ABSTRACT

The original iron sucrose complex (Venofer®) is used to treat iron deficiency anaemia, however ‘generic’ compounds known as iron sucrose similars are now available that have subtle structural differences. This study explores possible haemodynamic and functional differences between Venofer® and a Portuguese iron sucrose similar (Generis®). Thirty rats were divided into three groups and assigned to receive Generis®, Venofer® (ORIGINATOR) or isotonic saline solution (control). Five single intravenous doses of iron (40 mg iron/kg) or saline (equivalent volume) were administered every 7 days for 4 weeks. Blood and urine samples were collected for biological assessment prior to sacrifice (day 28) after which kidney, liver, and heart homogenates were collected for determination of antioxidant enzyme levels. Immunohistochemistry techniques were used to identify tissue ferritin and pro-inflammatory markers. Systolic blood pressure was significantly reduced in the Generis® group (liver, heart and kidney). Serum iron and percentage transferrin saturation were elevated in all groups (except control) (p<0.01) and no differences in haemoglobin concentration were observed. TNF-α and IL6 were significantly elevated in the Generis® group (liver, heart and kidney) compared with the ORIGINATOR and control on day 28 (p<0.01). These findings suggest significant safety and efficacy differences between the ORIGINATOR and Generis®, which may be attributed to the different stability of Generis®. Further studies are needed to investigate the safety profile of iron sucrose similar compounds.

Key-Words:
Anaemia; intravenous; iron sucrose; iron sucrose similar; oxidative stress; rats.

INTRODUCTION

Although oral iron is generally considered the first choice option for the treatment of iron deficiency anaemia, it may produce gastrointestinal and liver disorders1. Its use is also limited by poor absorption, poor compliance, low efficacy and the long time period required to replete iron stores2. Intravenous (IV) iron on the other hand is rapidly delivered to the bone marrow and appears to bypass many of the problems associated with oral
Iron, the frequency of adverse events is low, repletion of iron stores is rapid and high doses may be administered. IV iron is utilized worldwide for the treatment of patients with iron deficiencies including anaemia and is generally considered a well tolerated and convenient treatment. There are a variety of iron compounds available for IV administration including iron dextran, ferric gluconate and iron sucrose. However, the original iron sucrose complex is considered the safest treatment for iron deficiency in chronic kidney disease (CKD) patients amongst other subpopulations; hypersensitivity reactions are rare and haemoglobin levels are increased more effectively compared with oral iron.

The stability of the iron complex influences the safety of the product – weakly bound iron may dissociate from the complex and catalyse the generation of reactive oxygen species. Small differences in the structure of the iron carbohydrate complex may affect the stability of the IV iron preparation and, thus, determine the severity of oxidative stress and consequent tissue damage.

The original iron sucrose complex has been used in clinical practice for decades to provide rapid recovery from anaemic status and has become very important in the treatment of iron deficiency anaemia due to its favourable safety profile. In recent years, however, a number of iron sucrose similar (ISS) compounds have entered the market whose structures differ very slightly from that of the original iron sucrose complex. The physicochemical properties and pharmacological activity of iron complexes are highly dependent on the manufacturing process. Previous studies have suggested haemodynamic, tissue and functional differences between the original iron sucrose complex and ISS preparations such as Feriv® and Hemin®. However, a new ISS compound whose specification more closely resembles that of the original iron sucrose complex. It is therefore important to assess the effects that subtle structural modifications might have on safety and efficacy.

The present study examines potential differences in haemodynamic and oxidative stress parameters between the original iron sucrose complex and ISS in rats. The results confirm potentially deleterious effects of IV iron sucrose preparations that are similar but not identical to the original iron sucrose complex.

**SUBJECTS AND METHODS**

All experiments were approved by the Hospital Alemán Ethics Committee and the Teaching and Research Committee and performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1985). Fifteen male and fifteen female 2-month-old Sprague-Dawley rats (Laboratory of Experimental Medicine, Hospital Alemán, Buenos Aires, Argentina) weighing 210–230 g were randomised into three groups of 10 with an equal male/female distribution. Animals assigned to the control group received isotonic saline solution, those assigned to the ORIGINATOR group received iron sucrose (Venofer®, Vifor International, Switzerland) and those assigned to the ISS group received ISS (Generis®, Generis Co. Portugal, Lot.107984).

