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Assessment of the extent of oxidative stress induced by intravenous ferumoxytol, ferric carboxymaltose, iron sucrose and iron dextran in a nonclinical model

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Abstract

Intravenous (i. v.) iron is associated with a risk of oxidative stress. The effects of ferumoxytol, a recently approved i. v. iron preparation, were compared with those of ferric carboxymaltose, low molecular weight iron dextran and iron sucrose in the liver, kidneys and heart of normal rats. In contrast to iron sucrose and ferric carboxymaltose, low molecular weight iron dextran and ferumoxytol caused renal and hepatic damage as demonstrated by proteinuria and increased liver enzyme levels. Higher levels of oxidative stress in these tissues were also indicated, by significantly higher levels of malondialdehyde, significantly increased antioxidant enzyme activities, and a significant reduction in the reduced to oxidized glutathione ratio. Inflammatory markers were also significantly higher with ferumoxytol and low molecular weight iron dextran rats than iron sucrose and ferric carboxymaltose. Polarographic analysis suggested that ferumoxytol contains a component with a more positive reduction potential, which may facilitate iron-catalyzed formation of reactive oxygen species and thus be responsible for the observed effects. Only low molecular weight iron dextran induced oxidative stress and inflammation in the heart.

Key words

- Ferric carboxymaltose
- Ferumoxytol
- Inflammation
- Intravenous iron
- Iron sucrose
- Oxidative stress

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1. Introduction

Iron deficiency anemia (IDA) contributes to the symptom burden in many disease conditions including chronic heart failure [1], inflammatory bowel disease [2], chronic kidney disease (CKD) [3] and cancer [4]. Iron supplementation is often necessary to correct the hemoglobin (Hb) deficit and replenish body iron stores [5]. Although oral iron administration is the least expensive form of iron therapy [6], the intravenous (i. v.) route of iron administration has become more favored worldwide in recent years due to the gastrointestinal intolerance, low iron delivery rates, limited absorption and prolonged iron store repletion times associated with oral iron supplements [7]. I. v. iron is rapidly delivered to the bone marrow, bypassing many of the problems of oral iron treatment [8, 9]. Repletion of iron stores is rapid and relatively high single doses can be administered [8, 10, 11].

There are a variety of iron compounds available for i. v. administration including iron dextrans, ferric gluconate (FG), ferric carboxymaltose (FCM) and iron sucrose (IS). Recently, a number of iron sucrose similars (ISS) have entered the market. The physico-chemical properties and pharmacological activity of these ISS complexes are highly dependent on the manufacturing process [12–14]. In 2009, the FDA approved ferumoxytol (FMX), a semi-synthetic, polyglucose sorbitol carboxymethylether (i. e. a dextran derivative) coated superparamagnetic iron oxide nanoparticle for the treatment of IDA in adult patients with CKD [15].

All i. v. iron compounds comprise a polynuclear, nonionic iron(III)-hydroxide/oxide core shielded by a carbohydrate shell [12, 16]. These complexes can generally be classified according to their kinetics and thermodynamics variability, molecular weight, and side-effect profiles [12]. The most stable complexes are the iron

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Table 1: Size, stability and dosing of i. v. iron preparations.

Parameter	FG (Ferrlecit®)	IS (Venofer®)	LMWID	FCM (Ferinject®)	FMX (Feraheme®)
MW[a] (Da)	37,000	43,300	103,000[b]	150,000	185,000[c]
MW[d] (Da)	200,000	252,000	410,000[e]	N/A	731,000
Stability	Low	Medium	High	High	High
Maximum iron infusion dose	125 mg (EU 62.5 mg)	200 mg (some countries 500 mg)	20 mg/kg bw	1000 mg (15 mg/kg bw)	N/A
Infusion time (min)	60	30	360[f]	15	N/A
Maximum injectable bolus dose (mg iron)	125	200	100	200	510
Injection time	10 min	10 min	2 min	Bolus push	17 s[g]

N/A, not available; bw, body weight.
[a] Method according to USP iron sucrose injection, relative to a pullulan standard [18].
[b] Imferon®.
[c] This study.
[d] Method according to Balakrishnan *et al.* [19] relative to a protein standard.
[e] Unknown low molecular weight iron dextran.
[f] Total dose infusion.
[g] Patients should be observed for signs and symptoms of hypersensitivity for at least 60 min following each injection [20, 21].

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dextrans, FMX and FCM. The stability of IS is intermediate, whereas FG is the least stable of the available i. v. iron preparations (Table 1) [17].

Due to the potential of iron to induce oxidative stress, the safety profile of i. v. iron preparations must be carefully assessed. Typically, the more labile, low molecular weight compounds, such as FG, release larger amounts of iron into the circulation, saturating transferrin and generating non-transferrin bound iron (NTBI) [13, 22]. NTBI is taken up non-specifically by the endocrine system, the heart and the liver. In the tissues of these organs, it can catalyze a number of reactions that lead to oxidative stress and tissue damage [23, 24]. The extent of iron release determines the maximum single dose of each i. v. iron [25]. The more stable iron complexes, such as iron dextran, FMX and FCM, release only small amounts of iron and thus can be administered in higher single doses (Table 1).

In addition to the stability of the complex, another important aspect of the safety of i. v. iron preparations is their potential to cause dextran-induced anaphylactic reactions. Iron dextrans are associated with immediate, life-threatening anaphylactic reactions (especially allergic reactions) and deaths at a rate of 0.5–1 % [25–29]. IS shows no cross-reaction with dextran antibodies and is considered safe in patients intolerant to iron dextran [30]. FCM is free of dextran and its derivatives and thus has a low immunogenic potential [31]. FMX, which has a modified dextran shell, has been designed to have a low immunogenic potential and improved safety profile compared to other i. v. iron dextran preparations [19, 32, 33]. In a recent case, however, a clinically consistent anaphylactic reaction to FMX was reported, raising concerns about the safety of this i. v. iron preparation [34].

Using the same rat model as that employed in the current study, it has recently been possible to show that the levels of oxidative stress and inflammation induced by ISSs are significantly higher than those caused by the original IS, despite only minor structural differences [35, 36]. The objective of this nonclinical study was to assess the potential of FMX to induce oxidative stress in the kidneys, heart and liver, and to compare it to that of three marketed i. v. iron compounds [IS, FCM and low molecular weight iron dextran (LMWID)].

