Alpha-tocopherol supplementation decreases LDL(−) in HD patients


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Alpha-tocopherol supplementation decreases electronegative low-density lipoprotein concentration [LDL(−)] in haemodialysis patients

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Abstract

Background. Oxidative stress is a significant contributor to cardiovascular diseases (CVD) in haemodialysis (HD) patients, predisposing to the generation of oxidized low-density lipoprotein (oxLDL) or electronegatively charged LDL subfraction. Antioxidant therapy such as α-tocopherol acts as a scavenger of lipid peroxy radicals attenuating the oxidative stress, which decreases the formation of oxLDL. The present study was designed to investigate the influence of the α-tocopherol supplementation on the concentration of electronegative low-density lipoprotein [LDL(−)], a minimally oxidized LDL, which we have previously described to be high in HD patients.

Methods. Blood samples were collected before and after 120 days of supplementation by α-tocopherol (400 UI/day) in 19 stable HD patients (50 ± 7.8 years; 9 males). The concentrations of LDL(−) in blood plasma [using an anti-LDL− human monoclonal antibody (mAb)] and the anti-LDL(−) IgG auto-antibodies were determined by ELISA. Calculation of body mass index (BMI) and measurements of waist circumference (WC), triceps skin folds (TSF) and arm muscle area (AMA) were performed.

Results. The plasma α-tocopherol levels increased from 7.9 μM (0.32–18.4) to 14.2 μM (1.22–23.8) after the supplementation (P = 0.02). The mean concentration of LDL(−) was reduced from 570.9 μg/mL (225.6–1241.0) to 169.1 μg/mL (63.6–621.1) (P < 0.001). The anti-LDL(−) IgG auto-antibodies did not change significantly after the supplementation. The α-tocopherol supplementation also reduced the total cholesterol and LDL-C levels in these patients, from 176 ± 42.3 mg/dL to 120 ± 35.7 mg/dL (P < 0.05) and 115.5 ± 21.4 mg/dL to 98.5 ± 23.01 mg/dL (P < 0.001), respectively.

Conclusion. The oral administration of α-tocopherol in HD patients resulted in a significant decrease in the LDL(−), total cholesterol and LDL-C levels. This effect may favour a reduction in cardiovascular risk in these patients, but a
larger study is required to confirm an effect in this clinical setting.

**Keywords:** α-tocopherol; cardiovascular disease; electronegative LDL; haemodialysis

**Introduction**

Atherosclerotic cardiovascular disease is the leading cause of death in haemodialysis (HD) patients. In this regard, traditional risk factors alone, as dislipidaemia, obesity and smoking, cannot explain the high prevalence and incidence of CVD in this population. Inflammation has been suggested to be a significant contributor, which is interrelated to insulin resistance, wasting, endothelial dysfunction and oxidative stress [1,2]. Oxidative stress, the imbalance between generation of oxidant compounds and antioxidant defence mechanisms, leads to oxidation of carbohydrates, proteins and lipids [3], and the low-density lipoprotein’s (LDL) oxidative modification is by far the most studied aspect, which gave rise to the oxidative hypothesis of atherosclerosis [4]. LDL consists of a series of discrete particle subtypes of distinct physicochemical, immunological and hydrodynamic properties. Several studies support the hypothesis that the risk of atherosclerosis is associated with the proportion of these different LDL subfractions in blood and the amounts present in vascular lesions [5]. The presence of different forms of modified LDL in blood was established in the 1980s. In 1988, Avogaro and collaborators [6] reported the presence of an electronegatively charged LDL subfraction, the electronegative LDL [LDL(−)], found in blood plasma and with pro-inflammatory properties. LDL(−) exhibits oxidative changes, such as high content of conjugated dienes and lipid hydroperoxides and a reduced amount of α-tocopherol in comparison with native LDL [7].

LDL(−) has been suggested to be involved in atherogenesis as these particles induce cytotoxicity, apoptosis and the production of cytokines and chemokines, such as interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1) and vascular cell adhesion molecule 1 (VCAM-1) [8]. Ziouzenkova et al. [9] reported increased serum levels of LDL(−) in haemodialysis (HD) patients and suggested that radicals could be formed on haem proteins, following the oxidation with hydrogen peroxide, released from activated white blood cells. We have recently shown that the serum levels of LDL(−) in HD patients were higher than those in peritoneal dialysis (PD) patients, which were higher than levels from a population of healthy individuals [10].

