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# Involvement of the endothelin and nitric oxide systems in the pathogenesis of renal ischemic damage in an experimental diabetic model

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ABSTRACT

*Aims:* Ischemic acute kidney injury (iAKI) in experimental diabetes mellitus (DM) is associated with a rapid kidney dysfunction more than in non-diabetic rats. We hypothesize that this vulnerability is due to excessive endothelin-1 (ET-1) expression along with dysregulation of nitric oxide synthase (NOS) isoforms. The aim of the present study was to assess the impact of ischemia on renal function in diabetic rats as compared with non-diabetic rats, and to investigate the involvement of ET-1 and NO systems in the susceptibility of diabetic kidney to ischemic damage.

*Main methods:* DM was induced by Streptozotocin. iAKI was induced by clamping of left renal artery for 30 min. Right intact kidney served as control. 48 h following ischemia, clearance protocols were applied to assess glomerular filtration rate (GFR), urinary flow (V) and sodium excretion ($U_{Na}V$) in both kidneys. The renal effects of ABT-627, $ET_A$ antagonist; A192621.1, $ET_B$ antagonist; L-NAME, NOS non-selective inhibitor; 1400 W, inducible NOS (iNOS) inhibitor; and NPLA, neuronal NOS (nNOS) inhibitor, were assessed following ischemic renal injury in diabetic rats.

*Key findings:* Induction of iAKI in diabetic and non-diabetic rats caused significant reductions in GFR, V, and $U_{Na}V$, which were greater in diabetic than non-diabetic rats. While, treatment with ABT-627 decreased V and $U_{Na}V$, and increased GFR, A192621.1 decreased all these parameters. L-NAME, 1400 W, and NPLA improved GFR in the ischemic diabetic kidney.

*Significance:* Excessive vasoconstrictive effects of ET-1 via $ET_A$ and upregulation of iNOS, are partly responsible for the impaired recovery of renal function following ischemia in diabetic rats.



## Introduction

Endothelin-1 (ET-1) is produced by most renal cell types, including glomerular endothelial cells (ECs), mesangial cells, and epithelial cells. The highest concentrations of ET-1 are detected in the inner renal medulla (Kitamura et al, 1989; Markewitz and Kohan, 1995;Abassi et al., 2001; Kalani, 2008; Kon and Badr, 1991; Naicker and Bhoola, 2001). ET-1 affects three aspects of renal function: 1) hemodynamics of the kidney, 2) tubular handling of electrolytes and water, and 3) proliferation and mitogenesis of certain renal cell types such as mesangium and vascular smooth muscle (Kohan, 1997; Abassi et al., 2001). These actions are mediated by two receptor subtypes, $ET_A$ and $ET_B$. $ET_A$ receptor is localized in various nephron segments including glomerulus, vasa recta bundle, and arcuate artery (Terada et al, 1992). In contrast, $ET_B$ receptors are abundantly expressed in glomerular endothelial cells, vasa recta bundles, epithelial cells of thin segments of Henle's loop and collecting duct (Terada et al, 1992; Naicker and Bhoola, 2001). Systemic infusion of ET-1 markedly decreases renal blood flow (RBF) and glomerular filtration rate (GFR) due to a profound and sustained increase in renal vascular resistance (Kohan et al, 2011). The sustained renal vasoconstriction is often preceded by a transient vasodilatory response possibly due to $ET_B$ receptor mediated release of nitric oxide (NO) (Abassi et al., 2001; Kalani, 2008; Kon and Badr, 1991; Naicker and Bhoola, 2001; Wilhelm et al., 1999).

Elevated levels of ET-1 are found in patients with DM, where it contributes to the reduced insulin sensitivity and development of metabolic syndrome. Enhanced ET-1 production by vascular endothelium exists as an early phenomenon rather than a result of advanced stage of DM

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(Anfossi et al., 2007). Yamauchi et al. (1990) has reported that aortic ECs exposed to increased levels of glucose enhanced the production of ET-1. Similar results were reported by Park et al. (2000) in both retinal capillary ECs. In streptozotocin diabetes models, we have showed that kidney medullary endothelin converting enzyme-1 protein and gene expression were gradually increased 7–14 days following the induction of DM (Khamaisi et al., 2008). It is widely accepted that increased production of ET-1 plays a mandatory role in the pathogenesis of endothelial dysfunction and renal injury characterizing patients with DM (Brownlee, 2005; Goligorsky et al., 2001; Kalani, 2008; Neuhofer and Pittrow, 2006). Additionally, glucose, advanced glycated end products and induction of plasminogen activator inhibitor-1, have the ability to scavenge NO in the initiation phase of endothelial dysfunction, resulting in decreased expression of endothelial nitric oxide synthase (eNOS) and reduced generation of NO (Verbeke et al., 2000; Goligorsky et al., 2001).

