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Potential role of empagliflozin in prevention of nephropathy in streptozotocin-nicotinamide-induced type 2 diabetes: an ultrastructural study

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Abstract

Objectives: Diabetic nephropathy is a serious factor in end-stage renal disease worldwide. Sodium-glucose cotransporter 2 inhibitors, the most novel glucose-lowering drug, may have a nephroprotective effect by modulating blood glucose, blood pressure and autophagy. The present work aimed to study the possible protective effect of empagliflozin in Type 2 diabetic nephropathy with special considerations to oxidative stress, fibrosis and ultrastructural modulation including autophagy.

Methods: Thirty-six adult male Sprague-Dawley rats were divided into 3 groups; control, diabetic, and treatment. Type 2 diabetes was induced by pretreatment with nicotinamide followed by single low-dose of streptozotocin (40 mg/kg, i.p). The treatment group received empagliflozin (10 mg/kg/day, intragastric) for 4 weeks. At the end of 4 weeks, parameters of renal function and oxidative stress were analyzed. Kidney samples were collected for histological and ultrastructural studies.

Results: Empagliflozin significantly reduced hyperglycemia, blood urea nitrogen, serum creatinine and oxidative stress which were elevated in the diabetic group. It also decreased renal tissue injury and fibrosis; however, did not lower the increased kidney index and glomerular size. Beside the amelioration of ultrastructure changes, empagliflozin enhanced the autophagy in renal tubular cells, indicated by increased number of autophagic vacuoles.

Conclusion: Empagliflozin provided an efficient, but not complete protection against diabetic nephropathy in streptozotocin-nicotinamide-induced type 2 diabetic rat model. This effect could be related to reduction of hyperglycemia and improvement of cellular defense mechanisms, and reduction of glucose-induced oxidative stress and autophagy.

Keywords: autophagy; diabetes; nephropathy; empagliflozin

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Introduction

Diabetic nephropathy (DN) is a major complication of diabetes mellitus, and a serious factor in end stage renal disease (ESRD) worldwide.[1] Novel management of DN includes control of high blood pressure and blood glucose levels and suppression of the renin angiotensin system to eliminate proteinuria.[2] However, some patients suffer from deterioration in renal function causing ESRD. So, new effective line of therapy is a must for the control of ESRD.[3]

The pathogenesis of DN includes different stages; reversible glomerular hyperfiltration, normal glomerular filtration with normo-albuminuria, decreased glomerular filtration with microalbuminuria, decreased glomerular filtration with macroalbuminuria, and finally proteinuria with ESRD.[4] The mechanism of DN includes hemodynamic factors like oxidative stress and inflammation and metabolic factors. The inflammatory and fibrotic mediators result in loss of podocytes, hypertrophy of GBM, atrophy of tubules, interstitial and tubular inflammation, and fibrosis.[5]

Autophagy plays an essential role in the stress-response mechanism, the disturbance of which is included in the pathogenesis of diabetes-related complications.[6,7] Autophagy is responsible for the degradation of damaged proteins and organelles in the cell to preserve homeostasis and cell integrity in both normal and diseased conditions.[3] Autophagy was suggested to play a major role in

nephroprotection in some animal models, including aging and acute renal injury models.[8,9]

One of the most novel oral glucose lowering drugs in type 2 DM is sodium-glucose cotransporter 2 (SGLTI) inhibitors.[10] There are three available drugs of this family; empagliflozin, dapagliflozin and canagliflozin.[11] SGLT2 inhibitors were reported to disturb diabetic complications and their progression.[12] SGLT2 inhibitors might have nephroprotective effect by not only controlling the blood glucose level, but also glucose-independent actions such as lowering of the blood pressure.[13]

Normally, the filtered glucose by healthy kidneys is reabsorbed to circulation and the renal reabsorption of glucose occurs through SGLT2. In type 2 DM, enhanced activity of SGLT2 results in more glucose reabsorption and persistent high blood glucose levels. On the other hand, inhibition of SGLT2 reduced renal reabsorption of glucose up to 50 %.[14]

SGLT2 inhibitors also causes diuresis with reduction of blood pressure. These drugs cause an initial reduction in the glomerular filtration rate (GFR), followed by stabilization of the GFR.[15]

The present work aimed to study the possible protective effect of empagliflozin in type 2 DN with special considerations to oxidative stress, fibrosis and ultrastructural changes including autophagy modulation.

Materials and Methods

Thirty-six adult male Sprague Dawley rats, aging 10–12 weeks and weighting 200–250 g were used in this study, purchased from AL-Nile Experimental Animal Center, Mansoura, Egypt. The rats were housed in separate cages at a constant temperature of 20°C and 45% humidity. Rats had a free access to normal rodent chow and drinking water. All experiments were carried out after approval of the Institutional Research Board in Faculty of Medicine, Mansoura University. Streptozotocin (STZ, CAS no: 18883-66-4, Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.1mM citrate buffer (pH 4.4). Nicotinamide (NA, CAS no: 98-92-0, Sigma-Aldrich, St Louis, MO, USA) was dissolved in normal saline. Empagliflozin (Boehringer Ingelheim Charmaceutical Company, Biberach, Germany) was purchased from local pharmacy.

After acclimatization for one week, the rats were separated randomly into three groups 12 rats each: control group of non-diabetic rats (were given vehicle only); diabetic group of diabetic untreated rats; and empagliflozin treated diabetic rats that received a single daily dose of empagliflozin (10 mg/kg, intragastric) for four weeks.[16] Induction of type 2 diabetes was performed by intraperitoneal (i.p.) injection of freshly prepared NA solution (120 mg/kg), after 15 min, the rats were given single STZ injection (40 mg/kg, i.p).[17] The fasting blood glucose levels were measured seven days after the diabetic induction. The rats with fasting blood glucose more than 126 mg/dL were selected as diabetic.[18]

The rats were weighed and sacrificed at the end of 4th week under general anesthesia. Blood and kidney samples were collected immediately. The blood samples were centrifuged at 3000 rpm for 15 min. The serum was stored at -20°C till assessment for biochemical parameters. The kidney weights were measured and the kidney index [kidney weight ×100 / body weight] was calculated.[19] The right kidneys were fixed in 10% formaldehyde for histopathological examination. The left kidneys were used for electron microscopic examination.

