Vitamin E Attenuates Oxidative Stress Induced by Intravenous Iron in Patients on Hemodialysis

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Abstract. Intravenous iron application to anemic patients on hemodialysis leads to an “oversaturation” of transferrin. As a result, non-transferrin-bound, redox-active iron might induce lipid peroxidation. To test the hypothesis that vitamin E attenuates lipid peroxidation in patients receiving 100 mg of iron(III) hydroxide sucrose complex intravenously during a hemodialysis session, 22 patients were investigated in a randomized cross-over design, either with or without a single oral dose of 1200 IU of all-rac-α-tocopheryl acetate taken 6 h before the hemodialysis session. Blood was drawn before and 30, 60, 90, 135, and 180 min after the start of the iron infusion, and areas under the curve (AUC0–180 min) of ratios of plasma malondialdehyde (MDA) to cholesterol and plasma total peroxides to cholesterol (two markers of lipid peroxidation) were determined as the outcome variables. At baseline of the session without vitamin E supplementation, plasma α-tocopherol concentrations (27.6 ± 1.8 μmol/L) and ratios of α-tocopherol to ferritin, with the protein ligand apoferritin replaced by sucrose, can be used. Iron in nonionic form as a water-soluble ferric hydroxide complex is well tolerated and, being a large molecular complex of approximately 43 kD (product characterization, Vifor International, St. Gallen, Switzerland), it is not eliminated by the kidney or by hemodialysis treatment.

Normally, iron is safely sequestered in transport proteins such as transferrin and lactoferrin and stored in proteins such as ferritin and hemosiderin. In healthy subjects, transferrin saturation (TSAT), calculated from total serum iron and transferrin concentrations, is ≤45% (7). The doses recommended for iron supplementation in patients on chronic hemodialysis, i.e., 1 to 4 mg iron/kg body weight or 100 to 200 mg iron, lead to an “oversaturation” of transferrin (8,9). Peak serum iron concentrations depend not only on the dose, but also on the duration of the infusion: The higher the dose and the faster the application, the higher the peak iron concentrations (8). However, even infusion lasting 4 h led to an “oversaturation” of transferrin (8). High percentage TSAT was found to be associated with the presence of non-transferrin-bound, potentially redox-active iron, and iron complexed with citrate or acetate, i.e., low molecular weight complexes, was shown to be redox-active (10,11).

Redox-active iron is a potent pro-oxidant (8,10,11). Hydroxyl radical and lipid alkoxyl radical, formed by the Fenton reaction, is a potent pro-oxidant (8,10,11). Hydroxyl radical and lipid alkoxyl radical, formed by the Fenton reaction, were calculated from total serum iron and transferrin concentrations, is ≤45% (7). The doses recommended for iron supplementation in patients on chronic hemodialysis, i.e., 1 to 4 mg iron/kg body weight or 100 to 200 mg iron, lead to an “oversaturation” of transferrin (8,9). Peak serum iron concentrations depend not only on the dose, but also on the duration of the infusion: The higher the dose and the faster the application, the higher the peak iron concentrations (8). However, even infusion lasting 4 h led to an “oversaturation” of transferrin (8). High percentage TSAT was found to be associated with the presence of non-transferrin-bound, potentially redox-active iron, and iron complexed with citrate or acetate, i.e., low molecular weight complexes, was shown to be redox-active (10,11).
reaction, represent the reactive oxygen species that trigger iron-induced lipid peroxidation in the presence of hydrogen peroxide or lipid hydroperoxides. These, like any other oxygen free radicals, can initiate the chain reaction of lipid peroxidation by giving rise to the formation of a lipid radical from a polyunsaturated fatty acid (PUFA). In different in vitro models and in the intact animal, iron has been shown to initiate lipid peroxidation (12–16), the consequences of which are disturbances of tissue and organ functions (17,18). Evidence has accumulated that oxidative modification of LDL is causally involved in atherogenesis (19). Vitamin E is a potent antioxidant that terminates the chain reaction of lipid peroxidation (20). It has been demonstrated to inhibit lipid peroxidation in animals and human subjects (21) and to enhance the resistance of LDL to copper(II) ion-induced oxidation both in healthy subjects (22) and patients with impaired vitamin E status (23).