Acute kidney injury (AKI) is a term describing decrease in renal function with rapid deterioration of glomerular filtration rate (GFR), resulting in retention of urea, creatinine and other waste products (Schrier et al, 2004; Lameire et al., 2005; Lameire and Vanholder, 2004; Van Biesen et al., 2006). The ischemic renal damage is largely attributed to oxidative stress, inflammation and endothelial dysfunction. The latter is characterized by an imbalance between the release of ET-1 and NO (Goligorsky, 2005; Lameire and Vanholder, 2004; Bonventre and Yang, 2011). It has been shown that ET-1 concentrations are increased during the initial 24 h following reperfusion in experimental ischemic AKI (iAKI) (Wilhelm et al., 1999). Additionally, it has been reported that patients with AKI have elevated plasma concentrations of ET-1 (Naicker and Bhoola, 2001; Neuhofer and Pittrow, 2006; Wilhelm et al., 1999).

Diabetes has been recognized as a risk factor for the development of AKI in a variety of clinical settings, such as radio-contrast nephropathy or following cardiopulmonary bypass operations. The increased susceptibility of the kidney to AKI has been previously reported in patients with diabetes mellitus and experimental models of this disease (Bellomo et al., 2004; Shi et al., 2007; Woodrow et al., 1994). However, the mechanisms underlying the enhanced vulnerability of the kidney to ischemia in diabetes have not been clearly established. We assume that DM could augment renal damage induced by ischemia-reperfusion in part by aggravation of endothelial dysfunction. Therefore, in the present study we investigated the contribution of ET-1 and NO systems to the increased susceptibility of the diabetic kidney to ischemic insult, by using selective blockers of ET-1 receptors, and NOS inhibitors.

## Materials and methods

Studies were conducted on male Sprague Dawley rats, weighing ~300 g. All experiments were performed according to the guidelines of the Committee for the Supervision of Animal Experiments, Technion, IIT.

### *Animal model of diabetes*

The animals were rendered diabetic by a single intraperitoneal injection of Streptozotocin (STZ, 65 mg/kg body weight; obtained from Sigma–Aldrich Co., USA) dissolved in sodium citrate buffer (pH 4.5). Diabetes was confirmed 5 days later by blood glucose sampling from the tail tip by glucometer (Ascensia Elite XL, Milano, Italy).

### *Ischemia reperfusion injury*

Five days after STZ injection, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and placed on a thermoregulated table. Unilateral renal ischemia was produced by clamping the left renal artery for 30 min. To minimize dehydration of the exposed tissues, the abdominal area was covered with saline-soaked gauze. For reperfusion, the clamp was removed and blood flow to the kidney was reestablished with visual verification of blood supply. Subsequently the abdomen was sutured, and the animals were returned to their cages for 48 h.

### *Clearance studies*

Seven groups of rats were studied: 1) Control Rats, normoglycemic ($n=7$); 2) Diabetic rats ($n=8$); 3) Diabetic rats ± $ET_A$ receptor antagonist, pretreated with the selective $ET_A$ receptor antagonist ABT-627 (10 mg/kg P.O.) for 7 days immediately following STZ injection ($n=9$); 4) Diabetic rats ± $ET_B$ receptor antagonist, pretreated with the selective $ET_B$ receptor antagonist A192621.1 (10 mg/kg/day P.O.) for 7 days immediately following STZ injection ($n=7$); 5) Diabetic rats ± L-NAME, pretreated with the nonspecific NOS inhibitor L-NAME (15 mg/L in drinking water, Sigma–Aldrich) for 7 days immediately following STZ injection ($n=9$); 6) Diabetic rats ± 1400 W, pretreated with the iNOS inhibitor 1400 W (3 mg/kg S.C., Alexis Biochemicals) for 3 days starting 2 h before ischemia ($n=9$); 7) Diabetic rats ± NPLA, pretreated with the nNOS specific inhibitor $N^G$-propyl-L-arginine NPLA (3 mg/kg I.V. boluses through the tail vein, Alexis Biochemicals) for 3 days starting 2 h before ischemia ($n=6$).

On the day of the experiments, animals were anesthetized with Inactin (100 mg/kg, i.p.) and prepared for clearance studies (Fuhr et al., 1955). After tracheotomy, polyethylene tubes ($PE_{50}$) were inserted into the carotid artery and jugular vein for blood pressure monitoring and infusion of solutions, respectively. For urine collection, catheters were inserted through a supravesical incision into both the left ureter ($PE_{10}$) and the bladder ($PE_{50}$), to monitor the ischemic kidney and the control kidney, respectively. A solution of 2% inulin in 0.9% saline was continuously infused throughout the experiment. Mean arterial pressure (MAP) was continuously monitored with a pressure transducer (model 1050.1, UFI, Morro Bay, CA) connected to the carotid artery. After a 60 min. equilibration period, urine was collected separately from each kidney in timed periods and a reference blood sample was drawn between every two urine collections.

### *Chemical analyses*

Urine volume was determined gravimetrically. Sodium concentrations of in plasma and urine were determined by a flame photometer (model IL 943, Instrumentation Laboratories, Italy). Inulin concentrations in plasma and urine were measured by colorimetric methods. Glomerular filtration rate (GFR) was equated with the clearance of inulin ($C_{in} = U_{in} * V/P_{in}$).

