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Iron-induced oxidative stress in haemodialysis patients: a pilot study on the impact of diabetes

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Abstract
Background: Administration of intravenous iron preparations in haemodialysis patients may lead to the appearance of non-transferrin bound iron which can catalyse oxidative damage. We investigated this hypothesis by monitoring the oxidative stress of haemodialysis patients and the impact of iron and diabetes mellitus herein.
Materials and methods: Baseline values of serum iron and related proteins, transferrin glycation, non-transferrin bound iron, antioxidant capacity and lipid peroxidation (malondialdehyde) of 11 haemodialysis patients (six non-diabetic and five type 2 diabetes) were compared to those of non-haemodialysis control subjects (non-diabetic and type 2 diabetes). Changes in these parameters were monitored during haemodialysis before and after iron administration.
Results: Baseline values of malondialdehyde correlated with ferritin concentration ($r = 0.664$, $P = 0.036$) and were elevated to the same extent in non-diabetic and diabetic haemodialysis patients (median of 1.09 compared to 0.60 μmol/l in control persons, $P < 0.02$). After iron infusion, transferrin saturation increased more markedly in non-diabetic subjects from 28% to 185% vs. from 33% to 101% in diabetic patients ($P = 0.008$). This increase was accompanied by the appearance of non-transferrin bound iron (5.91 ± 1.33 μmol/l), a loss in plasma iron-binding antioxidant capacity and a further increase in malondialdehyde which was more pronounced in diabetic patients (from 0.93 ± 0.30 μmol/l to 2.21 ± 0.69 μmol/l vs. from 1.21 ± 0.42 μmol/l to 1.86 ± 0.56 μmol/l in the non-diabetic subjects, $P = 0.046$).
Conclusions: In haemodialysis patients, higher lipid peroxidation is determined by higher body iron stores. The increase induced by iron infusion is accompanied by a loss in iron-binding antioxidant capacity and is more pronounced in diabetes mellitus.

Keywords Haemodialysis · Iron · Oxidative stress · Diabetes

Abbreviations

ANOVA	Analysis of variance
CV	Coefficient of variation

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MDA	Malondialdehyde
NTBI	Non-transferrin bound iron
TAC-PI and TAC-TE	Total antioxidant capacity-percent inhibition and -Trolox equivalents
TIBC	Total iron-binding capacity
Tf	Transferrin

Introduction

Patients with end-stage renal disease are chronically exposed to oxidative stress which is mainly caused by uraemic toxicity and the activation of neutrophils induced by their interaction with dialysis membranes (Himmelfarb et al. 1991; Luciak and Trznadel 1991). This is evidenced by the increased concentration of lipid peroxidation products (Dasgupta et al. 1992; Loughrey et al. 1994; Peuchant et al. 1994) and decreased levels of antioxidants such as glutathione peroxidase (Lim et al. 1999), ascorbate (Loughrey et al. 1994) and α-tocopherol (Peuchant et al. 1994) in these patients. In addition, they are dependent on intravenous iron supplementation to maintain adequate iron stores for the effective treatment of renal disease anaemia with recombinant human erythropoietin (Sunder-Plassmann and Horl 1995; Macdougall et al. 1996). Although current iron formulations are highly effective and relatively free of short-term side-effects, little information is available on the safety limits of iron administration (Lim et al. 1999). Iron infusion may cause transient but repeated overload of the iron-binding capacity of the plasma transport protein transferrin (Tf) which eventually leads to the appearance of unbound plasma iron (NTBI) (Sunder-Plassmann and Horl 1995). NTBI is often considered as synonymous to free iron which is a powerful pro-oxidant, capable of generating very reactive hydroxyl radicals (Fenton reaction) that initiate chain reactions of lipid peroxidation (Gutteridge et al. 1982).

The resulting oxidative damage may be aggravated by the diabetic condition which is often present in patients with chronic renal failure. Hyperglycaemia-induced oxidative stress involves glycation of proteins in a process that may alter the protein function. Recently we demonstrated that in vitro glycation of Tf resulted in a decreased iron-binding capacity that was associated with an impairment of the antioxidant function that protects against iron-induced lipid peroxidation (Van Campenhout et al. 2003). This impairment was also observed in vivo (Van Campenhout et al. 2006a). We developed a method to measure in vivo Tf glycation and found this to be higher in diabetic patients (Van Campenhout et al. 2006b). Glycation is also increased in haemodialysis patients and it has been established that the concentration of advanced glycation end-products increases up to 10-fold during uraemia (Henle et al. 1999). This is the combined result of enhanced generation due to oxidative stress on the one hand (Miyata et al. 1997a, b) and the diminished or absent elimination of the glycated products by the kidneys on the other hand (Koschinsky et al. 1997). Moreover, these patients are generally older and glycated products are known to accumulate naturally over a life time (Papanastasiou et al. 1994).

In this study we describe the impact of diabetes and renal insufficiency on oxidative stress status of haemodialysis patients compared to healthy controls and diabetic patients. To investigate the hypothesis that the variable presence of free iron and related oxidative stress may be aggravated by the diabetic condition, we compared in a pilot study iron parameters and oxidative stress status before and after iron administration in a small group of non-diabetic and diabetic patients during haemodialysis.

