Free Radical Research, February 2006; 40(2): 207–212

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Vitamin E-coated filter decreases levels of free 4-hydroxyl-2-nonenal during haemodialysis sessions

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Accepted by Dr J. Vina

(Received 18 May 2005; in revised form 13 October 2005)

Abstract
Uraemic subjects undergoing chronic haemodialysis show increased oxidative stress. The use of non-biocompatible filters and reduced antioxidative defences are important sources of reactive oxygen species (ROS) release. The highly oxidative environment accelerates the onset and progression of tissue damage and atherosclerotic cardiovascular disease. The aldehyde 4-hydroxyl-2-nonenal (HNE) is probably the best marker of oxidative stress. In this study, the concentration of plasma HNE was evaluated in eight uremic subjects during two sessions of haemodialysis: the first using a standard biocompatible filter and the second using a filter coated with vitamin E. Baseline plasma levels of HNE were elevated, and dropped during haemodialysis. At the end of the session, however, low levels were maintained only when the vitamin E-modified filter was used. By contrast, a marked increase in HNE was recorded at the end of the session in all subjects who underwent haemodialysis with the conventional filter. This study provides evidence that the vitamin E-coated filter plays a role in counteracting oxidative stress. The chronic use of vitamin E-modified filters in haemodialysed subjects might help to counterbalance oxidative attack and, consequently, contribute to preventing cardiovascular disease.

Keywords: *Haemodialysis, oxidative stress, vitamin E-coated filter, 4-hydroxyl-2-nonenal*

Introduction

A common feature of chronic diseases is the presence of oxidative stress, which is involved in the onset and progression of disease-related complications. Renal failure is no exception [1,2].

Uremic patients display high levels of advanced glycoxidation and lipoxidation end-products, which are now regarded as true uraemic toxins that cause serious complications, including cardiovascular diseases. Moreover, as haemodialysed patients lack protective antioxidant mechanisms and exhibit greater generation/accumulation of free radicals and other pro-oxidants, they are prone to reactive oxygen species (ROS)-related complications.

Together with Maillard's reaction, oxidative stress is responsible for non-enzymatic modification of proteins, loss of cellular viability and tissue damage [3,4].

Oxidative stress is known to play a pivotal role in the pathogenesis of atherosclerosis and other complications in uremic patients undergoing haemodialysis (HD) by activating NFkB; this rapid-response nuclear transcriptional factor is involved in the modulation of cytokines, adhesion molecules and chemokines, and contributes to the release of inflammatory agents, to

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500445121

the triggering of leukocytes and to the promotion of endothelial dysfunction [1,5–7].

ROS-related clinical problems involve a series of pathophysiological steps which lead to the onset of various diseases, such as anaemia, oedema, dialysis-associated amyloidosis, atherosclerosis and cancerogenesis.

In particular, oxidative stress, increased lipid peroxidation and impaired function of antioxidant systems are known to contribute to the pathogenesis of atherosclerotic cardiovascular disease through the accelerated formation of atheroma (enhanced susceptibility to oxidation of LDL, with subsequent formation of foam cells and instability of the plaque, increased formation of fatty streaks by smooth muscle cells) and impaired endothelial function (with formation of peroxynitrites and vasomotor impairment) [8–10].

Atherosclerosis is the most frequent cause of death in patients with end-stage renal disease undergoing dialysis treatment, strongly correlating with a high comorbidity rate, increased mortality and a poor outcome.

Haemodialysis filter membrane materials that are not fully biocompatible activate the oxidative metabolism of polymorphonuclear leukocytes (PMN) and monocytes, which appears to be one of the most important sources of ROS generation through the promotion of a pathogenetic environment for cellular and tissue damage [11,12].

The problem of the bio-incompatibility of haemodialysis filters is still unsolved. However, in order to reduce reactivity, innovative filters have recently been introduced [11,12]. Among these, the vitamin E-bonded cellulose filter should endow the filter membrane with an antioxidant potential.

The present study evaluates the acute effect on plasma 4-hydroxyl-2-nonenal HNE concentration of a multilayer HD filter bonded with α-tocopherol in the hydrophobic part of the blood surface, in patients undergoing chronic HD, and compares this filter with the conventional biocompatible membrane.

As the markers of oxidative stress are extremely unstable, they are very difficult to detect in biological fluids. They have therefore been estimated indirectly by means of by-products such as isoprostanes, malondialdehyde, oxidised LDL or isolevuglandins.

However, HNE is probably one of the most active and best characterized lipoperoxidation end-products as it is considered to be one of the most reliable bioactive markers of oxidative stress and a potential causative agent in the atherosclerotic process [13,14]. Moreover, HNE was selected in the present study because it had not been previously determined by specific HPLC in the plasma of uraemic subjects and therefore provided an opportunity to extend our knowledge of clinical oxidative stress and its pathogenetic implications.

