Protective effect of N-acetylcysteine on cyclosporine A-induced changes in lipid hydroperoxide levels and renal dysfunction in rats

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Abstract

Introduction: One of the major adverse effects of long-term cyclosporine is chronic nephrotoxicity. Renal damage due to cyclosporine treatment is an important clinical challenge. N-acetylcysteine (NAC) is a potent antioxidant and has been shown to reduce free radical injury. The aim of this study was to investigate the possible protective role of NAC treatment on cyclosporine-induced renal damage using biochemical and histopathological parameters.

Material and methods: Adult male albino rats were randomly assigned to control (saline treated), cyclosporine (20 mg/kg/day), NAC alone (20 mg/kg/day) and cyclosporine + NAC (20 mg/kg/day) groups. Rats were sacrificed at the end of the experiment and serum was analyzed for urea, uric acid, creatinine and blood urea nitrogen (BUN). Total antioxidant level and lipid hydroperoxides were also estimated. Histopathological changes in the kidneys were assessed semiquantitatively.

Results: Cyclosporine treatment produced a significant increase in serum creatinine, urea, uric acid and BUN, indicating a marked renal injury. Treatment with N-acetylcysteine significantly reduced these changes. Total antioxidant level decreased significantly both in serum and kidneys after cyclosporine. Administration of NAC significantly prevented these changes. Lipid hydroperoxide level increased significantly with cyclosporine and the changes were reduced when supplemented with NAC. Cyclosporine treatment produced severe glomerular atrophy, blood vessel thickening and moderate tubular necrosis. N-acetylcysteine significantly prevented these histopathological changes in the kidneys.

Conclusions: Depletion of antioxidants and increased lipid hydroperoxides play an important role in cyclosporine-induced renal damage. N-acetylcysteine supplementation significantly reduced cyclosporine-induced structural and functional impairment of the kidneys. Concurrent use of antioxidant N-acetylcysteine may be of therapeutic value to minimize cyclosporine-induced nephrotoxicity.

Key words: cyclosporine, N-acetylcysteine, nephrotoxicity, antioxidants, lipid hydroperoxides.

Introduction

Cyclosporine A (CsA) is the most common immunosuppressive drug used for the prevention of allograft rejection [1]. Cyclosporine has improved patient and graft survival rate in solid organ transplantation and has been increasingly applied with considerable clinical benefits [2, 3]. Therapeutic benefits of cyclosporine are limited by the occurrence of chronic nephrotoxicity. Acute renal dysfunction with cyclosporine therapy was recognized at the time of its first use in clinical renal transplantation [4, 5]. The exact mechanism of CsA-induced nephrotoxicity remains obscure. Clinical and experimental studies have revealed that several mechanisms may be involved [4, 6, 7]. Cyclosporine-induced nephrotoxicity seems to be caused by reduction in renal blood flow caused by arteriolar constriction [8]. Cyclosporine A nephrotoxicity is characterized by progressive renal dysfunction, afferent arteriolopathy and inflammatory cell influx [9]. Several lines of evidence suggest that cyclosporine increases hypoxia, decreases glomerular filtration rate and increases intra-renal vascular resistance [10].

Cumulative data suggest a role for reactive oxygen metabolites as one of the postulated mechanisms in the pathogenesis of CsA nephrotoxicity [11-13]. It is known that CsA increases renal nerve activity, resulting in vasoconstriction [14]. In addition, CsA brings about vasoconstriction in isolated renal arterioles by direct actions [15]. The vasoconstriction is reported to be due to CsA action in blocking mitochondrial calcium release, inducing increased intracellular calcium which causes vasoconstriction. These changes could lead to renal hypoxia reoxygenation injury and production of reactive oxygen free radicals [16, 17]. *In vitro* and *in vivo* studies indicate that CsA reduces renal microsomal NADPH cytochrome P450 and renal reduced/oxidized glutathione ratio in the kidneys. The extent to which the adverse effects of cyclosporine are related to the immunosuppressive mechanisms of the drug is controversial [18]. Cyclosporine A is known to alter the production of many biologically active agents, such as endothelin, nitric oxide (NO) and transforming growth factor-β1 (TGF-β1), which have been implicated in adverse renal effects [6, 10, 18]. Endothelial dysfunction and reduced activity of endothelial derived nitric oxide might be one of the mechanisms underlining the functional effects of CsA on glomerular filtration [19].

