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RESEARCH ARTICLE

Bioavailability and stability of intravenous iron sucrose originator versus generic iron sucrose AZAD

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Abstract

Context: Severe iron deficiency requires intravenous iron supplementation to replenish iron stores. Intravenous iron sucrose has been used for decades for the treatment of anemia. New generic iron sucrose products are now marketed for the use in several countries and there is an ongoing discussion about the safety and efficacy of iron sucrose similars.
Objective: In this study, we compared the iron sucrose originator Venofer® and the generic iron sucrose AZAD (ISA) regarding bioavailability, toxicity and stability in human THP-1 cells and HepG2 cells.
Methods: The bioavailability of Venofer® and ISA was investigated in both cell types by a ferrozin-based assay. The release of incorporated iron was assayed by atomic absorption spectroscopy. Ferritin content was measured by enzyme-linked immunosorbent assay (ELISA). HepG2 cells were used to investigate the intracellular labile iron pool (LIP), which was measured by the fluorescent calcein assay. The amount of redox-active iron within the iron formulations was assayed using fluorescent dichlorofluorescein.
Results: We found no significant differences in all parameters between Venofer® and ISA in regard of bioavailability, toxicity and stability *in vitro*.
Discussion: ISA shows identical physico-chemical features and identical bioavailability *in vitro*. This study is a profound basis for future clinical tests with generic iron sucrose compounds.

Keywords

Bioavailability, biological stability, generic intravenous iron, iron sucrose, iron uptake, oxidative stress

History

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Introduction

The appropriate use of intravenous iron (IVI) is increasingly recognized as fundamental to the optimal management of iron deficiency anaemia in a number of settings including inflammatory bowel disease or patients with mal-absorption of iron[1–5].

Iron deficiency may also be caused by blood loss during dialysis, increased erythropoiesis following administration of erythropoietin and insufficient absorption of iron from the gastrointestinal tract. Most dialysis patients require IVI supplementation to replenish iron stores[6]. Venofer® (Vifor International Inc., St. Gallen, Switzerland) was first introduced in Switzerland in the early 1950s with numerous clinical trials, safety reviews and assessments. Recently generic iron sucrose copies of this iron sucrose originator so-called iron sucrose similars (ISSs) have been approved via the generic approach without the same degree of testing or number of patients exposed to the originator[5–7]. Venofer® and ISSs are complex macromolecules that belong to the class of non-biological complex drugs[7], whose structure is closely dependent on the manufacturing process. Differences in the manufacturing process raises potential concerns because it may lead to subtle structural modifications, which can affect the physicochemical properties of the drug. Such modifications can modify stability and redox properties, which affect its potential to influence cytokine activation and reactive oxygen species (ROS) generation[6,8].

Recent studies in rat models and patients have demonstrated differences between certain ISS preparations and the originator Venofer®[7,10] and also different safety and toxicity profiles of certain ISS versus originator were reported[7,10,12–14]. Therefore it is of great importance that new iron sucrose products are carefully studied to avoid side effects. In patients there is so far only scant information about possible differences in the frequency of side effects[11]. In a clinical study, it was shown that the various parenteral iron preparations (like iron dextran, ferric gluconate, ferric carboxymaltose and iron sucrose) significantly increase ROS production in hemodialysis patients to a similar extent, although the iron preparations are known to have quite different physico-chemical properties and stability characteristics[15]. In the same study, these authors found significant differences when patients were treated with the originator or a ISS—despite their much higher similarity compared to preparations with different carbohydrate shells[15].

Recently, a new iron sucrose generic from Azad Pharma AG has been developed. This generic iron sucrose AZAD (ISA) has identical physico-chemical features as the reference product Venofer® (including the size of the molecules). So far ISA showed *in vivo* no difference in tolerability compared to Venofer®[16]. A recent study in mice showed also that the biodistribution of administered iron is essentially similar for ISA and Venofer®[17].

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In this study, we compared the originator Venofer® and ISA in regard of bioavailability, toxicity and stability *in vitro* using a setting of methods to analyze properties of ISA compared to the originator. Our set up of analytical methods enables the quantification of available iron from iron compounds and its metabolic behaviour *in vitro*. As a cell model we used THP-1 cells as a model for macrophages and HepG2 cells as a model for liver cells.