Patients and methods

Eight non-diabetic uraemic patients (six males and two females) aged 63 ± 4 years and a group of ($n = 15$; age = 62 ± 4 years) age-matched healthy volunteers were studied. This latter group was used to compare the basal level of HNE with a non-uraemic group. The causes of renal failure were: cancer, polycystic kidney and unknown pathologies. All participants were non-smokers and in alcohol abstaining (Table I).

The study consisted of two separate phases: in the first phase, a blood sample was collected from each patient before, during (after 100–120 min of dialysis) and at the end of the HD session. All the patients underwent dialysis with a conventional membrane (Gambrane, Gambro, Italy; dialyser surface 1.3 m^2). After 5–7 days, the same group of subjects (patients) was switched to dialysis with the filter covered with vitamin E (α-tocopherol hydrophobically bonded to a cellulose membrane modified by covalent linkage of block copolymer to the hydroxyl group of cellulose. This bond is strong enough to prevent any risk of leakage into the bloodstream; Excebrane, Terumo Corp. Europe Branch, Rome, Italy; dialyser surface 1.2 m^2). Blood samples were drawn as for the conventional membrane.

A specific marker of lipoperoxidation, HNE, was determined in plasma before, during and after a single HD session (sampling times: before HD session; about 2 h later, in mid-session; after HD session). The results obtained by means of the conventional membrane and the vitamin E-bonded membrane in the same group of patients were compared.

HNE was selected because of its major biological activity and its high cytotoxicity, which make it a very

Table I. Characteristics of healthy and uraemic subjects.

Group	Controls	Chronic haemodialysis	*p*
n (male/female)	15 (8/7)	8 (6/2)	–
Age (years)	62 ± 4	63 ± 4	n.s.
Duration of dialysis (months)	–	52 ± 24	–
Smokers	0/15	0/8	–
Serum creatinine (mmol/l)	0.075 ± 0.01	0.899 ± 0.024	<0.001

sensitive marker of oxidative stress in the human body [13–15].

Quantitative determination of HNE was performed according to Esterbauer [16]. Briefly, 500 micromoles of plasma (mixed with 140 mM KCl in 20 mM HEPES, pH 7.4) were extracted twice with equal volumes of dichloromethane; the extract was collected in a flask containing acetate buffer (0.1 M, pH 3.0). Dichloromethane was evaporated under nitrogen stream, and the material was then applied onto a C18 solid-phase column (Bond-Elut, Varian-Superchrom, Milan, Italy): the non-polar material was first removed by elution with hexane, and HNE was then eluted with 80% methanol.

A volume of the sample was analysed by HPLC on an S-5 4.6 × 250 mm Spherisorb ODS column (Waters S.P.A, Milan, Italy), with 40% acetonitrile as mobile phase, and with UV detection at 220 nm. The intra- and inter-assay coefficients of variation were 12 and 15%, respectively.

The standard of HNE was produced by I.V. and B.T according to the method proposed by Esterbauer [17]: the separation of HNE from impurities was obtained by means of a reverse-phase C18 10 × 250 mm semi-preparative column (Vydac, Separation Co., Hesperia, CA, USA) using 30% acetonitrile in water as a mobile phase, with isocratic elution; purity was confirmed by mass spectrometry (HP5973 MSD system, Hewlett–Packard Co., Milan, Italy) on the final product after derivatization with bis (trimethylsilyl)trifluoracetamide (BSTFA).

The levels of HNE are expressed as micromoles per milligram of protein in order to exclude bias dependent on blood concentration/dilution.

Student's *t*-test was used to compare healthy and uraemic subjects; two-way analysis of variance (repeated measures) and post-test (Bonferroni's test) were used to evaluate statistical difference between the two treatments during the haemodialysis session.

The tests were performed using GraphPad Prism version 4.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. A probability level lower than 5% ($p < 0.05$) was considered significant. Data are expressed as mean ± standard error of mean.

Results

As expected, the level of free plasma HNE was higher in uraemic patients undergoing chronic HD than in normal age-matched subjects (7.6 ± 1.2 vs 3.0 ± 0.28 μmol/mg protein ($n = 15$, mean age 62 ± 4 years; $p < 0.01$).

When the conventional filter was used, a significant drop was observed during HD (basal 8.01 ± 1.24 μmol/mg protein, mid-session 4.09 ± 0.54 μmol/mg protein; −49%), followed by an evident rebound at the end of the session (13.81 ± 4.02 μmol/mg protein; +72%).

