Accelerated atherosclerosis in haemodialysis patients; correlation of endothelial function with oxidative DNA damage

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Abstract

Background. Accelerated atherosclerosis is the major cause of mortality in patients on chronic haemodialysis (HD). The aim of this study was to evaluate the relationship between oxidative DNA damage [8-hydroxy-2' deoxyguanosine/deoxyguanosine ratio (8-OHdG/dG ratio)], oxidative stress biomarkers and endothelial function in HD patients as an indicator of atherosclerosis.

Methods. Forty-four chronic HD patients without known atherosclerotic disease and 55 age- and sex-matched healthy individuals were included in the study. Plasma malondialdehyde (MDA) levels and 8-OHdG/dG ratio were determined as oxidative stress markers. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured as antioxidants. Endothelial function was assessed by ultrasonography.

Results. 8-OHdG/dG ratio and MDA levels were higher in HD patients than controls while SOD and GPx activities were lower in HD patients compared to controls. Flow-mediated dilatation FMD% in HD patients were lower than the control group (7.28 ± 0.79 versus 11.18 ± 0.82, P < 0.001). There was a significant negative correlation between FMD% and 8-OHdG/dG ratio (r = -0.678, P < 0.001) and MDA levels (r = -0.517, P < 0.01), while there was a significant positive correlation between FMD% and SOD (r = 0.538, P < 0.01) and GPx levels (r = 0.720, P < 0.01).

Conclusions. Our data have demonstrated that HD patients exhibit increased oxidative DNA damage and decreased antioxidant activity. We propose that endothelial function is negatively correlated with 8-OHdG/dG ratio and positively correlated with antioxidant enzymes. To our knowledge, this is the first study to demonstrate the inverse relationship between endothelial function and plasma oxidative DNA damage in HD patients.

Keywords: endothelial dysfunction; haemodialysis; oxidative DNA damage

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in end-stage renal disease (ESRD) patients and cardiovascular death rate of dialysis patients is ~30 times higher than in the general population [1]. Evidence shows that there is an increased incidence and accelerated worsening of atherosclerosis in patients on chronic haemodialysis (HD) [2, 3]. Although traditional risk factors such as hypertension, diabetes mellitus and hyperlipidaemia are highly prevalent in ESRD patients, these factors cannot fully explain the increased cardiovascular morbidity in this population [4]. Recently, there has been ample evidence to support the role of ‘non-traditional’ risk factors for cardiovascular events in ESRD patients such as oxidative stress and endothelial dysfunction [5, 6].

Oxidative stress occurs when there is an imbalance between free radical production and antioxidant capacity. This may be due to an increase in free radical generation and/or a decrease in normal antioxidant activity. Several studies emphasize the importance of antioxidant status in cardiovascular disease [7, 8]. It is also speculated that reduced antioxidant capacity might play a major role in the initiation of DNA damage [9, 10]. Several markers of oxidative damage in ESRD patients have been studied; however, most of them are either excreted in the urine or eliminated by dialysis; therefore, these biomarkers do not provide a good index of oxidative damage [11–13]. In this context, oxidative stress-induced DNA damage has been identified as a useful index and a possible indicator of cardiovascular disease and cancer risk. 8-hydroxy-2'-deoxyguanosine
(8-OHdG) is considered to be a sensitive DNA damage marker [10].

Endothelial dysfunction is an early initiated event in atherosclerosis as well as a risk factor for future cardiovascular disease [14]. The presence of endothelial dysfunction in ESRD patients has been shown by many investigators and oxidative stress is suggested as a risk factor for endothelial dysfunction [15–18].

In this study, we aimed to evaluate the relationship between endothelial dysfunction, antioxidant enzyme levels and oxidative DNA damage in HD patients.

Materials and methods

Chemicals

Proteinase, ammonium acetate, electrochemical (HPLC-ECD), Coomassie brilliant blue R-250, trihydroxymethyl amino methane–ethylene diamine tetraacetic acid (Tris–EDTA), wavelength detector (HPLC–UV), EDTA, sodium dodecyl sulphate (SDS), NaOH (Sigma–Aldrich Ltd, Poole, Dorset, UK). Sodium carbonate, sodium potassium phosphate, bicarbonate, 1,1,3,3-tetraethoxypropane standard solution, Tris, NaCl, sodium citrate dehydrate, HCl, acetic acid, propanol and thioctobarbituric acid (TBA) (Merek AG, Darmstadt, Germany). Analytical column (250 mm × 4.6 mm × 4.0 μm; Phenomenex, Torrance, CA and Merek AG). Methanol (34885), ethanol (34870), benzoquinon (B10358), lithium perchlorate (20 528-1), (Sigma–Aldrich, Taufkirchen, Germany), n-propanol (24135) (Riedel-de-Haën, Darmstadt, Germany) were used as supplemental chemicals.