The question of whether redox-active iron occurs as an immediate response to intravenous iron application with a frequently used therapeutic dose and mode of application and what the effects are on in vivo lipid peroxidation have not been addressed before in patients on chronic hemodialysis. Given that redox-active iron causes lipid peroxidation, it could represent the critical link between oversaturation of transferrin and lipid peroxidation. The “bleomycin assay,” developed by Guttridge et al. (24), allows quantification of bleomycin-detectable iron (BDI), a marker of non-transferrin-bound iron that has the potency of becoming redox-active. In contrast, iron bound to proteins is not detected. Among different indexes for assessing in vivo lipid peroxidation in human subjects, plasma malondialdehyde (MDA) concentrations are most frequently used (25). MDA is an end product of nonenzymatic, oxidative degeneration of PUFA containing three or more conjugated double bonds (26,27).

The purpose of this study was to test the hypothesis that a single oral dose of vitamin E taken before intravenous iron application attenuates lipid peroxidation, which occurs in patients receiving iron(III) hydroxide sucrose complex intravenously at a dose of 100 mg during a hemodialysis session. The effect of vitamin E on lipid peroxidation was studied in a two-period cross-over design, using areas under the curve for the 180-min study period (AUC0–180 min) because the amount of lipid peroxidation products formed and being present during the entire observation period was considered to be pathophysiologically relevant. The study further aimed to explore the time course of variables of iron status and lipid peroxidation in the absence and presence of iron application and vitamin E supplementation, and analyze relations between these variables.

Materials and Methods

Patients

We enrolled 22 patients, 11 men and 11 women, ages 56.6 ± 14.6 yr, who received hemodialysis treatment three times a week, 4.1 ± 0.4 h per session, for 3.5 ± 2.6 yr. All were on rhEPO therapy, which was administered intravenously during hemodialysis 2 to 3 times per week at a weekly dose of 2,000 to 30,000 IU (mean ± SD, 10,136 ± 7,266 IU). None took vitamin E supplements. Inclusion criteria were serum ferritin concentrations <100 μg/L and/or TSAT <20% before the start of this study. The study was approved by the Ethics Committee of the University Hospital and Faculty of Medicine, University of Graz, and informed consent was obtained from the patients.

Study Design

Study Aim A. All patients were investigated twice in a randomized, two-period cross-over design, 7 d apart with and without supplementation of a single oral dose of vitamin E 6 h before the start of the hemodialysis session. All patients received iron(III) hydroxide sucrose complex intravenously on each of the two occasions. The iron infusion was started 30 min after the hemodialysis session had begun and lasted for 20 min.

Study Aim B. To further explore the effect of iron treatment in the absence and presence of vitamin E supplementation, patients were also investigated during a hemodialysis session without iron application, using the same variables as in the other sessions. This session was performed 1 mo after the randomized cross-over trial, assuming that period effects are negligible.

Treatment

Iron. The iron preparation used (Venofer®, Vifor, Inc., St. Gallen, Switzerland) was a solution of iron(III) hydroxide sucrose complex of approximately 43 kD containing 2% iron (20 mg Fe per milliliter of injectable solution, pH 10.5 to 11.0). A very small proportion (0.14%) of the iron was found not to be present as a high molecular weight complex when we separated iron according to molecular weight, using an ultrafiltration membrane (Vivaspin 500, Vivascience Ltd., Binbrook, Lincoln, United Kingdom) that discriminates by molecular weight of 10 kD, and determined iron levels in the ultrafiltrate by the FerroZine method using a Hitachi analyzer. The content of the ampoules (5 ml) was diluted with sterile 0.9% NaCl solution to give a total volume of 50 ml that was administered slowly over 20 min by infusion via the venous line of the extracorporeal circuit, using Pilote C from Fresenius Vial SA (Brezins, France). A dose of 100 mg was chosen because similar doses are frequently applied to hemodialysis patients.

Vitamin E. A single oral dose of 1200 IU of all-rac-α-tocopheryl acetate (Vitamin E “ratiotharm”-Kapseln®, Ratiotharm Arzneimittel Ltd., Vienna, Austria), i.e., the esterified form of the synthetic all-racemic mixture of α-tocopherol, was taken 6 h before the hemodialysis session, along with a meal for proper absorption. This dose was chosen to achieve high plasma vitamin E concentrations, while avoiding possible unwanted gastrointestinal side effects as reported for extremely high doses of vitamin E (28). The time point was chosen on the basis of the results of a previous study, showing maximum plasma α-tocopherol concentrations at 6 to 9 h after ingestion of a similar dose (Winklhofer-Roob et al., unpublished data). This combination should allow for a maximum protective effect of vitamin E at the time when intravenous iron is being applied and throughout the observation period.