Methods

Materials

The human hepatoma HepG2 cells and THP-1 cells were obtained from CLS – Cell Lines Service (Eppelheim, Germany). The iron chelator deferiprone (L1) was a generous gift from Dr. Peter Nielsen (UKE, Hamburg, Germany). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and Calcein-AM was from Biotium Inc. (Hayward, CA). Isonicotinoyl salicylaldehyde hydrazone (SIH) was a generous gift from P. Ponka (Lady Davis Institute for Medical Research, Montreal, Canada). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Carl Roth GmbH + Co.KG (Graz, Austria). Serdolit CHE was from Serva (Vienna, Austria). 1-[3-Di-(ethylamino)-propyl]-3-ethylcarbodiimide was from Aldrich (Vienna, Austria). Nonident P-40 (IPEGAL CA630), ferrozine (3-(2-pyridyl)-5,6-bis(phenyl sulfonic acid)-1,2,4-triazine), and neocuproine (2,9-dimethyl(1,10-phenanthroline)), apotransferrin, thyroglobulin and ferrous ammonium sulfate were from Sigma (Vienna, Austria). Gentamycin was from GERBU Biotechnik GmbH (Wieblingen, Germany). Roswell Park Memorial Institute medium (RPMI) and Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine and trypsin were from PAA (Pasching, Austria). All other chemicals were obtained from Merck (Vienna, Austria). The preparations for testing were iron sucrose originator ''Venofer®'' (Lotnr. 9920002; Vifor, St. Gallen, Switzerland) and generic iron sucrose AZAD (ISA) (Lotnr. A1129; AZAD Pharma, Toffen, Switzerland).

Calculation of clinically relevant iron concentrations of iron sucrose for non-clinical studies

Frequently used doses which are physiologically active and recommended by the producers are 100 mg iron for Venofer® which, however, is not the maximum clinically used dose. In a clinical study with peritoneal dialysis patients, single doses of 300 mg iron sucrose were used[18]. From pharmacokinetic studies in healthy volunteers it is known that infusion of IVI sucrose leads to rapid high plasma iron levels and that the mean volume of distribution of the central compartment is 3 litres, hence close to the volume of plasma[19]. The expected plasma concentration of IVI after infusion of 100 mg IVI therefore is close to 600 µmol/l plasma and with 300 mg one can expect plasma concentrations close to 1800 µmol/l. The *in vitro* concentrations for the assays were calculated accordingly.

Cultivation of cells

HepG2 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and gentamycin (50 µg/ml) under standard tissue culture conditions (5% CO_2, 37 °C).

Human monocyte THP-1 cells were grown in suspension in RPMI containing 10% fetal calf serum, 2 mM L-glutamine and gentamycin (50 µg/ml) under standard tissue culture conditions (5% CO_2, 37 °C). For the experiments, THP-1 cells were differentiated to adherent macrophages, with phorbol myristate acetate (PMA) which was added to the RPMI-medium to give a final concentration of 160 nM. After four days of cultivation, the cells were used for the experiments.

Treatment of the cells with IVI preparations

HepG2 cells and THP-1 macrophages cultivated in 6-well or 96-well plates were incubated with various concentrations of IVI preparations (Venofer® or ISA) in medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, gentamycin (50 µg/ml) for the indicated time at 37 °C. Finally, the cells were washed on ice once with cold medium (4 °C) containing 50 µM DTPA and twice with pure cold medium (4 °C) to remove surface bound iron.

Iron uptake

For the measurement of the total cellular iron content the cells were lysed in NP-40 buffer (150 mM NaCl, 1% IPEGAL CA630, 50 mM Tris, 1 mM phenylmethyl sulfonylfluoride) (700 µl/well) and ultrasonicated. The iron content of the samples was assayed spectrophotometrically by the ferrozine method[18]. A standard curve was generated by using the standard atomic absorption iron solution from Sigma. The standard (20 µg/ml in 0.5 M HCl) and 500 µl of cell lysate were incubated with 5% (w/v) $KMnO_4$ and 1.2 M HCl for 2 h at 60 °C in the dark. Finally, the ferrozine reagent (6.5 mM ferrozine, 13.1 mM neocuproine, 5 M ammonium acetate and 2 M ascorbic acid) was added to the samples, vortexed and 200 µl aliquots were transferred to a 96-well plate and measured photometrically at 540 nm. The iron content was normalized to the protein content of each sample which was assessed by the standard procedure using the protein assay-reagent (Bio-Rad, Vienna, Austria).

Iron release

The cells were loaded with IVI as described above. 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 a graphite furnace atomic absorption spectroscopy Hitachi Z-8200 with a heated graphite tube and longitudinal Zeeman effect background correction was applied. Measurements were performed using a hollow cathode lamp (slit width 0.20 nm) and measured at 248.3 nm. 15 µl sample volume was used. The amount of iron was correlated to the amount of protein of the same sample.

Assessment of the labile iron pool (LIP)

The method was carried out according to Sturm et al.[21]. For the fluorescent calcein-assay, HepG2 cells were loaded with different concentrations of IVI. After the incubation, the cells were washed and finally loaded with 0.25 µM Calcein-AM in 20 mM Hepes buffered medium for 15 min at 37 °C and washed again. Finally, the cells were washed with medium and incubated with medium containing an anti-calcein antibody and 20 mM Hepes.