Subjects and methods

Patients and study design

Eleven patients at the University Hospital Antwerp (six non-diabetic and five insulin-treated type 2 diabetic subjects) on regular haemodialysis treatment of three sessions a week, 4 h per session, agreed to take part in this study. All of the patients were treated with recombinant human erythropoietin (Eprex®, Janssen Cilag) and the majority, except when serum ferritin >800 μg/l, received intravenous iron supplementation in the form of 100 mg dissolved iron saccharate (Venofer®, Byk Gulden Lomberg Chemische Fabrik GmbH) administered in the last 30 min of the dialysis session. Creatinine clearance was estimated using the Cockroft-Gault formula: [(140-age)× weight × gender factor]/[72 × serum creatinine (mg/*dl*)].

Baseline values from haemodialysis patients were compared to those of non-haemodialysis control

subjects without (56/44 males/females, median age of 46 years) or with type 2 diabetes (33/34 males/females, median age of 64 years).

To determine the effect of haemodialysis and iron-supplementation, blood was collected before the dialysis session (measure 1), just before iron administration at 30 min from the end of the session (measure 2) and immediately after the iron infusion at the end of the session (measure 3).

The ethical commission of the hospital approved the protocol (ID 3/25/91) and written informed consent was obtained from all participants.

Measurements of metabolic control and iron status

HbA_{1C} was measured by ion-exchange high performance liquid chromatography (ADAMS HA 8160 Menarini), (coefficient of variation, CV 0.7%). Glucose (CV 0.6%), serum iron (CV 3.0%) and total iron-binding capacity (TIBC) (CV 2.8%) were measured colorimetrically on the day of sampling (Vitros 950 AT, Ortho Clinical Diagnostic Inc.). Tf saturation was calculated by the formula: serum iron/TIBC. Levels of Tf (CV 2.8%), soluble transferrin receptor (CV 4.6%), ferritin (CV 2.2%), haemopexin (CV 6.0%), haptoglobin (CV 3.6%) and high sensitive C-reactive protein (CV 3.9%) were assayed using nephelometric kits (BNII Nephelometer, Dade Behring).

By measuring fructosamine concentration using the nitroblue tetrazolium assay (Johnson et al. 1983) adapted for 96 well-plates, glycation was determined in total serum proteins (CV 3.2%) and in Tf isolated from serum by immunocomplexation (CV 3.7%). Results are expressed as µmol fructosamine per g albumin and g Tf, respectively.

NTBI was measured by a competitive fluorescence-based assay according to Breuer and Cabantchik (2001). Briefly, in a 96-well-plate, 10 µl of serum was mixed with a solution containing 0.6 µmol/l fluorescein-labelled apotransferrin (kindly donated by EH Jansen, RIVM Utrecht, The Netherlands) which undergoes fluorescence quenching upon binding to iron. The fluorescence was determined at excitation/emission wavelengths of 485/538 nm after incubation in the dark for 1 h at room temperature (CV < 3%).

Measurements of oxidative stress status

Iron-binding antioxidant capacity of plasma was evaluated in a liposomal model containing iron and ascorbate to induce lipid peroxidation measured as thiobarbituric reactive substances (Gutteridge and Quinlan 1992). It is expressed as the percent inhibition of the production of these substances relative to the control value containing no plasma (CV 2.8%).

Total antioxidant capacity was evaluated by measuring the inhibition of chemiluminescence after the addition of plasma to a reaction mixture containing 75 µmol/l luminol and peroxyl radicals liberated by the thermal decomposition of 20 mmol/l 2,2-azo-bis(2-amidinopropane) hydrochloride (Lissi et al. 1995). Two antioxidant effects are distinguished: (1) the duration of the lagtime before appearance of the chemiluminescence signal was expressed as trolox-equivalents (TAC-TE), (2) the percent inhibition of the maximum chemiluminescence peak (TAC-PI).

Plasma α-tocopherol and retinol concentration were determined by high-performance liquid chromatography (Shimadzu) with a reversed-phase C18 column LiChrospher RP C18 (Alltech) with 100% methanol mobile phase and detection at 292 and 325 nm, respectively (CV 4.8% and 4.1%) (Caye-Vaugien et al. 1990). Plasma ascorbate was also measured by reverse-phase high-performance liquid chromatography with 2 mmol/l KCl mobile phase and electrochemical detection at 1,000 mV (Washko et al. 1989) (CV 6%). Reduced glutathione in whole blood and protein thiols in heparin plasma were measured by a colorimetric method using Ellman's reagent (Beutler 1975; Hall et al. 1982) (CVs 7% and 6%).

Plasma malondialdehyde (MDA) was analysed by a reverse-phase high-performance liquid chromatography with methanol/KH_2PO_4 10 mmol/l (40/60 v/v) as mobile phase and detection at 532 nm (Nielsen et al. 1997) (CV 9%).

