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Preconditioning with atorvastatin against renal ischemic-reperfusion injury in non-diabetic versus diabetic rats

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Abstract

Acute renal failure complicates renal ischemic-reperfusion (I/R) due to reactive oxygen species production. Atorvastatin (ATO) has anti-inflammatory and antioxidant properties. The current study investigated whether ATO alleviated damages induced by renal I/R injury in non-diabetic versus diabetic rat models. Thirty-six rats (18/group) were divided into non-diabetic and diabetic Groups, A and B, respectively: Group A1 (sham), Group A2 (I/R), Group A3 (ATO + I/R), Group B1 (sham), Group B2 (I/R), and Group B3 (ATO + I/R). All groups experienced 45 minutes of renal ischemia, bilaterally, followed by 24 hours of reperfusion. Groups A3 and B3 were treated with intraperitoneal single doses of ATO (10 mg/kg) 30 minutes before ischemia. Histological analysis of kidney tissues, immune expression of caspase 3 and CD44, kidney function tests, and oxidative stress markers, assessed tubular injury. Histological analysis revealed marked tubular damage in Groups A2 and B2 but improvement in Groups A3 and B3. Improvements were also found for immune expression of caspase 3 and CD44, kidney function tests, and oxidative stress markers. Our results suggested ATO may ameliorate renal I/R injury, differently between non-diabetic and diabetic rats.

Key words: atorvastatin, ischemic-reperfusion, diabetes, kidney, protection.
Introduction

Renal ischemic-reperfusion injury (IRI) is associated with acute kidney injury (AKI), which is frequently associated with high morbidity and mortality. The pathogenesis of AKI is related to oxidative stress, renal vasoconstriction, hypoxia, hypoperfusion, and hypotension (Kunzendorf et al. 2010). Eventually, tubular and endothelial cell injury and mitochondrial damage are inevitable. Yung et al. (2011) reported that increased reactive oxygen species (ROS) production was a key factor for the development of IRI and endothelial dysfunction.

Ischemic-reperfusion (I/R)-induced AKI is characterized by a reduction in the microvascular blood flow of the renal medulla due to decreased glomerular filtration rate, causing endothelial dysfunction (Hasegawa et al. 2010). Hasegawa et al. (2010) speculated that the overproduction of free radicals and nitric oxide (NO) played a role in the pathogenesis of I/R-induced AKI by altering the renal hemodynamics. However, the exact mechanism remains incompletely understood. Further, the epithelial cells of the renal tubules could suffer from a variable degree of necrosis or apoptosis causing tubular obstruction, dependent on the severity of renal ischemia (Hasegawa et al. 2010).

Diabetes may be a major contributing factor for renal IRI in a rat model where inflammation and apoptosis both coexist (Yuen et al. 2006). Caspase 3 is a crucial fragment of the processes of inflammation and apoptosis in cases of IRI (Yuen et al. 2006).

Statins may have protective effects against IRI; however, the mechanism of action remains unclear (Sarin et al. 2016). Atorvastatin (ATO) is a statin that works by inhibiting HMG-CoA (5-hydroxy-3-methylglutaryl-coenzyme A) reductase, which is the rate-limiting enzyme of endogenous cholesterol biosynthesis. Park et al. (2018) reported a protective effect of ATO on endothelial function in diabetes. ATO inhibited the production of ROS, correcting
oxidative stress and endothelial dysfunction (Caliman et al. 2013). Schouten et al. (2006) reported a connection between statin administration and the prevention of AKI via its antiinflammatory and antioxidant properties. Marques Dos Santos et al. (2018) reported that postconditioning with ATO minimized renal IRI in non-diabetic rats.

Thus, the aim of the current study was to investigate whether ATO alleviated the damages induced by renal IRI in non-diabetic versus diabetic rat models and the potential mechanism of action by which ATO produces such protection.

**Materials and methods**

**Rats**

The current study used 36 adult male Sprague-Dawley rats (180-220 g). They were raised in the Animal House at the Faculty of Medicine, Cairo University in Cairo, Egypt, housed in separate cages (4 rats/cage), and allowed ad libitum water and food throughout the study. Rats were acclimatized for 2 weeks before starting the experiment. All individuals involved in the study had received animal care training. All procedures, sampling, animal handling, and euthanization were approved by the ethical committee of the Anatomy Department, Faculty of Medicine, Cairo University and followed the Guide for the Care and Use of Laboratory Animals, 8th edition (National Research Council 2011).

**Experimental design**

The rats were divided into 2 diagnostic groups of 18 rats: Group A (non-diabetic) and Group B (diabetic). Each diagnostic group was then subdivided into 3 study groups of 6 rats: Group A1 (sham), Group A2 (I/R), Group A3 (ATO + I/R), Group B1 (sham), Group B2 (I/R), and Group B3 (ATO + I/R).

**Induction of diabetes**
To induce diabetes, 45 mg/kg Streptozotocin (STZ) (Sigma Chemical, St. Louis, MO, USA) was administered in a single intraperitoneal dose. STZ was prepared in a 0.1 M phosphate-citrate buffer (pH 4.5). Three days later, a blood sample was taken from the tail vein and tested for glucose to confirm the diabetic state. Rats with blood samples ≥ 250 mg/Dl glucose were classified as diabetic. All diabetic rats were confirmed to be diabetic and no rats died from the procedure. Rats were observed for 1 month before starting experiments, and blood glucose measurements were documented during that time In Group A1, each animal was injected with an equal volume of buffer, intraperitoneal, without inducing diabetes. (Guneli et al. 2008).

Renal ischemia-reperfusion model

Rats were anesthetized, prior to the laparotomy procedure, with intraperitoneal xylazine hydrochloride (10 mg/kg) and ketamine (50 mg/kg). Laparotomy was conducted to expose the renal pedicles bilaterally. Basal blood glucose was measured before abdominal incision, and rats were exposed to a heating lamp to maintain the rectal body temperature at 37°C during anesthesia. Occlusion of the renal pedicles was performed bilaterally for 45 min, using non-traumatic microvascular clamps to induce ischemia. Clamps were then removed to induce reperfusion of blood.

In Groups A1 and B1, the renal pedicles were exposed bilaterally without any further interference after laparotomy. In Groups A2 and B2, the clamps were removed after the 45 min of ischemia, and reperfusion of the kidneys was allowed for 24 h after which the abdominal walls were reopened. In Groups A3 and B3, rats were treated with a single intraperitoneal dose of ATO (10 mg/kg) 30 min before the induction of ischemia (Wu et al. 2014). ATO tablets (Global Napi Pharmaceuticals, Egypt) containing 10 mg of atorvastatin were used and dissolved
in 10 mL distilled water for treatment. After ATO administration, Groups A3 and B3 had the same treatment as Groups A2 and B2.