Blood glucose monitoring in experimental rats was performed by obtaining a drop of blood from the tail vein before STZ injection, one week after STZ injection to confirm diabetic glucose level and then on the 4th week after STZ injection. Glucose level was evaluated with the ACCU-CHEK glucose meter (Roche Diagnostic Co., Mannheim, Germany). The serum levels of creatinine and blood urea nitrogen (BUN) were evaluated using a spectrophotometric enzymatic kit (Thermo Trace-BECG-MAN, Germany). Oxidative marker superoxide dismutase (SOD) and lipid peroxidation marker malondialdehyde (MDA) were measured (absorbance 450 nm and 532 nm, respectively) using a kit (Biovision, cat. No. K335-100 and K739-100, San Francisco, CA, USA).

The formalin-fixed right kidneys were processed for paraffin block preparation. Serial sections were cut at 5 µm andn stained with haematoxylin and eosin (H&E) to detect histopathological changes.[20] Masson's trichrome staining[21] was used to demonstrate the accumulation of connective tissue in the nephrons. Also, 1 µm semi-thin sections were prepared from left kidneys and stained with toluidine blue to examine under the light microscope. Small pieces (1×2 mm) from cortexes of the left kidneys were cut and fixed in a solution containing glutaraldehyde (3%) and paraformaldehyde (2%) dissolved in 0.1 mol/L phosphate buffer. Specimens were washed in phosphate buffer, post-fixed in osmium tetroxide (1%), and embedded in epon. 1 µm semithin toluidine blue sections were prepared. Ultrathin sections of 50–70 nm were prepared and stained with uranyl acetate and lead citrate.[22] Those sections were examined with JEOL-100SX transmission electron microscope (TEM; Jeol Inc., Peabpdy, MA, USA) in Mansoura University.

Morphometric studies were done using NIH Image J program (National Institutes of Health, Bethesda, MD, USA), according to the instructions. The average glomerular diameter [(maximum perpendicular diameter+maximum transverse diameter)/2] was measured in H&E stained sections in randomly chosen 10 glomeruli per animal.[23] The mean width of glomerular (Bowman's capsule) space in H&E stained sections were measured in 10 non-overlapping fields for each group.[24] The percentage of blue stained area was measured in Masson's trichrome-stained sections in 10 non-overlapping random fields.[25] The thickness of GBM was measured and the mean value was calculated in 20 randomly selected ultrathin fields of cortex from each rat. The number of autophagic vacuoles in podocytes was calculated (×40.000) in 15 randomly selected fields.[26]

The data were expressed as mean±standard deviation (SD). Analysis of variance (ANOVA) followed by post-hoc Tukey test were used to compare the significance between different groups; $p<0.05$ was considered statistically significant. All statistics were carried out using IBM SPSS Statistics for Windows (Version 22, Chicago, IL, USA).

Results

The body weight was assessed at the beginning and by the end of the study, the kidney weights were measured and kidney indexes were calculated (**Table 1**). The diabetic group showed significant body weight loss compared to the control group. Treatment with empagliflozin significantly prevented diabetic induced weight loss (p=0.07). The kidney index was increased in both diabetic and treated groups compared to the control group (p=0.771).

Before the induction of DM, the fasting blood glucose level was nearly equal between the experimental groups. One week after diabetes induction, there was a significant increase in blood glucose of both diabetic and treated groups as compared to the control group. At the end of the study, the blood glucose of both diabetic and treated groups was significantly higher than the control group (p=0.009); however, the blood glucose in the treated group was significantly lower than the diabetic group (p=0.001) (**Table 2**).

To assess the effect of empagliflozin on the kidney function, the serum creatinine and BUN were measured in each group (**Table 3**). The diabetic group demonstrated a significant increase in serum creatinine and BUN compared to the control group, indicating a reasonable kidney tissue injury. Administration of empagliflozin achieved a renal protective effect evidenced by a significant decline in the serum creatinine and BUN in the treated group compared to the diabetic group (p=0.0771).

To investigate the protective effect of empagliflozin on diabetes-induced oxidative stress, the levels of antioxidant enzyme SOD and MDA (lipid peroxidation biomarker) were assessed in the renal tissues of each group (**Table 4**). The diabetic group showed a significant decrease in SOD and and increase in MDA levels compared to the control group. The level of SOD was decreased in the treated group compared to the control and diabetic groups. Interestingly, the treated group

Table 1
Comparison of the body weight and kidney index measurements (mean±SD; n=12).

	Control	Diabetic	Treated
Body weight (g) (beginning of experiment)	310±8	290±65	296±37
Body weight (g) (end of experiment)	320±7	200±25*	287±50†
Kidney index (kidney wt×100/body weight)	.279±.9	.422±.15*	.962±.30*,†

*p<0.05 compared to the control group; †p<0.05 compared to the diabetic group.

Table 2
Comparison of fasting blood glucose levels (mean±SD; n=12).

	Control	Diabetic	Treated
Before induction of DM	105.3±31	108.1±39	107.4±42
1 w after induction of DM	109.2±26	500.1±134.7*	495.3±126.9*
Day of sacrification	106.6±39.5	441.9±128.4*	216.2±91.2†

*p<0.05 compared with the control group; †p<0.05 compared with the diabetic group.

