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Effects of *Stevia rebaudiana* (Bertoni) Extract and *N*-Nitro-L-Arginine on Renal Function and Ultrastructure of Kidney Cells in Experimental Type 2 Diabetes

Cansu Ozbayer,[1] Hulyam Kurt,[1] Suna Kalender,[2] Hilmi Ozden,[3] Hasan V. Gunes,[1] Ayse Basaran,[1] Ecir A. Cakmak,[4] Kismet Civi,[5] Yusuf Kalender,[6] and Irfan Degirmenci[1]

Departments of [1]Medical Biology and [3]Anatomy, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey.
[2]Department of Science Education, Faculty of Education, and [6]Department of Biology, Faculty of Arts and Science, Gazi University, Ankara, Turkey.
[4]Department of Medical Biology, Faculty of Medicine, Gaziantep University, Gaziantep, Turkey.
[5]Pathology Laboratory, Inegol State Hospital, Bursa, Turkey.

ABSTRACT Diabetes is the leading cause of chronic renal failure. Our purpose was to determine the effects of *N*-nitro-L-arginine (L-NNA) and an extract of *Stevia rebaudiana* (Bertoni) (SrB) leaves on renal function in streptozotocin-nicotinamide (STZ-NA)–induced diabetic rats. Rats were divided into seven groups. Three of these groups were controls. Diabetes was induced by STZ-NA in the other four. Diabetic rats were treated with SrB (200 mg/kg), L-NNA (100 mg/kg), or SrB + L-NNA for 15 days after 5–8 weeks of diabetes. At the end of the experiments, urine and blood samples were collected from the rats, and kidney tissue samples were collected with the animals under ether anesthesia. Renal filtration changes were determined by measuring urine pH, urine volume, and serum and urine creatinine. Nitric oxide synthase (NOS) activity was measured in kidney homogenates. Alterations in kidney ultrastructure were determined by electron microscopy, and histological changes were examined by hematoxylin and eosin staining. No statistical differences were observed in urine creatinine or creatinine clearance. Even so, we observed higher NOS activity in SrB-treated diabetic rats. SrB-treated diabetic rats had less mitochondrial swelling and vacuolization in thin kidney sections than other diabetic groups. The control groups showed normal histological structure, whereas in the diabetic groups, membrane thickening, tubular epithelial cells, and cellular degeneration were observed. Thus, SrB has beneficial effects on diabetes compared with L-NNA. Our results support the validity of SrB for the management of diabetes as well as diabetes-induced renal disorders.

KEY WORDS: • *diabetes* • *kidney ultrastructure* • **N-*nitro*-L-*arginine*** • *renal function* • **Stevia rebaudiana (*Bertoni*)**

INTRODUCTION

DIABETES MELLITUS is a syndrome characterized by abnormal insulin secretion and derangement of carbohydrate and lipid metabolism and is diagnosed by the presence of hyperglycemia. Diabetes is also a risk factor for chronic renal disease. Once it occurs, chronic renal failure and end-stage renal disease increase mortality in those with type 2 diabetes. A decrease in glomerular filtration rate indicates the development of renal disease, and early identification of this event is important in subjects with type 2 diabetes.[1,2]

A new experimental diabetic syndrome has been developed in adult rats by administering streptozotocin (STZ) and partially protecting them with a suitable dose of nicotinamide (NA). This syndrome shares several features with human type 2 diabetes and is characterized by moderate stable hyperglycemia, glucose intolerance, and altered but significant glucose-stimulated insulin secretion.[3,4]

STZ, an *N*-nitroso-*N*-methylurea derivative of 2-deoxy-D-glucose, is a diabetogenic agent that acts through the selective destruction of pancreatic islet β cells. Insulin increases the transport of amino acids into the cell, increases the degradation of proteins, and causes changes in the levels of some amino acids in plasma. Thus, STZ influences blood and urine amino acid levels. In addition, STZ displays nephrotoxic and hepatotoxic activity. STZ causes cataracts, necrosis of kidney tubules, mesangial proliferation, and hyalines of vessels in rats.[5]

Stevia rebaudiana (Bertoni) (SrB) extracts have been used to treat diabetes in, for example, Brazil, although a positive effect on glucose metabolism has not been unequivocally demonstrated. In addition, oral intake of SrB extracts slightly suppresses plasma glucose during an oral glucose tolerance test in healthy subjects. A 35% reduction

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Address correspondence to: Dr. Hulyam Kurt, Department of Medical Biology, Medical Faculty, Eskişehir Osmangazi University, 26480 Eskişehir, Turkey, E-mail: hkurtayda@gmail.com

in blood glucose is also observed in diabetic subjects after oral intake of SrB extracts.[2,6–8]

One of the main constituents of the dry matter of SrB is the diterpene glycoside stevioside, which is 200–300 times sweeter than sucrose, and, in addition, steviosides have a caloric value of zero. Initial scientific studies have indicated that steviosides can regulate the blood glucose level and are safe for diabetics.[2,7,8] Because stevia extract promotes additional insulin secretion,[6,7] helps reverse diabetes,[2,6–8] is safe for phenylketonuria patients,[8] and helps reduce hypertension,[9] it can be used as a medicinal food.

Nitric oxide (NO) synthases (NOSs) are a family of enzymes that synthesize NO and citrulline from L-arginine. Three major subtypes have been identified; two of them, neuronal NOS (nNOS) and endothelial NOS (eNOS), are constitutively expressed and Ca^{2+}/calmodulin-dependent, whereas the cytokine-inducible isoform is calcium-independent.[10]

Changes in renal NO production have been associated with glomerular hyperfiltration, vascular permeability, albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis. Several studies have indeed demonstrated downregulated expression of NOS in experimental diabetic nephropathy. Although most evidence about the roles of NO in renal physiology and pathophysiology has been obtained by exploring the effects of nonspecific NOS inhibitors, more recent studies have used newly available specific inhibitors.[10,11]

N-Nitro-L-arginine (L-NNA), an analog of L-arginine, is an NOS inhibitor. The slow reversibility of L-NNA-mediated inhibition provides a degree of selectivity for nNOS and eNOS in long-term studies (for example, with cells or *in vivo*), but none of the compounds described to date can be used to target a specific isoform (for example, L-NNA shows only a twofold selectivity for nNOS over eNOS).[12,13]

The aim of the present study is to examine the effects on kidney function in type 2 diabetes of SrB and L-NNA.

MATERIALS AND METHODS

Treatment of rats

All animals received humane care according to the criteria outlined in the guidelines for the care and use of laboratory animals of the National Institutes of Health (Bethesda, MD, USA).

Female Sprague–Dawley rats, 2–3 months old and weighing 150–300 g (Medical Biology Animal Laboratory, Eskisehir, Turkey), were used. They were kept in an air-conditioned room, fed a standard commercial diet and tap water *ad libitum*, and left for 1 week for acclimatization before the start of the experiment. At the beginning of the experiment, the glucose level was measured in the tail vein, and then rats were divided into seven groups (Table 1).

Type 2 diabetes was induced by combined STZ-NA injection as described previously.[3,4] Rats were intraperitoneally administered 270 mg/kg NA (Sigma Chemical, St. Louis, MO, USA) dissolved in saline 15 minutes before intravenous injection of 60 mg/kg STZ (Sigma Chemical), which was dissolved in saline immediately before use. After administration of STZ and NA, the blood glucose level of all animals was determined every week from the tail vein during the next 7 weeks. The animals that showed stable hyperglycemia (range, 150–180 mg/dL) were used for experiments as diabetics.

