Protective effect of substituted biguanidine on rat diabetic nephropaty – histological study

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SUMMARY

Nephropathy remains an important complication of diabetes. This work was carried out to evaluate the protective effects of N-thioacetylbiguanidine on the diabetic rat kidney. Fifteen adult male *Wistar* rats were divided into two groups: Group I, control (n=5) and Group II, diabetic group (n=10). The latter was equally divided into two subgroups: IIa (diabetic control) and IIb (diabetic N-thioacetylbiguanidine treated). Specimens were taken from the renal cortex, processed and examined using light, immunohistochemical, ultrastructural and morphometric techniques.

The renal cortices from diabetic animals showed dilated glomeruli and tubules and thickening of the glomerular capillary basement membranes. The proximal tubules revealed partial loss of brush border and deposition of PAS-positive material in some distal tubules. iNOS-immunoreactivity was strongly expressed in the renal tubules and glomeruli. The juxtaglomerular (JG) cells revealed no intra-cytoplasmic secretory granules. The histological changes in renal glomeruli and tubules were improved in N-thioacetylbiguanidine treated group. Use of N-thioacetylbiguanidine characteristically reduced iNOS expression in kidney and renin secretion in JG cells.

In conclusion, N-thioacetylbiguanidine is effective in attenuating the histological changes of diabetic nephropathy reaching healing features, which resemble that of a normal kidney.

Key words: Diabetic nephropathy –

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N-thioacetylbiguanidine – Histopathology

INTRODUCTION

Diabetes mellitus (DM) is one of the most common endocrine diseases in the world characterized by the state of hyperglycemia. Diabetes mellitus patients are prone to some long-term complications like nephropathy, retinopathy and neuropathy (Nathan, 1993). The kidney is the main affected target organ, and diabetic nephropathy is one of the serious secondary consequences of diabetes resulting in end-stage renal disease (Schena and Gesualdo, 2005).

Multiple biochemical mechanisms have emerged to explain the adverse effect of hyperglycemia and the genesis of oxidative stress in both diabetic patients and diabetic experimental animals. These include glucose autooxidation, protein glycation, formation of advanced glycation endproducts (AGEs) and the polyol pathway (West, 2000). As a consequence of increased substrate (glucose) availability, AGEs accumulate at an accelerated rate in patients with diabetes, where they have been postulated to play a major role in the pathogenesis of the microvascular complications of diabetes (Kelly et al., 2001). The anti-glycation agent, aminoguanidine, inhibits AGEs formation both in vitro and in vivo (Ihm et al., 1999; Kousar et al., 2009) and received the widest interest from the perspective of clinical trial. However, a study by Bolton et al. (2004) indicated that aminoguanidines may have some toxicity when administered for diabetic nephropathy. This led scientists to search for some other guanidine compounds with anti-glycation activity.

Biguanidines and cyclic guanidines are one of

most active diabetic inhibitors and showed a wide range of biological activities. For example, it not only lowers blood glucose (Vaillancourt et al., 2001) and inhibits dihydrofolate reductase of opportunistic microorganisms but also acts as an anti-inflammatory (Sutherland et al., 2008). Besides, in an *in vitro* glycation reactions, biguanidines derivatives in aqueous medium, demonstrated marked antiglycation activity (Mohamed et al., in progress).

Therefore, this study has been conducted to evaluate the potency of a substituted guanidine containing a drug named N-thioacetylbiguanidine in ameliorating diabetic nephropathy in hyperglycaemic, streptozotocin-induced diabetic rats, possessing antidiabetic and antiinflamatory properties. The substituted biguanidine N (carbamimidoylcarbamimidoyl) ethanethioamide (N.car.) (IUPAC name) has been screened and evaluated using well established in vitro assay method (Mohamed, 2013).

MATERIALS AND METHODS

A total number of 15 adult (5-month-old) male *Wistar* rats (200 g body weight) were used in this study. They were purchased from the Central Animal House, Faculty of Medicine, Assiut University. All animal procedures were in accordance with the standards set forth in guidelines for the care and use of experimental animals by the Committee for the Purpose of Supervision of Experiments on Animals (CPCSEA), and according to the National Institute of Health (NIH) protocol, and approved by the Institutional Ethics Committee of Assiut University. The animals were housed in clean capacious cages under normal day and night cycles and appropriate temperature ($25 \pm 5°C$), fed rat chow (standard rat pellets) and water *ad libitum*.

