Oxidative Stress and Asymmetric Dimethylarginine Are Associated with Cardiovascular Complications in Hemodialysis Patients: Improvements by L-Arginine Intake

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Key Words
Chronic kidney disease · Oxidative stress · Hemodialysis · L-Arginine · Asymmetric dimethylarginine · Homocysteine

Abstract
Background/Aim: High incidence of cardiovascular disease (CVD) in chronic kidney disease (CKD) patients is a result of an interlaced relation between oxidative stress, endothelial dysfunction (ED) and inflammation. This study tries to investigate the development of these processes in CKD patients receiving conservative treatment or on hemodialysis (HD). We also examined the modulating effect of oral L-arginine in HD patients having CVD. Methods: The study included 12 healthy volunteers and 63 renal patients divided into 15 renal impairment, 18 HD free of CVD, and 30 HD suffering from CVD (HD+CVD). Of the latter, 15 patients were given oral L-arginine (15 g/day, 5 g t.i.d.) for 1 month. Blood levels of asymmetric dimethylarginine (ADMA), malondialdehyde (MDA), and homocysteine and myeloperoxidase activity (MPO) were estimated. Results: ADMA, MDA and homocysteine were significantly elevated in renal impairment group. HD and HD+CVD patients experienced higher levels, along with high MPO activity. Significant reduction by 21, 46, 11, and 26%, respectively, in the aforementioned parameters was observed in HD+CVD patients following L-arginine intake. Conclusion: We recommend considering ADMA, MDA, homocysteine and MPO as potentially important cardiovascular risk factors in CKD patients, and focus the attention to the cardiovascular advantages of L-arginine in these patients.

Introduction

Over the past two decades, despite the great technical advances in the management of chronic kidney disease (CKD), little progress has been made in the overwhelming problem of cardiovascular disease (CVD) in this group of patients [1]. Recently, oxidative stress, defined as the imbalance between formation of reactive oxygen species (ROS) and antioxidative defense mechanisms, has been implicated as an important etiologic factor in atherogenesis both in the general and uremic populations [2].

Endothelial dysfunction (ED) due to reduced bioavailability of nitric oxide (NO) is a key initial event in the development of atherosclerosis, and recently has been demonstrated in CKD patients. An increasing body of evidence suggests that oxidative stress accounts for a significant proportion of ED through overproduction of ROS which may cause the degradation of NO [3]. Additionally, NO production can be reduced by inhibition of
endothelial NOS activity (eNOS). Nitric oxide, which is synthesized from the amino acid precursor l-arginine by the action of the constitutive enzyme, eNOS, inhibits key processes of atherogenesis such as monocyte adhesion, platelet aggregation and vascular smooth muscle cell proliferation [4].

Vallance et al. [5] reported for the first time increased plasma concentration of an endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), in patients with CKD. Subsequent studies have shown that accumulation of ADMA may contribute to the high cardiovascular (CV) risk in patients with CKD [4, 6]. ADMA per se seems responsible for 52 and 34% increase in the risks of death and CV events, respectively, in dialysis patients [6]. Intake of high doses of the NO precursor, l-arginine, may constitute an interesting opportunity for the intervention on this putative CV risk factor. Beneficial effects of l-arginine supplementation have been reported in several models of chronic kidney disease including renal ablation, urethral obstruction, nephropathy secondary to diabetes, and salt-sensitive hypertension. However, the role of l-arginine in the pathogenesis and treatment of renal disease is not completely understood and remains to be established [7].

Hyperhomocyst(e)inemia has been recognized as an independent risk factor for atherosclerotic vascular disease [8]. Although the mechanism remains controversial, several studies suggest that homocysteine increases oxidative stress by stimulating production of ROS and inhibiting expression or function of key antioxidant enzymes such as superoxide dismutase [9].

It has been increasingly apparent that CKD patients exhibit an inflammatory burden that may play a pivotal role in the massive increase in the relative risk of CVD. Treatment of CKD patients with hemodialysis (HD) has been suggested to contribute to the development of oxidative stress through the activation of macrophages on the surface of dialysis membranes during the dialysis session [10]. Myeloperoxidase (MPO) is a heme protein released by activated leukocytes in response to inflammatory stimuli. MPO causes catalytic consumption of NO through the generation of diffusible oxidants and hence, limiting its bioavailability and function [11]. Thus, increased MPO activity could serve as one mechanistic link between inflammation, oxidative stress and ED in CKD [12].

In conclusion, this study was undertaken to test the correlation of several biomarkers with the development of cardiovascular complications in renal failure patients. Additional information about the influence of HD and l-arginine administration were investigated.