2. Materials and methods

2.1 Molecular weight determination

The molecular weight distribution of FMX was measured in the Analytical Development Laboratory of Vifor (International) Ltd. (St. Gallen, Switzerland) by gel permeation chromatography, as described previously [12, 18].

2.2 Reduction potential determination

The Fe(III)/Fe(II) reduction potentials were measured in the Analytical Development Laboratory of Vifor (International) Ltd. (St. Gallen, Switzerland) by polarography, as described previously [12]

2.3 Animals and treatments

All animal experiments were approved by the Hospital Alemán Ethic Committee and the Teaching and Research Committee and were undertaken according to the NIH Guide for the Care and Use of Laboratory Animals. Twenty-five male and 25 female non-anemic Sprague-Dawley rats (Laboratory of Experimental Medicine, Hospital Alemàn, Buenos Aires, Argentina) weighing 240–260 g were randomized into five groups with equal male-female distribution (n = 10/group). The control group received isotonic saline solution; group L received IS (Venofer®, LOT 9383, American Regent, Inc., Shirley, NY, USA);

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group O received FCM [Ferinject®, LOT 94300012, Vifor (International), Ltd., St. Gallen, Switzerland]; group P received FMX (Feraheme®, LOT 09060402, AMAG Pharmaceuticals, Inc., Lexington, MA, USA); and group R received LMWID (INFeD®, LOT 08W06A, Watson Pharma Inc., Morristown, NJ, USA). The investigators were blinded to the treatment groups.

Rats were housed in metabolic cages in a temperature-controlled room (22 ± 2 °C) subject to 12 h light/dark cycles (07.00 – 19.00). All animals had 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 i. v. dose by tail vein injection of the corresponding iron compound (40 mg iron/kg body weight) or control solution (equivalent volume) at the same time every 7 days for 4 weeks (a total of five administrations on days 0, 7, 14, 21 and 28). Treatment doses were adjusted each week according to the body weight of each animal.

Blood samples were obtained for biochemical assessment of Hb, serum iron and liver enzymes 24 h after the first i. v. iron dose and every seven days for four weeks (days 1, 8, 15, 22, and 29). Urine was also collected for 24 h after each i. v. injection as described previously [37]. Rats were sacrificed 24 h after the last i. v. injection (day 29) by subtotal exsanguination under anesthesia (sodium thiopental 40 mg/kg body weight intraperitoneal) according to institutional guidelines for animal care and use. Previously, blood samples were obtained for biochemistry determination. The liver, heart and kidneys of each rat were perfused with ice-cold saline through the abdominal aorta until they were free of blood and then removed for oxidative stress evaluation, microscopy and immunohistochemical analyses.

2.4 Blood pressure measurement

At baseline (day 0) and 24 h after each i. v. iron administration (days 1, 8, 15, 22, and 29), SBP and DBP were measured by a non-invasive pressure device with volume pressure recording, CODA 2 (Kent Scientific Co., Torrington, CT, USA) [38, 39].

2.5 Biochemical procedures

All animals were subject to 14 h of fasting before blood samples were collected from the tail vein in capillary tubes. Hb was determined by SYSMEX XT 1800i (Roche Diagnostic GmbH, Mannheim, Germany). Serum iron and liver enzymes, including AST, ALT and ALP, were assessed by colorimetric and UV methods, respectively, by using an Autoanalyzer Modular P800 Roche Diagnostic with the correspondent reagents (Roche Diagnostic GmbH, Mannheim, Germany). Serum transferrin was determined by radial immunodiffusion (Diffu-Plate, Biocientifica, S. A.). Transferrin saturation (TSAT) was calculated using the following equation: serum iron concentration (μg/l)/total iron-binding capacity (μg/l) × 100 = TSAT (%) [40, 41]. Aliquots of sera and urine were assayed for creatinine with the enzymatic UV method (Randox Laboratories Ltd., Crumlin, Northern Ireland). CrCl was determined by the standard formula and urinary protein excretion was determined by the sulphosalicylic acid method.

2.6 Evaluation of oxidative stress parameters in liver, heart and kidneys

Samples of the whole liver, heart and kidney were homogenized (1 : 3, w : v) in ice cold 0.25 M sucrose solution. GSH levels were determined in the 10,000 × g supernatant by methods described previously [42, 43]. Further samples of the corresponding perfused tissues were homogenized (1 : 10, w : v) in 0.05 M sodium phosphate buffer (pH 7.4) and used for the determination of malondialdehyde to evaluate lipid peroxidation by thiobarbituric acid reactive species (TBARS). The remaining homogenate was centrifuged at 4 °C for 15 min at 9,500 × g and the resulting supernatant was used to measure catalase activity. The remaining tissue samples were homogenized (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 Cu,Zn superoxide dismutase (Cu,Zn-SOD) and glutathione peroxidase (GPx) activity [44 – 46]. Enzyme units (U) were defined as described previously [13]. Specific activity was expressed as U/mg protein.

2.7 Light microscopy and immunohistochemical study

Decapsulated liver, heart and kidney samples were cut longitudinally, fixed in phosphate-buffered 10 % formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections were cut and stained. All observations were performed using a light microscope Nikon E400 (Nikon Instrument Group, Melville, NY, USA).

Immunolabelling of specimens was carried out by a modified avidin-biotin-peroxidase technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, CA, USA), as described previously [13]. Tissue ferritin was quantified with antiferritin monoclonal antibody (Biogen, San Román, CA, USA). Pro-inflammatory markers were quantified with monoclonal antibodies against rat tumor necrosis factor-alpha (TNF-α; R&D Systems, Minneapolis, MN, USA) and interleukin-6 (IL6; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilutions of 1 : 50 and 1 : 100, respectively (PBS diluting agent).

2.8 Morphometric analysis

Histological sections were studied in each animal with an image analyzer (Image-Pro Plus 4.5 for Windows, Media Cybernetics, LP, Silver Spring, MD, USA), as described previously [13].

2.9 Statistical methods

Values were expressed as mean ± SD. All statistical analyses were performed using absolute values and processed through GraphPad Prism, version 5.01 for Windows (GraphPad Software, Inc. San Diego, CA, USA). For parameters with Gaussian distribution, comparisons among groups were carried out by analysis of variance (ANOVA) and for parameters with non-Gaussian distribution by Kruskal-Wallis test (non-parametric ANOVA) and Dunn's multiple comparison test. A value of $p < 0.05$ was considered significant.