Blood samples were collected by cardiac puncture and immediately centrifuged to obtain serum samples. Aliquots were stored at −80°C for later biochemical analysis. Bilateral nephrectomies were then performed. Left kidneys were stored at −80°C for later immune histochemical and histochemical examination. Right kidneys were cut into small pieces and preserved in 10% formaldehyde for light microscopic examination (Ozbilgin et al. 2016). Rat sera were analyzed for kidney function tests. Kidney tissue samples were analyzed for histological analysis, immune histochemical examination of caspase 3 (for apoptosis) and CD44 (for tubular injury), histochemical examination of oxidative stress markers, and morphometric examination of the mean area percentages of caspase 3 and CD44 immune expression via image analyzer.

Light microscopic examination

Kidney tissue samples were dehydrated in ascending grades of ethanol (70%, 90%, and 100%) and were then cleared in xylene for 2 h. Impregnation and embedding were done in soft paraffin wax for 3 h at 50°C and in hard paraffin for 1 h at 60°C. Paraffin blocks were set and sections of 5 µm were stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) method (Bancroft and Gamble 2008).

The morphometric analysis of the histological study was performed by an independent, blinded observer to avoid bias. Ten measuring frames were examined per sample using Leica Qwin 500 image analysis software (Leica Microsystems Imaging Solutions, Wetzlar, Germany). Objective lens of magnification 40 (total magnification 400) was used. Images were captured live on the screen from sections under a light microscope (Olympus Bx-40, Olympus Optical,
Japan) with an affixed video camera (Panasonic color CCTV camera, Matsushita Communication Industrial, Japan).

For the H&E sections, quantitative morphometric assessments of the glomerular and tubular parameters were performed inside the measuring frames. The glomerular parameters measured the glomerular area and Bowman’s space. While the tubular parameters measured the luminal areas of the proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) and the epithelial height of the PCT.

For the PAS-stained sections, quantitative morphometric assessments of the basal laminae of renal tubules and glomerular capillaries and the brush border of the PCT were performed. The optical density was measured inside the measuring frames.

Immune histochemical examination for caspase 3 immune expression

Some of the 5 µm-sections in paraffin blocks were de-paraffinized, hydrated in alcohol, and exposed to anti-caspase 3 antibodies in acidic pH. Sections were cooled for 20 min, rinsed with distilled water, and placed in buffered saline. They were incubated for 20 min in 0.3% H$_2$O$_2$ solution (99 mL of TBS, 0.1 g of NaN$_3$, 1 mL of 30% H$_2$O$_2$) to block endogenous peroxidase. Sections were rinsed for 10 min in TBS, and the rabbit primary antibody was applied to them and incubated for 60 min at room temperature in 1% TBS/BSA (1:100).

The biotinylated secondary antibody against rabbit antibodies was then applied and incubated for 15 min, conjugated with streptavidin peroxidase that was incubated for 15 min. AEC substrate chromogen (reaction dye) was then added and incubated for 15 min. After chromogen staining, specimens were placed in hematoxylin solution for about 1 min, washed with distilled water, and covered with Aquatex fluid cover slip. Primary and secondary antibodies for caspase 3 immune expressions, streptavidin peroxidase, and AEC substrate were
obtained from Abcam (Cambridge, UK). The appearance of dark brown cytoplasmic granules indicated a positive immune expression for caspase 3.

Immune histochemical examination for CD44 immune expression

Some of the 5 µm-sections in the paraffin blocks were immune histochemically-stained using anti-CD44 (IM7.8.1, BD Pharmingen Franklin Lakes, NJ, USA), anti-active caspase 3 (Cell Signaling, Danvers, MA, USA), and anti-CD3 (BD Pharmingen). Hyaluronan was detected by biotinylated HA-binding protein (Millipore Sigma, Billerica, MA, USA). Slides were developed using HRP-labeled secondary antibody (DAKO, Agilent Pathology Solutions, Santa Clara, CA, USA) and DAB (Sigma-Aldrich, St. Louis, MO, USA) (Rampanelli et al. 2015). The appearance of dark brown cytoplasmic granules indicated a positive immune expression for CD44.

For the immune histochemically-stained sections, quantitative morphometric assessments of the mean area percentages for caspase 3 and CD44 immune expression were calculated inside the measuring frames.

Biochemical analysis

Rat sera were analyzed for blood urea nitrogen (BUN) at a wavelength of 578 nm using the enzymatic method and for creatinine at a wavelength of 492 nm using the Jaffe kinetic method. Assays, calibrations, and quality controls were performed according to manufacturer instructions using 1 reagent lot and 1 calibration lot for either parameter. BUN and creatinine were analyzed using a spectrophotometer (DTN 402, DIALAB Diagnostics, Wiener Neudorf, Austria) (Ozbilgin et al. 2016). Commercial reagents for kidney function tests that measure BUN and creatinine were purchased from DIALAB Diagnostics Co.

Histochemical examination: estimation of oxidative stress markers of renal tissue homogenate
Kidney tissue samples were homogenized to 10% (w/v) using Tris-HCl buffer (5 mmol/L comprising 2 mmol/L ethylenediaminetetraacetic acid at pH 7.4). Kidney homogenates were centrifuged at 1000 rpm for 10 min at 4°C. The supernatants were used to determine the oxidant-antioxidant status. To determine the extent of oxidative stress and lipid peroxidation in kidney tissue, malondialdehyde (MDA), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), and glutathione reductase (GR) were determined according to manufacturer instructions. Commercial kits and assays were obtained from Biodiagnostic Laboratories (Brooklyn, NY, USA).

**Data analysis**

The aim of this study required the answering of three statistical questions: (1) was there a difference in outcomes (histological, immune histochemical, biochemical, and histochemical) between the study groups (Group 1, Group 2, and Group 3) with the diagnostic groups (non-diabetic Group A and diabetic Group B), (2) was there a difference in outcomes between the diagnostic groups, and (3) were there differences in outcomes based on the assignment of treatment group and diagnosis of diabetes?

SPSS version 25 (IBM Corp., Armonk, N.Y., USA) was used to perform all statistical analyses. Descriptive statistics were summarized using means and standard deviations. To examine study group (Group1, Group 2, and Group 3) differences, a one-way ANOVA was conducted for each diagnostic group. If a statistical difference was found, a Dunnet T3 post-hoc was completed to determine between group differences. A two-way ANOVA was then completed to investigate the interaction between diagnostic groups and study group assignment. If the interaction was significant, a simple-effects analysis was completed. $P < .05$ was considered statistically significant.
Results

Light microscopic examination

Group A1 H&E-stained kidney sections revealed classic architecture of the renal cortex with glomerulus and proximal and distal tubules (Figs. 1A and 1B). The proximal tubules had prominent brush border (Fig. 1B). Group A2 showed necrotic changes in the renal cortices by dilation of the proximal and distal tubules with loss of brush border and hyaline cast formation (Fig. 1C). The corticomedullary region had dilation of the tubules and marked interstitial hemorrhagic exudate. The tubular cells appeared detached from the basement membrane with pyknotic nuclei and contained hyaline cast (Fig. 1D). Group A3 had remarkable improvement and restoration of the normal renal architecture with apparently normal renal glomerulus and proximal and distal tubules (Fig. 1E). The corticomedullary junction showed normal proximal tubules with preserved intact brush border, normal distal tubules, and minimal cast formation (Fig. 1F).