Animal groups

Animals were divided into two main groups. Group I (normal vehicle control) consisted of 5 rats that were intra-peritoneally (IP) injected with 0.5 mL of 0.1 M citrate buffer (purchased from Sigma Chemical Co, St Louis, MO, USA). Group II: Streptozotocin (STZ)-induced diabetic group, consisted of 10 rats that were fasted overnight 12 hours before STZ injection. Then each one received a single intra-peritoneal injection of STZ (purchased from Sigma Chemical Co, St, Louis, MO, USA) at a dose of 80 mg/kg freshly dissolved in 0.5 mL of 0.1 M citrate buffer at pH 4.5 (Kim et al., 2010).

After 3 days of diabetes induction, fasting blood glucose (FBG) level was measured by Dextrostix glucometer (Ames, Elkhart, Indianapolis, IN, USA). Rats with FBG above 300 mg/dL were considered diabetic (Han et al., 2004) and were equally divided into two subgroups: Groups IIa & IIb. Rats in group IIa were left without treatment for 3 months. Rats in group IIb were given N-thioacetylbiguanidine (Table 1) orally by a gastric tube once daily at a dose of 10 mg/ kg dissolved in 0.5 mL distilled water for 3 months (Mohamed, 2013).

At the end of the experiment, blood samples were obtained from the orbital vein through heparinized capillary tubes. FBG was measured in mg/dL using Dextrostix glucometer (Ames, Elkhart, Indianapolis, IN, USA). The animals were sacrificed and specimens from the kidney were processed to be examined.
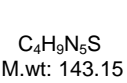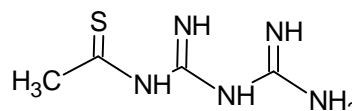
Light microscopic study

Three rats from each group were perfused intra-cardially with 10% formaldehyde solution. The specimens were immersed into in 10% formaldehyde solution to continue fixation for two more days. Paraffin sections (5 µm) were cut serially using a microtome (Leica RM 2125RT, Germany), mounted on glass slides, and every 10th section was stained with hematoxylin-eosin stain. In addition, some sections were processed for histochemical demonstration of polysaccharides using periodic acid-Schiff (PAS) method for neutral mucosubstances. Processing and staining techniques were carried out according to Drury and Wallington (1980).

Electron microscopic study

Two rats from each group were perfused intra-cardially with 4% glutaraldehyde in cacodylate buffer (pH7.4). The specimens were cut into thin slices (1×1 mm) and immersed in 4% glutaraldehyde in cacodylate buffer (pH 7.4) for 24 hours and post fixed in 1% osmium tetroxide in phosphate buffer for two hours. Tissues were rinsed in the same buffer, dehydrated with alcohol, cleared with propylene oxide and embedded in Epon-812 substitute (SPI- Pon Araldit Kit, Cat. no. 02635- AB., SPI- chem. USA). For Polymerization, the embedded samples were kept in the incubator at 35 ºC, 45 ºC and 60º C for one day each (Gupta, 1983). Semi-thin sections (0.5-1 µm) were cut with glass knives on the ultramicrotome (LKB Bromma 8800 Ultratome[R] III, 3518, Sweden) and stained with 1%

Table 1. The substituted biguanidine

Name	Structure	Formula
N-(carbamimidoylcarbamimidoyl) ethanethioamide (IUPAC name)	$C_4H_9N_5S$ M.wt: 143.15	

toluidine blue (pH 7.3) for examination on a light microscope (Olympus, Bx50. Model Bx50F-3, SC09160, Tokyo, Japan). Ultrathin sections (50-80 nm) were cut from selected areas of the blocks on a Reichert ultramicrotome (Leica WILDM3Z, 89386, Austria), placed on copper grids (G300, 3.05 mm, Polaron Equipment Ltd. Watford. England) and contrasted with uranyl acetate and lead citrate. These sections were examined using the transmission electron microscope (Jeol EM-100 CX11; Japanese electron optic laboratory, Tokyo, Japan) and photographed at 80 kV.