The plate was measured at Ex 485 nm/Em 535 nm (measurement A) with a fluorescence plate reader (Anthos Zenyth 3100, HVD Vienna, Austria). 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 represents the LIP.

Quantification of ferritin by a ferritin-ELISA

The cells were incubated with IVI for 3, 6 and 24 h, washed and finally lysed with NP40 buffer, sonicated and stored at −80 °C. Ferritin was determined by a human ferritin enzyme immunoassay test kit (BioCheck, CA).

Assessment of transferrin-chelatable iron

Transferrin-chelatable iron was assayed by iron free fluorescent-transferrin (fluorescent apo-transferrin; Fl-aTf), whose fluorescence is stoichiometrically quenched by iron which binds to the protein.

Fluorescent apo-transferrin was prepared according to the method of Breuer and Cabantchik[22].

Transferrin-depleted human serum (by ultrafiltration through a 20 kD cut-off filter, ICON-concentrator, Pierce, FL) was supplemented with 75 μM IVI ± 0.56 mM ascorbic acid for 1 h at 37 °C. To assay for transferrin-chelatable iron, 10 μl of the sample were placed in quadruplicates in black 96-well plates with clear, flat bottoms (Greiner-Bio-One GmbH, Kremsmünster, Austria). Two of the wells were be incubated with 180 μl reagent A (containing 0.6 μM Fl-aTf in HBS), the other two wells were incubated with 180 μl reagent B (containing 0.6 μM Fl-aTf, 5 mM EDTA in Hepes buffered saline (HBS)). HBS consisted of 150 mM NaCl and 20 mM Hepes pH 7.4. After incubation for 1 h, 2 h and 3 h in the dark at 37 °C, the fluorescence (Ex 485 nm/Ex 535 nm) was measured in a fluorescence plate reader (Anthos Zenyth 3100 HVD Vienna).

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

Assessment of redox-active iron

Redox-active iron was measured by the method of Esposito et al.[23], with slight modifications as reported by Schaller et al.[18]. To assess for redox-active iron, buffer (plasma like medium) or human serum (20 μl) was supplemented with various concentrations of IVI preparations and transferred in quadruplicates to black, clear bottom 96-well plates (Greiner Bio-one, Austria). Plasma like medium (20 mM Hepes, pH 7.4, 150 mM NaCl, 120 μM sodium citrate, 40 μM ascorbic acid, 1.2 mM Na_2HPO_4, 10 mM $NaHCO_3$ and 40 mg/ml bovine serum albumin) and HBS were rendered iron free before use by treatment with 1 g/100 ml Chelex-100 (Sigma). Two wells were incubated with iron free HBS containing 150 μM ascorbate and 5 μM dichlorofluorescein (DCF) 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). The kinetics of fluorescence increase at Ex 485 nm/Em 530 nm were measured in a fluorescence plate reader (Anthos Zenyth 3100, Perkin Elmer). Measurements between 120 and 375 min were used to calculate slopes of DCF fluorescence intensity over time. The fluorescence increase measured in the presence of L1 represents oxidation of DCF by several other oxidants, e.g. peroxidases or hypochlorous acid generated by myeloperoxidases. Therefore, the difference in the rate of oxidation of DCF with and without addition of the chelator L1 represents the redox-active component of NTBI. The duplicate values of the slopes with and without addition of L1 were averaged, and redox-active iron (in μM) was determined from calibration curves correlating the difference in slopes with and without L1 against the iron concentration.

Statistical analysis

Data were analyzed with the Graph Pad Prism software. Results are presented as means ± standard error of the mean (SEM). Differences were examined for statistical significance using the one-way analysis of variance (ANOVA). Differences with $p < 0.05$ were assumed to be significant. Significant differences are marked with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

