CHAPTER-2

TO UNRAVEL THE ROLE OF NITRIC OXIDE IN
CYCLOSPRINE A-INDUCED RENAL INJURY
INTRODUCTION:
Cyclosporine A (CsA), the first calcineurin inhibitor available for clinical use, was launched in the 1980s and it radically changed the field of organ transplantation. The incidence of acute rejection in solid organ transplantation decreased significantly and patient survival has increased to unparalleled levels. CsA also has been proven to be effective in bone marrow transplant immunosuppression and in the treatment of autoimmune diseases (De Mattos et al., 1996).

Pharmacological properties of cyclosporine A:
CsA is a cyclic endecapeptide, extracted from Tolypocladium inflatum Gams (Wenger, 1988). The drug has a low bioavailability (mean 30%), with great variations among patients for several reasons e.g. the ability of intestinal content to separate the drug from its vehicle, intestinal rhythm, liver function or presence of food in the stomach (Ptachcinski et al., 1985; Kahan and Grevel, 1988). CsA is metabolized through the super family of hepatic isoenzymes P-450; this is remarkable because several drugs can interfere with its metabolism. Its mean life is 6.4-8.7 h, although there is a wide range of inter individual variations. Ninety percent of the drug is withdrawn through biliary excretion and only 6% appears unchanged in the urine (Beveridge, 1982; Ptachcinski et al., 1985).

CsA inhibits interleukin-2 gene transcription and the transition of T lymphocytes from the G0 to G1 phase of the cell cycle. It binds to a cytoplasmic immunophilin called cyclophilin. The complex CsA-cyclophilin decreases calcium signaling and blocks calcineurin, a calcium-dependent enzyme responsible for the nuclear translocation and dephosphorylation of the cytosolic activating nuclear factor of T lymphocytes. The cytosolic activating nuclear factor of T lymphocytes regulates the transcription of genes responsible for several cytokines, including interleukins 2 (De Mattos et al., 1996). Like FK-506 or rapamycin, CsA is able to block the biosynthesis of some lymphokines produced by T lymphocytes; particularly, CsA blocks the interleukin-2 synthesis at transcriptional level (Graham, 1994). The initial mechanism of action of CsA in T lymphocytes is unknown and several hypotheses have been suggested (Borel, 1976; Harding et al., 1986; Citterio and Kahan, 1989; Erlanger, 1992; Schreiber and Crabtree, 1992). CsA can interact with cytoplasmic membrane and activate the intracellular
calcium pathway (Erlanger, 1992). Other authors propose that CsA binds to cytoplasmic proteins, such as cyclophilin, which can inhibit signal mechanisms from the cytoplasm to the cellular nucleus (Harding et al., 1986; Citterio and Kahan, 1989; Erlanger, 1992; Schreiber and Crabtree, 1992). Finally, CsA can exert its action through direct influences on the nucleus; the binding of CsA to nuclear receptors can induce the synthesis of lymphokines and several cytotoxic enzymes (Erlanger, 1992).

Although extensively used in renal transplantation, kidney dysfunction is the major complication of CsA treatment. About 30% of patients treated with CsA have moderate to severe kidney damage. CsA-induced nephrotoxicity is manifested in two particular and very distinct forms of renal injury. Acute CsA nephrotoxicity is a hemodynamically mediated event, characterized by the absence of permanent structural injury and by the normalization of renal function when CsA is decreased or discontinued. Acute CsA nephrotoxicity is a functional and reversible abnormality related to a renal imbalance of vasoconstrictor and vasodilators mediators. The main feature of this form of nephrotoxicity is an intense intrarenal vasoconstriction reflected by increased renal vascular resistance and reciprocal renal blood flow (RBF) decrease, followed by variable degrees of glomerular filtration rate (GFR) impairment. This vasoconstriction occurs preferentially in the afferent arterioles and also in adjacent small arteries, including the glomerular tuft (English et al., 1987; Potier et al., 1998). In early stages, decreased glomerular filtration rate and renal plasma flow have been documented as a result of alteration of intrarenal hemodynamics, mainly related to afferent arteriolar vasoconstriction (Barros et al., 1987; English et al., 1987). Even after 2 weeks of treatment, loss of proximal tubular cells brush border, proximal tubule dilatation, swelling, necrosis, and infiltration of white blood cells may appear in kidney cortex (Mihatsch et al., 1988). Tubular toxicity affects mainly the third segment of the proximal tubule (Berty and Adler, 1991).

The experimental model for acute CsA nephrotoxicity is consistent and has been shown after different doses and routes of CsA administration in animals. Similarly, acute impairment of renal hemodynamics and function has been observed after CsA administration to patients and healthy volunteers. Experimental or clinical histology changes are minimal and nonspecific or absent, even when renal dysfunction is striking.
Chapter 2

Pathophysiology of CsA Nephropathy

Intravascular Volume → Sympathetic Tone → Hypertension

↑ Capillary Permeability

Endothelial Injury → ↑ Endothelin, ↓ Nitric Oxide, ↑ Thromboxane, ↓ Prostaglandins

Renal and Systemic Vasoconstriction

Vascular Smooth Muscle Sensitivity to Vasoconstrictors

Stripped Interstitial Fibrosis

Cyclosporine-A

Angiotensin II

Tubular Cell Injury → ↓ GFR

Pathophysiology of CsA Nephropathy
Conversly, chronic CsA nephrotoxicity is an insidious lesion, characterized by an irreversible and progressive renal interstitial fibrosis, which may evolve to chronic or even end-stage renal disease (ESRD). Chronic CsA-induced nephropathy is characterized by irreversible tubulointerstitial fibrosis in a striped pattern beginning in the medulla and progressing to the medullary rays of the cortex, generally accompanied by some degree of renal dysfunction (Bennett et al., 1996). CsA-induced chronic nephrotoxicity was described in renal and nonrenal transplant recipient and in patients with autoimmune diseases receiving the drug for periods of 6 months or more (Mihatsch et al., 1995; Bennett et al., 1996).

