Impact of iron sucrose therapy on leucocyte surface molecules and reactive oxygen species in haemodialysis patients

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Original Article

Abstract

Background. It has been suggested that iron increases oxidative stress and that an excess of iron contributes to cardiovascular disease and infections in haemodialysis patients. In the present study, the effects of parenterally administered iron on leucocyte surface molecule expression and the production of reactive oxygen species (ROS) were evaluated.

Methods. Ten chronic haemodialysis (HD) patients without iron overload were studied. To each patient, four different regimens were applied: placebo; iron sucrose, either 30 or 100 mg, administered via the outflow dialysate line; and 100 mg of iron sucrose infused via the inflow dialysate line. Blood was sampled at different time points: before, during and after infusion and immediately before the next dialysis session. Levels of CD11b and CD45 expression on granulocytes and of CD11b, CD14 and CD36 on monocytes were determined using flow cytometric analysis. The generation of ROS was quantified using chemiluminescence with and without ex vivo stimulation by phorbol myristate acetate (PMA).

Results. No significant differences among the four different treatment regimes were found, neither in chemiluminescence activity nor in the expression of CD11b and CD45 on granulocytes, and of CD11b, CD14 and CD36 on monocytes.

Conclusions. Our results suggest that parenteral infusion of iron sucrose during haemodialysis in patients who have no signs of iron overload has no significant effect on the expression of leucocyte surface molecules and does not increase production of ROS.

Keywords: CD11b; CD14; CD36; CD45; iron sucrose; reactive oxygen species

Introduction

Most haemodialysis (HD) patients treated with recombinant human erythropoietin receive concurrent iron therapy. Although both oral and parenteral iron supplementation have been used in patients with stage V renal disease, the parenteral route has been found to be more effective. Polynuclear ferric hydroxide–carbohydrate complexes, such as iron dextran, iron sucrose and iron gluconate, have been used widely; nonetheless, lately, iron sucrose was considered to be one of the safest agents [1]. Iron overload and intravenous (IV) infusion of iron have been incriminated in the increased susceptibility for infection [2–5] and the accelerated rate of atherosclerosis [6,7], which are current complications in dialysis patients.

Leucocytes play a key role in the defence against infection and are important mediators in the progression of atherosclerosis. Their function relies at least in part on the expression of surface molecules such as CD11b, CD14, CD36 and CD45, and on the generation of mediators such as the reactive oxygen species (ROS). CD11b is mainly found on the surfaces of monocytes and granulocytes; CD14 is predominantly expressed on monocytes and macrophages. The surface molecule, CD36, belonging to the class B family of scavenger receptors, is expressed on monocytes, platelets, endothelial cells and on some lines of human tumour cells, but not on lymphocytes or granulocytes. Another leucocyte surface molecule, CD45, is abundantly found on granulocytes, lymphocytes, monocytes, eosinophils and basophils. These leucocyte surface molecules have many roles in inflammatory, chemotactic and immunological responses such as leucocyte and endothelial cell interactions, cell recognition, phagocytosis and atherogenesis [8–11]. Therefore, in the present study, all these surface molecules were studied, in order to cover a variety of points of action.

In the present study, the possible effects of IV infusions of iron sucrose on leucocyte expression of CD11b, CD14, CD36 and CD45, and on the
production of ROS were investigated in chronic HD patients without iron overload.

**Patients and methods**

**Patients**

Ten chronic HD patients (two females and eight males; mean age, 65.1 ± 8.9 years; mean dialysis duration 73.7 ± 64.4 months) participated in the study. Their baseline mean ferritin level was 104 ± 60 ng/ml, and mean transferrin saturation level 17.8 ± 3.8%. The causes of renal failure were renovascular disease (n = 3), immunoglobulin A (IgA) nephropathy (n = 2), chronic glomerulonephritis (n = 1), analgesic nephropathy (n = 1) and combined diabetes/renovascular disease (n = 1). No vitamin C or E, antibiotics, corticosteroids or non-steroidal anti-inflammatory agents were administered, and none of the patients had infectious or inflammatory conditions during the investigation period or in the month preceding the study. Likewise, no parenteral nutrition or mannitol was administered to any patient during this time frame.