Statistical analysis

Results were expressed as median [range] or as mean ± SD. Data were analysed using SPSS Version 11.0. Two-tailed *P*-values <0.05 were considered as significant. Baseline values between haemodialysis- and non-haemodialysis patients were compared by one-sample Student *t*-test and differences according

to the diabetic state were investigated by the Mann–Whitney test. Changes during haemodialysis and after iron supplementation were analysed by repeated measures analysis of variance (ANOVA) and confirmed using the non-parametric Friedman test. Both the changes during the course of the haemodialysis (within-subject comparison in the whole group) and the differences in these time-related changes between the diabetic and non-diabetic groups (between-group comparison) were analysed. Analysis of contrasts was applied to identify the significance of the change when compared to the value in the preceding measurement. In this way we can identify the effect of haemodialysis per se (measure 2 vs. 1) and that of iron administration (measure 3 vs. 2). Multiple linear regression and Pearson correlations were applied to identify the relationship between the various parameters. Ferritin values were log-transformed before use in the statistical tests.

Results

Influence of haemodialysis and diabetes on glycation, iron parameters and oxidative stress status

The clinical characteristics of the study subjects are shown in Table 1.

Baseline values were compared between haemodialysis patients and control subjects, taking into account the diabetic state (Table 2). HbA_{1C} was higher in diabetic patients and was significantly lower in non-diabetic haemodialysis patients when compared to non-diabetic control subjects ($P = 0.004$). In contrast, serum protein glycation was higher in all haemodialysis patients, this difference being most pronounced in the diabetic subjects ($P < 0.02$ for the effect of haemodialysis and $P = 0.035$ for the differences according to the diabetic state in haemodialysis patients). Tf glycation was higher in diabetic compared to

Table 1 Clinical characteristics of non-diabetic and diabetic haemodialysis patients

	Non-diabetic ($n = 6$)	Diabetic ($n = 5$)
Male/female	4/2	4/1
Age (years)	78 [34–82]	68 [57–73]
Body mass index (kg/m^2)	24.4 [21.2–26.6]	26.9 [23.2–32.7]
Systolic blood pressure (mmHg)	14 [12–16]	16 [15–17]
Diastolic blood pressure (mmHg)	7 [5–9]	8 [7–9]
Duration of diabetes (years)	Not relevant	20 [5–41]
Time spent on dialysis (months)	16 [8–41]	40 [12–240]
Recombinant erythropoietin dose (U/kg/week)	101 [73–300]	150 [53–247]
Intravenous iron supplementation (yes/no)	5/1	4/1
Serum total cholesterol (mmol/l)	4.19 [2.64–5.02]	4.58 [3.49–4.91]
Serum HDL cholesterol (mmol/l)	1.28 [0.10–1.66]	0.83 [0.83–1.09]
Serum triacylglycerol (mmol/l)	1.27 [0.69–2.00]	2.19 [1.40–2.30]
C-reactive protein (mg/dl)	1.00 [0.50–2.50]	0.50 [0.50–0.60]
Plasma ureum (mg/dl)	161 [137–185]	183 [98–195]
Plasma creatinine (μmol/l)	911 [769–1299]	902 [804–1043]
Creatinine clearance (ml/min)	5.48 [4.85–6.25]	7.92 [7.42–10.27]
Renal Pathology Nephro-angiosclerosis	3	
Polycystic nephropathy	1	1
Renal stenosis	1	
Glomerulonephritis	1	
Diabetic nephropathy		4

Values are expressed as median [minimum–maximum]. Routine biochemistry was conducted in non-fasting blood samples just prior to the dialysis session

Table 2 Baseline values for iron parameters and oxidative stress status according to haemodialysis and diabetes