Table 1. The Substrates Given to Control and Experimental Groups

Group	*Receiving substrates*
Control	
I	(Control) water + CDS
II	SrB (200 mg/kg, intragastrically) + water + CSD
III	L-NNA (100 mg/kg, intraperitoneally) + water + CSD
Diabetic	
IV	(Diabetic control) (60 mg/kg STZ + 290 mg/kg NA intraperitoneally) + water + CSD
V	(60 mg/kg STZ + 290 mg/kg NA i.p.) + SrB (200 mg/kg, intragastrically) + water + CSD
VI	(60 mg/kg STZ + 290 mg/kg NA intraperitoneally) + L-NNA (100 mg/kg, intraperitoneally) + water + CSD
VII	(60 mg/kg STZ + 290 mg/kg NA intraperitoneally) + SrB (200 mg/kg, intragastrically) + L-NNA (100 mg/kg, intraperitoneally) + water + CSD

CSD, commercial standard diet; NA, nicotinamide; L-NNA, *N*-nitro-L-arginine; SrB, *S. rebaudiana* (Bertoni); STZ, streptozotocin.

Each group of treated animals was paralleled by a group of controls receiving the vehicles of both substances (SrB and L-NNA). Animals were treated with SrB extract (Nu-Naturals, Inc., Eugene, OR, USA) and/or L-NNA (Sigma Chemical) 5–8 weeks after diabetes was induced.

This study was approved by the Ethics Committee of Eskisehir Osmangazi University.

Biochemical analysis

After administration of STZ and NA, blood glucose was measured every week during experiments with an Accu-Chek® Go glucometer (Roche Diagnostics, Mannheim, Germany) in all animals. After determination of the volume and pH (inoLab® pH 720 pH meter, WTW Laboratory, Weilheim, Germany) of collected urine samples, urine creatinine was measured spectrophotometrically (Jaffe's alkaline picrate reaction assay), and serum creatinine activity was measured spectrophotometrically by Jaffe's reaction in blood serum samples. Creatinine clearance was calculated from urine creatinine, serum creatinine, and the 24-hour urinary excretion volume. All spectrophotometric measurements were performed with a Shimadzu (Kyoto, Japan) model UV-1601 digital spectrophotometer.

NOS activity in kidney homogenates was determined using the Bioxytech® NOS assay kit (catalog number 22113, OXIS International Inc., Portland, OR, USA).

TABLE 2. URINE pH AND URINE VOLUME LEVELS

Group	n	*pH*	*Urine volume (mL)*
Control			
I	7	8.4 ± 0.43	2.50 ± 0.40
II	8	9.2 ± 0.05	2.03 ± 0.57
III	7	9.1 ± 0.16	2.14 ± 0.37
Diabetic			
IV	7	9.4 ± 0.06	4.00 ± 0.81
V	8	8.8 ± 0.23	4.56 ± 2.90
VI	10	9.1 ± 0.12	5.35 ± 2.61*
VII	8	9.0 ± 0.07	2.78 ± 1.33

Data are mean ± SD values.
*$P < .05$.

TABLE 4. NITRIC OXIDE SYNTHASE LEVELS

Group	n	*NOS (nmol/mL/second)*
Control		
I	7	0.307 ± 0.44
II	7	0.351 ± 0.76
III	7	0.414 ± 0.78
Diabetic		
IV	7	0.183 ± 0.01
V	7	0.945 ± 0.26***
VI	7	0.276 ± 0.02
VII	7	1.339 ± 0.26***

Data are mean ± SD values.
***$P < .001$.
NOS, nitric oxide synthase.

Electron microscopic analysis

Kidney tissues were removed and cut into small pieces. The samples were immediately prefixed in 2.5% glutaraldehyde prepared in sodium phosphate buffer (pH 7.2) for 3 hours. They were washed in the same buffer solution for 1 hour, and tissue was postfixed in 1% OsO_4 in the same buffer solution. Tissues were washed in sodium phosphate buffer for 1 hour. Specimens were then dehydrated and embedded in Araldite® CY212 (Serva, Germany). Samples were sectioned with a Reichert OM U3 ultra microtome (Leica) and stained with 2% uranyl acetate and lead citrate. Finally, samples were viewed and photographed on a JEOL (Tokyo, Japan) 100 CX II electron microscope.

Histopathological procedures

Fragments of kidney were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and then stained with hematoxylin and eosin.

Statistical analysis

The data are expressed as mean ± SD values and analyzed using measures analysis of variance. Tukey's test was used to test for differences among means when analysis of variance indicated a significant difference ($P < .05$). Differences were considered statistically significant if $P < .05$.

RESULTS

The biochemical results of this study are summarized in Tables 2–4. As shown in Table 2, no statistical differences were found in urine pH ($P > .05$), but urine volume was significantly increased in the diabetic L-NNA group compared with the control ($P < .05$).

In addition, in creatinine clearance and urine creatinine level (Table 3), we found no statistical difference between the control group and the other groups ($P > .05$). However, serum creatinine was different in the diabetic SrB and L-NNA vehicle groups compared with the non-diabetic control group ($P < .01$ and $P < .05$, respectively).

As shown in Table 4, we found decreased NOS activity in diabetic rat kidney, but SrB-treated diabetic rats showed higher NOS activity ($P < .001$).

In this study, no pathological changes were observed in the saline control, L-NNA vehicle, or SrB vehicle group (Figs. 1–3). Large vacuoles were observed in the cyto-

TABLE 3. URINE CREATININE AND SERUM CREATININE LEVELS AND CREATININE CLEARANCE

Group	n	*Urine creatinine (mg/dL)*	*Serum creatinine (mg/dL)*	*Creatinine clearance (mL/minute)*
Control				
I	7	31.51 ± 14.26	1.48 ± 0.04	0.040 ± 0.004
II	8	79.75 ± 36.72	2.10 ± 0.03**	0.057 ± 0.015
III	7	69.18 ± 24.11	1.94 ± 0.07*	0.052 ± 0.017
Diabetic				
IV	7	70.53 ± 15.45	1.84 ± 0.10	0.121 ± 0.026
V	8	35.94 ± 6.26	1.83 ± 0.06	0.068 ± 0.023
VI	10	47.08 ± 5.85	1.72 ± 0.15	0.103 ± 0.021
VII	8	55.82 ± 4.98	1.68 ± 0.04	0.073 ± 0.020

Data are mean ± SD values.
*$P < .05$, **$P < .01$.

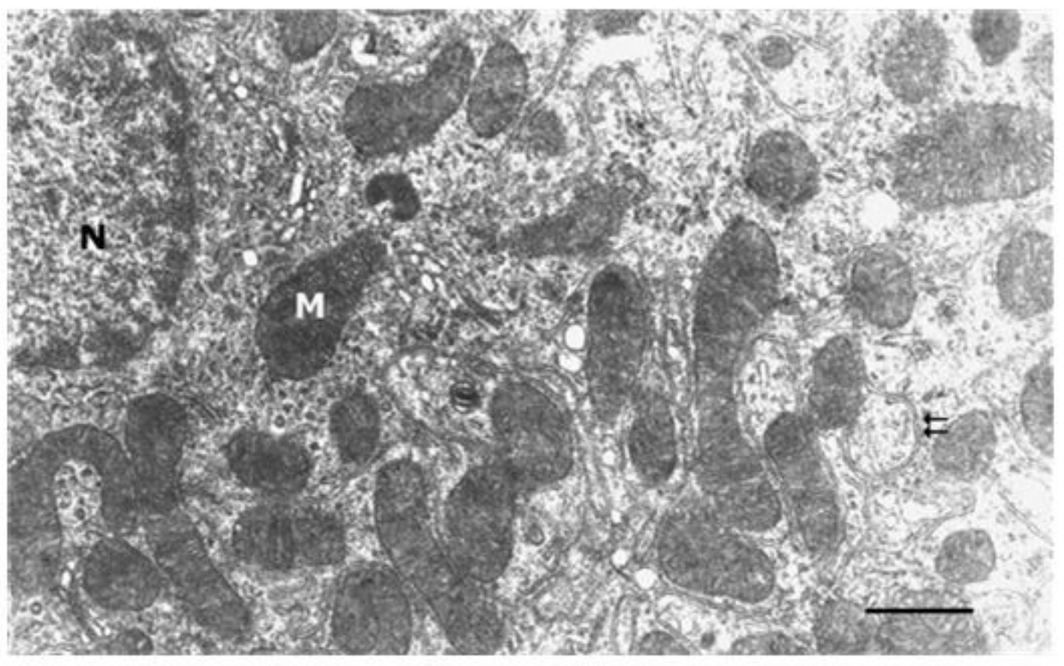

FIG. 1. Normal kidney cell structure observed in the control group. M, mitochondria; N, nucleus; double arrows, endoplasmic reticulum. Scale bar = 1 μm.

