Iron availability and complex stability of iron hydroxyethyl starch and iron dextran—a comparative in vitro study with liver cells and macrophages

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Abstract

Background. Intravenous iron (IVI) therapy is required in patients with end-stage renal disease (ESRD) under chronic haemodialysis (HD). In this in vitro study we investigated the availability and stability of iron hydroxyethyl starch (iron-Hes) compounds in THP-1 cells (macrophage phenotype) and liver cells (HepG2 cells) and compared it with the well-known iron dextran.

Methods. The uptake and release of these iron formulations by THP-1 cells (macrophage phenotype) and HepG2 cells were investigated with atomic absorption spectrometry (AAS). Ferritin was measured by ELISA. HepG2 cells were used to investigate effects of IVI on the intracellular labile iron pool (LIP), which was measured by using the fluorescent calcein assay. The amount of redox-active iron within the iron formulations was assayed using dichlorofluorescein as fluorescent probe.

Results. All iron preparations were taken up, stored in ferritin and released again by macrophages and HepG2-cells. This study shows that the availability and stability of iron-HES formulations in vitro are comparable with the well-known iron dextran compounds.

Conclusions. Our results indicate that these new iron formulations have a good stability and availability in vitro and are comparable with the well-known iron dextran complexes.

Keywords: HepG2; intravenous iron therapy; in vitro; iron dextran; iron hydroxyethyl starch; THP-1

Introduction

Parenteral iron formulations are widely used for the treatment of iron deficiency anaemia in patients with chronic renal failure. In haemodialysis (HD) patients treated with recombinant human erythropoietin (rHuEPO) intestinal iron absorption is insufficient to meet the enhanced iron requirement [1]. Therefore, iron supplementation is necessary to support erythropoiesis initiated by erythropoietin therapy to counteract renal anaemia [2].

Several parenteral iron formulations exist for the administration of intravenous iron (IVI) to patients with end-stage renal disease (ESRD) [3]. These different formulations are complexes of ferric iron with polymeric carbohydrates like dextran or organic compounds like sucrose or gluconate to form polynuclear complexes with the metal [4]. Recently, also ferric pyrophosphate (Fe-PP) has been used as a direct dialysis supplement [5].

These iron complexes are mainly degraded in reticuloendothelial cells, from where the iron is delivered to transferrin and further to the erythroblastic cells of the bone marrow. Furthermore it has been shown that parenteral iron formulations can be taken up by epithelial cells, such as the human hepatoma cells HepG2 [6,7]. In general, the various formulations are effective in correcting iron deficiency, but due to the potential toxicity of ferric compounds there are possible risks associated with IVI therapy. These risks include the possible role of iron in infectious diseases or atherosclerosis, its effect on endothelial cells, polymorphonuclear leucocytes and cytokines, as well as the occurrence of non-transferrin bound labile iron (NTBI) in the blood. Dependent on different carbohydrate moieties, the profiles of pharmacology and toxicology can vary between iron preparations [4]. The nature of the carbohydrate polymer shell appears to determine...
cellular iron uptake and the capability to induce cytotoxic effects [7].

The primary source of danger stems from the potential to release iron from the complex into the plasma as 'labile plasma iron' [8], especially with iron sucrose and iron gluconate compounds. In iron dextran complexes the binding between the components is very strong. The disadvantage of iron dextran is the risk of severe adverse reactions due to anaphylactic or allergic-type reactions [9]. High-molecular-weight dextran complexes alone are known to be antigenic by themselves [10]. Ferric sodium gluconate and iron sucrose have a lower incidence of allergic and anaphylactoid reactions.

Recently, newly synthesized iron hydroxyethyl starch compounds (iron-HES) were developed to minimize the risk of adverse reactions. Iron-HES is a colloidal suspension containing a ferric oxyhydroxide (FeOOH) core, surrounded by chains of low-molecular-weight hydroxyethyl starch. This type of compound binds iron very strongly within the FeOOH core, which suggests that the possible release of labile iron is minimized due to a strong binding of iron.