### *Western blot*

Kidneys were sectioned and the medulla and cortex were separated on ice. Tissues were homogenized within Ripa (Radio-Immunoprecipitation Assay) buffer containing protease and phosphatase inhibitors. 100 μg of protein was treated with sample buffer then electrophoresed on 7.5% polyacrylamide Tris-glycine gels and transferred electrophoretically to a nitrocellulose membrane. The membranes were blocked in 5% milk and incubated with 300-fold-dilutede eNOS, iNOS or nNOS polyclonal antibodies (Santa Cruz, sc-654, sc-651, and sc-648, respectively) overnight at a 4 °C, followed by washing the membranes three times with T-TBS. Then, the blots were incubated with 2000-fold-diluted peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma Chem1icals) for 60 min. Immunoreactive bands were visualized by Enhanced chemiluminescence detection system (Amersham). The density of autoradiographic signals were quantitated using Toolbar (Nonlinear Dynamics Ltd.,Newcastleupon Tyne, UK). Quantitated bands were normalized to β-actin densities.

*Morphologic evaluation*

Hematoxylin and Eosin (H & E) staining was performed in paraffin embedded longitudinal 5-μm kidney's sections. Morphological changes were noted in the kidneys subjected to ischemic and non-ischemic kidneys, and determined by a semi-quantitative analysis using a 0–4 score. The 4th grade reflecting en block infarction of the entire region examined, casts detected in all tubuli, extensive congestion and extravasation of red cells into the parenchyma, and extensive polymorphonuclear infiltration, respectively, as described by Kiris et al (2008).

*Statistical analysis*

Paired *t*-test was applied for comparisons between the ischemic kidney and the control kidney from the same experiment. One-way ANOVA, followed with Dunnett's test as post-ANOVA evaluation, was used for group comparisons (where the diabetic group serving as control group for the statistical tests). Data are expressed as mean ± SEM. *P*-value <0.05 was considered statistically significant.

## Results

*Effects of ischemia-reperfusion injury on renal function in diabetic rats*

Diabetic rats exhibited significant weight loss and dramatic increase in blood glucose levels (Table 1). Ischemia decreased GFR (Fig. 1A), urine flow rate (Table 1), and sodium excretion ($U_{Na}V$) (Fig. 1B) in both non-diabetic and diabetic rats. The decline in GFR, V, and $U_{Na}V$ following ischemia was more severe in diabetic rats compared to non-diabetic rats. These findings clearly demonstrate that the diabetic kidney is more susceptible to ischemic injury than the non-diabetic kidney.

*Effects of ischemia-reperfusion injury on renal morphology in diabetic rats*

Ischemia-reperfusion injury in normoglycemic rats was associated with necrosis and casts, mainly in the outer stripe of the renal outer medulla, and this effect was more profound in diabetic rats. A broader pattern of injury in the diabetic ischemic kidney was noted in the inner stripe of the outer medulla, including also congestion and inflammation, with all pathological parameters higher than 2.5 in the morphological score [0–4] (Fig. 2). It should be emphasized that diabetes alone had no histological effects on the non-ischemic kidney (data not shown).

*Effects of ABT-627, a selective ETA antagonist, on renal function after ischemic injury in diabetic rats*

ABT-627decreased MAP in diabetic rats from 122.1 ± 1.4 mmHg to 101.2 ± 2.3 mmHg ($P<0.001$), and decreased V and $U_{Na}V$ in the ischemic and normal kidney in diabetic rats. Blockade of $ET_A$ receptor did not affect GFR of the normal kidney in diabetic rats. In contrast, ABT-627 tended to increase GFR of the ischemic kidney by 2-fold in diabetic animals (from 26.35 ± 11.51 to 57.99 ± 35.47 μl/min in diabetic non-ischemic and ischemic kidney, respectively, (P = NS)) (Fig. 1A and B).

*Effects of A192621.1, a selective ETB antagonist, on renal function after ischemic injury in diabetic rats*

A192621.1 aggravated the loss of body weight (B.W.) but significantly reduced blood glucose levels (Table 1). At the renal level, $ET_B$ antagonist remarkably declined V in the ischemic and non-ischemic kidney. $ET_B$ blockade did not have any impact on $U_{Na}V$ in the normal kidney but decreased significantly $U_{Na}V$ in the ischemic kidney of diabetic rats. Noteworthy, the drug caused a remarkable decrease in GFR both in non-ischemic and ischemic diabetic kidneys (Fig. 1A and B).