Subjects

Forty-four non-diabetic HD patients without evidence of atherosclerotic disease and 55 age- and sex-matched healthy controls were included in the study. Atherosclerotic disease was excluded by patient history, physical, electrocardiographic and echocardiographic examinations. The study was approved by the local ethics committee and all participants gave written informed consent. Patients in the HD group all had creatinine clearance levels of <10 mL/min/1.73m² and had been on chronic HD treatment for at least 1 year. They were dialysed three times a week with synthetic membranes. None of the subjects received antibiotics, corticosteroids, anti-inflammatory drugs, cytotoxic drugs or vitamins during the study period.

Measurement of blood pressure

All study subjects were monitored with Spacelab1 90207 ambulatory blood pressure monitor (Spacelabs Medical, Redmond, WA) for 24 h, every 20 min from 07.00 am to 23.00 pm and every 30 min from 23.00 pm to 07.00 am. Ambulatory blood pressure monitoring data included 24-h systolic blood pressure (SBP), 24-h diastolic blood pressure (DBP), 24-h mean arterial pressure (MAP), daytime SBP, daytime DBP and daytime MAP measurements. Hypertension is defined according to JNC-7 criteria [19].

Determination of endothelial function

Endothelial function was measured non-invasively as the percentage of flow-mediated dilatation (FMD) of the brachial artery in the non-dominant or non-fistula arm, as described previously [20]. All study subjects were investigated in the morning after a 12-h overnight fast. The measurements were taken in the supine position after 15 min of rest in a temperature-controlled room. The arm was comfortably immobilized in an extended position in order to best visualize the brachial artery, which was scanned in a longitudinal section 3–5 cm above the antecubital fossa using a 10-MHz high-resolution linear array transducer. After optimal transducer positioning, skin was marked for reference for later measurements and the arm was kept in the same position throughout the study. The internal diameter of the brachial artery was assessed at end-diastole (timated by QRS complex) and the mean of three consecutive measurements was noted. Then, the cuff was inflated to 200 mmHg (or 50 mmHg higher than the SBP) for 5 min to create forearm ischaemia. Subsequently, the cuff was deflated and the arterial diameter was measured again at every 45–60 s after deflation. FMD of the brachial artery was expressed as the percentage change in the brachial artery diameter from baseline to reactive hyperaemia FMD%. Intra-observer variability of FMD% measurement was 3.6% in the current study.

Determination of DNA damage

Fasting blood samples were obtained from all subjects and collected into tubes without coagulant. Serum was obtained by centrifugation at 1000 g for 15 min and stored at –80°C until assayed.

Isolation and hydrolyzation of DNA

DNA isolation from blood was performed according to Miller et al. [21] with some modifications. Two millilitres of blood with EDTA was mixed with 3 mL of erythrocyte lysis buffer and incubated for 10 min in ice, which was followed by centrifugation (10 min at 2000 g). The supernatant was decanted and the pellet was thoroughly resuspended in SDS (10%, v/v), proteinase K (20 mg/mL) and 1.9 mL leucocyte lysis buffer. The mixture was incubated at 65°C for 1 h and then mixed with 0.8 mL of 9.5 M ammonium acetate. After centrifugation at 2000 g for 25 min, the clear supernatant (2 mL) was transferred to a new sterile tube and DNA was precipitated by addition of 4 mL of ice-cold absolute ethanol. DNA samples were dissolved in Tris EDTA buffer (10 mM, pH 7.4) and were then hydrolysed according to Shinemag’s method.