Before and during the investigation, all patients were dialysed three times weekly for at least 4 h. Bicarbonate dialysate (formula 4002, Bieffe Medital, Grosotto, Italy or B 714, Sterima NV, Kortrijk, Belgium) and single-use low-flux polysulfone membranes (F8, 1.8 m², Fresenius Bad Homburg, Germany) were used. All patients were taking epoetin beta (Recormon®, Roche, Hertfordshire, UK) at doses that maintained a stable haematocrit between 33 and 36%. None of the patients had received blood transfusions in the month prior to the study.

**Study protocol**

The investigation was conducted over four consecutive weeks. Each individual was subjected randomly to three different regimes of IV iron sucrose (Venofer® Vifor France SA, Levallois-Perret, France) administration and one placebo treatment. Every patient had one experimental session on the same day of each week, and the different regimens were applied in random order. The four forms of therapy were as follows: 100 ml of saline infused via the outflow (venous) dialyser blood line (placebo—treatment A); 30 mg iron sucrose dissolved in 100 ml of saline and infused via the outflow dialyser line (treatment B); 100 mg iron sucrose dissolved in 100 ml of saline and infused via the outflow dialyser line (treatment C) and 100 mg iron sucrose dissolved in 100 ml of saline and infused via the inflow dialyser line, upstream of the artificial kidney (treatment D). All placebo and iron sucrose infusions were started 60 min after the beginning of the session, and were administered over a period of 30 min. In the typical clinical setting, IV iron is usually administered during the last 30 min of the session. There were two reasons why we used a different time frame for the present study: (i) to allow evaluation of effects on leucocytes in a period at least 150 min after iron administration; this could be done by blood sampling after dialysis, without the need to recur to post-dialysis blood collections and (ii) to minimize the effect of potential membrane bioincompatibility on leucocyte activity, as this effect is most pronounced during the first 30 min of the session.

For each experimental treatment, blood samples were collected from the inflow dialyser blood line at six different time points: before the start of the session (t0i); after 60 min of dialysis (t1i); after 90 min (t2i); after 120 min (t3i); after 240 min (t4i) and before the start of the next session (t5i). We also collected blood from the outflow dialyser blood line 90 min after the start of the session (t2o). In each case, the sample at 60 min (t1i) was drawn just before the start of iron (or placebo) infusion. The samples at 90 min (t2i and t2o) were drawn just prior to the completion of iron (or placebo) infusion. Figure 1 illustrates the study design, including the sites of blood sampling at different time points and the two sites of infusion.

**Fig. 1.** An illustration of the study design showing the sites of blood sampling at different time points, and the two sites of infusion (i, inflow; o, outflow).
Flow cytometric analysis

The levels of expression of CD11b and CD45 on granulocytes and of CD11b, CD14 and CD36 on monocytes were evaluated by direct immunofluorescence staining, using a flow cytometer (FACScan™, Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). The following monoclonal antibodies (BD Bioscience, San Jose, CA, USA) were used: (i) phycoerythrin (PE)-conjugated anti-CD11b (Leu™-15); (ii) fluorescein isothiocyanate (FITC)-conjugated anti-CD36 and (iii) a mixture of FITC-conjugated anti-CD45 (anti-HLe-1) and PE-conjugated anti-CD14 (Leu™-M3) (Simultest™ LeucoGATE™). For testing, 20 μl of monoclonal antibody was placed in a Falcon polystyrene test tube (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) and 100 μl of whole blood was added. Each blood sample was incubated with the respective monoclonal antibodies for 20 min at 4°C. About 2 ml of 10 × diluted FAC$^R$ Lysing Solution (Becton-Dickinson) was then added to each tube, and this mix was incubated for 8 min. After washing the cells twice, the cell pellet was re-suspended in Hanks’ balanced salt solution (HBSS) (GIBCO BRL Life Technologies, Paisley, UK) and immediately analysed on a FACScan™. CaliBRIT£™ beads and AutoCOMP™ Software were used to set photomultiplier tube voltages, adjust fluorescence compensation and check detector sensitivity. The mean channel of fluorescence intensity was calculated. Saturating concentrations of monoclonal antibodies were used. Non-specific labelling was excluded by the Simultest™ Control FITC-conjugated IgG1/PE-conjugated IgG2a and FITC-conjugated IgM-κ (BD Bioscience). Monocytes and granulocytes were gated selectively for analysis by low-forward-angle light scatter (FSC) and right-angled light scatter (SCC).

