CHAPTER 1
TO STUDY THE ROLE OF NITRIC OXIDE IN
ISCHEMIC ACUTE RENAL FAILURE IN RATS
Chapter 1

INTRODUCTION:
The temporary discontinuation of renal blood supply is an important clinical syndrome such as during renal transplantation, surgical revascularization of renal artery, partial nephrectomy and treatment of suprarenal aortic aneurysms. Renal I/R is not only an invariable consequence of transplantation but also results from circumstances such as aortic cross-clamping and resuscitation following systemic hypotension (Weight et al., 1996). The acute renal failure observed after ischemia is characterized by decreased glomerular filtration rate (GFR), tubular necrosis and increased renal vascular resistance (Erbas et al., 2004). The prognosis is complicated by the fact that reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage (reperfusion injury). Despite significant advances in critical care medicine, acute renal failure (ARF) remains a major clinical problem, and mortality associated with ARF has not decreased substantially over the past 50 years (Thadhani et al., 1996; Star, 1998).

Although the pathophysiology of ischemia-reperfusion (I/R)-induced ARF is not completely understood, some key events leading to tissue injury and renal failure have been identified. Initial pathological events in the kidney following renal ischemia injury include vasoconstriction, endothelial-cell activation, tubular swelling, desquamation and necrosis of tubular epithelium and interstitial edema. Organ reperfusion increases endothelial expression of the adhesion molecules, and promotes leukocyte recruitment and activation, giving way to increased reactive oxygen species production and the development of the inflammatory process (Singbarti et al., 1998; Takada et al., 2000). NO being synthesized from L-arginine and molecular oxygen by the enzyme NO synthase (NOS), has been recognized as a potential link between ischemia-reperfusion (I/R) injury and rejection of the transplanted kidney (Ketteler et al., 1994). The constitutive enzyme (eNOS) is produced by endothelial cells in the kidney and modulates vascular tone. Since a renal transplant is denervated, a contributory role of neuronal NOS (nNOS) is much less likely. By contrast, NO produced in larger quantities by the inducible NOS (iNOS) enzyme, has been implicated as a mediator of alloimmune damage (Langrehr et al., 1993; Russell et al., 1995). Therefore the activity of NOS enzyme after I/R injury can influence the degree of ischemic damage and the rate of repair during the injury response and can interact with alloimmune molecules. During
RIR, ROS can disrupt the integrity of the endothelium and can affect NO production, resulting in increase in renal vascular resistance (Rhoden et al., 2001). Furthermore NO, can interact with $O_2^-$ to form the peroxynitrite free radical (ONOO$^-$), an important agent that causes lipid peroxidation (LPO) of cellular membranes (Rhoden et al., 2001). The role of NO in ischemic ARF is controversial. Lieberthal et al. (1991) found that decrease in renal blood flow (RBF) and GFR in rats with hypovolemic shock induced by hemorrhage, were to some extent overcome by the inhibition of NO production. NO synthase inhibitor was reported to prevent hypoxia/reoxygenation injury in rat proximal tubules, thereby suggesting that NO is synthesized in proximal tubules and is involved in tubular hypoxia/reoxygenation injury (Yu et al., 1994). In contrast, Chintala et al. (1993) noted that the inhibition of NO production with a NO synthase inhibitor significantly deteriorated renal function of the postischemic kidney in anesthetized rats, whereas pretreatment with the NO precursor, L-arginine abolished the NO synthase inhibitor-induced deterioration of renal function. Similar improvement by L-arginine against the decreased renal function in ischemic ARF was noted by Schramm et al. (1994), although they did not observe detrimental effects of the NOS inhibitor.

Several recent studies have demonstrated that oxygen-derived reactive species and nitric oxide are involved in renal I/R injury (Jassem et al., 2002; Oehlschlaeger et al., 2003), but the nature of the mediators is still controversial. Reactive oxygen species such as superoxide radical, hydroxyl radical and hydrogen peroxide can induce cellular injury through lipid peroxidation reaction in cell membranes (Paller et al., 1984). Interaction between superoxide and nitric oxide produces peroxynitrite which can cause lipid peroxidation. Furthermore peroxynitrite anion eventually degrades into peroxynitrous acid and the hydroxyl radical both of which can cause lipid peroxidation of cell membranes (Gross and Wolin, 1995; Akcetin et al., 1999).

With this background, the present study was designed to assess the ability of various nitric oxide donors or NOS upregulators to ameliorate the I/R-induced acute renal failure. Further, the efforts were made to explore the involvement of specific NOS in the development of I/R-induced renal injury in the rats.
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

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 (Jennessan 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

Rats were anesthetized with ketamine (50 mg/kg, intraperitoneally) and the abdominal region was shaved with a safety razor and sterilized with povidone iodine solution. A midline incision was made and both the kidneys were isolated. Renal ischemia was instituted by using two different sets of protocols. In one, both the renal pedicles were occluded, whereas, in the other, only left renal pedicle was occluded after right nephrectomy. The nephrectomy groups were employed to delineate the effect of contralateral kidney removal on I/R. Ischemia was induced for 45 minutes followed by reperfusion for 24 hours. After the surgical procedures the midline incision was sutured back with the local applications of povidone and neosporin. The animals were allowed to recover from anesthesia with free access to tap water and food, in the individual metabolic cages. The total water intake, and urine output was measured for each animal. At the end of reperfusion period, the blood and urine samples were collected and used for the measurement of renal function. The abdomen was reentered and bilateral nephrectomies were carried out; the left kidney was used for further enzymatic analysis, whereas the right kidney was stored in 10%
formalin for histological examination. Additional groups were employed for harvesting the left kidneys for histological analysis in nephrectomized animals.

