Citrate anticoagulation abolishes degranulation of polymorphonuclear cells and platelets and reduces oxidative stress during haemodialysis

Mareille Gritters¹,  Muriël P. C. Grooteman³,  Margreet Schoorl²,  Marianne Schoorl²,  Piet C. M. Bartels²,  Peter G. Scheffer⁴,  Tom Teerlink⁴,  Casper G. Schalkwijk⁴,  Marieke Spreeuwenberg⁵ and Menso J. Nube¹,³

¹Department of Nephrology and ²Department of Clinical Chemistry, Medical Center Alkmaar, Alkmaar, ³Department of Nephrology, ⁴Department of Clinical Chemistry and ⁵Department of Clinical Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, The Netherlands

Abstract

Background. During haemodialysis (HD), polymorphonuclear cells (PMNs) and platelets are activated and release various granule products, including myeloperoxidase (MPO) and platelet factor 4 (PF4). MPO triggers the generation of reactive oxygen species, leading to irreversible protein, carbohydrate and lipid modification. PF4 probably also contributes to oxidative stress. As previously shown, HD-induced PMN degranulation is almost completely abolished during citrate anticoagulation, most probably due to its calcium chelation ability.

Methods. In the present study, apart from HD-induced PMN and platelet degranulation, oxidative stress was analysed during three modes of anticoagulation. Heparin, dalteparin and citrate (HDhep, HDdal and HDcit) were compared in a randomized, crossover fashion in eight chronic HD patients. Multiple blood samples were taken during the third HD session of each modality, from both the afferent and efferent line. Besides the degranulation markers MPO and PF4, various markers of oxidative stress were measured, including oxidized low-density lipoprotein (ox-LDL), malondialdehyde (MDA) and carboxymethyllysine (CML).

Results. During HDhep and HDdal, marked degranulation was observed shortly after the start of HD. In contrast, during HDcit, PF4 and MPO levels remained unaltered, suggesting no release at all. After 1 week of HDcit, ox-LDL levels were markedly reduced [median 26% (3–65%), P = 0.01], if compared with HDhep and HDdal. As regards MDA and CML, no differences were found.

Conclusions. This study shows first, that HD-induced PMN and platelet degranulation are early, most probably calcium-dependent processes and, secondly, that the formation of ox-LDL is clearly dependent on the type of anticoagulant applied.

Keywords: anticoagulation; haemodialysis; oxidative stress; polymorphonuclear cells; thrombocytes

Introduction

Patients with end-stage renal disease (ESRD) have a 10- to 20-fold increased risk of cardiovascular mortality, if compared with healthy controls. Besides traditional risk factors, such as hypertension and dyslipidaemia, uraemia-related risk factors, including fluid overload, hyperhomocysteinaemia and disturbances of mineral metabolism, may play an important role. In addition, undesirable side effects of the haemodialysis (HD) treatment itself, commonly termed bio-incompatibility, may contribute to the extremely high risk of cardiovascular disease (CVD) in these patients [1]. As for the latter, both humoral systems, such as the coagulation pathway and the complement system, and cellular elements, including polymorphonuclear cells (PMNs), monocytes, lymphocytes and platelets, are activated during HD. With respect to PMNs, various granule products, such as myeloperoxidase (MPO) and lactoferrin, are released into the circulation shortly after the start of HD. During the respiratory burst, MPO triggers the formation of chlorinated oxidants, leading to irreversible protein and lipid modification [2]. Hence, it has been speculated that HD treatment itself contributes to the increased oxidative stress in ESRD patients and, consequently,
to endothelial dysfunction and CVD [3]. As mentioned, not only PMNs, but also platelets are activated during the course of HD. In this respect, release of the thrombocyte α-granule products platelet factor 4 (PF4), platelet-derived growth factor AB and β-thromboglobulin was demonstrated previously [4]. Interestingly, in vitro it was shown that PF4 promotes the formation of oxidized low-density lipoprotein (ox-LDL) by inhibition of the binding and degradation of LDL cholesterol through its receptor. Moreover, in cell cultures, it could be demonstrated that PF4 binds directly to ox-LDL, thereby increasing its binding to vascular cells and macrophages [5], thus enhancing the formation of foam cells. Hence, it is conceivable that both HD-induced PMN and platelet degranulation contribute to the oxidative stress and CVD in ESRD patients [6].

