[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

ORIGINAL ARTICLE

Exenatide protects renal ischemia reperfusion injury in type 2 diabetes mellitus

Jitendra Vaghasiya, Navin Sheth[1], Yagnik Bhalodia[2], Malaviya Shailesh[2], Nurudin Jivani[2]

Department of Pharmacetical Sciences, Jodhpur National University, Jodhpur, Rajasthan, [1]Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, [2]Department of Pharmacology, RBPMPC, Atkot - 360 040, Gujarat, India

Background: In view of the reported efficacy of glucagons-like peptide-1 (GLP-1) on ischemia reperfusion (I/R) injury, this study was designed to assess the effect of exenatide (GLP-1 receptor agonist) on renal I/R in type 2 diabetes mellitus (T2DM). **Materials and Methods:** T2DM in rats were induced by administration of nicotinamide (230 mg/kg, i.p.), 15 min prior to the single dose of streptozotocin (65 mg/kg, i.v.). *In vivo* renal I/R were performed in both T2DM and normal rats. **Results:** The lipid peroxidation, xanthine oxidase activity, and nitric oxide level in renal tissue were significantly increased after I/R in diabetic rats compared to I/R in normal rats. Antioxidant enzymes such as glutathione, superoxide dismutase, catalase, and glutathione peroxidase were significantly reduced after I/R in diabetic rats compared to normal rats. Exenatide treatment significantly normalized these biochemical parameters compared to diabetic I/R rats. Serums TNF-α level and myeloperoxidase activity in renal tissue and apoptosis were also significantly normalized after administration of exenatide. Furthermore, treatment with exenatide (10 mcg/kg) had preserved the normal morphology of the kidney compared to I/R performed in T2DM rats. **Conclusion:** In conclusion, exenatide protects exaggerated renal I/R injury in T2DM. These findings have major implication in the treatment of ischemic injury that prone to develop in T2DM.

KEY WORDS: Type 2 diabetes mellitus, exenatide, inflammation, ischemia reperfusion, kidney, oxidative stress

DOI: 10.4103/0973-3930.70863

Correspondence to: **Mr. Jitendra Vaghasiya**, Department of Pharmaceutical Sciences, Jodhpur National University, Jodhpur, Rajasthan, India. E-mail: j_vaghasiya@yahoo.com

Manuscript received: 27.08.2009; Revision accepted: 20.08.2010

Introduction

Type 2 diabetes mellitus (DM) is one of the leading causes of end-stage renal disease (ESRD).[1] Diabetics at a higher risk of an ischemic condition caused by decreased blood flow.[2] With increasing duration and severity of ischemia, however, greater cell damage can develop, with a predisposition to a spectrum of reperfusion-associated pathologies, collectively called reperfusion injury.[3] The recent study have demonstrated higher incidence of nephropathy in type 2 DM than in type 1 DM patients.[4] The mechanisms behind the injury in diabetic nephropathy are not fully understood despite intense research. Diabetic patients may need renal transplantation in their later life due to diabetic nephropathy. I/R injury is one of the dangerous complications of this procedure.

The short period of ischemia (30 min) in diabetes has been demonstrated to lead to reversible renal failure, leading to progressive injury with ESRD.[5] Reactive oxygen species (ROS) and nitric oxide (NO) play an important role in mediating cell damage during I/R injury.[6,7] Inflammation contributes substantially to the pathogenesis of I/R with a central role for particular cells, adhesion molecules, and cytokines.[8] Neutrophils are the inflammatory cells, which produce abundantly ROS during I/R injury. Myeloperoxidase (MPO) is found in neutrophils, which catalyze the formation of hypochlorous acid (HOCl), a toxic agent to cellular components, and initiates oxidative injury.[9] Renal I/R causes tissue injury by oxygen radicals, and oxidative stress caused by an imbalance between production of ROS and the antioxidant capacity.[10,11] Renal I/R injury may cause oxidative stress and increase lipid peroxidation in the tissue, and the rat tissue decreased shows antioxidant enzyme activities after renal I/R is well reported.[12]

Exenatide, a synthetic version of Exe-4 is presently the

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

first GLP-1 receptor agonist approved for clinical use by the Federal Drug Administration in the treatment of type 2 diabetes mellitus. Exenatide exerts insulinotropic and insulinomimetic properties via the G-protein-coupled GLP-1 receptor, which has also been reported to be expressed in various tissues like kidney.[13,14] GLP-1 have reported efficacy in ischemia reperfusion (I/R) injury.[15-17] So far, no study has proved that treatment with exenatide could protect renal I/R in T2DM, and therefore, this study was designed to investigate the effect of exenatide on renal I/R in diabetic rats.

Materials and Methods

Chemicals

Superoxide dismutase (SOD), crystalline beef liver catalase (CAT), 1,1,3,3-tetrahydroxy-propane, glutathione (GSH), and epinephrine hydrochloride were purchased from Sigma Aldrich, USA. A 1 kb DNA marker was obtained from Fermentas, Germany. R Nase A, ethidium bromide, and agarose were procured from Himedia Lab, Mumbai. Tris buffer, thiobarbituric acid, and trichloroacetic acid were purchased from Himedia Lab. Folin's phenol reagent was procured from S.D Fine Chemicals Mumbai, India, The diagnostic kits used for estimation of BUN, Creatinine and AST were purchased from Span Diagnostic Pvt. Ltd, India. All other chemicals used in the study were of laboratory grade.

