Research article

Cyclosporine A induced changes to plasma and erythrocyte antioxidant defences

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Organ transplant recipients develop pronounced cardiovascular disease, and decreased antioxidant capacity in plasma and erythrocytes is associated with the pathogenesis of this disease. These experiments tested the hypothesis that the immunosuppressant cyclosporine A (CsA) alters erythrocyte redox balance and reduces plasma antioxidant capacity. Female Sprague-Dawley rats were randomly assigned to a control or CsA treated group. Treatment animals received 25 mg/kg/day of CsA via intraperitoneal injection for 18 days. Control rats were injected with the same volume of the vehicle. Three hours after the final CsA injection, rats were exsanguinated and plasma analysed for total antioxidant status (TAS), α-tocopherol, malondialdehyde (MDA), and creatinine. Erythrocytes were analysed for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glucose-6-phosphate dehydrogenase (G6PD) activities, α-tocopherol, and MDA. CsA administration resulted in a significant (P < 0.05) decrease in plasma TAS and significant increases (P < 0.05) in plasma creatinine and MDA. Erythrocyte CAT was significantly (P < 0.05) increased in CsA treated rats compared to controls. There were no significant differences (P > 0.05) in erythrocyte SOD, GPX, G6PD, α-tocopherol or MDA between groups. In summary, CsA alters erythrocyte antioxidant defence and decreases plasma total antioxidant capacity.

Keywords: Cyclosporine A, reactive oxygen species, free radicals, antioxidants, erythrocytes, plasma

INTRODUCTION

The major cause of death following organ transplantation is a cardiovascular event secondary to atherosclerosis and the incidence is significantly increased compared to the general population.¹ In the general population, endothelial dysfunction is an independent predictor of cardiovascular events,² and convincing evidence is accumulating to implicating oxidative stress.² Indeed, it has been reported that transplant recipients exhibit endothelial dysfunction³ and a recent study shows endothelial dysfunction to be an independent predictor of cardiovascular disease (CVD) in transplant recipients.⁴

The blood is a tissue that comes into direct contact with the endothelium and previous in vitro studies have shown that erythrocytes can protect endothelial cells against oxidant-induced damage.⁵⁶ It is, therefore, possible that the extensive antioxidant defence of the erythrocyte may protect endothelial cells against oxidant induced damage in vivo. Available evidence suggests that the potent oxidative stress.²

Abbreviations: CAT, catalase; CsA, cyclosporine A; G6PD, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GSH, glutathione (reduced); MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TAS, total antioxidant status
antioxidant defence system of the erythrocyte is pivotal to the maintenance of the overall antioxidant status of the blood, as the erythrocyte performs a vital role in maintaining plasma antioxidants in the reduced state. The erythrocyte recycles oxidised extracellular vitamin C, which in turn recycles oxidised vitamin E in plasma lipoproteins. In addition, antioxidant enzymes present in the red cell neutralise superoxide and hydrogen peroxide derived from the plasma.

Epidemiological studies have shown associations between CVD and both plasma and erythrocyte levels of α-tocopherol. Increased plasma levels of α-tocopherol were associated with a decreased incidence of angina, myocardial infarction and mortality from CVD. Increased erythrocyte α-tocopherol was associated with a decreased thickening of the arterial wall, and low concentrations were associated with accelerated atherosclerosis. In transplant recipients, decreased thiol concentrations, increased production of ROS and MDA, and an increased susceptibility of low density lipoprotein (LDL) to oxidation. Furthermore, it has been shown that oxidised plasma LDL is a prognostic marker of transplant coronary artery disease.

Cyclosporine A (CsA) is the most common immuno-suppressive agent administered to organ transplant recipients to prevent rejection. CsA-induced oxidative stress has been implicated in tissue toxicity, hypertension, and endothelial dysfunction; however, non-oxidant mechanisms have also been implicated in CsA-induced pathology. In vitro, it has been shown that CsA increases MDA production when co-incubated with hepatic microsomal preparations. The CsA dose, vehicle, and administration period were approximately the same time of the day in both groups. Changes in body mass. All injections were performed at the chest cavity was opened and rats were exsanguinated via intracardiac puncture. About 5 ml of blood was collected in glass EDTA Vacutainer tubes; from this, 300 µl was used for analysis of peak CsA concentrations, and the remaining whole blood centrifuged at 4°C for 10 min at 800 g. The plasma was then removed, divided into peak CsA concentrations.

