Antioxidant Supplementation Enhances Erythrocyte Antioxidant Status and Attenuates Cyclosporine-Induced Vascular Dysfunction

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The aim of this study was to determine the effects of dietary antioxidant supplementation with \( \alpha \)-tocopherol and \( \alpha \)-lipoic acid on cyclosporine-induced alterations to erythrocyte and plasma redox balance, and cyclosporine-induced endothelial and smooth muscle dysfunction. Rats were randomly assigned to either control, antioxidant, cyclosporine or cyclosporine + antioxidant treatments. Cyclosporine A was administered for 10 days after an 8-week feeding period. Plasma was analyzed for \( \alpha \)-tocopherol, total antioxidant capacity, malondialdehyde and creatinine. Erythrocytes were analyzed for glutathione, methemoglobin, superoxide dismutase, catalase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, \( \alpha \)-tocopherol and malondialdehyde. Vascular endothelial and smooth muscle function was determined \textit{in vitro}. Antioxidant supplementation resulted in significant increases in erythrocyte \( \alpha \)-tocopherol concentration and glutathione peroxidase activity in both of the antioxidant-supplemented groups. Cyclosporine administration caused significant decreases in glutathione concentration, methemoglobin concentration and superoxide dismutase activity. Antioxidant supplementation attenuated the cyclosporine-induced decrease in superoxide dismutase activity. Cyclosporine therapy impaired both endothelium-independent and -dependent relaxation of the thoracic aorta, and this was attenuated by antioxidant supplementation. In summary, dietary supplementation with \( \alpha \)-tocopherol and \( \alpha \)-lipoic acid attenuated the cyclosporine-induced decrease in erythrocyte superoxide dismutase activity and attenuated cyclosporine-induced vascular dysfunction.

Key words: Antioxidant status, cyclosporine A, erythrocytes, oxidative stress, vascular function

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Introduction

Cardiovascular disease is significantly increased in organ transplant recipients and remains the largest single factor limiting long-term survival in these patients (1). In the general population, endothelial dysfunction is an independent predictor of cardiovascular disease (2), and convincing evidence implicates oxidative stress in its pathogenesis and progression (3). Inverse associations have been found between cardiovascular disease and antioxidants in the circulation, including plasma antioxidants, erythrocyte \( \alpha \)-tocopherol and erythrocyte glutathione peroxidase activity (4–6). It is therefore not surprising that transplant recipients show endothelial dysfunction (7) and altered plasma and erythrocyte redox balance (8,9). Cyclosporine A (cyclosporine) is an immunosuppressant commonly administered to prevent organ rejection and evidence suggests that this agent may be responsible for the endothelial dysfunction and altered plasma and erythrocyte redox balance observed in transplant recipients (7,10,11). Endothelial dysfunction is a loss of vascular homeostasis that may be caused by reduced nitric oxide (NO) bioactivity and is characterized by impaired NO mediated vasodilation (2). Enhanced oxidative stress causes a loss of vascular homeostasis by decreasing NO production and increasing NO elimination via increased formation of peroxynitrite (12).

