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Simvastatin ameliorates diabetic nephropathy by attenuating oxidative stress and apoptosis in a rat model of streptozotocin-induced type 1 diabetes

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ABSTRACT

Statins are HMG-CoA reductase inhibitors with lipid-lowering effect and commonly used to reduce cardiovascular risk in diabetic patients. The present study investigates the ameliorative effect of simvastatin (SIM) on diabetic nephropathy in rats, pointing to its anti-apoptotic and anti-oxidative stress efficacies. Diabetes was induced by a single intraperitoneal injection of 55 mg/kg body weight streptozotocin (STZ) and both control and diabetic rats received 10 mg/kg SIM for 90 days. Treatment with SIM diminished the body weight loss, blood glucose and, serum creatinine, urea and uric acid in diabetic rats. SIM improved the creatinine clearance rate and urinary levels of creatinine, urea and albumin in STZ-induced rats. Lipid peroxidation and nitric oxide (NO) were significantly increased in the diabetic kidney whereas reduced glutathione, SOD and catalase were declined. Bax and caspase-3 showed a significant increase and Bcl-2 was decreased in the kidney of diabetic rats. SIM administration reduced lipid peroxidation and NO, and improved antioxidants and the expression of apoptotic markers. Diabetic rats showed increased collagen deposition in the kidney, atrophied irregular renal corpuscles with collapsed glomeruli and tubules with degenerated epithelial lining, an effect that was reversed following treatment with SIM. In conclusion, SIM can protect against diabetic nephropathy by attenuating oxidative stress and apoptosis.

1. Introduction

Diabetes mellitus is a serious life-long disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The incidence of diabetes is dramatically increasing and the number of patients with diabetes is expected to increase to 693 million by 2045 [1]. Long-term hyperglycemia is associated with the micro- and macrovascular complications that affects multiple organs, including the eyes and kidneys. This has made diabetic nephropathy (DN) one of the most common complications in patients with diabetes [2]. It has been estimated that approximately a quarter of the diabetic patients develop nephropathy [3]. It is worth stressing that early treatment may keep the kidney disease from getting worse. If left untreated, the patient shows gradually declined glomerular filtration rate (GFR) and persistence of microalbuminuria which progresses to manifest proteinuria. Finally, the kidneys fail and end-stage renal disease (ESRD) usually follows. DN is one of the most important causes of ESRD which affects 15–25% of type 1 and 30–40% of type 2 diabetes patients [4].

The pathogenesis of DN is complex, multifactorial and involves many pathways. Hyperglycemia is the main driving force for the progressive destruction of the glomeruli in diabetes. Persistent elevated blood glucose produces mechanical tension and hemodynamic changes to the glomeruli, which lead to alteration in downstream transcription factors and gene expression [5]. These changes induce various pathways of oxidative stress and liberate numerous cytokines and growth factors [6]. Hyperglycemia-mediated increased generation of reactive oxygen species (ROS) activates various redox-sensitive signaling molecules, leading to cellular dysfunction and injury and ultimately micro- and macrovascular complications [7]. In addition, excessive ROS production can inactivate endogenous antioxidants, and provoke chromatin condensation, DNA fragmentation and accelerated apoptosis of renal epithelial cells [8]. Therefore, mitigating hyperglycemia-induced oxidative stress and apoptosis can prevent DN.

Statins are lipid-lowering medications often prescribed to help lower blood cholesterol levels [9]. This class of drugs are the first-line treatment widely prescribed for coronary artery disease and to reduce

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the risk of stroke and heart attack [9]. Statins work by inhibiting hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase and recently their beneficial effects have been attributed to their anti-inflammatory [10] and anti-oxidant properties [11]. Simvastatin (SIM) is a statin drug with pleotropic effects, including hypocholesterolemic, antineoplastic and anti-inflammatory [12–14]. Recently, we demonstrated the protective effect of SIM against diabetic cardiomyopathy in rats [15]. We showed that treatment of diabetic rats with SIM reduced hyperlipidemia and diminished oxidative stress, inflammation and apoptosis. Therefore, we aimed in the present study to evaluate potential of SIM to attenuate hyperglycemia-induced oxidative stress and apoptosis in a rat model of DN. Streptozotocin (STZ)-induced type 1 diabetes in rats has been suggested as a useful model to study early changes in DN [16]. In addition, STZ-induced type 1 diabetic rodents develop renal injury with similarities to human DN [17].

2. Materials and methods

2.1. Chemicals and reagents

STZ, SIM, malondialdehyde (MDA), pyrogallol, reduced glutathione (GSH), hydrogen peroxide, thiobarbituric acid and Griess reagent were purchased from Sigma-Aldrich (USA). Creatinine and urea assay kits were purchased from Quimica Clinica Aplicada (Amposta, Spain). Rat Albumin ELISA kit was supplied by Assaypro (St. Charles, Mo., USA). Anti-caspase-3 (ab13847) was supplied by Abcam (Cambridge, MA, USA), and antibodies against Bcl-2-associated X protein (Bax; Cat. No. sc-7480), B-cell lymphoma 2 (Bcl-2; Cat. No. sc-7382) and β-actin (Cat. No. sc-47778) were purchased from Santa Cruz Biotechnology (USA). All other chemicals and reagents were supplied by Sigma-Aldrich or other standard commercial suppliers.

2.2. Experimental animals and treatments

Adult male Wistar rats weighing 160–200 g, obtained from the Animal Care Center at the College of Pharmacy, King Saud University (Riyadh, Saudi Arabia), were included in the present study. The animals were maintained at normal atmospheric temperature (23 ± 2 °C) and relative humidity of 50–60% on a 12 h light/dark cycle. The rats were supplied a standard laboratory diet of known composition and water *ad libitum*. All animal procedures were approved by the Institutional Research Ethics Committee, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia).