A different pattern was observed when the filter bonded with α-tocopherol was used; while this filter also caused a significant mid-session decrease (basal level 7.13 ± 1.23 μmol/mg protein; mid-session 3.59 ± 0.58 μmol/mg protein; −49%), at the end of the session, no rebound occurred and a further decrease was recorded in all subjects (2.97 ± 0.41 μmol/mg protein; −58%).

Two-way analysis of variance revealed that the source of variation was significant for both the time ($F = 4.8$, $p < 0.02$) and the type of filter ($F = 10.0$, $p < 0.01$). Therefore, the variation in HNE levels observed during haemodialysis was remarkable and there was a significant difference between the two filters. The Bonferroni post-test revealed a significant difference between the basal and the final level of HNE ($t = 2.6$, $p < 0.05$) and between the mid-session and final HNE levels ($t = 4.4$, $p < 0.001$) only when the conventional filter was used (Figure 1).

Both filters (conventional and α-tocopherol-bonded) were effective in extracting uraemic toxins: the removal rate of urea was 70.4 ± 1.6 vs 68.9 ± 1.6%, phosphorus 51.5 ± 2.9 vs 46.1 ± 4.6%, uric acid 71.5 ± 1.2 vs 70.8 ± 1.2 and potassium 32.4 ± 2.5 *vs* 30.3 ± 2.6%. No side-effects were recorded during the time-sequence of the study.

The Qb (blood flow) and Qd 8 (dialysate flow) were similar during both sessions; the mean duration of an HD session was 226 ± 6 min.

The microbiological count of the dialysate was lower than 100 UFC for both treatments and good haemodynamic tolerance was observed for both filters.

Discussion

Haemodialysis is associated with an increase in ROS production mainly due to an activation of PMN cells. These cells exhibit a burst of oxygen consumption, producing a series of reactive substances (such as superoxide radicals, hydrogen peroxide, hypochlorous acid) and leading to oxidant-derived tissue injury [1,2,6,9]. The lack of antioxidant defences and of scavenger molecules against oxygen free radicals contributes to the unfavourable cellular environment, inducing molecular, cellular and tissue damage. The imbalance of redox status affects transmembranal signalling, leading to cellular death via apoptosis [2,18,19].

The consequences of this redox rearrangement might render uraemic subjects more prone to hypertension (e.g. inactivation of nitric oxide, generation of vasoconstrictive molecules, cardiovascular remodelling), anaemia (e.g. shortened erythrocyte lifespan, reduced erythropoietin release, impaired iron use) and accelerated atherosclerosis, even in the absence of traditional risk factors (e.g. endothelial

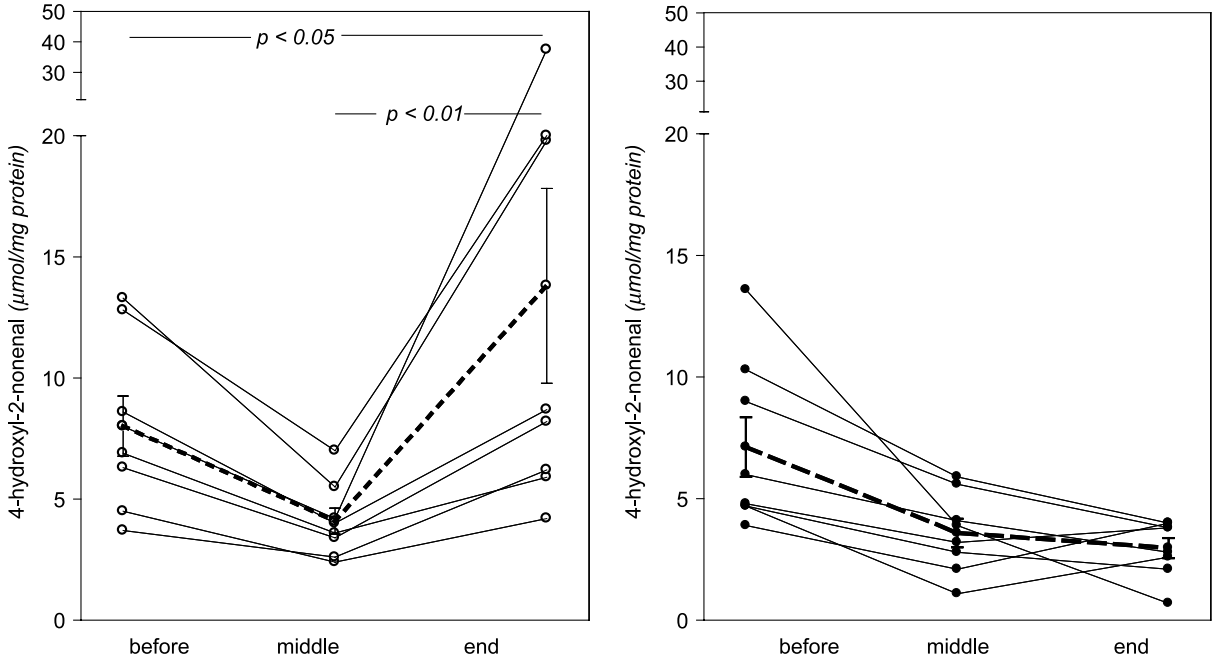

Figure 1. Plasma concentration of HNE in uraemic patients ($n = 8$) during a haemodialysis session with a conventional biocompatible membrane and with a vitamin E-coated membrane (Excebrane, Terumo). Dotted line represents mean level (± standard error). Probability level was calculated according to Bonferroni's post-test after two-way analysis of variance (see text).