Analysis of 8-OHdG and deoxyguanosine (dG) by the HPLC method

In the hydrolysed DNA samples, 8-OHdG and deoxyguanosine (dG) levels were measured, respectively, by high-performance liquid chromatography (HPLC) with electrochemical (HPLC-ECD) and variable wavelength detector (HPLC–UV) systems as previously described [22]. Twenty microlitres of final hydrolysate were analysed by HPLC–ECD (HP, Agilent 1100 modular system) and HPLC–UV (HP, Agilent 1049A ECD detector, Germany). Column: reverse phase-C18 (RP-C18) analytical column (250 mm × 4.6 mm × 4.0 μm; Phenomenex). The mobile phase consisted of 0.05 M potassium phosphate buffer (pH 5.5) containing acetoneitril (97:3 v/v) with a flow rate of 1 mL/min. The dG and 8-OHdG concentrations were monitored based on absorbance (245 nm) and electrochemical reading (600 mV), respectively. Levels of dG and 8-OHdG were quantified using the standards of dG (Sigma Chemical Co., St.Louis, MO) and 8-OHdG (Cayman, Ann Arbor, MI); the level of 8-OHdG was expressed as the number of 8-OHdG molecules per 10¹⁰ dG. The intra-assay coefficients of variation (CV) ranged from 3 to 7%, and inter-assay CV ranged from 4 to 9%; the lower numbers refer to the CV for the high standard and the higher numbers refer to the CV for the low standard.

Determination of the oxidative stress markers

Measurement of plasma malondialdehyde (MDA) concentration was performed according to Khoschsorur et al. [23]. Briefly, 50 μL of plasma sample was mixed with 0.44 M H₃PO₄ and 42 mM TBA and incubated for 30 min in a boiling water bath. After rapidly cooling on ice, an equal volume of alkaline methanol was added to the sample, vigorously shaken, centrifuged (1500 g for 5 min) and the aqueous layer was removed. Then, 20 μL of supernatant was analysed by HPLC (HP, Agilent 1100 modular systems with FLD detector, Germany). Column: RP-C18 (5 μm, 4.6 × 150 mm, Eclipse VDB-C18. Agilent); elution, methanol (40:60, v/v) containing 50 mM KH₂PO₄ buffer (pH 6.8); flow rate, 0.8 mL/min. Fluorometric detection was performed with excitation at 527 nm and emission at 551 nm. The peak of the MDA–TBA adduct was calibrated as a 1,1,3,3-tetraethoxypropane standard solution carried out in exactly the same process as with the plasma sample. The intra-assay and inter-assay CV were 3.5 and 5.2%, respectively.

Determination of antioxidant activity

Superoxide dismutase (SOD) enzyme activation was measured with Randox-Ransod enzyme kit with autoanlyser at 505 nm and 37°C. Ten microlitres were taken from packet of erythrocyte and 2500 μL 0.01 M buffer of phosphate (pH = 7.0) were mixed and later diluted 251 times with water (F = 251). Inhibition was obtained between 30 and 60%. The intra-assay and inter-assay CV for SOD were 3.4 and 5.9%, respectively.

Measurement of glutathione peroxidase (GPx) enzyme activation was performed according to Paglia and Valentine [24]. GPx catalysed oxidation of glutathione. When the oxide glutathione is reduced, NADPH is oxidized and it is turned into NADP+. This change was observed at 340 nm...
Descriptive statistics for continuous variables were expressed as mean ± SD. Student’s t-test was used to compare means of control and patient groups for oxidative DNA damage, oxidative stress markers and FMD% variables. Pearson’s correlation test was performed to explore the linear relationships between FMD% and oxidative stress markers. A multiple linear regression analysis was used to determine the independent predictor of endothelial function. A P-value < 0.05 was interpreted as statistically significant. All statistical analyses were performed with statistical analysis program (SPSS 13.0 for Windows).

Results

Clinical and demographic characteristics of the patient and control groups are given in Table 1. There were no significant differences in age, body mass index, gender, blood pressure parameters, glucose, mean total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) among groups. 8-OHdG/dG ratios, oxidative stress markers and antioxidant parameters measured are shown in Table 2. MDA levels as well as 8-OHdG/dG ratios were significantly higher in the HD group as compared to the control group (P < 0.001). The HD group also had significantly lower SOD and GPx levels compared to controls (P < 0.001 and P < 0.001, respectively). Although baseline brachial artery diameters were similar in both groups, FMD% in HD group was lower than in the control group (7.28 ± 0.79 versus 11.18 ± 0.82, P < 0.001) (Table 2). When the two groups were pooled for analysis, MDA levels (r = −0.517, P < 0.01) and 8-OHdG/dG ratios (r = −0.678, P < 0.01) were negatively correlated with FMD%, while SOD (r = 0.538, P < 0.01) and GPx (r = 0.720, P < 0.01) were positively correlated (Figures 1 and 2). However, when the two groups were separately analysed, there was a weak negative correlation between FMD% and 8-OHdG/dG ratios (r = −0.246, P = 0.108) and MDA levels (r = −0.272, P = 0.074) as well as a positive correlation between FMD% and SOD (r = 0.423, P = 0.004) and GPx (r = 0.475, P = 0.001) levels in the HD group. There were no significant correlations between FMD% and oxidative stress biomarkers in the control group.