The erythrocyte is pivotal to the overall antioxidant status of the blood, as the red cell functions to maintain plasma antioxidants in the reduced state (13), and eliminates superoxide and hydrogen peroxide derived from the plasma (14). Furthermore, the blood is a tissue that comes into direct contact with the endothelium and previous \textit{in vitro} studies have shown that intact erythrocytes can protect endothelial cells against oxidant-induced damage (15,16). Recent animal studies from our group showed that acute cyclosporine administration altered plasma and erythrocyte redox balance (10,11). Moreover, cyclosporine administration induces endothelial dysfunction (17–19) and smooth
muscle dysfunction (20–22) of rat thoracic aorta when measured in vitro. The aim of the present study was to investigate a strategy that could improve antioxidant defences in plasma and erythrocytes and attenuate cyclosporine-induced endothelial and smooth muscle dysfunction. It is widely believed that an optimum antioxidant supplement contains more than one nutrient (23), and the combination of α-tocopherol and α-lipoic acid has generated scientific interest (24,25). α-Tocopherol is the major lipid-soluble antioxidant in plasma lipoproteins and cell membranes (26). α-Lipoic acid is a naturally occurring cofactor within pyruvate dehydrogenase and a keto-glutarate dehydrogenase, and is soluble in both lipid and aqueous phases (27). Although very little free α-lipoic acid is thought to occur in unsupplemented conditions, free α-lipoic acid can recycle glutathione, α-tocopherol and ascorbic acid, and scavenge superoxide, hydrogen peroxide, hydroxyl and peroxynitrite (27). In vitro α-lipoic acid decreased plasma susceptibility to oxidation (28), was protective against hemolysis of human erythrocytes induced by peroxyl radicals (29), and increased the capacity of endothelial cells to generate NO (30). In humans, supplementation with α-lipoic acid improved endothelial dysfunction in diabetic patients (31). Therefore, α-lipoic acid supplementation may prevent or attenuate cyclosporine-induced endothelial dysfunction, and be protective against cyclosporine-induced oxidative stress in the circulation. Supplementation with α-tocopherol improved endothelial dysfunction in cholesterol-fed rabbits (32) and in humans with high cholesterol concentrations (33). Furthermore, the cyclosporine-induced increase in renal malondialdehyde was prevented when animals were supplemented with α-tocopherol (34). Despite previous animal studies failing to show a cyclosporine-induced decrease in erythrocyte α-tocopherol, it is hypothesized that α-tocopherol supplementation may improve cyclosporine-induced endothelial dysfunction and prevent the cyclosporine-induced increase in plasma malondialdehyde. Therefore, the present study was conducted to determine if a dietary combination of α-tocopherol and α-lipoic acid could improve antioxidant status in plasma and erythrocytes, and prevent cyclosporine-induced endothelial and smooth muscle dysfunction.

Materials and Methods

Animals and experimental design
Forty male Sprague Dawley rats (3 weeks) were randomly assigned (n = 10 per group) to one of four experimental groups: (1) control, (2) antioxidant, (3) cyclosporine or (4) cyclosporine + antioxidant. All rats were fed rat chow and water ad libitum and maintained on a 12-h light/dark photo-period with a room temperature of 21 ± 2 °C. The rat chow given to groups (2) and (4) was supplemented with d-α-tocopheryl acid succinate (α-tocopherol) (1000 IU/kg diet) and α-lipoic acid (1.6 g/kg diet). The supplemented diet was continued until sacrifice. After 8 weeks of antioxidant supplementation, cyclosporine-treated animals were administered with 25 mg/kg/day of cyclosporine A (Alexis biochemicals, San Diego, CA; dissolved 30 mg/mL in extralight olive oil and 4% ethanol) via intra-peritoneal injection for 10 days. Control and antioxidant groups were injected daily for 10 days with the same volume of the vehicle. Animal body weight was recorded daily and cyclosporine doses were adjusted to changes in body weight. All injections were performed at approximately the same time of the day. All experimental protocols were approved by the Animal Experimentation Ethics Committee of the University of Queensland under the guidelines of the National Medical and Health Research Council of Australia. This conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. Institutes of Health (NIH Publication No. 85–23, revised 1996).

The cyclosporine dose (25 mg/kg/day) and time period (10 days) used in the present study were based on previous animal studies showing cyclosporine-induced endothelial dysfunction using a similar dose and the same treatment period (20,21). The concentration of α-lipoic acid in the supplemented food was the same as a study by Coombes et al. (24) showing improved cardiac performance after ischemia in aged rats. The concentration of α-tocopherol (1000 IU/kg diet) in the supplemented rat chow was based on previous studies showing that this dose improved endothelial function in cholesterol-fed rabbits (35). The supplementation period was based on a study reporting significant increases in α-tocopherol concentration in the plasma and erythrocytes of rats after 8 weeks of α-tocopherol supplementation (1000 IU/kg diet) (36).