3. Results

3.1 Physico-chemical analysis

The molecular weight distribution of FMX was determined by gel permeation chromatography. The weight average molecular weight (Mw) was 185 kDa, the number average molecular weight (Mn) 153 kDa and the calculated polydispersity (P = Mw/Mn) 1.21. The chromatogram showed a distinct second peak of lower molecular weight.

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Table 2: Hematology parameters and creatinine clearance.

Mean ± SD	IS (n = 10)	FCM (n = 10)	FMX (n = 10)	LMWID (n = 10)	Control (n = 10)
Day 1					
Hb (g/dl)	16.1 ± 1.0	16 ± 1.1	15.9 ± 0.8	15.8 ± 1.1	15.8 ± 1.0
Serum iron (µg/dl)	365.2 ± 44.1	401.0 ± 25.3	425.2 ± 38.0[b]	382.1 ± 33.2	298.3 ± 17.0[a]
TSAT (%)	75.1 ± 6.2	77.8 ± 4.4	76.9 ± 4.1	78.7 ± 4.5	43.9 ± 2.9[a]
CrCl (ml/min)	2.7 ± 0.2	2.7 ± 0.1	2.7 ± 0.2	2.7 ± 0.3	2.8 ± 0.1
Day 8					
Hb (g/dl)	15.9 ± 0.9	15.9 ± 1.1	16.0 ± 1.0	16.1 ± 0.9	16.0 ± 1.1
Serum iron (µg/dl)	405.2 ± 42.0	417.3 ± 19.1	415.4 ± 32.5	395.0 ± 22.1	293.3 ± 21.0[a]
TSAT (%)	72.0 ± 5.1	75.9 ± 6.3	74.8 ± 5.6	78.1 ± 5.0	44.7 ± 3.2[a]
CrCl (ml/min)	2.8 ± 0.3	2.8 ± 0.1	2.7 ± 0.2	2.8 ± 0.5	2.9 ± 0.1
Day 29					
Hb (g/dl)	16.0 ± 1.1	15.8 ± 1.2	15.9 ± 0.7	15.9 ± 1.1	15.8 ± 1.0
Serum iron (µg/dl)	385.4 ± 37.3	410.0 ± 20.3	422.2 ± 38.1	415.4 ± 26.2	304.0 ± 16.1[a]
TSAT (%)	74.3 ± 4.0	74.2 ± 5.5	73.9 ± 4.8	76.7 ± 4.9	43.1 ± 3.2[a]
CrCl (ml/min)	2.6 ± 0.3	2.7 ± 0.2	2.6 ± 0.2	2.5 ± 0.3	2.9 ± 0.1

IS, iron sucrose; FCM, ferric carboxymaltose; FMX, ferumoxytol; LMWID, low molecular weight iron dextran; Hb, hemoglobin; TSAT, transferrin saturation; CrCl, creatinine clearance.
[a] $p < 0.01$ versus all groups, [b] $p < 0.05$ versus LMWID, [c] $p < 0.01$ versus FMX and LMWID.

The reduction potentials of FMX, determined by polarography, are expressed *vs.* Ag/AgCl 3M KCl, if not otherwise specified. Two distinct Fe(III)/Fe(II) transitions were observed at –462 mV [–255 mV *vs.* normal hydrogen electrode (NHE)] with a half-width of 209 mV, and at (989 mV (–782 mV *vs.* NHE) with a half-width of 166 mV.

3.2 Nonclinical analysis

Twenty five male and 25 female non-anemic Sprague-Dawley rats (Laboratory of Experimental Medicine, Hospital Alemàn, Buenos Aires, Argentina) weighing 240–260 g were randomized into five groups with equal male-female distribution (n = 10/group). Rats from each group received a single i. v. dose of the corresponding

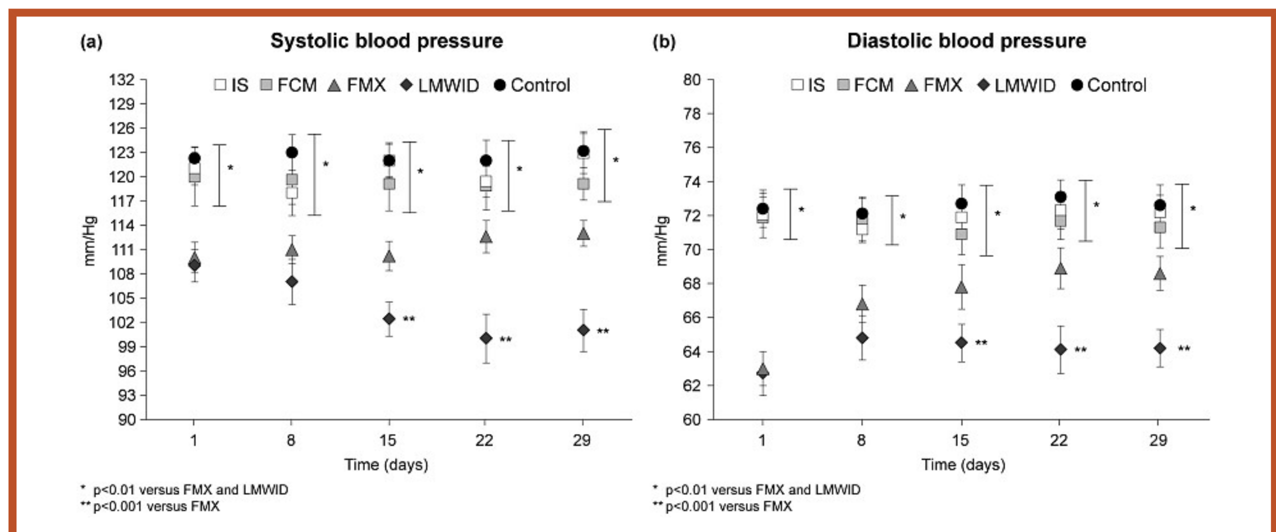

Fig. 1: Blood pressure in rats treated with different i. v. iron preparations. (a) Systolic blood pressure and (b) diastolic blood pressure at baseline (day 0) and 24 h after weekly (days 1, 8, 15, 22, and 29) i. v. iron administration (40 mg iron/kg body weight or equivalent volume) in iron sucrose (IS), ferric carboxymaltose (FCM), ferumoxytol (FMX), low molecular weight iron dextran (LMWID) and control groups.