**Assessment of renal function**

Serum samples were assayed for blood urea nitrogen (BUN) and serum creatinine by using standard diagnostic kits (Span Diagnostics, Gujarat, India).

**Estimation of urea**

Urea reacts with hot acidic diacetylmonoxime in the presence of thiosemicarbazide and produces a rose-purple coloured complex. The intensity of the colour is proportional to the concentration of urea in the solution and is measured spectrophotometrically at 525 nm.

\[
\text{CH}_3\text{COCCH}_3 + \text{H}_2\text{O} \xrightarrow{\text{Diacetylmonoxime, NOH}} \text{CH}_3\text{COCOCH}_3 + \text{NH}_2\text{OH}
\]

\[
\text{CH}_3\text{COCOCH}_3 + \text{H}_2\text{N} - \text{C} - \text{NH}_2 \xrightarrow{\text{Diacetyl, Urea}} \text{H}_3\text{CC} \xrightarrow{\text{Diazone}} \text{CCH}_3 + 2\text{H}_2\text{O}
\]

**Reagents:**

Reagent 1: Urea reagent  
Reagent 2: Diacetylmonoxime (DAM)  
Reagent 3: Working urea standard, 30 mg%  
Samples: Serum, Urine (Diluted with DW 1:20).

**Preparation of working solutions**

Solution 1: Dilute 1 ml of Reagent 1 to 5 ml with distilled water.
Procedure

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (Plasma/Urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td></td>
<td>0.01ml</td>
</tr>
<tr>
<td>Reagent 3</td>
<td>–</td>
<td>0.01ml</td>
<td>–</td>
</tr>
<tr>
<td>(Working urea standard, 30 mg %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent 2:</td>
<td>0.25ml</td>
<td>0.25ml</td>
<td>0.25ml</td>
</tr>
<tr>
<td>Diacetylmonoxime (DAM)</td>
<td></td>
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</tbody>
</table>

Mixed well and kept the tubes in the boiling water exactly for 10 minutes. Cooled immediately under running water for 5 minutes, mixed by inversion and measured the optical density within 10 minutes at 525 nm.

Calculations

1. Serum:
   \[
   \text{Urea Concentration (mg/dl)} = \frac{\text{OD of test}}{\text{OD of std.}} \times 30
   \]
   Blood urea Nitrogen (mg/dl) = Serum Urea (mg/dl) × 0.467

2. Urine:
   \[
   \text{Urea Concentration (g/l)} = \frac{\text{OD of test}}{\text{OD of std.}} \times \frac{30 \times 20}{100}
   \]
   Urea Nitrogen (g/l) = Urine urea (g/l) × 0.467
Chapter 1

**Estimation of Creatinine**

Creatinine in a protein free solution reacts with alkaline picrate and produces a red coloured complex, which is measured spectrophotometrically.

![Chemical reaction diagram](image)

**Reagents:**
- Reagent 1: Picric Acid
- Reagent 2: Sodium Hydroxide, 0.75 N
- Reagent 3: Stock Creatinine Standard, 150 mg%

**Preparation of working solution:**

**Working Standard:** Dilute one ml of Reagent 3 (Stock Creatinine Standard) to 10 ml with distilled water and mix well.

**Test Samples:**

- Serum
- Urine: Dilute 1 ml of urine to 25 ml with distilled water.

**Procedure**

For Creatinine, pipetted following in tubes.

**Step A. Deproteinization of test sample:**

- Serum / Dilute Urine: 0.5 ml
- Distilled Water: 0.5 ml
- Reagent 1: Picric acid: 3.0 ml

Mixed well, kept in boiling water exactly for one minute. Cooled immediately under running tap water and centrifuged.
Chapter 1

Step B: Colour Development:

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard</th>
<th>Test (Plasma/Urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant (from Step A)</td>
<td>—</td>
<td>—</td>
<td>2.0ml</td>
</tr>
<tr>
<td>Working standard</td>
<td>—</td>
<td>0.5 ml</td>
<td>—</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.5ml</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>1.5ml</td>
<td>1.5ml</td>
<td>—</td>
</tr>
<tr>
<td>(Picric acid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent 2</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>(Sodium hydroxide, 0.75 N)</td>
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</table>

Mixed well and allowed to stand at room temperature for 20 minutes and immediately measured the optical density of Blank (B), Standard (S) and Test (T) at 520 nm.

Calculations

Serum Creatinine in mg / 100 ml = \( \frac{OD_{test} - OD_{blank}}{OD_{std} - OD_{blank}} \times 3.0 \)

Urine Creatinine in g/litre, \( A = \frac{OD_{test} - OD_{blank}}{OD_{std} - OD_{blank}} \times 0.75 \)

Urine Creatinine in g/24 hr. collection = \( (A) \times 24 \) hours

Urea Clearance (ml/min):

\[
\left[ \frac{Urine\text{ Urea}(mg/dl) \times \text{Urine}(ml)(24\text{hrs})}{\text{Plasma Urea}(mg/dl)} \right] \div [24 \times 60]
\]
Creatinine Clearance (ml/min):
\[
\left( \frac{\text{Urine Creatinine(mg/dl)}}{\text{Plasma Creatinine(mg/dl)}} \right) \times \text{Urine(ml)(24hrs)} = \left[ 24 \times 60 \right]
\]

Estimation of urine nitrite and nitrate levels
Nitrite and nitrate are the primary oxidation products of NO subsequent to reaction with oxygen and therefore, the nitrite/nitrate concentration in urine was used as indicator of NO synthesis. Quantitation of nitrate and nitrite was based on the Griess reaction, in which a chromophore with a strong absorbance at 550 nm is formed by reaction of nitrite with a mixture of naphthylethylenediamine and sulphanilamide. The nitrate was reduced to nitrite by 30 minutes incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH). Total nitrite/nitrate concentration was calculated by using standard of sodium nitrate. Results were expressed as μmol/L.