In this study, we compared for the first time different iron dextran and iron-HES formulations with regard to the availability and stability of these compounds in vitro. The cell culture models we used were human HepG2 hepatoma cells and human macrophage-like THP-1 cells. Our results indicate that iron-HES formulations could be a good alternative to the well-known iron dextran for IVI therapy.

**Subjects and methods**

**Materials**

The iron chelator deferiprone (L1) was a generous gift from Dr Peter Nielsen (UKE, Hamburg, Germany). Nitrilotriacetic acid (NTA), ferrous ammonium sulfate (FAS), diethylene triamine pentaacetate (DTPA) and HEPES were from Sigma (Vienna, Austria), 2’,7’-dichloro-dihydrofluorescein diacetate (DCFH-DA), calcein and its acetoxyethylster (calcein-AM) from Molecular Probes Inc. (Eugene, OR, USA). The iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) was a generous gift from Dr. Prem Ponka (Lady Davis Institute for Medical Research, Montreal, Canada). The human ferritin enzyme immunoassay test kit was from BioCheck (California, USA) and the solution for the protein assay (Bradford method) from BioRad.

**Cell culture**

HepG2 cells were cultured in DMEM medium containing 10% fetal calf serum, 2 mM L-glutamine and antibiotics (gentamycin 50 μg/ml) under standard tissue culture conditions. Cells were treated with trypsin (1.25 x) and re-suspended in DMEM medium and seeded on six-well or 12-well plates at a density of 10⁶ cells/ml. After 2 days the cells were in the log-phase and were used for the experiments.

THP-1 cells (a gift of the Institute for Cancer Research, Medical University of Vienna, Austria) were cultured in RPMI medium containing 10% fetal calf serum, 2 mM L-glutamine and antibiotics (gentamycin 50 μg/ml) under standard tissue culture conditions. For the experiments, the THP-1 cells were differentiated to adherent macrophages by incubation with 160 nM phorbol myristate acetate (in DMSO) and seeded at a density of 5 x 10² cells/ml in six-well or 12-well plates. After 4 days the cells were used for the experiments.

**Iron preparations**

The following iron preparations were used:

(i) Iron dextran-heptonate (Ursoferran®), C01
(ii) CosmoFer® iron dextran (CosmoFer®), C02
(iii) Iron hydroxyethyl starch 1A (iron-HESA1), C03
(iv) Iron hydroxyethyl starch 1F (iron-HES1F), C04
(v) Iron hydroxyethyl starch 2A (iron-HES2A), C05
(vi) Iron hydroxyethyl starch 2F (iron-HES2F), C06

**Uptake of parenteral iron preparations**

HepG2 cells and THP-1 (macrophage typ) cells were incubated with 2 mM IVI (in DMEM or RPMI, 10% fetal calf serum, 2 mM glutamine) for 3 h at 37°C. Afterwards the cells were washed once with ice-cold medium containing 50 μM DTPA and again with ice-cold pure medium to remove all surface-bound iron. For investigation of iron uptake the cells were lysed with NP40 buffer (150 mM NaCl, 1% IGEPAL CA630, 50 mM Tris and 1 mM PMFS). Finally, the iron content was measured by atomic absorption spectroscopy (AAS). The amount of iron measured in each samples was then normalized to the amount of protein in the same sample as measured with the Bradford method.

**Release of cellular iron**

The cells were loaded with IVI as described earlier. Finally the cells were washed and apotransferrin (2.5 mg/ml) was added to the supernatant to initiate the release of iron. Aliquots from the supernatant were taken at different time points (0, 5, 15, 30, 60, 120 min) to determine the iron which was released from the cells into the supernatant. The amount of iron was measured with AAS and was correlated with the amount of protein of the same sample.

