Carbonyl stress induced by intravenous iron during haemodialysis

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Abstract

Background. Anaemic haemodialysis (HD) patients are treated with erythropoietin and intravenous iron for effective erythropoiesis. Since iron is a potent inductor and aggravator of pre-existing oxidative processes in HD patients, this study was aimed to evaluate the acute in vivo effect of two recommended iron doses on protein oxidation during the HD session.

Methods. Iron gluconate was intravenously administered to HD patients in doses of 62.5 or 125 mg per session. A dialysis session without iron administration served as a control for each patient. Carbonylated fibrinogen and iron profile parameters were monitored before and after each session. Plasma carbonylated fibrinogen levels from healthy subjects and HD patients before dialysis were compared. Protein associated carbonyls were identified in plasma by derivatization with 2,4-dinitrophenylhydrazine followed by western analysis and were quantified by densitometry.

Results. HD patients on maintenance iron showed elevated carbonylated fibrinogen compared with healthy subjects. During a HD session, carbonyls on fibrinogen further increased when 125 mg iron gluconate was administered, but no changes were detected with 62.5 mg iron gluconate or in the absence of iron. The changes in carbonylated fibrinogen during dialysis showed a significant linear correlation with the calculated values of transferrin saturation and free transferrin.

Conclusions. The significant acute increase in carbonylated fibrinogen with 125 mg iron gluconate suggests that this iron dose should be used with caution. As fibrinogen is highly susceptible to iron-induced oxidation in vivo, it may serve as a marker reflecting acute iron oxidative damage and as a tool in refinement of the existing clinical dose guidelines for intravenous iron therapy.

Keywords: carbonyl stress; fibrinogen; haemodialysis; intravenous iron therapy; iron gluconate; oxidative stress

Introduction

Patients on chronic haemodialysis (HD) are exposed to oxidative stress (OS) and inflammation [1]. In the presence of OS, oxidation of carbohydrates and lipids may lead to the formation of reactive carbonyl compounds (RCOs), resulting in the formation of advanced glycoxidation and lipoxidation end-products [1,2]. Recently, the term ‘carbonyl stress’ was introduced [2] to describe the association of RCOs with proteins, ending in the formation of carbonylated proteins in plasma and tissues, representing severe OS. A general increase in plasma protein-carbonyls in HD patients has been reported [3] and, more recently, carbonyl formation on specific plasma proteins, mainly albumin, has also been documented [4]. Carbonylation of proteins in vitro, beyond reflecting severe oxidation, may lead to protein dysfunction, including enzyme inactivation [5], decrease in immunoglobulin receptor binding [6], impairment of actin function [7] and inhibition of clotting by carbonylated fibrinogen [8].

Anaemic HD patients are treated with intravenous (i.v.) iron for adequate erythropoiesis, according to international guidelines [9,10]. However, increased risk of infection and cardiovascular complications has been reported in association with iron therapy [11]. Free iron may be generated from commercial formulations upon i.v. administration as iron dissociates from its carbohydrate moiety (gluconate, saccharate, dextran, etc.), depending on the degree of the patient’s transferrin saturation and probably on the dosage and speed of administration. Free iron, by virtue of its ability to flip between reduced and oxidized forms, is a potent inductor and aggravator of oxidative processes, leading to severe oxidation via the formation of hydroxyl radical by the ‘Fenton reaction’. Few studies
have shown that i.v. iron saccharate further aggravated the OS caused by the dialysis process *per se* [12] when administered during the dialysis session [13–15]. These studies used various timing protocols and different markers of OS, such as antioxidant enzymes [13,14], lipid peroxides [13–15] and red blood cell glutathione [14]. Recently, an *in vitro* study using various iron complexes demonstrated the direct hazards of parenteral iron, including lipid peroxidation and cytotoxicity [16].

We have designed a study to evaluate the *in vivo* oxidative effect of i.v. iron gluconate administered during HD on plasma-protein oxidation. Based on the well-established carbonyl stress in uremic patients, carbonylation of a specific protein can serve as an ultimate marker of severe OS induced by iron. Fibrinogen, beyond its role in coagulation, is an acute-phase protein and an independent cardiovascular risk factor whose plasma concentration increases during inflammatory states. HD patients have been shown to have significantly high levels of fibrinogen [17] as well as increased cardiovascular morbidity and mortality. Since fibrinogen is also highly susceptible to carbonyl formation *in vitro* [18], the oxidative effect of two clinically recommended doses of iron gluconate was evaluated by measuring the formation of carbonyl groups on fibrinogen.