As published previously [7], citrate anticoagulation almost completely abolishes HD-induced PMN degranulation, most probably due to its calcium-chelating ability. Moreover, in a perfusion system, it was shown that citrate anticoagulation induced less platelet aggregation and degranulation than heparin [8]. In the present study, we investigated whether citrate anticoagulation reduces not only the degranulation of PMNs and platelets, but also oxidative stress. Therefore, eight chronic HD (CHD) patients were randomly, crossover assigned to anticoagulation with citrate, heparin and low molecular weight heparin (LMWH; dalteparin). Besides the granule products platelet factor 4 (PF4), which are commonly used indices of platelet activation/desintegration, we also assessed indices of oxidative stress, such as malondialdehyde (MDA) and N^ε-(carboxymethyl)lysine (CML). In addition, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, which is markedly elevated in ESRD and related to CVD in these patients [9], was measured. ADMA may accumulate in oxidative stress, as the critical sulphydryl group in the active site of its metabolizing enzyme dimethylarginine dimethylaminohydrolase is oxidatively modified [9].

Subjects and methods

Patients

Eight stable patients, two females and six males [median age 64 years (23–80)], undergoing regular HD treatment for at least 1 year [median 35 months (12–168)], were included in the study. The aetiology of renal insufficiency was hypertensive nephrosclerosis in four patients, diabetic nephropathy in one and chronic pyelonephritis in two, whereas one patient suffered from adult dominant polycystic kidney disease. Criteria for exclusion were clinical signs of infection, autoimmune disease or malignancy as well as the use of drugs known to interfere with the immune system and/or platelet function, such as immunosuppressive drugs, non-steroidal anti-inflammatory drugs, coumarins, clopidogrel and aspirin. Written informed consent was obtained in all cases. The protocol was approved by the local Medical Ethical Committee.

Design of the study

Three modes of anticoagulation, heparin, dalteparin and citrate (HDhep, HDdal and HDcit, respectively), were compared in a crossover fashion, according to a computerized randomization model. Each anticoagulant modality was applied for 1 week during three consecutive HD sessions. Blood samples were taken before and after the last dialysis session of each week. During wash-out periods, which were instituted for 1 week between the three treatment periods, dalteparin, the standard anticoagulation modality in our centre, was used.

Blood was drawn from the fistula before dialysis (t₀) (prior to heparin or dalteparin infusion), from the afferent line after 30 (t₁₀) and 210 min (t₁₂₀) and from the efferent line after first passage (t₁), and after 5 (t₅), 30 (t₉₀), 60 (t₉₀) and 150 (t₁₅₀) min. All samples were corrected for changes in plasma volume, based on haematocrit (Ht) measurements; corrected value = value × [Ht₀/(1–Ht₀)] × [(1–Ht₁)/Ht₁]. Blood was taken from the afferent line proximal to the heparin or citrate infusion port. The sampling point in the efferent line was located prior to the calcium and magnesium infusion port in the case of HDcit.

Degranulation of PMNs and platelets within the extracorporeal circuit (ECC) was assessed by quantification of the differences between the MPO and PF4 concentrations in the afferent and efferent line. Ox-LDL, MDA, CML and ADMA were measured as parameters of oxidative stress. Before the start of HD (t₀), LDL-cholesterol concentrations were assessed.