The oxidation of LDL can be prevented by a large variety of antioxidant substances, including enzymes such as superoxide dismutase and glutathione peroxidase, as well as by the chain breaking antioxidants, such as ascorbate and tocopherols [11]. The α-tocopherol, one of the eight isoforms of vitamin E, is the most potent fat-soluble antioxidant known in nature. It is known that α-tocopherol acts as scavenger of lipid peroxyl radicals in lipoproteins protecting them against oxidation and avoiding the generation of oxidized low-density lipoprotein (oxLDL), being considered as a chief antioxidant for the prevention of atherosclerosis [12].

**Subjects and methods**

A group of 19 HD patients (age was 50 ± 7.8 years; 9 males and 10 females) from Centro Integrado de Nefrologia (CIN), Rio de Janeiro, Brazil, were studied. Inclusion criteria were age > 18 years and maintenance haemodialysis for at least 6 months. HD was performed three times a week, comprising 12 hHD/week. The dialyzers used were cellulose triacetate (Nipro medical Corporation, Osaka, Japan). Kt/V was calculated according to the Daugirdas equation and was kept above 1.2. No major changes were made in the dialysis treatment and schedules during the follow-up period. Thirty-five patients entered the study, and 19 were selected according to the exclusion criteria. Exclusion criteria were patients older than 65 years, suffering from cancer, diabetes mellitus, AIDS and immune disease, patients using catheters as vascular access, antioxidant drugs, alcohol consumption and smoking. None of the patients were taking hypolipidaemic drugs. Table 1 shows the demographic data of the study group. The Ethics Committee of Clementino Fraga Filho University Hospital reviewed and approved the study protocol. The patients were asked to sign the informed consent.

**Alpha-tocopherol supplementation**

The oral supplementation (400 IU of α-tocopherol) was kindly provided by Sigma Pharma Inc. (São Paulo, Brazil). The patients were advised to take a capsule per day and maintain their usual diet and activities during 16 weeks and to report any side effects immediately to the investigators. All the data and samples of blood were collected before and after 16 weeks of supplementation.

**Biochemical analyses**

Blood samples were drawn in the early morning after overnight fasting, in tubes containing EDTA (1.0 mg/mL blood). The blood samples were drawn from the arteriovenous fistula before the dialysis session. From each sample, the plasma was immediately separated by centrifugation at
600 g and a solution containing BHT (100 µM), aprotinin (10 µg/mL), benzamidine (10 µM) and PMSF (5.0 µM) was added to plasma before storing at −80°C until analysis.

The following biochemical parameters were also measured in blood using routine enzymatic methods: total cholesterol (TC), HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c) and triglycerides (TG). Analyses of haematocrit, serum levels of BUN, creatinine, calcium, phosphorus and PTH were done according to routine requirements for dialysis patients.

Assessment of nutritional status

BMI was calculated as the weight/(height)^2 ratio, and values between 18.5 and 25 were considered normal according to the criteria from the World Health Organization [13,14]. The dry weight was considered as the weight measured immediately after the HD session. Anthropometric measurements were made using standard techniques. Waist circumference, triceps skin folds and arm muscle area (AMA) were used in these analyses. The non-dominant arm was used for these measurements, except in the case where there was vascular access, when the other arm was used. TSF was measured with a Lange Skinfold Caliper (Cambridge Scientific Industries Inc., USA), and the arm muscle area (AMA) was calculated according to the following formula: AMA = [(MAC (cm) − π × TSF (mm))/10]²/4π − N, where N = 10 for males and 6.5 for females. The measurements were made after the dialysis session by a trained staff.

Electronegative LDL isolation

The LDL fraction was separated by ultracentrifugation of blood plasma from hypercholesterolaemic patients (TC = 6.44 ± 0.75 mmol/L), according to Griffin et al. [15]. The LDL(−) subfraction was isolated as described by Hodis et al. [16] with minor modifications. Briefly, LDL(−) was isolated from aliquots of LDL by FPLC (BioLogic Duo Flow®, BioRad Inc., Hercules, CA, USA) with a UNO™ Q-12 column (BioRad Inc., Hercules, CA) and a gradient constituted by using 20 mM TRIS, pH 7.8 (pump A) and 20 mM TRIS + 1 M NaCl pH 7.8 (pump B). The eluent was monitored at 280 nm, and LDL(−) aliquots were harvested and enriched with protease inhibitors and BHT. Salts were removed from samples with Econo-Pac 10DG columns (Bio-Rad Inc., Hercules, CA). Immunoglobulin and albumin were eliminated by using an Econo-Pac Serum IgG purification kit (Bio-Rad Inc., Hercules, CA). The protein content of the LDL(−) subfraction was determined according to the Lowry method before storage at −80°C. This purified LDL(−) subfraction was used as a standard and antigen in ELISA assays for detection of LDL(−) and autoantibodies reactive to LDL(−), respectively.