Blood collection and sample preparation
Animals were sacrificed approximately 24 h after the final cyclosporine injection. Just prior to exsanguination, rats were administered sodium pentobarbital (90 mg/kg) via intra-peritoneal injection. After reaching a surgical plane of anesthesia, the chest cavity was opened and rats were exsanguinated via the abdominal aorta. Approximately 5 mL of blood was collected in glass EDTA vacutainer tubes and aliquots of whole blood removed for analysis of cyclosporine concentrations and methemoglobin. The remaining whole blood was centrifuged at 4 °C for 10 min at 800 × g. The plasma was then removed, divided into aliquots and stored at −80 °C until biochemical analysis of total antioxidant capacity, α-tocopherol, malondialdehyde and creatinine. The buffy coat was then removed and discarded. Erythrocytes were subsequently washed three times with phosphate-buffered saline. An aliquot of washed cell suspension was used to determine the concentration of erythrocyte glutathione. Erythrocyte aliquots were stored at −80 °C until biochemical analysis of α-tocopherol and malondialdehyde, and the activities of superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase and glutathione peroxidase. Because plasma contains comparatively little superoxide dismutase, catalase and glutathione peroxidase compared to the erythrocyte (37), these measurements were made only in the erythrocyte.

Cyclosporine A
Trough blood cyclosporine levels were determined at Sullivan Nicolaides Pathology Service (Brisbane, QLD) using Cedia Immunoassay conducted on a Hitachi 911 Autoanalyser (Roche Diagnostics, Germany).

Erythrocyte glutathione
Erythrocyte glutathione was determined by the method of Beutler et al. (38). This method is based on the principle that glutathione reduction of 5,5-dithiobis (2-nitrobenzoic acid) forms a yellow colored anion which is measured spectrophotometrically at 412 nm (Unicam 5625 UV/VIS, Unicam Ltd., Cambridge, UK).

Methemoglobin
Methemoglobin was determined by the method of Evelyn and Mallory (39). Methemoglobin has an absorption peak at 630 nm, which disappears upon addition of potassium cyanide. The difference in absorbance is therefore proportional to the concentration of methemoglobin. Total hemoglobin

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was measured by conversion to cyannemethoglobin by the addition of potassium cyanide and potassium ferricyanide. The procedure was conducted using a spectrophotometer (Unicam 5625 UV/VIS, Unicam Ltd., Cambridge, UK).

Erythrocyte antioxidant enzymes
Glucose-6-phosphate dehydrogenase activity was determined using a modification of the Sigma Diagnostics kit method (procedure #345-UV) which is based on the spectrophotometric method of Lohr and Waller (40). Catalase, glutathione peroxidase and superoxide dismutase activities were determined based on the methods of Slaughter and O’Brien (41), Andersen et al. (42) and Madesh and Balasubramanian (43), respectively.

Malondialdehyde
High performance liquid chromatography (HPLC) was used to determine plasma and erythrocyte malondialdehyde using the method of Sim et al. (44). The principle of this method is that malondialdehyde contained in plasma or erythrocytes is derivatized with 2,4-di-nitrophenylhydrazine which forms stable hydrazones that can be easily separated by HPLC using diode array detection (Shimadzu, Kyoto, Japan).

α-Tocopherol
HPLC was used to determine erythrocyte α-tocopherol concentrations using a modification of the methods of Hatam and Kayden (45). Proteins were precipitated from hemolysate with HPLC grade methanol and α-tocopherol extracted from the supernatant using HPLC grade hexane. Plasma α-tocopherol concentrations were determined using a modification of the method of Taibi and Nicotra (46). Proteins were precipitated from solution with HPLC grade ethanol and α-tocopherol subsequently extracted using HPLC grade hexane. α-Tocopherol extracted from erythrocytes and plasma was measured using fluorometric detection (Shimadzu, Kyoto, Japan). Analysis of quality control samples showed that α-tocopherol was stable throughout the duration of HPLC analysis. α-Lipoic acid was not assayed in the present study as it has been shown to have a short elimination half-life and there is a wide inter-subject variation in time to peak concentration in plasma after oral administration (47). This is supported by unpublished observations from our laboratory in rats.

