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Effects of amlodipine and valsartan on oxidative stress and plasma methylarginines in end-stage renal disease patients on hemodialysis

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Patients with end-stage renal disease (ESRD) receiving hemodialysis (HD) treatment have a markedly shortened life expectancy in large part owing to cardiovascular disease (CVD), not explained by established risk factors. We tested the hypothesis that therapy with valsartan, an angiotensin receptor blocker and amlodipine, an antioxidant calcium channel blocker will reduce oxidative stress and the plasma levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase. We confirmed that compared with age- and gender-matched healthy controls, ESRD patients have excessive oxidative stress and arginine methylation as indexed by elevated plasma levels of oxidation products of lipids (13-hydroxyoctadecadienoic acid (13-HODE)), thiols (oxidized:reduced glutathione, oxidized glutathione (GSSG):GSH), proteins, and nucleic acids, and the methylation products ADMA and symmetric dimethylarginine (SDMA). We undertook a double blind, crossover study of equi-antihypertensive treatment with amlodipine and valsartan for 6 weeks each to test our hypothesis. Both treatments significantly reduced GSSG:GSH, 8-hydroxy 2-deoxyguanosine, ADMA, and SDMA levels and amlodipine reduced 13-HODE. We conclude that hypertensive patients with ESRD receiving HD have evidence of extensive oxidation of lipids, thiols, proteins, and nucleic acids and methylation of arginine that could contribute to CVD. Many of these changes can be reduced by short-term treatment with amlodipine and valsartan.

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Cardiovascular disease (CVD) accounts for over 50% of the annual mortality of patients with end-stage renal disease (ESRD) on maintenance dialysis.[1] The prevalence of the traditional cardiovascular risk factors, diabetes mellitus, hypertension, smoking, and dyslipidemias, is high in this patient population.[2] However, the HEMO study concluded that systolic blood pressure, smoking, or male gender were not associated with an increased risk of CVD, whereas diastolic blood pressure was actually inversely related.[3] Hypercholesterolemia was associated with an increased risk of coronary artery disease but not cerebrovascular or peripheral vascular disease. More recently, a controlled trial of lipid lowering with atorvastatin in hypercholesterolemic diabetic patients on hemodialysis (HD) reported no beneficial effect on cardiovascular events.[4] These findings have prompted an interest in nontraditional risk factors to explain the epidemiology of CVD in ESRD population. These risk factors include oxidative stress,[5] abnormal calcium and phosphate metabolism,[6] hyperhomocysteinemia,[7] malnutrition and inflammation syndrome, and the accumulation of the nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA).[8] ADMA is an independent predictor of endothelial dysfunction and CVD both in patients with ESRD and the general population. It is associated with a three-fold increased risk of future severe cardiovascular events and mortality in patients on HD[9] and a four-fold increased risk of acute coronary events in clinically healthy, nonsmoking men.[10] In patients with chronic kidney disease, ADMA is a strong predictor of progression of chronic kidney disease and mortality.[11,12] Therefore, it represents a novel therapeutic target for clinical interventions. The pathogenesis of elevated ADMA levels is not understood in patients with ESRD. Data from experimental models suggest that oxidative stress increases the activity of protein arginine methyltransferases (PRMTs)[13] which methylate arginine moieties on proteins and inhibits N^G, N^G-dimethylarginine-dimethylaminohydrolase (DDAH) activity[14] which specifically metabolizes ADMA. However, effects of oxidative stress reduction on elevated plasma levels of methylarginines in chronic kidney disease are not known.