Renal vasoconstriction, which is a hallmark of CsA nephrotoxicity, is attributed to an imbalance in the release of vasoactive substances such as thromboxane (Perico et al., 1986b), endothelin (Kon et al., 1990), and angiotensin II (Perico et al., 1986a); and on the other, a decrease in vasodilating factors such as prostacyclin (Perico et al., 1986a) and nitric oxide (NO) (Vaziri et al., 1998; Gossmann et al., 2001).

A few reports showed that administration of L-arg greatly ameliorated kidney dysfunction induced by CsA in rats (Amore et al., 1995; Yang et al., 1998). However, this has been recently disputed by Lassila et al. (2001) who showed that treatment with L-arg did not affect renal dysfunction-induced by CsA.

The present work was undertaken to investigate the role of nitric oxide in acute CsA nephrotoxicity. The ability of nitric oxide donors as well as the NOS upregulators was assessed in ameliorating the acute CsA nephrotoxicity. Moreover, an attempt was made to find out the involvement of specific NOS subtype in the progression of acute CsA nephrotoxicity.

**MATERIALS AND METHODS**

Male wistar rats (150-200g), bred in the central animal house of Panjab University (Chandigarh, India) were used. The animals were housed under standard conditions of light and dark cycle with free access to food and tap water. All the protocols were approved by the Institutional Animal Ethics Committee of the Panjab University.
Drugs
CsA (Panacea Biotech, Lalru, India) was dissolved in olive oil, to get a final concentration of 20 mg/kg. L-arginine (Himedia, Mumbai), Pravastatin (Ranbaxy Research Laboratories, Gurgaon, India), L-NAME (Sigma, USA), aminoguanidine (Himedia, Mumbai), L-NIO (Caymen Chemicals, USA) were dissolved in distilled water. Nebivolol (Jenessan Pharmaceutica, Belgium), resveratrol (Sigma, St. Louis, MI, USA), molsidomine (Caymen Chemicals, USA) were suspended in 0.25% carboxy methyl cellulose (CMC). All the drugs were freshly prepared at the beginning of each experimental protocol.

EXPERIMENTAL PROTOCOLS
CsA was injected at a dose of (20 mg/kg) for 21 days to produce nephrotoxicity. This dose of CsA and duration of 21 days has already been standardized in our laboratory (Satyanarayana et al., 2001). Body weights of the animals were measured every day. Systolic blood pressure (SBP) was measured from the tail of the animals using a blood pressure recorder (UGO Basile, Italy) on days 0 and 22 just before sacrificing the animals. The animals were placed in individual metabolic cages for 24 hr after the last dose for urine collection. On day 22, animals were anesthetized with ketamine (50 mg/kg, i.p.) and blood was collected in centrifuge tubes through abdominal aorta. The blood samples were centrifuged and serum was collected. A midline abdominal incision was performed and both the kidneys were isolated, the left kidney was deep frozen till further enzymatic analysis, whereas, the right kidney was stored in 10% formalin for the histological studies.

Assessment of renal function
Serum samples were assayed for blood urea nitrogen (BUN), urea clearance, serum creatinine & creatinine clearance by using standard diagnostic kits (Span Diagnostics, Gujarat, India).

Estimation of tissue and urine nitrite and nitrate levels:
As per chapter 1.

Post mitochondrial supernatant preparation (PMS)
As per chapter 1.
Chapter 2

Estimation of lipid peroxidation
As per chapter 1.

Estimation of antioxidant enzymes (AOE)
The AOE were estimated by the well established procedures already described in Chapter 1. Briefly, the non-protein sulfhydryl (NPSH) as a marker for reduced glutathione (GSH), was measured by the method of Jollow et al. (1974) and the yellow color developed by the reduction of Ellman’s reagent by –SH groups of NPSH was read at 412 nm. The Catalase (CAT) activity was assayed by the method of Claiborne (1985) and the rate of decomposition of H$_2$O$_2$ was followed at 240 nm. The superoxide dismutase (SOD) activity was assessed by the method of Kono (1978). The nitro blue tetrazolium (NBT) reduction by superoxide anion to blue formazan was followed at 560 nm.

Renal Histology
Kidneys were processed in the same way as described in chapter 1, but the renal sections were examined in blind fashion for hemorrhagic and hyaline casts, tubulointerstitial fibrosis, arteriolopathy epical blebbing and glomerular basement thickening in all treatments.

STATISTICAL ANALYSIS
Data are presented as means ± S.E.M. One way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was applied to calculate the statistical significance between various groups. A value of $p<0.05$ was considered to be statistically significant.