Plasma total antioxidant capacity
Total antioxidant capacity was determined by the method of Miller et al. (48), which is based on the inhibition by antioxidants of the absorbance of the radical cation 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS•+). The ABTS radical cation is formed by the interaction of ABTS with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin with hydrogen peroxide. Antioxidant compounds suppress the absorbance of ABTS•+ to an extent and on a time scale dependent on the antioxidant capacity of the plasma. The assay was carried out on an automated spectrophotometer (Cobas, Mira, Roche Diagnostics, Switzerland).

Plasma creatinine
Plasma creatinine was determined using the Jaffe reaction method. Absorbance was measured at 520 nm using an automated spectrophotometer (Cobas, Mira, Roche Diagnostics, Switzerland).

In vitro experiments
Immediately after exsanguination, the thoracic aorta was removed and placed in a dissecting dish containing cold gassed (95% O2/5% CO2) physiological solution. Prior to the introduction of each of the vasoactive drugs, aortic rings were equilibrated for 30 min in fresh physiological solution (changed seven times) and a resting tension of 10 mN applied. All vasoactive drugs were purchased from Sigma (Sydney, NSW, Australia). A cumulative concentration-response curve to norepinephrine was determined (final bath concentration 10⁻⁸ to 10⁻⁴ M) for each of the two rings to assess smooth muscle cell contractile function. After a 70% submaximal pre-contraction to norepinephrine, endothelial control of vascular relaxation (endothelium-dependent) was assayed on one ring by the addition of the muscarinic agonist acetylcholine (10⁻⁶ to 10⁻⁴ M). Smooth muscle cell vasorelaxant function (endothelium-independent relaxation) was assayed on the second ring by the addition of sodium nitroprusside (10⁻⁸ to 10⁻⁴ M). Maximal contraction to isotonic potassium chloride (100 mM) was also determined as an assessment of smooth muscle contractile function. The change in isometric force was measured by Grass FT03 force transducers (Grass, MA) connected to a PowerLab chart recording system using Chart 4.0 recording software (AD Instruments, Sydney, NSW, Australia).

Statistical analysis
Cyclosporine concentrations at sacrifice of the cyclosporine, and cyclosporine + antioxidant-treated groups were compared using an independent t-test. Comparisons between groups for animal body weight data, and for all markers of antioxidant status and oxidative stress were made by one-way analysis of variance (ANOVA). If significance was determined, a Tamhane’s post hoc test was used. All biochemical data are shown as the mean ± standard deviation (SD). Comparisons between groups for in vitro data were made by one-way ANOVA. If significance was determined, a Bonferroni post hoc test was used. All in vitro data are shown as the mean ± standard error (SEM). Significance was established at the 95% confidence level (p < 0.05).

Results
Changes in body mass throughout the 10-day cyclosporine administration period are presented in Figure 1. Antioxidant-supplemented animals continued to gain weight at the same rate as control animals (p > 0.05 control vs. antioxidant; 5.6% increase in control group, 5.2% increase in antioxidant group). Cyclosporine administration
Table 1: Measures of antioxidant status, oxidative stress and renal function in the plasma after 8 weeks antioxidant supplementation and 10 days cyclosporine/placebo administration

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Antioxidant</th>
<th>Cyclosporine</th>
<th>Cyclosporine &amp; antioxidant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha tocopherol (µM)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>29.4 ± 5.9</td>
<td>38 ± 14.2</td>
<td>22.1 ± 5.99ao</td>
<td>30.5 ± 11.4</td>
<td></td>
</tr>
<tr>
<td>Total antioxidant capacity (mM)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>1.6 ± 0.6</td>
<td>1.64 ± 07</td>
<td>1.59 ± 06</td>
<td>1.6 ± 02</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde (µM)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>30.3 ± 4.5</td>
<td>29.5 ± 5.3</td>
<td>31.3 ± 5.5</td>
<td>30.3 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Creatinine (µM)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>64.9 ± 10.1</td>
<td>63.6 ± 8.8</td>
<td>71.4 ± 7.7</td>
<td>66.4 ± 5.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. Significantly different: ao = different from antioxidant.