### Materials and Methods

**Animals and experimental design**

After approval from the Charles Sturt University’s Animal Care and Ethics Committee, the following experiments were conducted. Twenty-four female, 4-month-old Sprague-Dawley rats weighing 247 ± 22 g (mean ± SD) were randomly assigned to one of two experimental groups, CsA or control (n = 12 per group). Rats were fed rat chow and water *ad libitum* and maintained on a 12-h light/dark photoperiod with a room temperature of 21 ± 2°C. For about 7 days prior to the beginning of the experiments, animals were handled daily by laboratory technicians to reduce contact stress. Treatment group animals were administered daily for 18 days with 25 mg/kg of CsA (30 mg/ml dissolved in extra-light olive oil and 4% ethanol) via intraperitoneal injection. The control rats were sham injected daily for 18 days with the same volume of the vehicle. Animal mass was recorded daily and CsA doses were adjusted to changes in body mass. All injections were performed at approximately the same time of the day in both groups. The CsA dose, vehicle, and administration period were based on previous studies investigating CsA-induced oxidative stress in the plasma.

**Blood collection**

Three hours after the final CsA injection, rats were administered 90 mg/kg sodium pentobarbital via intraperitoneal injection. After reaching a surgical plane of anaesthesia, the chest cavity was opened and rats were exsanguinated via intracardiac puncture. About 5 ml of blood was collected in glass EDTA Vacutainer tubes; from this, 300 µl was used for analysis of peak CsA concentrations, and the remaining whole blood centrifuged at 4°C for 10 min at 800 g. The plasma was then removed, divided into peak CsA concentrations.
aliquots, and stored at –80°C until biochemical analysis of α-tocopherol, MDA, creatinine, and total antioxidant status (TAS). Theuffy coat was then removed and discarded. Erythrocytes were subsequently washed three times with phosphate-buffered saline. Erythrocyte aliquots were added to an equal volume of deionised water and stored at –80°C until biochemical analysis of α-tocopherol, MDA, and the activities of SOD, catalase (CAT), GPX, and glucose-6-phosphate dehydrogenase (G6PD). Because plasma contains comparatively little SOD, CAT, and GPX compared to the erythrocyte, these measures were made only in the red cell.

**Cyclosporine A**

Peak blood CsA concentrations were determined at the Hunter Area Pathology Service (Hunter Hospital, NSW, Australia) using the EMIT 2000 Specific Assay (Dade Behring – Syva, Deerfield, IL, USA). The method was based on a homogenous immunoassay and analysis was carried out on a Roche Cobas Mira chemistry system (Roche, Basel, Switzerland).

**Erythrocyte antioxidants**

G6PD activity was determined using a modification of the Sigma Diagnostics kit method (#345-UV) which is based on the spectrophotometric method of Lohr and Waller. CAT activity was measured using the method of Slaughter and O’Brien. GPX activity was determined using the method of Andersen et al., and SOD activity was determined based on the method of Madesh and Balasubramanian. All erythrocyte enzyme activities were normalised to hemoglobin concentration. High performance liquid chromatography (HPLC) was used to determine erythrocyte α-tocopherol using the method of Bieri et al.

**Plasma antioxidants**

Plasma total antioxidant status was determined by the method of Miller et al. Plasma α-tocopherol was determined using the method of Taibi and Nicotra.

### Table 1. Measures of antioxidant status and oxidative stress in the plasma of control and cyclosporine A treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cyclosporine treated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total antioxidant status (mM)</td>
<td>1.51 ± 0.05</td>
<td>1.43 ± 0.08</td>
<td>0.028*</td>
</tr>
<tr>
<td>α-Tocopherol (µM)</td>
<td>11.9 ± 3.7</td>
<td>9.6 ± 2</td>
<td>0.12</td>
</tr>
<tr>
<td>Malondialdehyde (MDA)</td>
<td>19.7 ± 2.1</td>
<td>24.8 ± 4.4</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

Values are mean ± SD; *significantly different (P < 0.05).

**Statistical analysis**

Comparisons between groups for the dependent variables were made by independent t-tests. Significance was established at the 95% confidence level (P < 0.05). All data are shown as the mean ± SD unless otherwise stated.