To induced type 1 diabetes, overnight fasted rats received a single intraperitoneal (i.p.) injection of STZ (55 mg/kg body weight), dissolved in 0.1 M cold citrate buffer (pH 4.5). The corresponding control rats received a single i.p. injection of citrate buffer. Seventy-two hr after STZ injection, blood glucose levels were determined using MEDISAFE MINI blood glucose reader (TERUMO Corporation, Tokyo, Japan) and rats with fasting glucose levels more than 200 mg/dl were selected for further treatments.

Sixteen control and 16 diabetic rats were divided into 4 groups, each comprising 8 rats, as following:

- (Control): Rats received 0.9% sodium chloride (NaCl) for 90 days by oral gavage.
- (Control + SIM): Rats received 10 mg/kg simvastatin [18] dissolved in 0.9% NaCl by oral gavage for 90 days.
- (Diabetic): STZ-induced diabetic rats received 0.9% NaCl by oral gavage for 90 days.
- (Diabetic + SIM): STZ-induced diabetic rats 10 mg/kg simvastatin dissolved in 0.9% NaCl by oral gavage for 90 days.

One day before the end of the study, the rats were housed in individual metabolic cages to collect the urine for 24 h. Urine samples were pooled to assay creatinine, urea and albumin levels. Overnight fasted rats were then sacrificed and blood samples were collected. Immediately after sacrifice, the kidneys were rapidly excised, rinsed in ice-cold saline and weighed. Each kidney was cut into two halves. One half was fixed in 10% neutral buffered formalin for histopathological and immunohistochemical studies. The other half was minced, and a 10% (w/v) homogenate was prepared using 0.1 M phosphate buffered saline (PBS; pH 7.4). The homogenate was centrifuged, and the supernatant was used to assay lipid peroxidation, GSH, nitric oxide (NO), catalase (CAT) and superoxide dismutase (SOD). for the estimation of renal antioxidant parameters. Other samples from the kidney were kept frozen in liquid nitrogen and stored at −80 °C for western blot analysis.

2.3. Biochemical assays

Creatinine and urea were assayed according to the methods of Larsen [19] and Coulombe and Favreau [20] respectively, using reagent kits purchased from Quimica Clinica Aplicada (Amposta, Spain). Serum and urine albumin levels were determined using ELISA kit supplied by Assaypro (St. Charles, Mo., USA), following the manufacturer's instructions.

Lipid peroxidation in the kidney homogenate of control and diabetic rats was determined by assaying MDA, according to the method of Ohkawa et al. [16]. Kidney NO levels were determined as nitrite using Griess reagent [21]. GSH levels, and activity of SOD and CAT were measured in the kidney homogenates following the methods of Beutler et al. [22], Marklund and Marklund [23], and Cohen et al. [24], respectively.

2.4. Histopathology and immunohistochemistry

Immediately after sacrifice, samples from the kidneys of control and diabetic rats were fixed in 10% neutral buffered formalin for 24 h. The fixed samples were processed to prepare 4-μm-thick paraffin sections. The prepared sections were stained with hematoxylin and eosin (H&E) to examine the structure of the kidney or with Masson's Trichrome stain for the detection of interstitial fibrosis.

Other sections from the kidney of control and diabetic rats were processed for the immunohistochemical detection of caspase-3. Briefly, the sections were blocked by immersion in 3% hydrogen peroxide (H_2O_2) solution for 5 min. After washing in Tris-buffered saline (TBS; pH 7.6) for 10 min, the slides were incubated with protein block (Novocastra, UK) for 5 min to block the non-specific binding of antibodies. The sections were probed with rabbit polyclonal anti-caspase-3 (1:100 dilution), washed in TBS three times and then incubated with biotinylated IgG (Novocastra, UK) for 30 min. After washing in TBS, diaminobenzedine (DAB) substrate was added and the sections were counterstained with Mayer's hematoxylin. The sections were mounted and examined. Negative control slides were processed through the same steps with omission of the primary antibody. Image analysis of caspase-3 immuno- staining was performed using ImageJ (NIH, USA), and values were presented as % relative to control.

2.5. Western blot

The effect of SIM on the apoptosis markers Bax and Bcl-2 in the kidney of control and diabetic rats was determined using western blotting as previously described [15]. Briefly, the frozen kidney samples were homogenized in RIPA buffer supplemented with proteinase inhibitors and the concentration of proteins was determined using Bradford reagent. Forty μg proteins were separated on SDS-PAGE and electrotransferred onto nitrocellulose membranes. After blocking in 5% skimmed milk in TBS/Tween20 (TBST), the membranes were probed with anti-Bax, anti-Bcl-2 and anti-β-actin (Santa Cruz Biotechnology, USA) overnight at 4 °C. After washing in TBST, the membranes were incubated with the secondary antibody and developed using enhanced chemiluminescence kit (BIO-RAD, USA). The developed blots were