We modelled a multiple linear regression analysis to define the independent determinants of FMD%. Age, systolic and diastolic blood pressure, total cholesterol, triglyceride, LDL cholesterol, HDL cholesterol and white blood cell counts were incorporated into the model as independent variables, in addition to 8-OHdG/dG ratios, MDA, SOD and GPx levels. The R² of the model was 0.679 with P < 0.001. The linear regression model revealed that 8-OHdG/dG was significantly and negatively correlated with FMD%. In addition, regression coefficient for DNA = −0.464 (P < 0.001) (Table 3). Of note, age, systolic and diastolic blood pressure, total cholesterol, triglyceride, LDL cholesterol, HDL cholesterol and white blood cell counts were not independent determinants of FMD%.

Discussion

In this study, we assessed the relationship between oxidative DNA damage (8-OHdG/dG ratio), oxidative stress biomarkers, antioxidant enzymes and the presence of early sub-clinical atherosclerosis in humans determined

### Table 1. Clinical and demographic characteristics of study groups

<table>
<thead>
<tr>
<th></th>
<th>HD group (n = 44)</th>
<th>Control group (n = 55)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41 ± 09</td>
<td>41 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>21/23</td>
<td>27/28</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.06 ± 4.23</td>
<td>25.01 ± 4.01</td>
<td>NS</td>
</tr>
<tr>
<td>Dialysis duration (months)</td>
<td>44 ± 28</td>
<td>23.02 ± 2.13</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>129.50 ± 23.02</td>
<td>123.64 ± 16.00</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75.05 ± 12.00</td>
<td>73.00 ± 8.06</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>95.20 ± 10.00</td>
<td>98.40 ± 12.00</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>90.40 ± 17.40</td>
<td>84.59 ± 13.20</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>196.20 ± 38.40</td>
<td>181.10 ± 27.80</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>105.70 ± 26.10</td>
<td>109.45 ± 25.00</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>35.20 ± 12.48</td>
<td>42.50 ± 17.64</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>172.50 ± 65.00</td>
<td>160.58 ± 58.40</td>
<td>NS</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>276.40 ± 54.50</td>
<td>42.50 ± 23.20</td>
<td>&lt;0.05</td>
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</tbody>
</table>

*Data are presented as mean ± SD. BMI, body mass index; PTH, parathormone.

### Table 2. Oxidative stress parameters of study groups

<table>
<thead>
<tr>
<th></th>
<th>HD group (n = 44)</th>
<th>Control group (n = 55)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OHdG/dG</td>
<td>2.39 ± 1.14</td>
<td>0.63 ± 0.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td>10.31 ± 5.63</td>
<td>4.18 ± 1.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SOD (EU/mL)</td>
<td>5.89 ± 1.94</td>
<td>11.82 ± 6.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GPx (EU/mL)</td>
<td>18.37 ± 16.96</td>
<td>72.91 ± 28.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Baseline diameters of artery (mm)</td>
<td>4.32 ± 0.35</td>
<td>4.22 ± 0.49</td>
<td>NS</td>
</tr>
<tr>
<td>FMD%</td>
<td>7.28 ± 0.79</td>
<td>11.18 ± 0.82</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.
by FMD%. The main finding of this study is the demonstration of a negative correlation between FMD% and 8-OHdG/dG ratio in HD patients.

Oxidative stress has been implicated in the pathogenesis of atherosclerosis, and there is mounting evidence for the presence of disordered oxidative and glycoxidative chemistry in patients who undergo HD which may contribute to poor cardiovascular and global outcome [25, 26]. DNA, in particular, is more susceptible to attack by reactive oxygen species than proteins and membrane lipids, which are protected by low-molecular weight antioxidants and antioxidant enzymes. Among many types of oxidative DNA damage, 8-OHdG is one of the most abundant oxidative products of cellular DNA [27].

We found that HD patients without known atherosclerotic disease have endothelial dysfunction assessed by FMD% and that it is negatively correlated with 8-OHdG/dG ratio. It was previously shown that peripheral blood leukocytes of HD patients are suitable for monitoring 8-OHdG in cellular DNA because they are not only the source but also the target of endogeneous reactive oxygen species [26, 28]. It was reported that leucocyte 8-OHdG levels are highest among HD patients, which is followed by chronic renal failure patients who have not yet received dialysis and then by healthy subjects [26]. Furthermore, leucocyte 8-OHdG levels are also higher in patients who undergo HD using cellulose membranes compared with those who undergo HD using synthetic membranes or vitamin E-bonded membranes; therefore, leucocyte 8-OHdG is considered to act as a surrogate marker of oxidant-induced DNA damage among HD patients [26, 28].