**Ferritin ELISA**

The cells were washed with medium (37°C) and then incubated with IVI in DMEM/RPMI containing 10% of fetal calf serum and 2 mM glutamine for 3,6 and 24 h, washed on ice, once with 50 μM DTPA and twice with medium alone and finally lysed with NP40 buffer, sonicated and stored at -80°C. Ferritin was determined by a human ferritin enzyme immunoassay test kit (BioCheck, California, USA).
Measurement of the labile iron pool (LIP)

The method was carried out according to Sturm et al. [7]. Briefly, HepG2 cells were cultured in 96-well plates (Greiner) and incubated with different concentrations of IVI in DMEM with 10% fetal calf serum and 2 mM glutamine. After the incubation, the cells were washed once with ice-cold medium containing 50 μM DTPA for 5 min and twice with ice-cold medium to remove surface-bound iron. Then the cells were loaded with 0.25 μM calcein-AM in 20 mM HEPES buffered medium for 15 min at 37°C and washed again. The plate was measured at 485 nm/535 nm (measurement A) with a fluorescence plate reader (ViktorII, HV, Vienna). Two minutes after addition of 100 μM SIH, a strong iron chelator, the plate was measured again (measurement B). The difference between measurement B and measurement A represented labile iron.

Measurement of non-transferrin-bound iron (NTBI)

Redox-active iron was measured with slight modifications as described by Esposito et al. [11]. Briefly, 0.5 ml of 1 mM DCFDA in water was mixed and incubated with 2 ml of 0.01 N NaOH for 30 min at room temperature and then neutralized with 10 ml of 25 mM Na-phosphate buffer (pH 7.2), 20 μl of the iron preparations diluted in plasma-like medium or serum were transferred in quadruplicates to black, clear-bottom 96-well plates (Greiner, Bio-one, Graz, Austria). Two wells were incubated with iron free HBS (20 mM Hepes, 150 mM NaCl, pH 7.4; Merck, Darmstadt, Germany) containing 150 μM ascorbate and 5 μM DCFH at 37°C in the dark. The other two wells were incubated with 180 μl of the same solution containing 50 μM of the iron chelator deferiprone (L1). Before use, the HBS was rendered iron free by treatment with 1 g/100 ml Chelex-100 (Sigma-Aldrich, Vienna, Austria). The kinetics of fluorescence increase were measured using a fluorescence multwell plate reader (Victor II, Perkin Elmer, Vienna, Austria) (excitation 485 nm, emission 535 nm). Measurements between 120 and 390 min were used to calculate slopes (r) of DCF fluorescence intensity over time. The fluorescence increase measured in presence of L1 represents oxidation of DCF by several other oxidants, e.g. in the presence of peroxidases or hypochlorous acid generated by myeloperoxidases. Therefore, the difference in the rate of oxidation of DCF with and without addition of the chelator represents the redox-active component of NTBI. The duplicate values of r with and without addition of L1 were averaged and redox-active iron (μmole) was determined from calibration curves correlating the difference in slopes with and without L1 against the iron concentration. The calibration curve was generated using various concentrations, ranging from 0 to 25 μM, of ferrous ammonium sulphate with NTA (ratio Fe:NTA of 1:7) in plasma-like medium.

Statistical analysis. Data were analysed with the GraphPad Prism software. Results are presented as means±SEM. Differences were examined for statistical significance using the paired t-test. Significant differences are marked in the figures with *P<0.05, **P<0.01 and ***P<0.001. Differences with P<0.05 were assumed to be significant.

Results

Uptake of intravenous iron by HepG2 and THP-1 cells

When incubated for 3 h at 37°C with 2 mM of the various iron formulations all of them were taken up by HepG2 cells and THP-1 cells, but there were differences between these two cell lines and between the different iron preparations. HepG2 cells took up much more iron than THP-1 cells. The amount of iron which was taken up by differentiated THP-1 cells was almost equal for all iron preparations whereas in HepG2 cells the two dextran complexes, C01 and C02, showed a lower uptake than the other iron formulations (Figures 1A and 1B).