Post mitochondrial supernatant preparation (PMS)
After sacrificing the animals, their kidneys were quickly removed, perfused immediately with ice cold normal saline and homogenized in chilled potassium chloride (1.17%) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 800 g for 5 minutes at 4°C in a refrigerated centrifuge to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 g for 20 minutes at 4°C to get the post mitochondrial supernatant (PMS) which was used for further enzymatic analysis.

Estimation of Lipid Peroxidation (LPO)
Malondiadehyde (MDA), an indirect index of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) (Ohkawa et al., 1979). MDA, formed from the breakdown of polyunsaturated fatty acids, serves as an index for determining the extent of the peroxidation reaction. MDA has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colored product absorbing at 535 nm.
The reaction mixture consisted of 0.2 ml of PMS, 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. The mixture was brought up to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol & pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm. TBARS were quantified using an extinction coefficient of $1.56 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$ and expressed as nmol of TBARS per mg protein. Tissue protein was estimated using Biuret method (1988) of protein assay and the renal MDA content expressed as nanomoles of malondialdehyde per milligram of protein.

**Estimation of reduced glutathione**

Glutathione (GSH) was estimated by the method of Ellman (Jollow et al., 1974), in which 5-5’dithiobis-2-nitrobenzoic acid (DTNB) is reduced by –SH groups to glutathione (GSH) in alkaline medium to produce one mole of 2-nitro-5-mercaptobenzoic acid per mole of –SH. Since the anion (2-nitro-5-mercaptobenzoic acid) has an intense yellow colour, it can be used to measure –SH group.

PMS (1.0 ml) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4°C for at least 1 hour and then subjected to centrifugation at 1200 g for 15 minutes at 4°C. The assay mixture contained 0.1 ml filtered aliquot and 2.7 ml phosphate buffer (0.1 M, pH 7.4) in a total volume of 3.0 ml. Absorbance of stable yellow colour produced was read at 412 nm after 2 minutes. The –SH group was calculated on the basis of molar extinction coefficient of yellow colored anion, 2-nitro-5-mercaptobenzoic acid ($13.6 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$).

**Catalase activity**

Catalase (Cat) activity was assayed by the method of Claiborne (1985). It catalyzes the reaction $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$. The UV absorption of $\text{H}_2\text{O}_2$ can be measured between 230 nm and 250 nm. On decomposition of $\text{H}_2\text{O}_2$ with catalase,
the UV absorption is recorded and from this decrease in optical density, the enzyme activity can be calculated.

Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml PMS (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of k/minutes.

**SOD activity**
Superoxide dismutase (SOD) activity was assayed by the method of Kono (1978). Superoxide anions are generated by the autoxidation of hydroxylamine hydrochloride. The rates of autoxidation is however negligible below pH 8.0 but sharply increased with increasing pH. The superoxide anions reduces the nitro blue tetrazolium (NBT) forming a blue formazan, which is measured at 560 nm. SOD inhibits this reduction of NBT and thus the enzyme activity is measured by monitoring the rate of increase in optical density at 560 nm.

The assay system consisted of EDTA 0.1 mM, sodium carbonate 50 mM and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 ml of above mixture, 0.05 ml hydroxylamine and 0.05 ml of PMS were taken and the auto-oxidation of hydroxylamine was observed by measuring the absorbance at 560 nm.

**Histopathology Study**
The kidney was excised and washed thoroughly with chilled normal saline. It was then fixed in a 10% formalin solution for fixation. This would preserve the cells and tissue in as life-like manner as possible. Fixative had hardening effect and allowed easy manipulation of soft tissues. Processing of tissue was done in a ‘Histokinette’ i.e., tissue was exposed to various concentrations of ethyl alcohol viz. 70%, 80%, 90% and absolute alcohol for one hour for dehydration. After alcohol treatment, the kidney was placed in xylene to replace alcohol for 3 hr replacing with fresh xylene every hour. This process is known as clearing. The tissue was embedded in paraffin wax maintained at 60°C. Blocks were prepared and 5 μm thick sections were cut using microtome. The sections were fixed on a slide smeared with a mixture of equal volume of glycerine and Mayers egg albumin. Whole wax was removed by placing the slides in xylene for 5 min. followed by successive treatments with fresh absolute alcohol for one minute each. The slide was
Chapter 1

rinsed with water and stained with hematoxylin for 3 min. The excess hematoxylin was washed with water. The slide was then counter-stained with 1% solution of eosin for 3 min., followed by washing with water for 1 minute. The slide was then washed with ascending grades of alcohol for dehydration and subsequently treated with xylene twice for 5 min. for clearing. It was then mounted with a drop of DPX (dibutyl phthalate- 5 ml, Xylol-35 ml, Disterene 8-10 g). The slide was allowed to dry and was examined under microscope.

The renal sections were examined in a blind fashion for tubular cell swelling, cellular vacuolization, pyknotic nuclei, medullary congestion and moderate to severe necrosis 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 30 minutes prior to the institution of renal ischemia. 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 and elevating total nitric oxide levels following I/R and therefore, this dose was used for the further studies (Fig.1a.)