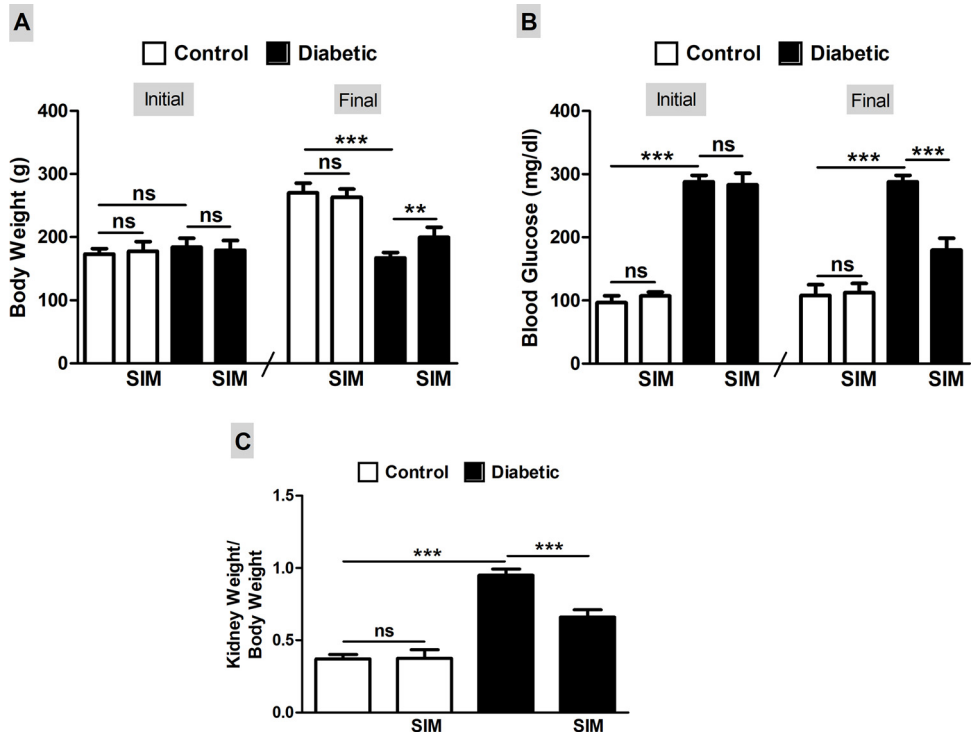


Fig. 1. Simvastatin improves body weight (A) and attenuates hyperglycemia (B) and renal hypertrophy (C) in STZ-induced diabetic rats. Data are Mean ± SEM (n = 8). ***P < 0.001. SIM, simvastatin; ns, non-significant.

Table 1
Effect of simvastatin on kidney function in control and diabetic rats.

	Control	SIM	Diabetic	Diabetic + SIM
Serum creatinine (mgdl)	0.62 ± 0.03	0.51 ± .0.02	1.36 ± 0.05**	0.75 ± 0.07$^{\#\#}$
Serum urea (mgdl)	48.20 ± 4.63	51.6 ± 3.7	106.23 ± 17.13**	86.46 ± 2.86$^{\#\#}$
BUN (mgdl)	22.51 ± 2.16	29.04 ± 2.3	49.61 ± 7.99**	36.36 ± 1.34$^{\#}$
Serum albumin (gdl)	5.50 ± 0.38	4.89 ± 0.42	3.53 ± 0.41*	4.81 ± 0.15$^{\#}$
Creatinine clearance (mlmin)	0.81 ± 0.04	0.74 ± 0.07	0.19 ± 0.01**	0.56 ± 0.04$^{\#}$
Urine creatinine (mg/day)	39.58 ± 4.90	33.56 ± 2.4	10.81 ± 0.94***	29.41 ± 4.73$^{\#\#\#}$
Urine volume (ml/day)	10.00 ± 2.19	9.25 ± 2.59	27.20 ± 6.46***	15.06 ± 4.24$^{\#\#}$
Urine albumin (mg/day)	4.21 ± 0.70	4.04 ± 0.57	25.28 ± 2.34***	15.42 ± 0.15$^{\#\#}$

Data are expressed as mean ± SEM, n = 8. *P < 0.05, **P < 0.01, ***P < 0.001 *versus* Control and $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 *versus* Diabetic. SIM, simvastatin; ns, non-significant.

scanned, and band intensity was quantified using ImageJ (NIH, USA). The obtained results were normalized to β-actin and presented as % of control.

2.6. Statistical analysis

All statistical comparisons were made by means of the one-way ANOVA test followed by Tukey's test *post hoc* analysis using GraphPad Prism (GraphPad Software, CA, USA). The results were presented as mean ± standard error of the mean (SEM), and A P value < 0.05 was considered significant.

3. Results

3.1. SIM improves body weight and attenuates hyperglycemia and renal hypertrophy

Diabetic rats showed a significant (P < 0.001) decline in body weight at the end of the experiment when compared with the control rats (Fig. 1A). Treatment with SIM significantly (P < 0.01) prevented the body weight loss in STZ-diabetic rats, while had no effect on the body weight of normal rats (Fig. 1A).

Diabetic rats showed a significant (P < 0.001) increase in fasting blood glucose levels when compared with the control group (Fig. 1B). After 90 days, diabetic rats exhibited significantly (P < 0.001) elevated fasting blood glucose levels. On the other hand, diabetic rats received SIM for 90 days showed a significant (P < 0.001) improvement in blood glucose levels as depicted in Fig. 1B. Normal rats received SIM for 90 days showed non-significant (P > 0.05) changes in blood glucose levels (Fig. 1B).

The relative kidney weight (kidney weight/body weight ratio) of normal rats treated with SIM for 90 days was not affected when compared with the control group. STZ-induced diabetic rats showed significantly (P < 0.001) increased kidney weight/body weight ratio, an effect that was markedly (P < 0.001) repressed by SIM (Fig. 1C).

