The glucagon-like peptide-1 receptor agonist Exendin-4, ameliorates contrast-induced nephropathy through suppression of oxidative stress, vascular dysfunction and apoptosis independent of glycaemia

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Summary
Contrast-induced nephropathy (CIN) is a leading cause of hospital-acquired acute kidney injury, particularly in diabetic patients. Previous studies have shown renoprotective effects of glucagon-like peptide-1 (GLP-1) signalling; however, its role in CIN remains unexplored. This study investigates the prophylactic effect of exendin-4, a GLP-1R agonist, against CIN in a rat model mimicking both healthy and diabetic conditions. Animals were randomly divided into 7 groups: a control sham group (n = 8), and 2 identical sets of 3 disease groups, one received exendin-4 before exposure to contrast medium (CM), while the other served as untreated control. The 3 disease groups represented diabetes (n = 8), CIN (n = 8), or diabetes and CIN combined (n = 8). Untreated groups showed deteriorating renal function as indicated by significantly higher levels of serum creatinine and blood urea nitrogen, malondialdehyde, and endothelin-1 and caspase-3 expression compared to the sham control group. This was accompanied by a significant decrease in tissue reserves of reduced glutathione, superoxide dismutase, nitrate and endothelin nitric oxide synthase as well as deteriorating renal histology. The CM-induced changes in diabetic rats indicate impaired renal function, oxidative stress, vascular dysfunction, and apoptosis, and were significance higher in intensity compared to non-diabetic rats. Pretreatment with exendin-4 ameliorated all the aforementioned CM-induced nephropathic effects independent of the glycemic state. To our knowledge, this is the first study describing the prophylactic renoprotective effects of exendin-4 against CIN. With the current pharmaceutical use of exendin-4 as a hypoglycaemic agent, the GLP-1R agonist becomes an interesting candidate for human clinical trials on CIN prevention.

KEYWORDS
contrast-induced nephropathy, diabetes, endothelin-1, eNOS, exendin-4

1 INTRODUCTION

Contrast-induced nephropathy (CIN) is the third leading cause of hospital-acquired acute kidney injury. With the wide use of iodinated contrast media in diagnostic and interventional procedures, the incidence rate of CIN ranges from 12% to 27% in the general population, and reaches up to 50% in high-risk patients suffering from chronic renal diseases or diabetes. CIN is typically associated with long-term suppression of renal function, necessity for dialysis, and higher risk for cardiovascular complications and fatality.

The mechanisms underlying the development of CIN are complex and not fully understood. Previous reports have suggested...
the involvement of various pathological cascades such as oxidative stress, renal ischaemia, endothelial dysfunction, inflammation, apoptosis and dysfunction of tubular transport. In addition, some risk factors can contribute to the development of CIN, most significantly diabetes. The latter potentially predisposes the kidney to CIN through several pathophysiological mechanisms, including the intensification of the renal hemodynamic changes affecting tubular transport activity and glomerular filtration rate, and the elevation of oxygen expenditure, medullary hypoxia and generation of reactive oxygen species (ROS).

Glucagon-like peptide-1 (GLP-1) is a gut incretin hormone that stimulates glucose-dependent insulin secretion from the pancreas. However, in addition to its insulinotropic effect, GLP-1 is also involved in the regulation of various physiological functions in non-digestive organs such as the brain, heart, lung and kidney. With regard to the kidney, experimental studies have shown GLP-1 or GLP-1R agonists to induce natriuresis and diuresis in the healthy state, while reducing albuminuria, glomerular sclerosis, oxidative stress, endothelial injury, and inflammation in models of diabetic kidney disease (DKD) and acute toxic renal injury. Despite this knowledge, the potential protective and curative benefits of GLP-1 signalling in CIN remains unexplored.

The majority of the studies investigating the clinical and therapeutic implications of the renal GLP-1 signalling have relied on GLP-1R agonists rather than GLP-1. This is particularly due to the short half-life of GLP-1, which is rapidly degraded in the circulation by the di-peptidylpeptidase-IV (DPP-4) enzyme. By contrast, GLP-1R agonists, including the widely explored exendin-4, are resistant to DPP-4 degradation and thereby exert a long-acting effect.

