Effects of antioxidant supplementation on blood cyclosporin A and glomerular filtration rate in renal transplant recipients

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Abstract

Background. Transplant recipients have elevated oxidative stress, which has prompted suggestions that supplementary antioxidants may be beneficial. However, only a small number of clinical trials have investigated antioxidant supplementation in transplant recipients, with very few data on their effects on patients’ immunosuppressive therapy.

Methods. A randomized placebo-controlled single-blind crossover trial was conducted in 10 renal transplant recipients (RTRs) taking cyclosporin A (CsA) as part of their immunosuppressive therapy. Each phase of the trial lasted 6 months, with a 6 month wash-out period in between. During one of the phases, patients consumed a tablet twice per day which delivered 400 IU/day of vitamin E, 500 mg/day of vitamin C and 6 mg/day of β-carotene.

Results. During antioxidant supplementation, there was no change in CsA dose. Antioxidant supplementation resulted in a significant decrease (P<0.05) in blood trough CsA by 24% (mean±SD, pre-127.3±38.9, post-97.2±30.7 μg/ml) compared with no change while taking the placebo (pre-132.2±50.6, post-138.6±56.0 μg/ml). The glomerular filtration rate was significantly (P<0.05) improved by 12% during antioxidant supplementation (pre-66.9±20.7, post-75.0±20.1 ml/min/1.72 m²), with no change during the placebo phase (pre-66.8±11.8, post-66.7±16.1 ml/min/1.72 m²). There were no significant differences (P>0.05) in markers of oxidative stress (malondialdehyde, susceptibility of plasma to oxidation) or plasma antioxidant enzymes.

Conclusion. In CsA-treated RTRs, antioxidant supplementation decreased blood CsA, which may affect adequacy of immunosuppression.

Keywords: antioxidants; cyclosporin A; oxidative stress; renal function

Introduction

Increased production of reactive oxygen species (ROS) and subsequent elevated oxidative stress have been implicated in the development and progression of a wide range of conditions, including diabetes, cardiovascular disease and renal disease [1]. There has been considerable interest in developing strategies to reduce oxidative stress, with many observational and animal studies supporting the use of antioxidant supplementation [2,3], leading many physicians to recommend supplements to patients. Although most, but not all, large-scale prospective clinical trials have shown little benefit with antioxidant supplementation, there is a strong belief that these supplements may be more beneficial in individuals with elevated oxidative stress or compromised antioxidant defences [4]. Indeed, the Secondary Prevention with Antioxidants of Cardiovascular Disease in End Stage Renal Disease (SPACE) trial reported that supplementation with 800 IU/day vitamin E in haemodialysis patients—patients predisposed to elevated oxidative stress—reduced composite cardiovascular disease end-points including myocardial infarction [5].

Renal transplant recipients (RTRs) have elevated oxidative stress, believed to be caused by the immunosuppressive therapy [6]. Although cyclosporin A (CsA) is credited with improved patient and organ–graft survival rates, its chronic nephrotoxicity is still a concern. CsA has been shown to increase the production...
of ROS in animal and in vitro models, leading to speculation that elevated oxidative stress may be involved in CsA-induced nephrotoxicity, as well as the elevated cardiovascular disease seen in RTRs.

Surprisingly, only five studies have investigated the effects of antioxidant supplementation in RTRs [7–11], with only three of these supplying the antioxidants for a prolonged period of time [8,10,11] and one reporting effects on blood CsA concentrations [8]. The major findings of these studies indicated that a variety of different antioxidants decreased markers of oxidative stress (see Blackhall et al. [12] for a detailed review). Recently, Barany et al. [13] reported that 6 weeks of vitamin E supplementation decreased the area under the CsA concentration curve by 21%, indicating a potential interaction between CsA and antioxidant supplementation. Given the importance in maintaining adequate immunosuppressant concentrations, the objective of this study was to determine the effects of antioxidant supplementation on blood CsA in RTRs. In addition, the effects of antioxidant supplementation on oxidative stress and endogenous antioxidants were also examined.

