Intravenous iron preparations and ascorbic acid: Effects on chelatable and bioavailable iron

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Background. There is growing interest to use ascorbic acid as adjuvant therapy for patients with recombinant human erythropoietin-hyporesponsiveness (rHuEpo). Several clinical studies showed the beneficial effect of ascorbic acid treatment on hematologic parameters in rHuEpo-treated hemodialysis patients with elevated or even normal iron stores. However, whether ascorbic acid directly affects stability and cellular metabolism of intravenous iron preparations (IVI) is not well understood.

Methods. The preparations for testing were iron sucrose (Venofer), ferric gluconate (Ferrlecit), and iron dextran (INFeD). HepG2-cells were used to investigate effects of ascorbic acid on iron bioavailability for the intracellular labile iron pool (LIP) from IVI by using the fluorescent calcein-assay, and cellular ferritin content was measured by enzyme-linked immunosorbent assay (ELISA). Transferrin-chelatable iron was assessed by fluorescent-apotransferrin, and cell toxicity was assayed by neutral red cytotoxicity test.

Results. The effects of vitamin C on different preparations do not reflect their known chemical stability (i.e., iron dextran > iron sucrose > ferric gluconate). Effects of ascorbic acid on the increase of the intracellular LIP, as well as on increasing mobilization to transferrin in serum, were limited to iron sucrose. Ascorbic acid did not increase cell toxicity and the amount of low molecular weight iron in serum.

Conclusion. We conclude that corrected ascorbic acid levels in hemodialysis (HD) patients could increase the amount of bioavailable iron from iron sucrose, but not from other classes of IVI. Vitamin C administration could therefore result in a lower need of iron sucrose to correct anemia.

Intestinal iron absorption is insufficient to meet the enhanced iron demand in recombinant human erythropoietin (rHuEPO) treated hemodialysis (HD)-patients [1]. Most of them require intravenous iron to sustain adequate erythropoiesis. Parenteral iron preparations are therefore widely used for the treatment of iron deficiency anemia in patients under chronic hemodialysis [2–5].

Several parenteral iron formulations exist for administration to patients with end-stage renal disease (ESRD) [6]. The preparations are complexes of ferric iron with polymeric carbohydrates like dextran or sugars like sucrose or gluconate-forming polynuclear complexes with the metal [7]. Recently, also ferric pyrophosphate (Fe-PP) has been used as a direct dialysis supplement [8]. These iron complexes are largely degraded in reticuloendothelial cells, from where the iron is delivered to transferrin and further to the erythroblastic cells of the bone marrow. The half-life of intravenous iron is several hours, depending on the molecular properties of the individual preparations [7, 9]. Thus, other tissues of the body are confronted with this form of iron at concentrations in the range between 10 and 500 μmol/L, depending on the dose used and the rate of infusion.

Iron therapy is important in anemic ESRD patients to achieve an adequate iron status defined as transferrin saturation (TS) higher than 20%, serum ferritin higher than 100 μg/L, and serum iron greater than 80 μg/dL [10]. Some HD patients with increased iron deposits exhibit rHuEPO hyporesponsiveness due to an inadequate iron mobilization and defective iron utilization [11].

Functional iron deficiency is characterized by both low TS and serum iron despite normal or elevated iron stores. TS shows circadian variations caused by wide fluctuations in reticuloendothelial iron release [12]. Nevertheless, TS remains a reliable parameter of iron availability. In recent studies, it has been demonstrated that TS <20% correlates with new markers of functionally iron-deficient erythropoiesis [13–15].

The potential role of adjuvant therapies in enhancing the effectiveness of rHuEPO in ESRD patients has received increasing attention in recent years. The important reason to search for adjuvant therapies is that they may help to overcome rHuEPO-hyporesponsiveness.
Plasma concentrations of ascorbic acid are often decreased in patients with iron overload. This may be due to increased vitamin oxidation catalyzed by iron. Moreover, subclinical ascorbic acid deficiency is frequently encountered in HD patients, owing to insufficient dietary intake, loss through the dialyzer, and uremia-associated metabolic derangement [16]. Unsupplemented dialysis patients reportedly have lower plasma levels of ascorbate in comparison to healthy controls, mostly due to a loss into the dialysate or, in case of nondialyzed patients, increased urinary loss [17].

Ascorbate represents one of the most prominent antioxidants both in plasma as well as intracellularly, exerting beneficial effects by an inhibition of lipid peroxidation and by reducing endothelial dysfunction [17–20]. In the presence of transition metals like iron, ascorbate may give rise to an increased generation of oxidants [17, 21–23]. However, other recent studies in humans and animals have provided no evidence in support of a pro-oxidant activity of vitamin C in vivo [24–29].