Immunohistochemical study

Expression of inducible nitric oxide synthase (iNOS) was detected in formalin- fixed paraffin-embedded sections (Yabuki et al., 2006). Sections (5 μm) were deparaffinized in xylene and rehydrated in alcohol. The (iNOS) Rabbit Polyclonal Antibody (Thermo Fisher Scientific, Fremont, CA 94538-6406, USA) was used at 1:100 dilution for 20 minutes at room temperature. Sections were boiled in 10mM citrate buffer, pH 6.0 for 15 minutes followed by cooling at room temperature for 20 minutes. Sections were processed according to the manufacture instructions using the universal kit (Ultra Vision LP system, HRP polymer & DAB plus chromogen, Thermo Fisher Scientific, Fremont, CA 94538-6406, USA). After completion of the reaction, counterstaining was done using Mayer's haematoxylin, dehydrated and coverslipped using DPX (Oxford laboratory reagents, Bombay, India).

Morphometric study

Using computer-assisted image analysis (Soft Imaging System, Analysis-2004, Olympus Company, Tokyo, Japan), the arbitary area of the glomeruli (renal corpuscles) was measured on H&E-stained sections (Kiran et al., 2012). Thirty glomeruli per animal group were measured using X40 objective lens.

Statistical analysis

The morphometric data of each animal group were statistically analyzed using the computer statistics Prism-5.0 package (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Newman-Kelus test as a post-test was employed to compare the studied animal group. The results were expressed as mean ± standard deviation (SD). *P*-value < 0.05 was considered significant.

RESULTS

Histological Examinations

Group I

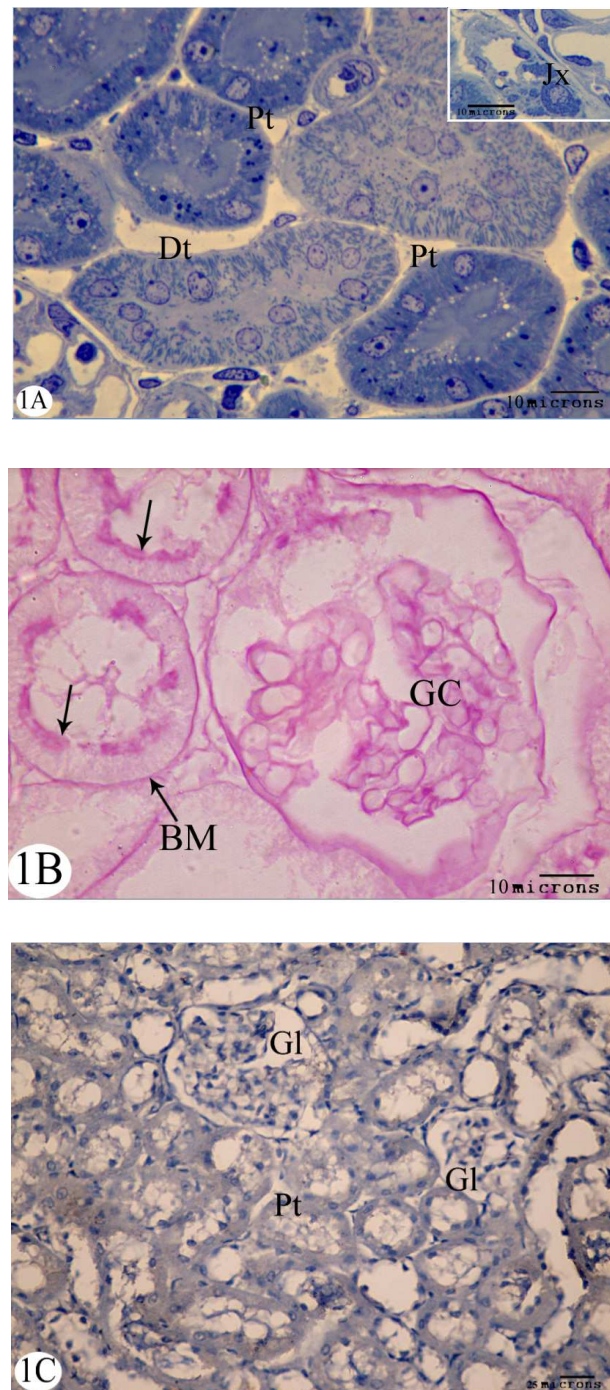Renal histological sections from control animals exhibited normal architecture. The proximal convo-