3.2. SIM ameliorates kidney function in diabetic rats

STZ-induced diabetic rats showed a significant (P < 0.01) increase in the circulating levels of creatinine, urea and BUN when compared with the control rats (Table 1). In contrast, serum albumin was significantly (P < 0.05) decreased in STZ diabetic rats. Treatment of the

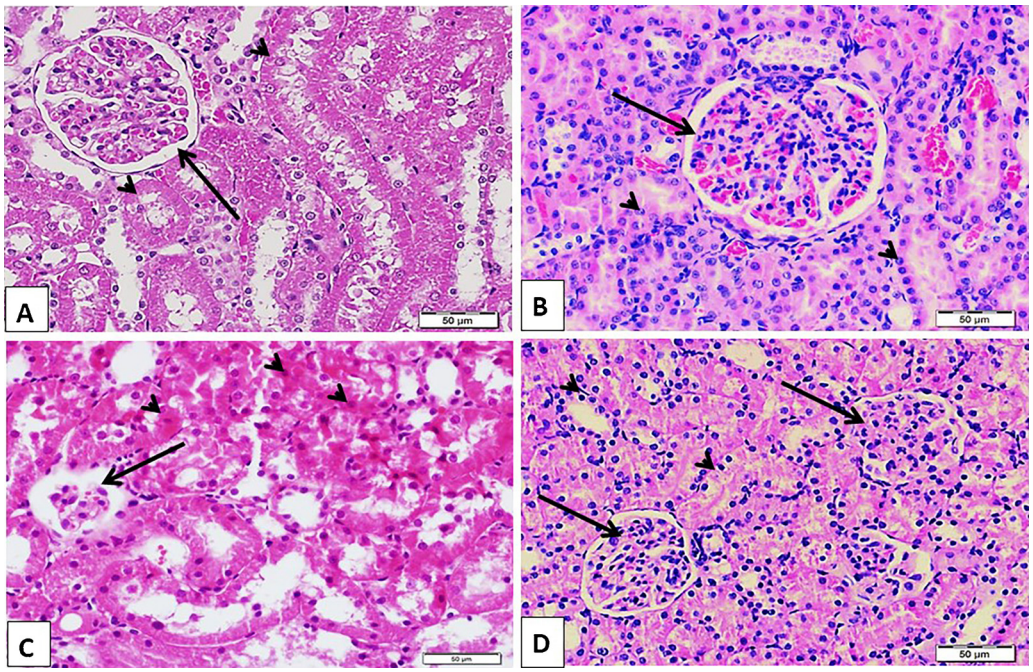


Fig. 2. H&E-stained sections in the kidney of (A) control and (B) control rats treated with simvastatin showing normal histological appearance of renal corpuscles (arrow) and renal tubules (arrow heads), (C) diabetic rats showing atrophied irregular renal corpuscle with collapsed glomerulus (arrow) and renal tubules with degenerated epithelial lining (arrow heads), and (D) diabetic rats treated with simvastatin revealing marked improvement in glomeruli (arrow) and renal tubules (arrow heads).

diabetic rats with SIM markedly alleviated the levels of creatinine ($P < 0.01$), urea ($P < 0.01$), BUN ($P < 0.05$) and albumin ($P < 0.05$).

Creatinine clearance rate and urinary creatinine were significantly decreased in STZ diabetic rats when compared with the control group. On the other hand, the volume of urine /day and urinary albumin were significantly increased in the STZ-induced diabetic rats (Table 1). Treatment of the diabetic rats with SIM for 90 markedly increased creatinine clearance and urinary creatinine levels, and decreased urine volume and albumin. Rats received SIM for 90 days showed non-significant changes in kidney function markers when compared with the control group (Table 1).

3.3. SIM prevents kidney damage and collagen deposition in diabetic rats

Histological examination of the H&E-stained kidney sections of control (Fig. 2A) and SIM-treated rats (Fig. 2B) revealed normal structure of the kidney with normal glomeruli and renal tubules. Sections in the kidney of diabetic rats showed several histological alterations, including enlargement of renal corpuscles and narrowing of the renal spaces due to the glomerular hypercellularity, severe cytoplasmic and nuclear degeneration in the tubular epithelial cells and desquamation into the lumen of the tubules particularly the proximal convoluted tubules (Fig. 2C) (Table 2). Diabetic rats treated with SIM showed an improvement in the structure of kidney as shown in Fig. 2D (Table 2).

To evaluate the effect of SIM on collagen deposition, sections from the kidney of control and diabetic rats were stained with Masson's Trichrome. Control and SIM-treated rats showed normal amount and distribution of interstitial collagen as depicted in Fig. 3A and B respectively. In contrast, the kidney of diabetic rats showed increased amount of deposited collagen (Fig. 3C) (Table 2). Diabetic rats treated with SIM for 90 days showed less collagen deposition when compared with the diabetic control group (Fig. 3D) (Table 2).

Table 2
Histopathological lesions in control and diabetic rats treated with simvastatin.

	Control	SIM	Diabetic	Diabetic + SIM
Focal necrosis of renal tubules	–	–	+ + +	+
Tubular degeneration	–	–	+ + +	+
Glomerular atrophy	–	–	+ +	–
Inflammatory cells infiltration	–	–	+ +	–
Interstitial fibrosis	+	+	+ + + +	+ +

3.4. SIM mitigates oxidative stress in the kidney of diabetic rats

The impact of SIM on the redox status of the normal and diabetic rats was evaluated through assessment the levels of MDA, NO and antioxidants. When compared with the control rats, rats received SIM for 90 days showed non-significant changes in MDA (Fig. 4A) and NO levels (Fig. 4B). On the other hand, STZ-induced diabetic rats exhibited a significant increase in kidney MDA ($P < 0.01$) and NO ($P < 0.001$) levels. Treatment with SIM significantly decreased MDA ($P < 0.01$) and NO ($P < 0.001$) levels in the kidney of diabetic rats.

SIM didn't affect the levels of GSH (Fig. 4C) or the activity of SOD (Fig. 4D) and CAT (Fig. 4E) in the kidney of the normal rats when compared with the control group. The kidney of STZ-induced diabetic rats showed significantly ($P < 0.001$) declined GSH, SOD and CAT. SIM markedly alleviated GSH ($P < 0.001$), SOD ($P < 0.01$) and CAT ($P < 0.001$) in the kidney of diabetic rats.