Oxidative stress is the outcome of complex interactions between oxidants and antioxidant defense pathways that damage thiols, lipids, proteins, and nucleic acids. Although the presence of oxidative stress is well documented in patients with chronic kidney disease,[15,16] there is currently no consensus on a single ideal marker of oxidative stress. Furthermore, effects of antihypertensive therapy on oxidative stress have not been studied prospectively in ESRD patients. Oxidation products of lipids, proteins, thiols, and DNA are the most commonly measured parameters in clinical studies.[15–19] 13-Hydroxyoctadecadienoic acid (13-HODE) is a linoleic acid peroxidation product generated by the action of 15-lipoxygenase and by non-enzymatic oxidation. It is a stable parameter of lipid peroxidation that has been implicated in the pathogenesis of atherosclerosis.[20] Although two trials of antioxidants reported a reduction in CVD events in HD patients, these trials did not measure the markers of oxidative stress or inflammation, thereby making their interpretation somewhat uncertain.[21,22]

Treatment of HD patients with antagonists of renin–angiotensin system reduces oxidative stress in observational studies.[23] Angiotensin II acting on AT_1 receptors increases superoxide generation in part by activating nicotinamide adenine dinucleotide phosphate oxidase.[24] The dihydropyridine calcium channel blocker (CCB) amlodipine exerts strong antioxidant actions *in vitro* in lipid-laden cell membranes independent of its CCB action,[25] but the relevance of this to treatment of oxidative stress *in vivo* has not been studied in ESRD patients. Although the antagonists of the renin-angiotensin system and CCBs are the most widely prescribed antihypertensive agents in patients on maintenance HD,[26] their antioxidant properties have not been prospectively studied. Furthermore, the effects of these antioxidant agents on methylarginine metabolism have not been explored in this population. Therefore, we hypothesized that in stable ESRD patients on maintenance HD, valsartan (an angiotensin receptor blocker) and amlodipine (a CCB) will lower oxidative stress, and that this will be associated with a reduction in the plasma levels of ADMA independent of blood pressure lowering. This was investigated in a double-blind crossover trial of equally antihypertensive therapy with these two agents.

RESULTS

A total of 30 patients were enrolled. Twenty-three patients completed the study; seven patients withdrew from the study owing to consent withdrawal (three), transfer to another facility (one), and hospitalization unrelated to the study (three). Four patients were excluded from the final analysis owing to administration of intravenous iron during one of the study phases. This left 19 patients who comprised the study population. They were matched with an equal number of healthy controls. The baseline characteristics of the patients and the controls are shown in Tables 1 and 2. There were no current smokers in either group. The maximum dose of valsartan (320 mg daily) and amlodipine (10 mg daily) was achieved in all study subjects.

After 6 weeks of therapy, both amlodipine and valsartan lowered 13-HODE by 29.7 and 14.4% ($P = 0.09$ for valsartan), 8-hydroxy 2-deoxyguanosine by 21 and 19.5%, ADMA by 38.1 and 39.2%, SDMA by 41.6 and 37.5%, and GSSG:GSH by 40 and 79%, respectively (Table 3). Neither treatment had any significant effect on plasma protein carbonyl content, high-sensitivity C-reactive protein, or L-arginine. A total of 10 patients received valsartan first and nine patients received amlodipine first. Data were also analyzed according to the order in which the study drugs were administered and we detected no carry-over effect. Additional antihypertensive drugs were used in all three phases of the study to maintain the target blood pressure level. During the amlodipine and valsartan phases, the drugs used in the run-in period were withdrawn as required to maintain a stable target BP, whereas the study drugs were titrated up to full dose. Drugs used in the run-in, amlodipine, and valsartan phases of the study were as follows: clonidine in

Table 1 | Patient characteristics

Variable	Patients (n=19)	Controls (n=19)	P-value
Age (years)	57.9±27	46.5±6	0.07
Gender	F=12 M=8	F=14 M=6	— —
Time on dialysis (months)	46±31	—	—
Etiology of ESRD			
Hypertension	12	—	—
Diabetes mellitus	4	—	—
HIV-AN	2	—	—
Lupus nephritis	1	—	—
Unknown	1	—	—

AN, associated nephropathy; ESRD, end-stage renal disease; F, female; HIV, human immunodeficiency virus; M, male.