Blood Sampling and Preparation

Blood was drawn immediately before (0 min, baseline value) and 30, 60, 90, 135, and 180 min after the start of the iron infusion. For determination of plasma concentrations of MDA, total peroxides, α-tocopherol, and additional antioxidants, blood was drawn on potassium ethylenediaminetetra-acetic acid (EDTA) (1.6 mg EDTA/ml
blood) (S-monovette KE; Sarstedt, Nürnberg, Germany) and lithium heparin (15 IU heparin/ml blood) (S-monovette LH; Sarstedt), respectively, and centrifuged immediately at 2000 × g at 4°C for 10 min. For determination of serum concentrations of cholesterol, triglycerides, albumin, total protein, total iron, transferrin, and BDI serum was obtained by centrifugation at 2000 × g for 15 min. All samples were kept at −80°C until analysis. Samples obtained from an individual patient at the different times of the hemodialysis sessions with and without vitamin E supplementation were analyzed in the same run.

Analytical Methods

Clinical routine methods were used for determination of serum iron (FerroZine method), total protein (Biuret method), cholesterol (CHOD-PAP test kit), and triglyceride concentrations (GPO-PAP test kit), using reagents from Boehringer Mannheim (Mannheim, Germany) and a Hitachi analyzer. Serum transferrin concentrations were measured nephelometrically, using the Behring nephelometer II (Marburg, Germany), and ferritin concentrations were determined with the fluorescence polarization immunoassay from Abbott Laboratories (Santa Clara, CA), using an AxSym analyzer. Plasma concentrations of α- and γ-tocopherol, β-carotene, lycopene, and retinol were determined by HPLC (29). Serum concentrations of BDI were determined by the method of Evans and Halliwell (30), with a minor modification concerning the sample volume (we used 5 μL instead of 15 μL). Plastic tubes were acid-washed and checked for possible iron contamination. Chelex 100 Resin (BioRad Laboratories, Vienna, Austria) was used as a trace metal chelator. Reagents were from Sigma-Aldrich (Vienna, Austria). The coefficient of variation was 2.5% within run and 10.2% from run to run, respectively; the detection limit was 0.5 μmol/L. Plasma MDA concentrations were measured after derivatization by thiobarbituric acid and separation on HPLC (31). The coefficient of variation was 4.4% within run and 6.9% from run to run, respectively, as reported previously (32). For determination of plasma total peroxide concentrations, we used the “Peroxide-activity” assay (POX ACT) from Tatzber KEG (Klosterneuburg, Austria), which is based on the reaction of horseradish peroxidase with plasma peroxides, using tert-amylethylbenzidine as the chromogen substrate. EDTA plasma (10 μL) was incubated with the reaction mixture, consisting of horseradish peroxidase, tetramethylbenzidine, and phosphate buffer, for 20 min, and absorbances were determined photometrically at 450-nm wavelength. Using a hydrogen peroxide (H2O2) standard curve, total peroxide concentrations were calculated and expressed as μmol H2O2 equivalents per liter plasma. The coefficient of variation was 5.3% within run and 9.1% from run to run, respectively.

Statistical Analyses

Study Aim A (Randomized Two-Period Cross-Over Trial). After checking for the appropriateness of parametric analysis, a paired t test was applied for analyzing differences in the AUC0–180 min of ratios of plasma MDA to cholesterol and AUC0–180 min of plasma total peroxides to cholesterol, respectively, between the sessions with iron application in the absence and presence of vitamin E supplementation.

Study Aim B (Exploratory Study). For baseline comparisons of variables of iron, lipid, and antioxidant status, as well as lipid peroxidation in the sessions with iron application in the absence and presence of vitamin E supplementation and in the hemodialysis session without treatment, two-way ANOVA with Tukey multiple comparisons test was applied. Repeated-measures ANOVA with Tukey multiple comparisons test was used to analyze changes for the 180-min observation period within treatment sessions in serum iron and BDI concentrations, TSAT, and ratios of α-tocopherol to cholesterol, MDA to cholesterol, and total peroxides to cholesterol. Linear regression analysis was applied for studying relations between TSAT and BDI concentrations as well as between BDI and MDA concentrations at the 30-min time point, i.e., the time point closest to BDI generation, of the sessions with iron application in the absence and presence of vitamin E supplementation. SigmaStat version 2.0 (Jandel Scientific Software, Erkrath, Germany) was used for all statistical procedures. Data are presented as mean ± SD unless otherwise stated. P < 0.05 was considered significant.