Expression of surface molecules on granulocytes

The levels of expression of CD11b and CD45 on granulocytes are illustrated in Figure 2.

Expression of surface molecules on monocytes

The levels of expression of CD11b, CD14 and CD36 on monocytes during the four treatment regimes are shown in Table 2.

Results

The laboratory results of pre-dialysis haematocrit, leucocyte counts and parameters related to iron stores, before and after the study, are presented in Table 1. Analysis revealed no significant differences between the findings at baseline and those at the end of the study.

Expression of surface molecules on monocytes

The levels of expression of CD11b, CD14 and CD36 on monocytes during the four treatment regimes are shown in Table 2.

CD11b: The iron treatment regimen had no significant effect on the expression of CD11b on granulocytes during dialysis. In each regimen, the level of expression at t20 was higher than the levels at the other time points.

CD45: The iron treatment regimen had no significant effect on the expression of CD45 on granulocytes during dialysis. In all the treatment regimens, the level of expression was slightly higher at t20 than at the other time points.

Table 1. Haematocrit, leucocyte counts and parameters of iron-metabolism before and after the study (n = 10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before</th>
<th>After</th>
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<tbody>
<tr>
<td>Haematocrit (%)</td>
<td>35.1 ± 2.6</td>
<td>34.7 ± 3.0</td>
</tr>
<tr>
<td>Leucocyte counts (×10⁶/mm³)</td>
<td>6600 ± 2158</td>
<td>6670 ± 2025</td>
</tr>
<tr>
<td>Iron (μg/dl)</td>
<td>52 ± 13</td>
<td>50 ± 13</td>
</tr>
<tr>
<td>Iron binding capacity (μg/dl)</td>
<td>296 ± 41</td>
<td>277 ± 45</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>17.8 ± 3.8</td>
<td>18.6 ± 5.7</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>104 ± 60</td>
<td>100 ± 55</td>
</tr>
</tbody>
</table>

At baseline vs final assessment: no statistically significant differences noted.
the expression at t2o was higher than the levels at the other time points.

CD14: The administration of iron also had no significant effect on the expression of CD14 during dialysis. The level of CD14 expression on monocytes did not change significantly throughout the observational period for any of the four regimes tested.

CD36: As with CD11b and CD14, the iron schedule did not significantly influence the CD36 expression during dialysis. There were no significant changes in CD36 expression on monocytes throughout the time periods analysed for any of the four regimes tested.

Chemiluminescence

The chemiluminescence results are shown in Table 3. The schedule of iron administration did not influence the levels of chemiluminescence (basal, PMA-stimulated and delta values) during the session. The levels of chemiluminescence (basal, PMA-stimulated and delta values) did not change significantly throughout the time periods analysed with any of the four treatment regimes.

Discussion

The objective of this study was to assess whether IV iron sucrose, administered at different doses and by different routes during HD, affects the expression of surface molecules on leucocytes and ROS production. The main finding is that the application of iron at the doses used in this study and in the type of patient evaluated in this study does not cause a major change, neither in baseline activity nor in stimulated response, as measured by the expression of leucocyte surface molecules and chemiluminescence.

The most critical concerns regarding iron therapy in HD patients are (i) the increased risk of infection and (ii) the potential to accelerate atherosclerosis, both of which seem related either to acute effects of iron administration or iron overload. The increased infection risk appears to be related to the effects of iron on microbial growth and on host immunity.
Under normal conditions, the human body has nearly all of its iron in an inactive form, bound to transferrin and lactoferrin, in order to decrease the access of microorganisms to iron. Microorganisms use free iron in the bloodstream to enhance their growth. In the presence of inflammation and fever, iron binding to lactoferrin increases and this complex is taken up by macrophages to sequester the iron in the reticuloendothelial system [12,13]. This event decreases free iron in the bloodstream and, hence, inhibits bacterial growth. IV iron administration might exceed transferrin-binding capacity in some HD patients, and the unbound circulating iron may increase bacterial growth [14–18]. It has been reported that IV iron administration increases infection risk and bacteremia episodes in dialysis patients [2–5,19]. Moreover, frequent administration of IV iron over a 6 month period was demonstrated to increase the risk of hospitalization and death [20]. Nevertheless, the relationship between IV iron therapy and infection is not consistent [21,22]. Similarly, no significant relationship between IV iron therapy and infection of hospitalization and death [20]. Nevertheless, the 6 month period was demonstrated to increase the risk of hospitalization and death [20].

