incubation MTT reduction, LDH release, and neutral red uptake assays were performed (20).

The MTT reduction colorimetric, NRU, and LDH release assays were performed using commercially available kits (TOX-1, TOX-4, and TOX-7, respectively) from Sigma-Aldrich according to the manufacturer's instructions, as previously described in the literature (20).

In the MTT and NRU assays, the cell viability was quantified spectrophotometrically compared to the control cells (the absorbance of the control cells as 100% viability). In the LDH release assay, a curve was drawn on the basis of the results. The IC_{50} (50% inhibitory concentration) value was calculated in all three assays. Per assay, six independent experiments were performed with three wells per treatment condition.

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

Results

As shown in Fig. 1, the BALB/3T3 and HepG2 cells displayed statistically significant decreases in cell viability after iron chloride and molybdenum trioxide treatment as assessed by MTT assay. However, in the case of iron chloride at concentrations of 100 and 200 μM, the tested compounds were not toxic, and even slightly stimulated cell viability. The IC_{50} values obtained by the MTT assay are shown in Table 1. An IC_{50} value was obtained in the MTT assay only when the HepG2 cells were exposed to iron chloride.

The toxic effects of iron chloride or molybdenum trioxide on membrane integrity in normal and cancer cells were examined by the LDH release assay. As shown in Fig. 2, in both cell lines, dose-dependent increases of lactate dehydrogenase release were observed after iron chloride treatment. However, in the case of molybdenum trioxide, similar effects were not observed. The IC_{50} values obtained by the LDH release assay are shown in Table 1. An IC_{50} value was obtained in the LDH release assay only when the BALB/3T3 and HepG2 cells were exposed to iron chloride.

The effects of iron chloride or molybdenum trioxide on cell viability in non-tumorigenic and cancer cells were examined by NRU assay. At concentrations of 100 and 200 µM experimental cell viability was slightly stimulated or it was the same as control cell viability in both cell lines and after treatment with both compounds (Fig. 3). Dose-dependent cytotoxic effects were observed after iron chloride treatment in the HepG2 cells and after molybdenum trioxide treatment in the BALB/3T3 cells. The IC_{50} values obtained by NRU assay are shown in Table 1. IC_{50} value was obtained in NRU assay only when the BALB/3T3 cells were exposed to molybdenum trioxide, and the HepG2 cells were exposed to iron chloride.

The mixture of iron chloride at the concentration of 200 µM and molybdenum trioxide at the concentration of 1,000 µM in the first case, and in the other case - the mixture of iron chloride at the concentration of 1,000 µM and molybdenum trioxide at the concentration of 200 µM caused synergistic effects which were observed in the decreased viability (assessed by MTT and NRU assays) and, at the same time, in the increased LDH release in both cell lines.

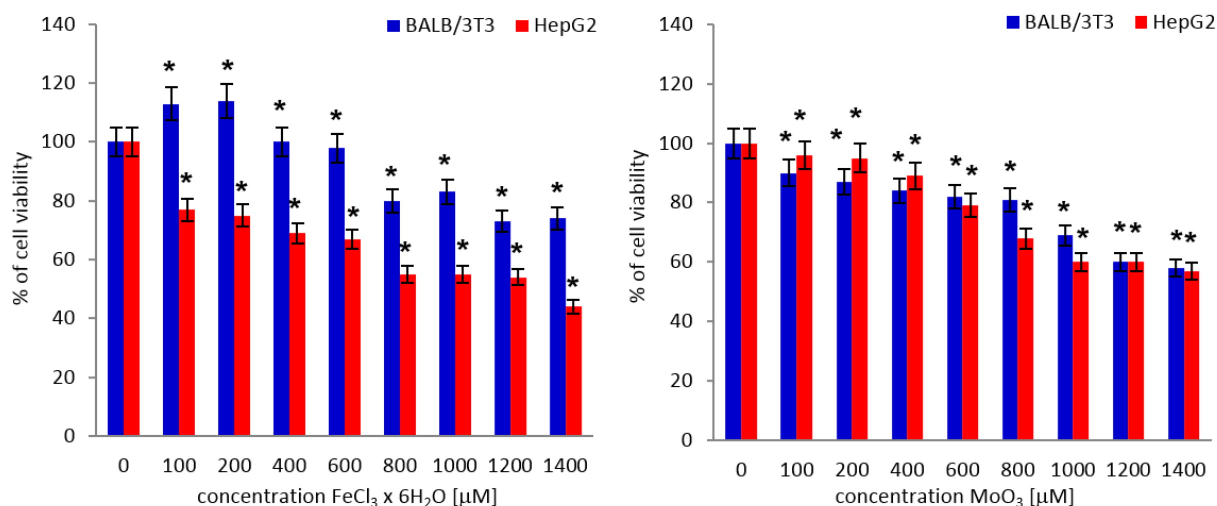

Fig. 1. Cytotoxicity of iron chloride and molybdenum trioxide in the BALB/3T3 and HepG2 lines detected with MTT assay, n = 6. Values are given as percentage of cell viability relative to control cells. *P < 0.05 compared to control cells