Release of iron by HepG2- cells and THP-1 cells

In a recent study, we showed the ability of parenteral iron preparations to deliver iron to cells others than the
reticuloendothelial cells, their effect on intracellular iron metabolism and indirectly on the LIP of the human hepatoma cells HepG2 [6]. HepG2 cells mainly take up iron and store it in ferritin. Under physiological conditions when more iron is needed, the stored iron should be able to be released to provide it to the circulation.

To measure the availability of the iron-HES compounds, we loaded THP-1 cells (macrophage phenotype) and HepG2 cells with the iron formulations for 3 h at 37°C. The release of iron was investigated with apotransferrin (2.5 mg/ml) in the supernatant. The amount of iron was determined by atomic absorption spectroscopy.

Interestingly, most of the iron was released within the first 5 min in both cell types and could only slightly increase with time. Whereas the THP-1 cells released almost all the iron which was taken up during the loading process, HepG2 cells only released about 25% of the iron.

Concerning the release of iron, all iron formulations showed similar release rates in both cell types. Only the two iron dextran formulations, C01 and C02, which showed lower iron uptakes in HepG2 cells, also showed slightly less iron release in these cells (Figures 2A and 2B).

**Labile iron pool**

The primary source of danger stems from the potential release of iron into the plasma as ‘labile plasma iron’ [8], as well as from the so-called cellular LIP, whose size mirrors all aspects of intracellular iron homeostasis. The chemical composition of the LIP has remained essentially elusive, but it may be implicated in generation of oxidative cell damage [12–14].

To assess the influence of the IVI preparations on the LIP in HepG2, the intracellular LIP was studied by the fluorescent calcein-assay [15]. We compared the influence of the iron formulations on the LIP in a concentration-dependent manner.

After exposure of HepG2 cells to the iron compounds for 3 h at 37°C with different iron concentrations ranging from 0 to 750 μM in complemented medium, the increase of labile iron, corresponding to the labile iron pool, could be detected by the calcein-AM assay. A dose-dependent increase in the LIP up to 200% of control levels was observed and there was no difference among the preparations. The LIP in macrophages was not measured due to technical reasons. Differentiated THP-1 cells form colonies in the state of macrophages. For LIP measurements we need a confluent adhesion from the cells which cannot be achieved with differentiated THP-1 (macrophage phenotype) cells. (Figure 3).

**Ferritin measurements**

Changes in the size of the LIP influence the expression of proteins like ferritin. An increase in the LIP should therefore result in increased ferritin synthesis [16].

![Fig. 2.](image)

**Fig. 2.** Release of iron by HepG2 and THP-1 cells after pre-incubation with intravenous iron. HepG2 and THP-1 cells were incubated with 2 mM intravenous iron (in DMEM/RPMI, 10% fetal calf serum, 2 mM glutamine) for 3 h at 37°C. Finally the cells were washed once with warm pure medium and then incubated for 3 h at 37°C with different iron concentrations, ranging from 0 to 750 μM in complemented medium (DMEM, 10% fetal calf serum, 2 mM glutamine). After 3 h the cells were washed once with ice-cold 50 mM DTPA and twice with ice medium to remove surface-bound iron. Then the cells were loaded with 0.25 mM calcein-AM in 20 mM HEPES-buffered medium for 15 min at 37°C. Afterwards the cells were washed with pure medium. Finally the medium with an anti-calcein antibody and 20 mM HEPES buffer was added to the cells and the plate was measured at 485nm/535nm (measurement A). Two minutes after the addition of 10 mM SH (a strong iron chelator), the plate was measured again (measurement B). The difference between measurement B and measurement A represented labile iron. [Unloaded control cells were set as (100%) fluorescence.] Data are represented as means ± SEM (n = 4).