Experimental groups and animals

Healthy adult Wistar rats (either sex) weighing 200–250 g were used. The experiment and protocol described in this study were approved by the Institutional Animal Ethics Committee (IAEC) of Smt. R.B.P.M.C. Atkot and with permission from committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The rats were divided into five different groups ($n = 6$). *Group-1*: Normal sham-operated (underwent all surgical procedures without I/R in normal rats); *Group-2*: Diabetic sham-operated (underwent all surgical procedures without I/R in diabetic rats); *Group-3*: In normal rats; on day 25, ischemia was produced for 30 min, followed by reperfusion of 24 h (I/R control); *Group-4*: After induction of diabetes; on day 25, I/R was produced (DM + I/R control), and *Group-5*: In diabetic rats; on day 14, exenatide (Sigma, St. Louis, MO) was injected in a dose of 10 mcg, subcutaneous, twice a day for 2 weeks, on day 24, I/R was produced (Exn + DM + I/R) [Figure 1].

Induction of T2DM and measurement of blood glucose level

T2DM in rats were induced by administration of nicotinamide (NAD) (230 mg/kg, i.p.), 15 min prior to the single dose of streptozotocin (STZ) (65 mg/kg, i.v.).[18] Control animals were received an equal volume of saline. The STZ solution contained STZ in saline with a sodium citrate buffer, pH 4.0. Food, water consumption, weight gain, and the blood glucose levels (by using standard diagnostic kits, Beacon Diagnostics Pvt. Lid.) were recorded to monitor the degree of diabetes. Four weeks elapsed in between the induction of diabetes and ischemic injury.

Renal I/R injury

Diabetic and normal rats were anesthetized with ketamine (60 mg/kg i.p.) and diazepam (5 mg/kg i.p). Body temperature was maintained throughout surgery at 37 ± 0.5°C. The skin on back was shaved and disinfected with povidone–iodine solution. All rats were undergoing surgical exposure of the left and right renal pedicles via midline incision. To induce renal ischemia, both renal pedicles were occluded for 30 min with vascular clamps. After 30 min of occlusion, the clamps were removed, and kidneys observed to undergo reperfusion for 24 h. At the end of each *in vivo* study, rats were killed, and the kidney quickly removed, placed into liquid nitrogen, and then stored at -70°C until assayed for oxidant and antioxidant parameters.

Renal function

Serum samples were assayed for blood urea nitrogen (BUN) (Jaffe's method), creatinine (DAM method), and aspartate aminotransferase (AST) by using standard diagnostic kits (Span Diagnostics, Gujarat, India).

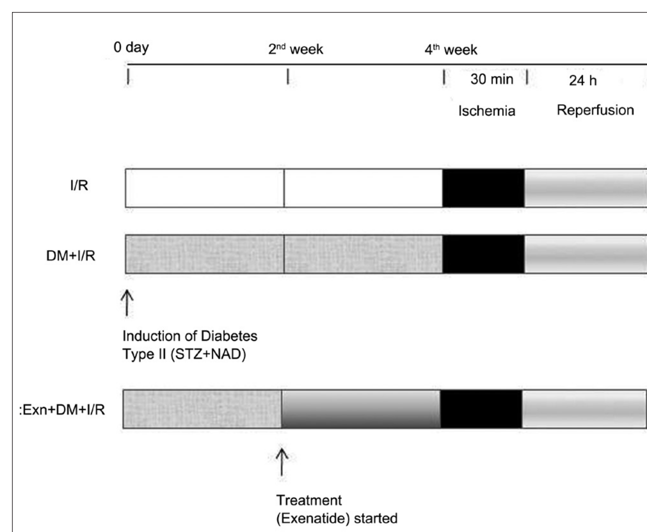

Figure 1: Scheme showing experimental protocols employed. Each protocol comprised following periods: ischemia 30 min, reperfusion 24 h, treatment period 14 days before induction of ischemia.

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

Lipid peroxidation and antioxidant enzymes

The kidney was removed and kept in cold conditions (precooled in inverted petridish on ice). It was cross-chopped with surgical scalpel into fine slices in chilled 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris–HCl buffer, pH 7.4 (10%w/v) with 25 strokes of tight Teflon pestle of a glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for assays of lipid peroxidation (MDA content) and endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and glutathione peroxidase (GSHPx). The MDA formation was estimated by the method of Slater and Sawyer.[19] Reduced GSH was determined by the method of Moran *et al*.[20] SOD was determined by the method of Mishra and Fridovich.[21] Catalase was estimated by the method of Hugo Aebi as given by Colowick *et al*.[22] GSH peroxidase was determined by the method of Paglia and Valentine.[23]

Xanthine oxidase activity

Tissue xanthine oxidase (XO) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm.[24] The phosphate buffer (pH 7.5) and xanthine were mixed with supernatant sample and then incubated for 30 min at 37°C. The reaction was stopped at 0 and 30 min by addition of 100% trichloroacetic acid. Then, the mixture was centrifuged at 5000*g* for 30 min. The activity was measured at 293 nm. One unit of activity was defined as 1 mmol of uric acid formed per minute at 37°C, pH 7.5.

Nitric oxide level

The nitrite (NO) was estimated by the method of Lepoivre *et al*.[25] To 0.5 mL of tissue homogenate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 min. The samples were then centrifuged at 5,000*g* for 15 min. The protein-free supernatant was used for estimating nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris–HCl buffer and mixed well. To this, 530 µL of Griess reagent was added and incubated in the dark for 10–15 min, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained. Standard curve was prepared by sodium nitrite solutions with concentrations in range 1–100 µM by diluting the nitrite standard solution.

Myeloperoxidase activity

MPO activity was measured in tissues in a procedure similar to that documented by Hillegas *et al*.[26] Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41,400*g* (10 min); pellets were suspended in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles with sonication between cycles, the samples were centrifuged at 41,000*g* for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM phosphate buffer, *o*-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g of tissue.

TNF-α quantification by ELISA

Levels of TNF-*α* in serum were determined by using an enzyme-linked immunosorbent assay (ELISA) (Endogen, mouse TNF-*α* kit, Pierce Biotech Int., Rockford, Illinois, USA) according to the manufacturer's instructions.