Table 3
Comparison of the results of renal function tests (mean±SD; n=12).

	Control	Diabetic	Treated
Serum creatinine	1.8±.32	3.7±.46*	1.3±.21*
BUN	18.4±.9	65.2±1.15*	40.3±1.46†

*p<0.05 compared to the control group; †p<0.05 compared to the diabetic group.

Table 4
Comparison of the levels of oxidative stress markers (mean±SD; n=12).

	Control	Diabetic	Treated
SOD %	85.7%±23.8	74.3%±31.7*	65.7%±28.3*,†
SOD concentration (μ/g)	918.2±167.4	796.1±148.3*	703.9±192.6*,†
MDA (nmol/g)	36.7±18.4	85.8±31.8*	48.5±20.1†

*p<0.05 compared to the control group; †p<0.05 compared to the diabetic group.

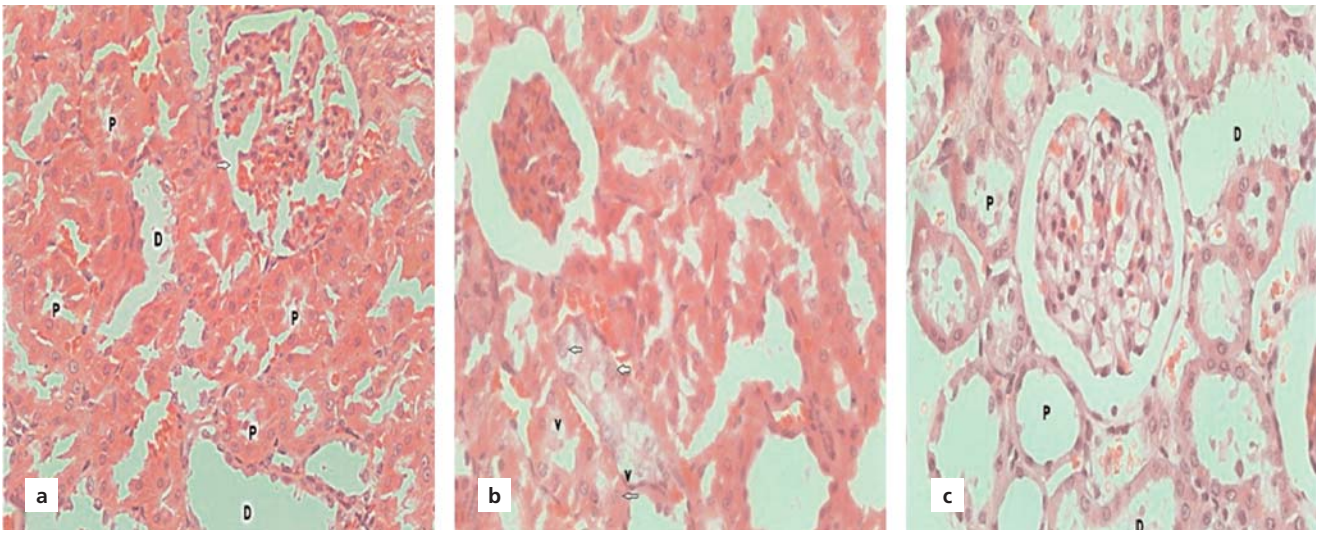

Figure 1. Photomicrographs of the renal cortex. (**a**) The control group shows renal corpuscle, glomerulus and Bowman's space (**arrow**), proximal convoluted tubules with cuboidal cells, vesicular nuclei and deeply stained eosinophilic cytoplasm. The distal convoluted tubules have a wide lumen, with cubical cells and pale eosinophilic cytoplasm; (**b**) The diabetic groups shows degenerated cells in the tubules (**arrows**) with cytoplasmic vacuoles; (**c**) The kidney tissues of treated rats shows renal tubules with normal cells and vesicular nuclei; proximal and distal convoluted tubules. **D:** distal convoluted tubules; **G:** glomerulus; **P:** proximal convoluted tubules; **v:** cytoplasmic vacuoles (H&E stain; ×100). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

showed significant decrease in the level of MDA compared to the diabetic group.

H&E stained sections of the renal cortex in the control group showed the normal renal corpuscles with glomeruli and glomerular spaces. While the proximal convoluted tubules were lined by tall cuboidal cells with deeply stained eosinophilic cytoplasm, the distal convoluted tubules had a wide lumen and lined by flat cubical cells with pale eosinophilic cytoplasm (**Figure 1a**). Degenerated tubular cells with cytoplasmic vacuoles and deeply stained nuclei were also detected in the diabetic group (**Figure 1b**). The diabetic groups showed a significant increase in the glomerular diameter (72.6±8 µm, $p<0.05$; **Figure 2**) and glomerular space (18.2±3.9 µm, $p<0.05$; **Figure 3**) compared to the control group (glomerular diameter: 54.7±10.1 µm, glomerular space: 7.6±2.7 µm). Kidneys of the treated rats showed normal renal corpuscles with a significant decrease in the glomerular diameter (83±16.2 µm, $p<0.05$; **Figure 2**) and glomerular space (10.4±2.1 µm, $p<0.05$; **Figure 3**) compared to the diabetic group, a large amount of apparent normal renal tubules and vesicular nuclei (**Figure 1c**).