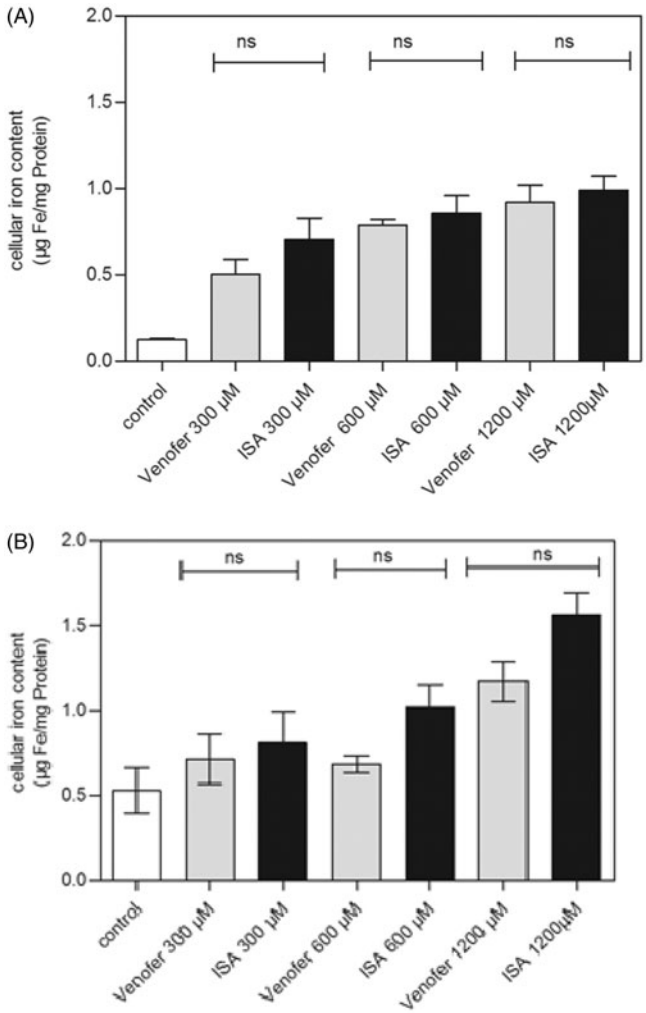

Figure 1. Uptake of intravenous iron by HepG2 cells (A) and THP-1 macrophages (B). HepG2 cells and THP-1 macrophages were incubated with 300, 600 and 1200 μM intravenous iron (Venofer® or ISA) in DMEM (10% fetal calf serum, 2 mM glutamine, gentamycin (50 μg/ml)) for 3 h at 37 °C. Finally, the cells were washed once with 50 μM DTPA, and twice with cold medium to remove surface bound iron. The cells were lysed with NP-40 buffer containing 1 mM of the protease inhibitor PMSF. The amount of iron within the cells was measured with the ferrozin method and then normalized to the amount of protein in the sample using the BioRad protein assay. Data are presented as means ± SEM (HepG2 cells: $n = 7$, THP-1 cells: $n = 6$). Differences were analyzed for statistical significance using one-way ANOVA. Not significant (ns) $p > 0.05$.

Results

Iron uptake

HepG2 cells and THP-1 macrophages were incubated with Venofer® and ISA at iron concentrations ranging from 300 to 1200 μM for 3 h. After the incubation period, the intracellular iron levels were measured using the ferrozine method following digestion of the incorporated iron complexes by high temperature acid permanganate digestion[20]. We found that HepG2 cells took up more iron than THP-1 macrophages, but we could not find significant differences between the uptake rates of Venofer® versus ISA (Figure 1).

Release of iron from iron sucrose loaded cells

To compare the availability of Venofer® and ISA, we loaded HepG2 cells and THP-1 cells with 1200 μM Venofer® or ISA for 3 h at 37 °C. Following removal of the incubation medium and several washings to remove surface bound iron, the release of iron