Fig. 1. Light micrographs, renal cortex, group I: **(A)** Semi-thin section stained by TB, proximal convoluted tubule (Pt), distal convoluted tubule (Dt). Inset: semi-thin section stained by TB, showing granules in juxtaglomerular cells (Jx). **(B)** Paraffin section, showing PAS+ reactions in brush border (↑) and basement membranes of proximal convoluted tubule (BM) glomerular capillaries (GC), and distal convoluted tubule. **(C)** Paraffin section, showing negative iNOS-immunoreactivity in the glomeruli (Gl) and renal tubules (Pt).

luted tubules (PTs) had an intact brush border, vesicular round nuclei and granular cytoplasm (Fig. 1A). The distal convoluted tubules (DTs) showed wider lumen with ill-defined brush border (Fig. 1A). The juxtaglomerular cells (JGCs) of the afferent arteriole revealed renin secretory granules in their cytoplasm (Fig. 1A, inset).

PAS stain demonstrated + reactions in the basement membranes of the parietal layer lining Bowman's capsule, the glomerular capillary loops, the mesangial matrix and the renal tubules, and the brush border of the PTs (Fig. 1B). Using immune-reactivity, no expression of iNOS was observed in the renal tissue (Fig. 1C).

In ultrastructure, the cells of the PTs showed characteristic numerous apical microvilli, ovoid euchromatic nuclei and basal in-foldings of plasma membrane with many basally located elongated mitochondria (Fig. 2A). The apical cytoplasm contained a few vacuoles (Fig. 2A). The lining cells of the DTs had a few luminal microvilli, and the nuclei were more apically located with less basal in-foldings of plasma membrane and elongated mito-chondria (Fig. 2B). The podocytes which lined the visceral layer of Bowman's capsule embraced the glomerular capillaries. They exhibited large and irregular nuclei (Fig. 2C), several primary cytoplasmic processes which arose from their body, and in turn gave secondary processes or pedicles covering the glomerular capillary basement membrane (Fig. 2C). The JGCs of the afferent arteriole revealed intra-cytoplasmic dark granules (Fig. 2D).