Masson-trichrome stained sections of the control kidney showed minimal fibrosis with normal collagen distribution within the renal corpuscle and between the tubules (**Figure 4a**). In the diabetic kidneys, there were massive collagen depositions between the tubules and within the

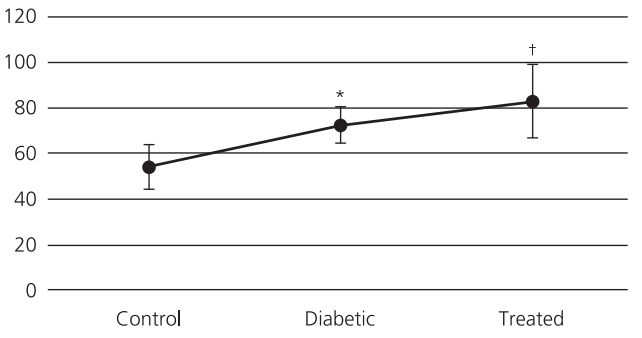

Figure 2. The mean glomerular diameter in control, diabetic and treated groups (mean±SD; n=12). *$p<0.05$ compared to the control group; †$p<0.05$ compared to the diabetic group.

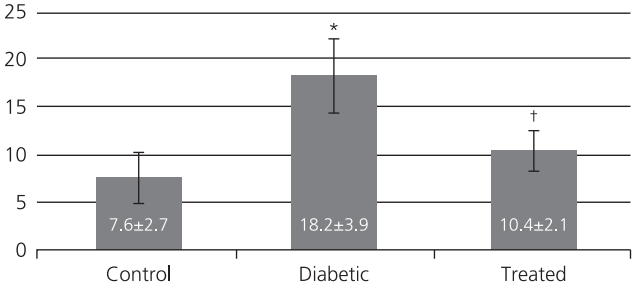

Figure 3. The mean glomerular space in control, diabetic and treated groups (mean±SD; n=12). *$p<0.05$ compared to the control group; †$p<0.05$ compared to the diabetic group.

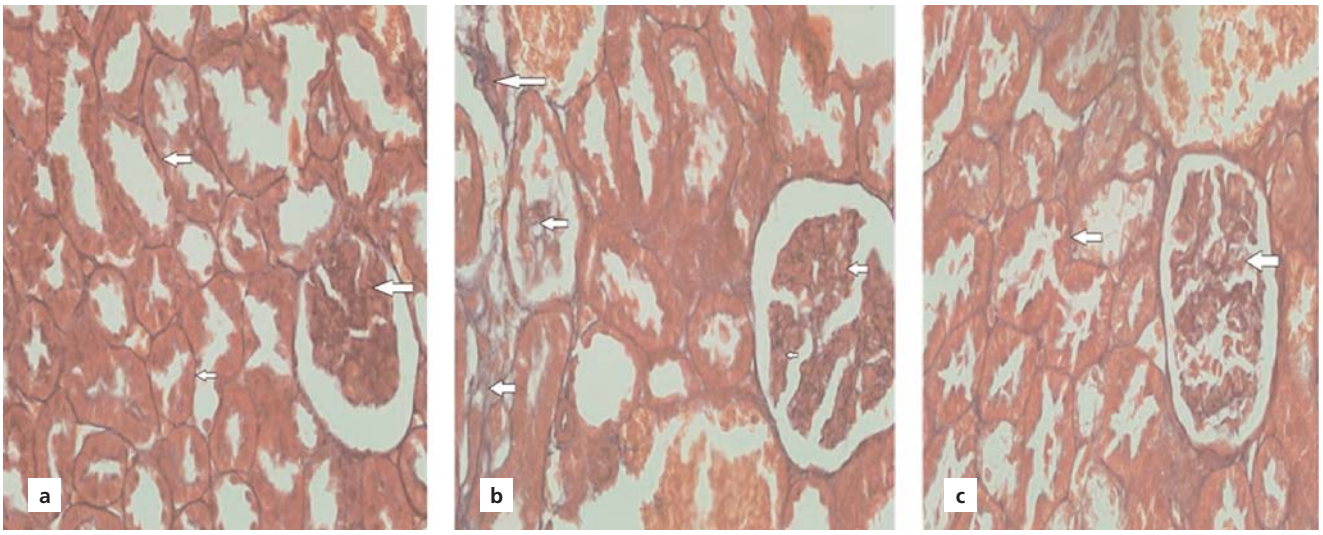

Figure 4. Photomicrographs of the renal cortex. (**a**) The control group shows minimal fibrosis within the normal area of collagen distribution within the renal corpuscle and among the tubules (**arrows**); (**b**) The diabetic kidney shows massive collagen deposition between the tubules and within the glomerulus (**arrows**); (**c**) The treatment kidney shows minimal fibrosis within the renal corpuscle and surrounding the tubules (**arrows**) (Masson's trichrome stain; ×100). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

glomerulus (**Figure 4b**) with a significant increased area percentage compared to the control group ($p<0.05$; **Figure 5**). The treated kidneys showed minimal fibrosis within the renal corpuscle and surrounding the tubules (**Figure 4c**) with a significant decrease in the area percentage as compared to the diabetic group ($p<0.05$; **Figure 5**).

Toluidine blue stained semithin sections in the control group showed normal renal corpuscles and glomeruli with mesangial cells and podocytes, capillaries and intact basement membrane. The renal tubule cells showed basal striations, vesicular nuclei, and apparent brush borders (**Figure 6a**). The diabetic kidneys showed glomeruli with podocytes, mesangial cells, renal tubules with absent of basal striation, lost brush border and areas of cytoplasmic vacuolation (**Figure 6b**). The treated kidneys showed the glomeruli with intact mesangial cells and podocytes. The renal tubule cells restored the basal striations and the apparent brush borders (**Figure 6c**).

TEM examination was performed to detect the ultrastructural changes of the renal tissues. For the glomeruli, ultrathin sections of the control group showed regular basement membrane, fenestrated endothelial cells and apparent podocytes of the foot processes (**Figure 7a**). The diabetic group showed significantly thickened basement membrane, a fusion of the foot processes and degenerated podocytes (**Figure 7b** and **7c**) compared to the control group. The treated group displayed regular basement membrane and apparent podocytes of the foot processes with a significant decrease in the basement membrane thickness compared to the diabetic group (1.6 µm, $p<0.05$) (**Figure 7d**, **Table 5**).