**RESULTS**

Changes in body weight over the 18-day drug administration period (measured as a percentage of initial body weight) were significantly different (P < 0.05) between groups. Control animals were 99.8 ± 3.2% after 18 days of vehicle administration, whereas treatment animals were 93.2 ± 4.2% after 18 days of CsA administration. Mean blood peak CsA concentrations in the treated animals at sacrifice were 5357 ± 962 µg/l. Plasma creatinine, measured as an assessment of renal function at sacrifice, was significantly increased (P < 0.05) in treatment animals (60.44 ± 7.68 µM) compared to controls (46.33 ± 1.66 µM).

Table 1 indicates that CsA administration resulted in a significant increase (P < 0.05) in plasma MDA, a significant reduction (P < 0.05) in plasma TAS, and no change in plasma α-tocopherol (P > 0.05).

Antioxidant enzyme activities and α-tocopherol were determined as an assessment of erythrocyte antioxidant status and are presented in Table 2. Erythrocyte CAT activity was significantly increased (P < 0.05) in the treatment group compared to the controls. In contrast, there were no significant differences (P > 0.05) in the activities of SOD, GPX,
Neoral, which contains the antioxidant DL-α-tocopherol. Erythrocyte α-tocopherol concentrations were not significantly different (P > 0.05) between groups. Erythrocyte MDA was measured as a marker of erythrocyte membrane lipid peroxidation, and was not significantly different (P > 0.05) between control and treatment groups.

**DISCUSSION**

The new findings of this study are that 18 days of CsA administration (25 mg/kg/day) resulted in decreased plasma antioxidant capacity and an altered erythrocyte antioxidant defence. In the erythrocyte, CsA administration caused an increase in the activity of CAT with no differences between groups in the activities of SOD, GPX, and G6PD. There were also no significant differences in erythrocyte α-tocopherol and MDA between groups. In the plasma, CsA caused a significant reduction in total antioxidant capacity, however, CsA treatment failed to alter plasma α-tocopherol.

The decreased plasma antioxidant capacity in CsA-treated animals suggests the presence of increased plasma ROS and an inability to reduce antioxidants at the rate of oxidation. These results are supported by studies showing lower plasma thiol concentrations in the plasma of renal transplant recipients, however, in other studies, plasma antioxidant capacity was unaltered in renal transplant recipients and in children who had undergone liver transplantation. The results of the latter studies must be interpreted with caution, as both patient groups were receiving the new CsA formulation, Neoral, which contains the antioxidant DL-α-tocopherol. Given that plasma antioxidant capacity was not significantly different between groups in the present study, increased oxidation of plasma antioxidants such as ascorbic acid, uric acid, thiols, bilirubin, and albumin may have occurred. Decreased plasma antioxidant capacity in transplant recipients is likely to be of physiological significance as epidemiological studies have consistently revealed an increased risk of ischemic heart disease and stroke at low plasma concentrations of antioxidants.

Given the findings of this study, CsA administration may be the cause of the decreased antioxidant capacity reported in the plasma of organ transplant recipients. The present study found a significant increase in erythrocyte CAT activity in the CsA-treated animals. The mechanism to explain the increased activity is not known; however, because erythrocytes lack a nucleus, the increased activity indicates that changes in inhibition or activation of the enzyme occurred. CAT functions to eliminate erythrocyte hydrogen peroxide (H$_2$O$_2$) by conversion to water and oxygen; therefore, it is possible that the increase in CAT activity is an adaptive response to increased concentrations of H$_2$O$_2$. Erythrocyte H$_2$O$_2$ is derived either from the enzymatic elimination of superoxide by SOD, or directly from the plasma. Given that SOD activity was not different between groups, it is likely that the increase in CAT activity was due to increased concentrations of H$_2$O$_2$ derived from the plasma. A potential source of plasma H$_2$O$_2$ is via endothelial production of superoxide. In support of this hypothesis, Longoni et al., reported an increased production of cellular oxidants after incubation of human umbilical vein endothelial cells with both pharmacological and clinical doses of CsA. A potential source of endothelial ROS is via NAD(P)H oxidase. Angiotensin II receptor (AT$_1$A) activation on endothelial cells causes increased superoxide production via activation of NADH oxidase, and a previous study has shown a CsA-induced up-regulation of the AT$_{1A}$ receptor in rat aortic endothelial cells. The increased CAT activity is likely to be a beneficial adaptive response, as excess H$_2$O$_2$ can lead to lipid peroxidation of the erythrocyte membrane along with oxidative damage to hemoglobin and membrane proteins. The mechanism responsible for the CsA-induced increase in erythrocyte CAT activity is an exciting area for future research.