Dialysis procedure and materials

The patients were on HD therapy for a median of 12 h (9–12) per week. Only first use high-flux polysulfone dialysers [F60, Fresenius Medical Care, Bad Homburg, Germany; ultrafiltration (UF) factor 40–55 ml/mmHg/h, surface area 1.3–1.8 m², steam sterilized] were used. According to individual needs, blood flow (250–300 ml/min) and UF rates (300–1000 ml/h) were kept constant. Bicarbonate dialysate was used with a dialysate flow of 500 ml/min. For dialysate preparation, tap water, purified by reverse osmosis, was used for the dilution of a concentrated bicarbonate solution to the following concentrations (mmol/l): 138 Na⁺, 2.0 K⁺, 1.50 Ca²⁺, 0.50 Mg²⁺, 109 Cl⁻, 2.5 CH₃COO⁻ and 32.5 HCO₃⁻ (SK-F 216/1; Fresenius Medical Care). During HDcit, a Ca²⁺-free dialysate solution was used with the following composition (mmol/l): 138 Na⁺, 2.0 K⁺, 0.50 Mg²⁺, 106 Cl⁻, 2.0 CH₃COO⁻ and 33.0 HCO₃⁻ (SK-F 219/0; Fresenius Medical Care). All dialysers were pre-rinsed with 1000 ml of 0.9% NaCl.

Dalteparin dialysis

The LMWH dalteparin (Fragmin®) is the standard anticoagulation modality in our centre. Individual doses are based on body weight and duration of dialysis, and are given as a bolus injection at the beginning of the dialysis session [median 5000 IE (4000–6000)]. In three of the participating patients, a second dose [median 2000 IE
Citrate anticoagulation in haemodialysis reduces oxidative stress (1000–2500 IE) was routinely administered during HD after 2–3 h.

**Heparin dialysis**

After a priming dose (2500 IU), heparin was given as a continuous infusion of 1000 IU/h. Heparin infusion was stopped 30 min before the end of the HD session.

**Trisodium citrate dialysis**

A sterile 15% trisodium citrate solution was infused continuously into the afferent line at a flow rate of 100 ml/h per 250 ml/min blood flow. A Ca$^{2+}$-free dialysate solution was used. After passage through the dialyser, Ca$^{2+}$ and Mg$^{2+}$ levels were corrected by the infusion of a calcium chloride (CaCl$_2$) (540 mmol/l)/magnesium chloride (MgCl$_2$) (240 mmol/l) solution into the efferent line at a rate of 28 ml/h per 250 ml/min blood flow.

**Analytical methods**

**MPO**. MPO was determined in serum. After collection, the samples were allowed to clot at room temperature for 1 h. Serum separation was achieved by centrifugation for 10 min at 1500 g. Immediately after separation, the samples were stored at −70°C until further analysis. All determinations were carried out in duplicate. A competitive radio-immunoassay was used according to the manufacturer’s procedure (Pharmacia Diagnostics, Uppsala, Sweden). The detection limit of this assay is 20 μg/l; the inter-assay variation is <8%.

**PF4**. PF4 concentration was determined in plasma. Blood samples were drawn into CTAD (citrate-theophylline-adenine-dipyridamol) tubes, cooled on ice and centrifuged at 2–8°C for 20 min at 2500 g to achieve plasma separation. Samples were stored at −70°C. PF4 was measured using a commercially available sandwich enzyme-linked immuno-sorbent assay (ELISA; Asserachrom PF4, Diagnostica Stago, Asnières, France). The detection limit of this assay is 1 IU/ml; the intra-assay and inter-assay coefficients of variation are 4.8 and 6.1%, respectively.

**Ox-LDL**. Ox-LDL was measured in plasma. Blood was collected in K$_3$EDTA (tripotassium ethylenediamine tetra-acetate) tubes and cooled on ice. After centrifugation for 10 min at 2500 g, the plasma fraction was stored at −70°C. Plasma levels of ox-LDL were measured by using a sandwich ELISA method (Mercodia, Uppsala, Sweden). This assay is based on a monoclonal antibody (4E6) directed against a conformational epitope in the apoB-100 moiety of LDL, generated as a consequence of the reaction of lysine residues with aldehydes [10]. The bound conjugate is detected by reaction with 3,3′,5,5′-tetrakis[tetramethylbenzidine and measured spectrophotometrically at λ = 450 nm. The detection limit is <1 mU/l; the intra-assay and inter-assay coefficients of variation are 4.0 and 4.2%, respectively.

**MDA**. MDA was measured in plasma. Blood was collected in K$_3$EDTA tubes and cooled on ice. After centrifugation for 10 min at 2500 g, the plasma fraction was stored at −70°C. The MDA concentration in plasma was determined as previously described [11]. Briefly, after an alkaline hydrolysis step, the plasma samples were incubated with thiobarbituric acid (TBA) and the resulting MDA–TBA adduct was measured by reversed phase high-performance liquid chromatography (HPLC) with fluorescence detection. The inter-assay coefficient of variation was 3.5%.