In addition, ascorbic acid, as a reducing agent, is able to release iron from ferritin and mobilize iron from the reticuloendothelial system to transferrin, which leads to increased iron availability and may prevent tissue iron overload [30, 31]. Berger et al showed that addition of iron to ascorbic acid-deficient plasma immediately resulted in the formation of lipid hydroperoxides, whereas endogenous and exogenous ascorbic acid in the plasma delayed the onset of iron-induced lipid peroxidation in a dose-dependent manner. These findings indicate that in iron-overloaded plasma, ascorbic acid acts as an antioxidant toward lipids [32].

In this study, we investigated the effects of ascorbic acid on the amount of chelatable and bioavailable iron from 3 different classes of IVI preparations in human hepatoma HepG2 cells. These cells are a suitable model because the liver is the main sink for iron. The preparations selected are commonly used in the clinical treatment of HD patients.

**METHODS**

**Materials**

Calcein and its acetoxymethylester (Calcein-AM) were obtained from Molecular Probes (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), and was prepared as 5 mmol/L stock solution in dimethyl sulfoxide (DMSO). Diethylene-triaminepentaacetic acid (DTPA), ferric-pyrophosphate (Fe-PP), ferrous ammonium sulfate (FAS), human apo-transferrin (apo-transferrin; aTF), and HEPES were from Sigma (Vienna, Austria), 5-(4,6-dichloro-triazinyl)aminofluorescein (DCTAF) from Molecular Probes, Inc. (Eugene, OR, USA).

**Iron preparations**

The preparations for testing were iron sucrose (Venofer) from Vifor, St. Gallen, Switzerland, ferric gluconate (Ferrlecit) from Rhone-Poulenc Rorer, A. Nattermann & Cie, and iron dextran (INFeD) from Schein Pharmaceuticals (Florham Park, NY, USA). The preparations were dissolved in phosphate-buffered saline (PBS: 137 mmol/L NaCl, 2.65 mmol/L KCl, 1.45 mmol/L Na2HPO4, 8.45 mmol/L NaH2PO4 × 12 H2O, pH 7.3) and freshly prepared for each experiment.

**Study design**

To assess whether ascorbic acid supplementation has effects on iron bioavailability we compared the increase of the intracellular labile iron pool (LIP) by parenteral iron in the presence and absence of ascorbic acid in a tissue culture model with human hepatoma HepG2 cells. The intracellular labile iron pool (LIP) was studied by the fluorescent calcein-assay [33]. The expression of several proteins important in iron metabolism is regulated by the iron regulatory proteins (IRPs). The activity of IRPs themselves depends on the size of the LIP. Changes in the size influence expression of proteins (e.g., ferritin), which are regulated by IRPs. An increase in the LIP should therefore result in increased ferritin synthesis. Therefore, we tested if ascorbic acid treatment influences ferritin expression in HepG2 cells.

One important concern in treatment of patients with ascorbic acid is the occurrence of possible pro-oxidant effects when iron is present. We therefore tested if ascorbic acid treatment increases cytotoxicity of iron preparations in HepG2 cells by using a neutral red (NR) cytotoxicity assay, which can distinguish between viable, damaged, or dead cells [34]. Alternations of the cell surface or the sensitive lysosomal membrane, which can be caused by cytotoxic compounds, lead to lysosomal fragility and other changes that gradually become irreversible. Such changes result in a decreased uptake and binding of NR.

To assess whether vitamin C directly affects chemical stability of the preparations, the amount of chelatable iron from parenteral iron preparations was assayed with 3 different in vitro tests: assay for transferrin chelatable iron, assay for ferrozine-chelatable ferrous iron, and assay for low-molecular-weight iron in serum.

In the assay for transferrin chelatable iron, fluorescent apotransferrin (which is the iron-free form of transferrin) was added to transferrin-depleted serum and used as a sensitive fluorescent probe to detect iron chelation by transferrin. When iron binds to fluorescent apotransferrin its fluorescence is stoichiometrically quenched [35]. This in vitro test for transferrin-chelatable iron is of physiologic importance because transferrin is also the
supplies the erythron with iron for hemoglobin synthesis.