Our study aims to investigate the possible prophylactic effects of exendin-4 pretreatment against CIN in a rat model of the disease, under both healthy and diabetic conditions. It also examines some of the pathological mechanisms underlying the incidence of CIN, including oxidative stress, apoptosis and haemodynamic alterations.

## RESULTS

### 2.1 Effect of exendin-4 on renal function and blood glucose

Injection of STZ resulted in a significant increase in blood glucose and urinary albumin excretion compared to the sham control group ($P < .05$). In addition, a non-significant increase in serum creatinine and blood urea nitrogen (BUN) was observed in STZ-injected groups together with a non-significant decrease in creatinine clearance (Table 1).

Injection of contrast medium (CM) caused a significant increase in serum creatinine, BUN and urinary albumin excretion, and a significant decrease in creatinine clearance compared to the sham control group ($P < .05$). These changes were similarly observed in both non-diabetic and diabetic CIN groups. However, the latter exhibited a significantly higher magnitude compared to the former ($P < .05$) (Table 1).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Control</th>
<th>CIN</th>
<th>CIN + Exendine-4</th>
<th>Diabetes</th>
<th>Diabetes + Exendine-4</th>
<th>Diabetes + CIN</th>
<th>Diabetes + CIN + Exendine-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>102 ± 6.5</td>
<td>101.8 ± 7.3</td>
<td>101.6 ± 5.7</td>
<td>342.8 ± 13.7</td>
<td>241.8 ± 8.9</td>
<td>242.4 ± 9.8</td>
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</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.66 ± 0.12</td>
<td>0.75 ± 0.13</td>
<td>0.72 ± 0.12</td>
<td>1.2 ± 0.4</td>
<td>0.73 ± 0.13</td>
<td>0.73 ± 0.13</td>
<td>0.73 ± 0.13</td>
</tr>
<tr>
<td>Serum BUN (mg/dL)</td>
<td>27.2 ± 4.7</td>
<td>29.9 ± 5.5</td>
<td>29.3 ± 4.3</td>
<td>56.1 ± 9.1</td>
<td>29.3 ± 4.3</td>
<td>29.3 ± 4.3</td>
<td>29.3 ± 4.3</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>1.16 ± 0.44</td>
<td>0.71 ± 0.33</td>
<td>1.07 ± 0.39</td>
<td>1.16 ± 0.44</td>
<td>0.71 ± 0.33</td>
<td>1.07 ± 0.39</td>
<td>1.07 ± 0.39</td>
</tr>
<tr>
<td>Urinary proteins (g/L)</td>
<td>0.13 ± 0.06</td>
<td>0.45 ± 0.17</td>
<td>0.23 ± 0.09</td>
<td>0.23 ± 0.09</td>
<td>0.23 ± 0.09</td>
<td>0.23 ± 0.09</td>
<td>0.23 ± 0.09</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD. Statistical differences were determined using 1-way ANOVA with LSD post hoc test. $P < .05$. 

$^a$: significant vs the control group; $^b$: significant vs the CIN group; $^c$: significant vs the CIN plus Exendine-4 group; $^d$: significant vs the diabetes group; $^e$: significant vs the diabetes plus Exendine-4 group; $^f$: significant vs the diabetes plus CIN group.
Treating the non-diabetic CIN group with exendin-4 before CM injection resulted in a significant decrease in serum creatinine, BUN and urinary albumin excretion, and a significant increase in creatinine clearance compared to the respective untreated group (P < .05) (Table 1). Similar protective effects were observed in the diabetic CIN group compared to the respective untreated group (P < .05). In addition, regardless of CIN involvement, exendin-4 treatment has resulted in a significant decrease in blood glucose levels in the diabetic groups compared to the respective untreated groups (P < .05) (Table 1).

2.2 | Effect of exendine-4 on oxidative stress and vascular dysfunction markers

Contrast media induced a significant increase in malondialdehyde (MDA) and endothelin-1 expression compared to the sham control group, with a significant decrease in nitrate, reduced glutathione (GSH), superoxide dismutase (SOD) and endothelial nitric oxide synthase (eNOS) expression (P < .05) (Table 2). The CM-induced changes were evident in both the diabetic and non-diabetic CIN groups, but with a significantly higher intensity in the diabetic group (P < .05). Similarly, a significant increase in MDA and endothelin-1 expression, with a significant decrease in nitrate, GSH, SOD and eNOS expression, were observed in the diabetic CIN-free group compared to the sham control group (P < .05) (Table 2).