PRELIMINARY STUDIES AND DOSE SELECTION

L-arginine treatment
L-arginine was dissolved in normal saline and was administered via intraperitoneal route 24 hr before CsA administration and continued along with CsA for 21 days. In the preliminary studies, dose range of 100 to 150 mg were tested, and the dose of 125 mg was found to be most effective in preservation of renal function following CsA administration and therefore, this dose was used for the further studies (Fig.1a.)
Chapter 2

**Molsidomine treatment**
Molsidomine was suspended in 0.25% sodium carboxy methyl cellulose (CMC) and was given 24 hr before CsA administration and continued along with CsA for 21 days. In the pilot studies, out of doses of 5 and 10 mg, 10 mg was found to be most effective in preservation of renal function following CsA administration (Fig.1b.).

**Nebivolol treatment**
Nebivolol was suspended in 0.25% sodium carboxy methyl cellulose (CMC) and was given orally 24 hr before CsA administration and continued along with CsA for 21 days. In our preliminary studies, dose ranges from 0.5 to 2 mg were tested, and the 1 mg was found to be most effective in preservation of renal function following CsA administration (Fig.1c.).

**Resveratrol treatment**
Resveratrol was suspended in 0.25% sodium carboxy methyl cellulose (CMC) and was given orally 24 hr before CsA administration and continued along with CsA for 21 days. Among the dose ranges (2 to 10 mg) tested, 5 mg was found to be most effective in preservation of renal function and total nitric oxide levels following CsA administration for 21 days. The higher doses of 10 mg did not show any additional advantage in the renal function preservation, therefore, the dose of 5 mg/kg was used for the further studies (Fig.1d.).

**Pravastatin treatment**
Pravastatin was dissolved in normal saline and was administered intraperitoneally (i.p.), 24 hr before CsA administration and continued along with CsA for 21 days. In pilot studies, dose ranges from 5 to 20 mg were tested, and the dose of 20 mg was found to be most effective in preservation of renal function and total nitric oxide levels following CsA administration for 21 days and therefore, this dose was used for the further studies (Fig.1e.).
Effect of various doses of L-arginine (100 mg/kg, 125 mg/kg, 150 mg/kg) on renal function and urinary total NO contents in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the control group; a p<0.05 as compared with the CsA group (one-way ANOVA followed by Newman Keuls test).
Fig. 1b. Effect of molsidomine (5 mg/kg, 10 mg/kg) on renal function and urinary total NO contents in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the control group; a p<0.05 as compared with the CsA group (one-way ANOVA followed by Newman Keuls test).
Fig. 1c. Effect of nebivolol (0.5 mg/kg, 1 mg/kg, 2 mg/kg) on renal function and urinary total NO contents in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the control group; a p<0.05 as compared with the CsA group (one-way ANOVA followed by Newman Keuls test).
Effect of resveratrol (2 mg/kg, 5 mg/kg, 10 mg/kg) on renal function and urinary total NO contents in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p<0.05 as compared with the control group; a p<0.05 as compared with the CsA group (one-way ANOVA followed by Newman Keuls test).

Fig. 1d. Effect of resveratrol (2 mg/kg, 5 mg/kg, 10 mg/kg) on renal function and urinary total NO contents in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p<0.05 as compared with the control group; a p<0.05 as compared with the CsA group (one-way ANOVA followed by Newman Keuls test).
Fig. 1e. Effect of pravastatin (5 mg/kg, 10 mg/kg, 20 mg/kg) on renal function and urinary total NO contents in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the control group; a p<0.05 as compared with the CsA group (one-way ANOVA followed by Newman Keuls test).
STUDY DESIGN

Animals were divided into 21 groups, each comprising of 6-8 animals. The animals were allowed free access to food and drinking water.

Group I (C) comprised of animals that received an equivalent volume of vehicle for CsA i.e., olive oil, subcutaneously (s.c.).

Group II (CsA) animals received CsA (20 mg/kg, s.c.) dissolved in olive oil for 21 days.

Groups administered NO precursor (L-arginine) and NOS inhibitors

Group III (L-arg+CsA) animals were given L-arginine (125 mg/kg, i.p.) 24 hr before administering CsA, and continued along with CsA for 21 days.

Group IV (L-NAME+L-arg+CsA) animals received L-NAME (10 mg/kg, i.p.) 30 minutes before L-arginine administration and continued for 21 days along with CsA.

Group V (L-NIO+L-arg+CsA) animals received L-NIO (5 mg/kg, i.p.) 30 minutes before L-arginine administration and continued for 21 days along with CsA.

Group VI (Amg+L-arg+CsA) animals received aminoguanidine (100 mg/kg, i.p.) 30 minutes before L-arginine administration and continued for 21 days along with CsA.

Groups administered NO donors (molsidomine, nebivolol) and NOS inhibitor

Group VII (Mol+CsA) animals were administered molsidomine (10 mg/kg, p.o.) 24 hr before CsA administration, and continued along with CsA for 21 days.

Group VIII (L-NAME+Mol+CsA) animals were administered L-NAME (10 mg/kg, i.p.) 30 minutes before molsidomine administration and continued with CsA for 21 days.

Group IX (Neb+CsA) animals received nebivolol (1 mg/kg, p.o.) 24 hr before CsA administration and continued with CsA for 21 days.
Group X (L-NAME+Neb+CsA) animals received L-NAME (10 mg/kg, i.p.) 30 minutes before nebivolol administration and continued with CsA for 21 days.

Groups administered NOS upregulators (resveratrol, pravastatin) and NOS inhibitors

Group XI (RVT+CsA) animals were administered resveratrol (5 mg/kg, p.o.) 24 hr before CsA administration and continued with CsA for 21 days.