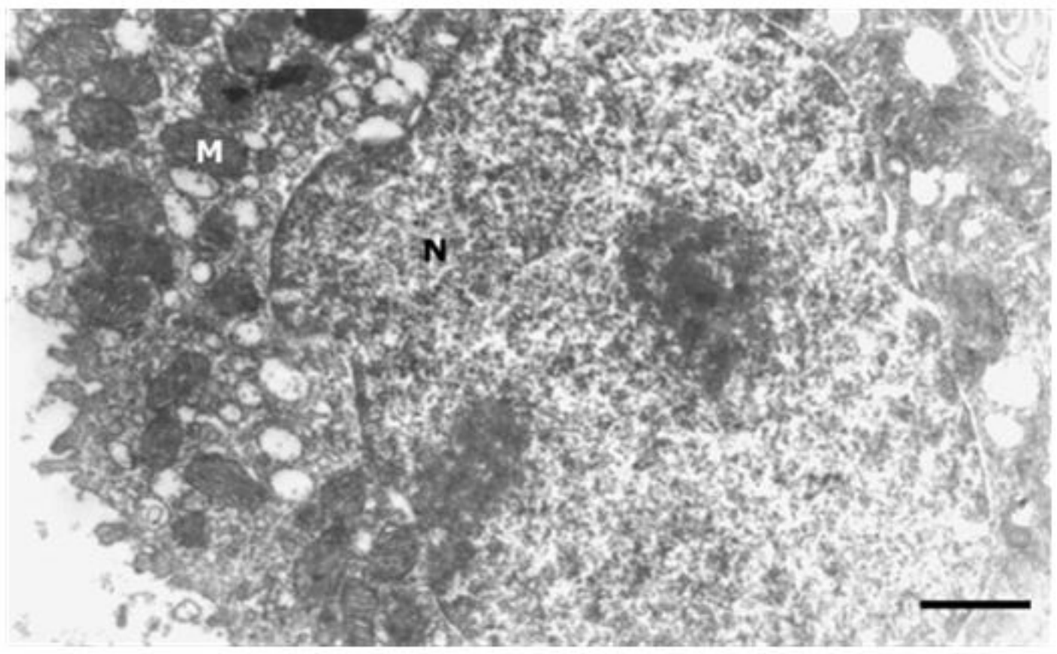

FIG. 2. Normal kidney cell structure observed in the stevia control group. N, nucleus. Scale bar = 1 μm.

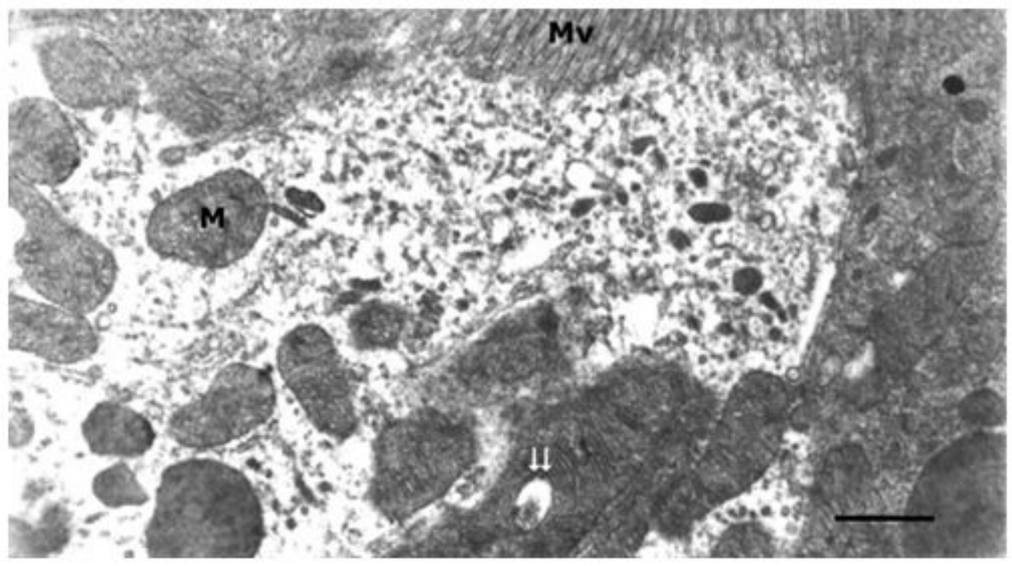

FIG. 5. Kidney tissue of the diabetic control group. Dissolution in the cytoplasm and vacuoles (double arrows) appears in the mitochondria (M) matrix. Mv, microvillus. Scale bar = 1 μm.

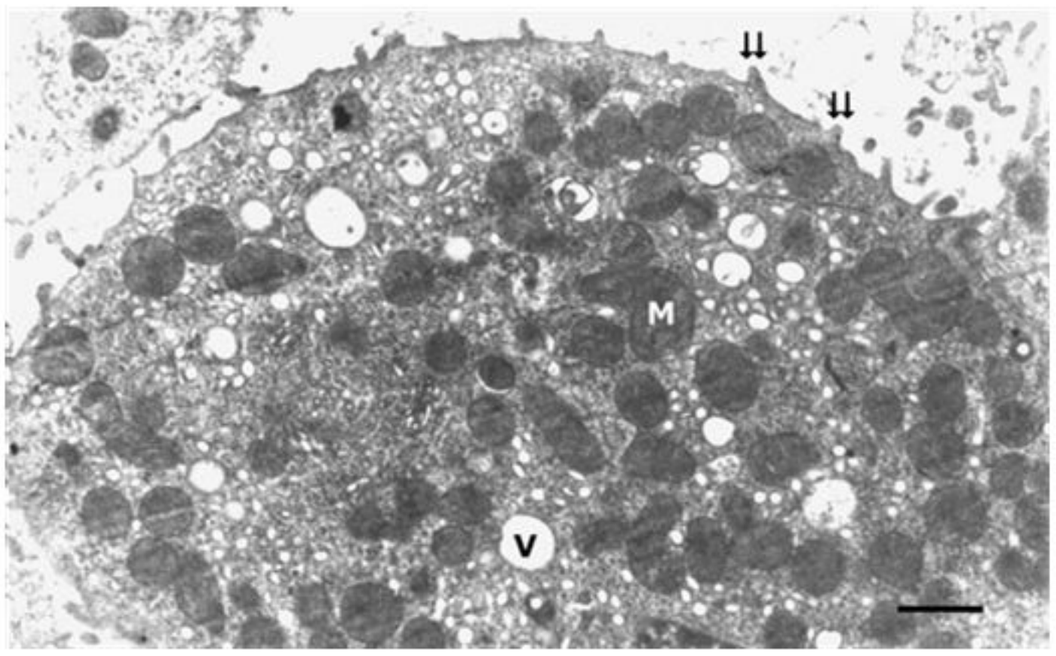

FIG. 3. Normal kidney cell structure observed in the L-NNA control group. M, mitochondria; V, vacuole; double arrows, microvillus. Scale bar = 1 μm.