Group B1 H&E-stained kidney sections revealed normal structure of the renal cortices (Fig. 2A). Group B2 had marked necrosis of the renal cortices and dilation and necrosis of the proximal and distal tubules. The lining tubular cells were detached from the basement membrane of the tubules. The interstitial tissue contained massive hemorrhagic exudate. The lumen of the tubules contained hyaline cast (Fig. 2B). The renal medulla showed marked dilation of the proximal and distal tubules, exfoliation of the lining epithelium, interstitial hemorrhagic exudate, and marked intratubular hyaline cast formation (Figs. 2C and 2D). Group B3 had mild improvement in the structure of the renal cortices. The tubules still appeared dilated with vacuolation of their lining cells, pyknotic nuclei, and disrupted brush border. The tubular lumen contained hyaline cast (Fig. 2E). The renal medulla revealed dilation of the tubules, necrosis, and
vacuolations of the lining tubular cells. The interstitial tissue contained hemorrhagic exudate (Fig. 2F).

Morphometric Analysis of the Histological Examination

For the H&E-stained glomerular and tubular parameters, a significant main effect of the study group was found on the glomerular area ($P < .001$, $\eta = .91$). Post hoc revealed that the glomerular area was less in Group A2 than in Group A3 (Table 1), less in Groups B2 and B3 than in Group B1 (Table 1), and greater in Group B3 than in Group B2 (Table 1).

The glomerular area was less in Group B ($M = 5555.52$) than in Group A ($M = 8145.28$, $\eta = .93$; Figure 3A). A significant interaction between the study groups and the diagnostic groups on the results of the glomerular area indicated that the study groups were affected differently by the diagnostic groups ($\eta = .83$; Figure 3A). Simple effects analysis indicated that the glomerular area was greater in Group A1 than in Group B1, greater in Group A2 than in Group B2, and greater in Group A3 than in Group B3 (Figure 3A).

A significant main effect of the study group was found on the area of Bowman’s space ($P < .001$, $\eta = .88$). Post hoc revealed that the area of Bowman’s space was greater in Group A2 than in Groups A1 and A3 (Table 1) and was greater in Group B2 than in Group B1 (Table 1).

The area of Bowman’s space was less in Group A ($M = 2248.18$) than in Group B ($M = 2482.12$, $\eta = .29$; Figure 3B). A significant interaction between the study groups and the diagnostic groups on the results of the area of Bowman’s space indicated that the study groups were affected differently by the diagnostic groups ($\eta = .56$; Figure 3B). Simple effects analysis indicated that the area of Bowman’s space was significantly greater in Group A3 than in Group A3 (Figure 3B).
A significant main effect of the study group was found on the luminal area of the proximal convoluted tubules (PCT) \((P < .001, \eta^2 = .85)\). Post hoc revealed that the luminal area of PCT was less in Group A1 than in Groups A2 and greater in Group A2 than in Group A3 (Table 1). Post hoc also revealed that the luminal area of PCT was less in Group B1 than in Groups B2 and B3 (Table 1) and greater in Group B2 than in Group B3 (Table 1).

The luminal area of PCT was greater in Group B \((M = 396.91)\) than in Group A \((M = 347.69, \eta = .12; \text{Figure 3C})\). An interaction between study group and diagnostic group was not found on the luminal area of PCT \((\eta = .07)\), indicating that the results of the study groups may not be affected differently by diagnosis.

A significant main effect of the study group was found on the luminal area of the distal convoluted tubules (DCT) \((P < .001, \eta^2 = .53)\). Post hoc revealed that the luminal area of the DCT was less in Group A2 than in Groups A1 and A3 (Table 1) and was greater in Group B1 than in Groups B2 and B3 (Table 1).

The luminal area of the DCT was greater in Group B \((M = 1105.94)\) than in Group A \((M = 900.25, \eta = .22; \text{Figure 3D})\). A significant interaction between the study groups and the diagnostic groups on the results of the luminal area of the DCT indicated that the study groups were affected differently by the diagnostic groups \((\eta = .20, \text{Figure 3D})\). Simple effects analysis indicated that the luminal area of the DCT area was greater in Group A1 than in Group B1 and greater in Group A3 than in Group B3 (Figure 3D).

A significant main effect of the study group was found on the epithelial height \((P < .001, \eta^2 = .85)\). Post hoc revealed that the epithelial height was less in Group A2 than in Groups A1 and A3 (Table 1), less in Group B2 than in Group B1 (Table 1), and less in Group B3 than in Group B1 (Table 1). The epithelial height was less in Group B \((M = 8.78)\) than in Group A \((M = 8.78)\).
A significant interaction between the study groups and diagnostic groups on the results of the epithelial height indicated that the study groups were affected differently by the diagnostic groups (\( \eta = .45 \); Figure 3E). Simple effects analysis indicated that the epithelial height was less in Group B1 than in Group A1 and significantly less in Group B3 than in Group A3 (Figure 3E).

Group A1 PAS-stained kidney sections revealed strong PAS reaction of the basement membrane of the cortical tubules and the brush border of the proximal tubules (Fig. 4A), Group A2 revealed weak positive reaction (Fig. 4B), and Group A3 revealed moderate reaction (Fig. 4C). Group B1 PAS-stained kidney sections revealed strong PAS reaction (Fig. 4D), Group B2 revealed weak reaction (Fig. 4E), and Group B3 revealed mild reaction (Fig. 4F).

For the PAS-stained sections, a significant main effect of study group was found on the optical density of the basal laminae of the renal tubules and glomerular capillaries (\( P < .001; \eta = .54 \)). *Post hoc* revealed that the optical density was less in Group A2 than in Groups A1 and A3 (Table 1) and was less in Group B2 than in Groups B1 and B3 (Table 1).

A significant interaction between the study groups and the diagnostic groups on the optical density of the basal laminae indicated that the study groups were affected differently by the diagnostic groups (\( \eta = .22 \); Figure 4G). Simple effects analysis indicated that the optical density was greater in Group A3 than in Group B3 (Figure 4G).

For the PAS-stained sections, a significant main effect of study group was found on the length of the brush border of the PCT (\( P < .001; \eta = .80 \)). *Post hoc* revealed that the optical density was less in Group A2 than in Groups A1 and A3 (Table 1) and was less in Group B2 than in Groups B1 and B3 (Table 1).
The length of the brush border of the PCT was less in Group A (M = 2.52) than in Group B (M = 3.07, \( \bar{\pi} = .41 \); Figure 4H). A significant interaction between the study groups and the diagnostic groups on the length of the brush border of the PCT indicated that the study groups were affected differently by the diagnostic groups (\( \bar{\pi} = .21 \); Figure 4G). Simple effects analysis indicated that the optical density was less in Group A3 than in Group B3 and less in Group A1 than in Group A2 (Figure 4H).