Results

Patient Characteristics

Patient characteristics are shown in Table 1. Compared with published data on healthy subjects, plasma MDA concentrations in untreated patients, i.e., at baseline of the sessions without vitamin E supplementation (1.20 ± 0.28 and 0.87 ± 0.17 μmol/L), were significantly higher than in healthy subjects (0.61 ± 0.22 μmol/L) (P < 0.001) (32), as were plasma total peroxide concentrations in patients (445 ± 285 and 422 ± 358 μmol H2O2 equivalents/L) compared with healthy subjects (315 ± 168 μmol H2O2 equivalent/L) (P < 0.05). Plasma α- and γ-tocopherol concentrations were well within the normal range (22,32), whereas plasma carotenoid (32) and vitamin C concentrations were low (33), and retinol concentrations were elevated (34), all of which are frequent findings in hemodialysis patients.

Study Aim A: Randomized Two-Period Cross-Over Trial

Both the intravenous application of the iron(III) hydroxide sucrose complex and the oral vitamin E supplementation were well tolerated by all patients without any overt side effects. As stated above, AUC0–180 min of ratios of MDA to cholesterol and AUC0–180 min of ratios of total peroxides to cholesterol have been chosen as the end points for testing the hypothesis that vitamin E reduces lipid peroxidation in patients receiving intravenous iron during a hemodialysis session. AUC0–180 min of ratios of MDA to cholesterol with vitamin E supplementation were significantly lower than those without (P = 0.004, paired Student t test was applied for analyzing differences in the AUC0–180 min of ratios of plasma MDA to cholesterol and AUC0–180 min of plasma total peroxides to cholesterol, respectively, between the sessions with iron application in the absence and presence of vitamin E supplementation.

Table 1. Baseline characteristics of 22 patients on chronic hemodialysisa

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>56.6 ± 14.6</th>
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<tbody>
<tr>
<td>Male/female</td>
<td>11/11</td>
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<tr>
<td>Weight (kg)</td>
<td>64.5 ± 13.8</td>
</tr>
<tr>
<td>Ultrafiltration (L/session)</td>
<td>2.05 ± 1.22</td>
</tr>
<tr>
<td>Blood flow (ml/min)</td>
<td>259 ± 40</td>
</tr>
<tr>
<td>Dialysis time (h/session)</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Duration of dialysis (yr)</td>
<td>3.5 ± 2.6</td>
</tr>
<tr>
<td>rhEPO dose (IU/wk)</td>
<td>10,136 ± 7266</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>10.2 ± 2.98</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>67.8 ± 17.2</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>7.31 ± 1.34</td>
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a Data are expressed as mean ± SD. rhEPO, recombinant human erythropoietin.
t test); the mean difference between the two sessions was 10.3 (μmol MDA/mmol cholesterol) × min (Table 2). AUC<sub>0–180 min</sub> of ratios of total peroxides to cholesterol were also significantly smaller with vitamin E supplementation than without (P = 0.002, paired t test); the mean difference was 3.18 (mmol H<sub>2</sub>O<sub>2</sub> equivalents/mmol cholesterol) × min. The response of variables of iron status to the intravenous iron infusion is shown in Figure 1, and the corresponding response of vitamin E status to the vitamin E dose is shown in Figure 2. The time courses of ratios of MDA to cholesterol and total peroxides to cholesterol are presented in Figure 3.

Study Aim B: Exploratory Study

Baseline Comparisons. In Table 3, data obtained for different biochemical variables at baseline of the sessions with iron application in the absence and presence of vitamin E supplementation and of the hemodialysis session without treatment have been compared with each other using two-way ANOVA with Tukey multiple comparisons test. Significant differences were found for plasma α-tocopherol concentrations and α-tocopherol to cholesterol ratios between the session with vitamin E supplementation and those without as an effect of the therapeutic intervention. Significant differences were also found for plasma MDA concentrations and ratios of MDA to cholesterol between the session without treatment and the two other sessions and for serum triglyceride concentrations between the session without treatment and the session with iron administration in the absence of vitamin E supplementation. Additional significant differences were observed for other variables. Some of these could be false-positive significances due to multiple comparisons.

Time Course of Variables of Iron Status. Serum iron concentrations increased rapidly in response to the infusion and peaked within 30 min, as did TSAT (Figure 1). Thirty minutes after the start of the iron infusion, the calculated TSAT was approximately 190%; it decreased thereafter, but still exceeded normal TSAT at 180 min. As depicted in Figure 1, there were no differences between the sessions with and without vitamin E supplementation in the presence of iron infusion, neither in peak values nor in the rate of elimination from serum. In both sessions, BDI was not detectable before the iron infusion, but showed a significant rise in response to intravenous iron (Figure 1). At 30 min, approximately 9% of total serum iron was BDI. Serum BDI concentrations at 30 min were strongly associated with TSAT both in the session without (r = 0.70, P < 0.001) (Figure 4, top panel) and with vitamin E supplementation (r = 0.78, P < 0.001) (Figure 4, bottom panel). In contrast to total serum iron, BDI concentrations did not show rapid elimination from serum from 30 min onward up to the end of the observation period; there were no significant differences between the sessions with and without vitamin E supplementation. No changes in serum iron, TSAT, and BDI concentrations were observed during the 180-min observation period in the session without iron application (Figure 1). BDI concentrations were below the detection limit in all but one patient (this patient’s BDI was 0.7 μmol/L and TSAT was 79% at the 0-min time point).