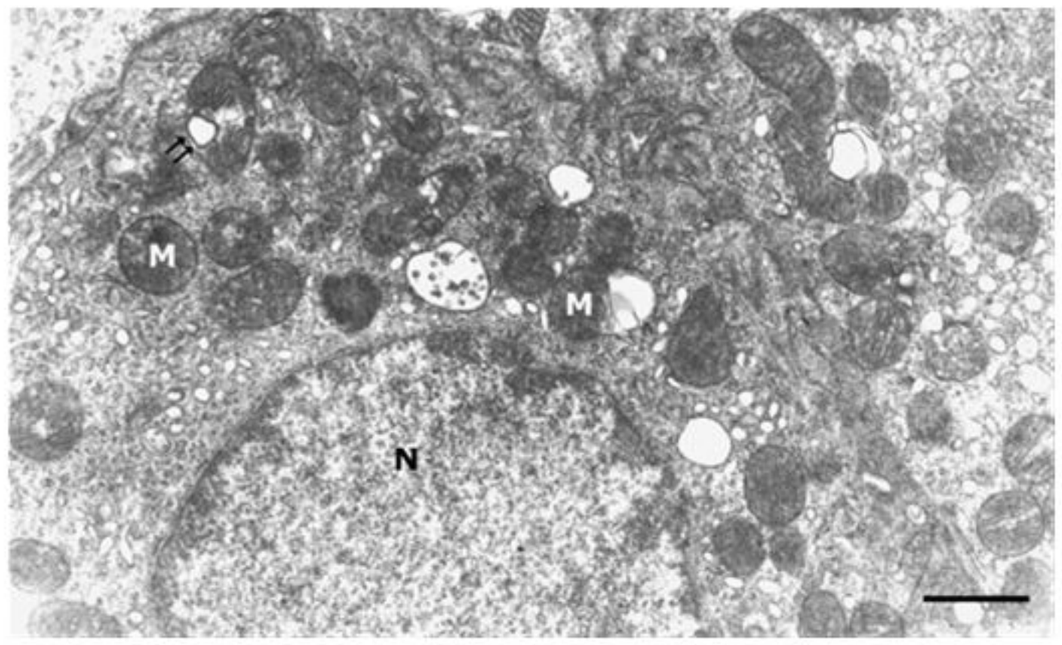

FIG. 6. Kidney tissue of the diabetic stevia group. Swelling and vacuolization (double arrows) appear of some mitochondria (M) in kidney cells. N, nucleus. Scale bar = 1 μm.

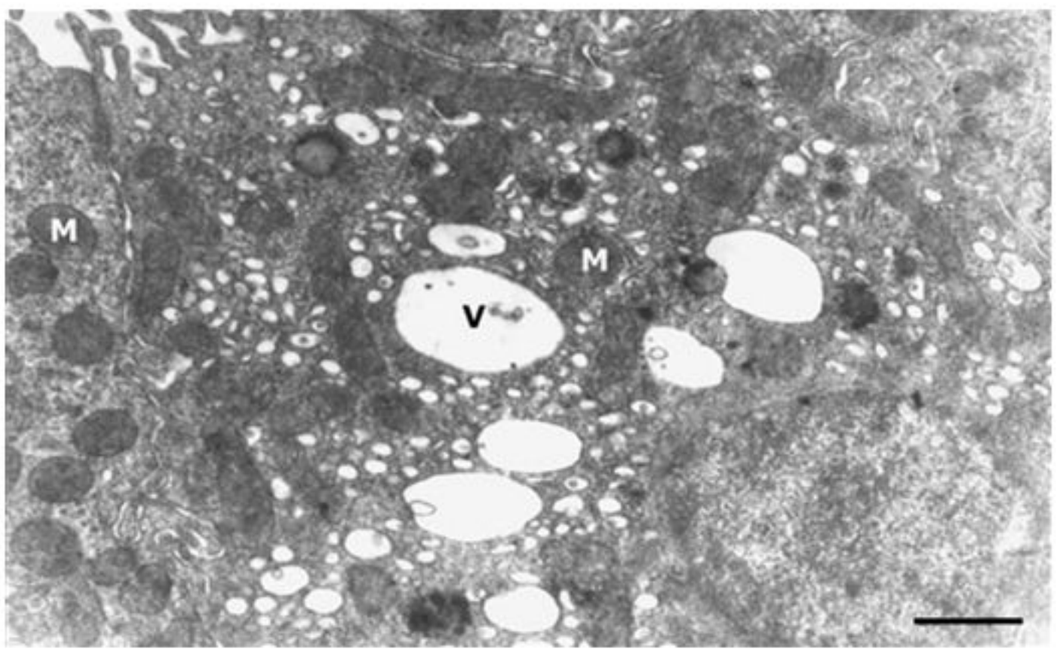

FIG. 4. Kidney tissue of the diabetic control group. Large vacuoles (V) appear in the cytoplasm of cells. M, mitochondria. Scale bar = 1 μm.

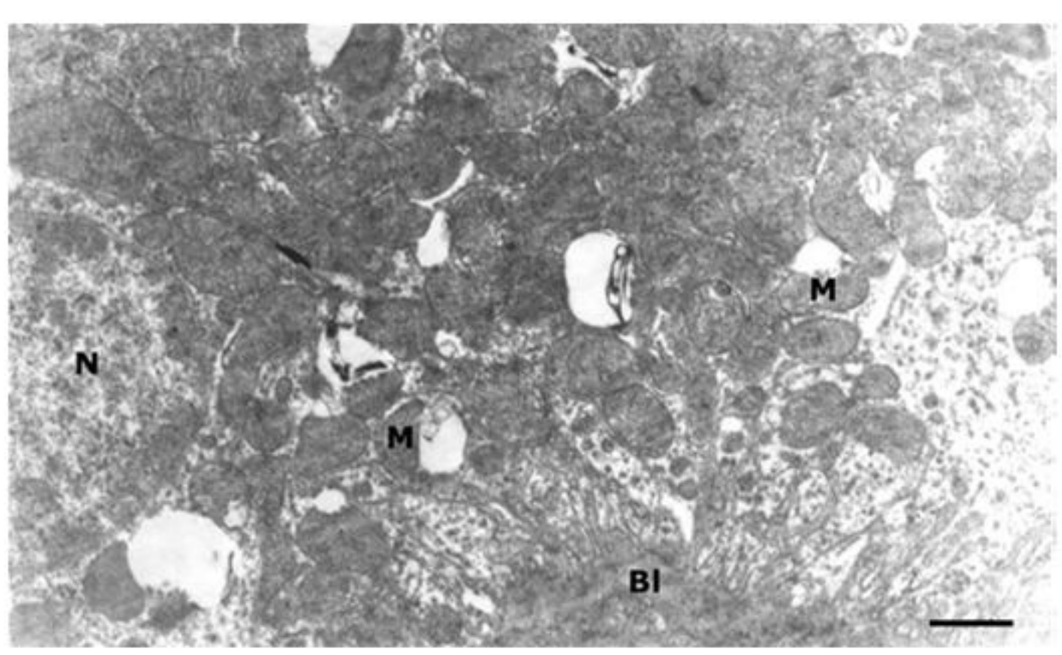

FIG. 7. Kidney tissue of the diabetic L-NNA group. Swelling and vacuolization appear in mitochondria (M) in the kidney cells. Bl, basal lamina; N, nucleus. Scale bar = 1 μm.