With the assay for ferrozine-chelatable ferrous iron ($\text{Fe}^{2+}$), another important aspect of direct interaction of ascorbic acid with iron preparations can be monitored; ascorbic acid not only has iron chelating properties, but is also a strong reductant. This test was used to measure the amount of reductive iron release from the iron preparations [all of them are polynuclear complexes of ferric iron ($\text{Fe}^{3+}$)] by ascorbic acid. The biological significance of such a reductive iron release seen in vitro is that ferrous iron could then be taken up by various cells expressing the divalent metal transporter DMT1 and, therefore, increase cellular iron uptake from the preparations by these cells. Ferrozine-chelatable iron chemically represents ferrous iron ($\text{Fe}^{2+}$), which can, in principle, catalyze Fenton reaction. Different amounts of ferrozine-chelatable iron in the preparations alone and in the presence of ascorbic acid could therefore cause differences in cytotoxicity.

Effects of ascorbic acid on the amount of low-molecular-weight iron could result in the appearance of potentially redox-active low-molecular-weight iron in the plasma, leading to generation of oxidative stress. To assay if ascorbic acid increases low-molecular-weight labile iron in serum, ultrafiltration experiments were performed using an ultrafilter with a 10 kD molecular weight cut-off. The amount of iron before and after ultrafiltration was measured by atomic absorption spectroscopy.

**Assessment of bioavailable iron (fluorescent calcein-assay)**

HepG2-cells were used to assay the effect of ascorbic acid on iron-bioavailability from parenteral iron preparations by the fluorescent calcein-assay [33]. In this assay the fluorescent metal binding dye calcein is used to measure intracellular iron levels. Calcein-acetoxyethyl ester permeates through cell membranes due to its lipophilic acetoxyethyl ester. Once inside the cell, the acetoxyethyl ester group is cleaved by nonspecific esterases, resulting in free fluorescent calcein, whose fluorescence is quenched by intracellular labile iron. To estimate the amount of the intracellular LIP, calcein fluorescence is measured before and after addition of the strong fast permeating iron chelator 1,10-phenanthroline (SIH) to the cells to obtain full fluorescence of calcein. The measured increase in calcein fluorescence is proportional to the size of the intracellular LIP.

The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)-medium containing 10% fetal calf serum, 2 mmol/L L-glutamine, and antibiotics (gentamycin 50 µg/mL). Cells were treated with trypsin (1.25%), and resuspended in DMEM-medium and grown on 48-well plates at a density of $1 \times 10^6$ cells/mL.

After 2 days the cells were in the log-phase and were used for the measurement of the LIP. To remove any surface-bound iron, the cells were washed with DMEM-medium containing 50 µmol/L DTPA and 2 more washings with DMEM-medium alone, and then loaded with 0.25 µmol/L calcein-AM for 15 minutes at 37°C in DMEM-medium, and buffered with 20 mmol/L HEPES. The cells were washed of excess calcein-AM and resuspended in DMEM-medium containing 20 mmol/L HEPES and a fluorescence-quenching anticalcein antibody ($10 \mu$L/mL medium) that was added to eliminate extracellular fluorescence. The anticalcein antibody was made by Dr. Marcela Hermann, Department of Medical Biochemistry, University of Vienna, Austria (method by Breuer et al, Hebrew University, Jerusalem, Israel) [36]. Calcein fluorescence is quenched by intracellular labile iron. Calcein fluorescence was measured in a fluorescence plate reader (Victor II) from Perkin Elmer (excitation 485 nm, emission 535 nm; Vienna, Austria) at 37°C. After stabilization of the signal, the amount of intracellular iron that was bound to calcein (Ca-Fe) was assessed by addition of 100 µmol/L of the fast permeating chelator SIH to remove all iron from calcein, and hence, to obtain maximal fluorescence. The increase in calcein fluorescence is proportional to the size of the intracellular LIP.

**Ferritin measurements**

Cells were incubated with 75 µmol/L of IVI between 0 and 24 hours, washed, harvested, and lysed on ice in NP-40 lysis buffer containing 150 mmol/L NaCl, 1% IGEPAL CA-630 (NP-40), 50 mmol/L Tris, pH 8, and 1 mmol/L PMSF. The lysates were centrifuged at 7500g for 10 minutes, and the supernatants were collected and stored at −80°C until use.

Measurements of cellular ferritin were performed on cell lysates using a human ferritin enzyme-linked immunosorbent assay (ELISA) test kit from BioCheck (Burlingame, CA, USA). The assay system utilizes 1 rabbit antiferritin antibody for solid phase immobilization and a mouse monoclonal antiferritin antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. Protein concentrations were determined with the Bradford method (BioRad, Hercules, CA, USA).