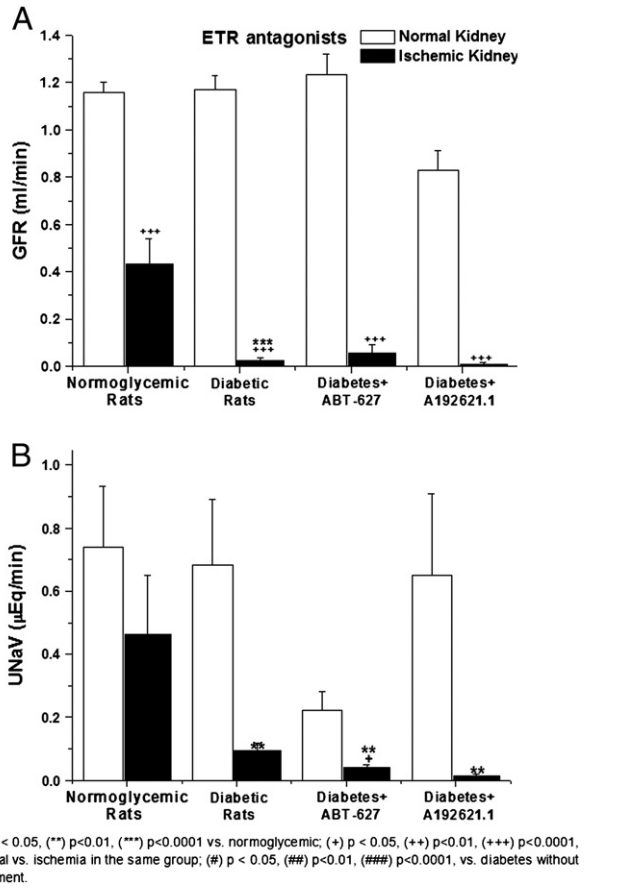
*Effects of ET-1 receptor blockers on renal morphology after ischemic injury in diabetic rats*

ABT-627 decreased necrosis, casts and inflammation in the outer stripe of the renal ischemic diabetic outer medulla. Likewise, A192621.1 decreased necrosis and casts but increased congestion and inflammation in the outer stripe of the renal ischemic diabetic outer medulla (Fig. 2A). Selective antagonists of ET-1 receptors had no effect on the injury in the inner stripe of the outer medulla, where the main injury occurred (Fig. 2B).

**Table 1**
Effects of the various treatments on % in B.W & K.W, blood glucose, mean arterial pressure (MAP), and urinary flow rate (V) of the experimental groups.

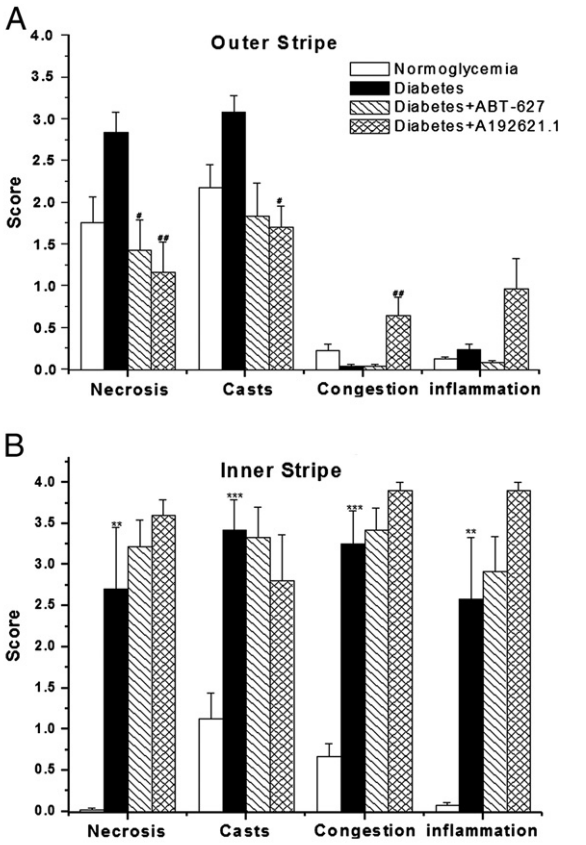
Effect of various treatments on B.W & K.W expressed as (% changed in B.W and K.W), blood glucose levels 5 days after inducing hyperglycemia, mean arterial pressure (MAP) in the test day. Urine flow rate (V) from each kidney along the clearance experiments, N; Normal Kidney I; Ischemic Kidney ($^{*}$) $P<0.05$, ($^{**}$) $P<0.01$, ($^{***}$) $P<0.0001$ vs. nomoglycemic; (+) $P<0.05$ , (++)$P<0.01$, (+++) $P<0.0001$, normal vs. ischemia in the same group; (#) $P<0.05$, (##) $P<0.01$, (###) $P<0.0001$ vs. untreated diabetes.