DNA fragmentation

Genomic DNA was extracted from renal cortices using DNA extraction kit (DNeasy kit, Axygen). Ten micrograms of DNA were loaded into 1.5% agarose gel containing 0.5 mg/mL ethidium bromide. DNA electrophoresis was carried out at 80 V for 1 to 2 h. DNA ladders, an indicator of tissue apoptotic nucleosomal DNA fragmentation, were visualized under ultraviolet light, and photographed for permanent records.

Renal histopathology

The kidneys fixed in 10% neutral-buffered formalin solution were embedded in paraffin, and were used for histopathological examination. Sections of 5 µm thickness were cut on a microtome and taken on glass slides coated with albumin. The hematoxyline-stained sections were stained with eosin for 2 min and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada Balsam. The stained sections were examined under Olympus BX40 photomicroscope and photographed. Either samples were coded to perform blind study or expert guidance from the veteran pathologist was sought to determine histopathological changes. A minimum of 10 fields for each kidney slide were examined and assigned for severity of changes using scores on a scale of none (–), mild (+), moderate (++), and severe (+++) damage.

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

Statistical analysis

All the values are expressed as mean ± SEM. Statistical significance between more than two groups were tested using one-way ANOVA followed by the Bonferroni multiple comparisons test using computer-based fitting program (Prism, Graphpad 5). Differences were considered to be statistically significant when $P < 0.05$.

Results

Effect of exenatide on renal function

Diabetic animals that underwent renal I/R exhibited significant increase in the serum concentrations of creatinine, urea nitrogen, and AST when compared with DM animals ($P < 0.001$), suggesting a significant degree of glomerular dysfunction mediated by renal I/R. Serum concentrations of urea nitrogen, creatinine, and AST were also significantly different in between I/R group and DM + I/R group ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively). Pretreatment of rats with exenatide (10 mcg/kg) produced, a significant reduction in the serum levels of creatinine, urea nitrogen, and AST compared to DM + I/R group ($P < 0.001$) [Table 1].

Effect of exenatide on lipid peroxidation and antioxidant enzymes

The renal tissue MDA content in the normal as well as in diabetic group was elevated after induction of I/R injury, compared to normal control ($P < 0.01$, $P < 0.001$, respectively); however, exenatide treatment in Exn + DM + I/R group, significantly decreased the I/R-induced elevation in renal MDA level compare to DM + I/R group ($P < 0.001$) [Figure 2A]. In accordance with that, I/R in diabetic rats demonstrated a significant decrease in renal tissue GSH ($P < 0.001$) and GSHPx ($P < 0.001$) level when compared to DM, while in the exenatide-treated Exn + DM + I/R group, renal GSH, and GSHPx content were found to be preserved ($P < 0.05$, $P < 0.001$), being not significantly different from that of the DM and NC [Figure 2B and C]. Diabetic animals that underwent renal I/R exhibited significant decrease in the SOD activity when compared with DM animals ($P < 0.01$), and I/R animals ($P < 0.01$). However, the activity of SOD in renal tissue was increased in the pretreatment with exenatide, in comparison with the DM + I/R ($P < 0.05$) [Figure 2D]. The CAT activity of DM + I/R group was decreased in comparison with the DM ($P < 0.001$), and I/R group ($P < 0.05$), whereas it was higher in the pretreatment with exenatide group than in the DM + I/R group ($P < 0.001$) [Figure 2E]. The XO activity was increased in I/R and DM + I/R groups in comparison with the NC group ($P < 0.001$). However, the XO activity was also found to be significantly different in between I/R and DM + I/R ($P < 0.05$). The XO activity was normalized by treatment with exenatide compared to DM + I/R ($P < 0.001$) [Figure 2F]. The levels of NO were increased in I/R and DM + I/R groups in comparison with NC group ($P < 0.001$). Exenatide treatment to DM + I/R groups demonstrated

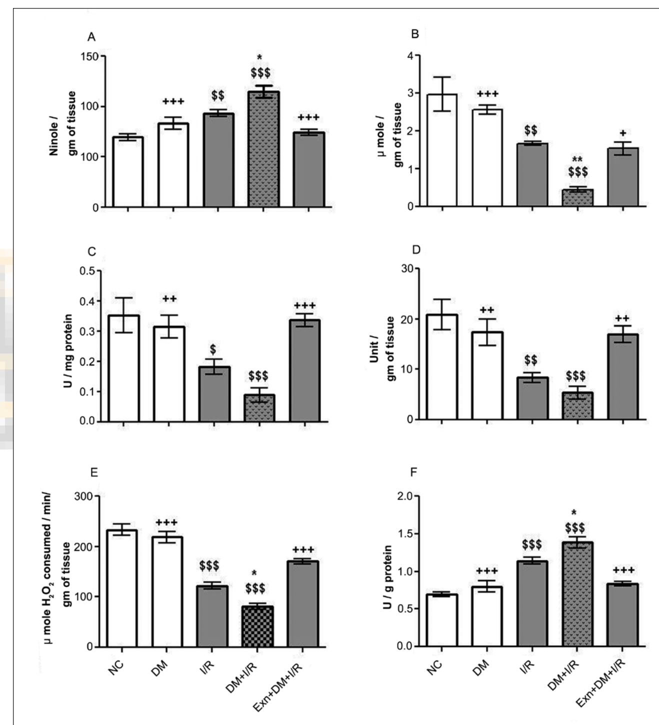

Figure 2: Effect of exenatide on lipid peroxidation (A); reduced glutathione (B); glutathione peroxidase (C); superoxide dismutase (D); catalase (E); and xanthine oxidase (F) in renal tissue after renal I/R in normal, and diabetic rats. Values are mean ± SEM. (n = 6), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. $^{\$,*,+}P < 0.05$; $^{\$\$,**,++}P < 0.01$; $^{\$\$\$,+++}P < 0.001$. $^{\$}$Compared with normal control, *compared with I/R, and +compared with DM + I/R group.