**CML**. CML was measured in plasma. Blood was collected in K$_3$EDTA tubes and cooled on ice. After centrifugation for 10 min at 2500 g, the plasma fraction was stored at −70°C. The CML content of plasma proteins was determined by stable isotope dilution tandem mass spectrometry [12]. Briefly, CML was liberated from plasma proteins by acid hydrolysis after the addition of deuterated CML as internal standard. Separation was performed by gradient elution reversed phase HPLC with nonfluoropentanoic acid as the ion-pairing agent. Mass transitions of m/z 205.1/84.1 and 209.1/88.1 for CML and the internal standard were monitored in positive ion mode on an API 3000 triple-quadrupole tandem mass spectrometer (Sciex, Applied Biosystems). The inter-assay coefficient of variation is <9%.

**ADMA**. ADMA was measured in plasma. Blood was collected in K$_3$EDTA tubes and cooled on ice. After centrifugation for 10 min at 2500 g, the plasma fraction was stored at −70°C. Sample clean-up was performed by solid-phase extraction on polymeric cation-exchange columns using monomethylarginine as internal standard. After derivatization with orthophthalaldehyde reagent containing 3-mercaptopyrroproionic acid, analytes were separated by isocratic reversed phase HPLC with fluorescence detection [13]. The inter-assay coefficient of variation is <3% and the upper limit of the reference range is 0.55 μmol/l.

**LDL-cholesterol**. LDL cholesterol was directly determined by the N-geneous™ assay (Genzyme, Cambridge, MA). The intra- and inter-assay coefficients of variation were 0.7 and 2.3%, respectively.

**Statistical analysis**

All analyses were performed with the SPSS 11.5 software system. Data are expressed as mean (±SD), or median (minimum – maximum), when appropriate. Although nearly all parameters were normally distributed, non-parametric tests were used to study differences between groups, as the number of observations was relatively small. Differences were considered significant at P < 0.05.

Since the study was set up in a crossover design, allowing subjects to serve as their own controls, Friedman’s test was used to analyse differences between the three modes of anticoagulation applied. A correction for multiple comparisons was performed, as four different parameters of oxidative stress were analysed. In the case of significant differences, Wilcoxon signed ranks tests were performed as post hoc analysis, with additional correction for multiple comparisons using Bonferroni’s method. Correlation coefficients were calculated with the Spearman’s rank method. To evaluate further the relative influence of LDL cholesterol and the use of citrate on the formation of ox-LDL, linear
regression analysis (Enter) was used. The use of citrate was introduced as a dummy variable.

## Results

### Degranulation of PMNs and thrombocytes

During **HDhep**, as well as **HDdal**, marked increases in MPO and PF4 concentrations were observed across the dialyser shortly after the start of HD (Table 1, Figure 1). For both modalities, MPO concentrations almost doubled, whereas the increase in PF4 was ~8-fold. In contrast, neither MPO nor PF4 release was found during **HDcit** at first passage. Noticeably, MPO levels in **HDhep** and **HDdal** remained elevated during the first 30 min, whereas PF4 levels were still elevated after 150 and 60 min, respectively (Table 1). MPO and PF4 gradients across the dialyser (**C**<sub>effenter line</sub>—**C**<sub>efffferent line</sub>) are shown in Table 1 and Figure 2. PF4 and MPO release were strongly correlated: \( r = 0.84 \) (\( P < 0.001 \)) (Figure 3).

### Oxidative stress

**Ox-LDL, MDA, CML and ADMA.** After 1 week of **HDcit**, ox-LDL \( t_0 \) values were reduced by 26\% (3–65\%) in comparison with both **HDhep** (\( P = 0.01 \)) and **HDdal** (\( P = 0.01 \)) (Table 2, Figure 4). Ox-LDL \( t_0 \) values were not significantly different between **HDhep** and **HDdal**. No differences were found for MDA, CML and ADMA between **HDcit** and the other two modalities (Table 2).