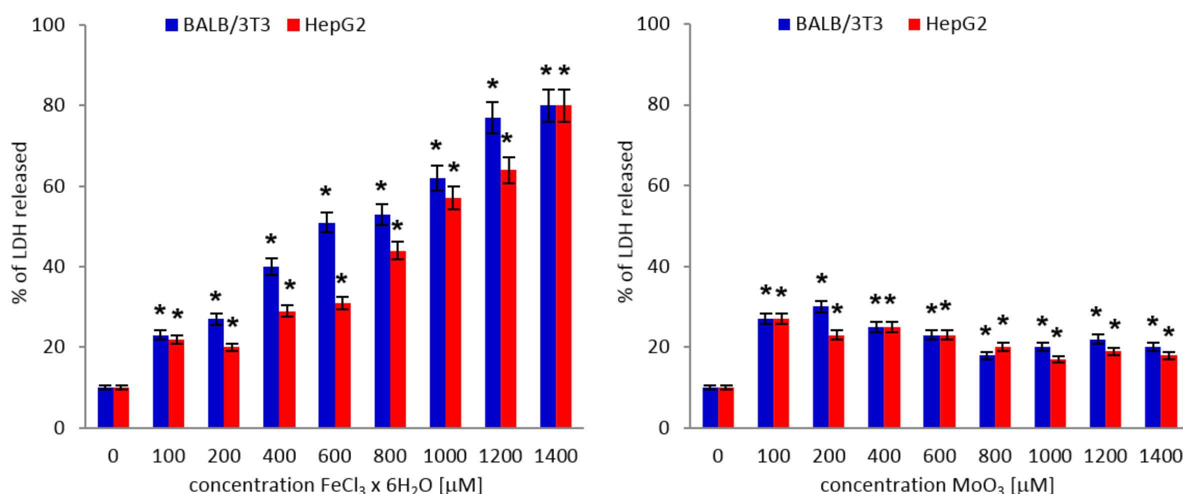

Fig. 2. Cytotoxicity of iron chloride and molybdenum trioxide in the BALB/3T3 and HepG2 lines detected with LDH assay, n = 6. Values are given as percentage of LDH released. *P < 0.05 compared to control cells

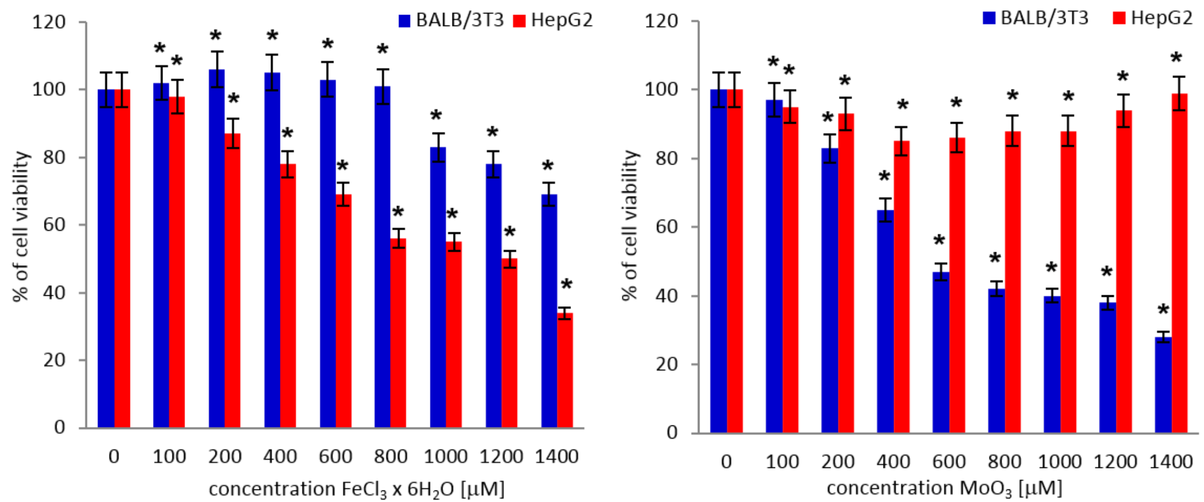

Fig. 3. Cytotoxicity of iron chloride and molybdenum trioxide in the BALB/3T3 and HepG2 lines detected with NRU assay, n = 6. Values are given as percentage of cell viability relative to control cells. *P < 0.05 compared to control cells