### Subjects and methods

#### Subjects

Seventeen HD patients (Table 1) on maintenance i.v. iron gluconate [62.5 mg iron (III) sodium gluconate complex, Ferrlecit®; Natterman, Rhone-Poulenc Rorer, Cologne, Germany] and erythropoietin [4000–12 000 U/week, s.c., Epoetin beta (recombinant human erythropoietin), Recormon®; Roche Diagnostics GmbH, Mannheim, Germany] treatment were studied according to the experimental protocol. All patients underwent HD three times a week for >1 year, 4 h per session, carried out on cellulose triacetate hollow fibre dialysers (Nipro; Nissho, Osaka, Japan). The water for dialysis was compatible with the Association for the Advancement of Medical Instrumentation standards. Patients who received blood transfusions within 3 months of the study and patients with evidence of infection, malignancy or severe hyperparathyroidism were excluded. Informed consent was obtained from all patients and the Institutional Helsinki Committee approved the protocol.

### Study protocol

Each patient included in the study underwent three dialysis sessions in which he randomly received intravenously 62.5 or 125 mg iron gluconate (Ferrlecit®) during the first 60 min or one session without iron administration. Blood samples were drawn before and after the dialysis sessions from the arterial line and analysed for transferrin, ferritin, total iron, haematocrit (Hct) and carbonylated proteins. For carbonyl measurements, heparinized plasma was separated immediately and stored at −70°C for a maximum of 4 weeks.

### Iron parameter calculations

The results of transferrin, ferritin and total iron before the dialysis are given as measured. In order to correct for the haemoconcentration caused by HD, the values of these parameters after dialysis were corrected according to Hct changes during the dialysis using the formula

\[
P_{\text{calc}} = P_{\text{meas}} \left( \frac{\text{Hct}_{\text{A}}}{\text{Hct}_{\text{B}}} \right)
\]

where \( P_{\text{calc}} \) is the calculated parameter, \( P_{\text{meas}} \) is the measured parameter after the session, \( \text{Hct}_{\text{A}} \) is the Hct after the session and \( \text{Hct}_{\text{B}} \) is the Hct before the session. Transferrin saturation (TSat) was calculated as \( 70 \times (\text{total iron}/\text{transferrin}) \) and free, unsaturated transferrin levels were calculated as \( T \times \{100 – \text{TSat}\}/100 \) where \( T \) is the measured transferrin (including saturated and unsaturated).

### Preparation and quantification of carbonylated fibrinogen standard

Fibrinogen (Sigma, St Louis, MO, USA) was oxidized *in vitro* by iron/ascorbate according to instructions of the OxyBlot-Protein oxidation detection kit (Intergene, Purchase, NY, USA) to yield a highly carbonylated protein. For quantification of carbonyl content following this oxidation, the oxidized fibrinogen was dissolved in 6% sodium dodecyl sulphate (SDS) solution and mixed with an equal volume of 20 mM 2,4-dinitrophenylhydrazine (DNPH; Fluka, Buchs, Switzerland) in 20% trifluoroacetic acid (TFA). After 15 min incubation at room temperature the mixture was neutralized by Tris buffer (Trizma base; Sigma, St Louis, MO, USA). Part of the sample was stored at 4°C for SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and the rest was precipitated with trichloroacetic acid. The protein pellet was washed three times in ethanol-ethyl acetate mixture (1:1, v/v) and resuspended in 6 M guanidine hydrochloride in 25 mM potassium phosphate adjusted to pH 2.0 with TFA. Insoluble material was removed by centrifugation, the absorbance of the sample was measured and the protein carbonyl content was calculated using the molar coefficients of 22 000 M⁻¹ cm⁻¹ for hydrazone at 370 nm and 9460 M⁻¹ cm⁻¹ for protein at 276 nm [18]. Using the above calculations, the amount of carboxyls on fibrinogen was expressed as nmol carbonyl/mg fibrinogen protein. A fixed amount of this carbonylated fibrinogen served as a standard on all gels.