**LDL cholesterol.** As expected, LDL cholesterol concentrations (\( t_0 \)) were similar in **HDhep** (3.0 ± 1.0 mmol/l), **HDdal** (3.0 ± 0.7 mmol/l) and **HDcit** (2.8 ± 1.0 mmol/l).

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**Table 1.** MPO (\( \mu g/l \)) and PF4 (IU/ml) concentrations and concentration gradients across the dialyser (**C**<sub>effenter line</sub>—**C**<sub>efffferent line</sub>) during heparin, dalteparin and citrate HD

<table>
<thead>
<tr>
<th></th>
<th>( t_{0(fistula)} )</th>
<th>( t_{1(eff)} )</th>
<th>( t_{30(eff)} )</th>
<th>( t_{210(eff)} )</th>
<th>( t_{1(eff)} - t_{0(eff)} )</th>
<th>( t_{30(eff)} - t_{30(eff)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HDdal</strong></td>
<td>63 (25–120)</td>
<td>152 (69–243)*</td>
<td>115 (72–166)*</td>
<td>103 (68–124)*</td>
<td>55 (46–113)</td>
<td>63 (39–157)</td>
</tr>
<tr>
<td><strong>HDcit</strong></td>
<td>74 (34–111)</td>
<td>58 (31–86)</td>
<td>45 (23–89)***</td>
<td>50 (26–113)</td>
<td>–13 (49 to 3)</td>
<td>5 (1 to 7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>( t_{0(fistula)} )</th>
<th>( t_{5(eff)} )</th>
<th>( t_{30(eff)} )</th>
<th>( t_{60(eff)} )</th>
<th>( t_{150(eff)} )</th>
<th>( t_{5(eff)} - t_{0(eff)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HDhep</strong></td>
<td>11 (2–55)</td>
<td>98 (67–113)***</td>
<td>57 (34–110)***</td>
<td>54 (16–139)**</td>
<td>63 (15–100)**</td>
<td>83 (36–99)</td>
</tr>
<tr>
<td><strong>HDdal</strong></td>
<td>13 (3–35)</td>
<td>100 (84–117)***</td>
<td>68 (33–96)***</td>
<td>66 (17–114)**</td>
<td>32 (13–96)</td>
<td>82 (48–103)</td>
</tr>
<tr>
<td><strong>HDcit</strong></td>
<td>9 (6–23)</td>
<td>6 (5–33)</td>
<td>19 (5–97)</td>
<td>29 (7–56)</td>
<td>15 (7–36)</td>
<td>–1 (10 to 10)</td>
</tr>
</tbody>
</table>

Values represent medians (minimum–maximum). Wilcoxon signed-ranks test for pairs \( t_x (1, 5, 30, 60, 150, 210) \) vs \( t_0 \), yielded \( P \)-values as shown: *\( P = 0.01 \); **\( P = 0.02 \); ***\( P = 0.03 \).

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**Fig. 1.** MPO (\( \mu g/l \)) (A) and PF4 (IU/ml) (B) concentrations in the ECC during heparin, dalteparin and citrate HD. Values represent medians. Wilcoxon signed-ranks tests for pairs \( t_1 \) vs \( t_0 \) in the case of MPO, and \( t_5 \) vs \( t_0 \) in the case of PF4 yielded \( P \)-values as shown: *\( P = 0.01 \); **\( P = 0.02 \); ***\( P = 0.03 \).
Correlation and regression

Univariate analysis revealed a significant correlation between the ox-LDL levels ($t_0$) and the LDL cholesterol concentrations at $t_0$ ($r=0.62$, $P=0.01$). No significant correlations were found between the ox-LDL levels ($t_0$) and the release of MPO (gradient $t_0-t_1$) and PF4 (gradient $t_0-t_5$) early in HD.

Additionally, a linear regression analysis was performed to study the relative influence of the anticoagulation modality and LDL cholesterol on ox-LDL formation. Ox-LDL $t_0$ levels were influenced by both the LDL cholesterol concentration ($\beta=0.51$, $P=0.004$) and the use of citrate ($\beta=0.40$, $P=0.023$) (adjusted $R^2=0.41$).