3.5. SIM prevents apoptosis in the kidney of diabetic rats

The expression of Bax, Bcl-2 and caspase-3 was determined to assess the potential of SIM to prevent apoptosis in the kidney of diabetic rats. The pro-apoptotic protein Bax was significantly increased in the kidney of diabetic rats when compared with the control rats (Fig. 5B). On the other hand, expression of the anti-apoptotic protein Bcl-2 showed a significant ($P < 0.001$) decrease in the kidney of diabetic rats (Fig. 5C). Treatment with SIM significantly decreased the expression of Bax ($P < 0.001$) while increased Bcl-2 ($P < 0.01$) in the kidney of diabetic rats. As the balance between Bax and Bcl-2 dictates susceptibility of the cells to apoptosis [25], we calculated the Bax/Bcl-2 ratio. Diabetic rats showed significantly ($P < 0.001$) increased Bax/Bcl-2 ratio when compared to control, an effect that was reversed in the kidney of SIM-treated diabetic rats (Fig. 5D). SIM administration didn't affect Bax and Bcl-2 expression in the kidney of normal rats.

The expression of caspase-3 showed marked ($P < 0.001$) increase in the kidney of diabetic rats when compared with the control rats

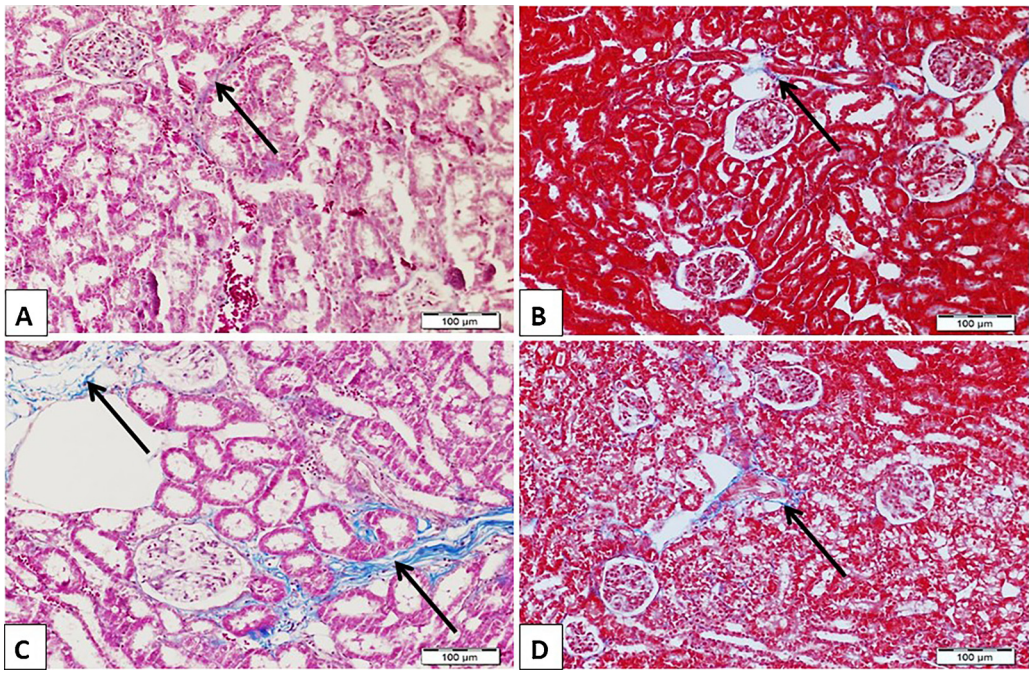


Fig. 3. Masson's trichrome-stained sections in the kidney of (A) control and (B) control rats treated with simvastatin showing normal amount and distribution of interstitial collagen (arrow), (C) diabetic rats showing increased amount of deposited collagen (arrow), and (D) diabetic rats treated with simvastatin revealing less collagen deposition (arrow) when compared with the diabetic control group.

(Fig. 6). Treatment of the diabetic rats with SIM significantly ($P < 0.001$) decreased the expression of caspase-3 as depicted in Fig. 6.

4. Discussion

Statins are lipid-lowering drugs that may offer renoprotective effects and therapeutic potential on pathologic albuminuria [26]. There is a growing interest in the beneficial therapeutic role of statins in DN [27]. In the present study, the protective effect of SIM against DN in STZ-induced rats was investigated. We provided an evidence that SIM ameliorates nephropathy *via* attenuation of hyperglycemia-mediated oxidative stress and apoptosis.

The management of blood glucose is the principal approach to control diabetes mellitus and its complications. Diabetic rats in the present study showed hyperglycemia and weight loss as we recently reported in a rat model of STZ-induced diabetes [15,28]. Diabetic rats treated with SIM showed significantly improved blood glucose levels and body weight. Accordingly, the studies of Mohamadin et al. [29] and Zhang et al. [30] have demonstrated the beneficial effect of SIM on blood glucose levels in type 1 and type 2 diabetic rodents, respectively. In addition, previous work from our laboratory showed improved blood glucose levels and body weight in STZ-induced diabetic rats treated with SIM [15]. Statins can protect the pancreatic β-cells against oxidative stress, increase insulin secretion and improve insulin sensitivity [31,32]. These pleotropic effects can explain the glucose-lowering effect of statins. Furthermore, inhibition of dipeptidyl peptidase IV (DPP-IV) [33] might has a role in mediating the glucose-lowering effect of statins. This notion is being supported by our previous findings where diabetic rats treated with the DPP-IV inhibitor sitagliptin showed improved blood glucose levels and body weight [28].