Moreover, frequent administration of IV iron over a 6 month period was demonstrated to increase the risk of hospitalization and death [20]. Nevertheless, the relationship between IV iron therapy and infection is not consistent [21,22]. Similarly, no significant relationship between IV iron therapy and infection of hospitalization and death [20]. Nevertheless, the 6 month period was demonstrated to increase the risk of hospitalization and death [20].

Table 3. Basal, PMA-stimulated and delta chemiluminescence results at each of the time points for each of the four treatment regimes

<table>
<thead>
<tr>
<th></th>
<th>t0i</th>
<th>t1i</th>
<th>t2i</th>
<th>t2o</th>
<th>t3i</th>
<th>t4i</th>
<th>t5i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.7±0.38</td>
<td>1.8±0.5</td>
<td>2.4±1.6</td>
<td>1.8±0.6</td>
<td>2.2±0.6</td>
<td>1.8±0.7</td>
<td>1.9±0.8</td>
</tr>
<tr>
<td>B</td>
<td>1.9±0.54</td>
<td>2.3±0.5</td>
<td>2.2±0.5</td>
<td>2.3±0.8</td>
<td>2.5±0.9</td>
<td>2.5±1.0</td>
<td>3.0±2.6</td>
</tr>
<tr>
<td>C</td>
<td>2.1±0.7</td>
<td>1.8±0.6</td>
<td>2.5±1.0</td>
<td>2.1±0.8</td>
<td>2.3±0.9</td>
<td>2.6±0.8</td>
<td>2.3±1.3</td>
</tr>
<tr>
<td>D</td>
<td>1.9±1.0</td>
<td>2.1±1.1</td>
<td>2.1±1.0</td>
<td>2.3±1.9</td>
<td>2.7±1.6</td>
<td>2.4±1.3</td>
<td>2.1±1.3</td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>41.7±12.6</td>
<td>44.4±13.0</td>
<td>45.4±12.4</td>
<td>37.1±12.1</td>
<td>44.4±13.3</td>
<td>44.3±9.2</td>
<td>48.6±13.9</td>
</tr>
<tr>
<td>B</td>
<td>39.5±14.5</td>
<td>43.8±15.0</td>
<td>44.4±18.6</td>
<td>38.8±16.0</td>
<td>44.4±12.5</td>
<td>46.6±15.9</td>
<td>42.5±15.5</td>
</tr>
<tr>
<td>C</td>
<td>45.1±20.8</td>
<td>41.7±10.8</td>
<td>47.3±14.1</td>
<td>38.4±11.9</td>
<td>47.0±12.3</td>
<td>45.2±13.5</td>
<td>46.3±18.2</td>
</tr>
<tr>
<td>D</td>
<td>36.3±18.6</td>
<td>41.9±17.0</td>
<td>43.6±18.9</td>
<td>38.1±16.1</td>
<td>47.5±18.6</td>
<td>43.8±17.9</td>
<td>44.2±17.6</td>
</tr>
<tr>
<td>Delta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>40.0±12.6</td>
<td>42.6±13.1</td>
<td>43.0±13.4</td>
<td>35.3±12.0</td>
<td>42.2±13.5</td>
<td>42.5±13.5</td>
<td>46.7±13.7</td>
</tr>
<tr>
<td>B</td>
<td>37.6±14.2</td>
<td>41.5±15.0</td>
<td>42.2±18.4</td>
<td>36.5±15.5</td>
<td>41.9±11.9</td>
<td>44.1±11.9</td>
<td>39.5±13.9</td>
</tr>
<tr>
<td>C</td>
<td>43.0±20.5</td>
<td>39.9±10.7</td>
<td>44.8±13.6</td>
<td>36.3±11.6</td>
<td>44.7±12.3</td>
<td>42.6±12.4</td>
<td>44.0±18.2</td>
</tr>
<tr>
<td>D</td>
<td>34.4±18.0</td>
<td>39.7±16.6</td>
<td>41.5±18.6</td>
<td>35.8±15.6</td>
<td>44.8±17.9</td>
<td>41.4±17.8</td>
<td>42.1±17.6</td>
</tr>
</tbody>
</table>

Data are integrals (calculated) × 10^3.