Methods

A randomized placebo-controlled single-blind crossover trial was conducted in 10 RTRs taking CsA (Neoral) as part of their immunosuppressive therapy. Any patient in Northern Tasmania who had received a renal transplant was invited to participate in the study. Patients were excluded if they were already taking an antioxidant supplement. All patients were recruited from the practices of the same nephrologist. The study received approval of the Ethics Committee of the Launceston General Hospital and patients provided written informed consent to participate.

Each phase of the trial lasted 6 months, with a 6 month wash-out period in between. During one of the phases, patients were randomly assigned to consume a tablet twice per day which supplied 400 IU/day of vitamin E, 500 mg/day of vitamin C and 6 mg/day of β-carotene. During the other phase, patients consumed a placebo tablet twice per day. Patients were instructed to take one tablet in the morning and one in the evening. Fasting blood samples and 24 h urine were collected from subjects before and at the completion of each phase.

Routine clinical chemistry measures

Routine clinical chemistry measures were completed in the National Accredited Testing Authority (NATA) Pathology Department at Launceston General Hospital. These included: full lipid profile [total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides], full blood count [haemoglobin, red blood cell (RBC) count, haematocrit, mean cell volume, mean cell haemoglobin, platelets, white cell count, neutrophils, lymphocytes, monocytes, eosinophils and basophils], liver function tests (aspartate aminotransferase, alanine transferase, bilirubin, γ-glutamyl transferase, alkaline phosphatase, total protein and albumin), serum creatinine, urinary metabolites (urea, urinary RBCs and urinary leukocytes), serum electrolytes (sodium, potassium and chloride), bicarbonate, calcium, magnesium, potassium and trough CsA. The majority of the measures were determined using an Olympus AU-600 (Mishima, Olympus Co., Japan) auto-analyser. Full blood counts were performed on a Coulter MaxM (Coulter, Luton, UK). Urinary RBCs and leukocytes were determined using microscopy. LDL cholesterol was calculated using the Freidwald formula. The glomerular filtration rate (GFR) was calculated using the Cockcroft and Gault equation. Whole blood CsA was determined using a monoclonal method (CEDIA Cyclosporin Plus Assay, Microgenics).

Susceptibility of plasma to oxidation

Susceptibility of plasma to oxidation was determined by the method of Kontush et al. [14] with slight modification. Briefly, the absorbance of conjugated dienes at 234 nm was followed spectrophotometrically (Varian 1E, Palo Alto, CA) for 20 h. The spectrophotometer was equipped with a six-position automatic sample changer in which the absorbance changes of all samples were compared with blank reference cells. Oxidation was initiated by 2,2’-azobis-(2-aminopropionate) hydrochloride (AAPH; 330 μM). Measures used to quantify plasma oxidizability were: (i) the lag time to conjugated diene formation (lag time)—this represented the hydrophilic antioxidant capacity and was obtained by non-linear regression analysis of absorbance data using PRISM data analysis software (GraphPad, San Diego, CA); (ii) change in absorbance at 234 nm—this represented the amount of lipid oxidation and was calculated by the difference between the maximum and minimum absorbance; and (iii) the slope of the oxidation curve during propagation (slope)—this represented the rate of lipid oxidation and was the linear trend line from the lag time to the end of the propagation phase.

Oxidative stress

Determination of plasma malondialdehyde (MDA) by high-performance liquid chromatography (HPLC) was used as the marker of oxidative stress. The principle of this method is that MDA is derivatized with 2,4-dinitrophenylhydrazine (DNPH) which forms stable hydrazones that can be easily separated by HPLC with diode array detection (Shimadzu, Kyoto, Japan). This assay has gained acceptance as being a valid and reliable measure of plasma oxidative stress.