Additional experiment

To ensure that heparin was released from platelets and not from the endothelium, an additional experiment was performed. In one of the participating patients, dalteparin (4000 IU) was injected into the shunt and flushed with 10 ml of NaCl 0.9%, 10 min before connection to the ECC and the start of HD ($t_0$). PF4 concentrations were measured at $t_{-10}$ (before injection), $t_0$ (before the start of HD), $t_5$, $t_{30}$, $t_{60}$ and $t_{150}$. Anti-Xa activity was measured to confirm the systemic activity of dalteparin. Despite evident systemic activity of dalteparin at $t_0$, the PF4 concentration rose < 2-fold from $t_{-10}$ to $t_0$ (Table 3), whereas after the start of HD a >15-fold increase was observed.
third HDhep, HDdal and HDcit session

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158 M. Gritters

... induced higher plasma levels of reciprocal activation. induced granulocyte–platelet aggregate formation and activation may occur independently of each other, the blood levels of MPO and PF4 were highly the calcium-chelating activity of citrate [16]. Of note, despite evident systemic activity of dalteparin... an additional experiment was performed. Secondly, as described above, in one of the participa-tion products were found. Linear regression analysis... 1 week of HDcit, significantly lower levels of this lipid peroxidation product were found. Linear regression analysis showed that besides citrate, baseline ox-LDL levels were dependent of the pre-dialysis (t0) plasma LDL concentrations. As the plasma LDL levels (t0) were similar in the three modalities, it is highly unlikely that this parameter underlies the differences in ox-LDL. However, since the number of observations was relatively small and the time span of the study was limited, our results should be interpreted with caution. Whether the lowering of ox-LDL resulted from the creation of an almost calcium-free environment within the ECC, the absence of heparin or a combination of these factors is not easily apparent from the present study.

In this respect, it is of note that circulating ox-LDL recently has been associated with CVD in non-diabetic, diabetic and renal patients. In addition, elevated MPO levels, both intracellular and circulating, have been related to the presence of CVD [17]. Moreover, it has been shown that MPO and PF4 [5] co-localize with ox-LDL in atherosclerotic lesions and that MPO contributes to LDL oxidation in vitro [18]. However, against our initial expectations, univariate analysis could not reveal MPO or PF4 as major determinants of plasma ox-LDL in our study. Besides considerable inter-individual differences, other factors may account for this unexpected finding. Regarding LDL, retention may occur as it binds to heparin, a glycosaminoglycan with a strong negative charge. In vitro it was shown that these heparin-bound LDL particles are more rapidly oxidized by MPO than native particles [19]. Moreover, in rats, it has been shown that heparin interferes with the uptake of ox-LDL, leading to a delayed plasma clearance [20]. Therefore, the mode of anticoagulation may have had over-riding effects over the degradation products MPO and PF4 in this respect. Considering the other markers of oxidative stress measured, such as MDA and CML, differences were not found between HDcit and the other two modalities. However, as the formation of these advanced lipoxidation and glycation end-products is a complicated process, involving a series of enzymatic and non-enzymatic reactions leading to the formation of various intermediate products [3,11], it is conceivable that a reduction can only be observed after a longer period of time.

Table 2. Concentrations of ox-LDL (U/l), MDA (µmol/l), CML (µmol/mmol lysine) and ADMA (µmol/l) at the beginning (t0) of the third HDhep, HDdal and HDcit session

<table>
<thead>
<tr>
<th></th>
<th>HDhep</th>
<th>HDdal</th>
<th>HDcit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox-LDL (U/l)</td>
<td>52 (31–88)*</td>
<td>49 (25–63)*</td>
<td>29 (24–55)</td>
</tr>
<tr>
<td>MDA (µmol/l)</td>
<td>11 (5–18)</td>
<td>11 (9–19)</td>
<td>10 (9–15)</td>
</tr>
<tr>
<td>CML (µmol/mmol lysine)</td>
<td>0.20 (0.13–0.23)</td>
<td>0.19 (0.12–0.23)</td>
<td>0.20 (0.13–0.24)</td>
</tr>
<tr>
<td>ADMA (µmol/l)</td>
<td>0.61 (0.53–0.75)</td>
<td>0.59 (0.50–0.75)</td>
<td>0.57 (0.53–0.80)</td>
</tr>
</tbody>
</table>

Values represent medians (minimum–maximum). Friedman's test (P = 0.002) followed by Wilcoxon signed-ranks test for comparison of HDhep and HDdal vs HDcit yielded P-values as shown: *P = 0.01.