	Haemodialysis patients		*P*-value	Non-haemodialysis controls	
	Non-diabetic (n = 6)	Diabetic (n = 5)		Non-diabetic (n = 100)	Diabetic (n = 67)
Plasma glucose (mmol/l)[b]	6.0 [5.9–8.0][a]	5.3 [3.8–7.9]	NS	4.7 [3.7–6.4]	7.0 [3.1–19.2]
HbA_{1C} (%)	5.0 [4.6–5.1][a]	7.2 [6.6–9.2]	0.006	5.3 [4.5–6.0]	7.0 [5.5–11.1]
Serum protein glycation (μmol/g albumin)	9.20 [7.35–10.83][a]	10.53 [8.46 –14.21][a]	0.035	5.55 [3.86–7.45]	6.34 [4.75– 10.22]
Tf glycation (μmol/g Tf)	0.77 [0.35–2.85]	2.16 [1.18–3.55][a]	0.035	0.67 [Not detectable–3.43]	1.01 [Not detectable–3.50]
Serum iron (μmol/l)	13 [8–21]	10 [8–23]	NS	18 [4–41]	16 [6–31]
TIBC (μmol/l)	49 [46 –63][a]	52 [44–58][a]	NS	68 [51–98]	69 [18–92]
Tf saturation (%)	23 [17–42]	20 [14–53]	NS	25 [5–72]	21 [8–130]
Tf concentration (g/l)	1.53 [1.15–2.23][a]	1.49 [1.32–1.99][a]	NS	2.65 [1.80–3.90]	2.67 [1.85–3.74]
NTBI (μmol/l)	−0.42 [−2.33–0.69][a]	−0.66 [−3.05–1.07]	NS	1.38 [−3.68–6.20]	0.61 [−4.15–5.20]
Ferritin (μg/l)	817 [423–1060][a]	770 [253–2010][a]	NS	102 [10–555]	112 [13–900]
Soluble transferrin receptor (mg/l)	1.31 [0.98–1.69]	1.47 [1.11–2.29]	NS	1.31 [0.92–2.15]	1.43 [0.96–2.55]
Haptoglobin (g/l)	1.44 [0.68–2.73]	1.62 [0.99–1.75]	NS	0.98 [0.28–2.34]	1.53 [0.75–2.70]
Haemopexin (g/l)	0.73 [0.65–0.87][a]	0.63 [0.30–1.10][a]	NS	0.88 [0.59–1.45]	1.14 [0.81–2.50]
Iron-binding antioxidant capacity (% inhibition)	91 [81–93]	88 [83–92]	NS	88 [70–98]	84 [73–94]
Plasma TAC-PI (% inhibition)	89 [86–91][a]	89 [87–91][a]	NS	76.09 [62–90]	80 [61–89]
Plasma TAC-TE (μmol/l)	337 [238–411][a]	286 [238–406][a]	NS	193 [120–281]	207 [69–362]
Plasma uric acid (μmol/l)	422 [345–500][a]	440 [315–482][a]	NS	291 [113–470]	327 [416–743]
Serum bilirubin (μmol/l)	6.0 [5.1–12.0][a]	6.8 [5.1–8.6]	NS	8.6 [3.4 –56.4]	6.8 [1.7–20.5]
Serum albumin (g/l)	34 [29 –38][a]	38 [37–39][a]	0.021	44 [33–51]	43 [36–50]
Plasma retinol (μmol/l)	7.19 [4.64–10.02]	9.11 [4.47–12.11]	NS	4.99 [0.94–16.06]	5.03 [2.23–12.36]
Plasma α-tocopherol (μmol/l/l)	25.3 [19.5–51.5]	33.8 [16.5–39.8]	NS	32.9 [14.5–72.7]	33.9 [17.1–83.0]
(μmol/mmol total lipid)	5.0 [3.3–8.4]	5.3 [3.4–5.5]	NS	5.4 [2.0–14.2]	4.7 [2.3–8.3]
Plasma ascorbate (μmol/l)	Not detectable (<10 μmol/l)			61 [4–115]	44 [5–92]
Plasma MDA (μmol/l)	1.17 [0.87–1.82][a]	0.93 [0.86–1.49][a]	NS	0.57 [0.21–1.22]	0.60 [0.24 –1.28]
(μmol/mmol total lipid)	0.21 [0.15–0.44][a]	0.18 [0.13–0.30][a]	NS	0.08 [0.04 –0.20]	0.08 [0.03–0.18]
Plasma protein thiols (μmol/g protein)	3.66 [1.99–4.52][a]	3.72 [2.75–4.48]	NS	4.81 [2.54–7.23]	3.58 [1.12–6.79]
Blood glutathione (μmol/g haemoglobin)	5.88 [4.93–6.62]	5.49 [4.89–7.64]	NS	5.56 [2.93–8.56]	5.49 [3.47–8.02]

NS = not significant

Results are expressed as median [minimum–maximum]. *P*-value denotes the difference between non-diabetic and diabetic haemodialysis patients and was calculated by Mann–Whitney comparison

[a] Denotes $P < 0.05$ for the comparison between each haemodialysis and non-haemodialysis study group (with or without diabetes) using the one-sample *t*-test

[b] Non-fasting in haemodialysis- and fasting in non-haemodialysis subjects

non-diabetic haemodialysis patients ($P = 0.035$). An additional independent effect of haemodialysis could be found in the diabetic group resulting in higher Tf glycation (2.16 μmol/g Tf) compared to the diabetic non-haemodialysis group (1.01 μmol/g Tf, $P = 0.05$).

Among the non-diabetic subjects NTBI was lower in the haemodialysis group ($P = 0.01$). None of the iron parameters were different according to the diabetic state in haemodialysis patients.

Concerning oxidative stress status, similar levels in the haemodialysis and control population were found for iron-binding antioxidant capacity, plasma α-tocopherol, retinol and glutathione concentrations. In contrast, total antioxidant capacity expressed as TAC-PI and TAC-TE was higher in the haemodialysis patients ($P < 0.03$). Serum albumin was lower ($P \leq 0.001$) and uric acid ($P \leq 0.05$) was higher in both non-diabetic as well as in diabetic haemodialysis patients. In contrast, plasma protein thiols were only decreased in non-diabetic haemodialysis patients ($P = 0.014$).