Molsidomine treatment
Molsidomine was suspended in 0.25% sodium carboxymethylcellulose (CMC) and was given orally 30 min before the induction of renal ischemia. In the pilot studies, doses of 5 and 10 mg were tested, and the range of 10 mg was found to be most effective in preservation of renal function and total nitric oxide levels following renal I/R with least effect on the hemodynamic parameters (Fig.1b.).
Chapter 1

Nebivolol treatment
Nebivolol was suspended in 0.25% sodium carboxymethyl cellulose (CMC) and was given orally 30 min before the induction of renal ischemia. In our preliminary studies, dose ranges from 0.5 to 2 mg were tested, and dose of 1 mg was found to be most effective in preservation of renal function and total nitric oxide levels following I/R with least effect on the hemodynamic parameters. The higher dose of 2 mg did not show any additional advantage in the renal function preservation, however, it produced a significant decrease (p<0.05) in systolic blood pressure and heart rate. Therefore, to rule out the contribution of hemodynamic parameters in the renoprotective effects, the 1 mg/kg dose was selected for the further studies (Fig.1c.).

Resveratrol treatment
Resveratrol was suspended in 0.25% sodium carboxymethyl cellulose (CMC) and was given orally 30 mins before the induction of renal ischemia. In pilot studies, dose ranges from 2 to 10 mg were tested, and the 5 mg was found to be most effective in preservation of renal function and total nitric oxide levels following I/R. 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.), 30 mins prior to renal I/R. 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 I/R and therefore, this dose was used for the further studies (Fig.1e.). At these doses (5-20 mg/kg), pravastatin did not alter serum cholesterol or triglyceride levels (Table 1.)
Fig. 1a. Effect of various doses of L-arginine on renal function and urinary total NO contents in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the sham group; a p<0.05 as compared with the I/R group (one-way ANOVA followed by Newman Keuls test).
Fig. 1b. Effect of two doses of molsidomine on renal function and urinary total NO contents in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the sham group; a p<0.05 as compared with the I/R group (one-way ANOVA followed by Newman Keuls test).
Fig. 1c. Effect of various doses of nebivolol on renal function and urinary total NO contents in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the sham group; a *p< 0.05 as compared with the I/R group (one-way ANOVA followed by Newman Keuls test).
Fig. 1d. Effect of various doses of resveratrol on renal function and urinary total NO contents in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the sham group; a p<0.05 as compared with the I/R group (one-way ANOVA followed by Newman Keuls test).
Fig.1e. Effect of different doses of pravastatin on renal function and urinary total NO contents in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p< 0.05 as compared with the sham group; a p<0.05 as compared with the I/R group (one-way ANOVA followed by Newman Keuls test).
Table 1. Effect of pravastatin (5-20 mg/kg) on serum cholesterol and triglyceride levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>150±18.6</td>
<td>70±7.9</td>
</tr>
<tr>
<td>Pra 5</td>
<td>148±16.1</td>
<td>67±7.1</td>
</tr>
<tr>
<td>Pra 10</td>
<td>146±15.8</td>
<td>65±7.6</td>
</tr>
<tr>
<td>Pra 20</td>
<td>145±16.4</td>
<td>67±</td>
</tr>
</tbody>
</table>

STUDY DESIGN
Animals were randomly distributed into twenty five groups, each consisting of fourteen animals (n=7, for bilateral ischemia, and n=7, for left renal ischemia after right nephrectomy).

Group I (Sham) consisted of sham operated animals, they underwent the exposure of both the renal pedicles, but did not receive any ischemia reperfusion, whereas the nephrectomized sham group consisted of animals that underwent the right nephrectomy, but the left renal pedicle was not occluded.

Group II (I/R) consisted of rats subjected to either (a) bilateral ischemia/reperfusion, in which both renal pedicals were occluded for 45 min followed by 24 hour of reperfusion, and (b) right nephrectomy, and after 10 minutes of stabilization, 45 minutes of left renal ischemia followed by 24 hour reperfusion

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

Group III (L-arg+I/R) animals received L-arginine (125 mg/kg, i.p.), 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group IV (L-NAME+L-arg+I/R) animals received L-NAME (10 mg/kg, i.p.), 60 minutes and L-arginine (125 mg/kg, i.p.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group V (L-NIO+L-arg+I/R) animals received L-NIO (5 mg/kg, i.p.), 60 minutes and L-arginine (125 mg/kg, i.p.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.
Chapter 1

Group VI (AMG+L-arg+I/R) animals received aminoguanidine (100 mg/kg, i.p.), 60 minutes and L-arginine (125 mg/kg, i.p.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Groups administered NO donors (molsidomine, nebivolol) and NOS inhibitors (L-NAME, L-NIO and AMG)

Group VII (Mol+I/R) animals received molsidomine (10 mg/kg, p.o.), 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group VIII (L-NAME+Mol+I/R) animals received L-NAME (10 mg/kg, i.p.), 60 minutes and molsidomine (5 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group IX (L-NIO+Mol+I/R) animals received L-NIO (5 mg/kg, i.p.), 60 minutes and molsidomine (5 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group X (AMG+Mol+I/R) animals received aminoguanidine (100 mg/kg, i.p.), 60 minutes and molsidomine (5 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XI (Neb+I/R) animals were treated with nebivolol (1 mg/kg, p.o.), 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XII (L-NAME+Neb+I/R) animals received L-NAME (10 mg/kg, i.p.), 60 minutes and nebivolol (1 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XIII (L-NIO+Neb+I/R) animals received L-NIO (5 mg/kg, i.p.), 60 minutes and nebivolol (1 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XIV (AMG+Neb+I/R) animals received aminoguanidine (100 mg/kg, i.p.), 60 minutes and nebivolol (1 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

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

Group XV (RVT+I/R) animals were administered resveratrol (5 mg/kg, p.o.), 30 minutes prior to renal ischemia and the rest of procedure was same as in Group II.
Group XVI (L-NAME+RVT+I/R) animals received L-NAME (10 mg/kg, i.p.), 60 minutes and resveratrol (5 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XVII (L-NIO+RVT+I/R) animals received L-NIO (5 mg/kg, i.p.), 60 minutes and resveratrol (5 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XVIII (AMG+RVT+I/R) animals received aminoguanidine (100 mg/kg, i.p.), 60 minutes and resveratrol (5 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XIX (Pra+I/R) animals were administered pravastatin (20 mg/kg, i.p.) 30 minutes prior to ischemic insult, and the rest of procedure was same as in Group II.