N-acetylcysteine (NAC) is a thiol-containing antioxidant agent. N-acetylcysteine scavenges oxidants directly and replenishes intracellular glutathione [20]. Stimulation of glutathione following administration of NAC results in greater supply of glutathione for detoxification of oxygen free radicals and other foreign substances [21]. There are few reports showing the protective effect of N-acetylcysteine against oxygen free radical mediated injuries in the liver, heart and lungs [21, 22]. N-acetylcysteine has proven to be renoprotective in toxic and ischaemic acute renal failure, although results have not been conclusive. It has been reported that antioxidant effects of NAC are able to prevent the increase in plasma peroxynitrite after ischaemia and NAC ameliorates the renal failure induced by inferior vena cava occlusion [23, 24]. N-acetylcysteine is reported to enhance the biological effects of nitric oxide and is known to have positive effects in reversing the haemodynamic disturbances in the renal circulation in acute renal failure [25]. A diet rich in natural substances reduces the risk of diseases associated with an increase in oxidative stress [26, 27].

The aim of this study was to observe the effect of NAC in reducing the cyclosporine-induced alterations in lipid hydroperoxides, total antioxidant levels and renal histopathology, and our research hypothesis was: NAC will increase the renal total antioxidant levels and ameliorate the cyclosporine-induced renal damage.

Material and methods

Animals

Adult male Sprague-Dawley rats weighing between 200 and 250 g were housed two per cage with food and water *ad libitum* for two weeks before the beginning of the experiment. The animals were kept on husk bedding with a 12-h dark/light cycle. The animals had free access to standard rodent food and water. The study was conducted between 15/10/2006 and 20/05/2007 at the International Medical University, Kuala Lumpur, Malaysia. All experiments were performed in accordance with institutional guidelines for the ethical care of animals. The study protocol was designed in accordance with the 1996 revised form of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH), United States, and the study received approval from the Institutional Ethics Committee.

Experimental groups

The rats were randomly divided into four groups (n = 8 in each group) and treated for 21 days. Group I (control group) received vehicle of CsA, i.e. olive oil orally. Group II rats were treated with CsA dissolved in olive oil orally (20 mg/kg body weight). This group served as a positive control. Group III received NAC alone intraperitoneally (20 mg/kg body weight). Group IV rats were treated with cyclosporine (20 mg/kg body weight) and NAC (20 mg/kg body weight). In group IV NAC was administered intraperitoneally 30 min before CsA administration. Cyclosporine A (Sandimmune from Novartis, Malaysia) was dissolved to give a final concentration of 10 mg/ml. N-acetylcysteine (Sigma-Aldrich, USA) was dissolved to give a final concentration of 10 mg/ml. The drugs were freshly prepared for administration.

Daily body weight and food intake were recorded throughout the experiment. After 24 h after the last treatment, body weight of the animals in all the groups were recorded and rats were sacrificed 24 h after the last dose (on the 22^{nd} day) using sodium pentobarbital anaesthesia (40 mg/kg body weight; Sigma Aldrich, USA) and blood samples were collected through cardiac puncture. Serum was separated and frozen at –20°C for biochemical analysis. A midline abdominal incision was done and both kidneys were removed and weighed; the left kidney was perfused with ice cold saline (0.9% NaCl) and homogenized in chilled phosphate buffer. The homogenate was centrifuged at 4000 rpm for 30 min at +4°C and the supernatant collected was stored at –20°C until analysis of lipid hydroperoxides and total antioxidants. The right kidney was stored in 10% neutral buffered formalin for histopathological studies. From the serum samples, creatinine, blood urea nitrogen (BUN), urea and uric acid assays were done by spectrophotometric methods using QuantiChrom Assay Kits (BioAssay Systems. USA). Lipid hydroperoxides and total antioxidants in the serum and kidneys were estimated using ELISA kits (Cayman Chemicals. USA).

Histopathological examination of kidneys: For light microscopic evaluation, portions of right kidney were sectioned and fixed in 10% neutral phosphate-buffered formaldehyde and embedded in paraffin. These specimens were cut into sections of 4 µm and these sections were stained with haematoxylin and eosin. A minimum of ten fields for each kidney section slide were examined blindly by a pathologist who was unaware of the treatment regimens used. The kidneys were examined for glomerular and tubular alterations, tubular casts, tubular necrosis, and blood vessel changes. All histopathological parameters were graded as follows: none (–), showing no meaningful histopathological changes; mild (+), with occasional glomerulus showing reduction in size, occasional blood vessel thickening, few foci of dilatation and casts; moderate (++), showing glomeruli beginning to undergo atrophy at different foci, thickened blood vessels, dilated blood vessels and tubular casts at different foci throughout the kidney; severe (+++), with marked histopathological changes showing extensive glomerular atrophy and tubular casts, tubular necrosis, thickened blood vessels, areas of congestion of interstitium and blood vessels, and very large areas of extravasation of red blood cells into the interstitium.