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iron compound (IS, FCM, FMX, LMWID; 40 mg iron/kg body weight) or isotonic saline solution every 7 days for 4 weeks (total of five administrations; days 0, 7, 14, 21 and 28). As expected for non-anemic rats, no significant differences were observed in Hb concentrations between the treated and control groups throughout the study (Table 2). Serum iron concentration and transferrin saturation (TSAT) were significantly ($p < 0.01$) higher in all treated rats compared to controls on days 1, 8, and 29 (Table 2). The small differences in serum iron and TSAT between the i. v. iron-treated groups did not show statistical significance at any point during the experiment.

Blood pressure values in the five groups were comparable on day 0. A significant ($p < 0.01$) decrease in systolic (SBP) and diastolic blood pressure (DBP) recordings was observed in the FMX and LMWID groups compared with the IS, FCM and control groups throughout the study (Fig. 1). Furthermore, a significant decrease ($p < 0.01$) in SBP and DBP recordings was observed in the LMWID group compared with the FMX group on days 15, 22, and 29.

Creatinine clearance (CrCl) was similar in all groups (Table 2). Urinary protein excretion was significantly increased, although to a variable extent, in the FMX and LMWID groups compared with the IS, FCM and control groups on days 15 ($p < 0.05$), 22 ($p < 0.01$) and 29 ($p < 0.01$) (Fig. 2 a). On day 22 and 29, proteinuria was also significantly increased ($p < 0.05$) in the FMX group compared with the LMWID group (Fig. 2 a). Aspartate transferase (AST), alkaline transferase (ALT) and alkaline phosphatase (ALP) were significantly increased ($p < 0.01$) in the FMX and LMWID groups throughout the study compared with the IS, FCM and control groups (Fig. 2 b–d).

Lipid peroxidation was evident mainly in the liver and kidneys in the FMX and LMWID groups, which showed a significant ($p < 0.01$) increase in malondialdehyde (TBARS) levels, GPx, catalase and Cu,Zn-SOD) activities and a significant ($p < 0.01$) reduction in the reduced to oxidized glutathione ratios (GSH : GSSG) when compared with the IS, FCM and control groups at the end of the study (Fig. 3).

On day 29, microscopy studies of the liver displayed significantly more ($p < 0.01$) positive staining for iron (Prussian blue) in rats treated with LMWID compared to rats treated with IS, FCM and FMX. In addition, the LMWID group had iron deposits not only in Kupffer cells, but also in hepatocytes and sinusoidal endothelial cells whereas FMX, IS and FCM groups had iron deposits only in Kupffer cells (Fig. 4 a). The area of ferritin staining in the liver was smaller ($p < 0.01$) in LMWID-treated rats than in IS, FCM and FMX-treated rats (Fig. 4 b).

On day 29, LMWID-treated rats showed a significantly larger ($p < 0.01$) area for iron staining (Prussian blue) in cardiomyocytes compared to that of the rats treated with other i. v. iron compounds (Fig. 4 a). Smaller ferritin deposits were observed by immunostaining in the

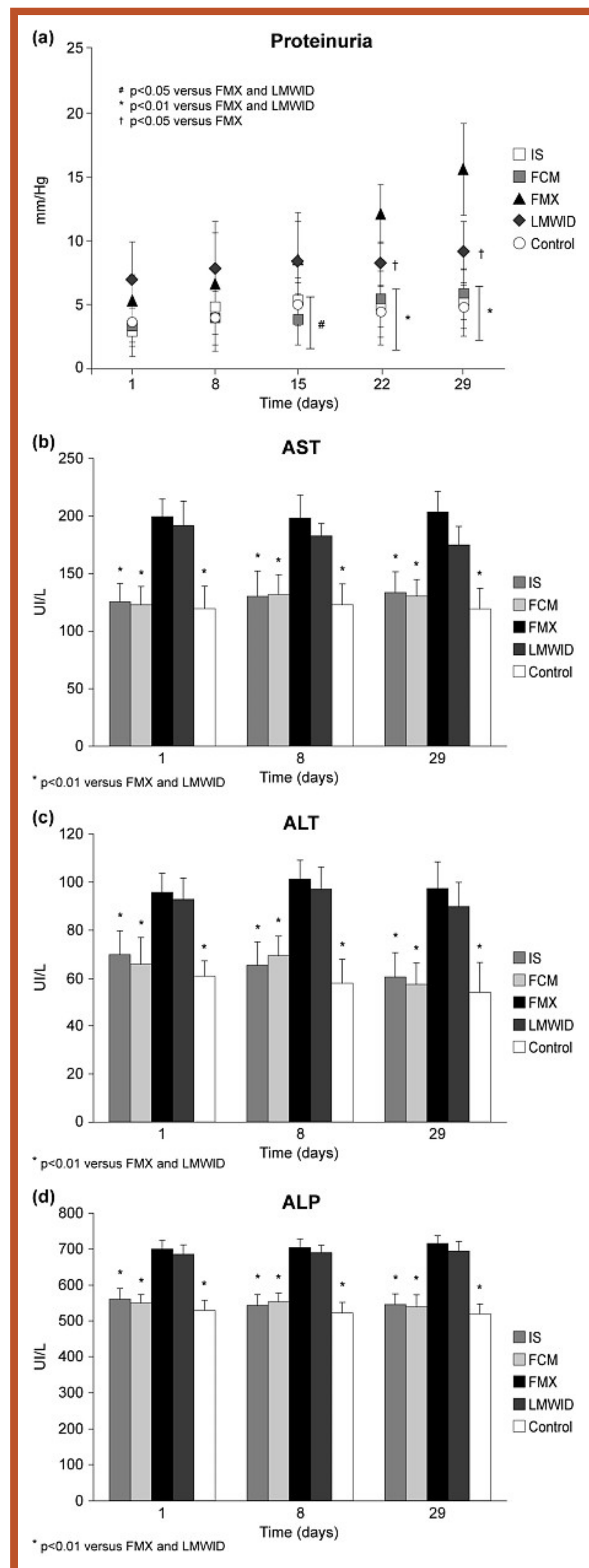

Fig. 2: Urinary protein excretion and liver enzyme levels in rats treated with different i. v. iron preparations. (a) Proteinuria, (b) aspartate transferase (AST), (c) alanine transferase (ALT) and (d) alkaline phosphatase (ALP) 24 h after weekly i. v. iron administration (40 mg iron/kg body weight or equivalent volume) in iron sucrose (IS), ferric carboxymaltose (FCM), ferumoxytol (FMX), low molecular weight iron dextran (LMWID) and control groups.