**Assessment of transferrin-chelatable iron**

Transferrin-chelatable iron was assayed by iron free fluorescent transferrin (fluorescent apo-transferrin; Fl-AMP, whose fluorescence is stoichiometrically quenched by iron that binds to the protein. Fluorescent apo-transferrin was prepared according to the method of Breuer et al [35]. In brief, 100 µmol/L 5-(4,6-dichlorotiazinyl) aminofluorescein (from a freshly prepared 10 mmol/L solution in dimethylsulfoxide) was added to a solution containing 8 mg/mL apo-transferrin.
in 100 mmol/L NaHCO₃, pH 8.4. After incubation for 30 minutes at 37°C in the dark, the coupling reaction was stopped by 5 mmol/L L-lysine, pH 8.0, and the protein was dialyzed against HEPES-buffered saline (HBS: 150 mmol/L NaCl, 20 mmol/L HEPES, pH 7.3) and stored at −20°C.

To assay for transferrin-chelatable iron, 10 μL of the sample was placed in quadruplicates in black 96-well plates with clear, flat bottoms (Greiner, Graz, Austria). Two of the wells were incubated with 180 μL reagent A (containing 1 μmol/L Fl-aTf in HBS), the other 2 wells were incubated with 180 μL reagent B (containing 1 μmol/L Fl-aTf, 5 mmol/L EDTA in HBS). After incubation (1 hour in the dark at 25°C), the fluorescence was measured in a fluorescence plate reader (Victor II) from Perkin Elmer (excitation 485 nm, emission 535 nm).

The ratio between the fluorescence of the samples obtained with reagents A (reading A) and B (reading B) was calculated, and the iron concentration was derived from a calibration curve with freshly prepared ferrous ammonium sulfate (FAS) in doubly deionized water.

**Measurement of ferrozine-chelatable ferrous iron**

Ferrozine can form a colored complex with ferrous iron (Fe²⁺) that can be determined photometrically at 540 nm. Ferric iron (Fe³⁺) in the preparations is firmly bound and has to be reduced and mobilized from the preparations to form the colored Fe²⁺-ferrozine complex.

The effect of ascorbic acid on the amount of ferrozine-chelatable iron in the preparations was assayed by mixing of 150 μL ferrozine reagents (containing 64 mg ferrozine, 64 mg neocuproine, and 7.76 g ammonium acetate dissolved in 20 mL doubly deionized water) with 400 μL of a solution containing 75 μmol/L IVI and 0 to 200 μmol/L ascorbic acid in PBS. The samples were incubated for 2 hours at 37°C in the dark, and the amount of ferrozine-chelatable iron was derived from a calibration curve with freshly prepared ferrous ammonium sulfate (FAS) in doubly deionized water.

**Measurement of low-molecular-weight iron**

Mobilization of low-molecular-weight iron from the iron preparations by ascorbic acid was assayed in human serum. Serum from a healthy person was incubated with 75 μmol/L IVI in combination with various concentrations of ascorbic acid at 37°C for 3 hours in the dark. Then the samples were centrifuged through a 10 kD molecular weight cut-off filter (Roth, Graz, Austria), and the iron content of the filtrate and the original sample was quantified for iron by AAS. The iron content of the original sample was set as 100%, and the percentage of low-molecular-weight iron concentration in the samples was calculated from the iron concentration in the original sample and in the filtrate.

**Neutral red cytotoxicity assay**

The neutral red (NR) cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red, a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by nonionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is therefore possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay [34].

The cells were incubated with DMEM alone (control) with DMEM containing 75 μmol/L IVI or with DMEM containing 75 μmol/L IVI and 150 μmol/L ascorbic acid for 6 hours at 37°C. After removal of the incubation medium, the cells were washed with DMEM and incubated with neutral red (50 mg/L in DMEM) for 3 hours at 37°C. The cells were washed 3 times with PBS and incubated with 200 μL of 50% ethanol (v/v), 1% acetic acid (v/v) in distilled water for 20 minutes at 37°C, and absorbance at 540 nm was read in a fluorescence plate reader (Victor II) from Perkin Elmer.

**Statistical analysis**

Results are presented as mean ± SEM from 3 independent experiments carried out in duplicates. Statistical analysis was performed with GraphPad Prism software. 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**

**Effect of ascorbic acid on iron bioavailability from IVI**

Recently, we showed by the fluorescent calcine-assay that parenteral iron preparations entered the LIP of HepG2 cells. We showed that the amount of bioavailable iron varies among different classes of IVI preparations [33]. The LIP increased lowest with iron dextran, which is known to be the iron preparation with the highest chemical stability [7]: iron sucrose was intermediate and ferric gluconate, the weakest iron complex, was most effective to increase the LIP within a short time.