Table I. Effect of cyclosporine (CsA) and N-acetylcysteine (NAC) treatment on creatinine, BUN, urea and uric acid levels. All values are mean ± SD

Parameters	Control	CsA	NAC	NAC + CsA
Creatinine [mg/dl]	1.798 ±0.167	3.916 ±0.390**◆◆	1.054 ±0.111*	2.427 ±0.392**✞✞◆◆
BUN [mg/dl]	11.061 ±0.894	25.454 ±1.626**◆◆	9.867 ±0.298	15.303 ±2.841**✞✞◆◆
Urea [mg/dl]	36.250 ±1.613	50.117 ±2.196**◆◆	30.687 ±2.316**	43.317 ±2.972**✞✞◆◆
Uric acid [mg/dl]	2.953 ±0.420	6.068 ±1.434**◆◆	1.886 ±0.216*	4.320 ±0.766**✞◆◆

***$p < 0.01$ – control group with cyclosporine and NAC + CsA groups*
✞$p < 0.05$, ✞✞$p < 0.01$ – cyclosporine with NAC + CsA group, ◆$p < 0.05$, ◆◆$p < 0.01$ NAC alone with CsA alone and NAC + CsA groups

Statistical methods

Data are presented as means ± SD. The statistical analysis was done using SPSS statistical software version 12. The non-parametric Kruskal-Wallis and Mann-Whitney tests were used as not all the parameters were normally distributed. Global comparison among the groups (control, cyclosporine alone, NAC alone and NAC with CsA) was done using the Kruskal-Wallis test. To further explore the effect of CsA on kidney toxicity and the nullifying effect of NAC, pair-wise comparisons were carried out using the Mann-Whitney test. A value of $p < 0.05$ was considered statistically significant.

Results

There was a significant increase in BUN, serum creatinine, urea and uric acid levels in rats treated with cyclosporine alone for three weeks compared to control or NAC alone groups ($p < 0.01$). The results show that the level of creatinine, BUN, urea and uric acid was highest in rats treated with cyclosporine for 21 days ($p < 0.01$) when compared to the control group (Table I). Treatment with NAC alone did produce a significant decrease in creatinine ($p < 0.05$), urea ($p < 0.01$) and uric acid ($p < 0.05$) levels compared to control groups. When NAC was treated with CsA it produced a significant decrease in these blood parameters, compared to the CsA alone group ($p < 0.01$ and $p < 0.05$). But the level of these parameters remained significantly higher than in the control group ($p < 0.01$) and NAC alone ($p < 0.01$) groups.

When the total antioxidant levels were compared between the groups, a statistically significant reduction in the antioxidant levels both in the kidney and serum was recorded in the cyclosporine alone group compared to the control ($p < 0.01$) and NAC alone group ($p < 0.01$) (Table II). N-acetylcysteine treatment alone significantly increased ($p < 0.01$) the total antioxidants in the kidneys and serum when compared to the control group. Treatment with N-acetylcysteine with CsA significantly increased the total antioxidant levels ($p < 0.01$) compared to the cyclosporine alone group and the increase was significantly more than the control total antioxidant levels ($p < 0.01$). No significant difference in total antioxidant levels was seen in the serum and kidneys when the NAC with CsA treated group was compared with the NAC alone group. There was a significantly greater decrease in the antioxidant levels with cyclosporine treatment in the serum (62%) than in the kidneys (49%). Compared to cyclosporine alone, there was a more than 600% increase in total antioxidant levels in the NAC with CsA group, indicating the significant antioxidant effect of NAC (Table II).

Lipid hydroperoxide levels in the serum and kidneys increased significantly with cyclosporine treatment ($p < 0.01$) when compared to control levels and NAC alone treatment ($p < 0.01$). Treatment with NAC alone was able to reduce the lipid hydroperoxides ($p < 0.05$) compared to control rats. Cyclosporine treatment for three weeks increased the lipid hydroperoxide levels 117% in the serum and above 300% in the kidneys compared to the control group. N-acetylcysteine treatment along with cyclosporine was able to decrease these changes significantly ($p < 0.01$). Lipid hydroperoxides in the serum and kidneys remained significantly higher than in the NAC alone group ($p < 0.01$) but there was no statistically significant difference in lipid hydroperoxide level in the serum and kidneys between the control and the NAC with CsA group, indicating the nullifying effect of NAC on cyclosporine toxicity (Table II).