Most remarkable was the absence of detectable (>10 μmol/l) ascorbate levels in the plasma of both diabetic and non-diabetic haemodialysis patients. Baseline levels of MDA as a measure of lipid peroxidation were significantly higher in both diabetic and non-diabetic haemodialysis patients (median of 1.09 compared to 0.60 in non-haemodialysis subjects, $P < 0.02$). Multiple regression containing variables of iron status (serum iron, Tf, Tf glycation, NTBI, ferritin) and antioxidants (Iron-binding- and total antioxidant capacity, bilirubin, uric acid, retinol, α-tocopherol, protein thiol content and glutathione) identified ferritin as the main determinant of MDA, explaining almost half of its variance (44%) and displaying a strong correlation with it ($r = 0.664$, $P = 0.036$, Fig. 1). This result was not influenced by introducing the factor diabetes indicating that ferritin is a diabetes-independent predictor of MDA in haemodialysis.

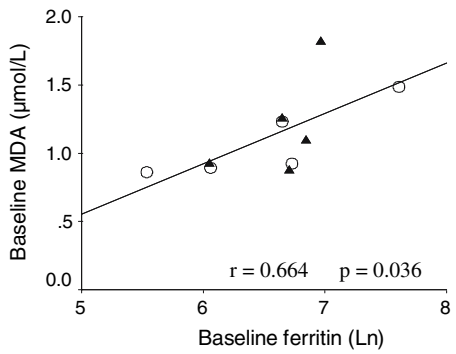

Fig. 1 Correlation between baseline serum ferritin levels and MDA in non-diabetic (▲) and diabetic (O) haemodialysis patients

Evolution of iron parameters and oxidative stress status during haemodialysis and after iron supplementation

Table 3 summarises the changes in iron parameters and oxidative stress status during the haemodialysis session (measure 2) and after iron infusion (measure 3).

During dialysis, TIBC, Tf saturation, NTBI, ferritin, soluble transferrin receptor, iron-binding antioxidant capacity, MDA and glutathione remained unchanged (measure 2 compared to measure 1 was not statistically different). Total serum protein glycation ($P = 0.018$ versus baseline) as well as Tf glycation ($P = 0.05$) decreased significantly during haemodialysis. The decrease in serum protein glycation was only seen in non-diabetic patients (from 9.05 ± 1.28 to 7.78 ± 0.83 vs. from 10.86 ± 2.28 μmol/g to 10.23 ± 1.17 μmol/g albumin in diabetic patients, $P = 0.012$ for the between-group difference). In contrast, the decrease in Tf glycation was less pronounced in non-diabetic patients (from 1.04 ± 0.90 to 0.70 ± 0.42 vs. 2.24 ± 0.98 μmol/g to 0.32 ± 1.51 μmol/g Tf in diabetic subjects, $P = 0.029$). TAC-TE and uric acid decreased ($P < 0.0005$) during the dialysis session and also to a lesser extent TAC-PI ($P = 0.009$). In contrast, protein concentrations of Tf ($P < 0.0005$), haptoglobin ($P = 0.004$), haemopexin ($P = 0.004$) and albumin ($P < 0.0005$) as well as serum iron ($P = 0.036$), plasma retinol ($P = 0.001$), α-tocopherol ($P = 0.012$) and protein thiol content ($P < 0.0005$) increased. In this limited study population, the changes were independent of the diabetic state except for plasma retinol and α-tocopherol in which the increases were only observed in the diabetic patients ($P = 0.005$ and $P = 0.034$, respectively for the differences according to the diabetic state).

After an additional half hour of haemodialysis during which iron was administered intravenously, measure 3 was carried out. Two patients were excluded from further statistical analysis because they did not receive iron. Serum protein glycation

Table 3 Changes in glycation, iron parameters and oxidative stress status during haemodialysis and after iron infusion

	Baseline Measure 1 (n = 11)	After haemodialysis Measure 2 (n = 11)	After iron infusion Measure 3 (n = 9)	P-value RMANOVA
Protein glycation (μmol/g albumin)	9.87 ± 1.95	8.89 ± 1.59[a,*]	8.63 ± 1.17[a,b]	0.040*
Tf glycation (μmol/g Tf)	1.59 ± 1.09	0.53 ± 1.02[a,*]	1.49 ± 1.1	0.040
Serum iron (μmol/l)	13 ± 6	16 ± 8[a]	158 ± 49[a,b]	0.001
TIBC (μmol/l)	50.9 ± 5.73	59.8 ± 13.15	108 ± 30[a,b]	0.007
Tf saturation (%)	26 ± 13	28 ± 17	154 ± 56[a,b,*]	0.001
Tf concentration (g/l)	1.60 ± 3.48	2.00 ± 0.37[a]	1.94 ± 0.51	<0.0005
NTBI (μmol/l)	−0.80 ± 1.32	−0.49 ± 2.11	5.91 ± 1.33[a,b]	<0.0005
Ferritin (μg/l)	830 ± 486	816 ± 295	854 ± 309	NS
Soluble transferrin receptor (mg/l)	1.41 ± 0.38	1.64 ± 0.48	1.71 ± 0.56	NS
Haptoglobin (g/l)	1.48 ± 0.55	1.78 ± 0.71[a]	1.80 ± 0.84[a]	0.020
Haemopexin (g/l)	0.72 ± 0.20	0.87 ± 0.16[a]	0.91 ± 0.17	0.030
Iron-binding antioxidant capacity (% inhibition)	88 ± 4	86 ± 10	−2 ± 69[a,b]	0.035
Plasma TAC-PI (% inhibition)	89 ± 2	87 ± 2[a]	88 ± 2	0.095
Plasma TAC-TE (μmol/l)	319 ± 65	119 ± 20[a]	115 ± 28[a,b]	<0.0005
Plasma uric acid (μmol/l)	419 ± 56	114 ± 24[a]	103 ± 27[a,b]	<0.0005
Serum albumin (g/l)	36 ± 3	42 ± 4[a]	42 ± 5[a]	0.001
Plasma retinol (μmol/l)	8.48 ± 2.72	10.96 ± 1.22[a,*]	11.35 ± 4.12	NS
Plasma α-tocopherol (μmol/l)	32.3 ± 11.1	42.9 ± 9.1[a,*]	45.4 ± 13.6	NS
Plasma MDA (μmol/l)	1.18 ± 0.33	1.09 ± 0.36	2.02 ± 0.61[a,b,*]	0.002
Plasma thiols (μmol/g protein)	3.65 ± 0.76	4.87 ± 0.69[a]	4.88 ± 0.72[a]	<0.0005
Blood glutathione (μmol/g haemoglobin)	5.82 ± 0.86	6.04 ± 1.21	5.70 ± 0.60	NS