Pretreatment of the CIN groups with exendin-4 resulted in a significant decrease in MDA and endothelin-1 expression with a significant increase in nitrate, GSH, SOD and eNOS expression. This was evident in both the non-diabetic and diabetic groups compared to the untreated diabetic group (P < .05) (Table 2). Exendin-4 exerted the same effects in the diabetic CIN-free group compared to the untreated diabetic group (P < .05).

2.3 | Effects of exendine-4 on the apoptosis marker caspase-3

Exposure to CM resulted in a significant increase in caspase-3 expression in the non-diabetic CIN group (Figure 1B) compared to the sham control group (Figure 1A). Treatment with exendin-4 caused a significant decrease in caspase-3 expression in the non-diabetic CIN group (Figure 1C) compared to the sham control group (Figure 1A) (Figure 1B). Similarly, a significant increase in caspase-3 expression in the non-diabetic CIN group (Figure 1C) was observed in both the non-diabetic and diabetic groups compared to the untreated diabetic group (P < .05) (Table 2). Pretreatment of the CIN groups with exendin-4 resulted in a significant decrease in caspase-3 expression compared to the untreated diabetic group (P < .05) (Table 2).

| TABLE 2 | Levels of oxidative stress and vascular dysfunction markers |
|-----------------------------------------------|
| **Sham control** | **CIN** | **CIN + Exendine-4** | **Diabetes** | **Diabetes + Exendine-4** | **Diabetes + CIN** | **Diabetes + CIN + Exendine-4** |
| MDA (nmol/g) | 1.9 ± 0.3 | 4.2 ± 0.8a | 2.1 ± 0.39b | 3.2 ± 0.63abc | 2.1 ± 0.45d | 8.5 ± 1.9bde | 2.7 ± 0.4bdf |
| GSH (mg/g) | 55.6 ± 8.7 | 25 ± 5.4a | 46 ± 7.4ab | 33.9 ± 6.5abc | 49.5 ± 9.31bd | 12.8 ± 3.33bcde | 49.2 ± 8.33def |
| SOD (u/g) | 7.6 ± 1.3 | 5.1 ± 0.9a | 7.2 ± 1.5b | 5.3 ± 0.8bc | 7.6 ± 1.6d | 3.1 ± 0.46bcde | 6.9 ± 1.44def |
| Nitrate (nmol/mg protein) | 30.7 ± 4.2 | 18.3 ± 2.5a | 27.4 ± 3.2b | 16.4 ± 3.3c | 26.4 ± 4.2bd | 10.2 ± 2.7bcde | 25.5 ± 5.3abdef |
| eNOS relative expression | 1.8 ± 0.04 | 0.45 ± 0.01a | 1.03 ± 0.04ab | 0.9 ± 0.02abc | 1.4 ± 0.02abcd | 0.3 ± 0.04bcdef | 1.3 ± 0.23bcdef |
| Endothelin-1 relative expression | 33.2 ± 7.4 | 67.2 ± 6.6a | 39.3 ± 8.5b | 56.5 ± 11.4abc | 34.2 ± 9.5bd | 83.3 ± 13.3bcde | 38.3 ± 10.6bdef |

MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; eNOS, endothelial nitric oxide synthase; a: significant vs the control group; b: significant vs the CIN group; c: significant vs the CIN plus Exendine-4 group; d: significant vs the diabetes group; e: significant vs the diabetes plus Exendine-4 group; f: significant vs the diabetes plus CIN group.

Data expressed as mean ± SD. Statistical differences were determined using 1-way ANOVA with LSD post hoc test (P < .05).
and 2.5 ± 0.38 vs 10.1 ± 1.1, 15.8 ± 0.89 and 4.6 ± 0.42, respectively; P < .05) (Figure 1H).