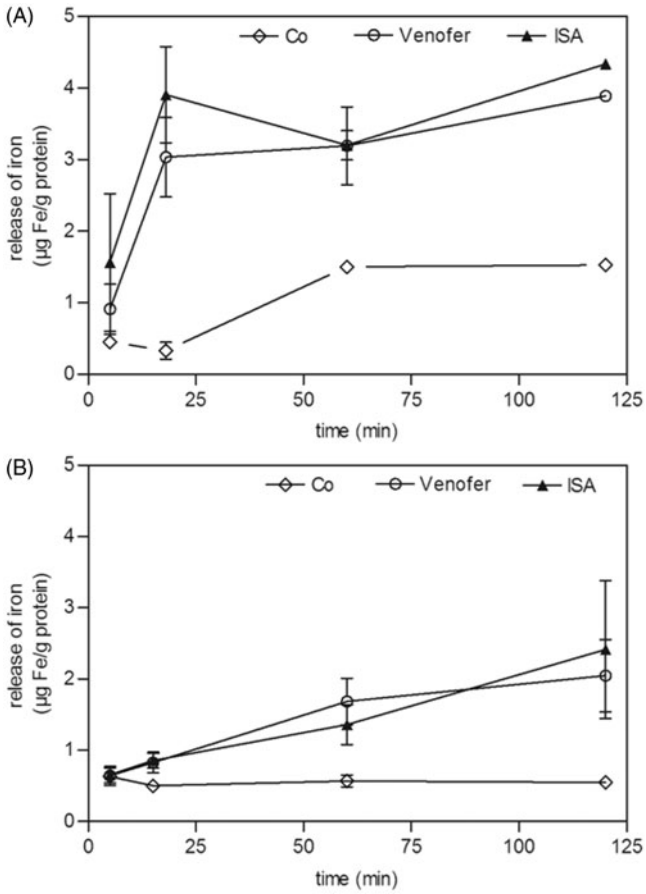

Figure 2. Iron release of intravenous iron by HepG2 cells and THP-1 macrophages. HepG2 cells (A) and THP-1 macrophages (B were incubated with 1200 µM intravenous iron (Venofer® or ISA) in DMEM (10% fetal calf serum, 2 mM glutamine, gentamycin (50 µg/ml)) for 3 h at 37 °C. Finally, the cells were washed once with 50 µM DTPA and twice with cold medium to remove surface bound iron. Then the cells were incubated with medium supplemented with 2.5 mg/ml apo-transferrin to promote cellular iron release. Control cells were not loaded with iron. The release of iron was measured in a time-dependent manner by atomic absorption spectroscopy (AAS). The amount of iron in the release medium was then correlated to the amount of cellular protein in the well using the BioRad protein assay. Data are presented as means ± SEM (HepG2 cells: $n = 4$, THP-1 cells: $n = 4$). Differences for statistical significance were analyzed using one-way ANOVA. Not significant (ns) $p > 0.05$.

was initiated by the addition of apo-transferrin (2.5 mg/ml) to the supernatant. Iron release from the cells was quantified in the release medium by atomic absorption spectroscopy.

In HepG2 cells there was a rapid release of iron within 5 min, whereas in THP-1 macrophages iron release was slower, but showed a constant rate over 2 h (Figure 2). Here again Venofer® and ISA showed the same iron release characteristics.

The intracellular LIP

The cytosolic LIP is a normal part of the total cellular iron, but it is tightly regulated by control mechanisms of cellular iron homeostasis. When this balance gets out of control, free iron can accumulate and cause oxidative damage, mainly by reaction with ROS like superoxide, hydrogen peroxide or organic peroxides[24–26]. The cellular iron pool consists of chelatable and redox-active iron which serves as a crossroad of cellular iron homeostasis, but does also promote the formation of ROS[27–29]. In HepG2 cells, the concentration dependent increase in the LIP with Venofer or ISA was similar (Figure 3). The LIP in THP-1 cells was not measured due to technical reasons.

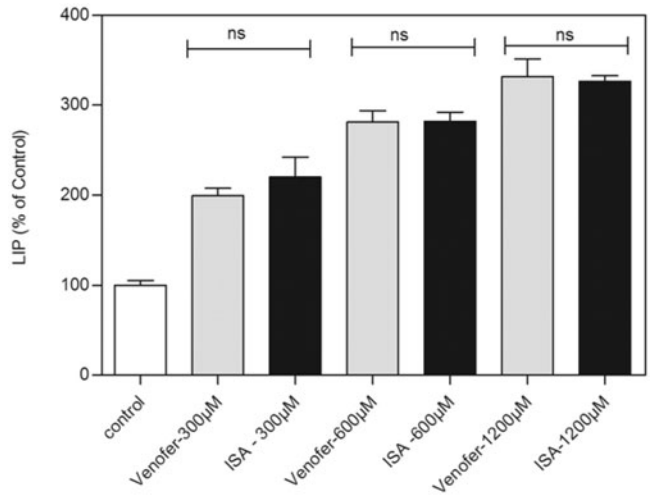

Figure 3. Increase of the labile iron pool following incubation with Venofer® or ISA. HepG2 cells were incubated with 1200, 600 and 300 µM intravenous iron (Venofer® or ISA) in DMEM (10% fetal calf serum, 2 mM glutamine, gentamycin (50 µg/ml)) for 3 h at 37 °C. Finally, the cells were washed with 50 µM DTPA and twice with 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. Then the cells were washed with medium and incubated with medium containing an anti-calcein antibody and 20 mM Hepes. Fluorescence was measured at Ex 488 nm/Em 517 nm (measurement A) in a fluorescence plate reader. Two minutes after the addition of 100 µM SIH, a strong permeant iron chelator, the plate was measured again (measurement B). The difference between measurement B and measurement A represents the labile iron pool. Cells not loaded with iron were set as 100% fluorescence of control. Data are shown as means ± SEM ($n = 12$). Differences were examined for statistical significance using the one-way ANOVA. Not significant (ns) $p > 0.05$.

Changes in ferritin content

Ferritin synthesis was investigated in HepG-2 cells and THP-1 macrophages after incubation with Venofer® or ISA. Time dependent incubation with 1200 µM Venofer® or ISA for 6, 12 and 24 h showed a comparable stimulatory effect on ferritin synthesis with both products. In HepG2 cells, we found a significant higher increase in ferritin after 6 h of incubation with Venofer®. However, after 24 h of incubation no such difference in ferritin levels between Venofer® and ISA could be observed any more (Figure 4).

Transferrin-chelatable iron

When iron is released from parenteral iron preparations to the plasma, it is potentially harmful when not firmly bound to transferrin. The chemical nature of ''released iron'' should allow that it is easily bound to apo-transferrin and therefore to be in a redox-inactive form[30].