Effect of Vitamin E Supplementation on Vitamin E Status. When patients had taken the vitamin E supplement 6 h before the start of hemodialysis treatment, plasma α-tocopherol concentrations and ratios of plasma α-tocopherol to cholesterol at baseline, i.e., immediately before the start of the iron infusion, were significantly higher than those in the sessions without vitamin E supplementation (P < 0.001, two-way ANOVA with Tukey multiple comparisons test) (Table 2). In individual patients, plasma α-tocopherol concentrations increased up to approximately 100 μmol/L. Figure 2 shows additional increases in ratios of α-tocopherol to cholesterol during the hemodialysis session with vitamin E supplementation (P = 0.007, repeated-measures ANOVA with Tukey multiple comparisons test), but not during those without.

Plasma Volume Changes during Hemodialysis. One of the therapeutic effects of hemodialysis is ultrafiltration of approximately 2 L per session. As a consequence, plasma volume is reduced. Between baseline and 180 min, serum cholesterol, triglyceride, albumin, and total protein concentrations, as well as plasma α-tocopherol (Figure 2), γ-tocopherol, β-carotene, lycopene, and retinol concentrations (data not shown), increased significantly. These changes in the range of 5 to 15% did not differ between the sessions with and without vitamin E supplementation except for α-tocopherol concentrations, which showed a more pronounced increase in the session with vitamin E supplementation (Figure 2). To correct for plasma volume changes, ratios of MDA to cholesterol and total peroxides to cholesterol have been chosen as the outcome variables.

<table>
<thead>
<tr>
<th>Outcome Variables</th>
<th>Hemodialysis Sessions</th>
<th>Paired t Test</th>
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<tbody>
<tr>
<td></td>
<td>+Fe − Vit. E</td>
<td>+Fe + Vit. E</td>
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<tr>
<td>Plasma MDA:cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0–180 min&lt;/sub&gt; (μmol/mmol) × min</td>
<td>66.6 ± 24.9</td>
<td>56.4 ± 19.2</td>
</tr>
<tr>
<td>mean difference (±SD)</td>
<td>−10.27 (±14.79)</td>
<td></td>
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<tr>
<td>Plasma peroxides:cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0–180 min&lt;/sub&gt; (mmol/mmol) × min</td>
<td>21.0 ± 10.1</td>
<td>17.8 ± 9.12</td>
</tr>
<tr>
<td>mean difference (±SD)</td>
<td>−3.18 (±4.09)</td>
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*Results are mean ± SD of 22 patients. AUC<sub>0–180 min</sub> area under the curve for the 180-min study period.
Time Course of Variables of Lipid Peroxidation. As shown in Figure 3, plasma MDA concentrations and ratios of MDA to cholesterol increased rapidly upon iron application, reached a maximum within 30 min after the infusion had been started, i.e., 10 min after completion of the infusion, and showed approximately linear elimination from plasma at a rate of $0.16 \pm 0.12$ mmol/L per h and $0.05 \pm 0.05$ mmol/mmol per h, respectively, in the session without vitamin E supplementation and $0.19 \pm 0.18$ mmol/L per h and $0.06 \pm 0.06$ mmol/mmol per h, respectively, in the session with vitamin E supplementation. There were no significant differences between the two sessions ($P = 0.48$, paired $t$ test). Ratios of MDA to cholesterol were significantly higher than baseline from 30 to 135 min in both sessions with iron application ($P < 0.001$, repeated-measures ANOVA with Tukey multiple comparisons test). In the session without iron application, there was no increase in ratios of MDA to cholesterol during the observation period. In contrast, a small but statistically significant decrease was noted ($P < 0.001$, repeated-measures ANOVA with Tukey multiple comparisons test) (Figure 3, top panel). Ratios of total peroxides to cholesterol were higher than baseline from 30 to 180 min in both sessions with iron application ($P < 0.001$, repeated-measures ANOVA with Tukey multiple comparisons test).
measures ANOVA with Tukey multiple comparisons test) (Figure 3, bottom panel). As depicted in Figure 3, bottom panel, ratios of peroxides to cholesterol did not decrease during the entire observation period. There was no significant increase in the ratios of peroxides to cholesterol in the absence of iron infusion.