Methods

Patients

The study population comprised 75 male subjects aged 45–66 years. Twelve of them were healthy volunteers serving as the control group. Sixty-three patients with CKD were recruited through the Department of Nephrology in Maadi and Kobry El-Obba Military Hospitals, Cairo, Egypt. Detailed medical history and drug treatments were obtained for all subjects. Subjects with diabetes mellitus, liver disease, and history of metabolic or other serious concomitant disease were excluded from the study. All study participants were nonsmokers. The study protocol was approved by the local university ethical committee and informed consent was obtained from all subjects in accordance with the principles of the Helsinki Declaration.

CKD patients comprised 15 nondiabetes patients on conservative treatment (renal impairment group) and 48 patients on maintenance HD, of whom 18 patients were free of CVD (HD group) and 30 patients suffered from CVD (HD+CVD group). CV events were defined as: acute myocardial infarction diagnosed by typical clinical and ECG changes, angina pectoris based on typical clinical characteristics or transitory ischemic events verified by echocardiography. Fifteen patients of the HD+CVD group were given oral l-arginine (15 g/day, 5 g t.i.d.) for 1 month [13]. Hemodialysis was performed three times per week; each session lasting for 4 h.

Sample Collection

Blood samples (5–10 ml) were collected in the morning after overnight fasting. For HD patients, blood samples were collected from arteriovenous fistula before the beginning of the first dialysis session of the week. Samples were divided into two parts: one part was added to Na2 EDTA-containing tubes for ADMA and homocysteine assays following plasma separation by centrifugation at 1,500 rpm for 15 min immediately after collection. The other part was added to vacutainer clotted tubes, where sera were separated within 30 min by centrifugation at 4,000 rpm, for 15 min, at 4°C (for MDA and MPO assays). Plasma and sera samples were kept frozen in aliquots at –80°C until assayed.

Assays

Before storage, sera aliquots were used for the measurement of creatinine, urea, total cholesterol (TC) and triacylglycerol (TAG) by standard enzymatic techniques using commercially available kits. HDL-C was determined after the precipitation of apolipoprotein B-containing lipoproteins. LDL-C was calculated according to Friedewald equation [14]. All spectrophotometric measurements were done by UV/Visible spectrophotometer Shimadzu, model no. 1650, USA.

MDA Measurement

Levels of MDA, an indicator of lipid peroxidation, were determined as thiobarbituric acid-reactive substances (TBARS) following a protocol described previously by Uchiyama and Miura [15].

MPO Determination

The method of measuring serum MPO activity was adopted from the reports of Manktelow and Meyer [16] and Grisham et al. [17]. Briefly, 0.2 ml of serum was mixed with 0.5 ml of phosphate-
buffered saline and 0.6 ml of Hanks’ balanced salt solution containing 0.25% bovine serum albumin. Then, 0.1 ml of 0.125% of dimethoxybenzidine (DMB) and 0.1 ml of 0.05% of hydrogen peroxide were added and vortexed. The reaction mixture was allowed to stand at room temperature for 15 min, and the enzymatic reaction was terminated by the addition of 0.5 ml of 1% sodium azide. DMB oxidized by MPO and hydrogen peroxide was measured spectrophotometrically at 460 nm. MPO activity was expressed as U/l.

ADMA and Homocysteine Determination
Plasma ADMA levels were determined by a validated ELISA technique using a kit purchased from DLD Diagnostika, Germany [18]. Plasma homocysteine levels were assayed using an ELISA kit purchased from Diazyme Laboratories, USA. All ELISA procedures were done by Hyprep® automated ELISA system, USA, according to the instructions of the manufacturer.

Statistical Analysis
All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 9 software. Data were represented as mean ± SD. Differences between groups were compared using a one-way analysis of variance (ANOVA) followed by LSD post-hoc analysis. A p value <0.05 was considered statistically significant. Effect of oral L-arginine administration in the HD+CVD group was analyzed by the paired-samples t test. Pearson correlation coefficient was used to determine the correlation between different parameters.

Results
Baseline Parameters
The age of the patient groups was comparable but significantly different from the normal control. The duration of dialysis was significantly higher for patients in HD+CVD group than those for patients in HD group, reaching almost 2-fold increase.

Serum creatinine and urea levels were significantly elevated in renal impairment patients when compared to healthy controls and dramatically elevated in HD patients with or without CVD reaching 350–400% of the corresponding levels of the renal impairment group. There were no significant differences in the serum creatinine and urea levels between HD and HD+CVD groups.

With regard to the lipid profiles (TC, TG, HDL-C and LDL-C), the differences between the renal impairment and control groups were nonsignificant for all parameters. In contrast, the profiles were significantly elevated in HD+CVD patients when compared to control and renal impairment groups with the exception of HDL-C level which was significantly lower. A detailed description of the baseline characteristics of the studied groups is depicted in table 1.