Table 1: Effect of exenatide on blood glucose concentration and renal function during the experiments

Groups	NC	DM	I/R	DM + I/R	Exn + DM + I/R
BG (mmol/L)	4.8 ± 0.94	34.26 ± 1.25	4.2 ± 0.84	32.58 ± 2.51$^{\$\$\$,***}$	6.4 ± 1.21^{+++}
AST (IU/L)	90.83 ± 8.207	84.47 ± 17.51^{+++}	503.2 ± 28.92$^{\$\$\$}$	796.5 ± 29.36$^{\$\$\$,***}$	352.3 ± 67.10^{+++}
BUN (mg/dL)	13.17 ± 2.638	16.33 ± 2.801^{+++}	36.33 ± 4.485$^{\$\$}$	56.33 ± 6.259$^{\$\$\$,*}$	22.00 ± 3.183^{+++}
Creatinine (mg/dL)	0.375 ± 0.027	0.475 ± 0.057^{+++}	0.963 ± 0.097$^{\$\$\$}$	1.433 ± 0.12$^{\$\$\$,**}$	0.6350 ± 0.057^{+++}

Values are mean ± SEM. (n = 6), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. $^{*}P < 0.05$; $^{\$\$,**}P < 0.01$; $^{\$\$\$,***,+++}P < 0.001$. $^{\$}$Compared with normal control, *compared with I/R, and + compared with DM + I/R group.

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

a significant decrease in NO level in comparison with DM + I/R group ($P < 0.01$) [Figure 3].

Effect of exenatide on myeloperoxidase activity

Myeloperoxidase activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in the kidney tissue of the DM + I/R group than that of the DC ($P < 0.001$) group and I/R group ($P < 0.01$). On the other hand, exenatide treatment significantly decreased renal tissue MPO level ($P < 0.001$) compared to DM + I/R, which was found to be not different than that of the NC and DM groups [Figure 4A].

Effect of exenatide on serum TNF-α

The serum TNF-α level was significantly increased in the I/R control as compared to NC group ($P < 0.001$). In DM + I/R rats, serum TNF-α level was significantly higher as compared to I/R control ($P < 0.01$). Exn + DM + I/R group had significantly lower serum TNF-α level as compared to the DM + I/R control ($P < 0.001$) [Figure 4B].

Effect of exenatide on DNA fragmentation

Apoptosis was evaluated by DNA fragmentation analysis. The typical DNA laddering activity was observed in the I/R control and in DM + I/R groups, which indicates cell necrosis. Treatment with exenatide in DM + I/R had decreased DNA fragmentation and necrosis in comparison to the DM + I/R group [Figure 5].

Effect of exenatide on renal histology

Histological damage ranged from normal (control and exenatide treatment groups) to mild (I/R group) and severe (DM + I/R group), with cortical rather than medullary tubules demonstrating the most marked changes. The histopathological changes were graded and summarized in Table 2. The control group did not show any morphological changes. By contrast, the kidneys of untreated I/R rats showed tubular cell swelling, interstitial edema, tubular dilatation, hyaline casts, and moderate-to-severe necrosis. Treatment with exenatide (10 mcg/kg) preserved the normal morphology of the kidney, and shows normal glomeruli with slight edema of the tubular cells [Figure 6].

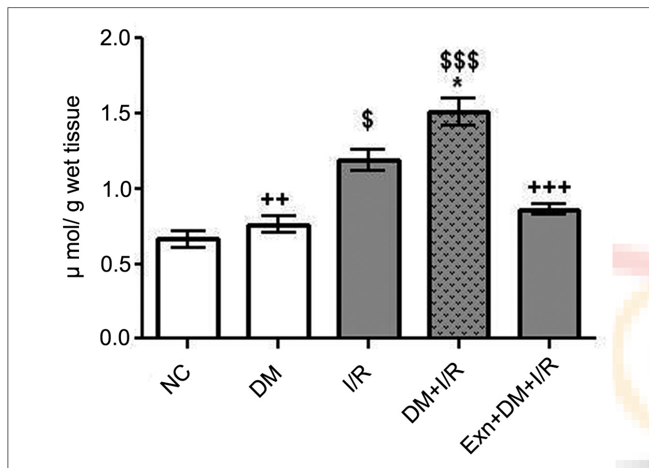

Figure 3: Effect of exenatide on nitric oxide after renal I/R in normal and diabetic rats. Values are mean ± SEM (n = 6), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. *$P < 0.05$; ++$P < 0.01$; $$$,+++$P < 0.001$. $Compared with normal control, *compared with I/R, and +compared with DM+I/R group

Discussion

In this study, we used 30 min of ischemia, the importance of the ischemic duration is well established,

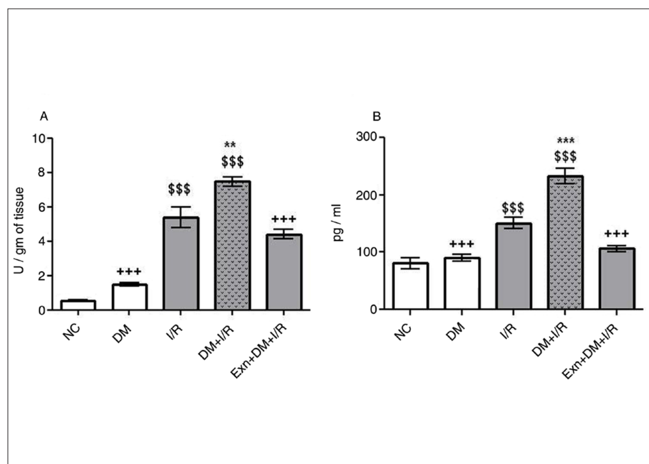

Figure 4: Effect of exenatide on MPO (A) and TNF-α (B) after renal I/R in normal and diabetic rats. Values are mean ± SEM (n = 6), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. **$P < 0.01$; $$$,***,+++$P < 0.001$. $Compared with normal control, *compared with I/R, +and compared with DM+I/R group.