Table 1. Inhibitory concentration (IC_{50}, µM) values for the BALB/3T3 and HepG2 cells following exposure to iron chloride and molybdenum trioxide

Metal compounds	Assay	BALB/3T3 cell line	HepG2 cell line
Molybdenum trioxide			
	MTT	–	–
	LDH	–	–
	NRU	500 µM	–
Iron chloride			
	MTT	–	1,300 µM
	LDH	550 µM	900 µM
	NRU	–	1,200 µM

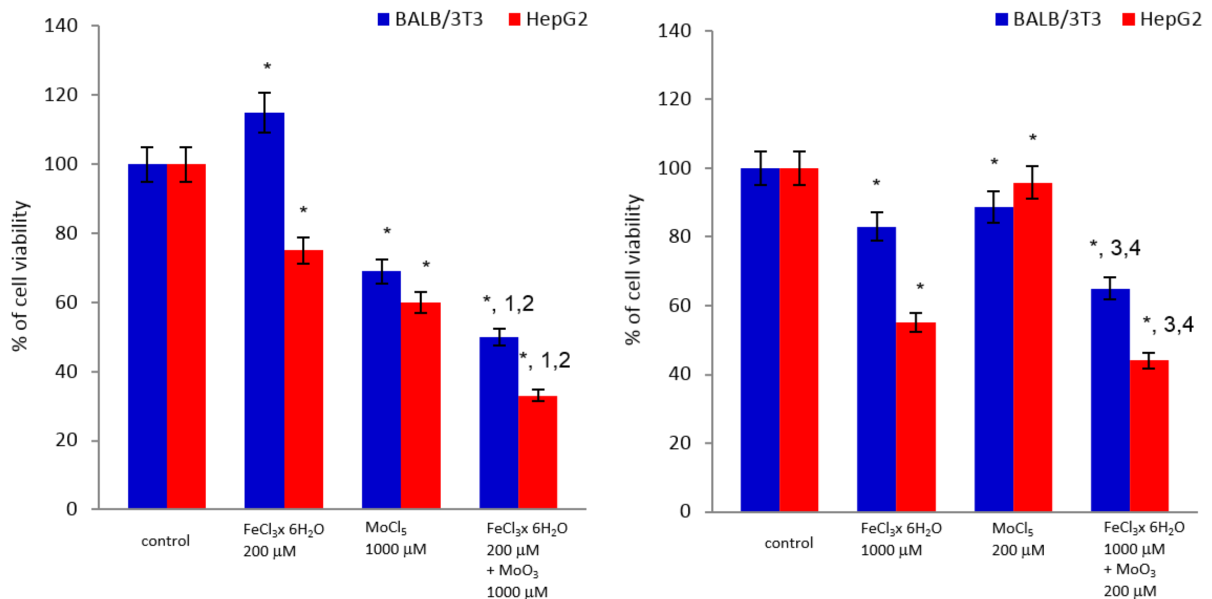

Fig. 4. Cytotoxicity of a mixture of iron chloride and molybdenum trioxide in the BALB/3T3 and HepG2 lines detected with MTT assay, n = 6. Values are given as percentage of cell viability relative to control cells

*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 molybdenum trioxide 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 molybdenum trioxide at concentration of 200 µM