Table 2 | Biomarkers of oxidative stress and L-arginine metabolism in patients with ESRD on maintenance HD and healthy controls

Parameter	Controls (n=19)	ESRD patients (n=19)	P-value
Plasma			
13-HODE (nmol/ml)	210.4±15.9	428.3±26.8	<0.0001
Protein carbonyl (ng/mg)	0.07±0.01	0.17±0.01	<0.0001
8-HO2dG (ng/ml)	1.88±0.14	2.85±0.22	0.0013
HS-CRP (mg/l)	0.07±0.008	0.84±0.36	0.034
L-arginine (μmol/l)	95.3±3.2	86.7±4.8	0.15
ADMA (μmol/l)	0.59±0.03	1.94±0.12	<0.0001
SDMA (μmol/l)	0.65±0.04	3.2±0.3	<0.0001
Whole blood			
GSSG:GSH (×100)	1.23±0.54	2.9±1.2	0.04

ADMA, asymmetric dimethylarginine; ESRD, end-stage renal disease; GSH, glutathione; HD, hemodialysis; 13-HODE, 13-hydroxyoctadecadienoic acid; 8-HO2dG, 8-hydroxy 2-deoxyguanosine; HS-CRP, high-sensitivity C-reactive protein; SDMA, symmetric dimethylarginine.

Table 3 | Effects of therapy with amlodipine and valsartan on parameters of oxidative stress and methylarginines in 19 patients on HD

Parameter	Baseline	Amlodipine	Valsartan	*P*-value (ANOVA)
Plasma				
13-HODE (nmol/ml)	428.3±26.8	301.0±14.0*	366.8±15.8	0.002
Protein carbonyl (ng/mg)	0.17±0.01	0.16±0.01	0.17±0.01	0.81
8-HO2dG (ng/ml)	2.97±0.22	2.34±0.2*	2.39±0.15*	0.028
HS-CRP (mg/l)	0.84±0.36	1.4±0.92	1.33±0.5	0.83
L-arginine (μmol/l)	86.7±4.8	78.2±4.8	79.3±5.5	0.4
ADMA (μmol/l)	1.94±0.12	1.2±0.16*	1.18±0.1*	<0.0001
SDMA (μmol/l)	3.2±0.3	1.87±0.24*	2.0±0.23*	0.001
Whole blood				
GSSG:GSH (×100)	2.9±3.1	1.8±1.3*	0.6±0.1*	0.02

ADMA, asymmetric dimethylarginine; ANOVA, analysis of variance; GSH, glutathione; HD, hemodialysis; 13-HODE, 13-hydroxyoctadecadienoic acid; 8-HO2dG, 8-hydroxy 2-deoxyguanosine; HS-CRP, high-sensitivity C-reactive protein; SDMA, symmetric dimethylarginine.
*Significant at $P<0.05$.

four, two, and two patients, labetalol in 12, 12, and 10 patients, and doxazosin in one, one, and one patient, respectively and were not statistically different. Blood pressure measured before HD three times a week was strictly similar during the run-in and intervention phases (Figure 1). Sixteen patients completed the 44-h ambulatory blood pressure monitoring. This confirmed that there was no difference in systolic (139±5.9 vs 137±5.9 mmHg), diastolic (80±3.7 vs 77.6±3.0 mmHg), or mean arterial pressure (102±3.8 vs 99.3±3.4 mmHg) between amlodipine and valsartan therapy, respectively (Figure 2).

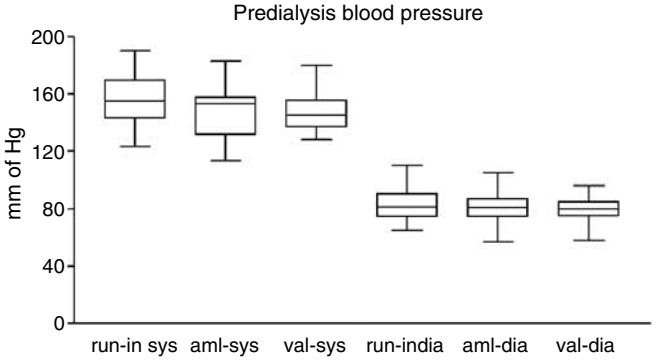

Figure 1 | Results of in-center pre-HD blood pressure. (sys, systolic; dia, diastolic; aml, amlodipine; val, valsartan).