During the progression of diabetes, nephropathies such atrophic changes in the glomeruli, tubules and interstitium are generally observed. This causes an increase in the kidney weight and elevation in BUN and serum creatinine [34]. In the present study, hyperglycemia induced kidney damage in the diabetic rats as evidenced by renal hypertrophy, increased circulating levels of creatinine, urea and BUN, and decreased creatinine clearance. Similar findings have been recently reported in low-dose STZ-induced diabetic rats [35]. The control of renal hypertrophy, an important pathological feature of early DN, is an important sign of the effectiveness of DN treatment. Creatinine clearance is an indicator for kidney function due to its positive correlation with the glomerular filtration rate. Additionally, urinary albumin levels and urine volume were increased. Albuminuria is as an initial predictor of kidney damage in DN and 24-hr urinary albumin excretion rate is used clinically to assess renal lesions in patients with DN [36]. Increased albumin excretion occurs as a result of podocytes hypertrophy and foot process effacement [37]. Hence, reduction of kidney damage and glomerular hyperpermeability can help ameliorating albuminuria. Diabetes associated nephropathy in this study was further confirmed by the histological changes, including cytoplasmic and nuclear degeneration in the tubular epithelial cells particularly the proximal convoluted tubules, and enlargement of renal corpuscles and narrowing of the renal spaces due to the glomerular hypercellularity. Interestingly, SIM prevented kidney damage, albuminuria and ameliorated BUN, serum and urinary creatinine, and creatinine clearance rate in STZ-induced diabetic rats. The ameliorative effect of SIM could be connected to the improved blood-glucose levels in diabetic rats. Studies have highlighted the beneficial role of good glycemic control in slowing kidney damage in diabetes [38]. In this context, several large trials have reported reduced albuminuria following glycemic control in type 1 and 2 diabetic patients [39,40].

Chronic hyperglycemia leads to the activation of several metabolic pathways that have been demonstrated to disrupt homeostasis regulatory mechanisms [41]. When altered, these metabolic pathways ultimately cause inflammation and fibrosis in the kidney [4,42]. Excessive generation of ROS and oxidative stress are the common denominator linking these pathways with the disrupted renal hemodynamics in the diabetic kidney. Hyperglycemia-induced ROS generation stimulates the production of growth factors, cytokines and transcription factors implicated in DN. ROS provoke renal fibrosis and tissue damage by triggering lipid peroxidation [43]. Diabetic rats in the present study showed a significant increase in kidney lipid peroxidation and NO levels, demonstrating oxidative/nitrosative stress. Increased kidney NO is a direct result of up-regulated inducible NO synthase (iNOS) [44]. NO can react with superoxide radicals to produce the versatile oxidant peroxynitrite, leading to DNA damage and cell death [45]. Renal oxidative stress occurs as a consequence to overproduction of ROS and concomitant depletion of cellular antioxidants. Herein, GSH and the antioxidant enzymes SOD and CAT were significantly reduced