| Experimental Group | B.W % change from baseline | K.W | Blood Glucose (mg %) | MAP (mmHg) | V (ml.min) | |
|---|---|---|---|---|---|---|
| **Normoglycemia** ($n=7$) | +2.37 ± 0.88 | +18.8 ± 3.8 | 112.28 ± 4.67 | 132.1 ± 4.2 | N | 7.22 ± 1.24 |
| | | | | | I | 5.5 ± 1.53 |
| **Diabetes** ($n=8$) | -13.9 ± 1.08$^{***}$ | +31.3 ± 5.2 | 503.1 ± 14.92$^{***}$ | 122.1 ± 1.4 | N | 19.6 ± 1.62$^{**}$ |
| | | | | | I | **1.48 ± 0.41$^{***}$** |
| **Diabetes + $ET_A$ antagonist** ($n=9$) | -11.33 ± 1.15 | +38.1 ± 5.7 | 446.5 ± 16.32$^{***}$ | 101.2 ± 2.3$^{***,\#\#\#}$ | N | 14.56 ± 1.1 |
| | | | | | I | 1.3 ± 0.52$^{***}$ |
| **Diabetes + $ET_B$** antagonist ($n=7$) | -16.7 ± 1.46 | +30.4 ± 7.2 | 409 ± 42.49$^{***,\#}$ | 117.3 ± 4.1$^{**}$ | N | 15.56 ± 2.9$^{*,\#}$ |
| | | | | | I | 0.29 ± 0.09$^{**}$ |
| **Diabetes + L-NAME** ($n=9$) | -15.96 ± 1.2 | +14.5 ± 2.6$^{**}$ | 458.92 ± 16.9$^{***}$ | 148.8 ± 6.4$^{\#\#\#}$ | N | 11.35 ± 1.2 |
| | | | | | I | 2.58 ± 0.59$^{***}$ |
| **Diabetes 1400W** ($n=9$) | -9.97 ± 0.86$^{*}$ | +29.3 ± 7.0 | 461 ± 18.6$^{***}$ | 148.8 ± 6.4$^{\#\#\#}$ | N | 12.96 ± 1.5 |
| | | | | | I | 3.72 ± 2.3$^{*}$ |
| **Diabetes + NPLA** ($n=6$) | -7.04 ± 1.12$^{***}$ | +24.5 ± 5.5 | 395 ± 20.4$^{***,\#\#}$ | 107 ± 3.8$^{*}$ | N | 24.49 ± 7.2 |
| | | | | | I | 11.64 ± 5.1$^{\#}$ |




(*) p < 0.05, (**) p<0.01, (***) p<0.0001 vs. normoglycemic; (+) p < 0.05, (++) p<0.01, (+++) p<0.0001, normal vs. ischemia in the same group; (#) p < 0.05, (##) p<0.01, (###) p<0.0001, vs. diabetes without treatment.

**Fig. 1. Effects of selective ET-1 receptors antagonists on GFR in diabetic rats subjected to ischemic renal injury.** Effect of selective $ET_A$ antagonist (ABT-627) and selective $ET_B$ antagonist (A192621.1) on GFR of ischemic and non-ischemic kidneys in diabetic rats (A). Urinary sodium excretion (B).




(*) p < 0.05, (**) p<0.01, (***) p<0.0001 vs. normoglycemic ischemic kidney; (#) p < 0.05, (##) p<0.01, (###) p<0.0001, vs. untreated diabetic ischemic kidney.

**Fig. 2. Effects of selective ET-1 receptors antagonists on morphological changes in the renal ischemic outer medulla.** Effect of selective ETA antagonist (ABT-627) and selective ETB antagonist (A192621.1) on morphological changes (necrosis, casts, congestion and inflammation) in the renal ischemic outer medulla.

*Effect of various NOS inhibitors on renal function after ischemic injury in diabetic rats*

iNOS expression increased 6 h following ischemia by 2 fold in normoglycemic and diabetic renal cortex. While iNOS upregulation persisted in normoglycemic kidneys after 18 h and 48 h following ischemic injury, it decreased in the diabetic kidney. The enhanced expression of iNOS was even more profound in the renal medulla, where it increased by 1.5 fold in diabetic non ischemic kidney, and 2 fold, and 3 fold in normoglycemic and diabetic kidney 6 h following ischemia. iNOS levels decreased after 18 h of AKI induction in all groups.

Cortical eNOS decreased by ~30%, 32% and 63% in diabetic non ischemic, ischemic normoglycemic, and ischemic diabetic kidneys 48 h after reperfusion, respectively. Similarly, diabetic medulla showed a 20% decrease in eNOS levels as compared to normoglycemic medulla at the same time point. Ischemic injury decreased eNOS level in normoglycemic and diabetic medulla by 66% and 54%, 6 h following ischemia, respectively. These decreases were even more profound 48 h after AKI, where eNOS levels declined by 70% in normoglycemic ischemic medulla and by 84% in diabetic ischemic medulla. In summary, the ischemic diabetic medulla was most affected, where iNOS increased in the early stages of AKI, and eNOS decreased through 48 h of AKI to 20% of the baseline levels.