Treatments: A: placebo; B: 30 mg iron sucrose via the outflow dialyser blood line; C: 100 mg iron sucrose via the outflow dialyser blood line and D: 100 mg iron sucrose via the inflow dialyser blood line. Sampling times: t0i, before the start of the session; t1i, 60 min after start of dialysis, before iron (or placebo) administration; t2i, 90 min after start of dialysis, at the end of iron (or placebo) administration; t3i, 120 min after start of dialysis; t4i, 240 min after start of dialysis; t5i, before the start of the next session and t2o, outflow dialyser blood line at 90 min after the start of the session.

Basal, chemiluminescence activity of whole blood without 1 phorbol 12-myristate 13-acetate (PMA) stimulation; PMA, with stimulation; Delta, subtracting the basal chemiluminescence activity from the PMA-stimulated chemiluminescence activity.
IV iron, leucocyte surface molecules and ROS

every week slightly decreased endogenous peroxide and oxidative stress [26]. Recently, it has been claimed that low iron levels were related to elevated hospitalization and mortality rates in HD patients, and IV iron up to 400 mg/month is associated with improved survival [35].

The incongruity of results from various studies might be due to a variety of factors, including: (i) the preparation of iron (dextran, sucrose or gluconate); (ii) iron dosage (from 10 up to 700 mg); (iii) iron infusion rate (bolus, 30 min or 4.5 h); (iv) the methods for determining infection risk and oxidative stress; (v) baseline iron indices such as transferrin saturation and ferritin levels; and (vi) the inflammation status of patients. Some results pertaining to iron toxicity come from in vitro experiments, in which the iron concentrations examined were much higher than those observed clinically in the serum of patients without iron overload [36,37].

Similar to the present study, the flow cytometric method and detection of ROS by chemiluminescence have generally been used to determine leucocyte functions in HD patients, and flow cytometric analysis has revealed the leucocyte surface molecule alterations associated with membrane bioincompatibility [24,38,39].

In our study, the expression of leucocyte surface molecules was highest in blood samples drawn from the outflow dialysate blood line (outlet HD membrane), demonstrating a high sensitivity to changes in the leucocyte surface molecules secondary to the HD membrane. Although the expression of leucocyte surface molecules was altered due to this membrane effect, these levels did not change from before to after iron sucrose infusion. In addition to low-dose iron administration, the low ferritin levels and transferrin saturation in our study population may be a reason that the expression of leucocyte surface molecules and chemiluminescence activity did not change significantly. It seems plausible that parenteral iron treatment during HD, in the setting of low transferrin saturation and serum ferritin levels, produces less chemiluminescence activity, because these patients have less free iron in circulation. Future studies need to compare patients with low, normal and high ferritin and transferrin saturation levels to confirm this hypothesis.

Our study has some limitations, including its small sample size. We also made no attempt to assess clinically relevant outcomes such as infection and death. The dose of iron sucrose infused, 30 and 100 mg, is certainly is lower than the one which others may use, and higher doses may have produced an observable effect on the expression of leucocyte surface molecule and ROS. Our findings indicate that parenteral administration of 30 or 100 mg iron sucrose during HD does not significantly change the expression of the leucocyte surface molecules CD11b, CD14, CD36 and CD45 in chronic HD patients without iron overload. Furthermore, these infusions do not appear to alter levels of ROS in this patient group. As the expression of these surface molecules and the generation of ROS are highly associated with leucocyte activation, the lack of effect of iron on these variables is an argument for a neutral effect of iron towards leucocyte activation. As the accelerated atherosclerosis and the propensity towards infection of haemodialysis patients are triggered by repetitive and inappropriate leucocyte activation, the results of this study do not point towards a causal effect of iron in these pathologies.

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Conflict of interest statement. None declared.

References


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