Group XX (L-NAME+Pra+I/R) animals received L-NAME (10 mg/kg, i.p.), 60 minutes and pravastatin (20 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XXI (L-NIO+Pra+I/R) animals received L-NIO (5 mg/kg, i.p.), 60 minutes and pravastatin (20 mg/kg, p.o.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XXII (AMG+Pra+I/R) animals received aminoguanidine (100 mg/kg, i.p.), 60 minutes and pravastatin (20 mg/kg, i.p.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Groups administered NOS inhibitors only

Group XXIII (L-NAME+I/R) animals received L-NAME (10 mg/kg, i.p.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XXIV (L-NIO+I/R) animals received L-NIO (5 mg/kg, i.p.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.

Group XXV (AMG+I/R) animals received aminoguanidine (100 mg/kg, i.p.) 30 minutes prior to the renal ischemia, and the rest of procedure was same as in Group II.
RESULTS

Animals that underwent renal I/R (mean of Bl/R and Np + Ul/R) exhibited significant increase in the serum concentrations of creatinine (2.5 ± 0.2 mg/dl) and blood urea nitrogen (BUN) (25.86 ± 3.5 mg/dl) as compared to sham operated animals (0.58 ± 0.095 mg/dl & 12.56 ± 1.21 mg/dl), suggesting a significant degree of glomerular dysfunction mediated by renal I/R (Fig. 2a, 2b). Renal I/R also produced a significant reduction in creatinine and urea clearance (0.066 ± 0.036 ml/min & 0.0829 ± 0.041 ml/min V/s 0.477 ± 0.08 ml/min & 0.451 ± 0.095 ml/min of sham) (Fig. 2c, 2d), which was used as an indicator of glomerular filtration rate and thus glomerular function. The urinary and tissue total NO (NO\(_2\) + NO\(_3\)) contents in renal I/R animals (7.19 ± 0.8 pmole/L and 1.4 ± 0.19 μmole/L) were significantly reduced as compared to sham-operated animals (24.12 ± 2.72 μmole/L and 6.5 ± 0.678 μmole/L) (Fig. 2e, 2f). Renal I/R produced a significant increase in TBARS as compared to sham-operated animals (85.56 ± 8.5 nmoles/mg protein V/s 38.34 ± 3.97 nmoles/mg protein) (Fig. 2g). The enzymatic activity of reduced glutathione, superoxide dismutase and catalase were significantly reduced in I/R treated animals as compared to sham-operated animals (Fig. 2h-j).

Effect of L-arginine, L-NAME, L-NIO and aminoguanidine on renal I/R:

Treatment of rats with L-arginine (125 mg/kg, i.p) produced a significant reduction in serum creatinine and urea nitrogen levels and a significant increase in creatinine and urea clearance associated with I/R. Administration of L-arginine (125 mg/kg, i.p.) to sham operated rats did not result in any alteration in the renal functions in comparison to non treated sham rats. 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 pretreatment significantly increased the urinary as well as the tissue total NO contents associated with I/R. The TBARS levels were significantly reduced in the L-arginine + I/R group, while the enzymatic activities of reduced glutathione, superoxide 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 have any effect on any of these parameters.

The administration of L-NAME and L-NIO in the I/R 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 (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on serum creatinine (Fig.2a.) and blood urea nitrogen (BUN) (Fig.2b.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to sham group, a p<0.05 as compared to I/R group, b p<0.05 as compared to L-arg + I/R 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, i.p.) and aminoguanidine (100 mg/kg, i.p.) on creatinine (Fig.2c.) and urea clearance (Fig.2d.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to sham group, a p<0.05 as compared to I/R group, b p<0.05 as compared to L-arg + I/R 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, i.p.) and aminoguanidine (100 mg/kg, i.p.) on urine (Fig.2e.) and tissue total NO contents (Fig.2f.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to sham group, a p<0.05 as compared to I/R group, b p<0.05 as compared to L-arg + I/R 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, i.p.) and aminoguanidine (100 mg/kg, i.p.) on lipid peroxidation (TBARS) (Fig. 2g.) and reduced glutathione (GSH) (Fig. 2h.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to sham group, a p<0.05 as compared to I/R group, b p<0.05 as compared to L-arg + I/R 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, i.p.) and aminoguanidine (100 mg/kg, i.p.) on superoxide dismutase (SOD) (Fig. 2i.) and catalase (Fig. 2j.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to sham group, a p<0.05 as compared to I/R group, b p<0.05 as compared to L-arg + I/R group (one-way ANOVA followed by Newman Keuls test).
Effect of molsidomine, nebivolol, L-NAME, L-NIO and aminoguanidine on renal I/R:

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 urea nitrogen levels and a significant increase in creatinine and urea clearance associated with I/R, however this was not observed in sham-operated animals. Pretreatment of animals with L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 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 protection observed in all above mentioned parameters was more in nebivolol treatment than that of molsidomine treatment. The pretreatment of L-NAME, L-NIO and aminoguanidine did not have any effect on all these parameters (Fig.3a-j.).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 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 (Fig.3a.) and blood urea nitrogen (BUN) (Fig.3b.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R 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 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on creatinine (Fig.3c.) and urea clearance (Fig.3d.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R 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 mg/kg, 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 exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R 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 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on TBARS (Fig.3g.) and reduced glutathione levels (Fig.3h.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R group (one-way ANOVA followed by Newman Keuls test).
Chapter 1

Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 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 SOD (Fig.3i.) and catalase levels (Fig.3j.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R group, (one-way ANOVA followed by Newman Keuls test).