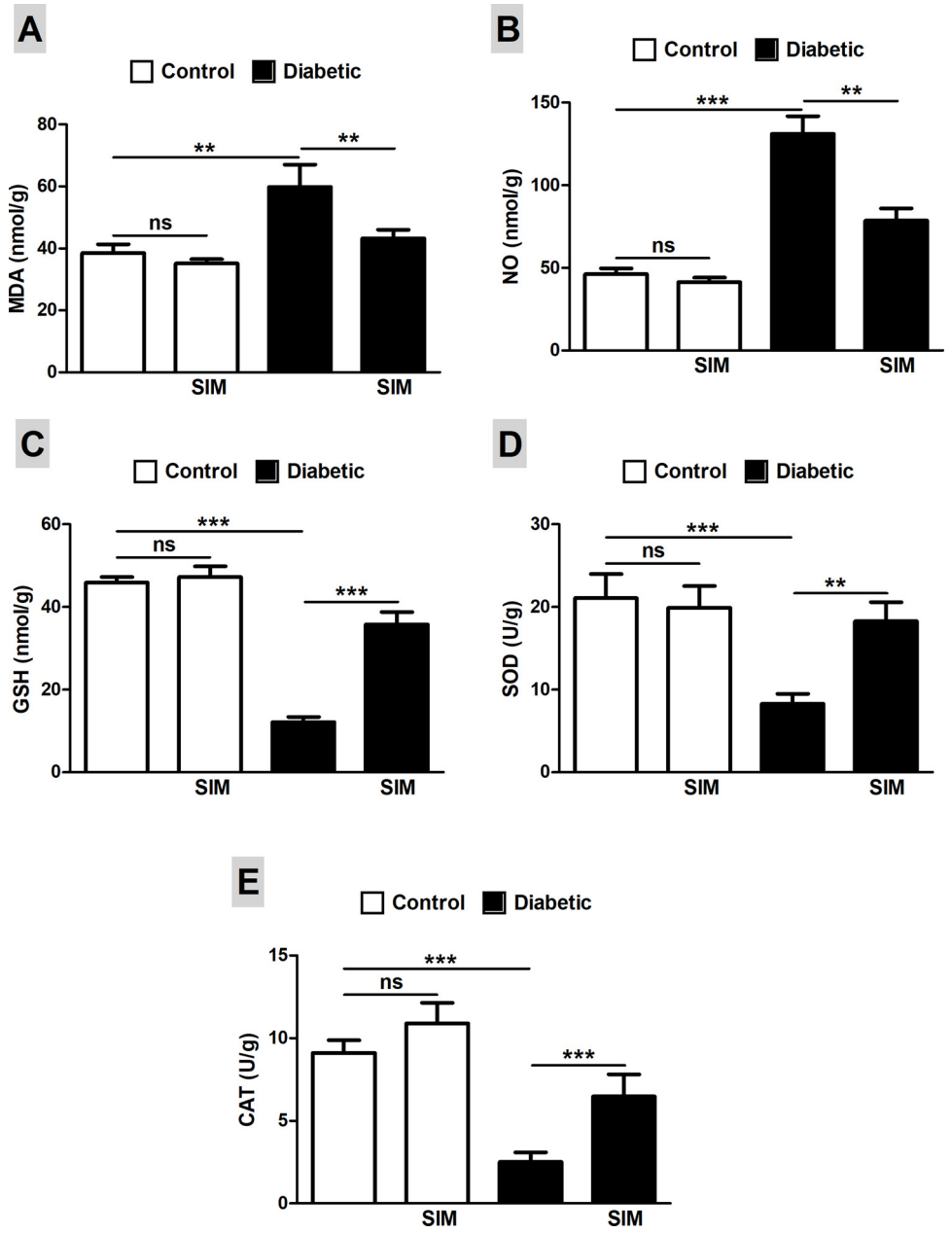


Fig. 4. Simvastatin mitigates hyperglycemia-induced oxidative stress in the kidney of diabetic rats. Treatment with simvastatin decreased renal MDA (A) and NO (B), and increased GSH (C), SOD (D) and CAT (E) in diabetic rats. Data are Mean ± SEM (n = 8). **P < 0.01 and ***P < 0.001. SIM, simvastatin; MDA, malondialdehyde; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; ns, non-significant.

in the kidney of diabetic rats. These findings added support to previous studies demonstrated diminished enzymatic and non-enzymatic defenses in the kidney of diabetic rats [35,46].

SIM supplementation decreased lipid peroxidation and NO, and boosted GSH, SOD and CAT in the kidney of diabetic rats. Accordingly, Mohamadin et al. [29] reported decreased lipid peroxidation and increased GSH in the kidney of diabetic rats treated with SIM for 5 weeks. Previous work from our laboratory showed that SIM prevented oxidative stress and enhanced antioxidant defenses in the heart of rat models of diabetes [15] and cardiac hypertrophy [18], supporting the role of SIM in mitigating oxidative stress. These results suggest that SIM exerts a renoprotective effect *via* attenuating oxidative stress and preventing the depletion of antioxidants. The beneficial role of statins in DN has been supported by studies demonstrated their inhibitory effect on nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), the main source of ROS. Pitavastatin down-regulated NOX4, diminished ROS generation, and ameliorated albuminuria and mesangial expansion in diabetic mice [47].

Given the role of SIM in minimizing hyperglycemia-induced oxidative stress, we assumed that it can protect the kidney against fibrosis. The present study showed increased deposition of collagen in the kidney of diabetic rats. Mesangial cell proliferation and excessive accumulation of extracellular matrix (ECM) occur in DN and can lead to chronic renal failure [48]. Collagen is one of the major ECM proteins that is often used as a marker of fibrogenesis in kidney diseases including DN [49,50]. Chronic hyperglycemia promotes the formation and accumulation of collagen I and IV and contributes to dysfunction of the kidney [51]. In addition, excessive ROS generation drives the recruitment of ECM-producing cells and promotes the progression of renal fibrosis [52]. ROS stimulate profibrotic pathways, including cytokines and growth factors which in association with ROS provoke excessive buildup of ECM and kidney injury [53]. Therefore,

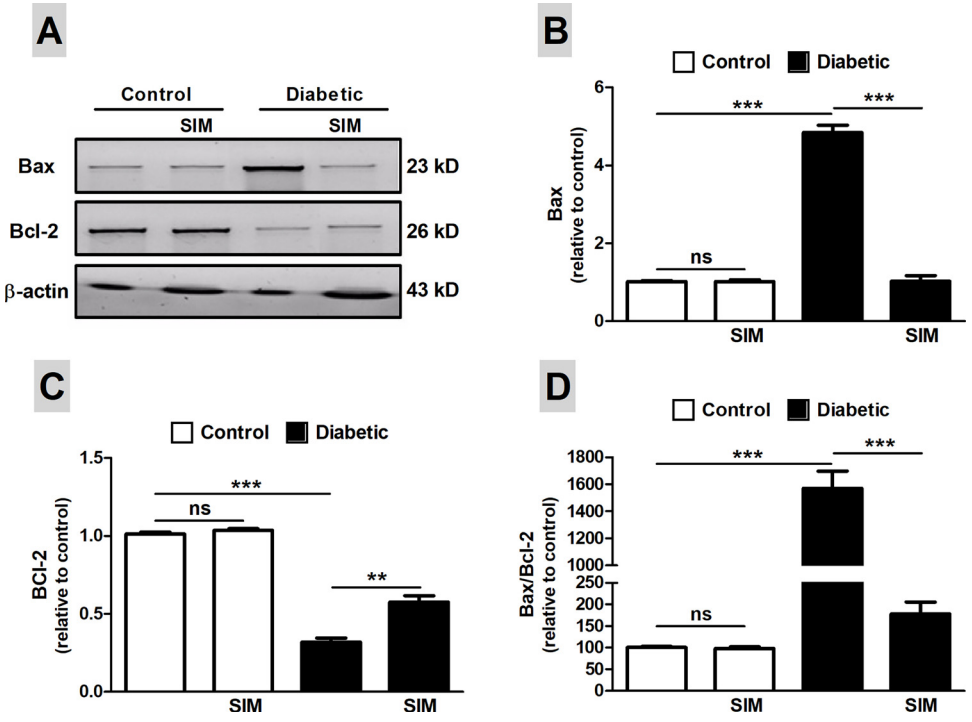
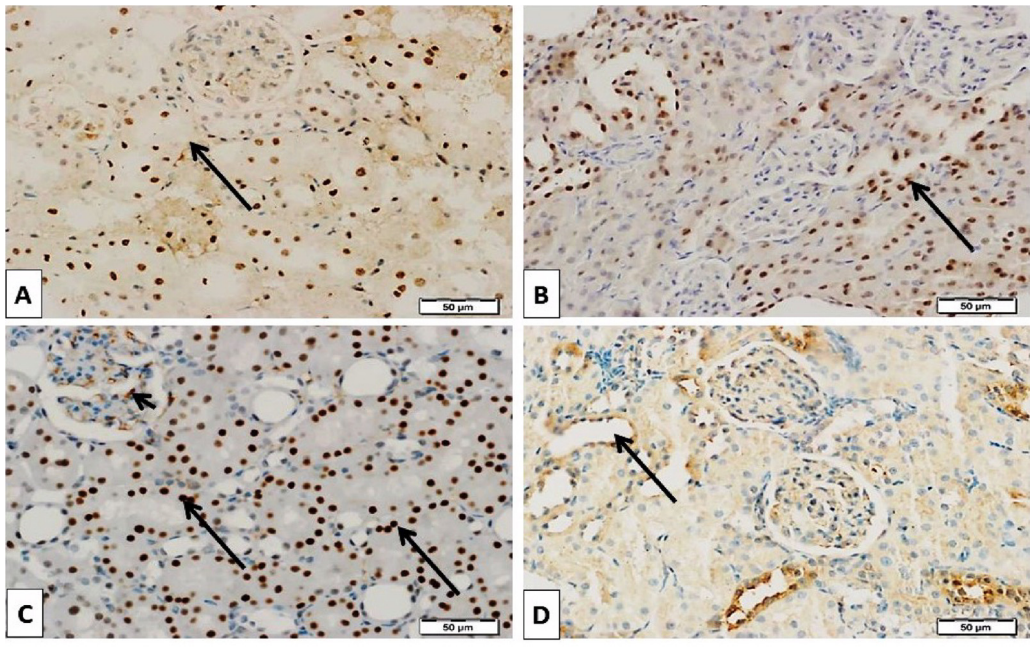