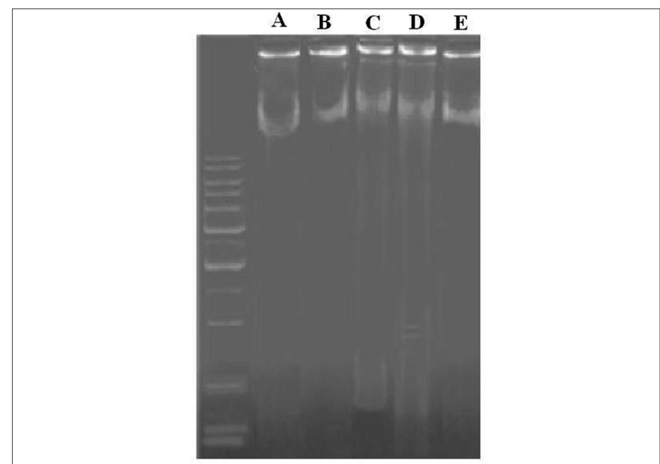

Figure 5: DNA fragmentation analysis revealed typical laddering of fragmentized DNA in I/R control and DM+I/R group. Exenatide treatment decreased the laddering pattern. (A) Normal control; (B) diabetic control; (C) normal rat exposed to renal I/R; (D) diabetic rat exposed to renal I/R; (E) exenatide (10 mcg/kg) pretreated diabetic rat exposed to renal I/R.

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

Table 2: Effect of exenatide (10 mcg/kg) pretreatment on morphological changes of kidneys, as assessed by histopathological examination of the normal rats, and diabetic rats exposed to renal I/R

Group	Tubular cell swelling	Interstitial edema	Tubular dilatation	Necrosis of epithelium	Hyaline casts
NC	–	–	–	–	–
DC	–	–	–	–	–
I/R	++	++	++	++	++
DM + I/R	+++	+++	+++	+++	+++
Exn + DM + IR	–	–	–	–	–

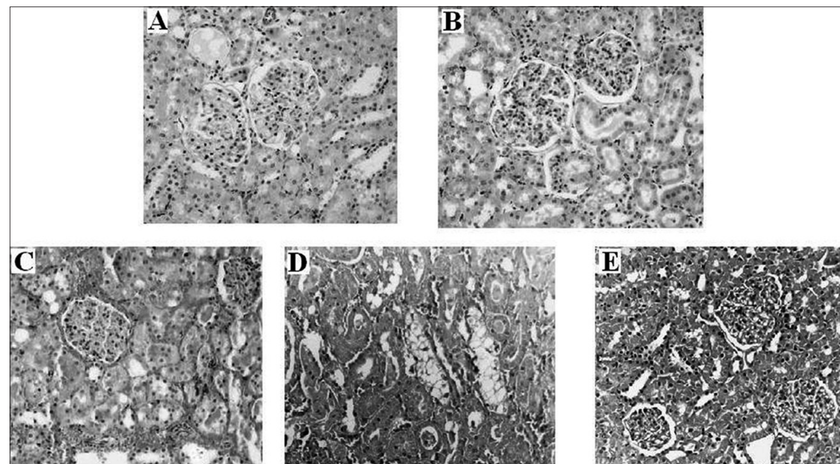

Figure 6: Microscopic observations of kidneys tissue sections of normal and diabetic rats subjected to 30-min ischemia and 24 h reperfusion with BIOXL light microscope showing morphological changes. Images were taken under light microscopy using (H&E, ×40): (A) Normal control; (B) diabetic control; (C) normal rat exposed to renal I/R; (D) siabetic rat exposed to renal I/R; (E) exenatide (10 mcg/kg) pretreated diabetic rat exposed to renal I/R.

and it has been previously shown that a longer period of ischemia causes a more severe injury.[5] The degradation of ATP to hypoxanthine and xanthine via inosine is enhanced with the duration of ischemia.[27] The restoration of ATP levels during reperfusion is slower after prolonged ischemia.[28] Temperature is a critical factor in ischemic injury. Hyperthermia, especially during the ischemic phase, leads to a more severe renal I/R injury. Raising the temperature from 37 to 39.5°C during ischemia leads to a 100% increase in BUN in a model using 30 min of ischemia and uninephrectomy.[29] To keep the temperature constant, we used a servo-controlled heating pad that kept the temperature in the rat at 37.5°. An important question in this work is how T2DM could cause the increased sensitivity to renal I/R, which observed in DM animals. Several possible explanations exist. The increased sensitivity to I/R could be due to hyperglycemia *per se*. Shortage of insulin could also be involved in the increased sensitivity to I/R. Secondary effects of hyperglycemia such as formation of advanced glycosylated end products, increased oxidative stress, hemodynamic alterations, and formation of NO could also be involved.

In our study, animals subjected to renal I/R demonstrated an increase in the renal tissue MDA levels, and attenuated the antioxidant enzyme pool. Renal I/R-induced oxidative stress in DM was associated with impaired renal function leading to a marked increase in serum creatinine, urea nitrogen, and AST levels. Pretreatment with exenatide prevented the renal I/R-induced lipid peroxidation and protected the kidneys from severe attenuation of antioxidant activity in rats exposed to the renal I/R in diabetes. Furthermore, histopathological evaluation of rat kidney demonstrated the renal functional damage in DM + I/R group of rats, which was significantly improved by exenatide treatment. Renal I/R in normal as well as in diabetic rats caused characteristic morphological changes, such as tubular cell swelling, tubular dilatation, necrosis of epithelium, and interstitial edema. In contrast, sections of the exenatide pretreated kidneys demonstrated architectural and cytological preservation of structure.