Results are expressed as mean ± SD of values taken before (measure 1), 30 min from the end of the haemodialysis session (measure 2) and after iron supplementation (measure 3). In measure 3, the results from 2 male patients (1 non-diabetic/1 diabetic) who did not receive iron supplementation were excluded. P-value was calculated by repeated measures (RM) ANOVA and confirmed using the non-parametric Friedman test

[a] Denotes $P < 0.05$ when contrasted to the baseline value

[b] When contrasted between measurement 2 and 3 and * when these changes were different according to the diabetic state, $P < 0.05$

decreased further (P = 0.021) but not Tf glycation. Iron administration resulted in significantly higher serum iron and thus Tf saturation ($P < 0.0005$), this increase being most pronounced in the non-diabetic subgroup (from 28 ± 17% to 185 ± 44% vs. from 33 ± 21% to 101 ± 22% in the diabetic subjects patients, P = 0.008 for the between-group comparison) (Fig. 2A). Although Tf concentration remained constant, TIBC doubled (P = 0.001). Despite this extra binding capacity, NTBI increased dramatically (from not detectable levels to values ranging from 3 μmol/l to 8 μmol/l, $P < 0.0005$) (Fig. 2B). This was accompanied by a substantial loss in iron-binding antioxidant capacity (P = 0.007). In some patients iron-binding antioxidant capacity even reached negative values indicating pro-oxidant properties rather than antioxidant properties in iron-infused plasma (Fig. 2C). All of these changes were similar in diabetic and non-diabetic haemodialysis patients.

After iron infusion there was no significant change in serum proteins and antioxidants except for a further slight decrease in TAC-TE and uric acid. In contrast, MDA concentrations increased to even double ($P < 0.0005$, power = 1.0), this effect being most pronounced in the diabetic subgroup (from 0.93 ± 0.30 μmol/l to 2.21 ± 0.69 μmol/l vs. from 1.21 ± 0.42 to 1.86 ± 0.56 in the non-diabetic subjects, P = 0.046, power = 0.55). There was no change in MDA in the patients not receiving iron supplementation (patient 6 and 11) (Fig. 2D). In contrast to the baseline, levels of MDA after iron supplementation were no longer determined by ferritin concentration at the end of the haemodialysis session.