Non-specific inhibition of NOS by L-NAME in diabetic rats significantly increased MAP and improved kidney swelling (as expressed by kidney weight (K.W)). L-NAME caused a decline in V and $U_{Na}V$ in normal kidney but improved V in ischemic kidney as compared with untreated diabetic rats. GFR significantly decreased in normal diabetic kidney after the administration of L-NAME. On the other hand, in the ischemic kidney, L-NAME increased GFR by 3 fold. Although it did not reach statistical significance, GFR ratio, defined as % change from non-ischemic kidney, was attenuated from a decline of 97.66 ± 1.14% in diabetic rats without treatment to 86.3 ± 4.1% decline in L-NAME treated rats (Fig. 3A and B).

Treatment with 1400 W, a specific inhibitor of iNOS, exerted a beneficial effect on V and UNaV in the ischemic kidney of the diabetic rats. Moreover, 1400 W improved GFR in ischemic kidney by almost 4 fold. Actually, percent decline in GFR improved from 97.66 ± 1.14% in untreated diabetic rats to 87.8 ± 3.96% in 1400 W treated animals (Fig. 3A and B).

Treatment with NPLA, nNOS inhibitor, attenuated the reduction in B.W, showed improvement in kidney weight and decreased blood glucose levels in diabetic rats during the experiment duration (Table 1). NPLA exerted a stimulatory effect on V and $U_{Na}V$ in non-ischemic and ischemic kidneys of treated rats as compared with diabetic non-treated rats (Fig. 3B). NPLA enhanced GFR in ischemic kidney by ~6 fold ($P<0.005$). This beneficial effect is demonstrated by a 59.4 ± 19% reduction in GFR (in the ischemic kidney relative to the normal kidney in rats treated with NPLA, compared with a GFR decline of 97.66 ± 1.14% in untreated diabetic rats (Fig. 3A).

*Effects of NOS blockers on renal morphology after ischemic injury in diabetic rats*

L-NAME decreased necrosis, and casts in both outer and inner stripe of the renal ischemic diabetic outer medulla. In addition, it decreased congestion and inflammation only in the inner stripe of the outer medulla. 1400 W and NPLA decreased necrosis and casts in

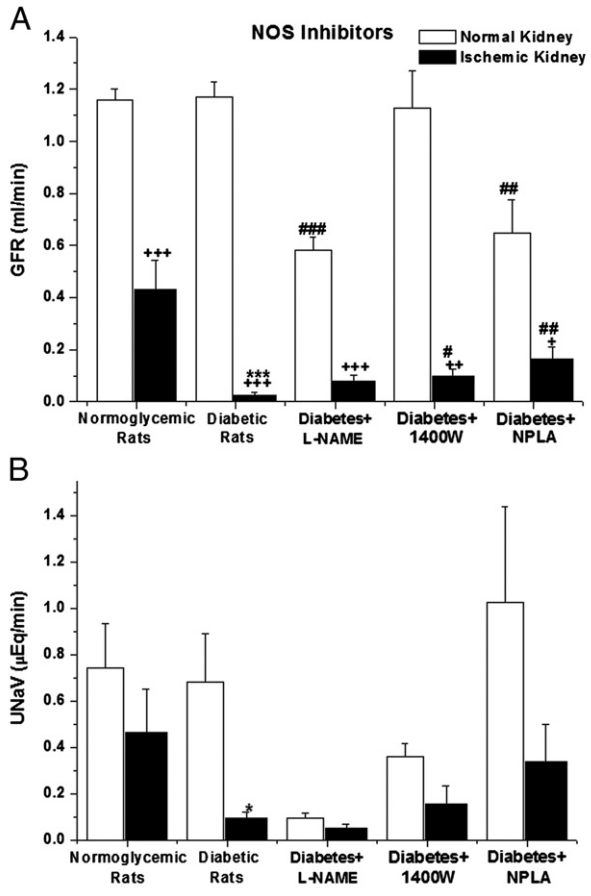



**Fig. 3. Effects of selective and non-selective NOS inhibitors on GFR in diabetic rats subjected to ischemic renal injury.** Effect of nonspecific NOS inhibitor (L-NAME), iNOS inhibitor (1400 W) and nNOS inhibitor (NPLA) on GFR of ischemic and non-ischemic kidneys in diabetic rats (A). Urinary sodium excretion (B).