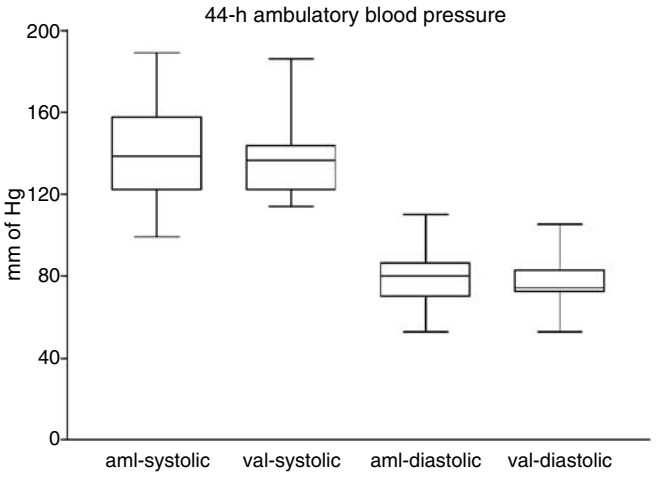

Figure 2 | Results of 44-h interdialytic ambulatory blood pressure monitoring. (aml, amlodipine; val, valsartan).

DISCUSSION

We confirmed previous reports of oxidative stress and elevated plasma levels of ADMA and SDMA in patients with ESRD.[15–19] We detected widespread oxidation of all major biomolecules (lipids, protein, thiols, and DNA). The main new finding is that equi-antihypertensive therapy with amlodipine and valsartan led to significant reductions in many parameters of oxidative stress, and in plasma levels of ADMA and SDMA.

We report 13-HODE as a new marker of lipid peroxidation in this patient population. High levels of 13-HODE cholesterol esters are found in atherosclerotic plaques in humans atheromas[20] where it may have a pathogenetic role. Linoleic acid is the most abundant polyunsaturated fatty acid in phospholipids including low-density lipoprotein, where its abundance is about seven times that of arachidonic acid.[27] Furthermore, it is the predominantly oxidized polyunsaturated fatty acid in phospholipids.[28] Oxidation of phospholipids is an early event in the pathogenesis of atherosclerosis. Once oxidized, phospholipids are recognized by the CD36 scavenger receptors on macrophages and smooth muscle cells[29] and their uptake by these cells results in the formation of foam cells. Low concentrations of oxidized phospholipids stimulate the proliferation of smooth muscle cells and high concentrations increase ceramide and smooth muscle cell apoptosis[30] and release cytochrome *c* from the mitochondria.[31] These events may contribute to the formation of a necrotic core in the plaque. Thus, oxidized phospholipids are involved in several steps of atherogenesis. Endothelial cells also synthesize 9- and 13-HODE.[32] We found that amlodipine was more effective in lowering plasma levels of

13-HODE than valsartan. Amlodipine is highly lipophilic and accumulates in cell membranes, where it can inhibit lipid peroxidation independent of its action as CCB.[25] Within the phospholipid bilayer of the cell membrane, the charged amino pole of amlodipine is positioned next to the oppositely charged groups whereas the hydrophobic pole is buried in the membrane hydrocarbon core, thereby aligning the dihydropyridine conjugated ring with phospholipid acyl chains which are an important target for peroxidation. Amlodipine scavenges free oxygen radicals by donating two abstractable hydrogen atoms associated with its aromatic ring, thereby breaking the lipid peroxidation chain reaction.[33] Evidence of an *in vivo* antioxidant action of amlodipine on lipids was provided by an approximately 30% reduction in the plasma levels of 13-HODE in our study.

The evidence of lipid oxidation in patients on HD was accompanied by evidence of oxidation of thiols, proteins, and nucleic acids. GSH is an important intracellular antioxidant that modulates vascular tone by scavenging free oxygen radicals. Intracoronary administration of GSH in high-risk individuals improves coronary endothelial vasomotor function.[34] We confirmed an increased oxidation of GSH in ESRD patients. We found a significant reduction in the oxidized fraction of GSH with valsartan and amlodipine, which indicates an improvement in the redox milieu. A similar reduction in GSH oxidation is also seen in patients with essential hypertension treated with candesartan and amlodipine.[35] Neither treatment had an effect on elevated plasma levels of high-sensitivity C-reactive protein, or protein carbonyl. The relatively short duration of our study may explain the lack of a reduction in these parameters. We limited the duration of each study arm to 6 weeks to avoid the high drop-out rate owing to concurrent illnesses and requirement for intravenous iron in this population, which could confound the oxidative stress parameters.[36] Compartmentalization of the oxidative processes within the cells may also explain some of the differences in the oxidative stress parameters and may limit the ability of these drugs to exert antioxidant effects.