<table>
<thead>
<tr>
<th>Groups/parameters</th>
<th>Control</th>
<th>Renal impairment</th>
<th>HD</th>
<th>HD+CVD</th>
</tr>
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<tbody>
<tr>
<td>Age, years</td>
<td>49.15 ± 1.9</td>
<td>55.9 ± 6.3a</td>
<td>53.4 ± 6.9a</td>
<td>57.77 ± 3.9a</td>
</tr>
<tr>
<td>Duration of dialysis, years</td>
<td>none</td>
<td>none</td>
<td>4.36 ± 1.8</td>
<td>8.2 ± 2.9c</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.87 ± 0.13</td>
<td>2.72 ± 0.9a</td>
<td>10.2 ± 1.5a</td>
<td>9.67 ± 1.7a</td>
</tr>
<tr>
<td>Urea, mg/dl</td>
<td>31.22 ± 3.9</td>
<td>81.9 ± 28.3a</td>
<td>127.27 ± 23.58a, b</td>
<td>125 ± 19a, b</td>
</tr>
<tr>
<td>TC, mg/dl</td>
<td>144.3 ± 16.9</td>
<td>150.2 ± 27</td>
<td>154.77 ± 22.3</td>
<td>163.92 ± 6.5a</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>145.4 ± 29.6</td>
<td>110.65 ± 33.3</td>
<td>134.14 ± 34.8a, b</td>
<td>143.72 ± 4.4a</td>
</tr>
<tr>
<td>HDL-C, mg/dl</td>
<td>44.5 ± 4.2</td>
<td>41.67 ± 7.6</td>
<td>39.22 ± 6.3a</td>
<td>31.42 ± 5.4a, c</td>
</tr>
<tr>
<td>LDL-C, mg/dl</td>
<td>78.5 ± 15.7</td>
<td>95.4 ± 25.5</td>
<td>87.5 ± 19.1</td>
<td>103.75 ± 24.8a, c</td>
</tr>
</tbody>
</table>

a, b, c Significant difference from healthy controls, renal impairment group, and HD group, respectively, at p < 0.05. Data are presented as mean ± SD.
The differences in MPO levels from control levels were significant only in the patients on hemodialysis. 25% increase in MPO activity was observed in the group with CV complications as compared to the HD group without complications.

Regarding plasma ADMA levels, gradual, but significant, increase was observed in all patient groups when compared to the normal control. The elevation reached 198, 302, and 348% of normal control levels for the renal impairment, HD, and HD+CVD groups, respectively. The differences among renal impairment, HD, and HD+CVD were also significant, complying with the severity of the renal failure status and duration of hemodialysis. The same pattern was observed for homocysteine plasma levels but with lower increase. The percentage elevations in renal impairment, HD, and HD+CVD were 159, 173, and 195% of the mean control value

**Effect of L-Arginine Administration**

Following 1 month of L-arginine treatment for a group of HD+CVD patients, significant reduction in oxidative stress and ADMA levels were observed in comparison with the corresponding pretreatment levels. The decrease was variable ranging from 46% in MDA to 26% in MPO, 21% in ADMA, and 11% in homocysteine levels. These changes are demonstrated in figure 2.

**Correlation Data**

Evaluation of the correlation coefficients of the biomarkers in CKD patients, comprising renal impairment, HD, HD+CVD groups, revealed a positive and significant associations of ADMA with MDA (fig. 3a), ADMA with MPO (fig. 3b), ADMA with homocysteine (fig. 3c), MPO with MDA (fig. 3d), and MPO with homocysteine (fig. 3e). The correlation between MDA and homocysteine in these groups of patients was not significant.

**Discussion**

The incidence of CVD is remarkably noticeable in CKD patients and accounts for the principal cause of death in this group. Among several classical and nonclassical risk factors, oxidative stress, ED and inflammation have emerged as important mechanisms conferring increased CVD risk.

The results of this study demonstrate the presence of oxidative stress among all CKD groups as represented by the elevation of the lipid peroxidation marker, MDA. The latter finding is consistent with earlier studies in animals and humans suffering from CKD and confirms the increased ROS activity in renal insufficiency [19]. There is good evidence indicating that uremia per se is associated with enhanced oxidative stress, and treatment of uremic
patients with dialysis has been suggested to particularly contribute to oxidative stress and reduced antioxidant levels in these patients [9]. This agrees with the results of the present study that showed a significant increase in MDA levels in HD patients with or without CVD when compared to renal impairment patients. Moreover, the HD+CVD group exhibited higher MDA levels than the HD group suggesting the involvement of oxidative stress in the development of atherosclerotic vascular disease in CKD.