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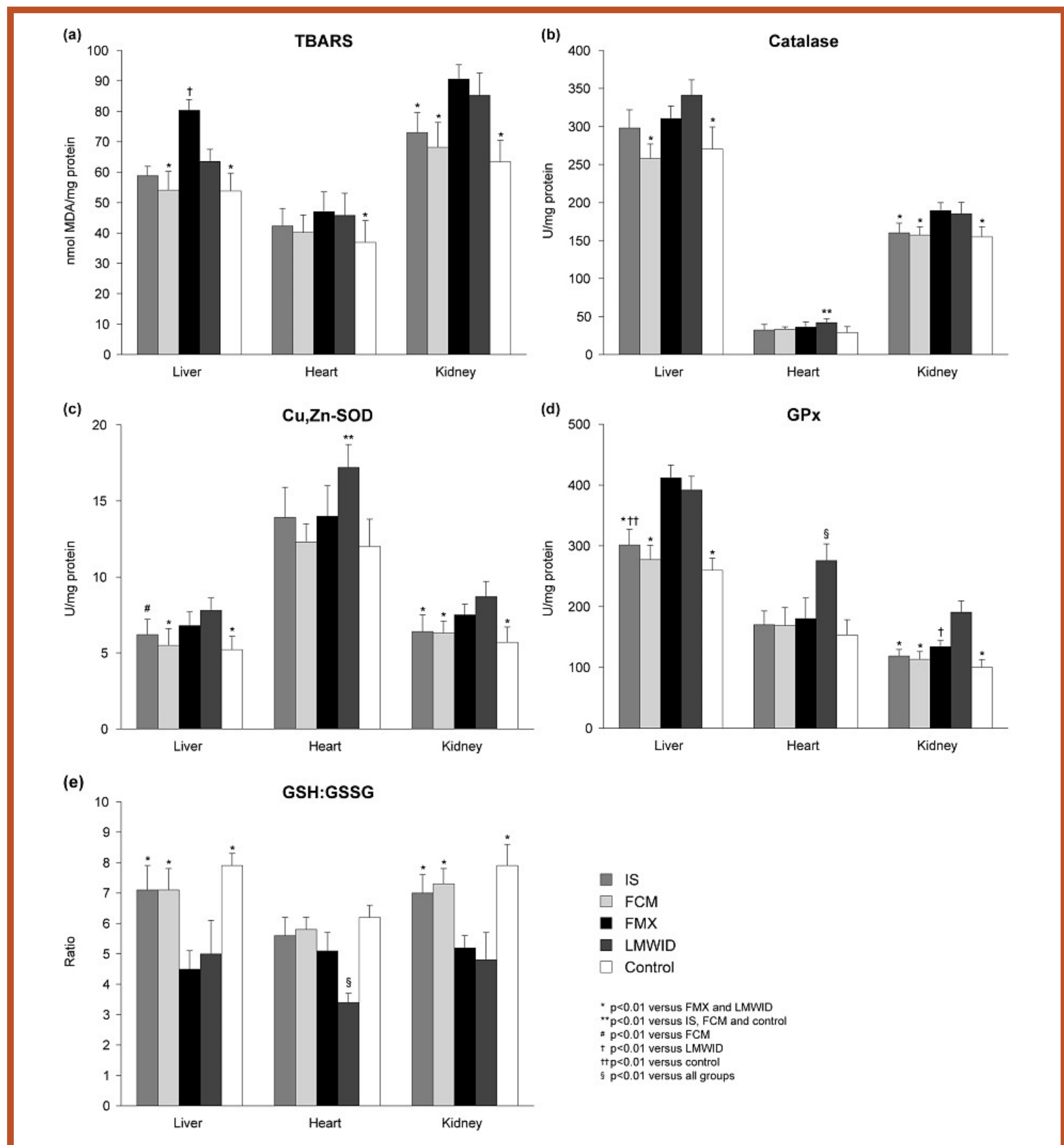

Fig. 3: Oxidative stress markers in rats treated with different i. v. iron preparations. (a) Thiobarbituric acid reactive species (TBARS), (b) catalase, (c) Cu,Zn superoxide dismutase (Cu,Zn-SOD), (d) glutathione peroxidase (GPx), (e) reduced to oxidized glutathione ratio (GSH:GSSG) in liver, heart and kidney homogenates 24 h after the last weekly (day 29) i. v. iron administration (40 mg iron/kg body weight or equivalent volume) in the iron sucrose (IS), ferric carboxymaltose (FCM), ferumoxytol (FMX), low molecular weight iron dextran (LMWID) and control groups.

LMWID group when compared with the IS, FCM and FMX groups ($p < 0.01$) (Fig. 4 b).

At the end of the study on day 29, the LMWID-treated rats showed a significantly larger ($p < 0.01$) area of positive staining for iron (Prussian blue) in tubular epithelial cells compared to that of the rats treated with the other i. v. iron compounds (Fig. 4 a). As seen in the liver and heart samples, the LMWID group presented a significantly smaller ($p < 0.01$) area of positive immunostaining for tissue ferritin compared to the groups treated with the other i. v. iron compounds (Fig. 4 b).

Upon completion of the experiment (day 29), levels of the inflammatory markers TNF-α and IL6 were both significantly increased ($p < 0.01$) in the liver and kidney samples of the FMX group as well as in the liver, heart, and kidney samples of the LMWID group compared to those of the IS, FCM and control groups (Fig. 5 and 6; see p. 406 and 407).