plasm of STZ-treated rat kidney cells (Fig. 4). The electron micrographs of this group showed cytoplasmic lysis and vacuolization in the mitochondrial matrix (Fig. 5). The kidney tissues of the SrB-treated diabetic group had either swelling or vacuolization and lysis in some mitochondria (Fig. 6). However, we observed both mitochondrial swelling and vacuolization in kidney tissue of the STZ + L-NNA group (Fig. 7), whereas the SrB + L-NNA diabetic group showed normal mitochondrial and organellar structure in kidney (Fig. 8). In the control group, the cortex

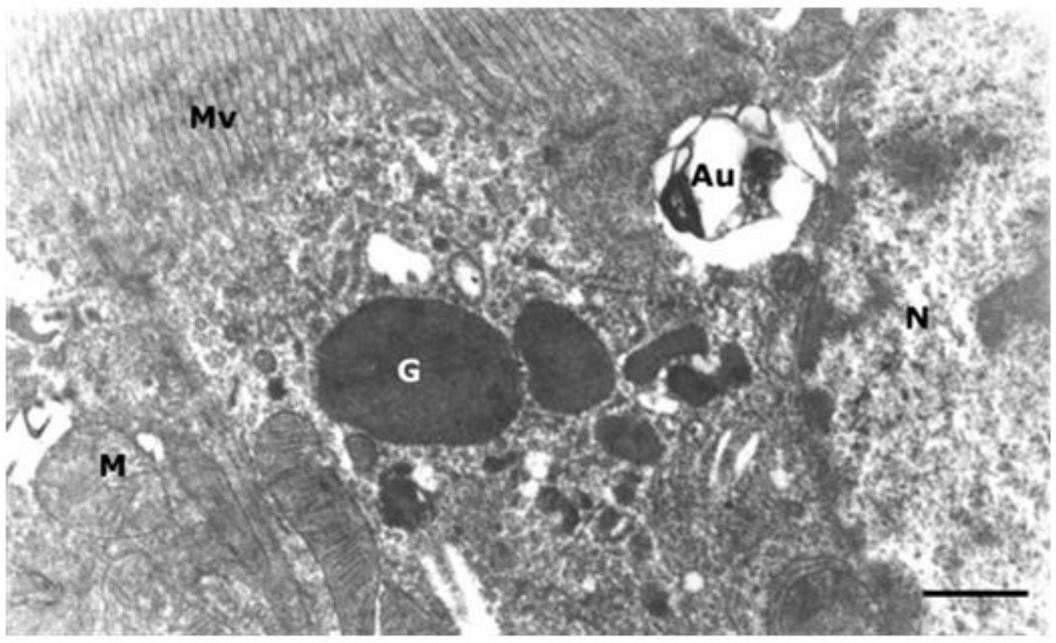

FIG. 8. Kidney tissue of diabetic stevia + L-NNA group. Normal structure of mitochondria (M) and other organelles. Au, autophagic vacuole; G, granule; Mv, microvillus; N, nucleus. Scale bar = 1 μm.

of renal corpuscles (glomerulus + Bowman's capsule structure) and distal and proximal tubule structures were histologically normal (Fig. 9). The kidney cortex and renal tubular structures in the stevia control group were generally normal, but in some preparations in this group, basal membrane thickening was seen (Fig. 10). Renal corpuscles in the L-NNA control group structure had nearly normal histological structure, but a slight degeneration in some tubules was seen (Fig. 11). In the experimental animals, basement membrane thickening, interstitial infiltration, and capillary dilation were also lower in kidney cortex; in tubular structures degeneration and tubular epithelial cell shedding were seen in the diabetic control group (Fig. 12). In the diabetic stevia group, partial capillary dilatations and hemorrhagic blood vessels (arrows) were seen. Tubular structures were observed in the nearly normal histological structure (Fig. 13). In the diabetic L-NNA group, renal tubular cell degeneration and epithelial cell spillover, albeit to a lesser extent than in the diabetic controls,

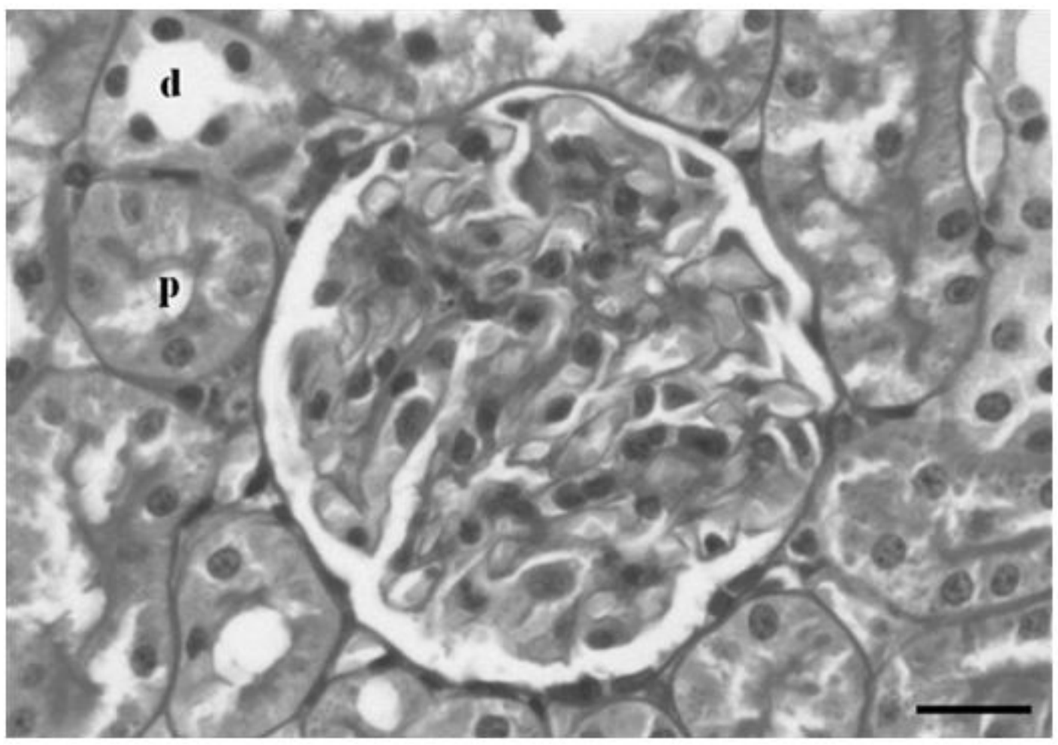

FIG. 9. Kidney samples in the control group. Renal cortex and distal (d) and proximal (p) tubule structures were seen in the normal histological structure. Scale bar = 20 μm. Hematoxylin and eosin, $\times$100.