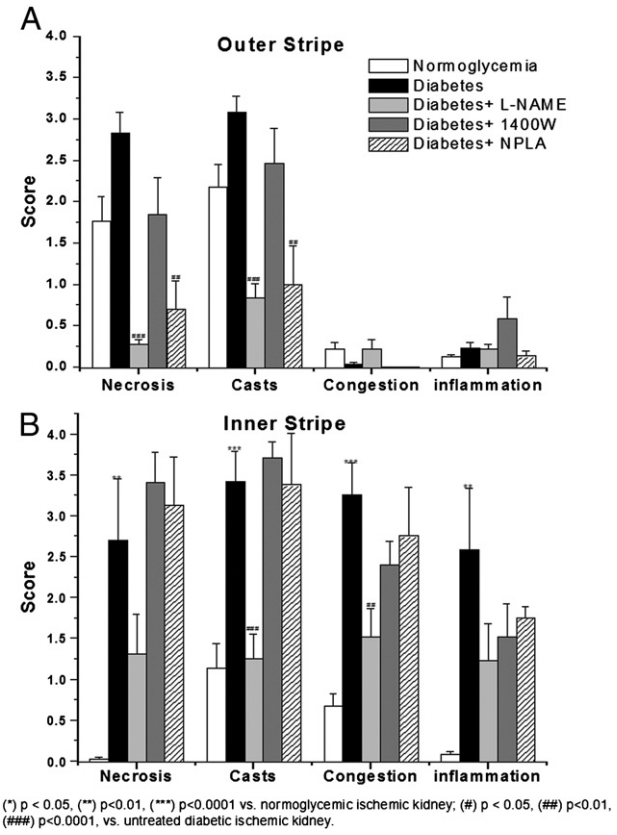



**Fig. 4. Effects of selective and non-selective NOS inhibitors on morphological changes in the renal ischemic outer medulla.** Effect of nonspecific NOS inhibitor (L-NAME), iNOS inhibitor (1400 W) and nNOS inhibitor (NPLA) on morphological changes (necrosis, casts, congestion and inflammation) in the renal ischemic outer medulla.

the outer stripe but not in the inner stipe of the renal ischemic diabetic outer medulla. Although all NOS inhibitors decreased cast and necrosis in the outer medullary stripe of the ischemic kidney, the effects of 1400 W did not reach statistical significance (Fig. 4A and B).

## Discussion

The findings of our study provides new insights into the mechanisms underlying the vulnerability of the diabetic kidney to ischemic damage, specifically on the role of ET-1 and NO systems in the pathogenesis of renal dysfunction in diabetic rats with and without ischemia. These findings may prove relevant for development of novel therapeutic approaches that rely on these mechanisms.

Reperfusion of the ischemic kidney may lead to massive cellular damage apart from the injury caused directly due to the occlusion itself. Previously, it has been shown that iAKI in rats increases ET-1 levels (Naicker and Bhoola, 2001; Neuhofer and Pittrow, 2006), iNOS expression (Lameire and Vanholder, 2004), and decreases eNOS abundance in the renal cortex and medulla. Therefore, it is reasonable to assume that the diabetic kidney is more susceptible to ischemic reperfusion injury than the non-diabetic kidney and imbalance between these NO and ET-1 systems may contribute to this phenomenon. Based on this assumption, we examined the effect of iAKI on renal function in diabetic rats and assessed the alterations in the expression of the various NOS isoforms and ET-1 system in the ischemic kidney as compared with the relevant controls.

Our findings demonstrate that ischemic injury in either normoglycemic or diabetic rats induced necrosis and cast formation mainly in the outer stripe of the outer medulla. However, ischemia in diabetic rats caused a more excessive increase in necrosis and casts in both the inner and outer stripe of the medulla, indicating that diabetic kidney is more susceptible than normoglycemic to morphological changes when subjected to AKI. Along these histological changes, ischemic kidney in nomoglycemic rats exhibited a 60% and 38% decreases in GFR and $U_{Na}V$ respectively as compared with basal values. These deleterious renal hypoperfusion and impaired excretory function of AKI are comparable to those reported by other groups who used a similar model of the disease. For instance, Lin et al. (1988) showed that rats that were subjected to 30 min of renal artery occlusion displayed after 5 days 50% reductions in GFR, V and $U_{Na}V$. Interestingly, when renal ischemia was induced in diabetic animals it resulted in more profound declines in GFR (by 98%) and inUNaV (by 87%), which were significantly greater than those obtained in ischemic normoglycemic rats. These findings are in line with reports by other groups. For example, Melin et al. (1997) showed that renal ischemia of 30 min in diabetic rats caused a substantial decrease in the clearance of inulin (GFR) to ~20% of that measured in ischemic normoglycemic animals. Similarly, Goor et al. (1996) demonstrated that renal artery clamping for 60 min resulted in a more severe acute renal damage in diabetic animals than that obtained in ischemic non-diabetic group. Specifically, creatinine clearance values in the diabetic rats were half of those measured in ischemic normoglycemic rats. Additionally, Shi et al. (2007) showed that renal ischemia in diabetic db/db mice results in impairment in renal reperfusion to both the cortex and the medulla during the early post-ischemic period, and that diabetic mice subjected to ischemic injury exhibit delayed recovery of

renal regional blood flow. Unfortunately, these authors did not examine the mechanisms responsible for the exaggerated sensitivity of the diabetic kidney to ischemic damage and have not evaluated the effects of potential therapeutic approaches.

In attempt to explain the severe worsening in renal function in ischemic diabetic kidney, we investigated the involvement of key regulators of kidney function, specificallyET-1 and NO, after a renal ischemic insult in diabetic rats.