The area percentage of Caspase 3 was less in Group A (M = 4.07) than in Group B (M = 4.98, \( P < .001, \bar{\pi} = .70 \)). A significant interaction between the study groups and the diagnostic groups on the area percentage of Caspase 3 indicated that the study groups were affected differently by the diagnostic groups (\( \bar{\pi} = .72 \)). Simple effects analysis indicated that the area percentage of Caspase 3 was significantly less in Group A2 than in Group B2 and was less in Group A3 than in Group B3 (Figure 5G).
Immune histochemical examination for CD44 immune expression

Kidney sections of the renal tubules in Group A1 revealed mild CD44 immune expression (Fig. 6A), Group A2 revealed strong positive immune expression (Fig. 6B), and Group A3 revealed negative immune expression (Fig. 6C). Group B1 revealed mild CD44 immune expression (Fig. 6D), Group B2 revealed intense strong positive immune expression (Fig. 6E), and Group B3 revealed moderate positive immune expression (Fig. 6F).

The only differences found in the mean percentage area of CD44 was between the study groups. Post hoc revealed that the area percentage of CD44 in Group A2 was greater than in Groups A1 and A3 (Table 1) and was greater in Group A3 than in Group A1 (Table 1). Group B2 was greater than Groups B1 and B3, and Group B3 was greater than Group B1 (Table 1).

A significant main effect of study group on Blood urea nitrogen (BUN) was found ($P < .001, \eta = .48$). Post hoc revealed only differences between the study groups in Group A for BUN; Group A2 was greater than in Groups A1 and A3 (Table 1). The BUN was greater in Group B ($M = 79.17$) than in Group A ($M = 55.08, = .50$; Figure 7A). A significant interaction between the study groups and the diagnostic groups was not found, indicating that the BUN results in the study groups may not differ by the diagnostic groups (Figure 7A).

Biochemical analysis

A significant main effect of study group on serum creatinine was found ($P < .001, \eta = .78$). Post hoc revealed that the serum creatinine was greater in Group A2 than in Group A1 (Table 1), greater in Group B2 than in Groups B1 and B3 (Table 1), and greater in Group B3 than in Group B1 (Table 1). The serum creatinine was greater in Group B ($M = 1.62$) than in Group A ($M = 88, \eta = .82$; Figure 7B). A significant interaction between the study groups and the diagnostic groups on serum creatinine was found, indicating that the study groups were affected differently.
by the diagnostic groups ($\eta = .57$, Figure 7B). Simple effects analysis indicated B2 was greater than A2 and A3 was greater than B3 (Figure 7B).

Histochemical examination: oxidative stress markers of renal tissue homogenates

A significant main effect of study group on malondialdehyde (MDA) was found ($P < .001$, $\eta = .92$). Post hoc revealed that MDA was greater in Group A2 than in Groups A1 and A3 (Table 1), and greater in Group B2 than in Groups B1 and B3 (Table 1). MDA was greater in Group B ($M = 38.58$) than in Group A ($M = 26.71$, $\eta = .67$; Figure 8A). A significant interaction between the study groups and the diagnostic groups on MDA was found, indicating that the study groups were affected differently by the diagnostic groups ($\eta = .32$, Figure 8A). Simple effects analysis indicated that Group B1 was greater than Group A1, Group B2 was significantly greater than Group B1, and Group B3 was significantly greater than Group A3 (Figure 8A).

A significant main effect of study group on inducible nitric oxide synthase (iNOS) was found ($P < .001$, $\eta = .70$). Post hoc revealed that iNOS was greater in Group A2 than in Groups A1 and A3 (Table 1) and greater in Group A3 than in Group A1 (Table 1). It also revealed that iNOS was greater in Group B2 than in Group B1 (Table 1) and greater in Group B3 than in Group B1 (Table 1).

iNOS was greater in Group B ($M = 5.70$) than in Group A ($M = 2.70$, $\eta = .41$; Figure 8B). A significant interaction between the study groups and the diagnostic groups on iNOS was found, indicating that the study groups were affected differently by the diagnostic groups ($\eta = .20$, Figure 8B). Simple effects analysis indicated that Group B1 was greater than Group A1, Group B2 was greater than Group A1, and Group B3 was significantly greater than Group A3 (Figure 8B).
A significant main effect of study group on inducible superoxide dismutase (SOD) was
found ($P < .001, \eta^2 = .93$). Post hoc revealed that the SOD was less in Group A2 than in Groups
A1 and A3 (Table 1) and less in Group A3 than in Group A1 (Table 1). It also revealed that SOD
was less in Group B2 than in Groups B1 and B3 (Table 1) and less in Group B3 than in Group
B1 (Table 1).

SOD was less in Group B ($M = 1.51$) than in Group A ($M = 2.27, \eta^2 = .59$; Figure 8C). A
significant interaction between the study groups and the diagnostic groups on SOD was found,
indicating that the study groups were affected differently by the diagnostic groups ($\eta^2 = .48$,
Figure 8C). Simple effects analysis indicated that Group A3 was significantly greater than Group
B3 (Figure 8C).

A significant main effect of study group on inducible glutathione reductase (GR) was
found ($P < .001, \eta^2 = .95$). Post hoc revealed that the GR was less in Group A2 than in Groups A1
and A3 (Table 1) and less in Group A3 than in Group A1 (Table 1). It also revealed that GR was
less in Group B2 than in Groups B1 and B3 (Table 1) and less in Group B3 than in Group B1
(Table 1).

GR was less in Group B ($M = 2.23$) than in Group A ($M = 2.45, \eta^2 = .18$; Figure 8D). A
significant interaction between the study groups and the diagnostic groups on GR was found,
indicating that the study groups were affected differently by the diagnostic groups ($\eta^2 = .35$ Figure
8D). Simple effects analysis indicated that Group A3 was greater than Group B3 (Figure 8D).

Discussion

Results of the current study suggested that administration of ATO alleviated renal IRI in
the renal cortices of non-diabetic rats, but such protection was limited in the renal cortices of
Results for all outcomes, excluding the optical density of the basal lamina of the renal tubules and glomerular capillaries and CD 44, indicated that protection differed by the presence of diabetes. These findings were revealed using histological, immune histochemical, biochemical, and histochemical analyses. No significant difference was found between Groups A3 (ATO + I/R) and A1 (sham) for epithelial height, luminal area of the PCT, luminal area of the DCT, area of Bowman’s Space, length brush border of the basal lamina, BUN, creatinine, and MDA, and no significant difference was found between Groups B3 (ATO + I/R) and B1 (sham) for optical density of the basal lamina of the renal tubules and glomerular capillaries, length brush border of the PCT, and BUN. These statistical finding suggested that administration of ATO alleviated renal IRI in the renal cortices of non-diabetic and diabetic rats.