Oxidative stress and inflammatory response might play a pathophysiological role in renal I/R injury in T2DM given the knowledge that oxidative stress is implicated both in the complications of T2DM and renal

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

I/R. Elevated oxidative stress has been demonstrated in cerebral[30] and intestinal[31] I/R in DM rats. The combined oxidative stress from two sources may thus increase the total level of ROS. Infiltration of inflammatory cells is one of the main features of renal I/R injury in T2DM rats. The infiltrate mainly consisted of cells identified as macrophages/monocytes and T-lymphocytes. The inflammatory response is increased acutely after I/R of the intestines in diabetic animals.[32] After a brief ischemia of the intestine, ROS are also increased and the increase is more pronounced in diabetes.[31] It is likely that inflammatory cells contribute to increased oxidative stress in T2DM kidneys after I/R. According to Sakr *et al.*, pretreatment with a single injection of tacrolimus, 24 h prior to 60 min of ischemia was able to decrease the renal injury, and the effect was associated with decreased levels of TNF-α.[33] Thus, we decided to estimate TNF-α and MPO. In our finding, the serum level of TNF-α was normalized in treatment with exenatide might be one reason of exenatide to protect against renal I/R in T2DM.

The cardiac MPO activity increased after renal I/R, consistently with leukocyte infiltration and activation. The active neutrophils show high MPO activity in the tissue as an inflammatory answer.[34] This study demonstrated that the high renal MPO activity after induction of I/R in T2DM rats is very important because it clearly shown high-leukocyte infiltration in the renal tissue. The neutrophils play a major role in oxidant injury via the mechanisms such as the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or MPO system. HOCl is produced largely from stimulated neutrophils by MPO activity. HOCl causes oxidation of other molecules such as proteins, amino acids, carbohydrates, nucleic acids, and lipids, expanding renal tissue damage.[35] In this study, increase in MPO activity was inhibited by exenatide treatment. This might also result in reduced lipid peroxidation and thus less accumulation of MDA, because activation of neutrophils might lead to the generation of more oxygen-reactive metabolites.

The NO system may be involved in the increased sensitivity to I/R in DM. There is evidence for increased NO-production in the STZ-DM kidney,[36] the reaction of NO with $O2^{\bullet-}$ results in peroxynitrite formation, a potent and aggressive cellular oxidant and causes the formation of 3-nitro-L-tyrosine.[37] Nitrite/nitrate levels, as the end products of NO conversion, were increased in blood plasma and aortic tissue in diabetic animals comparison with nondiabetic animals,[38] which was confirmed by elevated NO level in our study. Exenatide treatment with diabetic I/R animals had normalized the elevated tissue NO level that might be attributed to protective effect of exenatide against renal I/R in T2DM.

Several mechanisms might be responsible for the renoprotective effects of exenatide against renal I/R. First, the restoration of normoglycemia is the explanation for the beneficial effects of exenatide treatment in the present model [Table 1], because glucose metabolism is stimulated over fatty acid metabolism, which is more efficient with respect to oxygen consumption for adenosine triphosphate production,[39] and which might therefore reduce oxygen demand. Some previous works support the importance of BGC in I/R injury. In our study, we found severe renal injury when I/R performed in T2DM rats, in which blood glucose concentration (BGC) was higher than in the normal rats. Hyperglycemia, the elevated BGC during I/R could be deleterious for the kidney. An increased acute sensitivity to ischemia has been demonstrated when BGC was raised by dextrose infusion or intraperitoneal glucose injection in combination with renal I/R in both rats and dogs.[40] Numerous studies have investigated the influence of hyperglycemia and diabetes in cerebral ischemia. Diabetes is associated with a worse outcome after stroke in humans, and elevated blood glucose predisposes for a more severe cerebral injury even in non-DM patients.[41] There are conflicting evidences regarding the influence of hyperglycemia and diabetes on the degree of injury in experimental cerebral ischemia. DM or hyperglycemia in non-DM animals caused increased cerebral injury in most studies, especially when models with reperfusion were used.[42] Taken together, these studies suggested a role for reperfusion in the harmful effect of hyperglycemia in cerebral ischemic injury. In our study, BGC controlled by exenatide pretreatment could be a protective effect of exenatide against renal I/R in T2DM [Table 1].

Additionally, exenatide might reduce apoptosis and oxidative stress. Timmers *et al.* demonstrated myocardial expression of pAkt was increased after exenatide treatment and expression of active caspase 3 were reduced. Also, activity of the antioxidant enzymes SOD and catalase were higher in animals treated with exenatide, and nuclear oxidative stress was reduced.[43] In our study, decreased in MDA, XO activity, and increased in SOD, CAT, GSH and GSHPx in the treatment with exenatide demonstrated the reduction in nuclear oxidative stress. In addition, in response to

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

ROS, the outer membrane of mitochondria becomes permeabilized, resulting in the translocation of Bax from cytosol to the mitochondria and the release of cytochrome *c* occurs. The release of cytochrome *c* into the cytosol leads to form the apoptosome, which stimulates the activation of procaspase-9 and procaspase-3. Active caspase-3 activates the caspase-activated DNAase, leading to DNA fragmentation.[44,45] In this study, severe cell necrosis was observed in the I/R control and DM + I/R groups. Furthermore, decreased cell necrosis was observed in exenatide-treated rats in comparison to DM + I/R groups, and that confirm the finding of Timmers *et al*.[43]

In conclusion, T2DM had exaggerated renal I/R in STZ-NAD induced T2DM. Also, the exenatide treatment attenuated renal I/R in diabetic rats by modification in oxidative stress and inflammation. The treatment with exenatide for at least 2 weeks before I/R was a very efficient way to decrease the degree of renal injury in T2DM.