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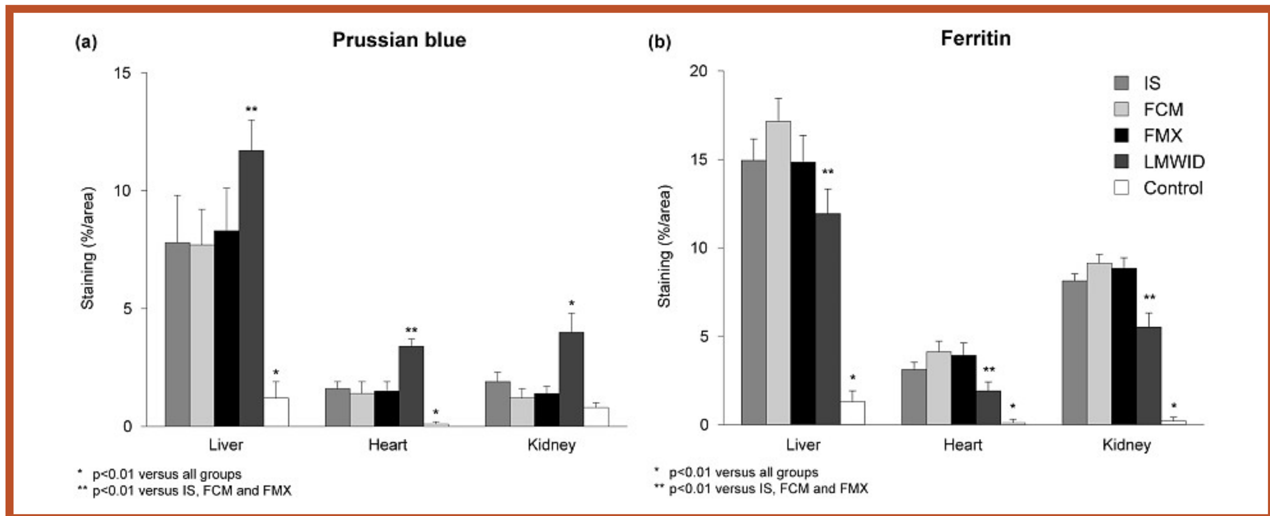

Fig. 4: Uptake and storage of iron in rats treated with different i. v. iron preparations. (a) Prussian blue staining of iron deposits and (b) ferritin immunostaining for stored iron in liver, heart and kidney samples taken from the iron sucrose (IS), ferric carboxymaltose (FCM), ferumoxytol (FMX), low molecular weight iron dextran (LMWID) and control groups 24 h after the last weekly (day 29) i. v. iron administration (40 mg iron/kg body weight or equivalent volume).

4. Discussion

An ideal i. v. iron compound should deliver sufficient amounts of iron in a readily available form to rapidly correct iron deficiency while causing no side effects. All i. v. iron compounds have the potential to cause oxidative stress, the extent of which mostly correlates with the amount of iron released from the complex. This, in turn, depends largely on the stability and the size of the complex [17]. Smaller and more labile compounds, such as FG, have been particularly associated with adverse events that may be caused by the presence of weakly-bound iron [17, 47, 48].

FMX consists of an iron oxide core stabilized by carboxymethylated dextran ligands [19, 49, 50]. FMX is a stable complex with a negligible release of iron into the circulation [19]. The reported Mw of FMX, measured relative to a protein standard, is 731 kDa [19]. However, in order to compare FMX with the other i. v. iron complexes in this study, the Mw of FMX was measured relative to a pullulan standard according to the method of The United States Pharmacopeia (USP) for iron sucrose injection [18]. With this method, the Mw of FMX was approximately 185 kDa, which is in the same range as that of FCM (Table 1). Analysis of the FMX chromatogram revealed a second, low molecular weight peak, most likely arising from a partly dissociated ligand.

Although a variety of i. v. iron preparations are currently used for the treatment of IDA, randomized controlled trials directly comparing their efficacy and safety are lacking. Comparisons of the relative risk of adverse events are only retrospective analyses [10, 26, 51, 52]. We have established a nonclinical model that allows distinguishing between the potential of IS and ISSs with similar physico-chemical properties to induce oxidative stress [35, 36]. Using the same model, we have recently shown that FG as well as high molecular weight iron dextran (HMWID) and LMWID have less favorable safety profiles than FCM and IS [13]. In the case of FG, this is most likely due to release of iron, while for HMWID and LMWID it is possibly due to improper iron distribution and utilization [53]. A similar model, albeit significantly less extensive, was recently used by another group to compare the potential of the original IS and an ISS to induce oxidative stress [54]. In the present study this nonclinical model was used to compare the newly approved dextran derivative FMX with IS, FCM, LMWID, each of which was retested to allow for direct comparison.

The cellular uptake, transient storage and subsequent utilization of iron is influenced in part by the form of iron administered and in part by the dosage, treatment regimen and physiological status of the subject [55]. Reticuloendothelial uptake of the injected iron carbohydrate complex is to indirectly indicate the safety of the preparation with regard to long-term effects on the parenchyma of various tissues [56, 57]. Tissue ferritin levels in the liver, heart and kidneys were similar in IS-, FCM- and FMX-treated rats but were higher than in LMWID-treated rats. The livers of the IS, FCM and FMX groups showed positive staining for iron only in Kupffer cells, whereas LMWID-treated rats showed positive staining also in hepatocytes and sinusoidal endothelial cells. Two similar studies with radiolabelled FCM and IS complexes have shown that the complexes are taken up and utilized similarly [56, 57]. High red blood cell utilization has demonstrated the efficacy of both IS and FCM [56, 57]. In contrast, it has been suggested that the iron from iron dextran is not entirely utilized in rats [13]. In man, transient iron dextran stores in the reticuloendothelial system (RES) become progressively unavailable over