In the current study, histological analysis of Group A2 (I/R) indicated necrotic changes in the renal cortices of non-diabetic rats from IRI and weak PAS reaction for the brush border of the renal tubules. These changes agreed with Bayrak et al. (2008), who reported a characteristic pattern of renal IRI that included extensive distortion of tubular architecture with loss of brush borders, desquamation of tubular epithelial cells, and tubular cellular necrosis with cast formation.

These necrotic changes in the tubular cells may cause cellular components to spill out into the tubular lumen through the cellular basement membrane because of the loss of the brush borders of the proximal tubular cells, thus obstructing the renal tubules. Bayrak et al. (2008) suggested that renal IRI was a progressive process involving free radical damage and hypoxia, which could eventually lead to acute renal failure (ARF).

Histological analysis of Group B2 (I/R) indicated marked necrotic changes in the renal cortices of diabetic rats from IRI compared with Group A2. These findings agreed with Vlad et
al. (2017), who reported that proximal tubule dysfunction usually occurred in parallel with glomerular changes in early diabetic nephropathy. Yuen et al. (2006) attributed these necrotic changes to increased activity of caspase 3 during hypoxia, which is more evident in diabetic rats, because of intracellular Ca2+ accumulation in ischemic tissues.

Histological analysis of Group A3 indicated remarkable improvement in structural changes in the renal cortices of non-diabetic rats and moderate PAS reaction from the IRI. These findings may be attributed to the anti-inflammatory properties of ATO, which may have increased endothelial nitric oxide synthase (eNOS). Wu et al. (2013) reported that ATO, by increasing eNOS, may indirectly protect against the damage of the endothelial vasculature by targeting indoxyl sulfate, which has a direct inhibitory effect on endothelial progenitor cells in AKI. Further, this mechanism of action may signify an innovative strategy to salvage endothelial progenitor cells and overcome impaired new vessel formation following AKI (Wu et al. 2013).

Histological analysis of Group B3 indicated a slight improvement in structural changes with milder PAS reaction in the renal cortices of diabetic rats from the IRI compared to Group A3. These findings contradicted Singh et al. (2016), who reported that ATO was remarkably effective at reducing AKI in diabetic nephropathy. The discrepancy in findings between this study (Singh et al. 2016) and the current study may be attributed to differences in experimental design or in a rat versus human model.

Immune histochemical examination of Groups A2 and B2 for caspase 3 immune expression found strong positive immune expression for both groups. These findings may be explained by Cakir et al. (2015), who reported widespread tubular necrosis and glomerular damage in their I/R group.
Immune histochemical examination of Groups A2 and B2 for CD44 immune expression also found strong positive immune expression for both groups. These findings may be explained by Rouschop et al. (2005), who suggested that inflammation may contribute to renal IRI, possibly causing renal dysfunction and increasing neutrophils. The authors (Rouschop et al. 2005) added that CD44 was expressed by neutrophils and was rapidly upregulated by capillary endothelial cells after IRI, suggesting that CD44 may contribute to the development of IRI.

Immune histochemical examination of Groups A3 and B3 for caspase 3 immune expression found mild immune expression for Group A3 and moderately positive immune expression for Group B3. This reduced response to ATO for apoptosis in Group B3 agreed with Miki et al. (2012), who reported a higher mortality rate in diabetic rats following IRI than in non-diabetic rats with similar treatment.

Immune histochemical examination of Groups A3 and B3 for CD44 immune expression found mild immune expression for Group A3 and moderately positive immune expression for Group B3. These findings may be explained by Ajamieh et al. (2015), who reported that ATO protected against hepatic IRI by increasing post-ischemic eNOS mRNA-protein, enhancing eNOS activity with a concomitant reduction in the recruitment of neutrophils. Maher et al. (2009) reported that ATO could significantly reduce neutrophil migration via its antiinflammatory properties. Therefore, understanding this possible mechanism of action of ATO for neutrophil migration may expand the use of ATO to excessive migration in serious inflammatory settings.

Biochemical analysis of Groups A1, A2, B1, and B2 indicated statistically significant differences between Groups A2 and A1 and between Groups B2 and B1. These findings agreed with Ragy and Aziz (2017), who reported an elevation in serum BUN and creatinine in
association with renal IRI. These findings could be attributed to the reduction in the microvascular blood flow due to decreased glomerular filtration rate causing endothelial dysfunction, a characteristic feature of IRI that progresses to AKI (Hasegawa et al. 2010).

Biochemical analysis of Groups A1, A3, B1, and B3 indicated non-significant differences between Groups A3 and A1 and between Groups B3 and B1. While BUN in Groups A2 and A3 were statistically different, there is slight overlapping of confidence intervals between the two Groups. Creatinine in Groups A2 and A3 was not significantly different. There was no difference between Groups B2 and B3 for BUN. However, results do indicate a clear significant decrease in serum creatinine level in Group B3. The mixed results prevent the authors from concluding that ATO preoperative treatment reduced BUN or serum creatinine in non-diabetic and diabetic rats. These findings agreed with Wu et al. (2014), who reported that treatment with ATO reduced elevated levels of serum creatinine and significantly increased the rate of creatinine clearance.

Sabbatini et al. (2004) reported that ATO increased the eNOS-mRNA expression in ATO-treated rats with ARF, protected against renal tubular cell injury, and enhanced availability of NO, thus improving kidney functions. Pretreatment with ATO also alleviated renal vasoconstriction in rats with renal IRI and restored the glomerular filtration rate (Sabbatini et al. 2004). Thus, it may be safe to prescribe ATO for diabetic patients without the risk of diabetes complications (Kim et al. 2017).

Histochemical examination of Groups A2 and B2 for oxidative stress markers in the renal tissue homogenate indicated a marked increase in MDA and iNOS and a marked decrease in SOD and GR. These findings may be explained by Johnson and Weinberg (1993), who reported that damaged tissues from IRI produced excessive amounts of ROS, causing oxidative stress. Consequently, the ROS caused changes in the mitochondrial oxidative phosphorylation process,
depleted adenosine triphosphate, increased intracellular Ca2+, and activated membrane phospholipid proteases (Johnson and Weinberg 1993). During the reperfusion phase of IRI, continual blood flow produced oxygen free radicals, causing peroxidation of membrane lipids, oxidative damage of proteins and DNA, and apoptosis (Kehrer 1993). Further, the pathogenesis of IRI may be attributed to downregulation of the antioxidant enzyme system, such as SOD, catalase, and glutathione peroxidase (Kehrer 1993). The aggravated increase in MDA and iNOS and marked aggravated decrease in SOD and GR in Group B2 in the current study may be explained by Miki et al. (2012), who reported that diabetes weakened regular protective effects against ROS, thus exacerbating IRI.

Histochemical examination of Groups A3 and B3 for oxidative stress markers in the renal tissue homogenate indicated an increase in MDA and iNOS and a decrease in SOD and GR. These findings suggested that ATO could not compensate for overexpression of iNOS through eNOS expression, resulting in a deficiency of NO (Caliman et al. 2013). These findings agreed with Ito et al. (2010), who reported that ATO did not affect expression of iNOS but increased expression of eNOS in the cortex and medulla of spontaneously hypertensive rats.