Group XII (L-NAME+RVT+CsA) animals received L-NAME (10 mg/kg, i.p.) 30 minutes before resveratrol administration and continued with CsA for 21 days.

Group XIII (L-NIO+RVT+CsA) animals received L-NIO (5 mg/kg, i.p.) 30 minutes before resveratrol administration and continued with CsA for 21 days.

Group XIV (Amg+RVT+CsA) animals received aminoguanidine (100 mg/kg, i.p.) 30 minutes before resveratrol administration and continued with CsA for 21 days.

Group XV (Pra+CsA) animals were administered pravastatin (20 mg/kg, i.p.) 24 hr before CsA administration and continued with CsA for 21 days.

Group XVI (L-NAME+Pra+CsA) animals received L-NAME (10 mg/kg, i.p.) 30 minutes before pravastatin administration and continued with CsA for 21 days.

Group XVII (L-NIO+Pra+CsA) animals received L-NIO (5 mg/kg, i.p.) 30 minutes before pravastatin administration and continued with CsA for 21 days.

Group XVIII (Amg+Pra+CsA) animals received aminoguanidine (100 mg/kg, i.p.) 30 minutes before pravastatin administration and continued with CsA for 21 days.

Groups administered NOS inhibitors

Group XIX (L-NAME+CsA) animals received L-NAME (10 mg/kg, i.p.) 24 hr before CsA administration and continued with CsA for 21 days.
Group XX (L-NIO+CsA) animals received L-NIO (5 mg/kg, i.p.) 24 hr before CsA administration and continued with CsA for 21 days.

Group XXI (Amg+CsA) animals received aminoguanidine (100 mg/kg, i.p.) 24 hr before CsA administration and continued with CsA for 21 days.

RESULTS

Chronic CsA-treated (20mg/ml, s.c., 21 days) rats lost the body weight as compared to those receiving vehicle significantly. This decrease in body weight was significantly improved by treatment with L-arg, molsidomine, nebivolol, resveratrol and pravastatin, however this improvement in bodyweight was reversed by treatment with L-NAME and L-NIO. CsA administration per se caused a significant rise in SBP, while with L-arg, molsidomine, nebivolol, resveratrol and pravastatin prior to CsA for 21 days prevented the increase in SBP following CsA administration, however this reduction in SBP was attenuated by prior treatment of animals with L-NAME and L-NIO.

Animals treated with CsA for 21 days showed significant increase in the serum concentrations of creatinine (1.91 ± 0.92 mg/dl) and blood urea nitrogen (29.45 ± 1.99 mg/dl) as compared to control group (0.558 ± 0.09 mg/dl & 13.68 ± 0.99 mg/dl). CsA administration also produced a significant reduction in creatinine and urea clearance (0.28 ± 0.061 ml/min & 0.228 ± 0.055 ml/min V/s 0.58 ± 0.05 ml/min & 0.608 ± 0.047 ml/min of control), which was used as an indicator of glomerular filtration rate and thus glomerular function. The urinary and tissue total NO (NO2 + NO3) contents in CsA treated animals (6.7 ± 1.8 pmole/L and 1.4 ± 0.199 pmole/mg protein) were significantly reduced as compared to control animals (22.11 ± 3.2 pmole/L and 6.5 ± 0.687 pmole/mg protein). CsA administration produced a marked increase in TBARS as compared to sham-operated animals (84.21 ± 9.2 nmoles/mg protein V/s 37.25 ± 4.9 nmoles/mg protein). The levels of reduced glutathione and enzymatic activities of superoxide dismutase and catalase were significantly reduced in CsA treated animals as compared to control animals.
Table 2: Effect of L-arg, molsidomine, nebivolol, resveratrol, pravastatin, L-NAME, L-NIO, aminoguanidine on body weight and systolic blood pressure in CsA treated animals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% change in body weight</th>
<th>change in blood pressure, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial (Day 0)</td>
</tr>
<tr>
<td>Control (vehicle treated)</td>
<td>0.56 ± 0.19</td>
<td>142±4.2</td>
</tr>
<tr>
<td>CsA</td>
<td>-5.89 ± 1.28*</td>
<td>140±3.5</td>
</tr>
<tr>
<td>L-arg+CsA</td>
<td>2.23 ± 0.09*a</td>
<td>135±4.5</td>
</tr>
<tr>
<td>L-NAME+L-arg+CsA</td>
<td>-3.56 ± 0.69*b</td>
<td>140±3.9</td>
</tr>
<tr>
<td>L-NIO+L-arg+CsA</td>
<td>-4.02 ± 0.76*b</td>
<td>145±3.2</td>
</tr>
<tr>
<td>Amg+L-arg+CsA</td>
<td>1.28±0.07b</td>
<td>140±4.8</td>
</tr>
<tr>
<td>Mol+CsA</td>
<td>4.56 ± 0.89*a</td>
<td>130±2.7</td>
</tr>
<tr>
<td>L-NAME+Mol+CsA</td>
<td>3.52 ± 0.92*b</td>
<td>140±5.0</td>
</tr>
<tr>
<td>Neb+CsA</td>
<td>1.13±0.05a</td>
<td>130±2.6</td>
</tr>
<tr>
<td>L-NAME+CsA</td>
<td>1.31±0.21a</td>
<td>140±3.1</td>
</tr>
<tr>
<td>RVT+CsA</td>
<td>0.23 ± 0.09a</td>
<td>130±2.5</td>
</tr>
<tr>
<td>L-NAME+RVT+CsA</td>
<td>-3.56 ± 0.89*</td>
<td>140±3.8</td>
</tr>
<tr>
<td>L-NIO+RVT+CsA</td>
<td>-3.10 ± 0.45*b</td>
<td>140±4.2</td>
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<tr>
<td>Amg+RVT+CsA</td>
<td>0.08±0.01</td>
<td>150±3.7</td>
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<tr>
<td>Pra+CsA</td>
<td>2.13±0.68a</td>
<td>140±4.8</td>
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<td>L-NAME+Pra+CsA</td>
<td>-1.36±0.32*b</td>
<td>140±4.1</td>
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<td>L-NIO+Pra+CsA</td>
<td>-1.41±0.36*b</td>
<td>140±3.8</td>
</tr>
<tr>
<td>Amg+Pra+CsA</td>
<td>0.23±0.05</td>
<td>140±4.1</td>
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<tr>
<td>L-NAME+CsA</td>
<td>-7.52 ± 1.86a</td>
<td>135±6.1</td>
</tr>
<tr>
<td>L-NIO+CsA</td>
<td>-8.56 ± 2.1a</td>
<td>140±4.8</td>
</tr>
<tr>
<td>Amg+CsA</td>
<td>-3.58±1.1a</td>
<td>140±4.2</td>
</tr>
</tbody>
</table>
Effect of L-arginine, L-NAME, L-NIO and aminoguanidine on CsA treatment:

Treatment of rats with L-arginine (125 mg/kg, i.p) produced a significant reduction in serum creatinine and blood urea nitrogen levels and a significant increase in creatinine and urea clearance in CsA treated animals. Administration of L-arginine (125 mg/kg, i.p.) to control rats did not result in any alteration in the renal functions. Pretreatment of animals with L-NAME (10 mg/kg, i.p.) and L-NIO (5 mg/kg, i.p.) significantly reversed the protective effect of L-arginine.

L-arginine treatment significantly increased the urinary as well as the tissue total NO contents in CsA administered rats. The TBARS levels were significantly reduced in the L-arginine + CsA group, while the enzymatic activities of reduced glutathione, super oxide dismutase as well as that of catalase was significantly improved with L-arginine treatment. The protective effect of L-arginine in case of urinary and tissue total NO contents, reduction in TBARS levels and the improvement in the enzymatic activities was significantly abolished by pretreatment with L-NAME and L-NIO, however aminoguanidine pretreatment did not had any effect on any of these parameters.

The L-NAME and L-NIO administration in CsA treated animals further worsened all the parameters mentioned above, however same was not the case with aminoguanidine (Fig.2a-j.).
Effect of L-arginine (125 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (and aminoguanidine (100 mg/kg, i.p.) on serum creatinine (Fig.2a.) and nitrogen (BUN) (Fig.2b.) in rats treated with CsA for 21 days. The values as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to L-arg + CsA group, b p<0.05 as compared to L-arg + CsA group (one-way ANOVA by Newman Keuls test).
Effect of L-arginine (125 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/ and aminoguanidine (100 mg/kg, i.p.) on creatinine (Fig. 2c.) and urea clearance ( in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. as compared to control group, a $p<0.05$ as compared to CsA treated group, b $p<$ compared to L-arg + CsA group (one-way ANOVA followed by Newman Keuls t
Effect of L-arginine (125 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on urine and tissue total NO contents in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to CsA treated group, b p<0.05 as compared to L-arg + CsA group (one-way ANOVA followed by Newman Keuls test).
Effect of L-arginine (125 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, and aminoguanidine (100 mg/kg, i.p.) on lipid peroxidation (TBARS) \( \text{Fig.2g.} \) and reduced glutathione (GSH) \( \text{Fig.2h.} \) in rats treated with CsA for 21 days. The value expressed as mean ± S.E.M. *\( p<0.05 \) as compared to control group, a \( p<0.05 \) compared to CsA treated group, b \( p<0.05 \) as compared to L-arg + CsA group (one ANOVA followed by Newman Keuls test).
Fig. 2i.

Effect of L-arginine (125 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 n and aminoguanidine (100 mg/kg, i.p.) on superoxide dismutase (SOD) (F catalase (Fig. 2j.) in rats treated with CsA for 21 days. The values are expressed ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to C group, b p<0.05 as compared to L-arg + CsA group (one-way ANOVA fc Newman Keuls test).
Effect of molsidomine, nebivolol and L-NAME on CsA treatment:

Treatment of rats with molsidomine (10 mg/kg, p.o.) and nebivolol (1 mg/kg, p.o.) produced a significant reduction in serum creatinine and blood urea nitrogen levels and a significant increase in creatinine and urea clearance in CsA administered rats, however this was not observed in control animals. Pretreatment of animals with L-NAME (10 mg/kg, i.p.) did not have any effect on the protective effect of molsidomine and nebivolol. Molsidomine as well as nebivolol pretreatment significantly improved the urinary as well as tissue total NO contents, reduced the elevated TBARS levels and significantly improved the enzymatic activities of reduced glutathione, superoxide dismutase and catalase. The pretreatment of L-NAME did not have any effect on all these parameters (Fig.3 a-j.).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAM i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on serum (Fig.3a.) and blood urea nitrogen (BUN) (Fig.3b.) in rats treated with CsA. The values are expressed as mean ± S.E.M. *p<0.05 as compared to cont p<0.05 as compared to CsA treated group (one-way ANOVA followed Keuls test).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on creatinin and urea clearance (Fig.3d.) in rats treated with CsA for 21 days. The expressed as mean ± S.E.M. *p<0.05 as compared to control group, a compared to CsA treated group (one-way ANOVA followed by Newman Keul):
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on urine (Fig. 3e.) and tissue total NO levels (Fig. 3f.) in rats treated with CsA for 21 days. The data are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a compared to CsA treated group (one-way ANOVA followed by Newman Keuls test).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (10 i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on TBARS (F) and reduced glutathione levels (Fig.3h.) in rats treated with CsA for 21 days. The are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0 compared to CsA treated group (one-way ANOVA followed by Newman Keuls tes
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (1 i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on SOD (Fig. 3j.) levels (Fig. 3i.) in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to treated group (one-way ANOVA followed by Newman Keuls test).
Effect of resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine on CsA treatment:

Treatment of rats with resveratrol (5 mg/kg, p.o.) and pravastatin (20 mg/kg, p.o.) produced a significant reduction in serum creatinine and blood urea nitrogen levels and a significant increase in creatinine and urea clearance in CsA treated animals. Pretreatment of animals with L-NAME (10 mg/kg, i.p.) and L-NIO (5 mg/kg, i.p.) significantly reversed the protective effect of resveratrol as well as that of pravastatin, however same was not the case with aminoguanidine pretreatment.

Resveratrol and pravastatin pretreatment significantly increased the urinary as well as the tissue total NO contents associated with CsA administration. The TBARS levels were significantly reduced in the resveratrol + I/R group and pravastatin + I/R group as compared to that of CsA administered group, while the enzymatic activities of reduced glutathione, superoxide dismutase as well as that of catalase was significantly improved with resveratrol and pravastatin treatment. The protective effect of resveratrol and pravastatin in case of urinary and tissue total NO contents, reduction in TBARS levels and the improvement in the enzymatic activities was significantly abolished by pretreatment with L-NAME and L-NIO, however aminoguanidine pretreatment did not affect any of these parameters (Fig.4a-j.).
Effect of resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, p.o.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on serum creatinine and blood urea nitrogen (BUN) in rats treated with CsA for 14 days. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control, †p<0.05 as compared to CsA treated group, b p<0.05 as compared to RVT + CsA CsA group (one-way ANOVA followed by Newman Keuls test).
Effect of resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, p.o.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on creatinine (Fig. 4d.) and urea clearance (Fig. 4d.) in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to CsA treated group, b p<0.05 as compared to RVT + CsA or Pr group (one-way ANOVA followed by Newman Keuls test).
Effect of resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, p.o.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on urine (Fig. 4e.) and tissue total NO levels (Fig. 4f.) in rats treated with CsA for 21 days. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to CsA treated group, b p<0.05 as compared to RVT + CsA or Pra + CsA group (one-way ANOVA followed by Newman Keuls test).
Effect of resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, p.o.), L-NAME (10 i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on TBARS (Fig.4h.) and reduced glutathione (GSH) levels (Fig.4h.) in rats treated with CsA for 21 da; values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a as compared to CsA treated group, b p<0.05 as compared to RVT + CsA or Pra group (one-way ANOVA followed by Newman Keuls test).
Effect of resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, p.o.), L-NAME (1 i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on SOD (Fig. 4i) in rats treated with CsA for 21 days. The values are expressed ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to Cs group, b p<0.05 as compared to RVT + CsA or Pra + CsA group (one-way followed by Newman Keuls test).
Effect of L-arginine, molsidomine, nebivolol, resveratrol, pravastatin, NAME, L-NIO and aminoguanidine on CsA-induced induced changes in renal morphology

The light microscopic findings of kidneys of control rats treated with olive oil for 21 days showed normal glomeruli, afferent arterioles and tubule cells. In contrast, the kidneys of rats treated with CsA showed marked histological changes in the cortex and outer medulla. The sections showed severe epithelial blebbing, hyaline casts and glomerular basement thickening. A marked tubulointerstitial fibrosis of stripped pattern in the cortex and arteriolo-arterioles with hyaline deposition within the tunica media of afferent arterioles, terminal portions of the interlobular arteries and terminal portions of the interlobular arteries were also observed.

Co-administration of L-arginine (125 mg/kg, i.p.), molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, p.o.) and pravastatin (20 mg/kg, i.p.) preserved the normal morphology of the kidney, and the normal glomeruli except for slight edema of the tubular cells. Pretreatment of animals with L-NAME and L-NIO abolished the protective effect of agents in case of renal morphology, however aminoguanidine pretreatment failed to do so. Administration of the same doses of the L-arginine, molsidomine, nebivolol, resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine in control rats did not result in any significant change in renal morphology.