Our data show that an $ET_A$ antagonist moderately improved GFR in ischemic kidney without affecting GFR in non-ischemic kidney of diabetic rats. This suggests that ET-1, via $ET_A$ receptor, mediates renal vasoconstriction and subsequently impairs GFR (Naicker and Bhoola, 2001). Furthermore, $ET_A$ antagonism decreased necrosis and casts in the outer stripe of the ischemic medulla, an effect that may have beneficial impact on kidney function. Unexpectedly, $ET_A$ antagonist worsened $U_{Na}V$ in both ischemic and non-ischemic kidneys. This effect is at odds with the stimulatory effects of $ET_B$ on sodium excretion (Naicker and Bhoola, 2001), but it may be a result of the hypotensive effects of $ET_A$ antagonism. Alternatively, ABT-627 may bind non-selectively to ETB during AKI, although we have no data to support this assumption. As expected, the $ET_B$ antagonist decreased GFR, V and $U_{Na}V$ in non-ischemic, and to a greater extent in ischemic kidneys of diabetic animals. These effects comply with the physiological roles of the $ET_B$ receptor in the kidney, where it promotes natriuresis and diuresis via NO and $PGI_2$ production, which also known to induce vasodilation (Kohan et al, 2011; Naicker and Bhoola, 2001). In line these findings, Pfab et al. (2006) reported that $ET_B$ deficiency in diabetic rats causes progressive renal failure, severe hypertension, albuminuria, and a mild reduction of creatinine clearance (Pfab et al., 2006).

Nonspecific inhibition of the three isoforms of NOS by L-NAME had a mild beneficial effect on GFR in the ischemic diabetic kidney, but not in the non-ischemic one. Additionally, L-NAME improved urine flow rate in the ischemic kidney but worsened it in the non-ischemic kidney. These effects may highlight the importance of the balance between the three NOS isoforms in the regulation of renal hemodynamics and excretory function. Accordingly, the deleterious effects of L-NAME on kidney function in the non-ischemic diabetic kidney as compared with its beneficial effects on the ischemic diabetic kidney fit our observation that renal iNOS is upregulated more intensively in the ischemic diabetic kidney as compared to the non-ischemic one. These beneficial effects were associated with remarkable morphological changes, where inhibition of NOS isoforms by L-NAME significantly decreased casts and necrosis in the outer and inner stripe of the ischemic medulla, in combination with decline in congestion and inflammation in the inner stripe. Although L-NAME inhibits all NOS isoforms, it is appealing to assume that these encouraging effects of L-NAME are due to its ability to inhibit iNOS, which is known to play critical role in inflammation and oxidative stress.

NPLA, a specific inhibitor of nNOS, exerted positive effects on the ischemic kidney's GFR, V and $U_{Na}V$, whereas in non-ischemic kidney it decreased GFR. In addition, NPLA derived morphological enhancement by decreasing casts and necrosis in the outer stipe of the outer ischemic medulla. Previous studies have demonstrated that inhibition of nNOS enhances tubulo-glomerular feedback (TGF) activity. Increased sensitivity of the TGF system reduces GFR, urine flow rate and sodium excretion rate (Blantz et al., 2002). It should be emphasized that these findings are in line with the well-established concept that nNOS plays a beneficial role in maintaining renal hemodynamics and kidney function under non-ischemic conditions (Ollerstam and Persson, 2002; Blantz et al., 2002). However, our finding that NPLA improves kidney function after ischemic insult in diabetic rats, suggest that nNOS may play an adverse role in renal dysfunction under this situation. Support for this notion comes from knock-out studies, where it was reported that knockout of nNOS reduced tissue damage in experimental models of stroke and cerebral ischemia (Vallance and Leiper, 2002).

As expected, 1400 W, a specific iNOS inhibitor, produced beneficial effects on the ischemic diabetic kidney's GFR. In addition, 1400 W increased $U_{Na}V$ and V, and attenuated the diuretic response characterizing hyperglycemia without affecting GFR in non-ischemic diabetic kidney. Thus, iNOS inhibition partly restored the impaired renal function parameters in the ischemic diabetic kidney. These encouraging findings probably are derived from the fact that iNOS is associated with inflammation and ROS production in the ischemic renal cells, and its inhibition ameliorates these adverse responses. However, the mild improvement in kidney function following iNOS inhibitor treatment, suggests that iNOS is only one player among many that contribute to the impaired renal function following ischemic insult. As evidenced, we have shown the involvement of another deleterious factor, namely ET-1, in the pathogenesis of renal damage under this condition.

## Conclusion

In summary, our findings show that ischemia alone in normoglycemic rats results in severe reduction in renal hemodynamic and excretory function, as expressed by remarkable decreases in V, $U_{Na}V$ and GFR. Interestingly, when the same ischemic procedure was applied in diabetic rats, the deleterious effects on renal function were greater than those obtained in normoglycemic animals and the kidney exhibited poor recovery. The exaggerated vulnerability of the diabetic kidney to the ischemic insult could be attributed to activation of the endothelin system. Moreover, upregulation of the harmful NOS isoform, iNOS, in parallel to down regulation of the beneficial isoform eNOS, may contribute to this phenomenon. Yet, additional studies are required to explore this issue.

### Conflict of interest

There is no conflict of interest.

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