Acknowledgments

We thank Dr. R. Balaraman (Head, Department of Pharmacology, M.S. University of Baroda) and Mr. Chintan Gandhi for advice regarding ischemic injury in rat, and B. T. Savani Kidney Foundation for secretarial support in this work.

References

1. Maisonneuve P, Agodoa L, Gellert R, Stewart JH, Buccianti G, Lowenfels AB, *et al*. Distribution of primary renal diseases leading to end-stage renal failure in the United States, Europe, and Australia/New Zealand: Results from an international comparative study. Am J Kidney Dis 2000;35:157-65.
2. Hokama JY, Ritter LS, Gorman GD, Cimetta AD, Copeland JG, McDonagh PF. Diabetes enhances leukocyte accumulation in the coronary microcirculation early in reperfusion following ischemia. J Diabetes Complicat 2000;14:96-107.
3. Yellon DM, Baxter GF. Protecting the ischaemic and reperfused myocardium in acute myocardial infarction: Distant dream or near reality? Heart 2000;83:381-7.
4. Yokoyama H, Okudaira M, Otani T, Sato A, Miura J, Takaike H, *et al*. Higher incidence of diabetic nephropathy in type 2 than in type 1 diabetes in early-onset diabetes in Japan. Kidney Int 2000;58:302-11.
5. Melin J, Hellberg O, Akyurek LM, Kallskog O, Larsson E, Fellstrom BC. Ischemia causes rapidly progssive nephropathy in the diabetic rat. Kidney Int 1997;52:985-91.
6. Basireddy M, Isbell TS, Teng X, Patel RP, Agarwal A. Effects of sodium nitrite on ischemia-reperfusion injury in the rat kidney. Am J Physiol Renal Physiol 2006;290:F779-86.
7. Noiri E, Nakao A, Uchida K, Tsukahara H, Ohno M, Fujita T, *et al*. Oxidative and nitrosative stress in acute renal ischemia. Am J Physiol Renal Physiol 2001;281:F948-57.
8. Ysebaert DK, De Greef KE, De Beuf A, Van Rompay AR, Vercauteren S, Persy VP, *et al*. T cells as mediators in renal ischemia/reperfusion injury. Kidney Int 2004;66:491-6.
9. Altunoluk B, Soylemez H, Oguz F, Turkmen E, Fadillioglu E. An Angiotensin-converting enzyme inhibitor, zofenopril, prevents renal ischemia/reperfusion injury in rats. Ann Clin Lab Sci 2006;36:326-32.
10. Erdogan H, Fadillioglu E, Yagmurca M, Ucar M, Irmak MK. Protein oxidation and lipid peroxidation after renal ischemia-reperfusion injury: Protective effects of erdosteine and N-acetylcysteine. Urol Res 2006;34:41-6.
11. Yildirim A, Gumus M, Dalga S, Sahin YN, Akcay F. Dehydroepiandrosterone improves hepatic antioxidant systems after renal ischemia-reperfusion injury in rabbits. Ann Clin Lab Sci 2003;33:459-64.
12. Emre MH, Erdogan H, Fadillioglu E. Effect of BQ-123 and nitric oxide inhibition on liver in rats after renal ischemia-reperfusion injury. Gen Physiol Biophys 2006;25:195-206.
13. Wei Y, Mojsov S. Tissue-specific expression of the human receptor for glucagon-like peptide-I: Brain, heart and pancreatic forms have the same deduced amino acid sequences. FEBS Lett 1995;358:219-24.
14. Vila Petroff MG, Egan JM, Wang X, Sollott SJ. Glucagon-like peptide-1 increases cAMP but fails to augment contraction in adult rat cardiac myocytes. Circ Res 2001;89:445-52.
15. Bose AK, Mocanu MM, Carr RD, Brand CL, Yellon DM. Glucagon-like peptide 1 can directly protect the heart against ischemia/reperfusion injury. Diabetes 2005;54:146-51.
16. Bose AK, Mocanu MM, Carr RD, Yellon DM. Glucagon like peptide-1 is protective against myocardial ischemia/reperfusion injury when given either as a preconditioning mimetic or at reperfusion in an isolated rat heart model. Cardiovasc Drugs Ther 2005;19:9-11.
17. Bose AK, Mocanu MM, Carr RD, Yellon DM. Myocardial Ischaemia reperõfusion Injury is Attenuated by Intact Glucagon Like Peptide-1 (GLP-1) in the *In Vitro* Rat Heart and may Involve the p70s6K Pathway. Cardiovasc Drugs Ther 2007;21:253-6.
18. Masiello P, Broca C, Gross R, Roye M, Manteghetti M, Hillaire-Buys D. Experimental NIDDM: Development of a new model in adult rats administered streptozotocin and nicotinamide. Diabetes 1998;47:224-9.
19. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogenoalkanes or peroxidative reactions in liver fractions *in vitro*. Biochem J 1971;123:805-14.
20. Moran MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochimica Biophysica Acta 1979;582:67-78.
21. Misra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay of SOD. J Biol Chem 1972;247:3170-5.
22. Aebi H. Oxidoreductases acting on groups other than CHOH: Catalase. In: Colowick SP, Kaplan NO, Packer L, editors. Vol. 105. Methods in Enzymology. London: Academic Press; 1984. p. 121-5.
23. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte peroxidase. J Lab Clin Med 1967;2:158-69.
24. Prajda N, Weber G. Malign transformation-linked imbalance: Decreased XO activity in hepatomas. FEBS Lett 1975;59:245-9.
25. Guevara I, Iwanejko J, Dembinska-Kiec A. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. Clin Chim Acta 1998;274:177-88.
26. Wei H, Frenkel K. Relationship of oxidative events and DNA oxidation in SENCAR mice to *in vivo* promoting activity of phorbol ester-type tumor promoters. Carcinogenesis 1993;14:1195-201.
27. Jan Willem J, Elisabeth K, Tom H, Bob S. Ischemic nucleotide breakdown increases during cardiac development due to drop in adenosine anabolism/catabolism ratio. *Journal of Molecular and*