Detection of LDL(−) in blood plasma

The concentrations of LDL(−) in blood plasma were determined by ELISA using an anti-LDL(−) human monoclonal antibody (mAb3D1036) produced in our laboratory. The mAb3D1036 recognizes an epitope formed due to slight loss of the apo-B100 secondary structure on minimally modified LDL particles as previously reported by Damasceno et al. [17]. Ninety-six-well microplates (Nunc™, MaxiSorp™ surface) were coated with 50 µL mAb3D1036 (1 µg/well) in a carbonate–bicarbonate buffer (pH 9.4, 0.1 M) and incubated overnight at 4°C. Then, the microplate was washed three times with a PBS buffer (Tris–HCl 50 mM and NaCl 150 mM, pH 7.4) containing Tween 20 (0.5%) and blocked with 5% non-fat dry milk for 2 h at 37°C. The microplates were washed again and incubated with 50 µL plasma for 2 h at 37°C. The plates were washed and incubated with the anti-LDL(−) monoclonal antibody biotinylated for 2 h at 37°C. After washing, the microplates were incubated with streptavidin–HRP conjugate (Tressgen Biotechnologies Corp., San Diego, USA) for 1 h at 37°C. Then, the HRP substrate solution (92.3 nM luminol, 0.9 mM p-iodophenol and 3.0 mM hydrogen peroxide) was added in each well. The chemiluminescence intensity was determined immediately after using a microplate reader (LumiCount, Packard; Medriden, CT, USA). The calibration curve was made with LDL(−) obtained from human plasma as previously described by Sevanian et al. [7]. All samples and standards were run in triplicate. The intraassay and interassay variations for this ELISA test were 8% and 15%, respectively. The reference value in healthy volunteers was 54.9 ± 33.3 µg/mL.

Detection of anti-LDL(−) Ig autoantibodies

Aliquots of native LDL and LDL(−) were diluted in the carbonate–bicarbonate buffer (0.1 M, pH 9.4) to 1.0 µg of protein/well and used to coat 96-well ELISA microplates (Costar, Cambridge, MA, USA) overnight at 4°C. Then, microplates were blocked in 5% fat-free milk in PBS pH 7.4, previously inactivated by heating (100°C) and incubated at 37°C for 30 min. After washing plates three times with PBS-Tween (0.05%), the plasma samples (50 µL/well) previously diluted in PBS-1% fat-free milk (1:10) were added and plates were incubated for 2 h at room temperature. After washing, as previously described, the peroxidase-conjugated affinity goat polyclonal antihuman IgG (Rockland, cod. 610-1302, diluted 1:2000 in PBS-Tween 20% (0.5%) and antigen in ELISA assays for detection of LDL(−) and autoantibodies reactive to LDL(−), respectively.

Determination of α-tocopherol concentration

Determination of plasma α-tocopherol concentration was measured in plasma by reverse-phase high-performance liquid chromatography following ethanol precipitation and hexane extraction as previously described [18].
**Table 2.** Lipid profile and levels of plasma α-tocopherol before and after vitamin E supplementation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Week 0</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>176 ± 42.3</td>
<td>120 ± 35.7*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>205 ± 189.6</td>
<td>191 ± 161.9</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>34 ± 8.5</td>
<td>37 ± 7.4</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>115.5 ± 21.4</td>
<td>98.5 ± 23.01*</td>
</tr>
<tr>
<td>Alpha-tocopherol (µM)</td>
<td>10.5 ± 3.3</td>
<td>14.6 ± 7.7*</td>
</tr>
</tbody>
</table>

*P < 0.05.

![Fig. 1. Serum LDL(−) concentrations before and after the α-tocopherol supplementation in HD patients (P < 0.001, compared to LDL(−) before the supplementation).](https://academic.oup.com/ndt/article-abstract/24/5/1587/1885637)

**Discussion**

Vitamin E supplementation has been found to be associated with beneficial effects on heart diseases [19–21] and to prevent lipid peroxidation in HD patients [22,23]. In this study, LDL(−) levels were high in HD patients, as previously reported [9,10], and α-tocopherol supplementation during 16 weeks significantly reduced the LDL(−) levels. On the other hand, the supplementation did not affect the concentrations of the serum IgG antibody reactive to LDL(−). This is the first study showing the influence of α-tocopherol supplementation on LDL(−) levels in HD patients. The patients presented good nutritional status related to BMI and AMA, but a low TSF value (a suggestive sign of malnutrition). This can be assumed to be a parallel feature of active disease and metabolic stress, as suggested by higher levels of LDL(−), but there was no correlation among the variables.