Here we show that the addition of ascorbic acid increased the amount of bioavailable iron from iron sucrose significantly (P < 0.015) (Fig. 1). After 4 hours of incubation with iron sucrose alone the LIP increased 2-fold compared to control, whereas a combined incubation with iron sucrose and ascorbic acid increased the LIP about 4-fold compared to control. According to the increased
bioavailability of iron with supplementary ascorbic acid treatment, we could also find a higher ferritin synthesis following combined preincubation with iron sucrose and ascorbic acid (see below).

Interestingly, with iron dextran, the iron complex with the highest chemical stability, but also with ferric gluconate, the weakest iron complex, the observed changes in iron bioavailability with supplementary ascorbic acid treatment were not significantly different from those with IVI alone.

Changes in ferritin synthesis in the presence of ascorbic acid and IVI

The ferritin content of HepG2 cells exposed to IVI (75 μmol/L) increased with time. Apparently, the higher the initial increase in the LIP, the faster the synthesis of ferritin is turned on, leading to a quicker disappearance of labile iron. Ferritin synthesis turned on first with ferric gluconate, followed by iron sucrose and iron dextran. Again, this sequence in increasing ferritin synthesis corresponds to the chemical stability of the IVI preparations and their ability to increase the LIP. This was already shown by Sturm et al [33] with IVI alone.

In this study, we could confirm this reported relationship between ferritin synthesis and LIP upon combined incubation with IVI and ascorbic acid. As expected from the observed increase of the cellular LIP with supplementary ascorbic acid treatment after exposure to iron sucrose (Fig. 1), ferritin synthesis significantly increased following combined preincubation with iron sucrose and ascorbic acid. Furthermore, the time course of ferritin synthesis corresponded to the increase in the LIP. As expected from the effects of ascorbic acid on iron bioavailability for the LIP (Fig. 1), a combined incubation of ascorbic acid with ferric gluconate and iron dextran did not influence ferritin content compared to incubation with IVI alone.

Table 1 data show that HepG2 cells were exposed to IVI (75 μmol/L) ± ascorbic acid (150 μmol/L) between 2 and 6 hours, washed of surface bound iron, lysed, sonicated, and stored at −80°C until use. The ferritin content was determined by ELISA as described in the experimental section and correlated to a standard curve. Data (ng ferritin/mg protein) are presented as mean ± SEM of 3 independent experiments, performed in duplicates (data from the measurements without ascorbic acid were published in Sturm et al [33]).

Effect of ascorbic acid on cytotoxicity

As shown in Figure 1, ascorbic acid could significantly increase iron bioavailability from iron sucrose. It was therefore interesting to test if increased iron bioavailability from the preparations with ascorbic acid is accompanied by increased cytotoxicity for the cells. Results from the neutral red cytotoxicity assay revealed that supplementary ascorbic acid treatment did not result in significantly increased cytotoxicity compared to control cells and cells treated with IVI alone (Fig. 2). The IVI concentrations that were used were in the range that can be expected in patients treated with IVI. In addition, there were no signs of cytotoxicity during an incubation period of 6 hours.
Effect of ascorbic acid on the release of ferrozine-chelatable iron

In the preparations, ferric iron (Fe\(^{3+}\)) is bound to a high-molecular-weight complex (e.g., dextran, gluconate, or sucrose). Since ascorbic acid has not only iron-chelating properties, but is also a strong reductant, it raised the question whether more ferrous iron (Fe\(^{2+}\)) may be generated during incubation of IVI in combination with ascorbic acid. The iron chelating and reducing properties of ascorbic acid could be important, since it is not known if cells take up IVI as a whole complex or following release of iron from the complex. Ferrous iron (Fe\(^{2+}\)) could then be taken up from the cells by the divalent metal transporter DMT1, which is up-regulated in IVI-treated HepG2 cells [37].

Ascorbic acid-dependent generation of ferrous iron (Fe\(^{2+}\)) from the preparations was assessed using ferrozine, which can form colored complexes with ferrous iron. Increasing concentrations of ascorbic acid significantly enhanced generation of ferrozine-chelatable iron from the complexes in vitro (Fig. 3). Ascorbic acid highly increased the amount of ferrozine-chelatable iron from iron sucrose (up to 2.5-fold), was less effective in the case of ferric gluconate, and even less effective in the case of iron dextran. Therefore, the ability for reductive iron release from the preparations markedly differs from their chemical stability [7].