**2.4 | Effects of exendine-4 on renal histology**

Exposure of the non-diabetic group to CM caused moderate glomerular swelling, haemorrhage and oedema (Figure 2B) (score 3.1 ± 0.16, Figure 2H) compared to normal renal histology and architecture in the sham control group (Figure 2A). The CM-induced damage was more intense in the diabetic group (Figure 2F) compared to the non-diabetic CIN group (Figure 2B), as evident by the severe haemorrhage, inflammatory cell infiltration and intratubular cast formation (score 4.9 ± 0.18 vs 3.1 ± 0.16, respectively; P < .05) (Figure 2H). The diabetic CIN-free group also showed moderate glomerular swelling, moderate oedema and inflammatory cell infiltration (Figure 2D) (score 1.1 ± 0.1) compared to the sham control group.

Administering exendine-4 before the exposure to CM ameliorated renal damage in all treated groups. The treated diabetic CIN group exhibited moderate edema and tubular vacuolization (Figure 2G) compared to the respective disease group (Figure 2F) (score 1.96 ± 0.06 vs 4.9 ± 0.18, respectively; P < .05, Figure 2H). Similarly, the non-diabetic CIN group only showed mild haemorrhage and vacuolar degeneration in the proximal convoluted tubules (Figure 2C) compared to the untreated CIN group (Figure 2B) (score 1.4 ± 0.09 vs 3.1 ± 0.16, respectively; P < .05; Figure 2H). Finally, the diabetic CIN-free group showed only mild oedema (Figure 2D) (score 1.1 ± 0.1, Figure 2A) compared to the sham control group.
3 | DISCUSSION

Contrast-induced nephropathy poses a significant risk of renal impairment in patients undergoing CM-based diagnostic procedures. The pathogenesis typically involves reduced renal perfusion, renal hypoxia, oxidative stress, and other systemic effects including pulmonary ventilation-perfusion mismatch, reduced cardiac output and altered blood rheology. The risk is even higher in diabetic patients, particularly due to the deteriorating effects of hyperglycaemia on renal function and physiology. Exendin-4 is a well-established...
hypoglycaemic agent with potential renoprotective effects against diabetic nephropathy as well as non-diabetic toxic renal injury. Indeed, our study shows that pretreatment with exendin-4 ameliorates the CIN-induced renal impairment, oxidative stress, apoptosis and cell injury. These renoprotective effects were evident in models representing both the healthy and diabetic state.

In our model, intravenous injection of CM caused a deterioration of renal function as indicated by high levels BUN and urinary albumin and reduced creatinine clearance, and enhanced oxidative stress as indicated by the elevated serum MDA, and reduced GSH and SOD. Following glomerular passage, CM is not reabsorbed by the renal tubules. This increases the concentration and viscosity of the tubular fluid, which subsequently increases the tubular hydrostatic pressure. As a result, the renal perfusion pressure and renal blood flux are diminished to eventually induce medullary hypoxia. Both impaired renal perfusion and hypoxia can increase oxidative stress, conceivably by triggering an excessive generation of ROS. The latter can further alter renal medullary oxygenation by enhancing tubular transport activity and oxygen consumption. In addition, CM seemed to compromise endothelial constriction and integrity as revealed by the high endothelin-1 expression and the low eNOS expression and nitric oxide release. Previous studies attributed the CM-induced expression of renal endothelin-1 by the activation of endothelin-converting enzyme-1 and by direct ROS-induced endothelial damage. Additionally, other studies attributed glomerular endothelial dysfunction, and reduced eNOS expression to the activation of the β isoform of protein kinase C by hyperglycemia. Exposure to CM also seemed to enhance apoptosis as indicated by the elevated caspase-3 expression. CM-induced apoptosis was previously explained by the disturbance of mitochondrial enzyme activity and mitochondrial membrane potential in tubular epithelium, or by the activation of shock proteins and the synchronous inhibition of cytoprotective enzymes and prostaglandins.

On the tissue level, exposure to CM caused vacular degeneration in tubular epithelium, which can be a result of direct cell toxicity through cell membrane damage.

With regard to the role of diabetes, all markers for renal function, oxidative stress, vascular dysfunction, and apoptosis as well as the renal histology were more adversely affected in the diabetic CIN group compared to the respective non-diabetic control. This outcome is not surprising, considering the vast evidence identifying the role of the diabetic hemodynamic changes, oxidative stress, and apoptotic hypoxia and oxidative stress in aggravating the pathophysiology of CIN.

