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Kakadiya and Shah

KINDEY DAMAGE INDUCED BY RENAL ISCHEMIA/REPERFUSION INJURY IN SPRAGUE DAWLEY DIABETIC RATS

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Summary

Present study was designed to evaluate in kidney damage induced renal Ischemia/reperfusion (I/R) induced renal damage in Sprague dawley diabetic rats. Hyperglycaemia is most probably a contributing factor in the development of ischaemic acute renal failure (ARF) in many patients. Both clinical and experimental data suggest that hyperglycaemia increases the risk of ARF. Type 2 Diabetes was induced in rats by a single intraperitoneal (i.p) injection of Streptozotocin (65 mg/kg, STZ) in overnight fasting rats followed by the i.p administration of Nicotinamide (110 mg/kg, NIC) after 15 minutes. After right nephrectomy, 0.5 % Sodium CMC was administered for 15 days. On the 16th day, ischemia was induced in contra lateral kidney for 45 min, followed by reperfusion for 24 hr. Renal function marker and oxidative parameter were estimated at the end of 24 hr reperfusion. At the end of experimental period the level of malondialdehyde formation/ lipid peroxidation (LPO) in kidney tissue and serum marker Creatinine, Urea and Uric acids were significantly increased. Whereas, the activity of biomarkers of oxidative stress such as reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were found to be decreased significantly compared to control rats. At the end of experimental period the level of nitrite in kidney tissue, serum marker Albumin and Blood urea nitrogen were significantly changed. Light microscopic evaluation of the kidneys of the diabetic rats with I/R only showed tubular cell swelling, interstitial edema, tubular dilatation, and moderate to severe necrosis. In conclusion, Kidney damage induced by renal ischamia/reperfusion injury Sprague dawley diabetic rats. So, it may be this model use in evaluation of some compound on renal damage induced by renal ischamia/reperfusion injury in diabetic rats or other word effect of some compound on renal complication in diabetic rats.

Keywords: Ischemia reperfusion injury; type 2 diabetes; Renal marker, histopathology
Hyperglycaemia is most probably a contributing factor in the development of ischaemic acute renal failure (ARF) in many patients. Both clinical and experimental data suggest that hyperglycaemia increases the risk of ARF (1-3). Hyperglycaemia also worsens the outcome in renal transplantation (4). Conversely, ischemia/reperfusion (I/R) combined with hyperglycaemia could also be important in the development of diabetic nephropathy.

Organ injury as a consequence of ischemia followed by reperfusion is a major clinical problem. Renal ischemia/reperfusion (I/R) injury is the most common cause of acute renal failure as seen after renal transplantation, major abdominal and vascular surgery, coronary bypass surgery, and in trauma and sepsis(5).

In the setting of loss of renal blood flow autoregulation that characterizes the post-ischemic kidney(6), Renal I/R injury is a major cause of acute renal failure(7), which is faced in many clinical situations such as kidney transplantation, partial nephrectomy, renal artery, angioplasty, aortic aneurysm surgery, and elective urological operations. In these conditions, I/R injury initiates a complex and interrelated sequence of events, resulting in injury to and the eventual death of renal cells (5, 8). Several factors have been implicated in the pathophysiological changes occurring while renal I/R injury including vascular or microvascular injury, endothelial dysfunction, accelerated cell necrosis, granulocyte activation, and modulation of nitric oxide/angiotensin II axis (9, 10).

The rennin-angiotensin system plays a pivotal role in regulation of blood pressure. Renin acts on angiotensinogen to form angiotensin I, which is converted to angiotensin II with the help of angiotensin-converting enzyme (ACE) (11). Angiotensin II is an important mediator in kidney injury. Accumulating evidence suggests that angiotensin II stimulates intracellular formation of reactive oxygen species (ROS) such as the superoxide anion and hydrogen peroxide that leads to kidney damage (12).

The present study, we investigated the reperfusion on renal marker and oxidative stress of kidney in diabetic rats and other word develop model experimentally induced ischemia/reperfusion induced renal damage in diabetic rats.
Materials and Methods

Drugs and Chemicals

STZ and NIC were obtained from SIGMA, St. Louis, MO, USA. All other chemicals and reagents used in the study were of analytical grade.

Experimental Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Dharmaj Degree Pharmacy College, Anand. Sprague Dawley rats (210 ± 15 g) were housed in-group of 3 animals per cage and maintained under standardized laboratory conditions (12- h light/dark cycle, 24°C) and provided free access to palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt., Pune) and purified drinking water ad libitum. The animal experiment was approved by Animal Ethical Committee of the Institute.