For proximal convoluted tubules, ultrathin sections of the control group showed proximal tubular cells with a regular basement membrane, apical microvilli, oval euchromatic nucleus, mitochondria, and apparent autophagic vacuoles (**Figure 8a**). The diabetic group showed thickened basement membrane, distorted apical microvilli, pyknotic nucleus. The cytoplasm showed vacuoles and swollen mitochondria (**Figure 8b**). The treated group showed normal nucleus, mitochondria, apparent microvilli, and autophagic vacuoles (**Figures 8c** and **8d**). The number of autophagic vacuoles was significantly less in the diabetic

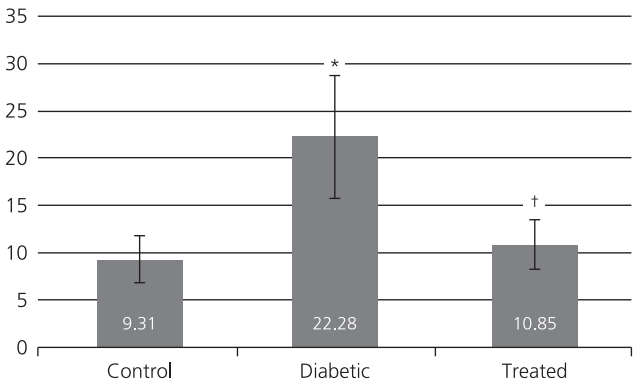

Figure 5. The mean fibrosis area in control, diabetic and treated groups (mean±SD; n=12). *$p<0.05$ compared to the control group; †$p<0.05$ compared to the diabetic group.

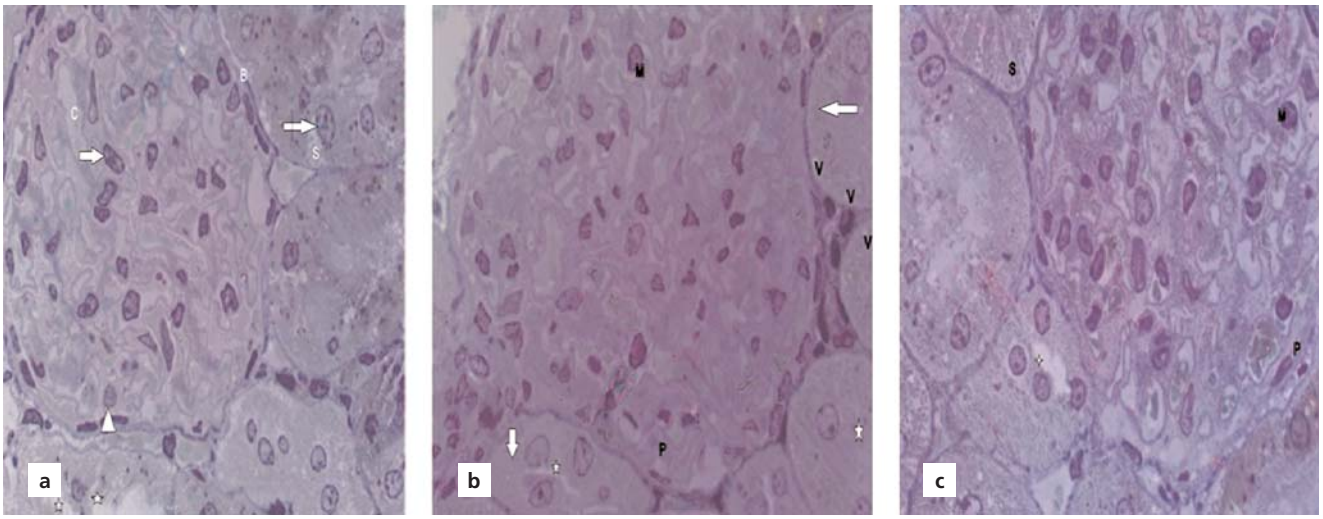

Figure 6. Photomicrographs of semithin sections of the renal cortex. (**a**) The control group shows normal renal corpuscle containing a glomerulus, with mesangial cells (**arrow**), podocytes (**arrowhead**), capillaries and intact basement membrane. The tubules' shows cells basal striations, apparent brush borders (*****) and large vesicular nuclei (**crossed arrow**); (**b**) The diabetic kidney shows glomerulus with podocyte, mesangial cells, renal tubules with absent basal striation (**arrow**), lost brush border (*****), areas of cytoplasmic vacuolation. (**c**) The treated kidney shows the glomerulus, with mesangial cells and podocytes. The tubuless cells shows restored basal striations, restored apparent brush borders (*****). **C:** capillaries; **m:** mesangial cells; **P:** podocytes; **S:** basal striations; **V:** cytoplasmic vacuoles (Toludine blue stain, ×1000). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

group as compared to both control and treated groups ($p<0.05$; **Table 5**).

Discussion

The present study provided evidence that empagliflozin induces an efficient, but not complete protection against DN. This conclusion is supported by our results which demonstrated that empagliflozin prevented kidney injuries occurred after 4 weeks in streptozotocin-nicotinamide-induced type 2 diabetic rat model. Empagliflozin significantly lowered the increased blood glucose, BUN, serum creatinine, and oxidative stress biomarkers that are known to be elevated in DN. Furthermore, it reduced renal tissue degeneration, fibrosis, and glomerular space; however, it did not reduce the increased kidney index and glomerular size. Beside the amelioration of ultrastructure changes occurred in the renal cortex, we reported for the first time that empagliflozin enhanced tautophagy in renal tubular cells which was indicated by increased number of autophagic vacuoles.

