Iron chloride cytotoxicity and cytokines (IL-1α and IL-6) production in BALB/c mice embryo fibroblasts cell line

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Abstract

The aim of this study was to examine the effect of the iron chloride on mouse fibroblasts. The experiments were performed on the BALB/c 3T3 (mouse BALB/c embryo fibroblasts) cell line. The cell viability was measured by three tests: the MTT reduction, LDH release and Neutral Red uptake. The type of cell death was determined by photometric enzyme immunoassay. Moreover, the IL-1α and IL-6 concentration was determined by the sandwich-linked immunosorbent assay.

The present study showed that iron chloride decreases cells viability (confirmed by three cell viability tests) after 24 hours of incubation. The suggested mechanism of iron action in mouse embryo fibroblasts can be related to the iron interaction with cell membrane, subsequntly with mitochondria and, finally with lysosomes. Moreover, iron decreases concentration of mono- and oligonucleosomes in the cytoplasm and IL-6 concentration secreted into the cell culture supernatant. However, the concentration of IL-1α increases after incubation with iron chloride.

Key words: *iron, cytotoxicity, necrosis, cytokines.*

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Introduction

Iron is an essential element playing a vital role in many cellular processes [1]. It is the key component of many cellular enzymes, such as oxidases, catalases, peroxidases, cytochromes, nitric oxide synthases, ribonucleotide reductases and aconitases. These enzymes are involved in many cellular processes, i.e. DNA and RNA synthesis, oxygen and electron transport, cell proliferation [2].

On the other hand, iron in its free form is a potential cytotoxic agent because of its property to form insoluble salts and to catalyze formation of free radicals [3, 4]. Iron participates in the oxidation-reduction reaction known as the Fenton reaction:

$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^{\bullet}$

$Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2$

Hydroxyl and superoxide radicals are highly toxic. The net effects are DNA damage, impaired synthesis of proteins, induction of proteases and altered cell proliferation. In addition, free iron can react directly with unsaturated fatty acids and cause cell death. This suggest that iron may play a role in the multi-step processes of carcinogenesis; pathogenesis of atherosclerosis; or neurodegenerative disorders, such as Parkinson's or Alzheimer diseases [2].

The aim of presented study was to investigate the influence of iron chloride on mouse embryo fibroblasts.

Material and methods

Chemicals and materials

The Dulbecco's Modified Eagle Medium (DMEM), the heat-inactivated Fetal Bovine Serum (FBS), antibiotic/antimycotic (penicillin, streptomycin, amphotericin B), iron chloride ($FeCl_3 \times 6H_20$), Neutral Red Kit and LDH Kit, were purchased from Sigma Chemical Co., (St. Louis, MO, USA). The MTT Kit, IL-1α and IL-6 ELISA Kits were obtained from R&D Systems Europe (UK). The Cell Death Detection ELISA Kit was obtained from Roche

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(Germany). Phosphate-buffered saline (PBS) and 0.25% trypsin were purchased from Biomed (Lublin, Poland) and tissue culture dishes were purchased from Nunc Brand Products (Denmark).

Cell culture and treatment

Mouse embryo fibroblasts (cell line BALB/c 3T3) kindly gifted by D. Śladowski (Department of Transplantology & Central Tissue Bank, Centre of Biostructure, The Medical University of Warsaw). The cells were cultured as adherent monolayers in plastic tissue culture dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS) and penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). Cells were maintained at 37°C in humidified incubator in atmosphere containing 5% CO_2. The cells were subcultured three times a week treating with 0.25% trypsin at 37°C for 5 min. Cells were used for cytotoxic assays during exponential phase of growth.

$FeCl_3 \times 6H_2O$ was dissolved in PBS at the concentration of 1 mM. The final concentration was obtained by the dilution in culture medium (DMEM) supplemented with FBS and antibiotics.