Group IIa

Renal sections of group IIa demonstrated changes consistent with early diabetic nephropathy, including tubular dilation and exfoliated epithelial casts in the lumina. The epithelial cells lining PT were distorted with vacuolated cytoplasm, numerous dark granules and loss of brush border (Fig. 3A). The DTs exhibited dark nuclei, vacuolated cytoplasm, disrupted and dilated capillaries (Fig. 3A). PAS reaction revealed a strong positive reaction in the basement membranes of the parietal layer lining Bowman's capsule, glomerular capillary loops, the mesangial matrix and the
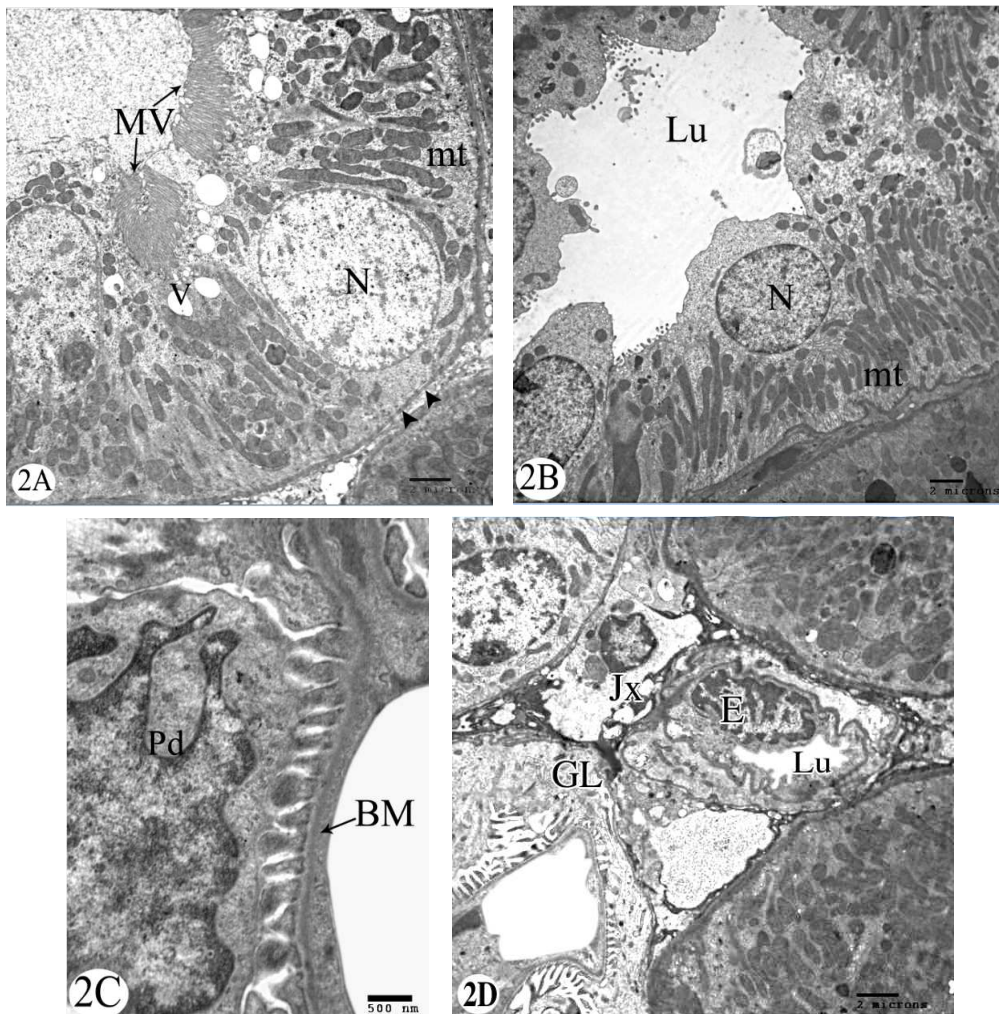

Fig. 2. Electron micrographs, renal cortex, group I: **(A)** PCT, microvilli (MV), nucleus (N), vacuoles (V), mitochondria (mt), basement membrane (▲). **(B)** DCT, lumen (Lu), nucleus (N), mitochondria (mt). **(C)** Podocyte (Pd), glomerular capillary basement membrane (BM). **(D)** Glomerulus (Gl), juxtaglomerular cells (Jx), endothelial cell (E), lumen (Lu).

renal tubules. The proximal tubules revealed partial loss of their brush border (Fig. 3B). Some distal tubules revealed deposition of PAS positive material (Fig. 3B). A strong iNOS cytoplasmic immune-reactivity was observed mainly in the renal tubules and in the periphery of the glomeruli (Fig. 3C). The glomeruli revealed hypertrophy and /or hyper-cellularity and dilated capillaries. The intra-cytoplasmic secretory granules were occasionally detected in the JG cells.

In ultrastructure, the proximal and the distal CTs had a thickened irregular basement membrane and exfoliated luminal cells. Cells lining the PTs exhibited disruption of the apical microvilli, extensive basal in-foldings of their plasma membrane and hyper-chromatic small nuclei. The cytoplasm contained scattered ovoid mitochondria and numerous basal vacuoles (Fig. 4A). Many DTs lining cells revealed loss of their normal architecture, which was replaced by homogeneous hyalinized material. Other DTs revealed apical irregular hyper-chromatic nuclei, numerous apical vacuoles, extensive basal in-foldings of their plasma membrane and extremely elongated mitochondria (Fig. 4B). Some DTs contained exfoliated luminal cells with dense nuclei and cytoplasm (Fig. 4C). The podocytes had electron-dense nuclei and cytoplasm (Fig. 4D), and some podocytic pedicles fused together along with a thickening of the glomerular capillary basement membrane (Fig. 4D). The JGCs of most of the afferent arterioles had electron-dense nuclei and cytoplasm that revealed no cytoplasmic renin granules (Fig. 4E).