### Preparation of plasma proteins for carbonyl detection

Plasma samples were prepared for carbonyl detection by derivatization with DNPH as described previously [19]. Briefly, plasma was diluted 1:30 in 6% SDS and mixed with 1 vol of 20 mM DNPH solution. The mixture was incubated for 15 min at room temperature, neutralized by 1.75 vol of

### Table 1. Baseline characteristics of the HD patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>17</td>
</tr>
<tr>
<td>Age (years range)</td>
<td>71.2 (50–84)</td>
</tr>
<tr>
<td>Gender (male:female)</td>
<td>9:8</td>
</tr>
<tr>
<td>Diabetic (type 2):non-diabetic</td>
<td>8:9</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>11.0 ± 0.2</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>32.6 ± 0.5</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.8 ± 0.03</td>
</tr>
<tr>
<td>Transferrin (mg/dl)</td>
<td>166.2 ± 3.7</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>326 ± 27</td>
</tr>
</tbody>
</table>
Detection and quantification of carbonyls on plasma proteins

Proteins were transferred to nitrocellulose filters in transfer buffer (25 mM Tris, 192 mM glycine) and used for western analysis according to the manufacturers instructions (OxyBlot kit). Carbonyl signal was detected on X-ray films using the chemiluminescence reagents of the EZ-ECL kit (Biological Industries, Beit-Haemek, Israel). Detection times were < 60 s. For the identification of the fibrinogen band, western analysis was performed with rabbit polyclonal anti-fibrinogen serum (ICN Pharmaceuticals, Aurora, OH, USA) and goat anti-rabbit–horseradish peroxidase (HRP) conjugate (OxyBlot kit). The immunoglobulins and albumin bands were identified by protein A–HRP conjugate (Sigma, St Louis, MO, USA) and specific albumin antiserum (Sigma, Israel), respectively.

The DNPH-derivatized samples were separated by SDS-PAGE together with the oxidized fibrinogen standard. The densities of the bands from the films of the western blots and the Coomassie blue stained gels were analysed with the BioCapt and Bio-Profil (Bio-1D) softwares. The content of carbonyls per fibrinogen was calculated in each gel relative to the standard fibrinogen sample.

Statistical analysis

All the results are given as the mean ± SE. The data [ratios and Δ (change in carbonylated fibrinogen during dialysis) from 17 patients] were slightly skewed and therefore Wilcoxon ranks sum test and the Kruskal–Wallis test were used for statistical evaluation of the iron dose effects. The Friedman test was used for analysis of the values before the dialysis session. A P-value of < 0.05 was considered significant.

Results

Detection of carbonyls on fibrinogen in HD patients and NC subjects

Carbonylation of plasma proteins was studied in HD patients on maintenance iron and NC subjects (Figure 1). Under the non-reducing SDS-PAGE conditions used in this study, the most carbonylated plasma protein was identified as fibrinogen. Fibrinogen identification was confirmed by several analyses: molecular mass determination, reactivity with specific anti-human fibrinogen antibodies and by subunits analyses (data not shown). The less carbonylated band was identified as immunoglobulins by similar analyses. However, detection of carbonyls on albumin was possible only after prolonged exposure of the X-ray films to the blots. Densitometry analysis of NC and HD plasma fibrinogen showed a 2-fold increase in the amount of the protein during HD and at least a 4-fold elevation in the carbonyl intensity. The mean value in HD patients was 1.37 ± 0.16 nmol carbonyl/mg fibrinogen compared with a significantly (P < 0.02) lower content of carbonyls (0.67 ± 0.13 nmol carbonyl/mg fibrinogen) in NC subjects. Since the expression of carbonyls on fibrinogen in nmol carbonyl/mg protein is corrected for the increase in plasma fibrinogen concentration, it represents only the degree of protein oxidation.

Iron profile

The mean transferrin value of the HD patients (before dialysis session) was 166.2 ± 3.7 mg/dl (Figure 2A), significantly (P < 0.0001) lower than the mean normal value of 280.1 ± 13.4 mg/dl. The mean transferrin levels were slightly elevated (174.4 ± 4.1 mg/dl) after dialysis, even after correction for haemoconcentration. Although these changes were small, they were significant (P < 0.01) and independent of iron administration, as it was also observed in the dialysis sessions without iron (Figure 2A).

Iron administration and the dialysis per se had no effect on ferritin levels (326 ± 27 ng/ml before and 351 ± 31 ng/ml at the end of the session).
Total iron (Figure 2B) and transferrin saturation values (Figure 2C) calculated at the end of the dialysis session showed significant dose-dependent increases only when iron gluconate was administered. However, transferrin saturation, calculated at the end of the session, did not exceed 100%, even when the high iron dose was administered.