In order to perform MTT, LDH, NR assays, cell death detection assay, IL-1α and IL-6 assays, the cells were cultured in 96-well plates (2×10^5 cells/ml) in 100 µl DMEM, supplemented with 10% FBS and antibiotics. After 24 hours of incubation the medium was exchanged and fresh DMEM supplemented with $FeCl_3 \times 6H_2O$ at final concentrations as follows: 0 (control), 50, 100, 200, 300, 400, 500, 600, 700 and 800 µM was added to the wells. Subsequently, MTT, LDH, NR assays and the cell death detection assay were performed. In the cultures supplemented with 0 (control), 50, 100, 300, 500 and 700 µM $FeCl_3 \times 6H_2O$ IL-1α and IL-6 concentration was measured. All concentrations mentioned above are the final concentrations in the incubations. After 24 hours of incubation, assays were performed according to the original manufacture's instruction. The absorbance in MTT, LDH, NR assays and the cell death detection assay were measured with the use of the microplate reader at 620 nm wavelength for MTT assay, 490 nm wavelength for LDH, NR, cell death detection assays, and 450 nm wavelength for IL-1α and IL-6 assays. All experiments were performed independently at least six times.

The MTT assay: the IC50 (concentration causing 50% reduction in growth compared with the control) value was calculated from the graph of the dose-response curve.

The type of cell death analysis: After the experiment the enrichment factor of mono- and oligonucleosomes released into the cytoplasm was calculated:

$$n \text{ (enrichment factor)} = \frac{\text{the absorbance of the sample}}{\text{the absorbance of corresponding negative control}}$$

Interleukin 1α and interleukin 6 cytokine measurement

Cytokines (IL-1α and IL-6) concentrations were measured by the sandwich-linked immunosorbent assay with the use of commercially available kits (R&D Systems) according to the manufacture's instruction. A standard curve was constructed by plotting the absorbance of each standard vs. the corresponding standard concentration and then, the cytokine levels of unknown samples were calculated. The sensitivities of assays were as follows: 2.5 pg/ml for IL-1α and 1.6 pg/ml for IL-6.

Statistical analysis of data

The results from MTT, LDH, NR, cell death and cytokines assays were analysed with the use of Student's *t*-test (Statistica program). The accepted level of significance was $p < 0.05$. All results are presented as mean values ± SD.

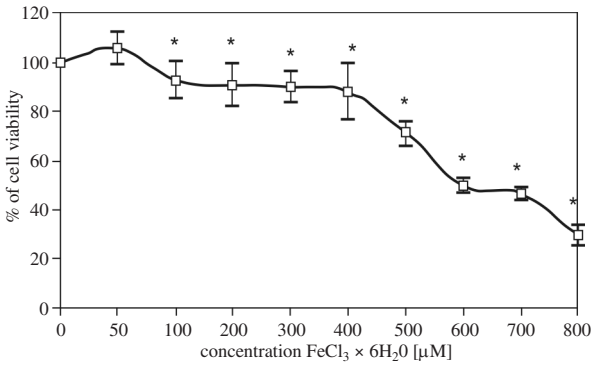

Fig. 1. Cytotoxic effect of iron chloride in BALB/c 3T3 line detected with the MTT reduction assay. Values are given as percentage of cell viability relative to cells without iron (control); *$p < 0.05$, significance of difference compared with control

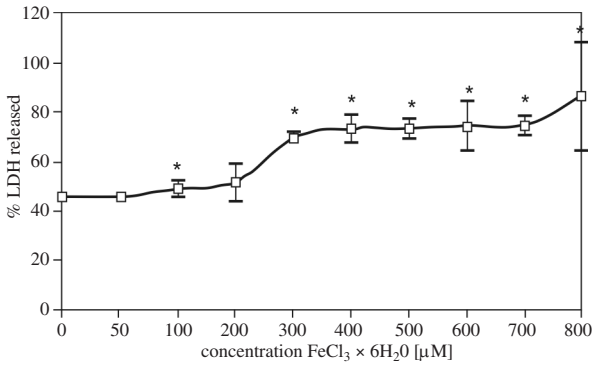

Fig. 2. Cytotoxic effect of iron chloride in BALB/c 3T3 line detected with the LDH release assay. Values are given as percentage of LDH release relative to cells without iron (control); *$p < 0.05$, significance of difference compared with control