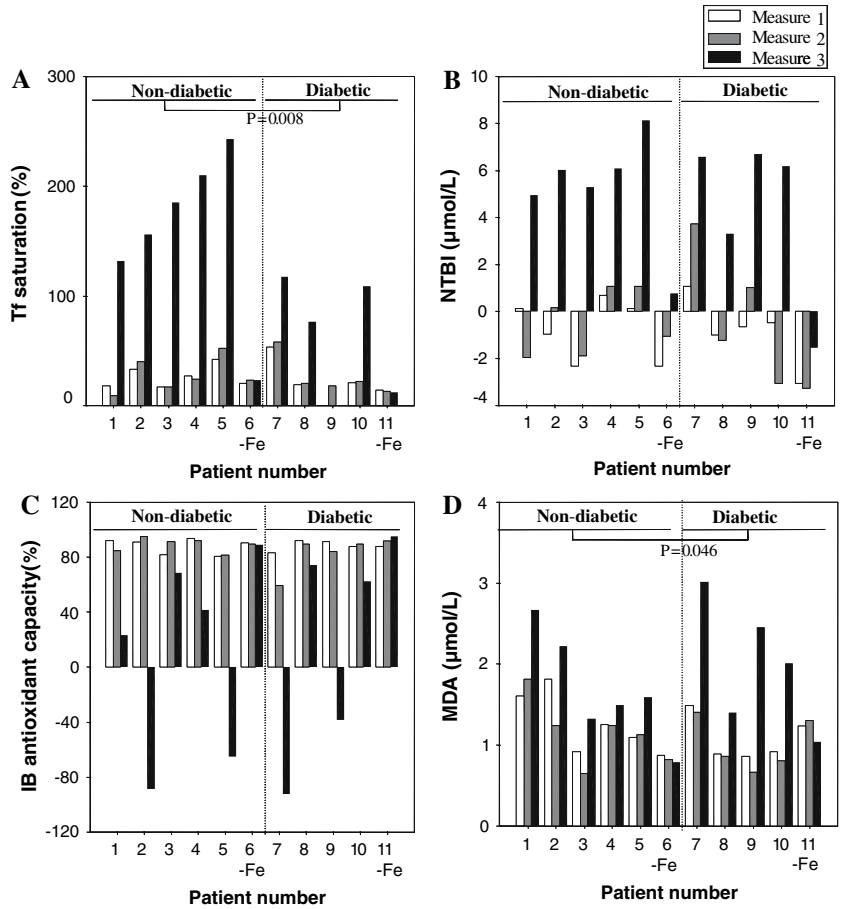

Fig. 2 Changes in Tf saturation (**A**), NTBI (**B**), IB antioxidant capacity (**C**) and MDA (**D**) in non-diabetic (nr 1–6) and diabetic (nr 7–12) dialysis patients from baseline (*white*), after the haemodialysis session (*grey*) and after iron infusion (*black*). Patient nr 6 and 11 did not receive iron infusion

Discussion

A recent report has shown that diabetes contributes to most of the increase in end-stage renal disease in the United States and that more research is needed to identify the interrelation between these pathologies (Jones et al. 2005). In this context, oxidative stress may play a central role since it is well-known to be involved in the pathogenesis of diabetes (Baynes 1991; Brownlee 2005) and is also a contributing factor in haemodialysis-related complications (Lim et al. 1999). A major source of oxidative damage may be the repeated intravenous iron infusions needed to maintain adequate iron stores for recombinant erythropoietin-stimulated erythropoiesis.

We observed that Tf levels were lower in haemodialysis patients with a median concentration of 1.5 g/l. Previously we demonstrated that Tf concentrations below 2 g/l result in a dose-dependent impairment of the protection against iron-induced liposome peroxidation in vitro (Van Campenhout et al. 2003). However, plasma iron-binding antioxidant capacity did not differ in haemodialysis patients compared to non-haemodialysis subjects. We have also previously reported that iron-binding antioxidant capacity is diminished in diabetic patients without end-stage renal disease (Van Campenhout et al. 2006a) but this was not observed in the diabetic subgroup of haemodialysis patients. Moreover, we did not observe detectable baseline values of NTBI in our study group in contrast to other investigations (Kooistra et al. 2002; Esposito et al. 2002).

Nevertheless, oxidative stress was already increased at this point as indicated by the elevated MDA levels in these subjects. This is in accordance with other reports invariably finding increased lipid peroxidation in the plasma and erythrocytes of uraemic patients (Toborek et al. 1992; Dasgupta et al. 1992; Paul et al. 1993; Loughrey et al. 1994; Lim et al. 1999). Moreover, multiple regression

analysis showed that higher ferritin levels, indicating elevated body iron stores, were a strong independent predictor of baseline MDA values, confirming findings by Lim et al. (1999). Another remarkable finding was the absence of detectable plasma ascorbate in our patients, even though α-tocopherol and glutathione were within acceptable levels as previously reported (Lim et al. 1999). This finding contrasts with reports of decreased glutathione (Ceballos-Picot et al. 1996) and α-tocopherol (Peuchant et al. 1994) in haemodialysis. We did find lower protein thiol content in haemodialysis patients but this difference was only significant in the non-diabetic group compared to non-haemodialysis controls. The diabetic non-haemodialysis group already displayed lower values, with no additional decrease associated with renal failure or haemodialysis. These observations point out to variable conditions which aggravate oxidative damage by depleting the antioxidants that are able to protect against the over-production of free radicals. In this limited number of patients we observed that the higher levels of total serum protein (but not Tf) glycation in haemodialysis patients were more pronounced in the diabetic subgroup. In contrast, the lower HbA_{1C} levels in the non-diabetic haemodialysis group might indicate a shorter exposure of haemoglobin to blood glucose as a result of erythropoietin treatment in these patients. Little is known on how these baseline differences in haemodialysis patients affect the relation between iron metabolism and oxidative stress during a haemodialysis session and an intravenous iron infusion.