Table 2: Measures of erythrocyte antioxidant status and oxidative stress after 8 weeks antioxidant supplementation and 10 days cyclosporine/placebo administration

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Antioxidant</th>
<th>Cyclosporine</th>
<th>Cyclosporine &amp; antioxidant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (U/gHb)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>(n = 8)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>24025 ± 2515</td>
<td>18306 ± 5334</td>
<td>20514 ± 1861c</td>
<td>21551 ± 4406</td>
<td></td>
</tr>
<tr>
<td>Catalase (U/gHb)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>5221 ± 1267</td>
<td>5022 ± 668</td>
<td>5988 ± 360ao</td>
<td>6449 ± 803ao</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (U/gHb)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>106.1 ± 7.2</td>
<td>128.8 ± 6.9c</td>
<td>116.7 ± 11.4</td>
<td>130.3 ± 6.7c, csa</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (U/gHb)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>25.3 ± 1.4</td>
<td>25.2 ± 2.6</td>
<td>24.3 ± 1.6</td>
<td>25.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Glutathione (mg/100 mL RBC)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>64.1 ± 10</td>
<td>62 ± 6</td>
<td>45.8 ± 10.8c, ao</td>
<td>52.4 ± 5.4c, ao</td>
<td></td>
</tr>
<tr>
<td>Alpha tocopherol (µg/mL packed cells)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>2.32 ± 0.11</td>
<td>3.8 ± 0.37c</td>
<td>2.27 ± 0.3ao</td>
<td>3.34 ± 0.39c</td>
<td></td>
</tr>
<tr>
<td>Met Hb (% total Hb)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>2.28 ± 0.49</td>
<td>1.96 ± 0.6</td>
<td>0.96 ± 0.52c, ao</td>
<td>1.02 ± 0.52c, ao</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde (µM)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>16 ± 2.1</td>
<td>15.7 ± 1.6</td>
<td>16.5 ± 3</td>
<td>15.5 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. Significantly different: c = different from control, ao = different from antioxidant, csa = different from cyclosporine.

resulted in a 6.7% decrease in body weight (p < 0.05 vs. all other groups). Body weight was maintained (100.7%) throughout the drug treatment period in animals receiving both cyclosporine and antioxidants (p < 0.05 vs. all other groups). Trough blood cyclosporine concentrations of the cyclosporine-treated, and cyclosporine + antioxidant-treated animals at sacrifice were not significantly different (6128 ± 996 µg/L, n = 10 and 5946 ± 1379 µg/L, n = 9, respectively).

Markers of plasma oxidative stress and antioxidant status are presented in Table 1. Antioxidant supplementation did not alter markers of oxidative stress and antioxidant status in the plasma. Plasma creatinine, measured as an assessment of renal function at sacrifice, was not significantly altered by antioxidant supplementation. Cyclosporine administration did not result in significant changes in plasma creatinine, or in markers of oxidative stress and antioxidant status in plasma.

Markers of erythrocyte antioxidant status and oxidative stress are presented in Table 2. Antioxidant supplementation resulted in significant increases in erythrocyte α-tocopherol concentration and glutathione peroxidase activity in both of the antioxidant supplemented groups. Cyclosporine administration caused significant decreases in glutathione concentration, methemoglobin concentration and superoxide dismutase activity. Antioxidant supplementation attenuated the cyclosporine-induced decrease in superoxide dismutase activity but failed to improve cyclosporine-induced decreases in glutathione concentrations.