Pretreatment with exendine-4 showed protective effects against all the aforementioned pathophysiological changes induced by CIN in diabetic rats (Tables 1 and 2, Figures 1 and 2). Exendin-4 is a GLP-1R agonist that exerts the same biological actions of GLP-1, including the glucose-dependent stimulation of insulin secretion, and inhibition of glucagon release. It is currently being used for glycaemic control in diabetic patients. Accordingly, it is plausible that the protective effect of exendin-4 is mediated through glycemic control, reducing the burden of hyperglycaemia on renal perfusion and medullary hypoxia and apoptosis. Indeed, our results show that exendin-4 pretreatment in diabetic rats challenged with CM injection have reduced blood glucose level, alongside the improved renal function and physiology. These findings are consistent with previous studies describing the protective effect of exendin-4 against diabetic renal injury, including mesangial expansion and albuminuria through reduction of renal oxidative stress, protection of glomerular endothelial cells and suppression of renal inflammatory cytokines in hyperglycaemic rats receiving high doses of STZ.

In addition to the discussed hypoglycaemic role of exendin-4 in renoprotection of diabetic rats against CIN, it exerted the same protective effects in non-diabetic or healthy rats. This effect does not seem to be glucose-dependent as these non-diabetic rats showed no change in blood glucose levels, which remained similar to the sham control group. A previous study has described the absence of hypoglycaemic effect on exendin-4 in rats with physiological blood glucose levels. Thus, in absence of hyperglycaemia, the renoprotective effects of exendin-4 could be attributed to a direct action on the kidney. The expression of the GLP-1R in not limited to the pancreas, and is present in other non-digestive organs including the kidney. This supports that the renoprotective effect of exendin-4 may extend beyond glycaemic control to a direct effect on the kidney. Indeed, previous studies have demonstrated the protective effect of exendin-4 against perfusion impairment induced by acute renal injuries in non-diabetic animal models. Furthermore, the direct effect of exendin-4 offers a plausible basis for the ability of exendin-4 to alleviate oxidative stress, and endothelial dysfunction, and apoptosis in our study. Although the exact localization of the GLP-1R in the kidney is unclear, several studies have reported its presence in tubular epithelium, glomerular capillaries and renal vascular walls. In this context, it has been proposed that GLP-1 signalling could upregulate cyclic adenosine monophosphate protein kinase A in these cells, which inhibit the production of ROS. With regard to endothelial dysfunction, it has been postulated that phosphorylation of nuclear factor-kappa B (NF-kB), a key nucleus transcriptional factor which regulates the expression of a number of genes may play a key role in the reduction in endothelin-1 expression as well as in the increase in eNOS expression by GLP-1R agonists in the endothelial cells. Finally, and with regard to apoptosis, it has been suggested that GLP-1R agonists exert renoprotective effects by targeting cAMP and phosphatidylinositol 3-kinase dependent signalling pathway, which is known to inhibit apoptosis. Other studies documented that anti-apoptotic effect of exendine-4 is by decreasing caspase-3 activity in renal tissue of renal ischaemia reperfusion models.

To our knowledge, our study is the first to demonstrate the renoprotective effects of prophylactic exendine-4 treatment against CIN by inhibiting oxidative stress and, apoptosis and maintaining normal vascular tone, and cell integrity. The effect is reproducible in both diabetic and non-diabetic condition through hypoglycaemic as well as direct effects on renal tubular and vascular cell. Bearing in mind the current pharmaceutical use of exendin-4 as a glycaemic control agent, our findings proposes exendine-4 as a
promising candidate for prophylactic therapy against CIN in humans, particularly diabetic and renal impairment patients.

4 | MATERIALS AND METHODS

4.1 | Animals

Male Sprague-Dawley rats, weighing 220 ± 20 g, were obtained from the Experimental Animal Unit of the Faculty of Agriculture, Moshtohor, Benha University. The rats were kept under 12:12 hour light: dark cycle and were given standard laboratory diet and water ad libitum. Animal handling and experimental procedures were performed with respect to the guide of the US national institute of health on use and care of laboratory animals. The experimental protocol was approved by the Animal Research Ethics Committee of the Faculty of Medicine, Benha University, Egypt.