We confirmed the reports of increased plasma levels of ADMA and SDMA, with unchanged L-arginine, in HD patients.[8] However, a new finding was the marked reduction in the plasma levels of these methylarginines during treatment with amlodipine and valsartan. Methylarginines result form methylation of the arginine residues in proteins by PRMTs using *s*-adenosylmethionine as a methyl donor.[37] Upon hydrolysis of the proteins, free methylarginines appear in the cytosol. The asymmetrically methylated arginines (ADMA and *N*-monomethyl-L-arginine), but not SDMA, inhibit nitric oxide synthase. ADMA, in addition, upregulates the expression of lectin-like OxLDL receptor in cultured human monocytes, leading to a 2.4-fold increase in the intracellular concentration of OxLDL.[38] DDAH hydrolyses ADMA to citrulline and dimethylamine which is its major route of its elimination, whereas SDMA is eliminated principally by renal excretion.[39] The absence of renal excretion accounts for the higher plasma levels of SDMA than ADMA in patients with ESRD. Both isoforms of DDAH (1 and 2) are inhibited by oxidation and nitrosation.[14] In this study, both amlodipine and valsartan were effective in lowering ADMA levels which might therefore be secondary to a reduction in oxidative inactivation of DDAH. However, a reduced inactivation of DDAH would not be expected to yield the parallel reduction in plasma SDMA levels. Rather, the finding of an equivalent and parallel reduction in both methylarginines by these drugs suggests a reduction in the activity of PRMTs, which may be redox-sensitive. Indeed, the gene expression of PRMTs in cultured endothelial cells is increased by native and oxidized low-density lipoprotein which can be blocked by the antioxidant pyrrolidine dithiocarbamate.[13] In a dog model of chronic renal failure, the plasma levels of ADMA can be lowered by inhibiting PRMT with adenosine dialdehyde.[40] This suggests that both amlodipine and valsartan may lower ADMA and SDMA by limiting the activity of PRMTs and this may be secondary to their antioxidant properties. Additionally, a reduction in the endothelial shear stress by these agents could also lower the oxidative stress and plasma ADMA levels. A mild-to-moderate degree of shear stress increases the release of ADMA from the human umbilical vein endothelial cells by upregulating PRMT-I expression.[41] This effect is mediated by the activation of nuclear factor-κB pathway and involves upregulation of redox-sensitive genes encoding endothelial adhesion molecules. On the other hand, shear stress at a higher magnitude reduces the ADMA release to the baseline by increasing DDAH activity. Therefore, therapy with antihypertensive agents may lower plasma ADMA levels in part by reducing shear stress. In fact, 1-week therapy with enalapril, eprosartan, and a combination of these agents lowered the plasma ADMA levels in essential hypertension by 16.5, 15.9, and 18%, respectively[42] for a similar blood pressure, except the combination phase in which the mean blood pressure was lower than the other phases. However, plasma SDMA and parameters of oxidative stress were not measured in this study. In another study in patients with essential hypertension, perindopril, losartan, and bisoprolol decreased blood pressure to a similar extent, but only perindopril and losartan, lowered the plasma ADMA levels, whereas bisoprolol had no such effect.[43] Findings from these studies suggest a beneficial effect of the antagonists of the renin–angiotensin system on plasma ADMA levels which appears to be independent of their blood pressure-lowering properties. However, in patients with type II diabetes and normal renal function, therapy with olmesartan lowered blood pressure, renal vascular resistance, 15(*S*)-8-*iso*-prostaglandin F_{2a} (a marker of lipid peroxidation) and improved renal plasma flow without lowering the plasma ADMA levels.[44] In our study, we could not entirely attribute the beneficial effects of valsartan and amlodipine on oxidative stress and methylarginine to blood pressure lowering alone, as the degree of blood pressure control was exactly similar in the run-in and intervention phases.