Experimental Induction of Type 2 Diabetes in Rats

Type 2 Diabetes was induced in rats by a single intraperitoneal (i.p) injection of Streptozotocin (65 mg/kg, STZ) in overnight fasting rats or mice followed by the i.p administration of Nicotinamide (110 mg/kg, NIC) after 15 minutes. STZ was dissolved in citrate buffer (pH 4.5) and NIC was dissolved in normal saline. After 7 days following STZ and NIC administration, blood was collected from retro-orbital puncture and serum samples were analyzed for blood glucose (14). Animals showing fasting blood glucose higher than 250 mg/dL were considered as diabetic and used for the further study.

Experimental Protocol

The rats were divided into three groups each consisting of six animals:

**Group 1**: Animals served as sham-operated (underwent all surgical procedures without ischemia reperfusion).

**Group 2**: After right nephrectomy on day 1, vehicle (0.5 % sodium CMC) was administered for 15 days; on day 16, ischemia was produced in the left kidney for 45 min, followed by reperfusion of 24 hr (I/R control).
Surgical Procedure

<table>
<thead>
<tr>
<th>Day</th>
<th>The progress of the experiment</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>Unilateral right nephrectomy</td>
</tr>
<tr>
<td>Day 15</td>
<td>Treatment</td>
</tr>
<tr>
<td>Day 16</td>
<td>45 minutes ischemia (left kidney)</td>
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<tr>
<td>Day 17</td>
<td>24 hr reperfusion</td>
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Right nephrectomy was performed through a right flank incision (2 cm) under general anesthesia, ketamine (100 mg/kg, i.p.). After right nephrectomy, several treatments were given as mentioned previously for 15 days. On day 16, ischemia was produced in the left kidney by performing a left flank incision and dissecting the left renal pedicle to expose the renal vessels. Non traumatic vascular clamps were used to stop blood flow (in artery and vein) for 45 min. Reperfusion was established by removing the clamp after 45 min ischemia. The abdominal wall (muscular layer and skin) was closed with 4.0 mononylon suture. At the end of reperfusion period (after 24 hr), blood samples were collected and used for the estimation of renal function (BUN and creatinine). The abdomen was opened, and the kidneys were harvested for the biomarkers of oxidative stress.

Characterization of Type 2 Diabetes Model

Type 2 diabetes was confirmed by measuring fasting serum glucose using standard diagnostic kit (SPAN diagnostics Pvt., India) and the degree of uncontrolled diabetic state was confirmed by measuring HbA1c (Ion Exchange Resin method). After seven day, diabetes was confirmed by measuring glucose and HbA1c as mentioned above. Blood urea nitrogen level was measured by assay kits (SPAN Diagnostics Pvt. India) and Serum Albumin levels were measured by assay kits (Crest Biosystems Ltd. India).

Estimation of kidney function marker

Blood was collected from the rats by retro-orbital puncture at the time of sacrify and was allowed to clot for 10 minutes at room temperature. Clots were centrifuged at 2500 rpm for 10 minutes to separate the serum. Serum creatinine and urea levels were measured by assay kits (SPAN Diagnostics Pvt. India) and Serum Uric acid levels were measured by assay kits (Crest Biosystems Ltd. India).
After sacrificing the animals, their kidneys were quickly removed, perfused immediately with ice cold hypertonic saline solution, weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000×g at 0°C for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assay of following antioxidant parameters. The levels of Lipid peroxidation (LPO) formation and the activities of endogenous antioxidant enzymes such as catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) were estimated by the method of Slater and Sawyer (15) Hugo Aebi as given by Hugo (16) Moron et al (17) and Mishra and Fridovich (18).

**Estimation of kidney Tissue Nitrite Levels**

The level of nitrite level was estimated by the method of Lepoivre et al. (19). To 0.5 mL of tissue homogenate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer and mixed well. To this, 530 µL of Griess reagent was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

**Histopathology**

For light microscopic evaluation, kidneys were fixed in 10% phosphate buffered formalin. Paraffin-embedded specimens were cut into 6 mm-thick sections and stained with hematoxylin and eosin (H&E). The kidneys were examined under a light microscope (Olympus Bioxl) for the presence of tubular changes and interstitial inflammatory cell infiltration by an observer blinded to the animal treatment group.