Table 5
Comparison of the glomerular basement membrane thickness, and the number of autophagy vacuoles in the proximal renal tubules (mean±SD; n=12).

	Control	Diabetic	Treated
Thickness (μm)	1.47	2.83*	1.6†
Number of autophagy vacuoles	.7/100 μm²	.4/100 μm²*	.6/100 μm²†

*$p<0.05$ compared to the the control group; †$p<0.05$ compared to the diabetic group.

Approximately half of cases with type 2 diabetes and one third of cases with type 1 diabetes develop DN.[27] Recent reports ensure that ten years mortality rates of cases with DN equal mortality rates of all cancers.[28,29] Therefore, there is an urgent motivation to develop effective medication to slow the progression of DN.

Early intensive glycemic control is a critical strategy for prevention and delay of DN progression. However, the currently used hypoglycemic drugs fail to achieve optimum blood glucose levels and are associated with hypoglycemia and weight gain.[30] Therefore, there is a need for new drugs that control the blood glucose level and have protective pathways. The SGLT2 inhibitors have recently being used widely.[31] Based on their insulin-independent action and effect in losing weight, SGLT2 inhibitors would be expected to be more beneficial than the current therapies of DM. Empagliflozin, one of selective SGLT2 inhibitors, attenuates DN[32] and cardiovascular diseases in patients with type 2 DM.[33]

A series of experimental studies have shown that empagliflozin exerts renoprotective benefits (**Table 6**). It was reported to decrease blood glucose and albuminuria and ameliorate glomerular hypertrophy, mesangial expan-

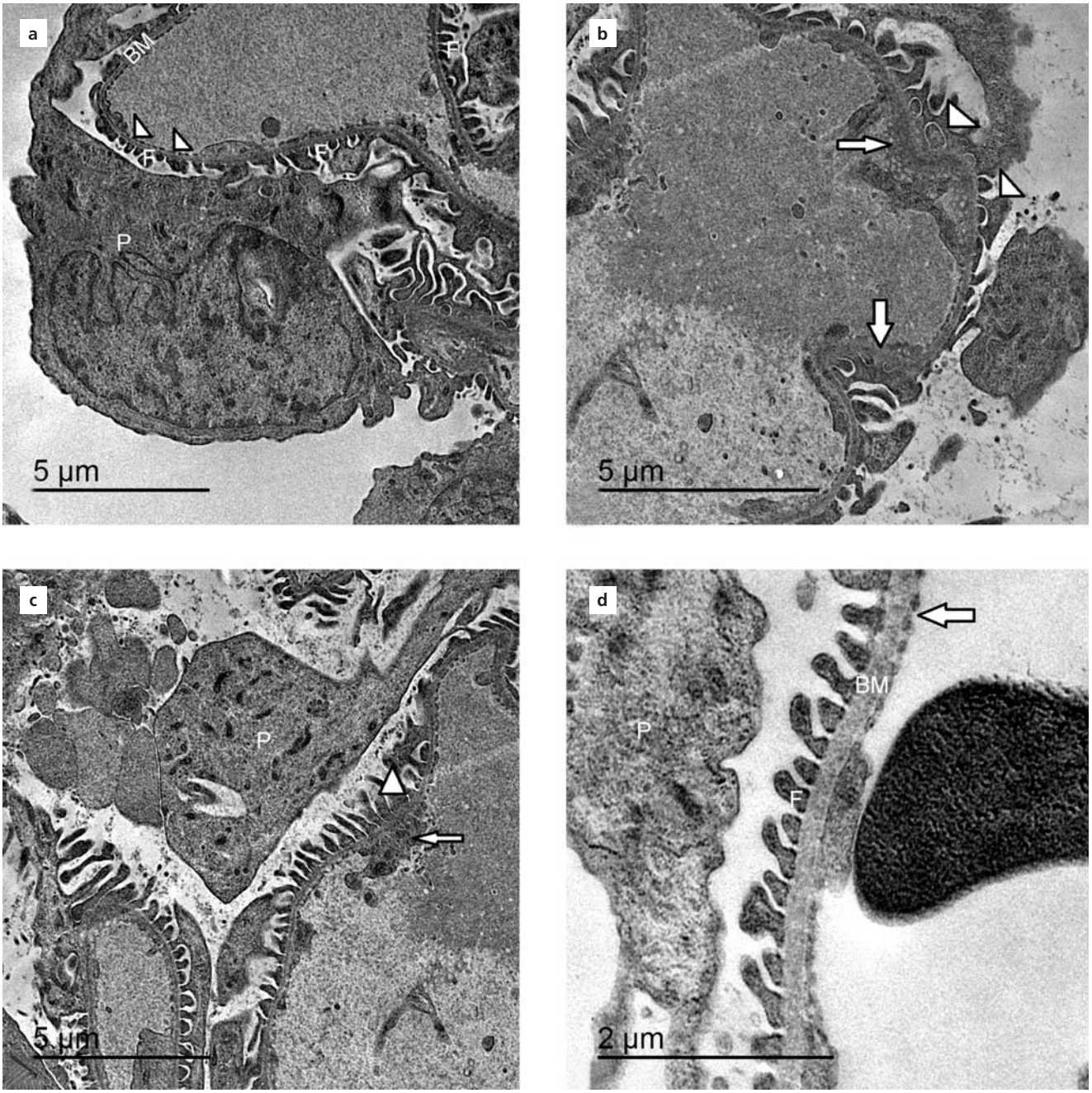

Figure 7. Photomicrographs of ultrathin section of the glomerulus. (**a**) The control group shows normal fenestrated endothelial cell (**arrowhead**); (**b**, **c**) Regular basement membrane, podocytes, foot processes of podocytes. The diabetic group shows areas of fusion of the foot processes (**arrowheads**) with thickened basement membrane (**arrows**) and degenerated podocytes. (**d**) The treated group shows regular basement membrane, foot processes of normal podocytes, intact endothelial lining (**arrow**). **BM:** basement membrane; **F:** foot processes of podocytes; **P:** podocytes.

sion, renal fibrosis, inflammatory and oxidative stress markers in different diabetic animal models.[16,25,30,34,35] However, none of these previous studies have demonstrated the protective effect of empagliflozin at the ultrastructural level. A summary of the effects of empagliflozin on experimental DN is shown in **Table 7**.