The mechanism of caspase 3 activation induced by IRI that was detected in the current study, was described by Gao et al. (2003) as an intracellular encoded cell death mechanism allowing the renal epithelial cells and glomeruli, injured by IRI and oxidative stress, to destroy themselves by reducing the mitochondrial membrane potential. Subsequently, that procedure released cytochrome C, causing apoptosome formation, activating caspase cascade and other intracellular proapoptotic proteins (Gao et al. 2003). Thus, caspases are widely well-thought-out as important effectors of the apoptotic mechanism (Tarantino et al. 2011).
CD 44 is a surface molecule having many properties that are imperative for the biological activities of normal cells. CD 44 is involved in cell migration, proliferation, and differentiation. It is important for angiogenesis and normal cell membrane function. CD 44 is also vital for growth factors and cytokines as well as for cell survival (Naor et al. 2002; Rouschop et al. 2006) reported that CD44 significantly subsidized to the migration of inflammatory cells, mainly neutrophils, into the inflamed kidney tissue after IRI causing its damage and consequently renal failure. The same authors (2005) assumed that CD44 might play a significant role in the development of I/R injury, since CD44 was expressed by neutrophils and was upregulated by the endothelial cells of the glomerular capillaries after I/R injury.

In the current study, morphometric examination of Groups A1, A2, B1, and B2 indicated statistically significant differences between Groups A2 and A1 and between Groups B2 and B1 for the mean area percentages of caspase 3 and CD44. These findings supported our immune histochemical findings for non-diabetic and diabetic rats, suggesting that IRI was associated with apoptosis from caspase 3 activation and with initiation of an inflammatory reaction from CD44 expression.

Morphometric examination of Groups A1 and A3 indicated non-significant differences between these groups for the mean area percentages of caspase 3 and CD44. These findings supported our immune histochemical findings in non-diabetic rats, suggesting that administration of ATO protected against renal IRI through its antiapoptotic and antiinflammatory properties, inhibited the production of ROS, and corrected oxidative stress and endothelial dysfunction (Caliman et al. 2013). Consequently, ATO prohibited the formation of apoptotic bodies, thriving the injured kidney tissue through this antiinflammatory property by decreasing the expression of caspase 3 as an apoptotic marker. These findings were also supported by Rouschop et al. (2005)
who correlated the protection renal function and morphology to a reduced influx of neutrophils in the recovering renal tissues after AKI. Hence, future therapies directed against CD44 may succeed in preventing or reducing renal IRI (Rouschop et al. 2005). Therapies targeting CD44 by tumor-specific agents could be a future therapeutic approach against various tumors, highlighting the therapeutic potential of therapies directed against CD44 (Naor et al. 2002).

Morphometric examination of Groups B1 and B3 indicated statistically significant differences, suggesting the protective effect of ATO against renal IRI in diabetic rats was inadequate. However, these findings disagreed with our immune histochemical examination of caspase 3 and CD44, which found a moderately positive immune expression for Group B3. These findings also disagreed with Park et al. (2018), who reported a protective effect of ATO for endothelial function in diabetes. Despite these results, we selected different techniques to compare the same outcomes to avoid discrepancies caused by diabetes on renal IRI because of differences in experimental design (Chen et al. 2017).

There were several limitations concerning this study. Although the results of the current study suggested that pretreatment with ATO protected the kidney from IRI in non-diabetic rats, the protection was less evident in diabetic rats. While the relatively small sample size of groups may have affected the outcomes of the statistical analysis, the positive morphological outcomes of the histological, immune histochemical, biochemical, and histochemical analyses indicated a possible protective effect of ATO against IRI in non-diabetic and diabetic rats. A future study, with larger sample size, could determine more definitively whether there are statistical significances between Groups A1 and A3, Groups B1 and B3, and Groups A and B Another suggested study of the effect of ATO on IRI analyzing the ultrastructural parameters of the renal tissues may support the outcomes of the current study. The electron microscopic analysis may
involve the following structures; thickness of the renal filtration barrier, diameter of filtration slits, and basal lamina of the PCT and renal corpuscles (Hassan et al. 2016). Further, another study could also examine the effect of different doses of ATO to determine whether increasing the dosage would increases the difference between various study groups. Finally, the authors used a rat model, and interpretations of these results in human are discouraged and clearly generalizations cannot be made.

Although recent clinical trials suggested statins might decrease the risk of AKI following cardiac surgery due its anti-inflammatory effect (Bellomo 2016), these trials have been contradicted by other recent clinical trials. Billing et al. (2016) could not find a difference between patients receiving high-dose of perioperative ATO, compared to patients receiving placebo. While Park et al. (2016) and Zhen et al. (2017) reported that although perioperative use of ATO could decrease the inflammatory response, it had no impact on clinical outcomes and it could not be associated with reduced risk of postoperative AKI. Therefore, the use of perioperative ATO to reduce the risk of AKI following cardiac surgery remains controversial.

In conclusion, the administration of ATO seemed to alleviate the histological, immune histochemical, biochemical, and histochemical degenerative changes caused by IRI to renal cortex. The nephroprotective mechanism of ATO could have achieved through its potent antiinflammatory and free-radical–scavenging effects, however, the exact mechanism is unknown. Thus, it is important to continue investigating new preventive strategies to protect against renal IRI in non-diabetic and diabetic patients. Additional studies and clinical trials are recommended to understand the pathophysiological changes caused by diabetes and confirm the beneficial effects of ATO for patients with various inflammatory conditions and following cardiac surgery.
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909