Animals administered CsA showed a significant increase in plasma MDA indicating increased ROS-induced lipid peroxidation. These results provide further support to previous animal studies reporting increased plasma and renal MDA as a result of CsA administration. Several authors have also reported increased concentrations of plasma MDA in transplant recipients, although other studies provide conflicting results.
results. McGrath et al. did not find a difference in plasma MDA between renal transplant recipients and controls, a finding also reported in juvenile liver transplant recipients. Recent studies in renal and liver transplant recipients have investigated additional markers of plasma oxidative stress such as LDL oxidation and carbonyl levels, and plasma oxidation and found no differences between CsA-treated patients and controls. This contrasts with the findings of earlier studies reporting enhanced oxidation of LDL in renal transplant recipients. The use of Neoral in the later studies, and the markedly lower immunosuppression used in stable liver transplant recipients may explain the conflicting findings. Indeed, Varghese et al. suggest that the DL-α-tocopherol in Neoral extends the oxidation lag time of LDL in vitro. Although the exact mechanism causing increased radical formation after CsA administration is not known, likely sources are the endothelium as described previously, and the superoxide producing liver and kidney cytochrome P450 enzyme systems. CsA is extensively metabolised by liver microsomal cytochrome P450 enzymes, and it has been shown that administration of CsA induces a several-fold increase in renal cytochrome P450. It is, therefore, possible in the present study that an up-regulation of the AT1 receptor on endothelial cells, and increases in cytochrome P450 enzyme activity in kidney and liver led to increased superoxide production, subsequent lipid peroxidation, and a resultant rise in plasma MDA.

In the present study, erythrocyte α-tocopherol and MDA concentrations were not different between groups. These findings indicate the presence of an intact erythrocyte membrane antioxidant defence, and an absence of CsA-induced erythrocyte lipid peroxidation. These results also indicate that the increased plasma MDA observed is derived from sources other than the erythrocyte. The limited number of significant findings in erythrocyte redox balance in the present study may be due the use of the female model. Previous studies have shown the ability of estrogen to improve the antioxidant status of erythrocytes and authors have hypothesised that this may partly explain the gender differences in the incidence of atherosclerotic heart disease. It is, therefore, of scientific interest to determine the effect of CsA on erythrocyte redox balance in male rats.

Although the drug dose of 25 mg/kg/day used in the present study is considered a high dose in humans, the same dose and doses of 50 mg/kg/day have been used previously to investigate CsA-induced oxidative stress in the plasma. Peak CsA concentrations measured in the present study are similar to those of previous findings using the same dose in the rat. The increase in plasma creatinine concentration observed in the CsA-treated rats has also been reported in previous animal studies and is not surprising given that kidney dysfunction is the main complication of CsA treatment. The CsA-treated animals failed to maintain body weight during the 18-day drug treatment phase of the study, a finding previously reported in this model. Evidence suggests that oxidative stress may play a role, as Wang and Salahudeen showed that co-administration of α-tocopherol with CsA administration limited the CsA-induced weight loss.

Given the high dose of CsA administered in the present study, the relevance of the present results to the clinical setting is unknown. In this regard, a recent in vitro study has shown that CsA alters endothelial cell function by different mechanisms when incubated at pharmacological doses versus clinical doses. At clinical doses, there was a change in cell morphology and an up-regulation of the anti-apoptotic factor Bcl-2; however, pharmacological doses caused Bcl-2 down-regulation and apoptotic cell death. Therefore, further animal studies that administer CsA at therapeutic levels are required in order to gain a better understanding of the relevance of the present results to the clinical setting.

CONCLUSIONS

The purpose of these experiments was to determine the effect of CsA on erythrocyte redox balance and plasma antioxidant capacity. The experiments demonstrate that CsA administration (25 mg/kg/day) for 18 days reduces the antioxidant capacity of the plasma and alters the erythrocyte antioxidant defence. These results suggest that CsA may be responsible for the decreased plasma antioxidant capacity and altered erythrocyte redox balance observed in transplant recipients. Further studies using male rats are required to confirm these results. In addition, to improve our understanding of the clinical relevance of the present results, additional animal studies using therapeutic doses are warranted.

ACKNOWLEDGEMENTS

This work was supported by grants from Charles Sturt University and the Clifford Craig Medical Research Trust. Technical assistance was provided by Gary Wilson at the University of Queensland.

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