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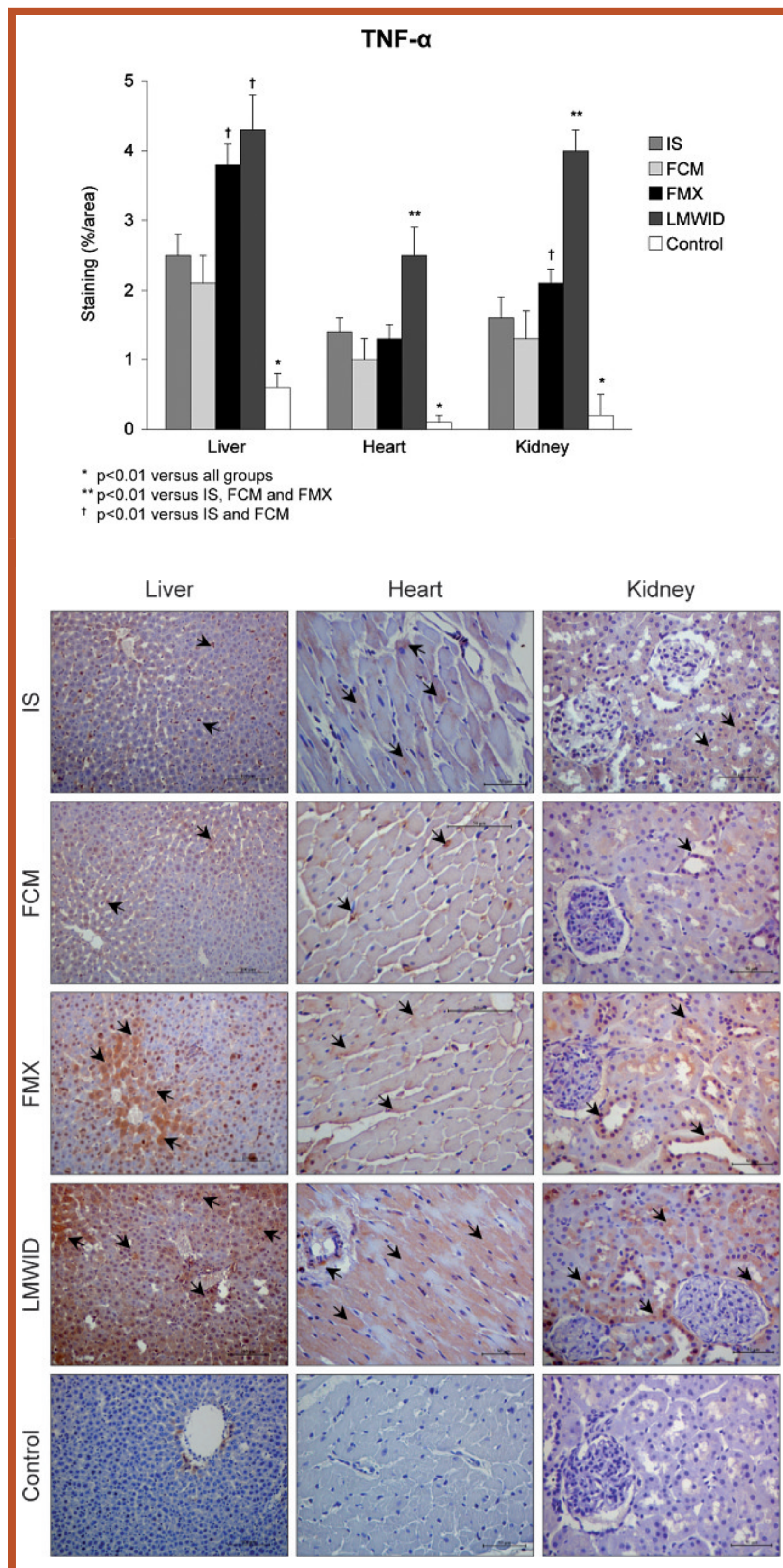

Fig. 5: TNF-α expression in rats treated with different i. v. iron preparations. TNF-α immunostaining in liver, heart and kidney samples taken from the iron sucrose (IS), ferric carboxymaltose (FCM), ferumoxytol (FMX), low molecular weight iron dextran (LMWID) and control groups 24 h after the last weekly (day 29) i. v. iron administration (40 mg iron/kg body weight or equivalent volume). Arrows indicate TNF-α localization.

time, possibly due to the physicochemical properties of the iron dextran complex [58]. Larger particles of iron dextran may be completely unavailable for iron release from the RES, especially sinusoidal endothelial cells, leading to a gradual deposition of unusable iron stores with repeated doses of dextran [53, 58–60]. The efficacy of iron utilization from FMX, a dextran derivative, has not yet been demonstrated by similar studies with radiolabelled FMX. These studies are important, because the higher thermodynamic stability of the polynuclear iron core of FMX, which is an iron oxide instead of an iron oxy-hydroxide as in all other iron carbohydrate complexes used for i. v. iron therapy, may lead to incomplete iron utilization.

Hypotension has previously been reported in animals [13, 61] and in CKD patients treated with various i. v. iron preparations [61]. Hypotension has been shown to be associated with NTBI and is more likely to occur when i. v. iron preparations are administered at the upper limits of recommended infusion doses and rates [62]. In this study, hypotension was observed in the groups receiving FMX and LMWID, both of which are dextran preparations. The SBP values of the LMWID group were lower in this study than in a previous study [13]. As shown previously [13], no decrease in SBP or DBP recordings was observed in response to IS or FCM.

The results of this study also showed that both FMX and LMWID caused proteinuria, which in the case of FMX significantly increased towards the end of the study. This suggests that, in contrast to IS and FCM, FMX and LMWID exert a negative effect on the kidneys. Moreover, increased levels of the liver enzymes AST, ALT and ALP in both the FMX and the LMWID groups indicate possible hepatic damage. In particular, the elevated AST level, which in rats requires relatively severe forms of liver necrosis [63], indicates that under the conditions of this study both dextran-containing