Group IIb

The renal sections from group IIb (diabetic bi-guanidine-treated) were markedly improved compared to non-treated diabetic group (Fig. 5A). The JG cells characteristically revealed numerous intra-cytoplasmic secretory granules (Fig. 5A, inset). The glomerular capillaries were less dilated compared to the non-treated diabetic group. In group IIb, the PAS reaction in the basement membranes of most of the glomeruli, the mesangial matrix and tubules were relatively reduced, and the brush border of the proximal convoluted tubules was less destructed in comparison with the diabetic-untreated group (Fig. 5B). The expression of iNOS was markedly reduced (Fig. 5C). Ultrastructure exhibited improved renal cortex structure compared with that of the non-treated diabetic group (Fig. 6A-C). The JGCs of the afferent arteriole revealed denser and more numerous dense renin granules compared with other groups (Fig. 6D).

Morphometry

In control sections the mean area of the glomeruli was $497.1 \pm 97.17 \ \mu m^2$, ranging from 300.7 to 660.6 μm^2 (Histogram 2). It significantly increased in group IIa (P < 0.05), (mean $1197 \pm 247.7 \mu m^2$) ranging from 901.1 to 1930 μm^2 (Histogram 1). In
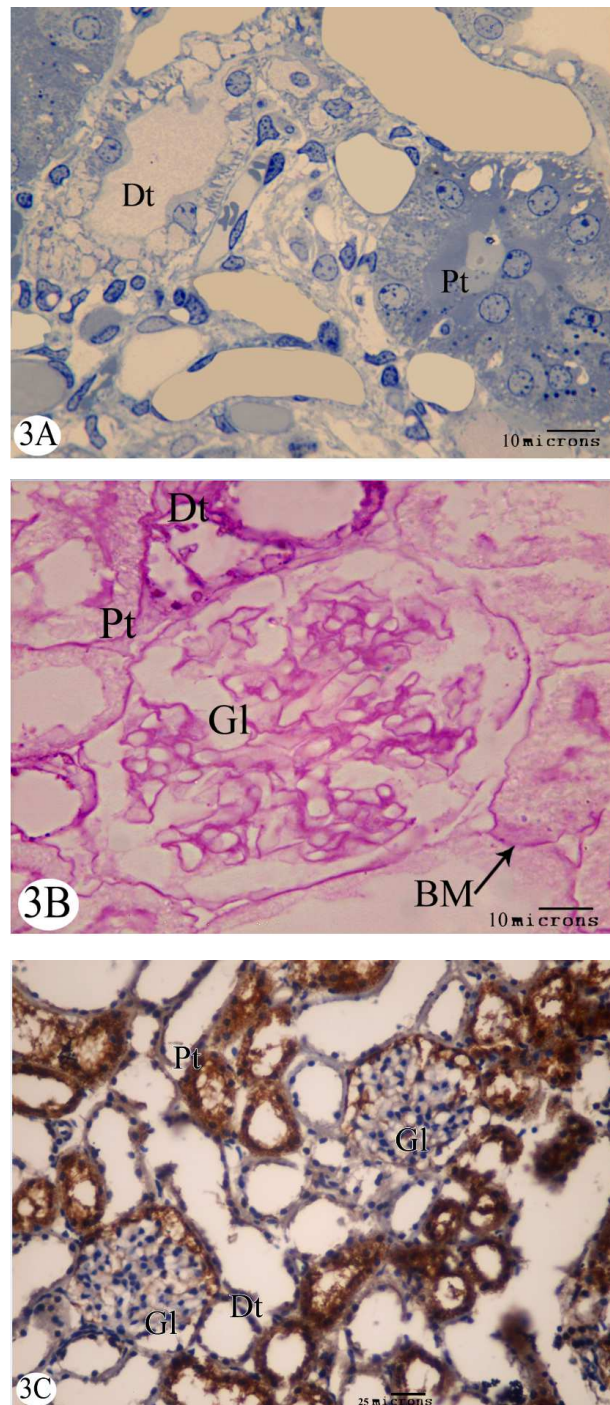