191-197


**Figure legends**

**Figure 1.** Histological examination of hematoxylin and eosin (H&E)-stained sections of rat kidney for the study groups in the diagnostic Group A (Non-Diabetic). A. Group A1 (sham) showing classic kidney structure of the renal corpuscle formed of glomerulus (G) and proximal (PT) and distal (DT) tubules (×100). B. Group A1 showing glomerulus (G), Bowman’s capsule (BC), and proximal (PT) and distal tubules (DT). The proximal tubules have prominent brush border (arrows) (×400). C. Group A2 (I/R) renal cortex showing the glomerulus (G) with dilatation of the proximal (PT) and distal (DT) tubules, loss of the brush border (arrow), and hyaline cast formation (C) (×400). D. Group A2 corticomedullary region showing dilatation of the proximal (PT) and distal (DT) tubules. There is marked interstitial hemorrhagic exudate (H). The cells are detached from the basement membrane with pyknotic nuclei (arrowheads). The lumen contains hyaline cast (C) (×400). E. Group A3 (ATO + I/R) renal cortex with remarkable improvement and restoration of normal renal architecture seen as apparently normal renal glomerulus (G), Bowman’s capsule (BC), and proximal (PT) and distal tubules (DT) (×400). F. Group A3 corticomedullary junction showing apparently normal proximal (PT) and distal (DT) tubules with preserved intact brush border (arrows) and some hyaline cast formation (C) (×400).
**Figure 2.** Histological examination of hematoxylin and eosin (H&E)-stained sections of rat kidney for the study groups in the diagnostic Group B (Diabetic). A. Group B1 (sham) showing normal kidney structure of the renal cortex formed of glomerulus (G) and proximal (PT) and distal (DT) tubules (×400). B. Group B2 (I/R) renal cortex showing dilatation and necrosis of the proximal (PT) tubules and distal tubules (DT). The lining tubular cells are detached from the basement membrane of the tubules and have pyknotic nuclei (arrows). The interstitial tissue contains massive hemorrhagic exudate (H). The lumen of the tubules contains hyaline cast (C) (×400). C. and D. Group B2 renal medulla showing marked dilatation of the proximal (PT) and distal (DT) tubules, exfoliation of the lining epithelium (arrows), and interstitial hemorrhagic exudate (H). Note marked intratubular hyaline cast formation (C) (×400). E. Group B3 (ATO + I/R) preserved renal cortex with glomerulus (G), proximal (PT) and distal (DT) tubules that still appear dilated with vacuolation of their lining cells, pyknosis of the nuclei (arrows), and disrupted brush border (arrowheads). The tubular lumen contains hyaline casts (C) and the interstitial tissue contains hemorrhagic exudate (E) (×400). F. Group B3 renal medulla shows dilated tubules (T), necrosis, and vacuolations of the lining tubular cells (arrows) and the interstitial tissue contains hemorrhagic exudate (E) (×400).

**Figure 3.** Comparison of morphometric analysis of the histological examination (H&E) for the study groups and diagnostic groups. A. Mean glomerular area. B. Mean Area of Bowman’s Space. C. Mean luminal area of (proximal convoluted tubules) PCT. D. Mean luminal area of distal convoluted tubules (DCT). E. Mean epithelial height of PCT. Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).
Figure 4. Periodic acid-Schiff (PAS)-stained sections of rat kidney for Groups A and B showing the basement membrane of the cortical tubules (arrow) and the brush border of the proximal tubules (arrow head). A. Group A1 (sham) revealed strong PAS reaction (x400). B. Group A2 (I/R) revealed weak positive reaction. C. Group A3 (ATO + I/R) revealed moderate reaction (x400). D. Group B1 (sham) revealed strong PAS reaction (x400). E. Group B2 (I/R) revealed weak reaction (x400). F. Group B3 (ATO + I/R) revealed mild reaction (x400). G. Mean optical density of basal laminae of tubules and glomerular capillaries. Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats). H. Mean length of the brush border of the proximal convoluted tubules (PCT). Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).

Figure 5. Immune expression of caspase 3 in the rat kidney for all study groups (caspase 3 ×400). A. Group A1 (sham) showing negative immune expression. B. Group A2 (I/R) showing strong positive immune expression in the tubules. C. Group A3 (ATO + I/R) showing mild immune expression. D. Group B1 (sham) showing mild immune expression. E. Group B2 (I/R) showing strong immune expression. F. Group B3 (ATO + I/R) showing moderate immune expression. G. Mean area percentages of Caspase 3. Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).

Figure 6. Immune expression of CD44 in the rat kidney for all study groups (CD44 ×400). A. Group A1 (sham) showing mild immune expression. B. Group A2 (I/R) showing strong positive
immune expression in the renal tubules. C. Group A3 (ATO + I/R) showing negative immune
eexpression. D. Group B1 (sham) showing mild immune expression. E. Group B2 (I/R) showing
intense strong positive immune expression. F. Group B3 (ATO + I/R) showing moderate positive
immune expression. G. Mean area percentages of CD44. Two-way ANOVA results and simple
effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R)
and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).

**Figure 7.** Comparisons of the biochemical study for the study groups and the diagnostic groups
A and B. A. Mean value of serum blood urea nitrogen (BUN). B. Mean value of serum
creatinine. Two-way ANOVA and simple effects analysis results comparing study groups
(Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-
diabetic rats, Group B = diabetic rats).

**Figure 8.** Comparisons of the oxidative stress markers for the study groups and the diagnostic
groups. A. Mean values of malondialdehyde (MDA). B. Mean values of inducible nitric oxide
synthase (iNOS). C. Mean values of superoxide dismutase (SOD). D. Mean values of glutathione
reductase (GR). Two-way ANOVA and simple effects analysis results comparing study groups
(Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-
diabetic rats, Group B = diabetic rats).
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<th>Outcome Measure</th>
<th>Units</th>
<th>Diagnostic Group</th>
<th>Study Group 1</th>
<th>Study Group 2</th>
<th>Study Group 3</th>
<th>P-value*</th>
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<td>Glomerular area</td>
<td>µm²</td>
<td>Non-diabetic</td>
<td>8743.79 (564.92) ab</td>
<td>7713.79 (380.77) ac</td>
<td>7978.27 (341.28) bc</td>
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<td>Diabetic</td>
<td>7467.81 (198.67) ab</td>
<td>2935.73 (356.89) ac</td>
<td>6263.03 392.18 bc</td>
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<tr>
<td>Area of Bowman’s Space</td>
<td>µm²</td>
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<td>1791.74 (104.80) a</td>
<td>3012.84 (211.65) ac</td>
<td>1939.98 (104.81) c</td>
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<td></td>
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<td>2948.19 (203.05) a</td>
<td>2747.65 (353.96) b</td>
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<td>Epithelial height</td>
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<td>6.82 (.88) a</td>
<td>8.00 (1.37) b</td>
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<tr>
<td>Luminal area PCT</td>
<td>µm</td>
<td>Non-diabetic</td>
<td>199.24 (9.96) a</td>
<td>566.79 (105.96) ab</td>
<td>277.05 (92.46) b</td>
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<td>599.29 (73.56) ac</td>
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<td>Luminal area DCT</td>
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<td>784.12 (136.45) a</td>
<td>1087.22 (166.78) ac</td>
<td>829.41 (129.29) c</td>
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<td>Diabetic</td>
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<td>1404.88 (345.09) a</td>
<td>1197.43 (280.85) b</td>
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<td>Optical Density of basal lamina</td>
<td>density</td>
<td>Non-diabetic</td>
<td>1.15 (.35) a</td>
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<td>1.34 (.31) c</td>
<td>.002</td>
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<td>.49 (.19) a</td>
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<td>Length Brush Border of PCT</td>
<td>µm</td>
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<td>3.00 (.28) a</td>
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<td>4.02 (.32) a</td>
<td>2.02 (.32) ac</td>
<td>3.19 (.65) c</td>
<td>&lt; .001</td>
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<td>Area Percentage of Caspase 3</td>
<td>area %</td>
<td>Non-diabetic</td>
<td>1.32 (.40) ab</td>
<td>10.12 (1.54) ac</td>
<td>4.07 (.32) bc</td>
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<td>Diabetic</td>
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<td>Area Percentage of CD44</td>
<td>area %</td>
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<td>1.84 (.47) ab</td>
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<td>BUN</td>
<td>mg/dL</td>
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<td>Creatinine</td>
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<td>.88 (.18) a</td>
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<td>MDA</td>
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<td>16.22 (3.73) a</td>
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<td>iNOS</td>
<td>nmol/mg</td>
<td>Non-diabetic</td>
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<td>5.28 (1.23) ac</td>
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<td>.58 (.49) ac</td>
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<td>1.12 (.36) ac</td>
<td>2.76 (.16) bc</td>
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Table 1. Results for outcome measures for all study groups.
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<th></th>
<th>Diabetic</th>
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<td></td>
<td>3.68 (.26) ab</td>
<td>.86 (.15) ac</td>
<td>2.14 (.19) bc</td>
<td>&lt; .001</td>
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**Note:** Values are reported as means (SD). Group 1 = sham, Group 2 = I/R, and Group 3 = ATO + I/R. The following significant pairwise comparisons were found between groups (P < .05): a, Group 1 versus Group 2; b, Group 1 versus Group 3; c, Group 2 versus Group 3.