4.2 | Induction of diabetes

Diabetes was induced by a single intraperitoneal (IP) injection of STZ (Sigma Aldrich, St. Louis, MO, USA), containing a dose of 50 mg/kg. The STZ solution was prepared in 0.1 mol/L sodium citrate solution, at pH 4.5. Prior to STZ injection, 1 mL of 50% aqueous glucose solution was orally administrated to prevent initial hypoglycaemia. After 48 hours blood samples were collected from the animals and blood glucose levels were measured using a glucometer and strips. Rats with blood glucose level of more than 250 mg/dL were considered diabetic and remained in the study.

4.3 | Induction of CIN

Contrast-induced nephropathy was induced by a single intravenous (IV) injection of iodinated CM containing 76% diatrizoate meglumine (XinYi, Shanghai, China), which is routinely used for renal imaging. As previously described by Duan et al., the commercial preparation was diluted in water to a concentration of 300 mg/mL, of which, 10 mL/kg was prepared in physiological saline and injected through the tail vein. CIN was diagnosed by measuring serum creatinine levels 48 hours after injection, with rats exceeding 1 mg/dL considered positive and included in the analysis.

4.4 | Experimental design and groups

The experimental animals were randomly allocated to 7 groups: a control group (n = 8), and 2 homogenous sets of 3 disease group, one received exendin-4 while the other served as untreated disease control. The 3 disease groups represented diabetes (n = 8), CIN (n = 8), or diabetes and CIN combined (n = 8). Animals undergoing the same experimental conditions were pooled till further allocation to an independent disease or treatment group.

A schematic illustration of the experimental design and interventional time-points is presented in Figure 3. In summary, the diabetic groups received a single IP injection of STZ at day 0, while the non-diabetic groups only received the sodium citrate solution. The induction of diabetes was confirmed by measuring blood glucose at day 2. The CIN groups received IV injection with the CM at day 12. The treatment groups received a daily subcutaneous (SC) injection with 25 mmol/kg exendin-4 from day 3 till day 13, while the nontreated groups only received the exendin-4's saline buffer. The sham control group received an IP injection with the sodium citrate solution at day 0, a daily SC injection with the exendin-4's saline buffer, and an IV injection with the CM's saline buffer.

During day 13, the rats were placed in metabolic cages and 24-hour urine was collected. At day 14, all rats were euthanized using thiopental sodium (5 mg/kg). Blood was drawn via cardiac puncture, and both kidneys were collected. The kidneys were further dissected into 2 longitudinal halves; the first one was placed into formalin 10% for histopathological and immunohistochemical staining, while the second one was rapidly frozen and stored in liquid nitrogen (−80°C) for measurement of oxidative stress and markers and real-time PCR.

4.5 | Measurements of blood glucose and renal function tests

Blood glucose and biochemical markers of renal function were measured by colorimetric commercial kits according to manufacturer instructions. Serum creatinine, urine creatinine and BUN were measured using respective kits from Diamond Diagnostics (Cairo, Egypt), while urinary protein extraction was measured by a respective kit from Fortress Diagnostics Limited (Antrim, UK). Blood glucose was estimated by the oxidase-peroxidase method using the GOD-POD kit (Biodiagnostics, Dokki, Giza, Egypt). Creatinine clearance was estimated through the following equation as described by Hussein et al.:  

\[
\text{Urine clearence (mL/min) = } \frac{\text{Urine creatinine (mg/dL)} \times \text{Urine output (mL/24h)}}{\text{Serum creatinine (mg/dL)} \times 1440 \text{(min)}}
\]

4.6 | Biochemical antioxidant assays

About 100 mg of kidney tissues were homogenized in 2 mL of an ice-cold buffer composed of 50 mmol/L potassium phosphates, 1 mmol/L EDTA, pH 7.5, using a mortar and pestle. The homogenate was then centrifuged for 15 minutes at 4°C, and the resultant supernatant was kept at −20°C until analysis. The biochemical antioxidant markers MDA, GSH, SOD were measured using a colorimetric method according to the manufacturer’s instructions (Biodiagnostics). Nitrate, the stable metabolic product of kidney tissues NO, was measured by Griess reaction for the simultaneous evaluation of nitrate concentrations according to Moshage et al.