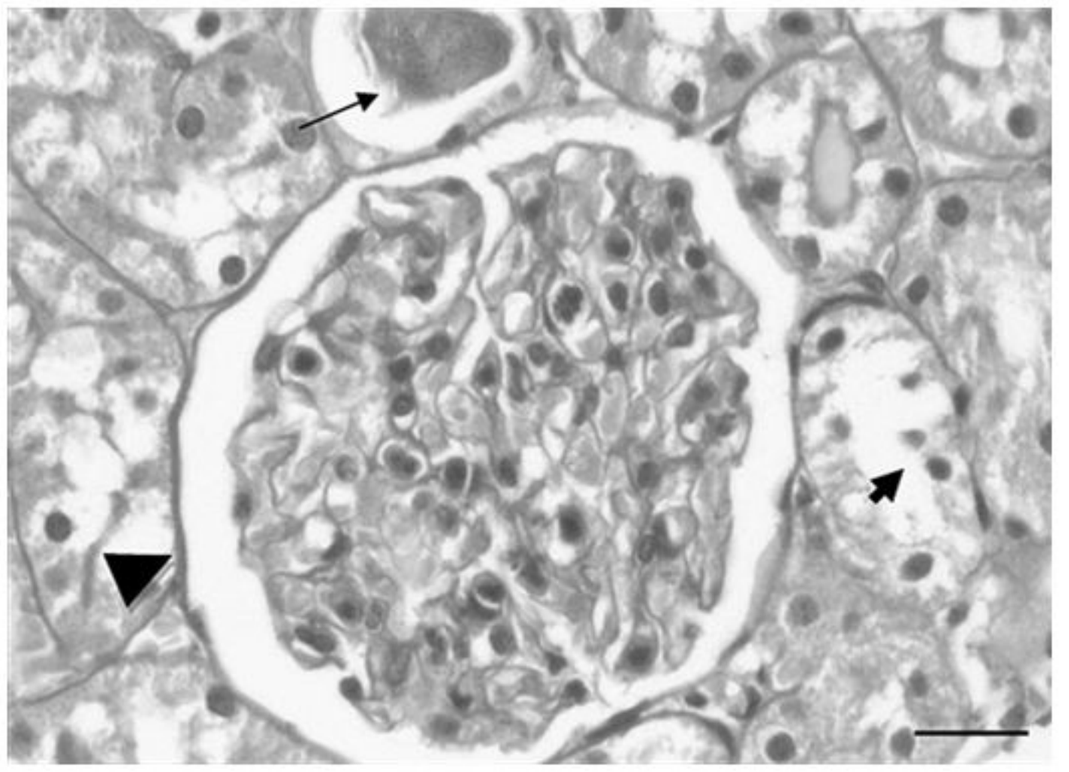

FIG. 10. Kidney samples in the stevia control group. In this group, preparations in the kidney cortex and renal tubular structures had near normal histological structure generally. However, in preparations for the diabetes group but not in this group, basal membrane thickenings (arrowhead) were seen. Tubular degeneration and renal tubules in this preparation for construction draw attention to hyaline casts (long arrow). Tubules are also seen in cellular loss (short arrow). Scale bar = 20 μm. Hematoxylin and eosin, $\times$100.

were noted (Fig. 14). In the stevia + L-NNA diabetic group, the renal cortex, renal bud (Bowman capsule's and glomerular structure), and tubular structures were largely normal (Fig. 15).

DISCUSSION

The kidneys are an important target organ of diabetes, and kidney failure often leads to death in diabetes. Diabetes causes glomerular lesions, atherosclerosis of renal veins,

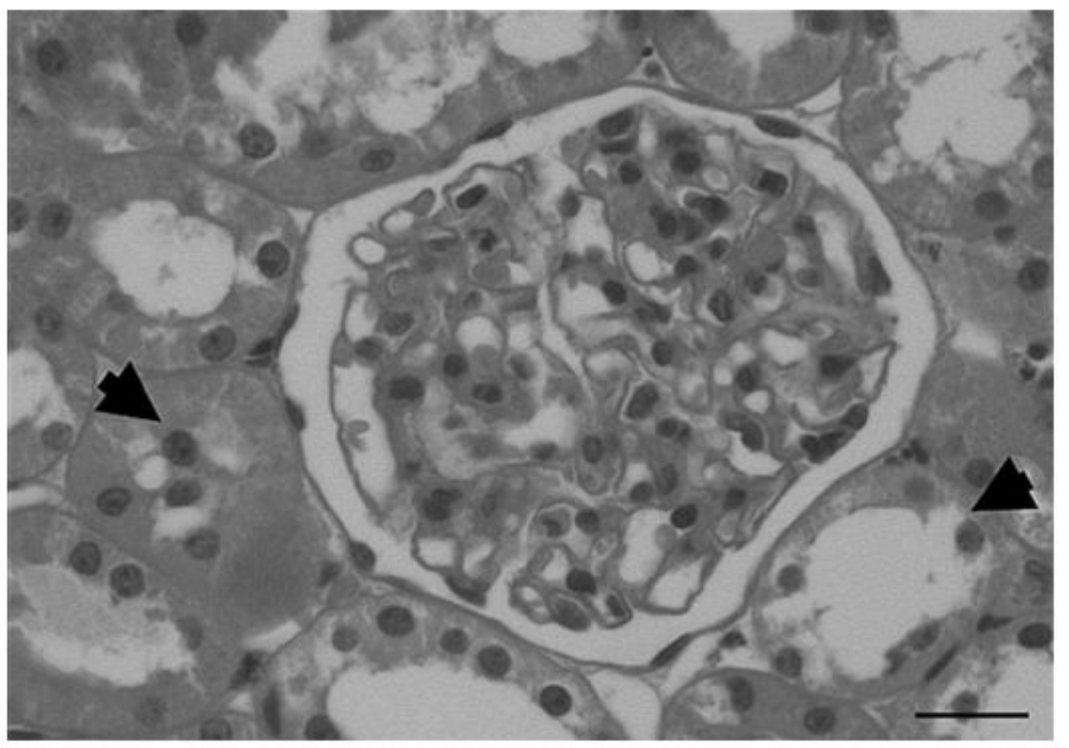

FIG. 11. Kidney samples in the L-NNA control group. Renal corpuscles in the preparations for the L-NNA-treated control group structure have near normal histological structure, but a slight degeneration in some tubules and epithelial cells shed by tubule (arrowheads) were seen. Scale bar = 20 μm. Hematoxylin and eosin, $\times$100.