The biostability corresponds to the chemical stability of IVI and can be tested by the ability to transfer iron directly to transferrin[31]. In this assay, fluorescent apo-transferrin was added to transferrin-depleted serum and used as a sensitive fluorescent probe to detect transferrin chelatable iron released from Venofer® and ISA. When iron binds to fluorescent apo-transferrin, its fluorescence is stoichiometrically quenched[22].

Additionally, we added ascorbic acid to Venofer® and ISA because ascorbic acid is considered as an adjuvant therapy to improve efficacy of IVI[32]. Therefore, the amount of transferrin-chelatable iron released from Venofer® or ISA was compared in the presence (Figure 5B) or absence (Figure 5A) of ascorbic acid. We could not find significant differences in the amount of transferrin-chelatable iron between Venofer® and ISA.

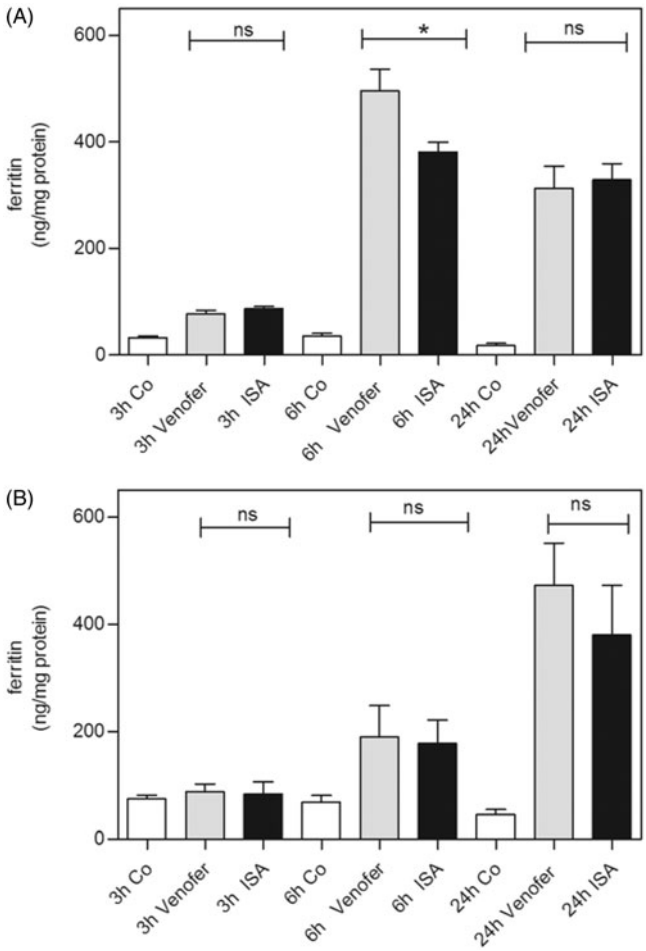

Figure 4. Effect of Venofer® and ISA on cellular ferritin levels in HepG2 cells and THP-1 macrophages HepG2 cells (A) and THP-1 macrophages (B) were washed with pure medium (37 °C) and then incubated with 1200 μM intravenous iron (Venofer® and ISA) in DMEM containing 10% of fetal calf serum, 2 mM glutamine and 10% gentamycin for 3, 6 and 24 h. At the indicated time points the cells were washed once with medium containing 50 μM DTPA and twice with pure medium. The cells were lysed with NP-40 buffer containing 1 mM PMSF. The amount of ferritin in the samples was measured by a human ferritin enzyme-linked immunosorbent assay (ELISA; BioCheck, Foster City, CA). The amount of ferritin was normalized to the amount of protein in the samples. Data are presented as means ± SEM (HepG2 cells: $n = 5$, THP-1 cells: $n = 4$). Differences were examined for statistical significance using the one-way ANOVA. Not significant (ns) $p > 0.05$, *$p < 0.05$.