**Statistical Analysis**

All of the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student’s t-test as appropriate using a computer-based fitting program (Prism, Graphpad 5). Differences were considered to be statistically significant when p < 0.05.
Table 1. Effect of Streptozotocin (65mg/kg/day, p.o) and Nicotinamide (110 mg/kg/day, p.o) on serum glucose and HbA1c changes level in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose</th>
<th>HbA1c</th>
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<tr>
<td>CON</td>
<td>101.8 ± 6.799</td>
<td>5.455 ± 0.3729</td>
</tr>
<tr>
<td>STZ + NIC</td>
<td>332.8 ± 9.167***</td>
<td>9.900 ± 0.6323***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM for six animals in the group. ***P<0.001 considered statistically significant as compared to respective Control group.

Results

Characterization of Type 2 Diabetes

Single intraperitoneal (i.p) injection of Streptozotocin (65mg/kg) followed by i.p administration of Nicotinamide (110 mg/kg) to rats produced severe hyperglycemia and increased HbA1c in 70 to 80 % the animals (Table 1).

Figure 1. Effect of reperfusion serum Creatinine (A), Urea (B), and Uric acid (C) in the diabetic rats.

Values are expressed as mean ± SEM for six animals in the group. ns = Non Significant, *P<0.05, **P<0.01, ***P<0.001 considered statistically significant as compared to respective Sham group.
Figure 2. Effect of reperfusion on Superoxide dismutase (A), Catalase(B), reduced glutathione (C) and lipid peroxidation (D) in the diabetic rats.

Values are expressed as mean ± SEM for six animals in the group. ns = Non Significant, *P<0.05, **P<0.01, ***P<0.001 considered statistically significant as compared to respective Sham group.

Effect of reperfusion on kidney function marker

The six rats which underwent renal I/R exhibited a significant increase in the serum concentrations of creatinine (P<0.001), urea (P<0.001), and uric acid (P<0.001) compared with the sham control animals, suggesting a significant degree of glomerular dysfunction mediated by renal I/R (Fig. 1).

Effect of reperfusion on antioxidant activity

Renal I/R group of diabetic rats showed significantly decreased enzymatic activity of superoxide dismutase (P<0.001), catalase (P<0.001), and reduced glutathione (P<0.001) when compared with the sham control rats. These declining trends were significantly (P<0.05, Sham control Group) decreased in the group treated with HES compared with those in the I/R-only group (Fig. 2).

Effect of reperfusion on Albumin and Blood urea nitrogen

The six rats which underwent renal I/R exhibited a significant increase in the serum concentrations of Albumin (1.950 ± 0.1746 mg/dL tissue, p<0.001, n = 6) and blood urea nitrogen (66.55 ± 3.32 mg/dL tissue, p<0.001, n = 6) compared with the sham control animals (4.133 ± 0.1532, 25.96 ± 3.396 mg/dL tissue, respective Sham control, p<0.001, n = 6), suggesting a significant degree of glomerular dysfunction mediated by renal I/R (Fig. 3).
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Figure 3. Effect of Reperfusion on serum Albumin (A) and Blood Urea Nitrogen (B) in the diabetic rats exposed to renal ischemia/reperfusion (I/R) injury.

Values are expressed as mean ± SEM for six animals in the group. * P<0.05, **P<0.01, ***P<0.001 considered statistically significant as compared to respective Sham group.

Figure 4. Effect of Reperfusion on Tissue nitrate level in kidney tissue in diabetic rats exposed to renal ischemia/reperfusion (I/R) injury.

Values are expressed as mean ± SEM for six animals in the group. * P<0.05, **P<0.01, ***P<0.001 considered statistically significant as compared to respective Sham group.

Effect of reperfusion injury on kidney Tissue Nitrite Levels

Renal I/R resulted in a significant decrease in the tissue levels of nitrite (127.5±7.68 nmol/gm tissue, p<0.05, n = 6) as compared to values obtained from the tissue of sham-operated animals (156.5 ± 9.68, n = 6 (Fig.4).

HISTOPATHOLOGICAL ANALYSIS

Light microscopic evaluation of the sham-operated groups revealed a regular morphology of renal parenchyma with well-designated glomeruli and tubuli. The sham control group of rats
did not show any morphological changes. By contrast, the kidneys of the diabetic rats with I/R only showed tubular cell swelling, interstitial edema, tubular dilatation, and moderate to severe necrosis (Fig. 5).

**Figure 5.** Morphological Changes Assessed by Histopathological Examination of Kidney by reperfusion in diabetic Rats. **Sham:** kidney section of a rat in the sham operation group shows normal glomeruli and tubuli, **I/R:** Kidney section of a rat exposed to bilateral renal ischemia/reperfusion shows severe interstitial hemorrhage surrounding the glomeruli and tubuli. Tubular epithelial degeneration is apparent. **G** = Glomerul, **T** = Tubuli.