![Fig. 3.](image)

**Fig. 3.** Amount of labile iron in HepG2 loaded with intravenous iron. HepG2 cells were cultured in 96-well plates (Greiner, Bio-one, Graz, Austria), were washed once with warm pure medium and then incubated for 3 h at 37°C with different iron concentrations, ranging from 750 to 500 μM in complemented medium. After 3 h the cells were washed once with ice-cold 50 mM DTPA and twice with ice medium to remove surface-bound iron. Then the cells were loaded with 0.25 μM calcine-AM in 20 mM HEPES-buffered medium for 15 min at 37°C. Afterwards the cells were washed with pure medium. Finally the medium with an anti-calcein antibody and 20 mM HEPES buffer was added to the cells and the plate was measured at 485nm/535nm (measurement A). Two minutes after the addition of 10 μM SIH (a strong iron chelator), the plate was measured again (measurement B). The difference between measurement B and measurement A represented labile iron. [Unloaded control cells were set as (100%) fluorescence.] Data are represented as means ± SEM (n = 4).
The ferritin content in HepG2 cells and macrophages was investigated after incubation of the cells with 2 mM iron from different iron preparations with a ferritin ELISA. As shown in Table 1 the amount of ferritin in HepG2 cells increased with time. Again the dextran formulations showed higher ferritin values despite lower amounts of iron uptake (Figure 1A). The ferritin content of THP-1 macrophages showed no increase after incubation with different iron formulations (Table 1).

Redox-active labile plasma iron

Oxidative stress in vivo is the result of an imbalance between the production of oxidants and the respective defence systems of an organism [17]. Free iron presents a danger for the generation of reactive oxygen species. If the iron within the iron formulations is weakly bound, free redox-active iron can occur. Therefore, we tested the iron formulations for the presence of redox-active iron by the dichlorofluorescein method. All iron formulations showed very low concentrations of redox-active iron which only represented a negligible part of the whole iron content, used in this assay. Compared with the other iron preparations, C01 and C02, the iron-dextran compounds, showed slightly higher amounts of redox-active iron but the concentrations were also negligible. (Figure 4).

Discussion

Patients with ESRD undergoing HD receive rHuEPO to correct anaemia. During rhuEpo therapy these patients can acquire absolute or functional iron deficiency due to increased haemoglobin synthesis. Therefore, the administration of parenteral iron preparations is necessary to counteract iron deficiency.
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and anaemia. Furthermore parenteral iron preparations influence the response to rhuEpo positively and can lower its doses down to 40% [18].

For parenteral iron therapy in ESRD multiple iron formulations exist [3]. The preparations are complexes of ferric iron with polymeric carbohydrates like dextran or organic compounds like sucrose or gluconate forming polymeric complexes with the metal [4]. In vivo, after injection, the iron-carbohydrate complex is metabolized, largely by the reticuloendothelial system. Iron gets released and binds to transferrin in the plasma while the rest of the carbohydrate shell is metabolized in the liver. The degradation of the various iron preparations is different. However, the smaller the molecular mass, the faster the release of iron can be mediated. This also represents the limiting factor for the maximal single dose of an iron preparation because a fast release of high amounts of iron leads to oversaturation of transferrin and might generate free iron [19]. On the other hand, high-molecular-weight iron dextran preparations show the highest risk for adverse drug events like anaphylactic reactions whereas non-dextran formulations appear to be safer [20].

However, we recently showed that parenteral iron preparations also deliver iron to cells other than the reticuloendothelial cells [6]. HepG2 cells, an accepted model for human hepatocytes with respect to iron metabolism, mainly take up iron and store it in ferritin. During an increased need of iron, stored iron can be released from the cells and is then provided to the circulation.

In this study, new iron preparations which contain a FeOOH core surrounded by low-molecular-weight hydroxyethyl starch chains were investigated. This new complex of iron-HES seems to provide a possible alternative to iron dextran formulations. We compared iron-HES with iron dextran with regard to their availability and safety in human hepatoma HepG2 cells, and THP-1 cells differentiated to macrophages and in human serum.