A
B
Hematoxylin-Eosin stained longitudinal sections of kidney of normal rat (A) and treated with olive oil (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of CsA treated rat (C), L-arg+CsA treated rat (D), L-NAME+L-arg+CsA treated rat (E), L-NIO arg+CsA treated rat (F), Amg+L-arg+CsA treated rat (G) and Mol+CsA treated rat (H) (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of L-NAME+Mol+C treated rat (I), Neb+CsA treated rat (J), L-NAME+Neb+CsA treated rat (k), RVT+CsA treated rat (L), L-NAME+RVT+CsA treated rat (M) and L-NIO+RVT+CsA treated rat (N) (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of Amg+I treated rat (O), Pra+CsA treated rat (P), L-NAME+Pra+CsA treated NIO+Pra+CsA treated rat(R), Amg+Pra+CsA treated rat (S) and L-N treated rat (T) (312.5X)
DISCUSSION

The present investigation revealed that administration of CsA (20 mg/kg, for 21 days) resulted in an overt nephrotoxicity as evidenced by marked renal dysfunction and significant increase in blood pressure. In addition, the tissue and urine nitric oxide levels were markedly reduced after 21 days along with significant depletion of renal oxidative enzymes. These findings were further reinforced by deranged renal morphology.

To study whether the CsA-induced changes are related to the hemodynamic effects of this immunosuppressive drug, we evaluated the effect of the concomitant administration of the NO precursor, L-arg. Our results showed that L-arg as well as molsidomine were able to prevent renal dysfunction as well as changes in tissue and urine nitrite levels along with the renal oxidative stress induced by CsA. The protection afforded by these agents was blocked by co-treatment with L-NAME, a non-selective NOS inhibitor, L-NIO, a specific eNOS inhibitor. Some findings of this study are in concurrence with the previous reports demonstrating that exogenous supplementation of L-arg is effective in reducing renal damage induced by CsA, possibly through the NO pathway (Amo et al., 1995; Yang et al., 1998). In addition, Assis et al. (1997) and De Nicola et al. (1997) showed that oral supplementation of L-arg prevents nephrotoxicity induced by chr

Hematoxylin-Eosin stained longitudinal sections of kidneys of L-NIO+CsA treated rat (U) and Amg+CsA treated rat (V) (312.5X)
Chapter 2

Administration of CsA due to formation of more NO which may enhance vasodilation and consequently reduce the kidney function impairment. Oriji and Keiser (1998) reported that CsA administration inhibits the endothelial NO activity and this inhibition can be overcome by parenteral administration of L-arg. Therefore, the protective effect of L-arg against kidney dysfunction may be related to its reported vasodilatory effect. However, the results of present study revealed that L-arg prevented CsA-induced renal oxidative stress as well as normalized the altered renal morphology. Thus it seems that the protective effect induced by L-arg against CsA-nephrotoxicity may involve an additional non-haemodynamic cytoprotective effect. However, it is difficult to assess the exact contribution of these properties in the protective effect induced by L-arg. In addition, we showed that L-arginine had a beneficial effect at a much lower dose, as compared to the dose used in previously reported studies. The protection afforded by L-arg was attenuated by pretreatment with L-NAME and L-NIO, indicating that NO, particularly the eNOS is involved in the protection afforded by this agent.

Molsidomine, a potent vasodilator, and nebivolol, a new selective β1-adrenergic blocking agent, endowed with peripheral vasodilating property (Mangrella et al., 1998), significantly reduced the elevated SBP, improved the renal dysfunction, significantly increased the urinary and tissue total NO levels and abrogated the renal oxidative stress. Prior treatment of L-NAME failed to abolish the protective effect observed with molsidomine as well as with nebivolol, indicating that NOS is not involved in the protective effect observed with these two agents. The protection observed with nebivolol (1 mg/kg) was greater than with molsidomine, however, both the agents exhibited the reversal of morphological damage to a similar extent. To further confirm that the protective effect observed with nebivolol is due to its nitric oxide donating property and not due to lowering of blood pressure, propranolol-treated group was employed (10 mg/kg, i.p.), where propranolol decreased the blood pressure to a significant extent, but it failed to show any protection in any of the other parameters, thus confirming that effect produced by nebivolol is due to its nitric oxide donating property.

Resveratrol, a polyphenolic phytoalexin, has structural similarity to the synthetic estrogen diethylstilbestrol. There is some evidence that resveratrol interacts with the vascular NO system. Resveratrol caused relaxation of the phenylephrine-precontracted rat aorta, an
Effect that was endothelium dependent and mediated by NO (Chen and Pace-Asciak, 1996; Orallo et al., 2002). Recently, Wallerath et al. (2002) have also shown that resveratrol enhances the expression and activity of endothelial nitric oxide synthase (eNOS). Interestingly, resveratrol shows many common physiological functions with NO. For example, both resveratrol and NO possess anti-inflammatory and antiplatelet activities (Ma et al., 1993; Jang et al., 1997) and can exert vasodilatory effect on blood vessels (Naderali et al., 2000). Similar to NO, resveratrol is a potent scavenger for peroxyl radicals (Das et al., 1999; Ray et al., 1999). NO exists as a free radical scavenger in vitro, but both of them possess potent antioxidant activity in vivo and can attenuate lipid peroxidation. The fact that resveratrol augments NO availability and both of them share common physiological function strongly suggests that resveratrol exerts its renoprotective effect through NO. In the present study, treatment of rats with resveratrol (5 mg/kg, p.o.) along with CsA, renders rats less susceptible to kidney damage induced by treatment with CsA. This protection was evidenced in the serum as the elevated levels of both BUN and creatinine were markedly lowered below those elicited by CsA. In addition, the urea and creatinine clearance were markedly improved as compared to CsA treated rats.