In summary, we report high plasma levels of methylarginines and oxidation of proteins, lipids, thiols, and DNA in patients with ESRD on HD. Therapy with amlodipine and/or valsartan lowered ADMA and SMDA levels equally independent of blood pressure lowering and both effectively reduced, but did not normalize parameters of oxidative stress. These findings point to the need for clinical trial of these agents to determine if this reduction in oxidative stress and arginine methylation translates into an improvement in the cardiovascular end points in patients with ESRD.

MATERIALS AND METHODS

Study design

This was a randomized, double-blind crossover study conducted on stable patients on maintenance HD between November 2002 and August 2004 at Georgetown University Hospital affiliated dialysis facilities. The study protocol was approved by the Institutional Review Boards of Georgetown University and the respective dialysis units. Patients aged 18 years or above established on regular three times weekly maintenance HD for at least 3 months with single-pool K_t/V of >1.2 who required antihypertensive therapy were eligible to participate. The exclusion criteria were: patients with active infection, malignancy, absolute requirement for antagonists of the renin–angiotensin system or CCBs, use of antioxidant or herbal medications, predialysis serum potassium (S_K) concentration of >5.0 mEq/l within the previous 3 months, a history of an adverse reaction to valsartan or amlodipine, and hematocrit $<32\%$. Informed consent was obtained from the study participants. After enrollment, there was a 2-week run-in period during which angiotensin converting enzyme inhibitors, angiotensin receptor blockers, or CCBs were withdrawn from the patients receiving these agents and substituted with labetalol, clonidine, or doxazosin to achieve a predialysis blood pressure of $<150/90$ mmHg. This level was selected as it was an achievable target BP in this patient population. Blood for the baseline labs was drawn at the end of this run-in period before the start of HD and heparin administration. Patients were then randomized to therapy with either valsartan 160 mg daily or amlodipine 5 mg daily in a double-blind manner for 6 weeks. A computer-generated block-randomization method was used to establish the order of therapy for each subject. During each intervention phase, the dose of valsartan or amlodipine was titrated to a maximum of 320 or 10 mg daily, respectively. The antihypertensive agents used during the run-in period were withdrawn as needed to achieve an equivalent pre-HD blood pressure control of $<150/90$ mmHg. At the end of 6 weeks, each patient had an ambulatory blood pressure monitoring performed for 44 h of interdialytic period to confirm equivalent blood pressure control. The predialysis blood sample for the second set of labs was drawn at the conclusion of this 6-week period. This was followed by a 2-week washout period during which the study drug was discontinued and the doses of other antihypertensive agents were titrated as needed in the same manner as during the run-in phase. Patients were then treated with the second study drug for 6 weeks with the same blood pressure goal. A second 44-h ambulatory blood pressure monitoring preceded the last set of lab draws, which concluded the study.

Patients were excluded from the final analysis if they (1) required intravenous iron, (2) had an infection that required treatment with antibiotics, (3) had a hospitalization, or (4) had a predialysis S_K elevation to >6 mEq/l, or (5) had a K_t/V <1.2. A group of healthy age- and sex-matched volunteers who were taking no medications served as control.

Sample collection and handling

Blood for the biochemical analyses was drawn in the dialysis facility into pre-chilled vacutainers containing either heparin or ethylenediaminetetraacetic acid before initiation of a midweek dialysis session before heparin administration. The samples were immediately centrifuged at 4°C, aliquoted, frozen, and transported on dry ice. They were stored at −80°C until the analyses. All biochemical analyses were performed in two batches with a known concentration of internal controls. All samples were run in triplicates.