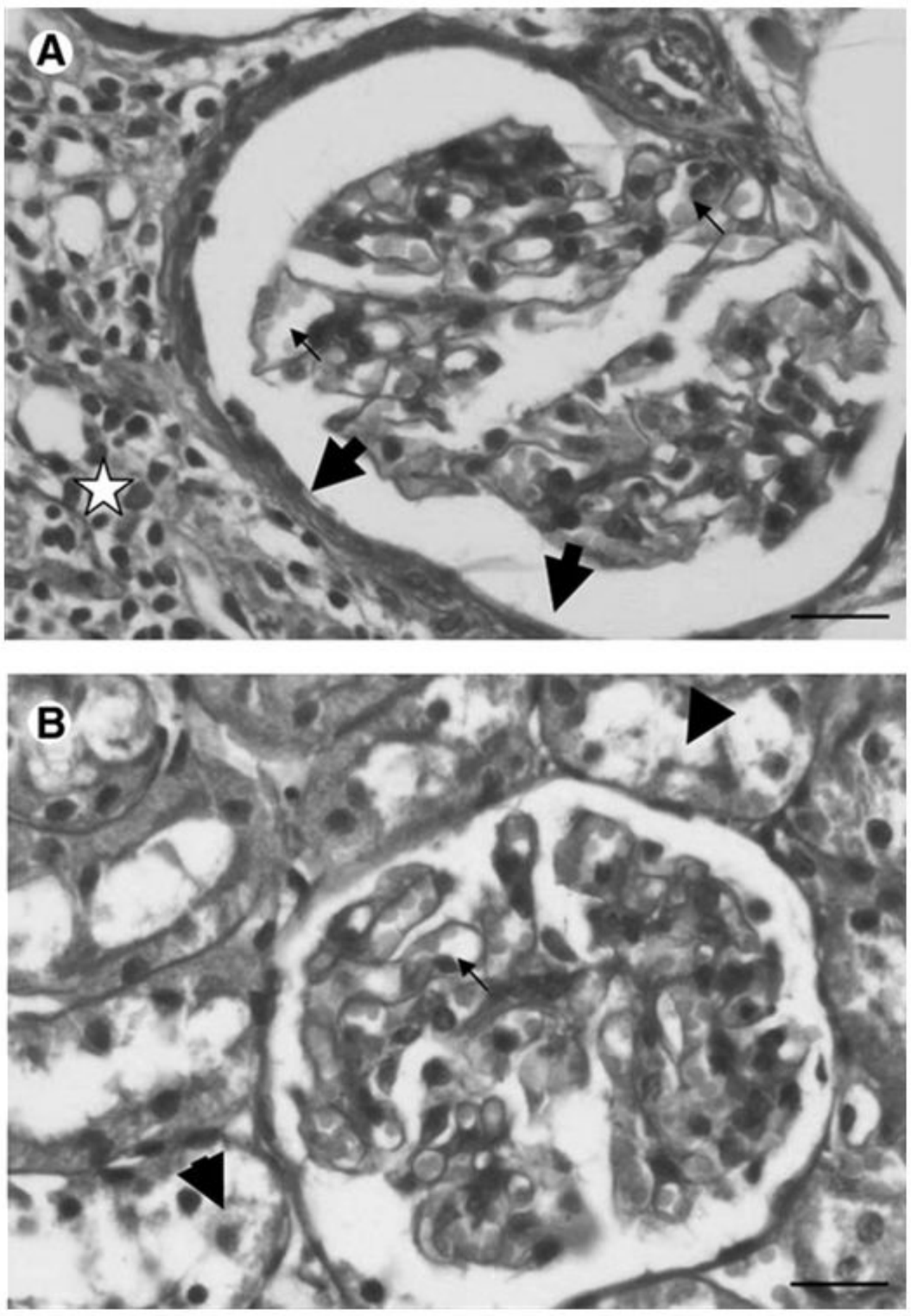

FIG. 12. Kidney samples in the diabetic control group. (**A**) Indicated are basement membrane thickening (arrows), interstitial infiltration (☆), and capillary dilation (thin arrow). (**B**) Degeneration of tubular structures and shedding of tubular epithelial cells shed in kidney cortex (arrowhead). Scale bars = 20 μm. Hematoxylin and eosin, × 100.

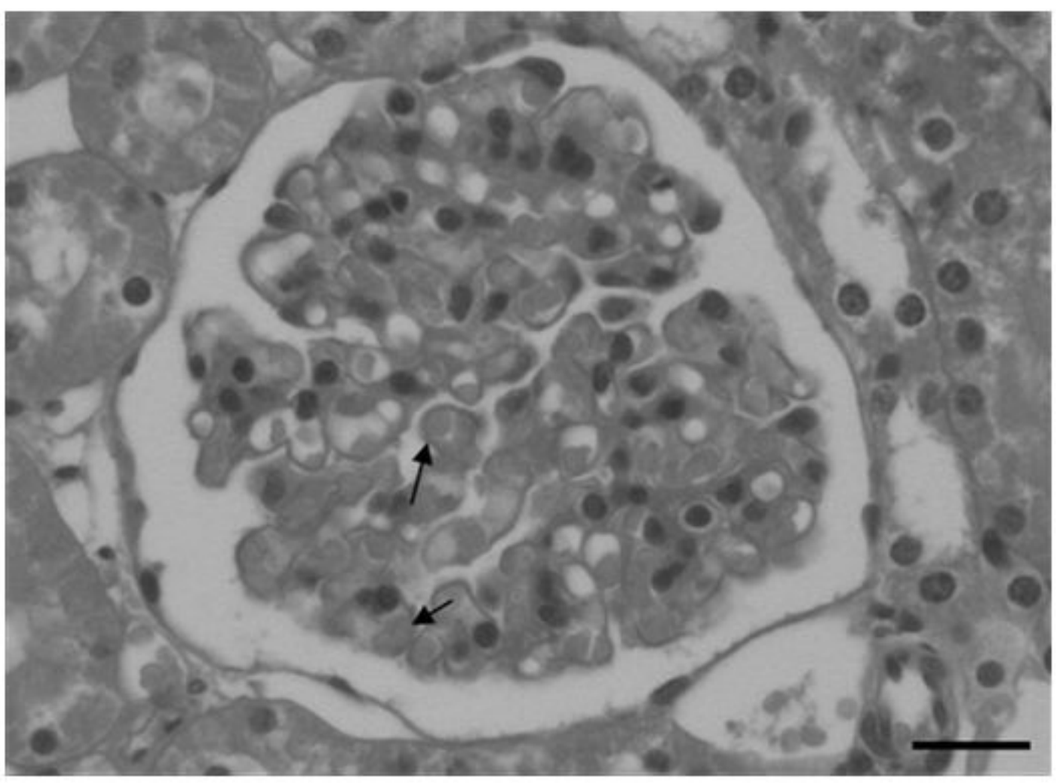

FIG. 13. Kidney samples in the diabetic stevia group. The kidney preparations of this group showed partial capillary dilatations and hemorrhagic blood vessels (arrows). Tubular structures were observed in the near normal histological structure. Scale bar = 20 μm. Hematoxylin and eosin, × 100.

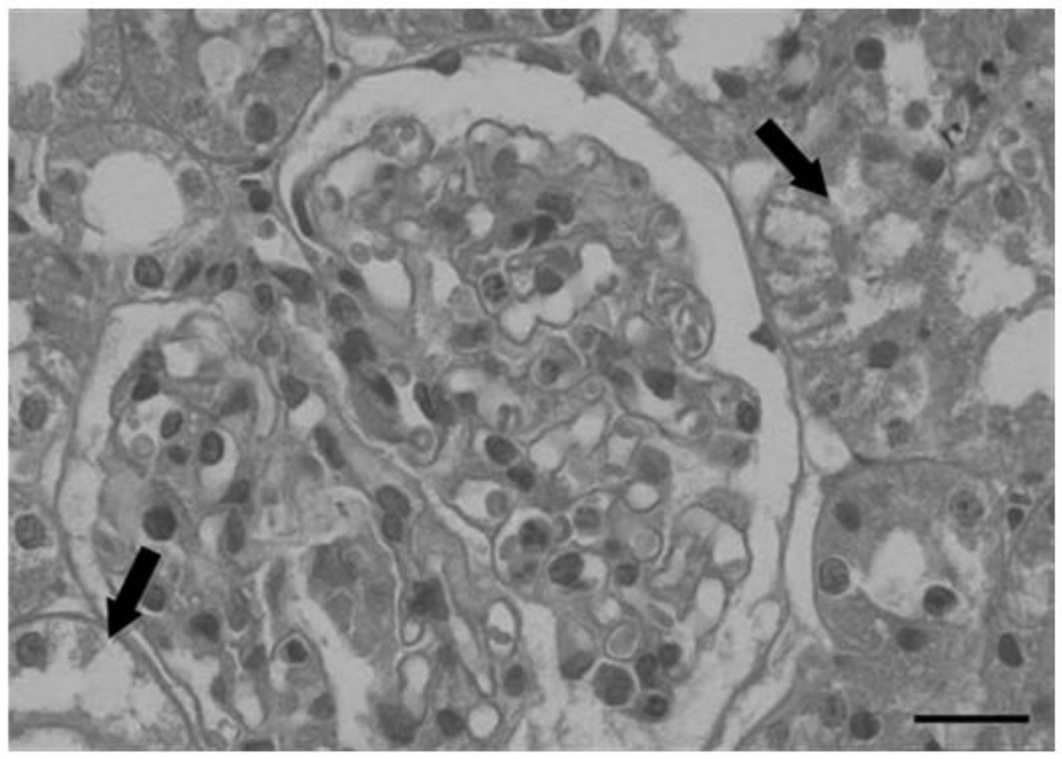

FIG. 14. Kidney samples in the diabetic L-NNA group. The samples of this group showed renal tubular cell degeneration and epithelial cells, albeit fewer spills are noted (arrows). Scale bar = 20 μm. Hematoxylin and eosin, × 100.

pyelonephritis, and nephropathy.[1,2,10,14,15] Diabetes can also increase urine volume and creatinine clearance.[1,5,12]

Our study found increased urine volume in L-NNA-treated diabetic rats, but there were no statistical differences among the other groups. In addition, urine volume in the diabetic control groups was higher than in other groups. No reports exist about the effects of SrB and L-NNA on urine volume in type 2 diabetes, but diabetes does cause increased urine volume. In addition, decreased renal NOS levels are associated with type 2 diabetes.[1,16] Consistent with this, L-NNA, as an inhibitor of NOS, caused higher urine volumes in diabetic rats.