Ascorbic acid-dependent mobilization of iron from IVI to transferrin

Ascorbic acid mobilized iron from IVI in vitro (Fig. 3). Since transferrin is the physiologic iron donor for erythropoiesis, it was of interest if serum transferrin could bind iron, which was mobilized by ascorbic acid.

Human serum, which was at first depleted from transferrin by using a 50 kD cut-off filter, was supplemented with iron free fluorescent transferrin (fluorescent apo-transferrin; FL-aTf), whose fluorescence is stoichiometrically quenched through the binding of iron to transferrin [35]. After addition of IVI (± ascorbic acid), we found a small but significant \(P < 0.05\) increase in iron binding to transferrin with ferric gluconate in the presence of ascorbic acid after 1 hour of incubation. Ascorbic acid increased iron mobilization from iron sucrose to transferrin approximately 2-fold \(P < 0.002\) at all time points. In contrast, ascorbic acid had no effect on iron mobilization from iron dextran (Fig. 4).

The ability of ascorbic acid to mobilize iron from the preparations to transferrin is therefore markedly different from their chemical stability [7].

### Table 1. Changes in cellular ferritin content in the presence of IVI and ascorbic acid

<table>
<thead>
<tr>
<th>Time</th>
<th>Ascorbic acid</th>
<th>2 hours</th>
<th>4 hours</th>
<th>6 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+</td>
<td>1.16 ± 0.15</td>
<td>1.33 ± 0.19</td>
<td>1.41 ± 0.28</td>
</tr>
<tr>
<td>+ AA</td>
<td></td>
<td>1.66 ± 0.10</td>
<td>1.75 ± 0.03</td>
<td>2.55 ± 1.17</td>
</tr>
<tr>
<td>Iron Dextran</td>
<td>1.74 ± 0.67</td>
<td>1.75 ± 0.03</td>
<td>4.67 ± 0.08</td>
<td>10.03 ± 0.42</td>
</tr>
<tr>
<td>Iron Sucrose</td>
<td>3.20 ± 0.30</td>
<td>4.56 ± 0.05</td>
<td>9.76 ± 0.19</td>
<td>13.71 ± 0.50</td>
</tr>
<tr>
<td>Ferric Gluconate</td>
<td>1.60 ± 0.37</td>
<td>1.90 ± 0.06</td>
<td>3.09 ± 0.30</td>
<td>4.60 ± 1.95</td>
</tr>
</tbody>
</table>

*Cellular ferritin content of control cells at time t = 0 h: control (− ascorbic acid): 1.29 ± 0.10 ng ferritin/mg protein. Control (+ ascorbic acid): 1.55 ± 0.27 ng ferritin/mg protein.

![Fig. 2. Effect of ascorbic acid on cytotoxicity.](image)

HepG2 cells were plated in 96-well plates and incubated with 75 \(\mu\)mol/L of IVI ± 150 \(\mu\)mol/L ascorbic acid for 6 hours at 37°C. Then, the cells were washed and incubated with neutral red for another 3 hours. Finally, the cells were washed with PBS and incubated with 200 \(\mu\)L of a mixture of 50% ethanol, 1% acetic acid in distilled water. The plates were carefully shaken and after 20 minutes’ absorbance was measured at 540 nm. Data are represented as mean ± SEM of 3 independent experiments, performed in duplicates. Some of the error bars are almost as thin as the upper line of the bar.

![Fig. 3. Effect of ascorbic acid on the release of ferrozine-chelatable ferrous iron (Fe\(^{2+}\)) in vitro.](image)

Seventy-five \(\mu\)mol/L of IVI ± 0 to 200 \(\mu\)mol/L ascorbic acid (AA) was dissolved in PBS buffer and incubated for 2 hours at 37°C. The release of ferrozine-chelatable iron was detected photometrically with the ferrozine assay described in Methods. Data are represented as mean ± SEM of 3 independent experiments, performed in duplicates. Some of the error bars are almost as thin as the upper line of the bar. *\(P < 0.05\) vs. 0 \(\mu\)mol/L AA; **\(P < 0.01\) vs. 0 \(\mu\)mol/L AA; ***\(P < 0.001\) vs. 0 \(\mu\)mol/L AA.
**Low-molecular-weight iron in human serum supplemented with IVI and ascorbic acid**

The previous data show that ascorbic acid can mobilize iron from IVI. Theoretically, this could result in the appearance of potentially redox-active low-molecular-weight iron in the plasma, leading to the generation of oxidative stress.