*One-way ANOVA to test for intergroup differences*
Figure 1. Histological examination of hematoxylin and eosin (H&E)-stained sections of rat kidney for the study groups in the diagnostic Group A (Non-Diabetic). A. Group A1 (sham) showing classic kidney structure of the renal corpuscle formed of glomerulus (G) and proximal (PT) and distal (DT) tubules (×100). B. Group A1 showing glomerulus (G), Bowman’s capsule (BC), and proximal (PT) and distal tubules (DT). The proximal tubules have prominent brush border (arrows) (×400). C. Group A2 (I/R) renal cortex showing the glomerulus (G) with dilatation of the proximal (PT) and distal (DT) tubules, loss of the brush border (arrow), and hyaline cast formation (C) (×400). D. Group A2 corticomedullary region showing dilatation of the proximal (PT) and distal (DT) tubules. There is marked interstitial hemorrhagic exudate (H). The cells are detached from the basement membrane with pyknotic nuclei (arrowheads). The lumen contains hyaline cast (C) (×400). E. Group A3 (ATO + I/R) renal cortex with remarkable improvement and restoration of normal renal architecture seen as apparently normal renal glomerulus (G), Bowman’s capsule (BC), and proximal (PT) and distal tubules (DT) (×400). F. Group A3 corticomedullary junction showing apparently normal proximal (PT) and distal (DT) tubules with preserved intact brush border (arrows) and some hyaline cast formation (C) (×400).

61x61mm (300 x 300 DPI)
Figure 2. Histological examination of hematoxylin and eosin (H&E)-stained sections of rat kidney for the study groups in the diagnostic Group B (Diabetic). A. Group B1 (sham) showing normal kidney structure of the renal cortex formed of glomerulus (G) and proximal (PT) and distal (DT) tubules (×400). B. Group B2 (I/R) renal cortex showing dilatation and necrosis of the proximal (PT) tubules and distal tubules (DT). The lining tubular cells are detached from the basement membrane of the tubules and have pyknotic nuclei (arrows). The interstitial tissue contains massive hemorrhagic exudate (H). The lumen of the tubules contains hyaline cast (C) (×400). C. and D. Group B2 renal medulla showing marked dilatation of the proximal (PT) and distal (DT) tubules, exfoliation of the lining epithelium (arrows), and interstitial hemorrhagic exudate (H). Note marked intratubular hyaline cast formation (C) (×400). E. Group B3 (ATO + I/R) preserved renal cortex with glomerulus (G), proximal (PT) and distal (DT) tubules that still appear dilated with vacuolation of their lining cells, pyknosis of the nuclei (arrows), and disrupted brush border (arrowheads). The tubular lumen contains hyaline casts (C) and the interstitial tissue contains hemorrhagic exudate (E) (×400). F. Group B3 renal medulla shows dilated tubules (T), necrosis, and vacuolations of the lining tubular cells (arrows) and the interstitial tissue contains hemorrhagic exudate (E) (×400).
Figure 3. Comparison of morphometric analysis of the histological examination (H&E) for the study groups and diagnostic groups. A. Mean glomerular area. B. Mean Area of Bowman’s Space. C. Mean luminal area of (proximal convoluted tubules) PCT. D. Mean luminal area of distal convoluted tubules (DCT). E. Mean epithelial height of PCT. Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).
Figure 4. Periodic acid-Schiff (PAS)-stained sections of rat kidney for Groups A and B showing the basement membrane of the cortical tubules (arrow) and the brush border of the proximal tubules (arrow head). A. Group A1 (sham) revealed strong PAS reaction (x400). B. Group A2 (I/R) revealed weak positive reaction. C. Group A3 (ATO + I/R) revealed moderate reaction (x400). D. Group B1 (sham) revealed strong PAS reaction (x400). E. Group B2 (I/R) revealed weak reaction (x400). F. Group B3 (ATO + I/R) revealed mild reaction (x400). G. Mean optical density of basal laminae of tubules and glomerular capillaries. Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats). H. Mean length of the brush border of the proximal convoluted tubules (PCT). Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).
Figure 5. Immune expression of caspase 3 in the rat kidney for all study groups (caspase 3 ×400). A. Group A1 (sham) showing negative immune expression. B. Group A2 (I/R) showing strong positive immune expression in the tubules. C. Group A3 (ATO + I/R) showing mild immune expression. D. Group B1 (sham) showing mild immune expression. E. Group B2 (I/R) showing strong immune expression. F. Group B3 (ATO + I/R) showing moderate immune expression. G. Mean area percentages of Caspase 3. Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).
Figure 6. Immune expression of CD44 in the rat kidney for all study groups (CD44 ×400). A. Group A1 (sham) showing mild immune expression. B. Group A2 (I/R) showing strong positive immune expression in the renal tubules. C. Group A3 (ATO + I/R) showing negative immune expression. D. Group B1 (sham) showing mild immune expression. E. Group B2 (I/R) showing intense strong positive immune expression. F. Group B3 (ATO + I/R) showing moderate positive immune expression. G. Mean area percentages of CD44. Two-way ANOVA results and simple effects analysis comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).

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Figure 7. Comparisons of the biochemical study for the study groups and the diagnostic groups A and B. A. Mean value of serum blood urea nitrogen (BUN). B. Mean value of serum creatinine. Two-way ANOVA and simple effects analysis results comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).
Figure 8. Comparisons of the oxidative stress markers for the study groups and the diagnostic groups. A. Mean values of malondialdehyde (MDA). B. Mean values of inducible nitric oxide synthase (iNOS). C. Mean values of superoxide dismutase (SOD). D. Mean values of glutathione reductase (GR). Two-way ANOVA and simple effects analysis results comparing study groups (Group 1 = sham, Group 2 = I/R, Group 3 = ATO + I/R) and diagnostic groups (Group A = non-diabetic rats, Group B = diabetic rats).