Fig.3i.

Fig.3j.

Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 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 SOD (Fig.3i.) and catalase levels (Fig.3j.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R group, (one-way ANOVA followed by Newman Keuls test).
Effect of resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine on renal I/R:

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 urea nitrogen levels and a significant increase in creatinine and urea clearance associated with I/R. 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 I/R. The TBARS levels were significantly reduced in the resveratrol + I/R group and pravastatin + I/R group as compared to that of I/R group, while the enzymatic activities of reduced glutathione, super oxide 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 exert any effect on 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 (Fig. 4a.) and blood urea nitrogen (BUN) (Fig. 4b.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R group, b p<0.05 as compared to RVT + I/R and Pra + I/R 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.4c.) and urea clearance (Fig.4d.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R group, b p<0.05 as compared to RVT + I/R and Pra + I/R 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 exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to I/R group, b p<0.05 as compared to RVT + I/R and Pra + I/R 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 TBARS (Fig.4g.) and reduced glutathione (GSH) levels (Fig.4h.) in rats exposed to renal I/R. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a /K0.05 as compared to I/R group, b /p<0.05 as compared to RVT + I/R and Pra + I/R 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 i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on SOD (Fig.4i.) catalase (Fig.4j.) in rats exposed to renal I/R. The values are expressed as mean ± S. *p<0.05 as compared to control group, a p<0.05 as compared to I/R group, b p<0.1 compared to RVT + I/R and Pra + I/R group (one-way ANOVA followed by Newman Keuls test).
Effect of L-arginine, molsidomine, nebivolol, resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine on renal I/R induced changes in renal morphology

The histopathological changes were graded and summarized in the sham-operated group did not show any morphological changes. By contrast, the kidneys of untreated ischemic rats showed tubular cell swelling, vacuolization, pyknotic nuclei, medullary congestion and moderate necrosis. Treatment with L-arginine (125 mg/kg, i.p.), molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, i.p.), pravastatin (20 mg/kg, i.p.) preserved the normal morphology of the kidney and showed normal glomeruli except for slight edema of the tubules. Pretreatment of animals with L-NAME and L-NIO abolished the effect of these agents in case of renal morphology, however aminoguanidine failed to do so.

Administration of the same doses of the L-arginine, molsidomine, resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine operated rats did not result in any significant change in the renal morphology.

Hematoxylin-Eosin stained longitudinal sections of kidney of sham-operated and rat subjected to Ischemia/Reperfusion (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of L-arg+I/R treated rat (C), L-NAME+L-arg+I/R treated rat (D), L-NIO+L-arg+I/R treated rat (E), Amg+L-arg+I/R treated rat (F), Mol+I/R treated rat (G), L-NAME+Mol+I/R treated rat (H) (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of L-NIO+M treated rat (I), Amg+Mol+I/R treated rat (J), Neb+I/R treated rat (K), NAME+Neb+I/R treated rat (L), L-NIO+Neb+I/R treated rat (M), Amg+ treated rat (N) (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of RVT+I/R treated (O), L-NAME+RVT+I/R treated rat (P), L-NIO+RVT+I/R treated rat (Q) Amg+Neb+I/R treated rat (R), Pra+I/R treated rat (S), L-NAME+RVT+I/R treated rat (T) (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of L-NIO+ treated rat (U), Amg+Pra+I/R treated rat (V), L-NAME+I/R treated rat (X), NIO+I/R treated rat (X), Amg+I/R treated rat (Y) (312.5X)
DISCUSSION
Renal failure caused by ischemia is a complex syndrome involving renal vasoconstriction, extensive tubular damage and glomerular filtration failure (Rhoden et al., 2001). In the clinical setting, renal I/R is a consequence of systemic hypoperfusion with subsequent circulatory resuscitation such as following aortic cross-clamping or renal transplantation (Weight et al., 1998). This is especially the case in renal transplants from non heartbeating donors, which consequently have an impaired renal function (Castelao et al., 1993; Wijnen et al., 1995). Thus I/R injury is a non-immune factor that is thought to contribute to both short-and long-term dysfunction of the graft. Much of this tubular and glomerular dysfunction has been postulated to occur during the reperfusion period following anoxia, and generation of ROS has been postulated as one of the major factors contributing to this reperfusion injury (Paller et al., 1984; Baker et al., 1995). Singh and Chopra (2004) have shown that during I/R, there is significant increase in renal oxidative stress that leads to both structural as well as functional damage in the rat kidney. Mashiach et al. (1998) reported abnormally low levels of NO in I/R injury. NO seems to have a beneficial role during renal ischemia reperfusion due to its vasodilatory action. In the reoxygenation phase, NO can react with $O_2^-\cdot$, impeding the chain of reaction for additional production of ROS such as ·OH and $H_2O_2$ (Kobayashi et al., 1995). However, the interaction between NO and $O_2^-\cdot$ generate peroxynitrite free radical ('ONOO'), which could cause LPO of cellular membranes (Moncada and Higgs, 1993). If the ratio of $O_2^-\cdot$/NO increases via overproduction of $O_2^-\cdot$ or impairment of NO synthesis, $O_2^-\cdot$ will produce $H_2O_2$ and promote the activation of phospholipase A$_2$, thereby synthesizing proinflammatory lipid mediators such as PAF (platelet activating factors) and LTB$_4$ (Leukotrienes B$_4$), which increases the leukocyte adhesion and oxidative injury caused by tissue I/R (Grisham, 1995). Furthermore, Clancy et al., (1992) found that NO decreases superoxide anion production in neutrophils by inhibiting NADPH oxidase activity.
On the other hand, NO also diminishes the leukocyte adhesion, neutrophil infiltration and the formation of inflammatory mediators during tissue I/R (Kobayashi et al., 1995) and it is a potential vasodilator which has been shown to decrease the renal vascular resistance and improve recovery of renal function after ischemic damage (Shoskes et al., 1997). A basal production of NO is necessary for maintaining an adequate glomerular function, since the inhibition of NO synthesis may increase both efferent glomerular arteriolar resistance and glomerular capillary pressure as well as induce significant changes in renal histology (Waz et al., 1998). Chintala et al. (1993) have demonstrated that NO inhibition may exacerbate renal dysfunction, while NO precursors may improve renal function. Lopez-Neblina et al. (1994) found a protective effect on serum creatinine levels in rats treated with nitroprusside. Recently several studies have demonstrated that administration of exogenous L-arginine has been shown to protect the kidney against toxic or ischemic injury Exogenous L-arginine supplementation for 14 days in a model of ischemic ARF has been shown to have a beneficial effect on GFR, RBF, and led to reduced $O_2^{-}$ production (Lopau et al., 2000; Valdivielso et al., 2000; Schramm et al., 2002).