Table 3. Concentrations of PF4 (IU/ml) and anti-factor-Xa activity prior to and during HD with dalteparin

<table>
<thead>
<tr>
<th></th>
<th>t-10</th>
<th>t0</th>
<th>t5</th>
<th>t30</th>
<th>t60</th>
<th>t150</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF4 (IU/ml)</td>
<td>3.6</td>
<td>6.1</td>
<td>100.3</td>
<td>35.8</td>
<td>43.3</td>
<td>35.7</td>
</tr>
<tr>
<td>Anti-Xa (U/ml)</td>
<td>-</td>
<td>1.07</td>
<td>0.87</td>
<td>0.83</td>
<td>0.75</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Dalteparin was injected at t-10; HD was started at t0.

Discussion

Our study clearly shows that both HD-induced PMN and platelet degranulation, as assessed by the release of MPO and PF, respectively, are early processes, occurring mainly in the ECC. As comparable results have been described before by us [7] and others [4] during treatment with different types of membranes, our data seem to be representative of routine HD.

Although heparin has also been associated with the release of endothelial-bound MPO [14] and PF4 [15], two key findings in our study argue strongly against this option. First, the steep rise of MPO directly after first passage indicates release within the ECC. Secondly, as described above, in one of the participating patients an additional experiment was performed. Of note, despite evident systemic activity of dalteparin at t0, PF4 levels rose <2-fold from t-10 to t0, whereas after the start of HD a >15-fold increase was seen. Therefore, we conclude that dalteparin did not elicit major PF4 release from the endothelium and that the rise observed at t5 most probably resulted from HD-induced platelet activation.

Interestingly, not only PMN degranulation, but also platelet degranulation was almost completely abolished during HDcit. The absence of MPO and PF4 release during HDcit is most probably attributable to the Ca**2+**-free status of the ECC, resulting from the calcium-chelating activity of citrate [16]. Of note, the blood levels of MPO and PF4 were highly correlated. Therefore, although PMN and platelet activation may occur independently of each other, our data are compatible with the concept of HD-induced granulocyte–platelet aggregate formation and reciprocal activation.

The present study convincingly showed that both HDhep and HDdal induce higher plasma levels of ox-LDL (t0) than HDcit. In fact, after only 1 week of HDcit, significantly lower levels of this lipid peroxidation product were found. Linear regression analysis showed that besides citrate, baseline ox-LDL levels were dependent of the pre-dialysis (t0) plasma LDL concentrations. As the plasma LDL levels (t0) were similar in the three modalities, it is highly unlikely that this parameter underlies the differences in ox-LDL. However, since the number of observations was relatively small and the time span of the study was limited, our results should be interpreted with caution. Whether the lowering of ox-LDL resulted from the creation of an almost calcium-free environment within the ECC, the absence of heparin or a combination of these factors is not easily apparent from the present study.

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Citrate anticoagulation in haemodialysis reduces oxidative stress

To summarize, in the present study, we showed that release of the degranulation products MPO and PF4 during HD$_{hep}$ and HD$_{dal}$ was almost completely abolished during HD$_{cit}$. After only 1 week of HD$_{cit}$, ox-LDL levels ($t_0$) were considerably reduced in comparison with both HD$_{hep}$ and HD$_{dal}$. The reduction in ox-LDL could not be explained by differences in LDL cholesterol, or the release of MPO and PF4. Although long-term HD treatment with citrate anticoagulation is more time consuming and laborious than HD with LMWH, the present findings are promising. Our study clearly showed that the HD-induced increase in oxidative stress in CHD patients, as measured by ox-LDL levels, can be influenced and reduced to a considerable extent. Whether these reduction leads to a decrease in cardiovascular morbidity and/or mortality remains to be established.

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References


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