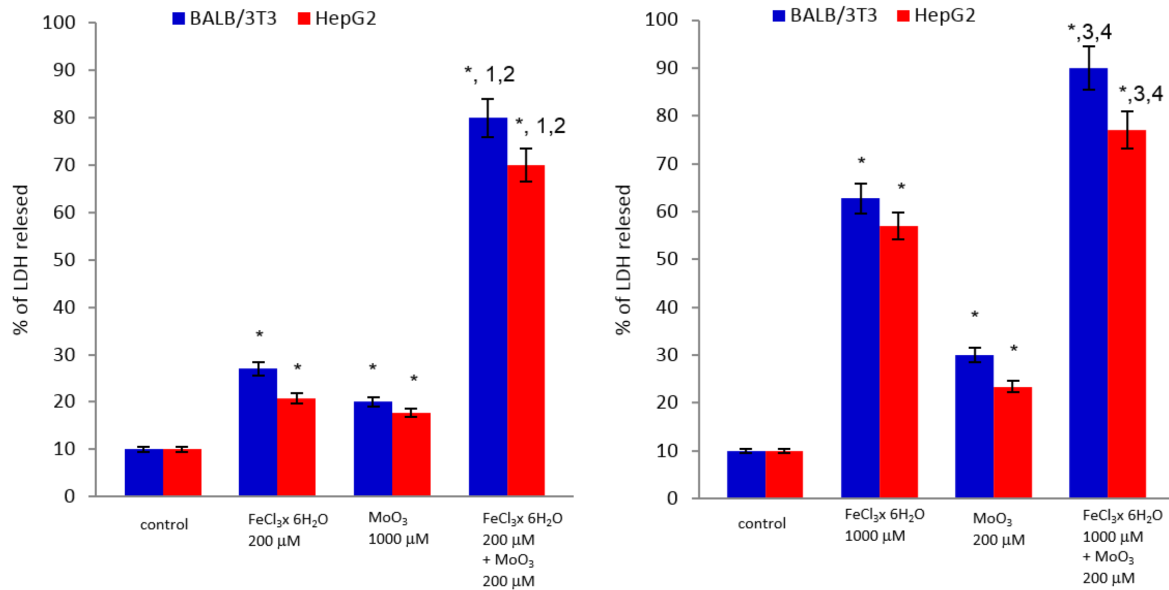

Fig. 5. Cytotoxicity of a mixture of iron chloride and molybdenum trioxide in the BALB/3T3 and HepG2 lines detected with LDH assay, n = 6. Values are given as percentage of LDH released
*P < 0.05 compared to control
[1]P < 0.05 compared to iron chloride at concentration of 200 µM
[2]P < 0.05 compared to molybdenum trioxide 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 molybdenum trioxide at concentration of 200 µM

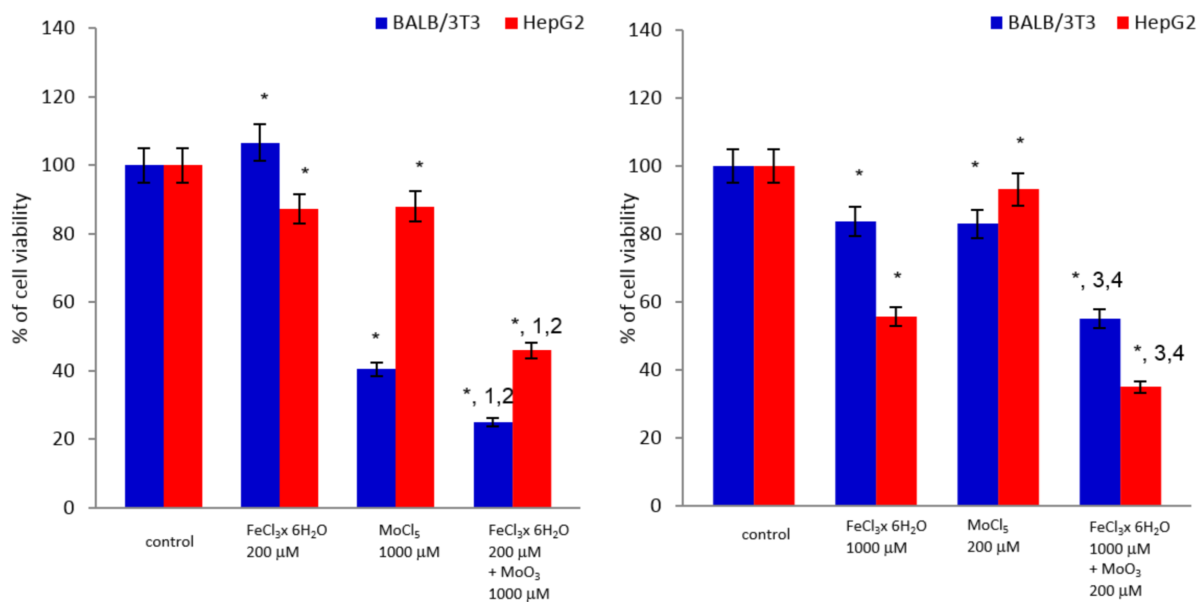

Fig. 6. Cytotoxicity of a mixture of iron chloride and molybdenum trioxide in the BALB/3T3 and HepG2 lines detected with NRU assay, n = 6. Values are given as percentage of cell viability relative to control cells
*P < 0.05 compared to control
[1]P < 0.05 compared to iron chloride at concentration of 200 µM
[2]P < 0.05 compared to molybdenum trioxide 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 molybdenum trioxide at concentration of 200 µM