CsA induces a marked oxidative stress in the kidneys by directly enhancing the generation of H$_2$O$_2$ in the mesangial cells and glomeruli, by altering Ca$^{2+}$ homeostasis and by uncoupling CYP 450 system (Ahmed et al., 1995). ROS can disrupt the integrity of the endothelium and affect NO production, resulting in increment of renal vascular resistance. Further, NO can interact with O$_2^{-}$ to generate peroxynitrite, a potent oxidant which causes lipid peroxidation of cellular membrane (Gross and Wolin, 1995). Resveratrol, due to its amphiphatic character, protects the cellular and cellular and subcellular components (Sun, 1997) and it is an efficient scavenger of 'OH$^-$ and O$_2^{-}$ (Hung et al., 2000). Various assays have also confirmed the copper chelating properties of resveratrol as well as it inhibited NF-kB activation (Leonard et al., 2003). Inline with these observations, resveratrol treatment ameliorated reduced levels in GSH, SOD and catalase activities and prevented the rise in lipid peroxides in kidney tissues. In addition, the tissue and urinary excretion of nitric oxide levels were significantly enhanced by treatment with resveratrol as compared to CsA treated rats. These findings may indicate a
possible protective effect of resveratrol against nephrotoxicity induced by CsA treatment. The protection afforded by the resveratrol was ameliorated to a significant extent in the animals co-treated with L-NAME, thus indicating that resveratrol does have NO releasing property. These findings were further confirmed by the histopathological studies. The animals with resveratrol treatment showed a significant improvement in renal morphology as compared to CsA treated animals; however co-treatment with L-NAME prevented this improvement in renal morphology. L-NIO, a specific eNOS inhibitor, was able to prevent the protection afforded by resveratrol to a significant extent, and since aminoguanidine, a specific iNOS inhibitor was not able to revert the protection afforded by the resveratrol in this model, it can be concluded that resveratrol exerts the protective affect in CsA nephropathy, both via its antioxidative property as well as due to its eNOS upregulating property.

Statins are the most potent and widely prescribed lipid-lowering agents used in the management of dyslipidemia and hypercholesterolemia (Rosenson and Tangney, 1998). The mechanism by which pravastatin attenuates nephropathy associated with CsA, may be multifactorial, but two possibilities should be considered. First, chronic CsA-induced nephropathy is associated with afferent arteriolopathy (Franceschini et al., 1998b), which ultimately leads to hypoxia-induced renal tubulointerstitial inflammation and tubulointerstitial fibrosis (TIF). Statins modulate vascular remodeling by inhibiting smooth muscle cell proliferation, migration, and extracellular matrix synthesis (Guijarro et al., 1998; Komukai et al., 1999; Riessen et al., 1999). Second, endothelial NO dysregulation has been linked with CsA-related vasoconstriction, inflammatory reaction, and fibrosis (Yang et al., 1998; Heeringa et al., 2000). Recently, in vivo and in vitro studies have confirmed that statins improve endothelium function through enhancing eNOS expression and its activity (Jorge et al., 1997; Laufs et al., 1998; Ni et al., 2001). Here, we found that pravastatin significantly counteracted the CsA nephrotoxicity and arteriopatp, and since this effect of pravastatin was blocked by L-NIO (a specific eNOS inhibitor), therefore effects of pravastatin in this study may be associated with its effects on endothelial NOS.

In the present study, we chose to compare two doses of pravastatin (5 or 20 mg/kg). Interestingly, the lower dose only improved renal morphology, whereas the higher dose
improved both histopathology and renal function. However, there were no significant differences in lipid parameters in any treatment groups (data not shown). This observation suggests that, although a low dose of pravastatin (5 mg/kg) failed to overcome the hemodynamic changes caused by CsA, it was still effective in preventing the progression of CsA-induced nephropathy. The functions of pravastatin also extend to immunomodulation, as demonstrated by the inhibition of the expression of class II major histocompatibility antigens and of natural killer cell activity (Weitz-Schmidt et al., 2001).

Indeed, experimental and clinical studies in cardiac and renal transplant recipients show that the administration of pravastatin successfully decreases acute or chronic rejection episodes and improves graft survival (Kobashigawa et al., 1995; Katznelson et al., 1996; Ji et al., 2002). Furthermore, combined treatment with pravastatin and CsA produced additional immunosuppression in patients with kidney transplants (Katznelson et al., 1996), which was related to the synergistic inhibition of cytotoxic T lymphocyte activity (Katznelson et al., 1998).

Several lines of evidence now indicate that CsA nephrotoxicity is associated with infiltration and activation of macrophages and neutrophils (Young et al., 1995; Thurman et al., 1997). The effects of NO on neutrophils have been delineated. NO effects on neutrophils include decrease in neutrophil chemotaxis, decrease in NAD(P)H oxidase activity and downregulation of both neutrophil and endothelial cell adhesion molecule expression (Ghielli et al., 1998). Statins have been shown to inhibit NAD(P)H oxidase expression (Wassmann et al., 2001) and activity (Christ et al., 2002), suggesting that NAD(P)H oxidase dependent ROS production is the causal event and thereby statins exert their antioxidative effect (Vacchione and Brandes, 2002).

In conclusion, the results of present study indicate that CsA impairs the NO pathway and the agents which can increase the nitric oxide release or the agents which can upregulate specifically the eNOS enzyme, have a potential as a possible therapy for attenuation of CsA nephrotoxicity.