The effect of iron administration on fibrinogen carbonyls

Administration of 125 mg iron gluconate during the first hour of the session caused a significant increase in carbonyls on fibrinogen assayed at the end of the dialysis session ($P < 0.002$; Figure 3A). According to statistical analysis, the carbonyl levels on fibrinogen at the start of each protocol were similar ($P = 0.59$). No significant changes were detected when the low dose (62.5 mg) of iron was administered or in the absence of iron. The average increase in carbonyls on fibrinogen following the high iron dose was $26 \pm 7.3\%$ (Figure 3B). This significant carbonylation was also demonstrated when the carbonyl changes were analysed as the change ($\Delta$) in carbonylated fibrinogen (after HD–before HD; Figure 3C). The increase in carbonyls level in the high iron dose protocol was significant compared with the two other study protocols (Figure 3B and C).

The baseline levels of carbonylated fibrinogen measured pre-dialysis in the diabetic and non-diabetic HD patients showed no statistical difference. Within the diabetic group, fasting blood glucose levels were well controlled in half of the patients ($< 100$ g/dl, four patients) and poorly controlled in the other patients.
Administration of 125 mg (but not 62.5 mg) iron gluconate induced significant increase in carbonylation of fibrinogen at the end of the session \( (r = 0.27, P < 0.03; \text{Figure } 4A) \). The change in carbonyls on fibrinogen during the session also correlated inversely with the calculated values of free transferrin at the end of the session and, interestingly, also with the calculated free transferrin values before the dialysis session (Figure 4B and C).

**Discussion**

This study was designed to examine the acute effects of two doses of i.v. iron on protein oxidation during a dialysis session in HD patients. Iron-induced OS was assessed by protein oxidation reflected by carbonyl groups on plasma fibrinogen. In general, fibrinogen carbonyls in HD patients were significantly greater than in healthy controls, with further increase after administration of 125 mg of iron gluconate. The increase in fibrinogen carbonyls during dialysis correlated with T\(_{Sat}\) and free transferrin.

This study also demonstrates that fibrinogen is the most sensitive protein target for carbonylation. Among other plasma proteins, immunoglobulins and albumin also carry carbonyls, but to a much lesser extent per molecule. This unique fibrinogen susceptibility to carbonylation *in vivo* is supported by a previous study where fibrinogen was the only carbonylated protein in plasma of smokers and lung cancer patients [20]. This conclusion is supported by an *in vitro* study where oxidation of plasma samples generated more carbonyls on fibrinogen than on any other major plasma protein [18]. Himmelfarb and McMonagle [4], by using a similar western analysis system, have recently claimed that albumin is the major target for carbonylation in plasma of HD patients. In their study, fibrinogen and albumin from HD patients carried the same amounts of carbonyls determined by densitometry units. Since fibrinogen is far less abundant in plasma, we think that correction per protein amounts would have shown that fibrinogen carries more carbonyls than albumin; hence it is more susceptible to oxidation. Our contradictory conclusions may also originate from the differences in the experimental conditions, as we used a modified DNPH reaction with improved specificity and sensitivity for protein carbonyl detection according to Shacter [19].

Based on our data, we suggest that the oxidation of fibrinogen may partially contribute to the impaired clotting activity observed in HD patients. This assumption is supported by data showing that oxidized fibrinogen *in vitro*, either by iron or by peroxynitrite, caused inhibition of clotting activity [8,21].

In addition, the HD patients in this study showed high levels of fibrinogen (as observed in the gels after protein staining) and low concentrations of transferrin. Both fibrinogen and transferrin are acute-phase proteins, hence this observation is compatible with the inflammatory state of HD patients, as previously described [12,17,22].

**Correlation between transferrin and carbonylated fibrinogen**

The results derived from all the study protocols showed that the change in carbonyls on fibrinogen during dialysis correlated linearly with transferrin saturation at the end of the session \( (r = 0.27, P < 0.03; \text{Figure } 4A) \). The change in carbonyls on fibrinogen during the session also correlated inversely with the calculated values of free transferrin at the end of the session and, interestingly, also with the calculated free transferrin values before the dialysis session (Figure 4B and C).