Relation between MDA and BDI. At 30 min (i.e., the time point closest to BDI generation) of the session without vitamin E supplementation, MDA was significantly related to BDI concentrations ($r = 0.53$, $P = 0.01$, MDA = 1.29 + 0.075 × BDI) (Figure 5, top panel). From the positive slope of the regression line, MDA = 1.29 + 0.075 × BDI, it can be seen that BDI concentrations explained the MDA concentrations. The intercept of 1.29 μmol/L, i.e. the MDA concentration that was not explained by BDI, corresponded well with baseline MDA concentrations of 1.20 ± 0.28 μmol/L, indicating that only the increase over baseline values in MDA concentrations was explained by the increase in BDI concentrations over baseline values; the latter were below the detection limit, as shown in Table 3.

A significant dependence of MDA on BDI concentrations was no longer observed in the presence of vitamin E supplementation ($r = 0.35$, $P = 0.11$, MDA = 1.37 + 0.04 × BDI) (Figure 5, bottom panel).

Discussion

The results of this study demonstrate that a single oral dose of 1200 IU of vitamin E taken 6 h before a hemodialysis session with intravenous iron application that leads to high TSAT and the occurrence of BDI significantly reduces in vivo lipid peroxidation. The total amount of plasma peroxides present during the observation period, as estimated by the AUC$_{0-180}$ min, was significantly lower in the presence than in the absence of vitamin E supplementation. Vitamin E supplementation also reduced the MDA load significantly, as estimated by AUC$_{0-180}$ min of ratios of MDA to cholesterol. However, ratios of MDA to cholesterol still increased significantly in response to the iron infusion, indicating that vitamin E did not exert full protection. In contrast to total peroxide concentrations, MDA concentrations were not reduced to those observed in the same patients 1 mo later when they did not receive intravenous iron. Patients had normal vitamin E status before the study, even though they did not take vitamin E supplements, which is a frequent finding in patients on chronic hemodialysis (35,36). When patients had taken the vitamin E supplement, plasma α-tocopherol concentrations were 1.7-fold higher than without supplementation, allowing us to study the effect of “above average” vitamin E status. Ratios of α-tocopherol to cholesterol showed a further increase during the observation period, suggesting that the vitamin E dose could also be taken more than 6 h before the iron infusion for efficient protection. The protective effect of vitamin E observed in this study is in agreement with a study in rats in which iron was injected into brain, and lipid peroxidation was compared with and without administration of vitamin E (37). Intravenous iron application, although associated with marked lipid peroxidation, is essential for hemodialysis patients on rhEPO therapy. Vitamin E supplementation before scheduled iron infusions represents a new approach for attenuating this oxidative stress.