Kidneys of the cyclosporine alone group showed severe glomerular atrophy. There was thickening of blood vessels and congestion of vessels and interstitium (Table III, Figure 1A). Many tubules showed casts and moderate areas of necrosis and hyalinization. N-acetylcysteine alone treatment showed normal kidney histology as in the control rats. In the NAC with CsA treated groups, most of the glomeruli appeared normal (Table III, Figure 1B). There was occasional reduced glomerular size compared to the control and NAC alone group. Minimal blood vessel thickening was present and areas of congestion were also reduced. Concomitant treatment with NAC showed only mild tubular atrophy, and occasional presence of tubular casts. Moderate glomerular atrophy and mild interstitial oedema were also seen in the NAC with CsA group (Table III).

Discussion

In the present study, treatment of rats with cyclosporine for a period of three weeks resulted in a significant increase in blood urea nitrogen, creatinine, urea and uric acid level, suggesting significant functional impairment in the kidneys. These observations are in agreement with earlier studies where significant alterations in the level of BUN and creatinine were reported following chronic CsA treatment [28, 29]. Treatment of rats with NAC alone had resulted in improvement in renal function which was reflected by a significant decrease in blood urea, creatinine and uric acid levels when compared to control rats. This effect on renal function with NAC alone in normal rats supports the hypothesis that NAC improves the renal haemodynamics [23, 24]. When NAC was

Table II. Cyclosporine (CsA) and N-acetylcysteine (NAC) treatment on total antioxidants and lipid hydroperoxide levels. All values are mean ± SD

Parameters	Control	CsA	NAC	NAC + CsA
Total antioxidants [nmol]	0.050 ±0.003	0.019 ±0.005**◆◆	0.07 ±0.001*	0.133 ±0.069**††◆◆
Kidney [nmol]	0.049 ±0.003	0.025 ±0.016**◆◆	0.075 ±0.001*	0.170 ±0.096**††◆◆
Lipid hydroperoxides [mM]	1.672 ±0.336	3.630 ±0.539**◆◆	0.892 ±0.102*	2.490 ±0.473**††◆◆
Kidney [mM]	1.318 ±0.345	4.125 ±0.480**◆◆	0.908 ±0.134*	2.628 ±0.426**††◆◆

***$p < 0.01$ – control group with cyclosporine and NAC + CsA groups †$p < 0.05$, ††$p < 0.01$ – cyclosporine with NAC + CsA groups, ◆$p < 0.05$, ◆◆$p < 0.01$ NAC alone with CsA alone and NAC + CsA groups*

Table III. Cyclosporine (CsA) and N-acetylcysteine (NAC) treatment on histopathological changes in the kidney

Groups	Glomerular atrophy	Blood vessel thickening	Interstitial oedema	Tubular casts	Tubular necrosis
Control	–	–	–	–	–
NAC	–	–	–	–	–
CsA	+++	+++	++	++	++
NAC + CsA	++	+	++	+	+

'–' – no morphological changes in histology, + – mild, ++ – moderate, +++ – severe morphological changes in histology

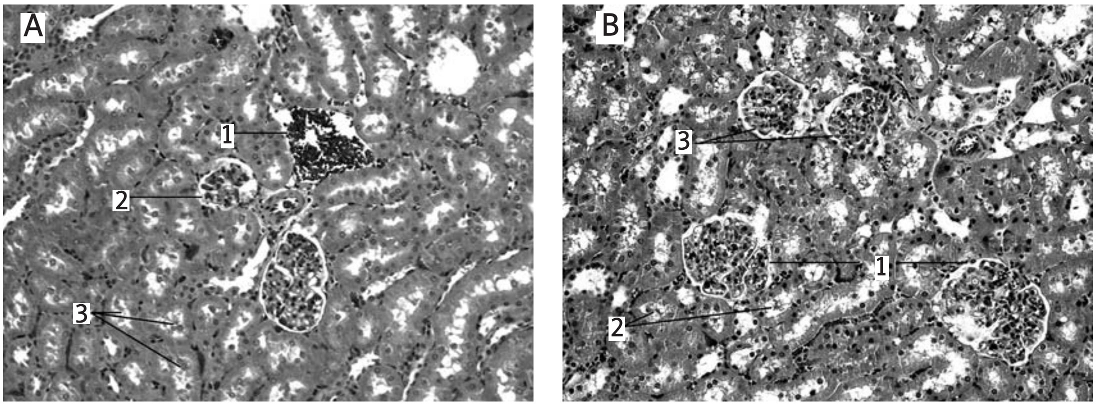

Figure 1. Photomicrographs of kidney sections stained with haematoxylin and eosin stain: A – represents a section taken from the kidney of a rat treated with cyclosporine (20 mg/kg) for 21 days showing extravasation of blood cells (1), severe glomerular atrophy (2) and extensive tubular casts (3) (magnification 200×), B – represents a section taken from the kidney of a rat treated with N-acetylcysteine (20 mg/kg) along with cyclosporine (20 mg/kg) for 21 days showing normal glomerulus (1), occasional tubular casts (2) and mild glomerular atrophy (3) (magnification 200×)