**Fig. 4.** The correlation between transferrin and the changes in carbonylated fibrinogen. The correlation between the change in carbonyls on fibrinogen (expressed as the ratio after HD before HD) and transferrin saturation values at the end of the dialysis session (A), free transferrin \( (T_A) \) at the end of the dialysis session (B) and free transferrin \( (T_B) \) at the start of the dialysis session (C).

\( \sim 250 \text{ g/dl, four patients} \). Administration of 125 mg (but not 62.5 mg) iron gluconate induced significant increase in carbonylation of fibrinogen at the end of the session in both groups \( (P = 0.023 \text{ for diabetic and } P = 0.02 \text{ for non-diabetic}) \). To explore the different effects of dialysis and iron supplementation with respect to diabetes, larger groups of patients should be studied.
The pre-existing OS of HD patients was exacerbated by i.v. iron administration. The occurrence of free iron during dialysis becomes especially harmful in the presence of reactive oxygen species, which may initiate the Fenton reaction, resulting in the generation of hydroxyl radicals. Increased OS during HD sessions due to activation of neutrophils has been reported by us and by others [12,23]. Based on these data, it appears that the conditions for a severe in vivo protein oxidation are already present when iron complexes are administered and, indeed, the acute increase in carbonyl levels on fibrinogen following i.v. iron administration support this idea.

As each administration of a high iron dose caused an average increase of 26% in carbonyls per fibrinogen, one may expect that with time, carbonyls would accumulate in HD patients to a greater extent than observed, as these patients continue to be on maintenance iron therapy for many years. One of the possible explanations could be that in vivo mechanisms limiting this accumulation exist but are not yet understood. The increased levels of fibrinogen carbonylation in the HD patients before the dialysis session are probably the result of chronic i.v. iron administrations, as these anaemic patients are on maintenance i.v. iron and erythropoietin therapy.

The administration of iron to dialysis patients via the i.v. route is universally accepted as the preferred method to treat functional iron deficiency in anaemic uraemic patients. Clinical trials have shown that iron preparations, such as gluconate and saccharate [24–26], have comparable efficacy to iron dextran with significantly fewer side effects. However, safety in these trials was considered in terms of scarcity of immediate side effects, such as anaphylactic reactions, arthralgia–myalgia syndromes and hypotension. This side effects were found to correlate with transferrin oversaturation [27], which is considered as a marker of free iron in plasma. The dilemma of whether free iron is present when transferrin saturation is below maximum was recently resolved: it was shown that catalytically active iron [28] or non-transferrin bound iron [29] existed in plasma of HD patients receiving i.v. iron formulations, even when transferrin saturation values were <83%. In our study, transferrin saturation increased to maximum of 83% following 125 mg iron gluconate and to 62% following 62.5 mg. Carbonyl formation during a HD session showed a positive correlation with transferrin saturation at the end of the dialysis session. This correlation supports the notion that free iron was probably present in the plasma following iron glucenate administration, although transferrin was not oversaturated. As $T_{Sat}$ was measured 3 h after iron administration, we assume that oversaturation may have occurred at an earlier timepoint during the session. The occurrence of free iron can be assumed also by the fact that labile iron is present in the parenteral iron formulations administered to the patients [29]. Nevertheless, the negative correlation between carbonyl formation and free transferrin throughout the dialysis session suggests that unsaturated transferrin has a critical role in iron-dependent oxidation. When fewer iron binding sites are available, free iron catalysed reactions are favoured. Studies resembling our iron administration protocols showed controversial results for transferrin saturation: in some studies oversaturation was determined [15,27], but not in others [13,28]. Since i.v. iron administration resulted in a dose-dependent increase in carbonyls on fibrinogen, carbonylated fibrinogen should serve as a better indication for iron-induced damage than transferrin oversaturation.

Several variables can be addressed by using carbonylated fibrinogen as a marker: safety of iron doses, rate and timing of administration and the interpretations of transferrin saturation. Unfortunately, it is not possible at the moment to indicate a ‘safe’ dose or procedure; nevertheless, based on our study, high doses of iron gluconate (125 mg) should be avoided. Carbonylated fibrinogen, as described in this study, once again raises concern for the potential hazards associated with i.v. iron oxidative damage. Carbonylated fibrinogen should be considered for refinement of the existing clinical guidelines and should be used for further prospective studies in order to clarify the issue of chronic, long-term damage of i.v. iron therapy.

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