Ten minutes after the end of the 20-min infusion of 100 mg of iron(III) hydroxide sucrose complex, serum iron concentrations were more than sixfold higher than at baseline. Values could have peaked before this time point and at a higher level,
but this could not be investigated because of limitations of the total amount of blood that could be drawn from the patients. Serum iron concentrations decreased between 30 and 180 min, but did not reach half-maximal concentrations. Healthy volunteers who received a similar dose of the same iron preparation by intravenous injection showed peak serum iron concentrations 10 min after the injection that were more than 10-fold higher than baseline values with a terminal half-life of 5.3 h (9). Ten minutes after the end of the intravenous iron infusion, TSAT in the hemodialysis patients was approximately 190%; 2.5 h after the end of the iron infusion it was still approximately 130%. TSAT is a calculated index that is frequently used in clinical practice. High TSAT values indicate that there is excess iron (expressed as moles of iron) that numerically exceeds the available binding sites according to the moles of transferrin present. The calculation of TSAT is based on the fact that 1 mole of transferrin is able to bind 2 moles of iron and the assumption that transferrin binds iron in the first instance and until saturation of all binding sites; only thereafter would iron be loosely bound to other plasma proteins. TSAT, however, does not indicate whether iron is indeed bound to transferrin or any other plasma proteins or, possibly, is still within the high molecular iron-sucrose complex, if the latter is present in a form that is detected by the method used for determination of serum iron. The high TSAT values found in this study upon iron infusion suggest that the capacity of transferrin to bind and transport the available iron has been exceeded over the entire observation period. As a consequence, high TSAT was associated with the occurrence of BDI. While BDI was not detectable in the serum of the study patients before, it increased rapidly upon infusion of the iron(III) hydroxide sucrose complex. The initial increase in serum BDI concentrations paralleled the increase in serum iron concentrations and TSAT. At 30 min, BDI concentrations correlated with total iron concentrations and TSAT. In the session without iron application, a single patient who showed TSAT of 79% but this could not be investigated because of limitations of the total amount of blood that could be drawn from the patients. Serum iron concentrations decreased between 30 and 180 min, but did not reach half-maximal concentrations. Healthy volunteers who received a similar dose of the same iron preparation by intravenous injection showed peak serum iron concentrations 10 min after the injection that were more than 10-fold higher than baseline values with a terminal half-life of 5.3 h (9). Ten minutes after the end of the intravenous iron infusion, TSAT in the hemodialysis patients was approximately 190%; 2.5 h after the end of the iron infusion it was still approximately 130%. TSAT is a calculated index that is frequently used in clinical practice. High TSAT values indicate that there is excess iron (expressed as moles of iron) that numerically exceeds the available binding sites according to the moles of transferrin present. The calculation of TSAT is based on the fact that 1 mole of transferrin is able to bind 2 moles of iron and the assumption that transferrin binds iron in the first instance and until saturation of all binding sites; only thereafter would iron be loosely bound to other plasma proteins. TSAT, however, does not indicate whether iron is indeed bound to transferrin or any other plasma proteins or, possibly, is still within the high molecular iron-sucrose complex, if the latter is present in a form that is detected by the method used for determination of serum iron. The high TSAT values found in this study upon iron infusion suggest that the capacity of transferrin to bind and transport the available iron has been exceeded over the entire observation period. As a consequence, high TSAT was associated with the occurrence of BDI. While BDI was not detectable in the serum of the study patients before, it increased rapidly upon infusion of the iron(III) hydroxide sucrose complex. The initial increase in serum BDI concentrations paralleled the increase in serum iron concentrations and TSAT. At 30 min, BDI concentrations correlated with total iron concentrations and TSAT. In the session without iron application, a single patient who showed TSAT of 79%
had detectable BDI. This is in agreement with other studies in which healthy subjects did not show BDI (37), but patients with idiopathic hemochromatosis (38) and patients with leukemia undergoing chemotherapy did (39). In these studies, BDI became detectable when total serum iron concentrations exceeded 40 μmol/L (38) and BDI concentrations accounted for up to 32% of total iron concentrations (38). Thirty minutes after the start of the iron infusion, BDI concentrations in our patients were approximately 9% of total iron concentrations, as determined by the FerroZine assay. BDI concentrations did not decline between 30 and 180 min, suggesting that BDI was not handled in the same way as was transferrin-bound iron, which is rapidly transported to its destination, the hematopoietic cells of the bone marrow. In rats, non-transferrin-bound iron was avidly taken up by hepatocytes, whereas transferrin-bound iron was poorly absorbed by the liver (40). Hepatic uptake of non-transferrin-bound iron is thought to contribute to liver injury in chronic iron overload. In our patients, no overt signs of acute toxic effects of BDI and increased lipid peroxidation have been observed. However, this does not rule out that free radical-mediated damage, for instance to DNA and proteins, might have occurred.

Baseline plasma MDA concentrations were significantly higher in patients on hemodialysis than in healthy subjects. This was true for all three sessions, although significant differences were observed between the two sessions of the randomized trial and a hemodialysis session without treatment 1 mo later. Significant within-subject variation in plasma MDA concentrations over 3-mo periods has been reported in a different clinical setting, where patients showed simultaneous changes in both vitamin C and inflammatory status (33). Changes in these and additional variables did not occur in the

Figure 4. Regression of serum BDI concentrations on TSAT at 30 min in the sessions with iron application in the absence (top panel) and presence of vitamin E supplementation (bottom panel) for 22 patients.

Figure 5. Regression of plasma MDA on BDI concentrations at 30 min in the sessions with iron application in the absence (top panel) and presence (bottom panel) of vitamin E supplementation for 22 patients. n.s., no significant dependence of MDA on BDI.
present study, but it cannot be ruled out that other as yet unknown variables may have contributed to these differences. That plasma MDA concentrations are increased in patients on hemodialysis has been observed before (35,41) and can be explained by oxidative stress due to uremia (42), hemodialysis treatment (43), and impaired antioxidant status (41,44). In a small group of hemodialysis patients who received iron on a regular basis, both superoxide dismutase activities and PUFA concentrations in erythrocytes were lower, but MDA concentrations did not differ from those in patients not receiving iron or in healthy subjects (44). In our patients, plasma concentrations of vitamin C, a potent antioxidant (45), were extremely low (20.5 ± 17.3 μmol/L) compared with healthy subjects (69.5 ± 16.6 μmol/L) (33). This is a common finding in patients on hemodialysis not taking vitamin C supplements to compensate for losses of vitamin C during hemodialysis treatment (46). Low plasma vitamin C concentrations may have contributed to increased lipid peroxidation in the study patients. Because intake of high doses of vitamin C has been found to be associated with hyperoxalemia (47), vitamin C supplements had not been part of the routine management in our hemodialysis center.