Fig. 3. Light micrographs, group IIa, renal cortex: **(A)** Semi-thin section stained by TB, proximal convoluted tubule (Pt), distal convoluted tubule (Dt). **(B)** Paraffin section, note the thickening of the basement membranes of glomerular capillaries loops (Gl), partial loss of brush border of PCT (Pt), PAS-positive material deposited on DCT (Dt). BM (basement membrane). **(C)** Paraffin section, strong iNOS- immunoreactivity in the glomeruli (Gl) and proximal convoluted tubules (Pt). Dt (distal convoluted tubule).
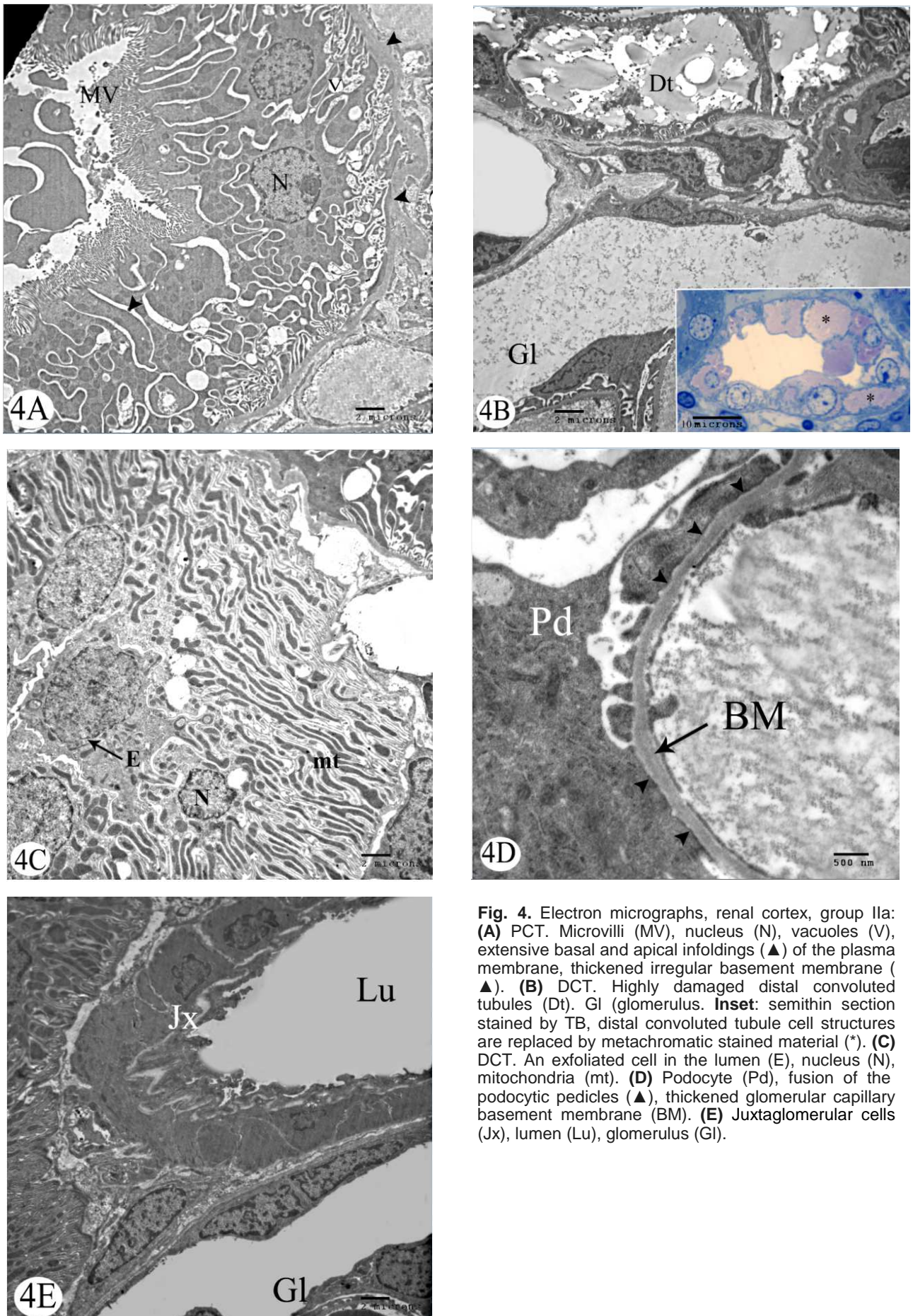
Fig. 4. Electron micrographs, renal cortex, group IIa: **(A)** PCT. Microvilli (MV), nucleus (N), vacuoles (V), extensive basal and apical infoldings (▲) of the plasma membrane, thickened irregular basement membrane (▲). **(B)** DCT. Highly damaged distal convoluted tubules (Dt). Gl (glomerulus. **Inset**: semithin section stained by TB, distal convoluted tubule