Rats were housed in metabolic cages in a temperature-controlled room (22 ± 2°C) and subjected to 12 hour light/dark cycles (7 AM–7 PM). All animals received free access to tap water and were fed standard rat chow (16–18% protein, Cooperación, Argentina) ad libitum throughout the study. Rats from each experimental group received a single IV dose by tail vein injection of the corresponding iron compound (40 mg iron/kg) or control solution (equivalent volume) at the same time every 7 days for 4 weeks (total of five applications). Doses were adjusted each week according to the body weight of each animal.

Blood samples were obtained for biochemical assessment of haemoglobin (Hb), serum iron and percentage transferrin saturation (TSAT) after each IV iron dose at baseline (after 24 hours), and every 7 days for 4 weeks. Urine was also collected for 24 hours every 7 days in accordance with methods described previously. Rats from each experimental group received a single IV dose by tail vein injection of the corresponding iron compound (40 mg iron/kg) or control solution (equivalent volume) at the same time every 7 days for 4 weeks (total of five applications). Doses were adjusted each week according to the body weight of each animal.

Blood samples were obtained for biochemical assessment of haemoglobin (Hb), serum iron and percentage transferrin saturation (TSAT) after each IV iron dose at baseline (after 24 hours), and every 7 days for 4 weeks. Urine was also collected for 24 hours every 7 days in accordance with methods described previously. Rats were sacrificed on day 28 by subtotal exanguination under anaesthesia (sodium thiopental 40 mg/kg intraperitoneal) according to institutional guidelines for animal care and use. The liver, heart and kidneys of each rat were perfused with ice cold saline solution.
through the abdominal aorta until they were free of blood and then removed for oxidative stress evaluation, microscopy and immunohistochemical study.

- **Blood pressure measurement**

  Systolic blood pressure (SBP) was measured by tail-cuff plethysmography every 7 days for 4 weeks. Rats were restrained in a plastic chamber without anaesthesia and cuff pressure was determined by a Pneumatic Pulse Transducer using a Programmed electro-sphygmomanometer PE-300 (Narco Bio-Systems, Austin, Texas). Pulses were recorded on a Physiograph MK-IIIS (Narco Bio-Systems, Austin, Texas); a minimum of three measurements were taken at each session and SBP was calculated as the average of the three readings.

- **Biochemical procedures**

  All animals were subject to 14 hours of fasting before blood samples were collected from the tail vein in capillary tubes; Hb concentration was determined by SYMEX XT 1800i (Roche Diagnostic), serum iron was determined by radial immunodiffusion (Diffu-Plate, Biocientifica, S.A.) and percentage TSAT was obtained using traditional chemical methods. Liver enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), were also assessed in the blood samples by colorimetric and ultraviolet (UV) methods respectively using an Autoanalyzer Modular P8oo and corresponding reagents (Roche Diagnostic GmbH, D-68298 Mannheim). Aliquots of urine were assayed for creatinine using the enzymatic UV method (Randox Laboratories Ltd., Crumlin, N. Ireland). Creatinine clearance was determined by the standard formula and proteinuria was determined using the sulphosalicylic acid method.

- **Evaluation of oxidative stress parameters in liver, heart and kidney**

  Samples of the liver, heart and kidney were homogenised (1:3, w:v) in ice-cold 0.25 M sucrose solution. Glutathione (GSH) levels were determined in the 10,000 ×g supernatant using methods described previously. Further samples of the corresponding perfused tissues were homogenised (1:10, w:v) in 0.05 M sodium phosphate buffer solution (pH 7.4) and used for the determination of malondialdehyde to evaluate lipoperoxidation by thiobarbituric reactive species (TBARS). The remaining homogenate was centrifuged at 4°C for 15 min at 9,500 ×g and the supernatant was used to measure catalase activity. Finally, the remaining tissue samples were homogenised (1:3, w/v) in ice-cold sucrose solution (0.25 M). The supernatant obtained after centrifugation at 105,000 ×g for 90 min was used to measure CuZn-superoxide dismutase (CuZnSOD), GSH peroxidase (GPx) activity and the ratio of reduced to oxidized glutathione (GSH:GSSG). Enzyme units (U) were defined as the amount of enzyme producing 1 nmol of product or consuming 1 nmol of substrate (catalase) under the standard incubation conditions. Specific activity (Sp Act) was expressed as U/mg protein. One unit of CuZnSOD was defined as the amount of CuZnSOD capable of inhibiting the rate of NADH oxidation measured in the control by 50%.