According to our findings, L-NNA had no effect on urine pH in diabetic rats, but SrB extract caused a decrease of a

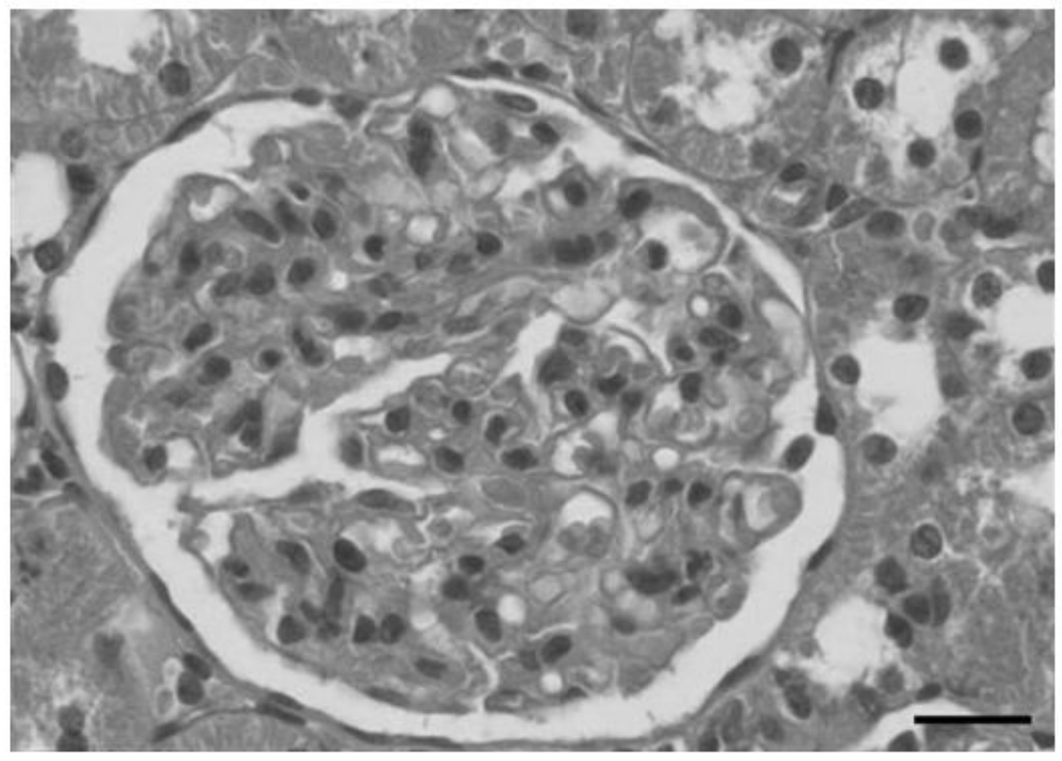

FIG. 15. Kidney samples in the diabetic stevia + L-NNA group. In this group, renal cortex, renal bud (Bowman's capsule and glomerular structure), and tubular structures were observed with near normal histological structure. Scale bar = 20 μm. Hematoxylin and eosin, × 100.

minimal level, within an acceptable range. SrB decreased urine glucose level, thereby decreasing urine pH in diabetic rats.

In addition, we found no statistical difference in urine creatinine level among the control group and other groups. To our knowledge, the effects of SrB and L-NNA on urine creatinine have not been reported.

Serum creatinine was different in the SrB and L-NNA vehicle control groups compared with the non-diabetic controls. There are also no reports on the effects of SrB and L-NNA on serum creatinine. STZ exhibits nephrotoxic and hepatotoxic activity, and it increases serum creatinine.[1,5] Serum creatinine concentration is inversely correlated with glomerular filtration rate, and increased serum creatinine indicates kidney failure and tissue necrosis.[5,17,18] Serum creatinine is a good measure of kidney function but was not increased significantly in our experimental diabetic rats, which may have been due to the short experimental time or the protective effects of NA.

We did not find any significant differences in creatinine clearance among the control and experimental groups. According to other studies related to diabetes, renal failure and renal disorders occur as a result of hyperglycemia. Decreased urine creatinine indicates renal failure. Previous studies have shown that creatinine values are increased in STZ-induced diabetes.[14,17,18]

Glomerular filtration rate is determined by measuring creatinine clearance, and a decrease in creatinine clearance indicates glomerular degeneration.[14.17,18] Renal filtration changes in diabetes with hyperglycemia, so STZ increases creatinine clearance. However, in our study, this increase did not occur, possibly because of the short experimental time or to the antihyperglycemic effects of SrB and L-NNA.

Clinical and animal studies have shown reduced nitric oxide production in chronic renal disease and end-stage renal disease.[1,16] We also found decreased NOS in diabetic rat kidney.

Stevia contains phenolic compounds and flavonoids at 24.01 and 18.93 mg/g dry weight of leaves, respectively. These substances have been suggested to have beneficial effects on health. Aqueous and methanolic extracts of stevia leaves have antioxidant properties equivalent to gallic acid and butylated hydroxyanisole.[19] Phenolic compounds such as resveratrol, quercetin, epicatechin gallate, and epigallocatechin gallate enhance NO levels by increasing the amount of NOS enzymes.[20,21] In our study, the SrB-treated diabetic group showed a higher NOS level. Therefore, the phenolic compounds of SrB might have induced NOS production in the diabetic rat kidney. As a NOS inhibitor, L-NNA decreased the NOS level in the diabetic rat kidney but increased NOS in healthy rat kidney.

After the onset of diabetes, diffuse glomerulosclerosis develops within 1–2 years in humans, characterized by mesangial cell proliferation with increased mesangial matrix. Thickening of the capillary basal membrane is also observed. STZ-induced diabetes leads to thickening of the glomerular basal membrane.[5]

In our study, we observed a normal histological structure of the kidney samples in the controls and SrB-treated diabetic groups. We observed glomerular basal membrane thickening, distal tubular epithelium thickening, cytoplasmic clear cell alteration, and small artery medial thickening in the diabetic control and L-NNA-treated diabetic groups, but these effects were lesser in the SrB + L-NNA diabetic group.

In the electron microscopic analysis, we observed fewer glycogen granules in cells, degeneration of nuclei, dilation of granular endoplasmic reticulum, dilation of the intracellular area, and aggregation of lipid in STZ diabetic rats.[5,22]

Although the kidney cells of control groups showed normal structure, SrB-treated diabetic groups had less mitochondrial swelling and vacuolization in thin kidney sections than diabetic control and L-NNA-treated diabetic groups. However, SrB + L-NNA-treated diabetic groups had normal mitochondria and organellar structure. SrB and L-NNA treatment protected the kidney cells by decreasing blood glucose.

In conclusion, the extracts of SrB leaves have beneficial effects on diabetes-induced histological, ultrastructural, and biochemical changes. L-NNA is less efficient in treating type 2 diabetes than SrB. Further studies on SrB for treating diabetes appear warranted.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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