**Discussion**

The present study was undertaken with the objective of exploring evaluate Hesperidine on renal marker in I/R induced renal damage in diabetic rats. The transient discontinuation of renal blood supply is encountered in many clinical situations such as kidney transplantation, partial nephrectomy, renal artery angioplasty, aortic aneurysm surgery, and elective urological operations (5, 8). This transient discontinuation causes renal I/R injury which results in decreased glomerular filtration and renal blood flow and increased urine output characterized by natriuresis and impaired concentrating ability. 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.

Intracellular oxidative stress, due to an increased production of superoxide by the electrontransport chain in the mitochondria, has been proposed as a unifying explanation for most metabolic alterations in diabetes (19). I/R are also a state where oxidative stress has been implied (20).
Moreover, the levels of endogenous antioxidant (SOD, CAT and GSH) were reduced and lipid peroxidation increased in Sham group showing increased oxidative stress. In renal I/R injury, ROS are capable of reacting with lipids leading to lipid peroxidation of biological membranes, which in turn impacts enzymatic processes, such as ion pump activity, inhibiting transcription and repair of DNA. If lipid peroxidation remains unchecked, it will ultimately result in cell death (21). The finding that hyperglycaemia increases renal I/R injury may have implications for the understanding of diabetic nephropathy as well. Ischaemia has been suggested in the development of diabetic nephropathy (22).

In our study, animals subjected to renal I/R demonstrated an increase in the renal MDA and at tenuated antioxidant enzymes pool. Lipid peroxidation and antioxidant enzymes are important indexes of oxidant injury (23). Demonstrations of lipid peroxidation as indexes for oxidative damage may help us better understand the effects of ROS on the cellular components(24). Renal I/R-induced oxidative stress was associated with impaired kidney function, leading to a marked increase in serum creatinine, urea, and uric acid levels.

The rennin-angiotensin system plays a pivotal role in regulation of blood pressure. Renin acts on angiotensinogen to form angiotensin-I, which is converted to angiotensin-II with the help of angiotensin-converting enzyme. Accumulating evidence suggests that angiotensin-II stimulates intracellular formation of ROS such as superoxide anion and hydrogen peroxide that leads to kidney damage (12). Generation of ROS has been postulated as one of the major factors contributing to this reperfusion injury. Oxidative stress can result from increased ROS production, and/or from decreased ROS scavenging capability. The ROS attach to the polyunsaturated fatty acids in the membrane lipids and result in peroxidation, which may lead to disorganization of cell structure and function. After reperfusion and reoxygenation, the imbalance between restoration of oxygen supply and mitochondrial respiratory function results in massive generation of superoxide anion in mitochondria (25). Under these conditions, the defensive system, which is known as antioxidant or antioxidant enzymes, cannot prevent the escape of ROS, especially in mitochondria, and their effects on other intracellular sites. This cascade of events is known as reperfusion injury (25).

Previous studies have shown that peroxynitrite level in the heart increases greater than 10-fold in the first minute of reperfusion (26). In this study, there was a significant decrease in tissue nitrite levels in kidney of I/R group animals as compared to sham-operated group.
Moreover, peroxynitrite could initiate lipid peroxidation, which damages the proximal tubular cells, nitration of tyrosine residues (nitro tyrosine), and nitration of cellular proteins, with a subsequent loss of protein structure resulting in reduction of the kidney function (27, 28). Similar results were obtained in this study as well. There was a significant increase (3 fold) in the levels of serum BUN and significant decrease (3 fold) in the levels of serum albumin in I/R control group.

In the clinical settings, renal I/R are a consequence of systemic hypoperfusion with subsequent circulatory resuscitation, such as following aortic cross-clamping or renal transplantation (29). There is good evidence from both in vivo and in vitro studies that the formation of NO plays an important role in I/R (30). One of the important mechanisms for I/R injury is excessive ROS, which scavenges NO and cumulates in reduced NO bioavailability (31). Endothelial cells produce less bioactive NO in the presence of higher oxidative stress (32, 33).

In this study, renal I/R increased oxidative stress products including tissue MDA and depleted the antioxidant enzymes pool, as is evident from the declined activity of superoxide dismutage, catalase, and reduced glutathione.

**Conclusions**

Our data support a role for renal Ischamia/reperfusion injury in attenuation of kidney damage in an animal model. In conclusion, Kidney damage induced by renal ischamia/reperfusion injury Sprague dawley diabetic rats. So, it may be this model use in evaluation of some compound on renal damage induced by renal ischamia/reperfusion injury in diabetic rats or other word effect of some compound on renal complication in diabetic rats.

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