The cumulative-concentration contractile response of isolated thoracic aortic rings to norepinephrine was not significantly different between groups (Figure 2). The maximal contractile response (mN) of isolated thoracic aortic rings to potassium chloride (100 mM) was not significantly different between control and cyclosporine-treated groups.
control vs. cyclosporine); however, the relaxation response in animals treated with cyclosporine + antioxidants was not significantly different from control animals. The relaxation response to acetylcholine was not significantly different between cyclosporine and cyclosporine + antioxidant-treated groups.

**Discussion**

These experiments show that cyclosporine administration compromised the erythrocyte antioxidant defence. Dietary supplementation with $\alpha$-tocopherol and $\alpha$-lipoic acid increased erythrocyte $\alpha$-tocopherol concentration and glutathione peroxidase activity, and attenuated the cyclosporine-induced decrease in superoxide dismutase activity. Antioxidant supplementation also attenuated cyclosporine-induced endothelial dysfunction and vascular smooth muscle dysfunction.

In the present study, cyclosporine administration caused a 15% decrease in erythrocyte superoxide dismutase activity and a 29% decrease in erythrocyte glutathione concentration. Superoxide dismutase is the first line of defence against superoxide radicals (49), and glutathione, a key erythrocyte antioxidant with the ability to reduce reactive oxygen species non-enzymatically, is integral to the functioning of glutathione peroxidase, and recycles $\alpha$-tocopherol and ascorbic acid (27,49,50). This is relevant to vascular injury as in vitro studies have shown that erythrocyte antioxidant defences protect endothelial cells against oxidant-induced damage (15,16). A decreased erythrocyte antioxidant defence may therefore compromise the ability of the erythrocyte to protect the endothelium against oxidant-induced damage, leading to endothelial dysfunction.
The significant increase in erythrocyte \( \alpha \)-tocopherol may prove to be important for transplant recipients, as epidemiologic studies have shown that increased erythrocyte \( \alpha \)-tocopherol is associated with a decreased thickening of the arterial wall, and low concentrations are associated with accelerated atherosclerosis (6). Indeed, it has been reported that transplant recipients show decreased concentrations of erythrocyte \( \alpha \)-tocopherol (51). Although none of the acute animal studies from our laboratory have shown a cyclosporine-induced decrease in erythrocyte \( \alpha \)-tocopherol (10,11), longer term studies are required to determine the effect of prolonged cyclosporine administration on erythrocyte \( \alpha \)-tocopherol levels.

Erythrocyte glutathione peroxidase activity was significantly increased in both antioxidant-treated groups, indicating an increased ability of erythrocytes to neutralize hydrogen peroxide. The mechanism to explain the increased activity is not known, although, because glutathione peroxidase activity in the antioxidant-only group was also significantly increased compared to control, it appears to be directly related to the antioxidant supplementation. Antioxidant supplementation with dietary selenium increased erythrocyte glutathione peroxidase activity by 64% in cyclosporine-dependent human transplant recipients and was associated with a 50% reduction in plasma lipid peroxidation and a 15% reduction in low density lipoprotein oxidation (8). The increased erythrocyte glutathione peroxidase activity shown in the present study may be of clinical relevance as a recent investigation of patients with suspected coronary artery disease showed that a low level of erythrocyte glutathione peroxidase activity was independently associated with an increased risk of cardiovascular events (4).

In the present study, antioxidant supplementation attenuated the cyclosporine-induced decrease in erythrocyte superoxide dismutase activity, indicating a maintained ability of the erythrocyte to remove superoxide enzymatically. Supplementation with \( \alpha \)-tocopherol and \( \alpha \)-lipoic acid may have provided an additional mechanism for the removal of superoxide, thus sparing erythrocyte superoxide dismutase in a time of enhanced oxidative stress. Future studies designed to test this hypothesis via the direct measurement of superoxide are warranted. Richards et al. (15) found that the coincubation of human umbilical vein endothelial cells with increasing erythrocyte concentrations resulted in a dose-dependent protection of endothelial cells against oxidant-induced damage. Importantly, when erythrocyte glutathione peroxidase activity was inactivated, or the ability of superoxide to cross the erythrocyte membrane was inhibited, the extent of endothelial damage increased (15). Therefore, the higher glutathione peroxidase and superoxide dismutase activity of erythrocytes from cyclosporine + antioxidant-treated animals in the present study may have provided enhanced antioxidant protection to endothelial cells, potentially explaining the attenuation of cyclosporine-impaired endothelial function.