To test this hypothesis, we incubated human serum with IVI in combination with different concentrations of ascorbic acid. After 3 hours of incubation, the samples were analyzed for low-molecular-weight iron by ultrafiltration through a 10 kD cut-off filter.

Although we could find an ascorbic acid-dependent mobilization of iron from IVI in buffer (Fig. 3), no increase in low-molecular-weight iron could be observed (Fig. 5). When normal human serum was incubated with 75 µmol/L Fe-PP (which represents pure low-molecular-weight iron and was used as a standard) for 3 hours, more than 90% of the iron was bound to serum components. Less than 10% could be filtered through a 10 kD cut-off filter, which means that about 10% was low-molecular-weight iron. Interestingly, the addition of up to 300 µmol/L ascorbic acid did not change the amount of low-molecular-weight iron in the serum.

**DISCUSSION**

The inability to mobilize iron from iron storage sites in quantities sufficient to match the demands constitutes the mechanism of functional iron deficiency in ESRD patients. On treatment with recombinant human erythropoietin (rHuEPO), they develop functional or absolute iron deficiency if they are not adequately supplemented with iron [2]. Therefore, iron supplementation enhances the efficacy of rHuEPO therapy. It has been shown that intravenous iron therapy allows rHuEPO dose reduction by approximately 40%, and is much more effective than oral iron treatment [10].

There is a need for an adjuvant therapy to improve iron supply for hemoglobin synthesis and to increase rHuEPO response in iron-overloaded dialysis patients. Adjuvant treatments other than iron supplementation have been investigated in combination with rHuEPO in iron-overloaded hemodialysis patients. Gastaldello et al [38] and Tarng et al [31] found intravenous ascorbic acid administration to be effective in these cases. Deicher et al [39] found evidence that vitamin C plasma levels account, at least partly, for the response to rHuEPO.

Vitamin C deficiency has been reported in HD patients, owing to inadequate dietary intake, loss through the dialyzer, and uremia-associated metabolic derangement [42, 43]. A daily dose of 100 to 200 mg is recommended in some reports [42–44], but the prescription of vitamin C in different dialysis facilities varies between 55 and 1000 mg daily [42–44].

On the other hand, concerns against vitamin C treatment are raised from the potential risk of uremia-related oxalosis [45, 46]. Moreover, due to the pro-oxidant effect of ascorbate and potentially harmful interactions...
between ascorbate and iron, which may cause oxidative damage to biological macromolecules, concerns have been raised about supplemental vitamin C intake. Especially in individuals with high iron status or clinical iron overload [22, 47], but also in dialysis patients with high serum ferritin, ascorbate administration can yield prooxidant effects [23]. The notion that ascorbate acts as a pro-oxidant in the presence of transition metal ions is based on the ability of ascorbate to enhance metal ion-dependent hydroxyl and alkoxyl radical formation in vitro by Fenton chemistry. However, due to the presence of various metal binding plasma proteins such as transferrin, ferritin, and ceruloplasmin, the availability of “free” redox-active metal ions in plasma and other biological fluids is very low [48].

In this study, we investigated the effects of ascorbic acid on 3 IVI preparations. It is the first report about direct effects of ascorbic acid on intravenous iron preparations. Recently, we showed in a cell culture model with HepG2 cells that all IVI preparations could increase the intracellular LIP, where ferric gluconate was most effective followed by iron sucrose and iron dextran [33]. This order corresponds to the known chemical stability of these intravenous iron preparations, which is highest with iron dextran, intermediate with iron sucrose, and lowest with ferric gluconate [7].

The most important findings of this study are that the effects seen with ascorbic acid on the size of chelatable and bioavailable iron from the preparations show a different order. Whereas ascorbic acid could further increase the amount of bioavailable iron from iron sucrose in HepG2 cells, which was mirrored by enhanced ferritin synthesis, ascorbic acid had no additional effects on the LIP with ferric gluconate and iron dextran. Ascorbic acid mobilized iron from all IVI preparations in vitro, but could increase the amount of bioavailable iron for the tissue culture model from iron sucrose only. Moreover, toxicity of IVI in the presence of ascorbic acid was not increased in HepG2 cells.