Antioxidants

Plasma concentrations of vitamin E (α-tocopherol) and total carotenoids were determined by HPLC. Briefly, proteins were precipitated and lips extracted with hexane, and carotenoids and vitamin E were measured using fluorometric detection. Plasma superoxide dismutase (SOD) activity was measured spectrophotometrically (Titertek Multiskan, Helsinki, Finland), determining the ability of the enzyme to inhibit reduction of the tetrazolium dye XTT. One unit of SOD is defined as the amount of SOD required to inhibit the XTT reduction by 50%. Plasma glutathione peroxidase (GPX) activity was measured via the oxidation of NADPH
to NADPH. The procedure was carried out on an automated spectrophotometer (Cobas, Mira, Roche Diagnostics, Switzerland) with one unit of GPX activity defined as 1 μmol NADPH oxidized/min/ml of plasma. The coefficients of variation of these assays were all <5%.

Data analysis

Paired Student t-tests were used to compare differences between pre- and post- mean values in the placebo and antioxidant-supplemented phases and the differences between the changes (pre- to post-) between placebo and antioxidant supplemented. Pearson correlational analyses were also used to determine relationships between changes in variables. For all statistical tests, significance was assumed if $P < 0.05$ (two sided). All data are presented as mean ± SD.

Results

Demographic and baseline data are provided in Table 1. Reasons for transplantation were chronic renal failure from: polycystic kidney disease ($n = 2$), presumed glomerulonephritis (GN) ($n = 1$), extra- and intra-capillary GN ($n = 1$), focal and segmental proliferative GN ($n = 1$), interstitial nephritis ($n = 1$), reflux nephropathy ($n = 1$), mesangial proliferative glomerulonephritis (IgA disease) ($n = 1$) and unknown ($n = 1$). Medications were as follows: CsA ($n = 10$), calcium channel blocker ($n = 7$), lipid-lowering therapy ($n = 7$), azathioprine ($n = 5$), hyperacidity reflux and ulcer therapy ($n = 3$), angiotensin-converting enzyme (ACE) inhibitor ($n = 6$), β-blocker ($n = 3$), adrenal steroid hormone (prednisolone) ($n = 2$), anticoagulant ($n = 3$), vasodilator ($n = 3$), mycophenolate mofetil ($n = 1$), agents used in gout and hyperuricaemia ($n = 1$), hypoglycaemic agents ($n = 1$), angiotensin II antagonist ($n = 1$), agent affecting calcium and bone metabolism ($n = 1$), antidepressant ($n = 1$), central acting antihypertensive ($n = 1$), mineral ($n = 1$), diuretic ($n = 1$) and muscle relaxant ($n = 1$). The median number of medications per patient was five.

There were no reports of adverse events or side effects during the trial. During the antioxidant phase, there were no changes in the CsA dosages (mean ± SD, 212.5 ± 63.7 mg/day), with one person taking 350 mg/day, three taking 250 mg/day, two taking 200 mg/day, one taking 175 mg/day and three taking 150 mg/day. During the placebo phase, one of the patients had their dosage reduced from 350 to 300 mg/day, one reduced from 300 to 275 mg/day and one from 200 to 175 mg/day. The CsA dosages in the placebo phase were pre- 217.5 ± 66.7 and post- 207.5 ± 54.1.

Table 2 shows the effects of the antioxidant supplements on exogenous and endogenous antioxidants. During the antioxidant phase, there were significant ($P < 0.001$) increases in plasma vitamin E (77%) and total carotenoids (145%). There were no significant differences ($P > 0.05$) between pre- and post-values or between the differences between antioxidant or placebo phases in the plasma antioxidant enzyme activities of GPX and SOD. Table 3 indicates that there were no significant ($P > 0.05$) effects of the antioxidant supplements on MDA or measures of the susceptibility of plasma to oxidation.