Biochemical analyses

Plasma ADMA, SDMA, and L-arginine were measured by high-performance liquid chromatography. Samples were diluted and applied to a preconditioned chlorobenzoic acid (CBA) solid-phase extraction cartridge. After elution with methanol:ammonia, the dried extracts were derivatized and injected onto a C18 high-performance liquid chromatography column (25×4.6 mm). ADMA and SDMA were quantified by reference to their fluorescence emissions. Data were acquired onto a HP Chemstation® and peak areas determined. The intra- and inter-assay coefficients of variability (CVs) were 3.1 and 3.6%, respectively for plasma ADMA and 2.6 and 3.1% for plasma SDMA.

Plasma 13-HODE was measured by high-performance liquid chromatography. Plasma was acidified with acetic acid to pH of 3 and extracted with hexane-isopropanol. After degassing, the samples were vortexed for 30 s. After centrifugation, the organic layer was transferred to a clean vial and evaporated under nitrogen. Samples were reconstituted with 100 μl of methanol and diluted 1/1 with mobile phase. Fifty microlitres was injected onto the high-performance liquid chromatography (25×4.6 mm column- Spherisorb C18). The mobile phase consisted of 25 mM sodium sulphate and methanol (40/60) at 1.25 ml/min. Quantification was achieved through integration of areas using Chemstation®. Peaks were monitored by ultraviolet at 236 nm (±2 nm) with a 400 nm (±50 nm) background subtract. The intra- and inter-assay CVs were 3.2 and 4.1%, respectively.

Serum high-sensitivity C-reactive protein was measured by nephelometry (Dade Behring Inc., Deerfield, IL, USA). The intra- and inter-assay CVs were 3.8 and 5.0%, respectively. Whole-blood reduced (GSH) and oxidized glutathione (GSSG) and GSSG/GSH ratio were measured using a method (Oxisresearch, Portland, OR, USA) that uses 5,5′-dithiobis-2-nitrobenzoic acid as described by Tietze.[45] 1-Methyl-2-vinylpyridinium trifluoromethanesulfonate was added to freshly drawn whole blood to scavenge GSH for GSSG measurement. The whole-blood GSH assay had intra- and inter-assay CVs of 3.6 and 8.4%, respectively. The whole-blood GSSG assay had intra- and inter-assay CVs of 6.4 and 9.6%, respectively.

Plasma levels of *protein carbonyl* were measured by an enzyme-linked immunosorbent assay (ZenTech, Dunedin, New Zealand) with the method of Buss *et al.*[46] Briefly, samples containing protein are reacted with dinitrophenylhydrazine. The protein is nonspecifically adsorbed to an enzyme-linked immunosorbent assay plate. Unconjugated dinitrophenylhydrazine and non-protein constituents are washed away. The adsorbed protein is probed with biotinylated anti-dinitrophenylhydrazine antibody followed by streptavidin-linked horseradish peroxidase. Serum albumin containing increasing

proportions of hypochlorous acid is used as standard curve. Intra- and inter-assay CVs were 7.5 and 12.5%, respectively.

Plasma 8-hydroxy 2-deoxyguanosine was measured by enzyme-linked immunosorbent assay.[47] A monoclonal immunoglobulin G (N45.1) specific for 8-hydroxy 2-deoxyguanosine was used in this enzyme-linked immunosorbent assay in combination with horseradish peroxidase-conjugated anti-mouse polyclonal immunoglobulin G and substrate *o*-phenylenediamine. Plasma was filtered using Millipore Ultrafilters® (Billerica, MA, USA), with 10 000 dalton molecular weight cutoff before the assay. The intra- and inter-assay CVs were 4.6 and 6.2%, respectively.

Data analysis

All values are expressed as mean $\pm$ s.e.m. All parameters were tested for normal distribution using Kolmogorov–Smirnov test and log-transformed if not normally distributed. An unpaired Student's *t*-test was used to compare values between HD patients and healthy controls. Repeated-measures analysis of analysis of variance was used to compare the effects of both treatments with the baseline values. A paired *t*-test was used to compare the effects of treatment among both interventions and the baseline. Wilcoxon-matched pairs test was used to compare groups when data were not normally distributed. A *P*-value <0.05 was considered statistically significant. To eliminate carry-over effect, the data were further analyzed according to the order in which the study drugs were administered.

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