![Fig. 1.](image1.png) **Fig. 1.** (A) Correlation between FMD% and 8-OHdG/dG ratio. (B) Correlation between FMD% and serum MDA levels.

![Fig. 2.](image2.png) **Fig. 2.** (A) Correlation between FMD% and serum GPx levels. (B) Correlation between FMD% and serum SOD levels.
8-OHdG levels were studied in patients who undergo HD using synthetic membranes and FMD% was used as a marker of sub-clinical atherosclerosis in patients without overt clinical atherosclerosis. This study provides, for the first time, evidence that oxidative DNA damage, in terms of 8-OHdG/dG ratio, does exist in HD patients with evidence of sub-clinical atherosclerosis.

Cangemi et al. [29] reported that 8-OHdG and FMD are inversely correlated in patients with the metabolic syndrome and vitamin C improved endothelial dysfunction and oxidative stress. Engler et al. [30] also reported that 6 weeks of treatment with antioxidant vitamins C and E improve endothelial function in children with familial hyperlipidaemia without an effect on urine 8-OHdG levels. The authors suggested that the combination of vitamins C and E has been shown to protect endothelial cells from cytotoxic effects of oxidized LDL [30]. To the best of our knowledge, no previous report for correlation between FMD% and 8-OHdG/dG ratio in HD patients exists in the literature. It is important to consider that oxidative DNA damage may change genes and result in problems for offspring of those with DNA damage. In this study, we demonstrated that 8-OHdG/dG ratio may be a good biomarker for risk assessment of accelerated atherosclerosis in HD patients.

MDA is an end product of lipid peroxidation of membrane polyunsaturated fatty acids by free radicals and is an indicator of oxidative damage. The second novel finding of the study is the negative correlation between FMD% and serum MDA levels. Higher MDA levels indicate increased production of reactive oxygen species in HD patients compared to healthy controls. Our results are particularly different from the following data in the current literature [17, 31]. The relatively higher serum MDA levels in our study population may have resulted from differences between demographic characters of the study groups such as younger patient age and lengthy dialysis duration. Previous reports have shown that oxidative stress is associated with cardiovascular morbidity and mortality in HD patients [32, 33]. In our study, we found that oxidative stress is negatively correlated with FMD%, which supports the role of oxidative stress in the development of accelerated atherosclerosis in these patients.

Antioxidants (SOD, GPx) are compounds that dispose, scavenge and suppress the formation of free radicals or oppose their actions. We found a decrease in SOD and GPx levels in HD patients compared to healthy controls and there is a positive correlation between FMD% and serum SOD and GPx activities. Our data indicate that HD patients have an impaired antioxidant response, particularly due to antioxidant enzyme deficiency. Our data suggests that oxidative stress and disturbances in antioxidant enzymes may facilitate the development of endothelial function abnormalities; and oxidative DNA damage may occur before development of overt atherosclerosis.

Due to the cross-sectional design of the study, the results should be interpreted with caution and causal relationship cannot be suggested. It would be interesting to assess the evolution of oxidative DNA damage and endothelial function in a long-term follow-up study and analyse the possible relationship with the clinical atherosclerosis of chronic renal failure patients over time.

As a conclusion, present data show that HD patients without known atherosclerotic disease have increased levels of genetic damage, which correlates with oxidative stress and endothelial dysfunction. Further prospective, randomized controlled studies are needed to determine the possible beneficial effects of antioxidant therapy on oxidative DNA damage and endothelial function abnormalities among HD patients.

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Conflict of interest statement. None declared.

References

Table 3. Regression analysis for defining the independent determinants of FMD% (*).

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>β (coefficient)</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OHdG (nmol/L)</td>
<td>−0.464</td>
<td>0.128</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>−0.078</td>
<td>0.030</td>
<td>0.012</td>
</tr>
<tr>
<td>SOD (EU/mL)</td>
<td>0.074</td>
<td>0.027</td>
<td>0.007</td>
</tr>
<tr>
<td>GPx (EU/mL)</td>
<td>0.019</td>
<td>0.005</td>
<td>&lt;0.001</td>
</tr>
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* R² = 67.9%, P < 0.001.

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