- **Light microscopy and immunohistochemical study**

  Kidney, liver and heart samples were cut longitudinally, fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections were cut and processed for immunohistochemical study. All observations were made using a light microscope Nikon E400 (Nikon Instrument Group, Melville, New York. USA).

  Immunolabelling of specimens was carried out using a modified avidin-biotin-peroxidase technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, CA). The sections were washed in phosphate buffer saline (PBS) for 5 min following deparaffinization and rehydration and then incubated for 30 min in 1% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After washing in PBS (pH 7.2) for 20 min, the sections were incubated with blocking serum for a further 20 min. Thereafter, the sections were rinsed in PBS and incubated with Biotynilated Universal Antibody for 30 min. After washing in PBS a final time, the sections were incubated for 40 min with Vectastain Elite ABC reagent (Vector Laboratories, CA) and exposed for 5 min to 0.1% diaminobenzidine (Polyscience, Warrington, Pa., USA) and 0.2% hydrogen peroxide.
in 50 mM Tris buffer (pH 8). Tissue ferritin was quantified using anti-ferritin monoclonal antibody (Biogen, San Román, CA). Pro-inflammatory markers were also quantified using monoclonal antibodies against rat tumour necrosis factor-α (TNF-α) (R&D Systems, Minneapolis, MN) and interleukin-6 (IL6) (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:50 and 1:100 respectively (PBS diluting agent).

■ Morphometric analysis

Histological sections were studied in each animal with an image analyzer (Image-Pro Plus version 4.5 for Windows, Media Cybernetics, LP. Silver Spring, MD, USA) and morphological analyses were performed at a magnification of x 100 or x 400 depending on the tissue being evaluated. In all cases, the observer was blinded to the animal treatment group. In liver, heart and kidney, tissue ferritin and inflammatory markers (TNF-α and IL6) were evaluated by the percentage of ferritin immunostaining and TNF-α or IL6 immunostaining/mm², respectively, using light microscopy; mean percentages were calculated for each rat.

■ Chemical analysis

A chemical analysis was performed by the quality control laboratory of Vifor International (Switzerland) on ISSG samples (Lot. 107133, 107970 and 107984) to enable comparison with the known specifications of iron sucrose. Molecular weight, polarography, pH, titratable alkalinity and the turbidity point were assessed using methods described previously²⁰,²⁶.

■ Results

Chemical analysis revealed that ISSG does not comply with the specifications of the ORIGINATOR as well as the US Pharmacopeia (USP) Monograph for iron sucrose (see Table I). In particular, ISSG has a higher turbidity point. Polarographic experiments also identified a different iron(III)/iron(II) reduction potential for ISSG which was more positive (–682 mV) than that given for the ORIGINATOR (–750±50 mV). Moreover, as shown in Figure 1, the shape of the polarogram of ISSG is not identical to that of the ORIGINATOR, further confirming the dissimilarities between the two complexes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Shelf life tolerance for ORIGINATOR</th>
<th>USP Monograph: Iron Sucrose Injection</th>
<th>ISSG (Lot. 107133)</th>
<th>ISSG (Lot. 107970)</th>
<th>ISSG (Lot. 107984)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
<td>Dark brown, opaque aqueous solution</td>
<td>–</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
</tr>
<tr>
<td>Iron(II) content</td>
<td>0.4% (m/V)</td>
<td>0.4% (m/V)</td>
<td>0.2% (m/V)</td>
<td>0.2% (m/V)</td>
<td>0.2% (m/V)</td>
</tr>
<tr>
<td>pH</td>
<td>10.5–11.0</td>
<td>10.5–11.1</td>
<td>10.9</td>
<td>10.9</td>
<td>11.0</td>
</tr>
<tr>
<td>Titratable alkalinity</td>
<td>0.5–0.8 ml</td>
<td>0.5–0.7 ml</td>
<td>0.6 ml</td>
<td>0.7 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>Turbidity point</td>
<td>4.7–5.3</td>
<td>4.4–5.3</td>
<td>5.2</td>
<td>5.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>Mw=34,000–60,000 Da, Mn=24,000 Da</td>
<td>Mw=34,000–60,000 Da, Mn=24,000 Da</td>
<td>Mw=34,000–60,000 Da, Mn=34,000 Da, p=1.3</td>
<td>Mw=38,100 Da, Mn=31,500 Da, p=1.2</td>
<td>Mw=38,500 Da, Mn=31,900 Da, p=1.2</td>
</tr>
</tbody>
</table>
SBP recordings in the ISSG group were significantly lower than those in the control and ORIGINATOR groups throughout the study (p<0.01) (see Fig. 2A). Conversely, serum iron concentration was significantly elevated in animals from the ISSG and ORIGINATOR groups compared with control on days 7, 14, 21 and 28, respectively (p<0.01) (Fig. 2B). Percentage TSAT was also significantly higher in the ISSG and ORIGINATOR groups compared with control on days 7, 14, 21 and 28 (p<0.01) (Fig. 2C). The ORIGINATOR group showed significantly lower values in both serum iron concentration and percentage TSAT compared with the ISSG group on days 7, 14, 21 and 28 (p<0.01). No significant differences were observed in Hb concentration between the ISSG, ORIGINATOR and control groups throughout the study.