In agreement with the previous studies, the present work demonstrated maintainance of body weight, significant reduction in blood glucose, and improvement in the kidney function after 4 weeks empagliflozin treatment. The only exception is the study of Lee et al.[30] who demonstrated significant reduction in body weight after 24 weeks

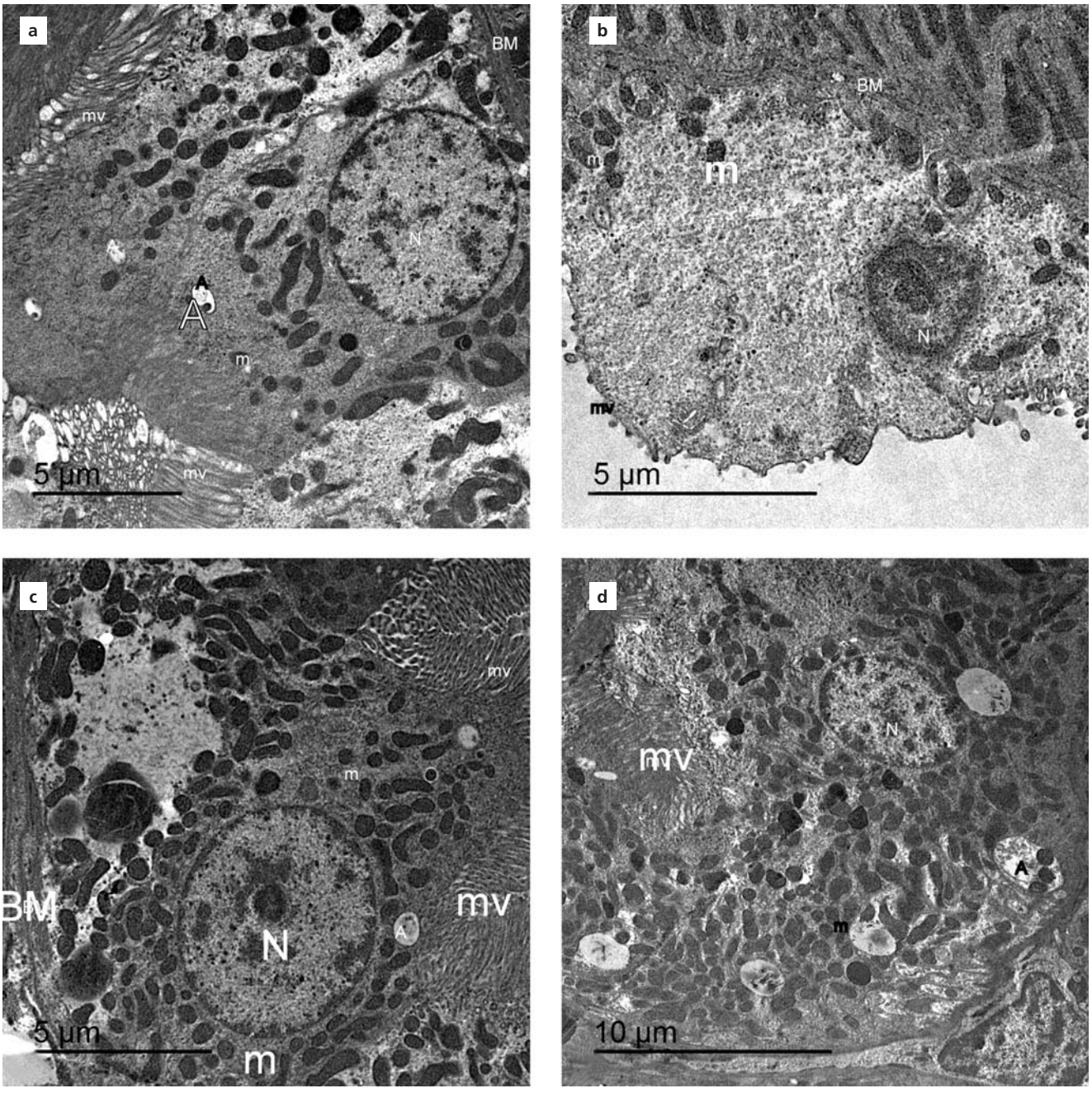

Figure 8. Photomicrographs of ultrathin sections of the proximal convoluted tubules. (**a**) The control group shows normal proximal tubular cells, regular basement membrane, apical microvilli, the nucleus appeared rounded and euchromatic, numerous mitochondria and autophagy vacuoles; (**b**) The diabetic group shows thickened basement membrane, distorted apical microvilli, pyknotic nucleus, the cytoplasm with vacuoles and swollen mitochondri; (**c**, **d**) The treated group shows thin basement membrane, normal nucleus, mitochondria, apparent microvilli and autophagic vacuoles. **A:** autophagy vacuoles; **BM:** basement membrane; **m:** mitochondria; **mv:** microvilli; **N:** nucleus.

empagliflozin treatment compared to the diabetic group. This may be related to longer duration of their study.