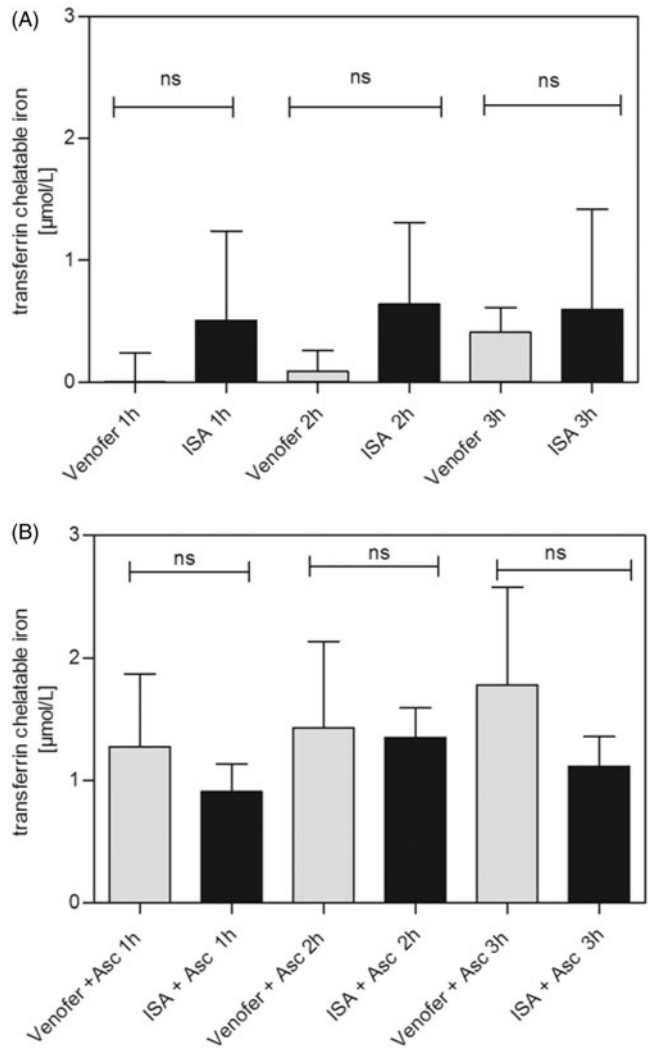

Figure 5. Transferrin-chelatable iron. Transferrin-depleted human serum was incubated with 75 μM intravenous iron (Venofer® or ISA) alone (A) or in the presence of 0.56 mM ascorbic acid (Asc) (B) for 1 h at 37 °C. The samples were then mixed with reagent A (HBS containing 0.6 μM fluorescein-labelled apo-transferrin, Fl-aTf) or reagent B (reagent A containing 5 mM EDTA) and incubated at 37 °C in the dark. After 1, 2 and 3 h the fluorescence was measured at Ex 485 nm/Em 535 nm in a fluorescence plate reader. The ratio between the incubation with and without EDTA (reading B/A) was calculated and correlated to a standard curve generated with ferrous ammonium sulfate at concentrations ranging from 0 to 20 μM. Data are presented as means ± SEM ($n = 4$). Differences were examined for statistical significance using the one-way ANOVA. Not significant (ns) $p > 0.05$.

Redox-active iron

Oxidative stress *in vivo* is the result of an imbalance between the production of oxidants and the respective defence systems of an organism[33]. Free iron presents a dangerous source for the generation of ROS. If the iron within the iron formulations is weakly bound, free redox-active iron can occur.

Therefore, we tested both products for the presence of redox-active iron. In plasma like medium, there was a comparable amount of redox-active iron at all concentrations tested (Figure 6A) while in serum (Figure 6B) no detectable redox-active iron could be found, which reflects the ability of serum proteins to minimise the risk of the generation of ROS mediated by iron.

Discussion

Due to some evidence that certain ISSs differ from the iron sucrose originator in safety and efficacy profiles, it seems prudent for physicians as well as patients who require IVI to have available data on therapeutic equivalence of new ISS preparations versus the originator[34]. It is very important that new IVI products are evaluated regarding bioavailability, stability and potential toxicity *in vitro*. In former studies, we have compared some of the IVI compounds which are available on the market and found significant differences among the different classes of IVI products[21,32,35–37].

The use of intravenous iron for the treatment of anaemia can cause potential danger. First there exists the danger of excess iron after intravenous iron infusion which can increase the potential to generate ROS. Therefore, the quality of parenteral iron preparations is also determined by the presence of redox-active iron. Additionally there is evidence for the occurrence of tissue iron overload (e.g. liver) in long-term treatment with intravenous iron[38]. *In vitro* studies with HepG2 cells can show the uptake rate of various intravenous iron compounds in liver cells. Also the