Additionally, the study revealed a marked elevation in MPO activity in CKD patients treated by HD. MPO is released from neutrophils in response to inflammatory stimuli. Many aspects of dialysis treatment contribute to inflammation such as vascular access, back leak of dialysate and exposure of blood to nonbiological surfaces [20]. Moreover, other data suggested that the reduction of renal function per se may be associated with an inflammatory response [21]. The higher levels of MPO in HD+CVD group in comparison with HD group may well agree with a previous study that identified high MPO serum level as a strong predictor for acute coronary disease syndromes [11]. MPO may contribute to CVD by promoting ED through the generation of diffusible oxidants causing catalytic consumption of NO [22]. In support, positive and significant correlation between MPO and MDA was revealed. This is consistent with Zhang et al. [23] who reported that MPO serves as a major enzymatic catalyst of lipid peroxidation at sites of inflammation, and thereby promoting atherogenesis. These findings may link inflammation to increased oxidative stress and ED in CKD.

CKD patients demonstrate markedly elevated ADMA levels compared to healthy controls. ADMA is excreted by the kidneys [5]; however, more recent data showed that this accounts only for about 20% of ADMA elimination, whereas the major metabolic pathway is degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyses ADMA into dimethylamine and L-citrulline, accounting for 80% of ADMA elimination [24]. It is thus highly suggestive that elevated ADMA levels in renal disease may be due to impaired ADMA degradation by DDAH rather than reduced renal excretion [25]. The positive association between ADMA and MDA as well as ADMA and MPO demonstrated in this study suggests a possible relation between ADMA accumulation and oxidative stress. DDAH contains a sulfhydryl group that is critical for its activity, and this group is very sensitive to oxidative stress [26, 27]. Hence, oxidative stress in the CKD patients could attenuate DDAH activity, causing ADMA to accumulate and to block NO synthesis. Moreover, elevated ADMA levels may contribute
to the development of oxidative stress possibly by uncoupling eNOS, resulting in NOS-derived oxygen-derived free radical formation.

Positive correlation was also demonstrated between ADMA and homocysteine. Homocysteine could mount an oxidative attack on DDAH to form a mixed disulfide, inactivating the enzyme, leading to accumulation of ADMA [26, 27]. All groups of CKD patients had higher homocysteine plasma levels than control subjects. This is possibly due to altered homocysteine metabolism as a result of uremia rather than to decreased renal excretion. The elevated plasma homocysteine levels in HD+CVD suggests a role for hyperhomocysteinemia in the CKD-associated CVD. More than one mechanism can be inferred; for example, several studies suggest that homocysteine is associated with ED and increased oxidative stress. Others referred to the ability of homocysteine to generate hydrogen peroxide and superoxide anion through the formation of disulfides and thereby increase oxidative degradation of NO [27]. Finally, methyl groups, generated during the biosynthesis of homocysteine from methionine, may be transferred to L-arginine, resulting in the formation of ADMA [28].

It is becoming increasingly apparent that impaired NO bioavailability, by oxidative stress, elevated ADMA or ED, is an important mechanism conferring increased CV risk in CKD. Hence investigation of the effect of L-arginine, the precursor of NO biosynthesis in HD+CVD patients became one of our aims. Results supported our speculation by the significant improvement of all biochemical risk parameters studied when compared to pretreatment levels. This finding are in support of Lin et al. [29] who found that L-arginine supplementation can prevent elevations in MPO activity and favorably influences pulmonary antioxidant defense systems. While L-arginine itself does not result in diminished ADMA formation, the reduced ADMA levels after L-arginine treatment may have been the result of decreased oxidative stress and/or homocysteine levels. This may explain the previous findings that L-arginine significantly improved endothelial function and flow-dependent vasodilatation in patients with peripheral arterial occlusive disease [30]. Beneficial effect of L-arginine intake in renal failure has been deduced from a number of studies including Tome et al. [31], Sanders [32], and Wakabayashi and Kikawada [33].

In conclusion, the salient findings of our study are as follows:

(1) Markers of endothelial dysfunction, inflammation and oxidative stress (ADMA, homocysteine, MDA and MPO) are well correlated to the presence of CVD in CKD patients.

(2) Oxidative stress and ED persist or are even aggravated under dialysis treatment.

(3) CKD patients with established CVD complications have higher values of the risk parameters (ADMA, homocysteine, MDA and MPO).

(4) Oral L-arginine administration improves oxidative stress and ED in HD patients suffering from CVD.

Our main recommendations are to further evaluate the role of ED, oxidative stress and inflammation in the CVD-associated CKD and their management regimens and to consider L-arginine supplementation as a reasonably safe and tolerable therapy that can modulate the atherogenic profile of renal patients.

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References