Fig. 5. Simvastatin ameliorates the expression of Bax and Bcl-2 in the kidney of diabetic rats. (A) representative bands showing the effect of simvastatin on the expression of Bax and Bcl-2. Simvastatin decreased the expression levels of Bax (B) and increased Bcl-2 (C). (D) Bax/Bcl-2 ratio was significantly declined in the kidney of diabetic rats treated with simvastatin. Data are Mean ± SEM (n = 8). **P < 0.01, ***P < 0.001. SIM, simvastatin; ns, non-significant.



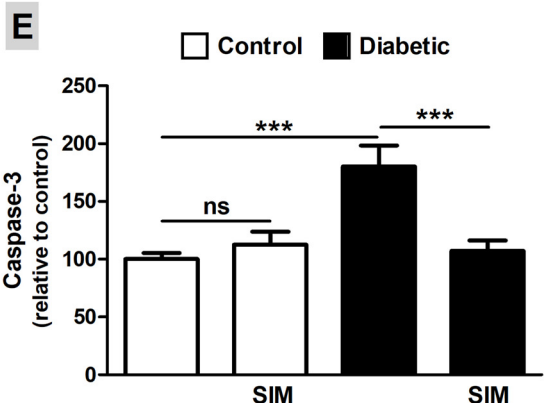


Fig. 6. Simvastatin down-regulates the expression of caspase-3 in the kidney of diabetic rats. (A–D) Photomicrographs of caspase-3 immuno-stained kidney sections of (A) control rats, (B) control rats treated with simvastatin showing immuno-positive nuclei of few tubular epithelial cells and some interstitial cells (arrow), (C) diabetic rats showing marked increase in the number of immuno-positive stained nuclei of both tubular epithelial and interstitial cells (arrows), and the cytoplasm of glomerular epithelial cells showing immune reaction (arrow head), and (D) diabetic rats treated with simvastatin showing immune reaction similar to that of the control (arrow). (E) Image analysis of caspase-3 immuno-staining represented as percentage relative to control. Data are Mean ± SEM (n = 8). ***P < 0.001. SIM, simvastatin; ns, non-significant.

attenuating ROS production can play a key role in preventing renal fibrosis in diabetes.

The renoprotective effect of SIM might be also explained in terms of reduced inflammation. The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) has been demonstrated to promote ROS production and hence is positively correlated with the progression of renal disease. Nuclear factor-kappaB (NF-κB), the main transcription factor that initiates inflammation in diabetes [54], has been shown to be up-regulated in the diabetic kidney and to activate mesangial cells to cause renal tissue injury [55]. Recently, we have shown that the treatment of STZ-induced diabetic rats with SIM for 90 days diminished serum TNF-α and cardiac expression levels of NF-κB [15]. SIM reduced collagen deposition and fibrosis in the heart of diabetic rats *via* its dual efficacy to diminish oxidative stress and inflammation [15]. As oxidative stress and inflammation work in concert to induce apoptosis [56,57], attenuation of these processes represents the mechanism behind the diminished apoptosis in the kidney of diabetic rats treated with SIM. The pro-apoptotic protein Bax was increased while the anti-apoptotic protein Bcl-2 was decreased in the kidney of diabetic rats. The balance between the pro- and anti-apoptotic Bcl-2 family members dictates the susceptibility of cells to apoptosis [25]. The association between apoptosis and the development and progression of DN has been well-acknowledged [5]. Hyperglycemia-mediated overproduction of ROS has been demonstrated to increase the levels of Bax [57] and induce apoptosis in podocytes and tubular and mesangial cells [57–59]. The protective effect of SIM against apoptosis in the kidney of diabetic rats was confirmed by the reduced expression of caspase-3.

In conclusion, these results indicate that SIM exhibits renoprotective effects in STZ-induced diabetic rats. These effects occurred *via* attenuation of hyperglycemia-induced oxidative stress, fibrosis and apoptotic pathways. Since the use of SIM preserved renal function and retained normal morphology in type diabetic rats, further studies are recommended to investigate the exact underlying mechanisms.

Conflicts of interest

The authors state no conflicts of interest.

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