The present study also demonstrated improvement in renal histology indicated by decreased renal tubular degeneration and glomerular space in the empagliflozin treated group. However, in contrast with the previous studies,[34–36] we couldn't demonstrate a reduction in the increased glomerular size or kidney index after 4 weeks of empagliflozin treatment which indicated partial protection. This may be explained by shorter duration or

Table 6
Literature review of the effects of empagliflozin on experimental DN.

Experimental model	Dose of empagliflozin	Duration of study	Main effects	Study reference
BTBR ob/ob mice	Diet with 300 ppm of empagliflozin	12 weeks	= Body weight ↓ Blood glucose ↓ Albuminuria ↓ Glomerular hypertrophy ↓ Inflammation ↓ Mesangial matrix expansion	[34]
STZ-diabetic rats	10 mg/kg	4 weeks	↓ Blood glucose ↓ Oxidative stress ↓ Inflammation ↓ Fibrotic gene markers ↓ Tubular injury	[16]
Akita mice	300 mg/kg	15 weeks	↓ Glomerular hyperfiltration ↓ Albuminuria ↓ Kidney Weight ↓ Inflammation	[35]
db/db mice	10 mg/kg	10 weeks	= Body weight ↓ Kidney weight ↓ Blood glucose = Albuminuria ↓ Profibrotic gene markers ↓ Collagen IV ↓ TGF-β	[36]
STZ-diabetic rats	3 mg/kg	24 weeks	= Blood glucose ↓ Body weight ↓ Albuminuria = Glomerulosclerosis = Mesangial matrix expansion	[30]
	10 mg/kg	24 weeks	↓ Blood glucose ↓ Body weight ↓ Albuminuria ↓ Glomerulosclerosis ↓ Mesangial matrix expansion	[30]

smaller dose in our study, as well as the different animal model.

Another mechanism that may explain the nephroprotective effect of SGLT-2 inhibitor is the improvement of renal hypoxia in diabetic kidneys.[37] It was reported that hyperglycaemia-induced changes in intracellular metabolism, like glycation end products accumlation, and free radical are main factors in the development of DN.[31]

In agreement with Ojima et al.[16] the present study demonstrated significant reduction in oxidative stress marker MDA in empagliflozin treated group compared to the diabetic group. This could be explained by increased glucose uptake in the kidney in diabetes which leads to increase intracellular glucose levels and in turn stimulates reactive oxygen species (ROS) production.[38,39] Also, proximal convoluted tubule is the site of reabsorption of organic solutes and electrolytes, which are oxygen dependent processes that cause a reduction of oxygen tension in the kidney tissue. SGLT-2 inhibitors decrease the reabsorption of sodium and glucose and therefore reduce tubular work load and improve renal oxygenation, resulting in improvement of tubular structure and function.[40]

Inflammation, fibrosis and oxidative stress are involved in the initiation and progression of kidney disease.[41,42] The present work demonstrated significant reduction in surface area of Masson's stained collagen fibers in the empagliflozin treated group. This finding

was in agreement with Gallo et al.[36] and other studies which linked SGLT-2 inhibitors with reduction in antioxidant and antifibrotic markers.[43]

Another cellular insult from hyperglycaemia is altered autophagic response due to cellular stress which is supposed to have a fundamental role in initiation and progression of DN.[44] Autophagy is a highly regulated lysosomal pathway involved in the removal of damaged organelles and protein aggregates to keep cell integrity.[45] It acts as an important role in maintenance of glomerular and tubular hemostasis. Therefore, insufficient autophagy against stresses such as hyperglycaemia may cause renal cells injuries.[31] Recent studies highlighted the role of autophagy in the pathogenesis of DN.[46–48] Reduction of autophagy results in podocyte degeneration, glomerular endothelial changes and increased collagen deposition.[49]

Until the current study, whether empagliflozin exerted any ameliorating effect in the autophagy of diabetic kidney was unknown. To address this question, we used TEM to monitor the appearance of autophagosomes and check changes in renal tubular and glomerular structures.

In agreement with the previous studies,[26,50] ultrastructural examination of diabetic kidney revealed degenerated renal tubular cells with occasional autophagic vacuoles, pronounced podocyte foot process fusion and GBM thickening. On the other hand, the tubular and podocyte injuries were greatly improved, thickness of the GBM was markedly reduced, and a significant higher number of autophagic vacuoles were detected in the proximal tubular cells in the empagliflozin treated group. Similarly, administration of another SGLT2 inhibitor, dapagliflozin, to db/db mice resulted in reduction podocyte foot process diameter by 44.9% and GBM thickness by 37.7%.[22] It is worth mentioning that Vallon et al.[51] demonstrated that a lack of SGLT2 gene resulted in reduced renal accumulation of p62 which is an indicator of active autophagy in SGLT2-deficient diabetic mice.

In hyperglycaemia, there is increased mTORC1 expression in podocytes and decreased autophagic activity with renal injury and fibrosis. So, activation of autophagy in the tubular cells could maintain the cellular homeostasis, stress resistance, prevents tubulointerstitial fibrosis. Therefore, the autophagy improvement may be a novel therapy for the suppression of DN.[31]

In the light of our results, it seems that empagliflozin might be protective against DN through the restoration of autophagy activity in diabetic kidneys. This will stimulate further studies in the future to declare this cellular pathway as a new pathway where SGLT2 inhibitor empagliflozin is involved in renal protection.

Conclusion

The use of new anti-diabetic SGLT2 inhibitors creates a new era of DM treatment and reduction of its complications. Our experimental model revealed that treatment with empagliflozin significantly improved renal function and ameliorated the tubular and glomerular changes induced by diabetes in NA-STZ-treated rats. We hypothesize that its protective effect is related to reduction of hyperglycemia and improvement of cellular defense mechanisms by other effects of high glucose such as oxidative stress and autophagy induction.

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