Creatinine clearance was significantly reduced in rats from the ISSG group throughout the study com-
pared with the ORIGINATOR and control groups (Fig. 3); values did not differ significantly between the ORIGINATOR and control groups. The ISSG group also showed significant proteinuria (p<0.01) on days 7, 14, 21 and 28 compared with the ORIGINATOR and control groups, which showed no differences throughout the study (Fig. 4).

Liver enzymes (AST, ALT and ALP) were markedly increased (p<0.01) in the ISSG group after 28 days relative to baseline compared with the ORIGINATOR and control groups which showed no differences throughout the study (Fig. 5A-C). Lipoperoxidation was also evident in the liver as well as the heart and kidney tissues of the ISSG group, which showed a significant increase in TBARS after weekly treatments (p<0.01) compared with the ORIGINATOR and control groups (Fig. 6A). In addition, the antioxidant enzymes, catalase, GPx and CuZnSOD, were markedly increased in the ISSG group (p<0.01) (Fig. 6B–D). However, a marked reduction in the GSH/GSSG ratio was observed in the liver, heart and kidney tissues of the ISSG group (p<0.01) compared with the ORIGINATOR and control groups, which showed no differences throughout the study (Fig. 6E).

After the last IV iron administration (day 28), microscopy studies of the liver showed a significantly smaller area of ferritin deposits in the ISSG and control groups compared with the ORIGINATOR group (p<0.01) (Fig. 7A–C). The percentage of positive staining is also represented in the corresponding bar chart. Similar results were observed in the
heart and kidney; immunostaining for ferritin deposits in myocardial tissue was significantly greater in the ORIGINATOR group compared with the ISSG and control groups after 4 weeks (p<0.01) (Fig. 7B). The ORIGINATOR group also showed significant immunostaining for ferritin deposits in the proximal tubular epithelial cells of the kidney compared with the ISSG and control groups after 4 weeks (p<0.01) (Fig. 7C).

Levels of the inflammatory markers TNF-α and IL6 were markedly increased in the liver samples taken from the ISSG group compared with both the ORIGINATOR and control groups after 4 weeks (p<0.01) (Fig. 8A). Immunostaining for TNF-α and IL6 were also significantly elevated in heart (Fig. 8B) and kidney samples (Fig. 8C) from the ISSG group (p<0.01).
Figure 8
Bar chart and corresponding micrographs to show IL6 and TNF-α immunostaining in liver (A), heart (B) and kidney (C) samples taken from the ISSG, ORIGINATOR and control groups on day 28.


discussion

In vitro tests show that commonly available IV iron preparations have differing capacities to saturate transferrin, reflecting the different amounts of weakly bound iron. The structure of the iron carbohydrate complex determines its stability and, thus, the potential toxicity of the IV iron preparation. Under physiological conditions, iron is capable of redox cycling resulting in the production of reactive oxygen species that can damage tissues. The original iron sucrose complex has a good safety record and has been utilized to treat iron deficiency anemia for several decades. However, ISS preparations that are not identical structurally to the original iron sucrose complex, have recently become available and their use may present potential safety and efficacy concerns.

The results of the present study suggest that the ISS preparation Generis® causes deleterious haemodynamic and oxidative effects compared with the original iron sucrose complex. Hypotension was recorded in the ISSG-treated animals throughout the study up to day 28, whereas SBP was not affected in the ORIGINATOR and control treatment groups. This result is consistent with the vascular reaction described in response to ISS preparations used previously and supports the favourable safety profile of the original iron sucrose complex. Serum iron concentration and percentage TSAT were significantly elevated in all treatment groups. However, animals receiving the ORIGINATOR showed a lower, moderated increase of these parameters.