Cyclosporine-treated animals lost body mass during the drug treatment protocol; however, body mass was maintained in animals treated with cyclosporine and antioxidants. Previous work has shown that control and antioxidant-treated rats consume a similar amount of food (24), and weight loss occurs in cyclosporine-treated rats pair-fed with control animals (52). Furthermore, Wang and Salahudeen (52) showed that \( \alpha \)-tocopherol supplementation limited the cyclosporine-induced weight loss in pair-fed animals. It is therefore possible that antioxidant supplementation also prevented the cyclosporine-induced weight loss in the present study. The mechanism to explain the apparent oxidant-induced weight loss is currently unknown and would be an interesting area for future research.

Cyclosporine administration had no effect on concentrations of plasma creatinine and plasma malondialdehyde in the present study. Given that cyclosporine-induced increases in both plasma malondialdehyde (10,11,52) and creatinine (10,11,53) have been shown in previous animal studies, these results were unexpected. It is possible in the present study that a reduction in creatinine clearance was masked by an absolute reduction in creatinine production due to a lower muscle mass in the cyclosporine-treated rats. Trough blood concentrations of the drug for the cyclosporine-treated groups demonstrate that malabsorption and/or low bioactivity of the drug in the present study can be ruled out. Although it has not been a consistent finding, high cyclosporine levels in the absence of impaired renal function have previously been reported in the rat (54). The results of the present study, combined with those of previous studies, indicate that plasma malondialdehyde is not necessarily an indicator of increased vascular reactive oxygen species. Increases in plasma malondialdehyde may be more indicative of lipid peroxidation in organs such as the kidney.

Several studies have shown that cyclosporine administration in vivo caused impaired endothelium-dependent relaxation of rat thoracic aorta in vitro (17–19). The results of the present study showed that 8 weeks of dietary supplementation with \( \alpha \)-tocopherol and \( \alpha \)-lipoic acid attenuated the cyclosporine-impaired relaxation response to acetylcholine, thereby implicating oxidative stress in cyclosporine-induced endothelial dysfunction. This is supported by the improvement in the cyclosporine-impaired response to acetylcholine following 5 weeks of dietary supplementation with selenium (17). Furthermore, incubation with either superoxide dismutase or selenium improved the relaxations to acetylcholine in animals treated with cyclosporine alone (17). Oriji and Schanz (55) reported a cyclosporine-induced inhibition of aortic endothelial NO formation, and Kim et al. (22) showed that preincubation of the NO substrate \( l \)-arginine with aortic rings from cyclosporine-treated rats improved endothelium-dependent relaxation to acetylcholine. These findings indicate that reduced NO bioactivity due to increased
superoxide may be responsible for cyclosporine-induced endothelial dysfunction of rat thoracic aorta.

In the present study, the relaxation response to sodium nitroprusside was significantly reduced in cyclosporine-treated animals, and this was attenuated by antioxidant supplementation. These findings indicate that cyclosporine-impaired vascular smooth muscle relaxation may be due to an increased production of reactive oxygen species. A cyclosporine-induced blunted relaxation response to sodium nitroprusside in the thoracic aorta of the rat has been reported in some studies (20–22), but not in others (19,56). In contrast to our results, dietary supplementation with selenium, an important component of glutathione peroxidase, had no effect on cyclosporine-impaired aortic smooth muscle relaxation to sodium nitroprusside (17). The mechanism to explain the conflicting findings is not known, although it is possible that the antioxidant combination used in the present study provided a greater antioxidant protection than the supplements used in the study by Berkenboom et al. (17). Because erythrocytes do not come into direct contact with the vascular smooth muscle layer, it is hypothesized that α-tocopherol and α-lipoic acid were directly responsible for attenuating cyclosporine-induced smooth muscle dysfunction.