administered to the cyclosporine treatment groups, there was a significant improvement in renal function in the rats and the level of urea, BUN, uric acid and creatinine decreased significantly compared to the CsA treatment group. But the level of these substances remained higher than the normal control and NAC alone groups, indicating that NAC could not totally nullify the effect of CsA on renal function.

Increased production of lipid hydroperoxides and a significant decrease in total antioxidants with CsA confirms the role of oxidative stress in CsA-induced nephrotoxicity. Parra *et al.* reported that treatment of CsA increased oxygen free radical production [30]. These oxygen free radicals modulate the filtration in the glomerulus and also influence the renal blood flow [31]. The endogenous antioxidant system operates to combat the oxygen free radicals and these antioxidants scavenge the oxygen free radicals. Decreased total antioxidants after CsA treatment proves that there is increased production of oxygen free radicals and endogenous antioxidants are being used up for scavenging the wide variety of these free radicals including O_2^-, H_2O_2 and OH^- [32].

Treatment with N-acetylcysteine along with CsA increased the total antioxidant level both in the serum and kidneys. N-acetylcysteine with CsA also decreased the lipid hydroperoxide levels. Treatment of rats with NAC alone showed a significant improvement in the total antioxidant levels and decreased the lipid hydroperoxide levels compared to control rats. The mechanism of action of NAC in reducing renal damage caused by CsA is not clear [33]. Protective effects of NAC against oxidative stress induced tubular damage might involve various chemical mechanisms. N-acetylcysteine directly scavenges superoxide radicals. As a precursor of glutathione synthesis NAC significantly increases intracellular redox potential and improves the reductive states of critical regulatory protein thiol groups [23, 34, 35]. Marked protective effects of NAC administration against CsA-induced renal damage may be strongly associated with amelioration of the effects of oxidative stress [25]. N-acetylcysteine is also reported to stimulate vasodilatation in the kidneys, thereby reducing the renal vascular resistance and hypoxia/reoxygenation-induced formation of oxygen free radicals [36]. CsA-induced nephrotoxicity is associated with accumulation of cellular calcium, and calcium channel blockers have been shown to reduce CsA-induced kidney damage. N-acetylcysteine have also been shown to block calcium channels, maintain calcium homeostasis and improve renal function [37, 38].

There were characteristic morphological findings with CsA treatment, such as glomerular atrophy, blood vessel thickening and hyaline casts in the tubules. Concomitant treatment with NAC attenuated the CsA-induced structural and functional changes in the kidneys. Reactive oxygen species mediate peroxidation of lipid structures of the tissues, resulting in subcellular damage, as observed by histopathological examination in this study with CsA treatment for 21 days [4, 39]. Vasoconstriction produced by CsA produces local ischaemia which may lead to a number of cellular changes such as deterioration of membrane integrity and distinct histological changes in the renal structures [29]. Treatment with NAC along with CsA significantly reduced the pathological changes in the kidneys, suggesting the protective role of NAC in CsA-induced renal morphological damage. Decrease in structural damage with NAC treatment could be attributed to the NAC-induced decreased vascular resistance and enhanced tissue perfusion [36, 40]. Improved tissue perfusion with

NAC decreased the formation of oxygen free radicals and minimized the CsA-induced cellular damage in renal tissues [41].

Thus, cyclosporine decreased the total antioxidants and increased the lipid hydroperoxides, causing severe renal damage. There was also a significant histopathological change in the kidneys with three weeks of cyclosporine treatment. Concomitant treatment with NAC (20 mg/kg body weight) could prevent the toxic renal damage of CsA by increasing total antioxidants and reducing the lipid hydroperoxides in the kidneys. In conclusion, our study confirms the hypothesis that oxidative stress is the main cause of renal damage induced by cyclosporine, and N-acetylcysteine, through its marked antioxidant activity and positive haemodynamic effects, significantly reduces the renal damage due to cyclosporine treatment. N-acetylcysteine may be considered for pharmacological therapy for cyclosporine-induced nephrotoxicity in humans.

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