Discussion

The purpose of this study was to evaluate the cytotoxicity of molybdenum trioxide or iron chloride and their mixtures on the BALB/3T3 and HepG2 cell lines. The cytotoxicity assays used in this study employed mitochondrial function (MTT), lysosome function (NRU), and membrane integrity (LDH release).

The concentrations of iron chloride and molybdenum trioxide were chosen on the basis of other reports and our earlier investigations (10, 19, 20). At 200 μM concentration in both trace elements, no degradation in cell viability was 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 observed (20).

The BALB/3T3 cell line was chosen for our investigations because it was suggested as a cellular model for study of the morphological and biochemical changes induced by biometals (10, 15). Human cell lines are sensitive tools for high-throughput toxicity screening which can potentially reduce toxicological testing in animals (11). Human hepatoblastoma HepG2 cells have been well characterised and frequently used as an *in vitro* toxicity model. They have also been used to distinguish between toxic and non-toxic substances (6, 16, 17).

The results observed in this study from biochemical endpoints showed that molybdenum trioxide was relatively non-toxic. When the BALB/3T3 cells were exposed to molybdenum trioxide, an IC_{50} value was only obtained in the NRU assay. Moreover, it was iron chloride rather than molybdenum trioxide which revealed the HepG2 cells to be more sensitive than the BALB/3T3 cells.

Molybdenum is an essential trace element for microorganisms, plants, and animals. Cells need Mo only in small amounts (12), and in higher concentrations, it may be toxic. Our study showed that MoO_3 was non-toxic. Similar results were obtained by Hussain *et al.* (8), who showed that MoO_3 was not cytotoxic up to 100 μg/mL in BRL 3A rat liver cells. However, the study conducted by Braydich-Stolle *et al.* (2) showed that MoO_3 was toxic at 50 μg/mL and at higher concentrations in mammalian germline stem cells that was in agreement with a study by Siddiqui *et al.* (18). A concentration and time-dependent statistically significant decrease in cell viability percentage (assessed by MTT and NRU assays) in L929 cells was observed. The same authors suggested that oxidative stress may be the major mechanism of Mo-induced toxicity. Molybdenum induces ROS production and decreases antioxidant enzyme activity.

Iron sulphate reduces cell viability as assessed by MTT assay in the human glioblastoma astrocytic cell line U343 MG-a (1). Concurring results were observed by He *et al.* (7) as an iron chloride-induced decrease in cell viability in Caco-2 cells. The same authors showed an increase in LDH leakage and malondialdehyde concentration. MDA is the final product of lipid peroxidation induced by ROS (7). In our study, iron chloride was most toxic against cell membranes. The IC_{50} value was lowest in the LDH assay and the IC_{50} values in the three assays used established their sensitivity hierarchy as LDH>NRU>MTT for iron chloride in the HepG2 cells. Basing on our study and other reports it can be concluded that damage to cell membrane stability can be inflicted by iron (III). The main mechanism responsible for that is production of free radicals in the Fenton and Haber-Weiss reactions: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-}$ and $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^{\bullet} + H^{+}$ (5). Fe (III) also binds to nitrilotriacetate (at molar ratios of 1:1) and consequently decreases membrane stability of Caco-2 cells (7). Moreover, the same study showed that iron (III) decreases antioxidant enzyme activity. These mechanisms lead to cell membrane destruction.

In cells treated with iron chloride at a concentration of 200 μM and molybdenum trioxide at a concentration of 1,000 μM and in the same cells treated with iron chloride at a concentration of 1,000 μM and molybdenum trioxide at a concentration of 200 μM, a synergistic effect was observed in all the assays applied. It has been proved that these elements decrease cell viability when used alone. The cell viability degradation was caused by ROS production and impairment of antioxidant activity. These two mechanisms lead to cell death. As a result, a synergistic deleterious effect on cell viability was observed.

These initial findings indicate the need to carry out further investigations to identify the different mechanisms of interaction between trace elements. Such greater understanding could be useful to define the influence of these elements on human and animal health.

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