dysfunction, oxidised LDL, accumulation of glycoxidation end-products, generation of cytokines). Indeed, it should be pointed out that the main cause of death in uraemia is cardiovascular disease (>50%) [2,4,9,10,20,21].

HNE is an aldehydic molecule generated by lipoperoxidation processes. It is believed to cause cellular and nucleic acid toxicity through a well-known pro-oxidant pro-inflammatory activity affecting signal transduction; HNE activates several signalling systems that affect cell survival mechanisms. Several HNE-adducts have been found in atherosclerotic plaques, in neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis) and in metabolic or hepatic diseases [13–15]. There is a general consensus that HNE levels increase in all the pathophysiological states characterised by oxidative stress, and that HNE is a reliable *in vivo* biomarker, as suggested by several reports [13–15].

In the present study, we observed that the level of HNE, as reported for other oxidative stress markers, is higher (twofold) than that seen in age-matched subjects, a finding that supports the notion of marked activation of oxidative stress.

The stability of this lipoperoxidation end-product in the two sessions in every single subject is noteworthy, the mean variability being 16 ± 3%; as the data indicate that HNE production was continuous during the study period, the two sessions were comparable.

The mid-session decline in HNE cannot be ascribed to a volume modification, as the levels of the aldehyde were calculated on the basis of plasma protein content. In our opinion, the reduction is easily explained by a favourable removal of toxins and small reactive molecules, as we have already described for advanced glycation end products [22,23]. We suppose that this efficient removal is based on the physical properties of the membrane. However, at the end of the session with the conventional filter, an unequivocal rebound of HNE was observed, indicating a recurrence of oxidative stress. The low biocompatibility of the haemodialysis filter plays a pivotal role in the activation of leukocytes, thereby promoting the generation of ROS [11,12].

The treatment of upregulated oxidative stress in uraemia involves two approaches: controlling ROS production and/or restoring antioxidant defences.

Since it is hard to slow down the release of ROS, many authors have utilised oral antioxidants in an attempt to lower oxidative stress, and have achieved apparently beneficial effects.

Tocopherols are the most widely used antioxidants because they work as powerful scavengers against lipoperoxidation attack on plasma lipids and cell membranes; moreover, they are physiological molecules that have low toxicity and a wide therapeutic range, and are reported to be endowed with several positive properties [24–26].

The use of a cellulose membrane bonded with α-tocopherol is an innovative therapeutic strategy that exploits both approaches: the presence of α-tocopherol should improve the biocompatibility of the filter, thereby inhibiting the priming action on blood cells, and should restore the redox balance, functioning as

a site-specific scavenging device. When this membrane was used in the present study, HNE showed a mid-session drop similar to that observed with the conventional filter. At the end of the session, however, the low HNE level was maintained in all subjects ($p < 0.01$). The most likely interpretation is that the α-tocopherol in the blood-exposed surface of the filter both prevents the blood cell activation associated with the release of free radicals [11,12,19] and restores the antioxidant defence by regenerating circulating tocopherols, glutathione and other antioxidant molecules [27–29].

Our results are in agreement with those of previous studies reporting that this membrane is effective in reducing the markers of oxidative stress (ascorbyl free radical, malondialdehyde, 8-hydroxy-2-deoxyguanosine, hydroperoxide and others) [30–33] or glycoxidation end-products (pentosidine) [23,34].

After long-term use of the vitamin E-bonded filter, the reduction in oxidative markers has usually been significant [29,32,33], though in some cases it has been reported to be negligible [34]. It is reasonable to suppose that, after the haemodialysis session, oxidative stress rises again, and that the effect of the filter fades.

It would be interesting to associate the vitamin E-bonded filter with oral antioxidant integration during the inter-dialysis period in order to extend the action of the filter.

A timely scavenging function against oxygen free radicals, as accomplished by the vitamin E-bonded filter, should engender a more favourable cellular environment, thereby reducing molecular, cellular and tissue damage and the most common complications of uraemia. The use of this filter is therefore an interesting first step in fighting an important pathogenic factor for overall cardiovascular risk in uraemic patients.

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