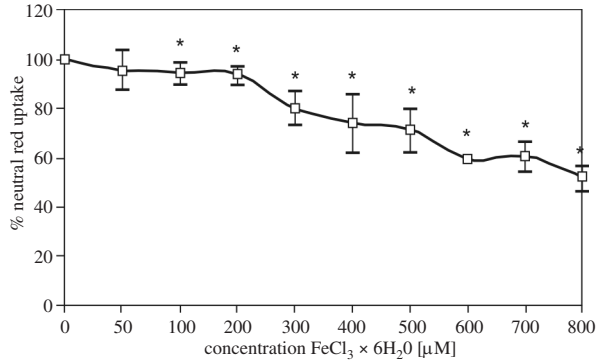

Fig. 3. Cytotoxic effect of iron chloride in BALB/c 3T3 line detected with the Neutral Red uptake assay. Values are given as percentage of neutral red uptake relative to cells without iron (control), $*p < 0.05$, significance of difference compared with control

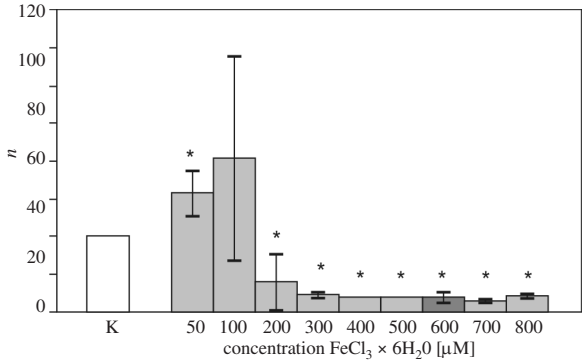

Fig. 4. *n* factor values after incubation BALB/c 3T3 cell line with iron chloride, $*p < 0.05$, significance of difference compared with control

Results

The results of the cell viability after incubation of cells with iron chloride, at various concentrations, are displayed on Figures 1-3. Figure 1 shows that there was a concentration-dependent decrease in the cell viability (statistically significant). Iron chloride was toxic in the concentration of 100, 200, 300, 400, 500, 600, 700, 800 μM. At concentration of 50 μM iron chloride slightly stimulated the cell viability.

The cell viability was also determined by LDH release assay (Fig. 2). BALB/c 3T3 fibroblasts exposed to iron chloride demonstrated a concentration-dependent increase of LDH release from the cells. Iron chloride was toxic in the concentration of 100, 300, 400, 500, 600, 700 and 800 μM.

The cell viability was also determined by Neutral Red uptake assay. The results are expressed at Figure 3. The concentration-dependent decrease (statistically significant) in Neutral Red uptake was observed.

Figure 4 demonstrates decrease of n factor after incubation with 200, 300, 400, 500, 600, 700 and 800 μM iron chloride.

Figure 5 shows IL-1α levels in culture medium supplemented with iron chloride. The iron chloride significantly increases IL-1α concentration after incubation with 50, 100, 300, 500, 700 μM of iron chloride, whereas iron chloride significantly decreases IL-6 concentration (Fig. 6).

Discussion

The aim of presented work was to investigate the effect of iron chloride on mouse embryo fibroblasts. The BALB/c cell line was chosen as a cellular model for studying morphological and biochemical changes induced by chemical substances [5]. Moreover, this cell line is recommended for cytotoxicity assays by European Union (Council directive 86/609/EEC).