Transferrin is the physiologic iron donor for erythropoiesis. Ascorbic acid-dependent iron mobilization from parenteral iron complexes to transferrin would therefore increase iron availability for erythropoiesis. Ferric gluconate, which is the most efficient donor of iron to serum transferrin, showed a small but significant increase in iron binding to transferrin upon incubation with ascorbic acid for 1 hour. Iron sucrose, which is a less efficient donor, showed an increase in iron binding to transferrin at all time points in the presence of ascorbic acid. In contrast, ascorbic acid had no additional effect on iron mobilization from iron dextran. Moreover, there was no increase of potentially harmful low-molecular-weight iron in the serum after combined incubation of iron sucrose with ascorbic acid. These results indicate that iron mobilized from IVI by ascorbic acid would mainly be scavenged by serum transferrin and other serum components in the circulation and should, therefore, be considered as nontoxic.

Taken together, our results show that bioavailability of iron from iron sucrose was improved by ascorbic acid, which resulted in an increase in the LIP, enhanced ferritin synthesis, and increased transfer of iron from iron sucrose to transferrin. In general, ferric gluconate and iron dextran did not show significant effects upon the addition of ascorbic acid. A possible explanation for the differences seen with ascorbic acid on additional iron availability from the preparations could be related to its iron chelating and iron reducing properties. One could hypothesize that ferric gluconate, the weakest iron complex, does not benefit from an additional iron mobilizing agent, like ascorbic acid, to make its iron more bioavailable. With this iron preparation alone the increase in the LIP, reflecting iron bioavailability, has already reached a maximal value, and no further increase seems possible. This was already shown by Sturm et al [33], where effects of ferric gluconate on the LIP were compared with effects seen with low-molecular-weight iron (ferric pyrophosphate). Ferric gluconate showed roughly the same quantitative effects on the LIP as low-molecular-weight iron.

**Fig. 5.** Low-molecular-weight iron in human serum supplemented with IVI and ascorbic acid. Human serum was incubated with 75 μmol/L IVI in combination with ascorbic acid (0–300 μmol/L) at 37°C in the dark. After 3 hours the samples were analyzed for low-molecular-weight iron by ultrafiltration through a 10 kD cutoff filter. Iron in the samples and in the filtrate was quantified by AAS. Data are represented as mean ± SEM of 3 independent experiments, performed in duplicates. Some error bars are smaller than the symbols. **P < 0.01 vs. control (no IVI and AA).**
iron. Iron bioavailability from iron sucrose alone did not reach this maximal LIP value, but in the presence of ascorbic acid iron was mobilized and increased the size of the LIP. Ascorbic acid is also a strong reductant that generates ferrous iron (Fe^{2+}) by reductive iron release from iron sucrose in vitro. Ferrous iron could then, in principle, enter the cells via the divalent metal transporter DMT1, a Fe^{2+} ion transporter at the plasmamembrane and increase the intracellular LIP. Bioavailability from iron dextran, which has also the longest half life time in plasma after intravenous administration, was lowest in HepG2 cells, which was reflected by a very slow increase in the LIP. Vitamin C was largely ineffective to mobilize iron from iron dextran in vitro as well as in HepG2 cells. This could be related to the inability of ascorbic acid to get access to the polynuclear iron core, resulting in a much lower reductive iron release (ferrozine-chelatable Fe^{2+}) in Fig. 3) compared to other IVI preparations.

The exact cellular metabolism of the iron preparations is not known. However, there is growing direct or indirect evidence that tissues other than the reticuloendothelial system are also able to incorporate IVI [37, 49, 50]. The iron preparations are high-molecular-weight complexes, and cells, including macrophages, take them up by a yet unknown mechanism, possibly by vacuolar endocytosis. It is therefore quite possible that some IVI ends up as whole complex inside the cells.

Ascorbic acid treatment was used in several studies to overcome functional iron deficiency in rhuEpo hyporesponsive HD patients. In view of our results, it is therefore also tempting to predict a better response to ascorbic acid treatment in patients previously treated with iron sucrose. So far, IVI therapy is considered to be safe but efficacy still needs optimization.

CONCLUSION

We conclude that corrected ascorbic acid levels in HD patients could increase the amount of bioavailable iron from iron sucrose, but not from other classes of IVI. Vitamin C administration could therefore result in the need of lower iron sucrose dosages to correct anemia. Further studies are necessary to determine safety and efficacy of different classes of IVI in correlation to patients’ plasma ascorbic acid levels.

ACKNOWLEDGMENTS

We are grateful to Prof. Bernhard Gmeiner, who supplied us with human serum. This work was supported by the Austrian Research Found (EWF # P147842-PAT) and the Hochschuljubilaeumstiftung der Stadt Wien (# H-83/2000).

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