In response to intravenous application of the iron(III) hydroxide sucrose preparation, plasma MDA concentrations increased significantly. MDA concentrations at 30 min were 1.5 times those at baseline. In the absence of vitamin E supplementation, BDI concentrations at 30 min explained the increase over baseline in MDA concentrations. The intercept at the y-axis, 1.29 μmol/L MDA, was in agreement with mean (± SD) MDA concentrations of 1.20 (±0.28) μmol/L at baseline, when BDI was not detectable. This suggests a pathophysiological relationship between redox-active iron and lipid peroxidation. In the session with vitamin E supplementation, plasma MDA concentrations were not significantly related to BDI concentrations, perhaps as a result of the effect of vitamin E on MDA concentrations.

MDA concentrations did not increase in the absence of iron application but showed a significant decrease at an approximate rate of 0.03 (μmol/L) per h from 0 to 180 min. Because the same was true for ratios of MDA to cholesterol, this decrease was considered to not result from plasma volume changes but rather from elimination of MDA by hemodialysis treatment. When patients had received iron, MDA elimination from plasma was linear from 30 min onward at an approximate rate of 0.16 (μmol/L) per h. Elimination may have started even before the 30-min time point, but this has not been investigated. Free MDA, due to its relatively low molecular mass, can be removed by hemodialysis. We recently detected small quantities in the dialysate (Roob et al., unpublished data). Although an end product of lipid peroxidation, MDA is further metabolized. In rats, approximately 70% of an oral dose of 14C-labeled MDA was expired as 14CO2 within 12 h (48). In rodents, MDA became rapidly distributed throughout all major organs, and acid-labile MDA metabolites, with very little free MDA, were excreted in urine (49). In rats receiving 100 mg of iron dextran intravenously, MDA concentrations increased in plasma, spleen, and heart 3 h after the injection. The MDA concentrations correlated with the respective iron concentrations and persisted in liver and spleen until day 28 (14). MDA is not only an indicator of in vivo lipid peroxidation, but has cytotoxic properties and, for instance, the ability to derivatize apoB-100, the protein moiety of LDL, thereby producing chemical aducts that are potent immunogens considered to play a role in atherogenesis (19).

While ratios of plasma total peroxides to cholesterol did not change in the absence of iron(III) hydroxide sucrose infusion, they increased rapidly upon iron application, reached a plateau at 30 min, and did not decline during the entire observation period. With the assay used, plasma peroxides of the general chemical structure ROOH have been detected. Because their chemical nature is not further defined, it is difficult to speculate about their metabolism. However, the time profiles indicate that these peroxides were not efficiently eliminated from plasma. Persistence of elevated peroxide concentrations, in the presence of transition-metal ions, might facilitate the generation of highly reactive oxygen species from ROOH. This underscores the possible benefit of reduction of plasma peroxide concentrations by vitamin E.

In summary, a single oral dose of 1200 IU of vitamin E efficiently reduces oxidative stress in patients receiving 100 mg of iron(III) hydroxide sucrose intravenously during a hemodialysis session, when taken 6 before the hemodialysis session. This beneficial effect was demonstrated in patients on hemodialysis with normal vitamin E status before supplementation, indicating a protective effect of high plasma vitamin E concentrations. Close correlations between calculated TSAT and BDI concentrations on one hand and BDI and MDA concentrations on the other suggest that lower doses of the iron(III) hydroxide sucrose complex may limit the extent of BDI and subsequent MDA formation in the first instance. Indeed, a recent abstract reported BDI only in two of 15 patients receiving doses ≤50 mg (50). It remains to be investigated whether iron doses lower than those applied in the present study in combination with vitamin E supplements could fully prevent iron-induced lipid peroxidation. Also, correction of impaired vitamin C status may potentiate the vitamin E effect through regeneration of vitamin E from the vitamin E radical formed during the antioxidant action of vitamin E. The effect on lipid peroxidation of intravenous iron application not in association with or at a later time point of a hemodialysis session could prove to be different owing to possible removal of lipid peroxidation products by hemodialysis. Finally, different iron preparations may behave differently, as suggested by higher rates of adverse events reported for instance for the iron dextran (6). The final goal of antioxidant supplementation in this clinical setting is to prevent long-term side effects associated with increased lipid peroxidation as a result of repeated exposure to iron-induced oxidative stress.

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References