First we measured the uptake rates of the two groups to see whether there is a difference in their efficacy to overcome iron deficiency anaemia. We found a higher uptake with iron-HES (ranging from 220 to 280 μg/g protein) than with iron dextran (ranging from 100 to 170 μg/g protein) in HepG2 cells, whereas in THP-1 cells the uptake rate was similar. HepG2 cells also took up more iron than THP-1 cells (macrophage phenotype).

After entering the cells, the iron should be available to be released into circulation. Therefore, we measured the release of iron after loading the cells with the two groups of iron formulations. Addition of transferrin to the supernatant promoted the release of iron. Slightly less iron was released from the iron dextran complex which was explained by the decreased uptake rate compared with iron-HES. Most of the iron was released within the first 5 min and reached its limit after 30 min whereas the THP-1 cells released almost all the iron which was taken up during the loading process, the HepG2 cells only released about 25% of the freshly loaded iron. This suggests that the iron in HepG2 cells was already stored in ferritin and the iron in THP-1 cells was still easily accessible. The time dependence of the release should be considered in the dosage regimen. With higher dosage, a larger part may become unavailable for erythropoiesis due to stable storage in the liver.

Ferritin is the main storage protein for iron in the human body and is regulated by the iron regulatory protein. At high intracellular iron concentrations, when the intracellular labile pool (LIP) is augmented, ferritin levels increase. Under these conditions high iron concentrations can be scavenged by binding of iron to ferritin and therefore protect the cells and membranes against oxidative damage.

The two iron dextran preparations showed lower uptake rates in HepG2 cells than the iron-HES formulations, but on the other hand, the amount of ferritin was higher in the iron dextran formulations. In THP-1 cells no significant increase in ferritin could be observed which can be explained by the fact that all the iron which was loaded into the cells was quickly released again. These results mirror the physiological role of macrophages, namely iron distribution in the body to tissues with high iron demand [21], which is also reflected by the unchanged amount of ferritin concentrations after uptake of parenteral iron.

The LIP is a small part (<5%) of the total iron content and is defined as transient redox-active and labile iron. Uptake of transferrin or non-transferrin-bound iron leads to an increase of the LIP, resulting in regulation of iron homeostasis by iron regulatory proteins [16]. The LIP is important in cellular iron homeostasis and associated with the production of reactive oxygen species (ROS). The chemical composition of the LIP has remained essentially elusive, but it may be implicated in generation of oxidative cell damage [12–14]. We investigated the influence of the iron-HES and iron-dextran complexes on the LIP in HepG2 cells. The two dextran formulations showed the highest increase of the intracellular labile iron compared with the iron-HES formulations. Furthermore the increase in intracellular labile iron was dependent on the iron concentration and seemed to be saturated with high concentrations of iron formulations. The higher increase of the LIP with iron dextran preparations might explain the higher concentrations of ferritin in HepG2 cells after uptake of iron formulations.

The danger of excess iron after parenteral iron infusion exists. Free iron has the potential to generate ROS. Therefore, the quality of parenteral iron preparations is also determined by the presence of redox-active iron.

To investigate the potential of the new iron-HES preparations to generate reactive oxygen species, which cannot be scavenged by serum components, an in vitro dichlorofluorescein assay was performed using different concentrations of iron formulations diluted in pooled human serum. Former studies have shown that
after an administration of 100 mg of IVI sucrose, a certain amount of this iron was not completely scavenged by transferrin or other serum components and therefore remained redox-active [22]. Our results indicate that iron from both groups of iron formulations can almost completely be scavenged by serum components. The two iron dextran compounds showed slightly higher values for redox-active iron compared with the iron-HES preparations we used but this amount remains negligible in relation to the total dose of iron.

In conclusion, our results indicate that the new iron-HES compounds have a good stability and availability in vitro. All tested aspects show a similar behaviour of iron-HES and iron dextran. This study is the first in vitro study with iron-HES preparations and presents a basis to motivate other groups to further investigate these compounds for a possible use in the treatment of anaemia.

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