4.7 | Real-time PCR

Kidney tissues (50-100 mg) were disrupted in 1 mL of Trizol to collect total RNA according to the manufacturer’s instructions (Invitrogen, Grand Island, NY, USA). Reverse transcription was performed using 1 μg total RNA and a cDNA kit (high-capacity cDNA archive kit). The
concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm. The amount of eNOS and ET-1 mRNA was determined with ABI Prism 7000HT quantitative real-time PCR (Applied Biosystems, Foster City, CA, USA). The primer sequences for the targeted genes are listed in Table 3. The thermal cycle was set as follows: inactivation of reverse transcriptase at 95°C for 10 minutes, followed by 45 cycles of 95°C for 30 second, 60°C for 1 minute, and 72°C for 30 second. The specificity of the PCR results was confirmed by dissociation curve analysis. Using ABI’s SDS software, the data were depicted as sigmoid shaped amplification plots in which the number of cycles is plotted against fluorescence on a linear scale. The threshold cycle serves as a tool for calculation of the starting template amount in each sample. Because the samples of control group and also samples of the treated group are used as calibrators, the expression levels are set to 1. Because the relative quantities of eNOS and ET-1 genes are normalized against the relative quantities of the endogenous control glyceraldehyde-3-phosphate dehydrogenase, gene expression fold-changes are calculated using the equation $2^{-\Delta\Delta ct}$.

### 4.8 Histopathology

Renal tissue specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and then cut in sequential sections of 4-5 mm thickness. These sections were stained with routine

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Primers used for real-time PCR</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Forward</td>
</tr>
<tr>
<td>eNOS</td>
<td>5’-GGACCCAAAGTTTCCTCGAGTAA-3’</td>
</tr>
<tr>
<td>ET-1</td>
<td>5’-CCTGGACATCATCTGGGTC-3’</td>
</tr>
<tr>
<td>GADPH</td>
<td>5’TATCCGGACGCTGTAC-3’</td>
</tr>
</tbody>
</table>

eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

**FIGURE 3** Schematic presentation of the experimental design and protocol. STZ, streptozotocin; CM, contrast media; IP, intraperitoneal; IV, intravenous; SC, subcutaneous; *, diabetes is confirmed through blood tests; ∞, collection of 24-hour urine; †, sacrifice and blood and renal tissue collection; ‡, serum creatinine is performed to confirm CIN.
H&E. The criteria of injury and necrosis were scored according to the method described by Ahmad et al., as follows: 0, normal histology; 1, minor oedema and minor cell swelling; 2, moderate haemorrhage, moderate oedema, moderate cells swelling and vacuolization; 3, moderate haemorrhage, moderate oedema, moderate cells swelling and vacuolization; 4, severe oedema, severe cells swelling and vacuolization; 5, severe oedema, severe cells swelling and vacuolization with intratubular cast formation. The renal injury was evaluated using a scoring system grading tubular dilatation, tubular necrosis, loss of brush border, and cast formation in 10 randomly chosen, non-overlapping fields. The severity of renal injury was semi-quantified by the following criteria: 0, none; 1, 0%-10%; 2, 11%-25%; 3, 26%-45%; 4, 46%-75%; 5, 76%-100%, as described previously.

4.9 | Immunohistochemistry

Immunohistochemistry was used to localize the caspase-3 antigen in renal tissue as described previously. Caspase-3 antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were put at 4°C overnight. The appropriate horseradish peroxidase conjugated secondary antibodies were added at dilution of 1:6000, followed by 1 hour incubation at room temperature. Caspase-3 staining-based apoptotic index was estimated as previously described. The mean area percentage of caspase-3 immunostaining was quantified in 10 images of high-power magnification (×400) for each group using Image-Pro Plus software version 6.0 (Media Cybernetics, Bethesda, MD, USA).

4.10 | Statistical analysis

All the data are presented as mean ± standard deviation (SD). Comparisons among groups were performed with 1-way ANOVA with post hoc test (LSD) using SPSS 19.0 software. A P value below .05 was considered significant.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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