The influence of NO on lipid peroxidation and oxidative damage depends on the relative concentration of NO and $O_2^{-}$ and organic peroxy radicals (ROO') present in the immediate vicinity. First, when the concentration of NO increases, so does the rate of reaction between NO and oxygen to form oxidant (ONOO'), thus stimulates lipid peroxidation. But, when concentration of NO exceeds the $O_2^{-}$, lipid peroxidation can be inhibited by NO, with a concurrent formation of nitrated lipid radical termination products. NO protects lipids from oxidation by terminating lipid radical mediated chain propagation reactions (i.e. ROO' + NO→ ROONO). NO-mediated inhibition of lipid peroxidation consumes two molecules of NO per ROO'. With oxidizing linoleic acid, NO first reacts with LOO' to form an unstable organic peroxynitrite (LOONO) intermediate, that quickly decomposes to generate an alkoxy radical (RO') and NO$_2$ as a caged radical pair (RO'-NO$_2$). Then a
second molecule of NO can react with RO\textsuperscript{'} to form alkyl nitrite, RNO\textsubscript{2}, thus inhibiting the lipid peroxidation and subsequent oxidative damage.

In this study, renal I/R caused an increase in the renal TBARS levels and depleted the antioxidant enzyme pool, as is evident from the declined levels of reduced glutathione, catalase, superoxide dismutase enzymes. Renal I/R induced oxidative stress was associated with impaired renal function leading to a marked increase in serum creatinine, blood urea nitrogen and a marked fall in the creatinine clearance. There was a significant decrease in the urine and tissue total nitric oxide levels in the I/R treated animals. Moreover, the kidney of rats that underwent I/R (+ contralateral nephrectomy) showed characteristic morphological changes such as tubular cell swelling, cellular vacuolization, pyknotic nuclei, medullary congestion and moderate to severe necrosis.

Pretreatment with L-arginine prevented the renal I/R induced lipid peroxidation and restored the severe depletion of antioxidant enzyme pool in the renal I/R treated rats. Furthermore, the nitric oxide levels and renal functional & morphological damage was significantly improved. In contrast to several recent studies demonstrating the protection against toxic or ischemic renal injury by exogenous L-arginine, we have observed protection at a much lower dose. However, pretreatment with L-NAME blocked the protective effect of L-arginine, indicating that NO has a beneficial role in the normal functioning of the kidney. Furthermore, pre-treatment of animals with L-NIO, significantly abolished the protective effect observed with L-arginine, whereas aminoguanidine did not have any effect suggesting that eNOS has an important role to play in preserving the renal function in I/R injury. Moreover L-NAME or L-NIO per se accentuated the injury in animals subjected to renal I/R.

Molsidomine (MOL) is a prodrug, and a potent vasodilator, has been used widely as an antianginal agent. In the liver, it decarboxylates enzymatically to form SIN-1 (Kukovetz and Holzmann, 1986). Molsidomine, a NO-yielding compound relaxes vascular smooth muscle by stimulating guanylate cyclase and thus by increasing cyclic GMP (cGMP) levels (Gruetter \textit{et al.}, 1979). Previous studies have shown a protective effect of molsidomine in experimental uremia (Garcia-Criado \textit{et al.}, 1998; Benigni \textit{et al.}, 1999). In the present study, pretreatment with molsidomine prevented the renal I/R induced lipid peroxidation and protected the severe depletion of antioxidant enzyme pool in the renal
I/R-induced rats. Furthermore, the nitric oxide levels and renal functional as well as morphological damage was significantly improved. Pretreatment of animals with L-NAME as well as L-NIO, did not block the protective effect of molsidomine clearly indicating that NOS is not involved in the protection afforded by this compound.