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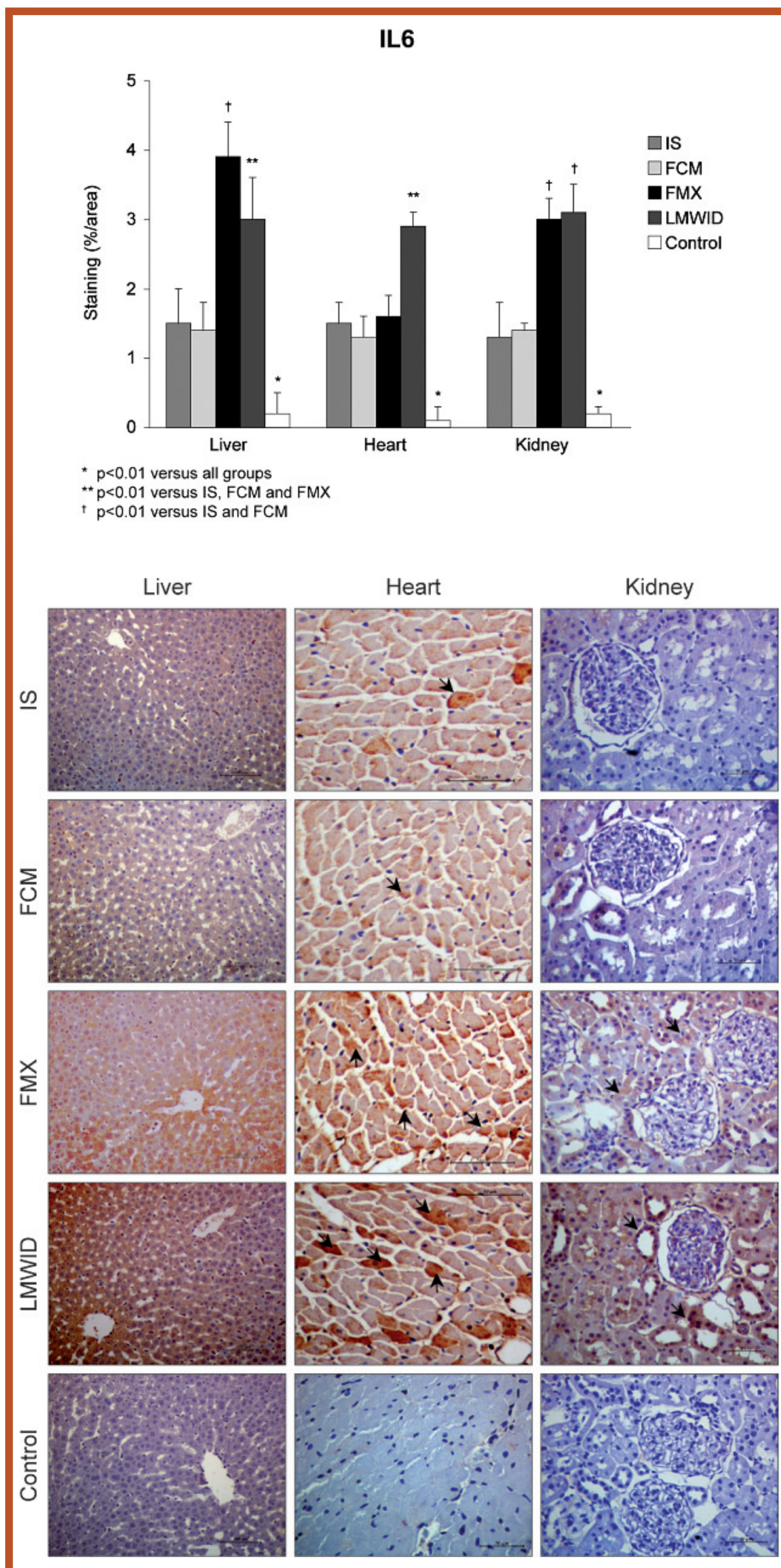

Fig. 6: IL6 expression in rats treated with different i. v. iron preparations. IL6 immunostaining in liver, heart and kidney samples taken from the iron sucrose (IS), ferric carboxymaltose (FCM), ferumoxytol (FMX), low molecular weight iron dextran (LMWID) and control groups 24 h after the last weekly (day 29) i. v. iron administration (40 mg iron/kg body weight or equivalent volume). Arrows indicate IL6 localization.

complexes induced irreversible liver damage. No liver damage was evident in rats treated with IS or FCM.

We have shown in a previous study that the concentration of oxidative stress markers in liver, heart and kidney tissue homogenates increases in response to LMWID (and HMWID), but not in response to FCM or IS [13]. In the present study, severe lipid peroxidation was indicated by a significant increase in malondialdehyde and in antioxidant enzyme activities in the liver and kidneys in response to FMX, and in the liver, kidneys, and heart in response to LMWID. Moreover, the reduced GSH : GSSG ratio, mainly in the liver but also to some extent in the kidneys of the FMX group and in the liver, heart, and kidneys of the LMWID group, reflects the consumption of antioxidants during oxidative stress. The concentration of GSH correlates closely with the degree of renal failure and it may also contribute to the progression of liver injury [64]. A reduction in GSH levels may lead to an increase of reactive oxygen species (ROS) and, thus, oxidative stress. It has been suggested that oxidative stress-induced lipid peroxidation may be involved in the pathogenesis of lipid-induced glomerulosclerosis in rats [65].

Oxidative stress can further increase the risk of endothelial damage and inflammation [66]. All the i. v. iron preparations studied were associated with an increase, of some degree, in the levels of inflammatory markers in the liver, heart and kidneys. However, the levels of both TNF-α and IL6 were significantly higher in the liver and kidney samples from FMX-treated animals and the liver, heart, and kidney samples of LMWID-treated rats compared to tissues from IS or FCM-treated rats. IL6 induces hepcidin expression, which, by binding to ferroportin and inducing its internalization, can prevent the export of iron from macrophages and hepatocytes [67, 68], thus affecting the utilization of iron.

The toxicity of i. v. iron has been attributed both to effects of oxida-

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tive stress and to the relative protective and allergenic effects of the carbohydrate shields, especially dextran. In the case of FG, which is a rather weak complex, there is a significant generation of NTBI, leading to oxidative stress and inflammation [13]. Although it has been shown that the release of catalytic iron from FMX is minimal [1, 2, 19], FMX-treated rats showed more severe lipid peroxidation in the liver and kidneys and higher antioxidant enzyme levels in the liver than FCM, IS or LMWID-treated rats. It is conceivable that oxidative stress is induced directly by the FMX complex. Indeed, the polarogram of FMX showed the presence of a component with a rather positive reduction potential (–255 mV *vs.* NHE). For comparison, the Fe(III)/Fe(II) reduction potentials of IS, FCM, and iron dextran are approximately −526, −390 and −475 mV, respectively [5]. The reduction potential of a complex indicates how easy it is to reduce Fe(III) to Fe(II) and thus provide the basis for iron-catalyzed ROS formation. A reduction potential below −320 mV *vs.* NHE indicates that biological reductants cannot reduce Fe(III) and that redox cycling is unlikely to take place [69].

In the polarogram of FMX, a second distinct Fe(III)/Fe(II) transition was found at −782 mV *vs.* NHE, suggesting that at least two types of Fe(III) centers with substantially different environments may exist in the complex. It seems feasible that the Fe(III) on the surface of the FMX complex has a more positive reduction potential than the Fe(III) that is surrounded by highly negatively charged O^{2-}-ions within the iron oxide core. Thus, the Fe(III) centers on the surface of the complex may be responsible for ROS formation and consequently for the elevated level of oxidative stress observed in some of the tissues.

In conclusion, this nonclinical study demonstrated signs of hypotension, renal and liver injury, oxidative stress and inflammation in the liver and kidneys of rats treated with FMX and LMWID but not in those treated with FCM or IS.

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Conflict of Interest

Vifor (International) Ltd. financially supported this study but did not contribute to the study design. Professor Jorge E. Toblli has received research grants and consultancy fees from Vifor (International) Ltd. The other authors have no conflicts of interest to declare.

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