In the present study, the cumulative-concentration contractile response to norepinephrine was not different between groups. These results support previous studies investigating the effects of acute cyclosporine administration on aortic contraction in vitro (20,22) and indicate that aortic smooth muscle contraction mediated by alpha adrenergic receptors is unaffected by cyclosporine. The maximal contractile response to potassium chloride was also not different between groups. This has also been shown previously (57) and suggests that depolarization induced contraction of the thoracic aorta was unaffected in the cyclosporine-treated rats.

Potential sources of vascular superoxide production include both endothelial and smooth muscle cells. Angiotensin II receptor (AT1A) activation on endothelial cells and vascular smooth muscle cells increases superoxide production via activation of NAD(P)H oxidase (58), and previous studies have shown a cyclosporine-induced up-regulation of the AT1A receptor in rat aortic endothelial and smooth muscle cells (59,60). In a more recent study, cyclosporine administration (30 mg/kg/day) to rats for 3 weeks increased aortic AT1 receptor expression and superoxide production (61). Concurrent administration of the AT1 receptor antagonist, valsartan, prevented the cyclosporine-induced increase in aortic superoxide (61).

Given that vascular dysfunction was only partially restored by antioxidant treatment, it is possible that additional mechanisms are involved in cyclosporine-impaired relaxation. A recent study by Ramzy et al. (62) showed that cyclosporine administration to rats increased the sensitivity of the thoracic aorta to endothelin. The authors suggest that endothelin blockade may be important for preserving endothelial dysfunction in cyclosporine-treated rats (62). Vascular structural changes associated with cyclosporine therapy may also have contributed to cyclosporine-impaired relaxation.

Peak cyclosporine concentrations in human transplant recipients are approximately 1500 μg/L just after transplantation, and are gradually reduced to 600–800 μg/L (63). A common adverse effect of cyclosporine therapy is renal dysfunction and it is estimated that 30% of cyclosporine-dependent patients experience renal toxicity (53). Although the trough cyclosporine levels achieved in the present study are higher than those used in human transplantation, it is difficult to extrapolate these findings to humans as it has previously been reported that rats are more resistant than humans to the toxic effects of cyclosporine (54). It is well established that endothelial dysfunction occurs in transplant recipients, and available evidence implicates cyclosporine therapy as a potential cause (7). In the present study, the aortic relaxation response to the maximum acetylcholine dose was 19% and 48% of control values for cyclosporine, and cyclosporine + antioxidant-treated animals, respectively. Although extrapolation of these findings to humans is difficult, a recent study showed that relatively small differences in coronary vessel vasoreactivity to acetylcholine resulted in significant differences in the incidence of cardiovascular events during long-term follow-up in humans (64). Intra-coronary infusion of 10−6 mol/L acetylcholine elicited vasodilation in 34% of the patients and vasoconstriction (due to the direct effect of acetylcholine on smooth muscle muscarinic receptors) in 66% of patients (64). The mean epicardial luminal area change was approximately −8% in patients without a cardiovascular event and approximately −26% in patients with a cardiovascular event during long-term follow-up (64). It is therefore possible that the modest increase in vasodilation of the cyclosporine + antioxidant-treated animals observed in the present study may be of therapeutic significance.

In summary, these experiments showed that dietary supplementation with α-tocopherol and α-lipoic acid increased erythrocyte α-tocopherol concentration and glutathione peroxidase activity, and attenuated the cyclosporine-induced decrease in superoxide dismutase activity. Antioxidant supplementation also attenuated cyclosporine-induced endothelial dysfunction and vascular smooth muscle dysfunction. Although the relevance of these findings to the clinical setting is yet to be established, the results suggest that antioxidant supplementation with α-tocopherol and α-lipoic acid may be beneficial in the management of cyclosporine-dependent patients by preventing or delaying vascular dysfunction.
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References