Nebivolol, a new selective β1-adrenergic blocking agent, is endowed with peripheral vasodilating properties mediated by the modulation of the endogenous production of nitric oxide (NO), as well as additional antioxidative effects (Mangrella et al., 1998). In particular, it has been demonstrated that nebivolol vasodilates human forearm vasculature via the L-arginine/NO pathway (Mangrella et al., 1998). Similar to molsidomine, nebivolol prevented the I/R renal dysfunction, significantly increased the urine and tissue total NO levels, protected the severe depletion of antioxidant enzyme pool, reduced the elevated TBARS levels and preserved the normal morphology of the kidney. Pretreatment of animals with L-NAME as well as L-NIO could not abolish the beneficial effect of nebivolol clearly indicating that NOS is not involved in the protection afforded by this compound as it directly donates NO.

Resveratrol is a naturally occurring polyphenol which can scavenge peroxyl radicals, is not a very potent antioxidant in vitro (Maulik et al., 1995). However, it functions as a potent antioxidant in vivo. Previous studies have shown that resveratrol prevented lipid peroxidation (Frankel et al., 1993) and lipid peroxidation-induced cell death (Sun et al., 1997). Bertelli et al. (1996) has also demonstrated reduction in MDA levels in the ischemic reperfused myocardium of resveratrol-treated rats. 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 acts 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. Pretreatment with resveratrol attenuated the acute renal dysfunction, increased the tissue and urine total nitric oxide levels, significantly improved the levels of renal antioxidant
enzymes and preserved the normal morphology of kidney. However, co-treatment of resveratrol with L-NAME as well as L-NIO attenuated the protection mediated by resveratrol indicating that resveratrol exerts its protective effect through release of nitric oxide, whereas, pretreatment with aminoguanidine did not affect RVT's protection.

HMG CoA reductase inhibitors have been shown to have direct renal hemodynamic effects independent of their lipid-lowering properties (Auer et al., 2002). These effects are thought to be mediated by the ability of the statins to vasodilate blood vessels via NO (Davignon and Laaksonen, 1999; Laufs et al., 2002). Unlike lipophilic HMG-CoA reductase inhibitors, pravastatin is hydrophilic, and its metabolism is independent of that of cytochrome P-450 3A4 in the liver (Williams and Feely, 2002). As a result, pravastatin may have fewer toxic effects than other statins. Experimental work has demonstrated that these agents exert beneficial effects on endothelial cell, macrophage, platelet, and smooth muscle cell function (Tannous et al., 1999; Laufs et al., 1999; Pruefer et al., 1999). Moreover, in vivo models have confirmed putative improvements in flow-mediated dilatation with increased bioavailability of NO (O'Driscoll et al., 1997; Endres et al., 1998). Stowe et al. (1996) have shown that three weeks of lovastatin treatment dilates preglomerular arterioles and increases renal blood flow in normal rats. Laufs et al., (1998) has reported that HMG CoA reductase inhibitors can enhance endothelial NO production by directly upregulating eNOS expression and activity. In this study we tested the hypothesis whether an HMG CoA reductase inhibitor, pravastatin, attenuates renal injury in I/R model of renal injury. Animals in the I/R group exhibited marked impairment of renal function, reduction of total nitric oxide levels, oxidative stress and significant alterations in the histopathologic features. Pretreatment of animals with pravastatin significantly attenuated the renal dysfunction, increased the urine and tissue total NO levels, reduced the oxidative stress and preserved the normal morphology of the kidney. L-NAME as well as L-NIO abolished the protective effect of pravastatin alone in I/R treated animals.

NO has a biphasic role in I/R injury involving eNOS being upregulated in the early phases after I/R injury and iNOS upregulated in the later phase (Packer and Murphy, 1995). Since we evaluated effect of pravastatin in the early phase i.e. 24 hr after induction of ischemia, further stimulation of eNOS must have favoured enhanced NO production
maintaining renal blood flow during the reperfusion period, thereby preserving urine adequate glomerular filtration. This preservation of blood flow is critical to ensure function of the sensitive proximal tubular cells. In our experimental model, the beneficial effects on renal vasculature occurred independent of any change in serum lipid levels. The inhibition of oxidative stress by pravastatin may also contribute significantly to its protective effect. Statins are able to prevent lipid oxidation (Giroux et al., 1993; Aviram et al., 1998; Suzumura et al., 1999) and they decrease AT-1 receptor dependent ROS generation (Wassmann et al., 2001). Statins also inhibit NAD(P)H oxidase expression (Wassmann et al., 2001), activity (Christ et al., 2002), and assembly by preventing isoprenylation of the small p21 rac protein (Wagner et al., 2000). All of these effects will increase NO bioavailability by preventing its reaction with O$_2^{-}$ but there is an evidence that statins also directly increase eNOS activity. These data suggest that HMG CoA reductase inhibitors may ameliorate renal impairment and allow earlier recovery from IR injury. This may have implications for modulating renal function in the clinical setting.

Recent findings have shown that endothelin mRNA is markedly enhanced in the postischemic kidney (Firth and Ratcliffe, 1992) and endothelin receptor antagonists prevents postischemic renal damage (Chan et al., 1994). It has also been stated that an inhibitor of NOS exerts an increased release of endothelins from cultured endothelial cells, thereby suggesting a role for endogenous NO as an inhibitory modulation on endothelin production (Boulanger and Luscher, 1990). Thus direct NO donors as well as eNOS upregulators might be attenuating endothelin production in the ischemic kidney and thus improve ischemic renal failure.

With the data in hand, it can be postulated that decreased formation and/or increased degradation of endogenous NO may occur during ischemia-reperfusion. The pharmacological modulation of renal I/R injury with the agents, that can either release NO directly or can upregulate the eNOS enzyme, may find application in the prevention and treatment of renal I/R exposing procedures in clinics.