Some studies showed increased LDL(−) levels in patients at high cardiovascular risk [24], familial hypercholesterolaemia [25,26], hypertriglyceridaemia [27–30] and type 1 and 2 diabetes mellitus [31–38]. The previous study by Ziouzenkova et al. [9] reported that LDL(−) levels in HD patients were significantly higher than in healthy subjects and they proposed a mechanism for LDL(−) formation in blood during HD, where haemoglobin could be oxidized to its met and/or ferryl states by reacting with lipid hydroperoxides or, readily, with H2O2. The latter is generated under inflammatory conditions by erythrocytes and platelets or during the auto-oxidation of haemoglobin. Thus, it is possible that LDL(−) is formed, at least partially, via covalent binding of haemoglobin fragments to apo B-100 and that the haemoglobin-mediated pathway may be a major mechanism of LDL(−) formation in HD patients [9]. It is noteworthy...
that in 2002, Ziouzenkova et al. [39] showed that oxidative modification can be inhibited by specific antioxidants during haemodialysis (an alternative dialysis procedure, which introduces antioxidants in the form of liposome circuits). The presence of both vitamin E and vitamin C in the dialysate produced the most effective overall antioxidant protection. After 4 h of HLD using circuit{\textsuperscript{Vit E&C}}, LDL(−) levels were ~46% lower than that observed in control HD.

Several studies documented the protective effect of α-tocopherol supplementation. Lubrano [40] observed that the administration of α-tocopherol acetate in HD patients decreased the oxidative damage. Panzetta et al. [41] showed that 50 IU/day α-tocopherol supplementation reduced the susceptibility of LDL to oxidation in haemodialysis patients. Boaz et al. [19] had investigated the effect of the supplementation with vitamin E in cardiovascular diseases in HD patients and observed the reduction of myocardium failure.

The supplementation with α-tocopherol can prevent several heart diseases in HD patients, probably through the reduction of oxidative stress [20,21]. Roob et al. [42] observed that a single oral dose of vitamin E attenuates lipid peroxidation in patients on haemodialysis receiving intravenous iron. Sato et al. [43] suggested that the microcirculatory disturbance in HD patients seems to be associated with endothelial damage caused by oxidative stress and combined supplementation with vitamins C and E may be clinically beneficial to improve the cutaneous microcirculation by reducing oxidative stress. In addition, there are some studies that support the efficacy of the use of vitamin E-coated cellulose membranes for the reduction of markers of oxidative stress in dialysis patients [23,44–46].

Dyslipidaemia is an independent risk factor associated with atherosclerosis, and HD patients may have the classic pattern of dyslipidaemia, characterized by increased serum triglycerides, decreased HDL-C, but high serum total and LDL cholesterol levels are relatively uncommon [47,48]. In the present study, we found that lipid alterations are present in HD patients and after the α-tocopherol supplementation, the levels of total cholesterol and LDL-C decreased significantly without affecting HDL and triglycerides. However, there is a paucity of data on the effect of the α-tocopherol supplementation on lipid abnormalities in HD patients [22,49]. Khajehdehi [50] showed that supplementations during 3 months with vitamin C (200 mg), E (200 mg) and D{\textsubscript{3}} (50 000 IU) are safe and beneficial for treatment of lipid abnormalities in HD patients.

The previously reported studies support a positive effect of α-tocopherol supplementation on oxidative stress markers in CKD patients. As far as we know, there are no studies showing the effects of α-tocopherol on LDL(−) levels. The α-tocopherol supplementation decreased the levels of LDL(−) in HD patients and this may have clinical implications since this particle has been shown to be proinflammatory and atherogenic contributing to morbidity and mortality in CKD patients [8,32,35].

There are several limitations in our study. The first is the lack of explicit laboratory markers of inflammation such as C-reactive protein (CRP). Moreover, biochemical and anthropometric analyses were not performed after the supplementation. Nevertheless, this study reinforces and gives support to those clinical studies that show the beneficial role of vitamin E supplementation on the population of patients on haemodialysis. These patients have been documented to present high levels of LDL(−), which is strongly correlated with cardiovascular risk, active disease, as tightly linked to oxidative stress.

In conclusion, this study revealed a significant decrease of circulating LDL(−) in HD patients after the α-tocopherol supplementation (400 I.U. for 16 weeks). The decrease of this atherogenic lipoprotein subfraction may have a preventive role in patients undergoing HD. Further studies will be necessary to investigate the effect of α-tocopherol on surrogates of clinical atherosclerosis.

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Conflict of interest statement. None declared.

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