In the next part of this study, we addressed this question and evaluated the changes of these parameters during a dialysis session just before (measure 2) and immediately after administration of iron saccharate (Venofer®) in the last 30 min of the session (measure 3). Haemodialysis resulted in slightly decreased global plasma antioxidant capacity (TAC-PI) and a strong decrease in TAC-TE, explained by the decrease in concentration of filterable antioxidants, in particular uric acid which is extensively cleared during the session. This was accompanied by a decrease in fructosamine which was more pronounced in non-diabetics for the total protein and in diabetics for the Tf moiety. Larger studies are needed to confirm if these differences represent less efficient clearance of glycated albumin products (in contrast to Tf) in diabetic patients. In this regard, data about the ability of dialysis to remove glycation products is controversial. Discrepant results may in part be due to the different methodologies reflecting the heterogeneity of glycated products (Papanastasiou et al. 1994; Odetti et al. 1995; Friedlander et al. 1995). To this effect, it has been reported that dialysis is able to reduce plasma advanced glycated end-products but not the fructosamine which consists of glycated proteins (Henle et al. 1999). Since proteins are not filtrated through the dialysis membrane, the increase in the concentrations of albumin, Tf, haptoglobin and haemopexin as well as serum iron, plasma retinol and α-tocopherol we found at measure 2 is most probably due to a haemo-concentration effect as a consequence of drainage of accumulated fluid.

After intravenous iron infusion during the last 30 min of the session, we observed a marked increase of serum iron concentration accompanied by oversaturation of Tf, in concordance with other reports of Tf oversaturation after intravenous iron gluconate (Zanen et al. 1996) and iron saccharate (Sunder-Plassmann and Horl 1996; Kooistra et al. 2002) in dialysis patients. The higher increase in saturation in the non-diabetic population was related to the combined result of slightly higher serum iron and lower TIBC in these patients. The main question is whether this oversaturation indeed represents 'labile' or 'free' iron not forming a stable complex with Tf or whether the iron excess binds to other molecules which make it less available to act as a catalytic agent in the formation of the damaging hydroxyl radical (Gutteridge et al. 1982). In this regard it is important to notice that not only saturation but also TIBC is increased after iron infusion as the saccharate network will be able to bind most iron until it is cleared by the mononuclear phagocytic system for reuse in erythropoiesis (Kooistra et al. 2002). Iron, which is not tightly bound to Tf or other molecules (NTBI) could be detected in serum samples using a fluorescence-based competitive assay (Breuer et al. 2000). Despite the extra binding capacity, we found appreciable concentrations of NTBI in iron-infused haemodialysis patients. The appearance of NTBI in serum after iron infusion is highly variable and could be either due to different intrinsic properties of iron preparations [concentration (Sengoelge et al. 2004) and form (Zager et al. 2002, 2004)] to different handling of iron by patients or to a combination of both. Intra-individual factors may include Tf concen-

tration as previously shown by Sunder-Plassmann and Horl (1996) who found oversaturation of Tf by infusion of 100 mg iron only if Tf levels were lower than 1.8 g/l. In our limited study population with Tf concentration ranging from 1.4 g/l to 3.1 g/l, we found no significant correlation between Tf concentration and Tf oversaturation or NTBI. In addition, we previously demonstrated impaired iron-binding in in vitro glycated Tf solutions (Van Campenhout et al. 2003). However, higher Tf glycation in the diabetic haemodialysis subgroup was not associated with a higher occurrence of NTBI. It is a matter of speculation in what form this iron exists. Besides the heterogeneous mixture of iron associated with low-molecular weight ligands (ATP, ADP, GTP, citrate, phosphate, albumin,...), it is conceivable that there is a proportion of iron bound to the surface of the saccharate network. This iron may be relatively free for donation to other ligands. The content of a small but significant fraction (approximately 1%) of redox-active iron in parenteral iron formulations has already been demonstrated (Esposito et al. 2002). It is also possible that during the measurement, some of the iron is detached by mobilising agents. In both cases it may involve iron that is available for the generation of oxidative stress. This was illustrated by the decreased iron-binding antioxidant capacity of iron-infused plasma, even leading to the stimulation of liposome peroxidation in some patients.

In line with these results and in accordance with other studies (Lim et al. 1999; Salahudeen et al. 2001; Muller et al. 2004; Mimic-Oka et al. 2005), lipid peroxidation products (MDA) increased significantly after iron infusion. Despite the limited patient numbers (power 0.55) we detected a more pronounced iron-induced increase in diabetic patients. The underlying reasons remain unclear since it was not accompanied by any differences in glycation, Tf saturation or NTBI. Other authors have also observed increases in protein (Anraku et al. 2004; Mimic-Oka et al. 2005) and DNA damage (Muller et al. 2004; Yoshimura et al. 2005) in haemodialysis patients upon iron infusion. The leading cause of mortality in the dialysis population is cardiovascular disease related to coronary atherosclerosis (USRDS 1997). Since repeated iron infusion can promote lipoprotein oxidation, it may therefore be involved in the accelerated atherosclerosis in dialysis population (USRDS 1997).

In conclusion, our study demonstrates decreased antioxidant defence and increased oxidative damage in the plasma of haemodialysis patients. Baseline ferritin levels were an independent predictor for higher lipid peroxidation. Iron infusion led to oversaturation of Tf and the appearance of NTBI which was accompanied by a further aggravation of oxidative damage that was more pronounced in diabetic patients.

The limiting factor in our current study is the small number of patients involved. The diabetes-associated differences that we highlighted in this pilot study require confirmation by conducting clinical trials on a larger study population (aiming to achieve a statistical power of at least 0.80) but investigating a less extensive but more focussed battery of relevant parameters.

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