Figure 1 indicates that blood CsA concentrations were significantly ($P < 0.05$) decreased by 24% when patients were taking the antioxidant supplements. In addition, there was a significant ($P < 0.05$) improvement from pre- to post-supplementation in GFR and a decrease in serum creatinine in patients taking the antioxidants (Table 4). Furthermore, there was a significant ($P < 0.05$) difference in the changes from pre- to post- between antioxidant and placebo phases in these patients. Surprisingly, there was no significant correlation ($P > 0.05$) between blood CsA concentration and GFR ($r = -0.28$, $P = 0.59$), although the lack of correlation may be due to the small number of subjects.
There were no significant differences between pre- and post-values (data not shown) in either the antioxidant or placebo phases in lipid concentrations, full blood count variables, liver function, urinary metabolites or circulating electrolytes.

**Discussion**

The main finding of this study was that antioxidant supplementation with vitamin E, vitamin C and β-carotene significantly decreased blood CsA concentrations by 24% and improved GFR by 17% in RTRs. This finding may have significant implications for RTRs taking CsA who use antioxidant supplements.

**Antioxidant supplementation and blood cyclosporin**

In support of the findings in the present study, Barany et al. [13] reported that after 6 weeks of vitamin E supplementation (800 IU/day), a single dose of CsA in healthy subjects was associated with a 21% lower area under the CsA curve. The authors stated that the finding was unexpected and cannot readily be explained. They did not speculate on a mechanism and suggested that future studies are required to confirm this finding. Possible mechanisms that may explain the decreased concentrations of CsA include (i) increased intestinal CsA metabolism; (ii) decreased intestinal CsA absorption; (iii) increased CsA metabolism after absorption; or (iv) changes in CsA distribution.
Intestinal metabolism and absorption of CsA are controlled by the cytochrome P450 IIIA4 (CYP3A4) pathway and P-glycoprotein. When the lipid-lowering antioxidant probucol was co-administered with CsA, the blood concentration of CsA significantly decreased [15]. Jiko et al. [16] investigated the mechanism of the interaction between probucol and CsA, and concluded that there is decreased intestinal absorption of CsA due to its lowered solubility in the presence of probucol. Results from Sugimoto et al. [15] supported these findings when they reported that blood CsA concentrations were not affected by probucol given via intravenous injection. Interestingly, in all of these studies, the potential mechanisms proposed did not take into account the antioxidant effects of probucol.

An interaction between CsA and a water-soluble vitamin E (α-tocopheryl polyethylene glycol 1000 succinate) has been reported [17]. However, contrary to the present findings, these studies found that the vitamin E analogue increased CsA absorption. The compound has been shown, at relatively low concentrations, to interact with P-glycoprotein and increase intestinal CsA excretion [17], raising the possibility that this mechanism may be reversed at higher concentrations of alpha tocopherol and intestinal CsA excretion may be inhibited.

Further mechanisms explaining the CsA-lowering effects of the antioxidant supplement used in this study include increased metabolism after intestinal absorption or changes in distribution. Approximately 40–60% of CsA in blood is bound to cholesterol-rich lipoproteins. Furthermore, the entrance of CsA into cells may be facilitated by the LDL receptor. Therefore, changes in lipid concentrations can alter CsA disposition and tissue distribution. In the present study, there were no significant changes in lipid concentrations when patients were taking antioxidants, suggesting that this mechanism was not responsible for our findings.

Non-antioxidant metabolic functions of the supplements may also explain the findings. Most research into non-antioxidant properties of these compounds has focused on vitamin E. At the post-translational level, vitamin E inhibits protein kinase C, 5-lipoxygenase and phospholipase A2, and activates protein phosphatase 2A and diacylglycerol kinase. Some genes (e.g. scavenger receptors, α-tropomyosin, matrix metalloproteinase-19 and collagenase) are modulated by vitamin E at the transcriptional level. Furthermore, vitamin E also inhibits cell proliferation, platelet aggregation and monocyte adhesion. These effects are unrelated to the antioxidant activity of vitamin E, and possibly reflect specific interactions of α-tocopherol with enzymes, structural proteins, lipids and transcription factors. Therefore, it is plausible that non-antioxidant metabolic functions of the supplements may be responsible for the findings.