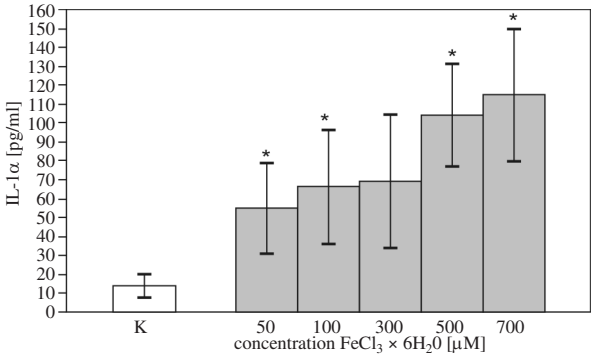

Fig. 5. The interleukin IL-1α concentrations after incubation with iron chloride, $*p < 0.05$, significance of difference compared with control

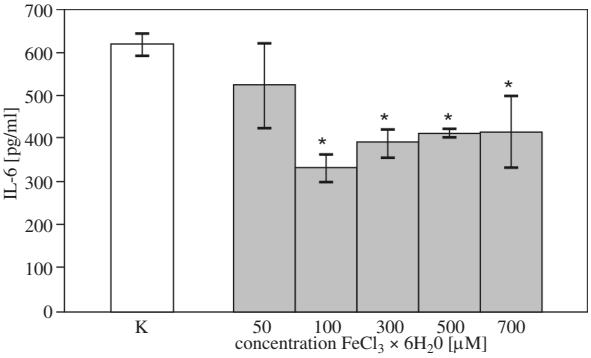

Fig. 6. The interleukin IL-6 concentrations after incubation with iron chloride, $*p < 0.05$, significance of difference compared with control

In vitro cytotoxicity assays can be used to predict toxicity of chemicals. In this work we used three different assays (MTT reduction, LDH release and Neutral Red uptake) to discover the mechanism of iron action in BALB/c mice cells. MTT reduction assay is based on the enzymatic conversion of MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) by the metabolically active cells in mitochondria. As a result, the MTT assay is a marker of mitochondrial function [6]. The LDH leakage assay is based on release of the enzyme in the culture medium, after cell membrane damage [6]. Therefore, LDH release assay can be used as an indicator of cell membrane integrity and cell viability. Whereas, Neutral Red release assay is a useful tool to detected lysosomal damage, based on ability of viable cells to incorporate and bind neutral red. In contrast, dead cells did not incorporate dye. Therefore, this test allows to distinguish between viable, damaged or dead cells [6]. The suggested mechanism of iron action in mouse embryo fibroblasts can be related to the iron interaction with cell membrane, subsequntly with mitochondria and, finally with lysosomes. Moreover, the present study has shown, that iron chloride decreases cell viability after 24 h of the incubation. Similar results were obtained by Zödl *et al*. in Caco-2 cells incubated with iron at concentration of 1.5 mM using LDH release test [7]. Moreover, investigations performed by these authors have shown, that iron chloride in dose mentioned above, did not affect cell viability detected in MTT and trypan blue tests [7].

The cell death was determined by the photometric enzyme-immunoassay. The assay is based on a quantitative sandwich-enzyme-immunoassay principle with the use of mouse monoclonal antibodies directed against DNA and histones, respectively. This test is based on a specific reaction between DNA and histones (H1, H2A, H2B, H3, H4), which are released during apoptosis. Our investigations have shown the decrease of *n* factor (enrichment of mono- and oligonucleosomes released into cytoplasm) after incubation with iron chloride compared with control. That suggests necrosis in cells.

Moreover, the influence on IL-1α and IL-6 production was determined by ELISA assays. The present study has shown that iron chloride increases IL-1α concentration and decreases IL-6 concentration secreted into the cell culture supernatant. Investigations performed by other authors have found a higher release of IL-6 from mouse epidermal JB6 cells induced by Fe^{3+} when compared with control cells [8]. The investigations performed by Bergman *et al*. are in contrast with our investigations. They have shown that peripheral blood mononuclear cells (PBMC) incubated with 50 and 100 μg% iron have secreted significantly lower amounts of IL-1β than control cells. Moreover, iron at concentrations of 50, 100 and 200 μg%. had no effect on IL-6 release from these cells [9]. These discrepancies may be explained by the different cells models.

Iron salts have been used as pharmaceutical agent for many years. The exact mechanism, by which iron compounds coordinate immune and inflammatory processes is not entirely clear. Moreover, it is very important to get to know the mechanisms of iron action in different cell types. Our investigations have shown the mechanism of iron action and cytokines production in fibroblasts. However, these problems still demand a lot of investigations.

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