Depending on their physicochemical properties, IV iron preparations have differing capacities to saturate transferrin and generate so-called non-transferrin-bound iron (NTBI). Weak or unstable iron complexes liberate iron ions quickly and to a large extent and consequently saturate transferrin at a rapid rate. When TSAT exceeds 60%, the plasma contains NTBI that is only weakly and non-specifically bound to plasma components. NTBI is a potential catalyst for the generation of reactive oxygen species such as loss of membrane structure and function. The incidence of adverse events following IV iron administration has been shown to correlate with TSAT and the amount of 'free iron'/NTBI. The moderate increase in percentage TSAT observed in the ORIGINATOR group suggests a decreased rate of ionic iron release into the circulation and implies an improved tolerability compared with ISSG. Furthermore, the results of the chemical analysis showed that some of the parameters for ISSG did not comply with the USP for iron sucrose, which may thus affect stability and safety. In particular, the iron(III)/iron(II) reduction potential of ISSG is more positive than that of the original iron sucrose complex. This result indicates that the ISSG complex can be reduced more easily under physiological conditions and is therefore more likely to catalyse the production of reactive oxygen species.

Renal function explored by creatinine clearance was reduced in the ISSG group over the 4-week treatment period. Proteinuria was also marked in this group by comparison with the ORIGINATOR and control groups indicating impaired renal function; proteinuria has previously been reported in rats treated with IV iron to induce iron overload. ISSG caused an accumulation of creatinine and reduced absorption of serum proteins from the urine, lending further support to the potentially deleterious effects of this compound. Furthermore, a significant increase in liver enzymes was recorded in the ISSG group in agreement with the 2006 results described previously. Leakage of ALT and AST into the blood is indicative of acute liver damage and in the present study, levels were significantly elevated relative to the ORIGINATOR and control groups over 4 weeks. ALP levels were likewise elevated in the ISSG group signifying the increased risk of hepatic injury associated with ISS treatments.

A severe process of lipoperoxidation was recorded in response to ISSG administration, indicated by an increase in the concentration of oxidative stress markers. IV iron leads to the generation of malondialdehyde, one of the end products of the peroxidation of polyunsaturated fatty acids, which reacts with thiobarbituric acid (TBARS assay). Antioxidant enzymes and TBARS levels were in turn significantly increased in liver, heart and kidney tissues. Conversely, the ORIGINATOR and control groups showed no changes in these parameters throughout the study, which supports the favourable safety profile of the original iron sucrose complex.
complex. A substantial reduction in the GSH/GSSG ratio, a marker of the antioxidant defence mechanism, was also observed in the ISSG group but not the ORIGINATOR or control groups over 28 days. GSH concentration is closely correlated with the degree of renal failure, and deficiency of GSH may lead to an elevation of reactive oxygen species and oxidative stress. These results confirm that ISSG causes significant oxidative stress in liver, heart, and kidney tissues, and that treatment with the original iron sucrose complex is comparatively safer.

Ferritin deposits were markedly increased in the liver, heart, and kidney samples from the ORIGINATOR group in parallel with the 2006 results, whereas ferritin deposits in the ISSG group were comparatively less. A lower expression of ferritin in this group correlates with a higher concentration of NTBI, which may consequently be associated with an inflammatory response. Accordingly, high levels of TNF-α and IL6 were recorded in the ISSG group. This effect was not observed in the ORIGINATOR and control groups and provides further evidence of ISSG toxicity. Pro-inflammatory cytokines produced in response to oxidative stress induce hepcidin synthesis in the liver and in turn augment the inflammatory response. High levels of hepcidin reduce intestinal iron absorbance and limit iron release from stores.

In conclusion, the results of this study indicate that ISSG significantly alters haemodynamic, functional, and tissue responses compared with the original iron sucrose complex (Venofer® [the ORIGINATOR]). Structural modifications of the original compound present significant risk factors, which raise safety concerns around the use of similar, but compound present significant risk factors, which raise safety concerns around the use of similar, but

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Conflict of interest statement and acknowledgements.

No financial competing interests. Research supported by funding from Vifor (International). Editorial support provided by Dr Kate Stooke at ScopeMedical Ltd.


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