[Downloaded free from http://www.ijddc.com on Monday, October 11, 2010, IP: 59.183.141.47]

Cellular Cardiology. 1990; 22(10): 1065-70
28. Stromski ME, Cooper K, Thulin G, Gaudio KM, Siegel NJ, Shulman RG. Chemical and functional correlates of postischemic renal ATP levels. Proc Natl Acad Sci USA 1986;83:6142-5.
29. Zager RA. Hyperthermia: Effects on renal ischemic/reperfusion injury in the rat. Lab Invest 1990;63:360-9.
30. Aragno M, Parola S, Brignardello E, Mauro A, Tamagno E, Manti R, *et al*. Dehydroepiandrosterone prevents oxidative injury induced by transient ischemia/reperfusion in the brain of diabetic rats. Diabetes 2000;49:1924-31.
31. Salas A, Panes J, Elizalde JI, Granger DN, Pique JM. Reperfusion-induced oxidative stress in diabetes: Cellular and enzymatic sources. J Leukoc Biol 1999;66:59-66.
32. Panes J, Kurose I, Rodriguez-Vaca D, Anderson DC, Miyasaka M, Tso P. Diabetes Exacerbates Inflammatory Responses to Ischemia-Reperfusion. Circulation 1996;93:161-7.
33. Sakr M, Zetti G, McClain C, Gavaler J, Nalesnik M, Todo S, *et al*. The protective effect of FK506 pretreatment against renal ischemia/reperfusion injury in rats. Transplantation 1992;53:987-91.
34. Kelly KJ. Distant effects of experimental renal ischemia/reperfusion injury. J Am Soc Nephrol 2003;14:1549-58.
35. Arnhold J, Osipov AN, Spalteholz H, Panasenko OM, Schiller J. Effects of hypochlorous acid on unsaturated phosphatidylcholines. Free Radic Biol Med 2001;31:1111-9.
36. Goor Y, Peer G, Iaina A, Blum M, Wollman Y, Chernikovsky T, *et al*. Nitric oxide in ischaemic acute renal failure of streptozotocin diabetic rats. Diabetologia 1996;39:1036-40.
37. Yagmurca M, Erdogan H, Iraz M, Songur A, Ucar M, Fadillioglu E. Caffeic acid phenethyl ester as a protective agent against doxorubicin nephrotoxicity in rats. Clin Chim Acta 2004;348:27-34.
38. Sudnikovich EJ, Maksimchik YZ, Zabrodskaya SV, Kubyshin VL, Lapshina EA, Bryszewska M, *et al*. Melatonin attenuates metabolic disorders due to streptozotocin-induced diabetes in rats. Eur J Pharmacol 2007;569:180-7.
39. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, *et al*. Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 1993;42:801-13.
40. Moursi M, Rising CL, Zelenock GB, D'Alecy LG. Dextrose administration exacerbates acute renal ischemic damage in anesthetized dogs. Arch Surg 1987;122:790-4.
41. Pulsinelli WA, Levy DE, Sigsbee B, Scherer P, Plum F. Increased damage after ischemic stroke in patients with hyperglycemia with or without established diabetes mellitus. Am J Med 1983;74:540-4.
42. Sutherland GR, Peeling J, Sutherland E, Tyson R, Dai F, Kozlowski P, *et al*. Forebrain ischemia in diabetic and nondiabetic BB rats studied with 31P magnetic resonance spectroscopy. Diabetes 1992;41:1328-34.
43. Timmers L, Henriques PS, De Kleijn PV, Hans DeVries J, Hans Kemperman, Cees WJ, *et al*. Exenatide Reduces Infarct Size and Improves Cardiac Function in a Porcine Model of Ischemia and Reperfusion Injury. J Am Coll Cardiol 2009;53:501-10.
44. Chen H, Xing B, Liu X, Zhan B, Zhou J, Zhu H, *et al*. Ozone oxidative preconditioning inhibits inflammation and apoptosis in a rat model of renal ischemia/reperfusion injury. Eur J Pharmacol 2008;581:306-14.
45. Hyo JK, Ki WL, Mi-Sung K, Hyong JL. Piceatannol attenuates hydrogen peroxide- and peroxynitrite-induced apoptosis of PC12 cells by blocking down-regulation of Bcl-XL and activation of JNK. J Nutr Biochem 2007;7:459-66.

Source of Support: Nil, **Conflict of Interest:** None declared

Author Help: Online submission of the manuscripts

Articles can be submitted online from http://www.journalonweb.com. For online submission, the articles should be prepared in two files (first page file and article file). Images should be submitted separately.

1) **First Page File:**
Prepare the title page, covering letter, acknowledgement etc. using a word processor program. All information related to your identity should be included here. Use text/rtf/doc/pdf files. Do not zip the files.
2) **Article File:**
The main text of the article, beginning with the Abstract to References (including tables) should be in this file. Do not include any information (such as acknowledgement, your names in page headers etc.) in this file. Use text/rtf/doc/pdf files. Do not zip the files. Limit the file size to 1024 kb. Do not incorporate images in the file. If file size is large, graphs can be submitted separately as images, without their being incorporated in the article file. This will reduce the size of the file.
3) **Images:**
Submit good quality color images. Each image should be less than **2048 kb (2 MB)** in size. The size of the image can be reduced by decreasing the actual height and width of the images (keep up to about 6 inches and up to about 1800 x 1200 pixels). JPEG is the most suitable file format. The image quality should be good enough to judge the scientific value of the image. For the purpose of printing, always retain a good quality, high resolution image. This high resolution image should be sent to the editorial office at the time of sending a revised article.
4) **Legends:**
Legends for the figures/images should be included at the end of the article file.