Improvement in GFR

RTRs taking the antioxidant supplement had significantly improved GFR and serum creatinine. Given the knowledge that CsA is nephrotoxic, it is not surprising that patients had improved GFR along with the decreased CsA. The lack of correlation between CsA and GFR is probably due to small patient numbers, although an alternative hypothesis is that the antioxidants improved GFR and decreased CsA independently of each other. As CsA is believed to exert its nephrotoxic effects through an increase in cytochrome P450 activity resulting in elevated oxidative stress [18], it is possible that the antioxidant activities of the supplements are responsible for the changes in GFR and serum creatinine independently of their effect on CsA. Indeed, there is evidence that suggests a link between oxidative stress and renal function. Renal artery stenosis, usually due to atherosclerosis, is one of the most common vascular nephropathies and renovascular diseases, accounting for over a third of all cases of renal dysfunction. ROS can modulate renal haemodynamics and function both directly, by leading to vasoconstriction, and indirectly, by inducing renal inflammation and tissue growth [19]. The superoxide anion can also react with nitric oxide, decreasing its availability and resulting in the formation of peroxynitrite. This can impair intra-renal vascular and glomerular function. Furthermore, ROS have been shown to interfere with renal oxygen usage for tubular sodium transport and enhance tubuloglomerular feedback [20]. The ability of antioxidant supplementation to improve renal function in RTRs is supported by the only two studies that have reported renal function changes while administering antioxidants to RTRs. Rabl et al. [7] found that following antioxidant infusion, patients had significantly lower serum creatinine and higher creatinine clearance post-transplantation, and Vela et al. [10] reported a modification of the inverse creatinine curve following supplementation with 500 mg of α-tocopherol for 6 months. The notion that antioxidant supplementation may independently improve GFR in RTRs is currently being investigated by our research group in a larger cohort over a longer period of time. In summary, as mentioned above, a more likely explanation for the improved GFR is the decreased CsA.

Antioxidants and oxidative stress

Plasma MDA from the patients was ~16–17 μmol/l, which is within the range of values taken from samples from our laboratory from healthy individuals (usual range 12–17 μmol/l). It has been suggested that antioxidant supplementation is beneficial only when elevated oxidative stress is present [4] and, as our patients did not have elevated oxidative stress, this may explain why the antioxidant supplements had no effect on our marker of oxidative stress. The inclusion of a control group would have provided a better opportunity of addressing this postulate.

The finding that antioxidant supplementation had no effect on oxidative stress appears to contradict the postulate that the antioxidants were responsible for the improved renal function. However, it is possible
that the supplementary antioxidants may have used their antioxidant properties to improve GFR rather than decrease circulating MDA. It is believed that the majority of plasma MDA is derived from the oxidation of polyunsaturated fatty acids in cell membranes throughout the body, with CsA reported to have a significant effect on these concentrations. Therefore, the supplementary antioxidants may not have been able to reduce the overall impact of CsA on MDA, but were still able to exert an effect on renal function.

**Summary**

Using a randomized placebo-controlled single-blind design, we report that antioxidant supplementation with vitamin E, vitamin C and β-carotene decreases blood CsA concentration and improves GFR in RTRs. This finding, if confirmed, will have important implications for transplant physicians monitoring blood CsA concentrations, as reduced immunosuppression is the main reason for organ rejection. The mechanism responsible for our findings may be due to either the antioxidant properties or the pleiotropic effects of the administered compounds and is an interesting area for future research. The improvement in GFR is probably due to the decreased CsA. In summary, nephrologists should be aware of the potential CsA-lowering effects of antioxidant supplements when optimizing CsA dosages in RTRs.

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