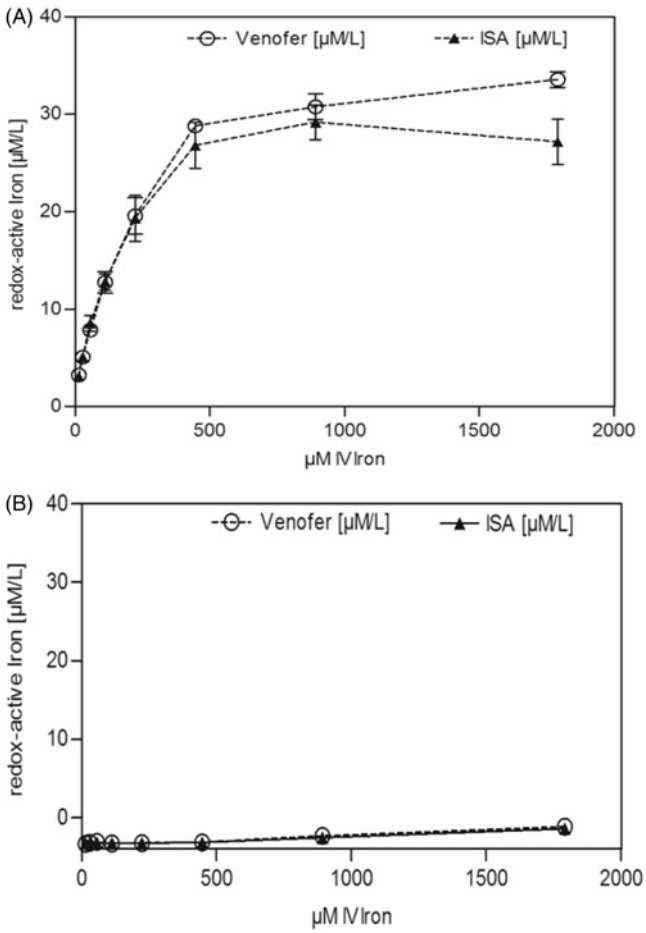

Figure 6. Redox-active labile plasma iron generated from iron sucrose compounds in plasma like medium (A) and human serum (B). Plasma like medium (A) or human serum (B) (20 µl) supplemented with various concentrations of intravenous iron (0–1750 µM) was transferred in duplicates to black, clear bottom 96-well plates. Two wells were incubated with HBS buffer, pH 7.4, containing 150 µM ascorbate and 5 µM DCF at 37 °C in the dark. The two other wells were incubated with the same solution containing 50 µM of the iron chelator deferiprone (L1). The kinetics of fluorescence increase was measured in a fluorescence plate reader (Ex 485 nm/Em 530 nm). Measurements between 120 and 375 min were used to calculate slopes of DCF fluorescence intensity over time. The fluorescence increase measured in the presence of L1 represents oxidation of DCF by oxidants. Data are presented as mean of two experiments.

determination of redox-active iron within the compounds reflects the potential of the compounds to generate ROS *in vivo*. In this study, we compared the iron sucrose originator Venofer® and the iron sucrose generic ''Iron Sucrose AZAD'' (ISA) in a set of different assays designed to find out possible iron related differences regarding the bioactivity, toxicity and biological stability.

First, we measured the uptake rates of iron from the iron sucrose compounds and analyzed whether there is a difference in their efficacy to overcome iron deficiency anaemia. We found no significant difference in the uptake rates between both compounds in HepG2 cells and in THP-1 macrophages.

In vivo after entering the cells the iron should be available to be released into the circulation. Therefore, we measured the release of iron after loading the cells with Venofer® or ISA. We added transferrin to the release medium to initiate the release of iron from the iron loaded cells. HepG2 cells released more iron than THP-1 macrophages. But overall there was no significant difference between Venofer® and ISA.

In HepG2 cells, most of the iron was released within the first 5 min and reached its limit after 30 min. In contrast, THP-1 cells showed a release of iron over a time period of 2 h. This suggests that the iron in HepG2 cells was easier accessible than the iron in THP-1 cells.

Ferritin is the main storage protein for iron in the human body and is regulated by the iron regulatory protein. During 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 storing iron into ferritin and therefore protect the cells and membranes against oxidative damage. In our study, we could not find differences in ferritin synthesis between the two products.

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[39]. The LIP is important for cellular iron homeostasis and associated with the production of ROS. We measured the amount of iron that enters the LIP in HepG2 cells from Venofer® and ISA. The increase in intracellular labile iron was dependent on the iron concentration and was similar with both products.

Therapy with IVI has to be strongly controlled due to the danger of excess iron after iron infusion. Excess iron has the potential to generate ROS and therefore the amount of redox-active free iron within the IVI products is of great importance.

Transferrin can also be considered as an iron buffer in the plasma, keeping redox-active iron low and avoiding adverse reactions. The transferrin binding capacity was tested and no differences were found. We also found that the amount of redox active iron within both products is very low.

Our results are in accordance with the study from Elford et al.[17] where they studied the biodistribution of the two compounds in mice. In general, they also found no significant differences in tissue iron levels (plasma, spleen, bone marrow, liver, heart, stomach, kidneys, liver or lungs).

Conclusion

We found no differences between the two products regarding bioavailability, stability and toxicity *in vitro* which indicate that ISA has a comparable behaviour than the originator Venofer®.

Declaration of interest

The study was funded by Azad Pharma AG. The funders had no role in data collection, analysis and interpretation of the data, decision to publish, or preparation of the manuscript. All authors declare no competing interest. This work was also supported by a FFG grant (Barbara Scheiber-Mojdehkar, TALENTE, No. 2441987-1).

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