cell structures are replaced by metachromatic stained material (*). **(C)** DCT. An exfoliated cell in the lumen (E), nucleus (N), mitochondria (mt). **(D)** Podocyte (Pd), fusion of the podocytic pedicles (▲), thickened glomerular capillary basement membrane (BM). **(E)** Juxtaglomerular cells (Jx), lumen (Lu), glomerulus (Gl).
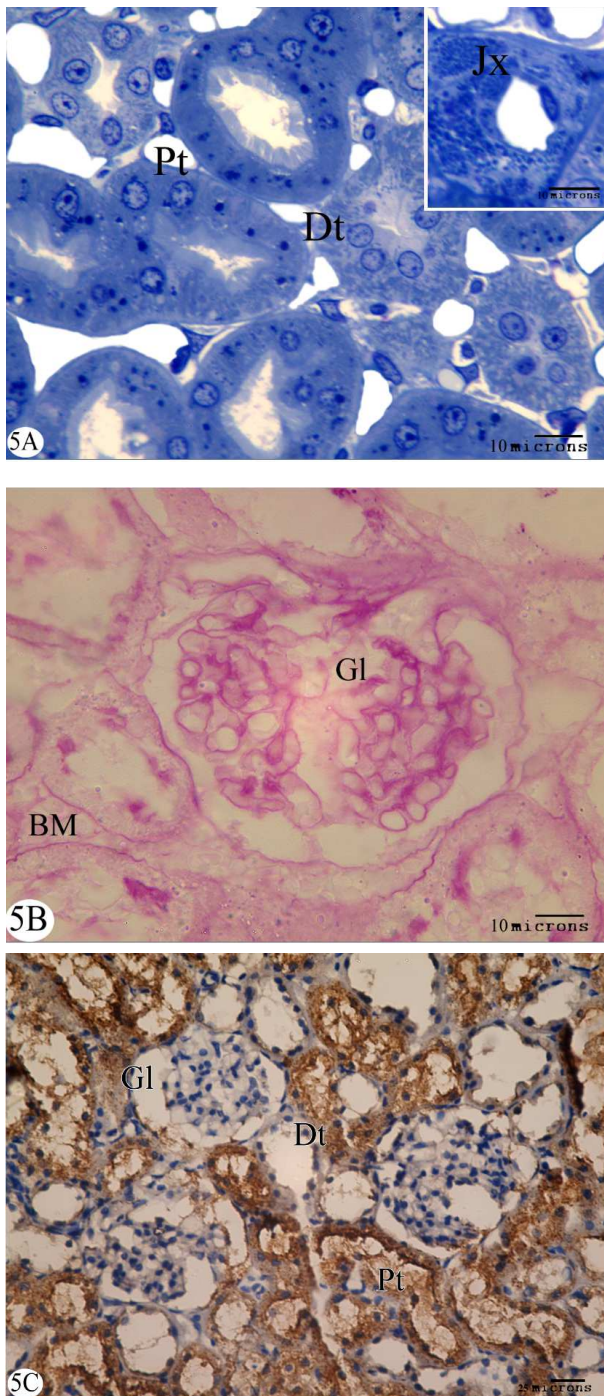
Fig. 5. Light micrographs, group IIb, Renal cortex: **(A)** Semi-thin section stained by TB, renal cortex, proximal convoluted tubule (Pt), distal convoluted tubule (Dt). **Inset**: semi-thin section stained by TB, showing granules in juxtaglomerular cells (Jx). **(B)** Paraffin section, Reduced PAS + reaction in the glomeruli (Gl) and basement membranes (BM). No PAS-positive material deposited on DCT. **(C)** Paraffin section, Reduced iNOS - immunoreactivity in the glomeruli (G1) and the proximal convoluted tubules (Pt). Dt (distal convoluted tubules).
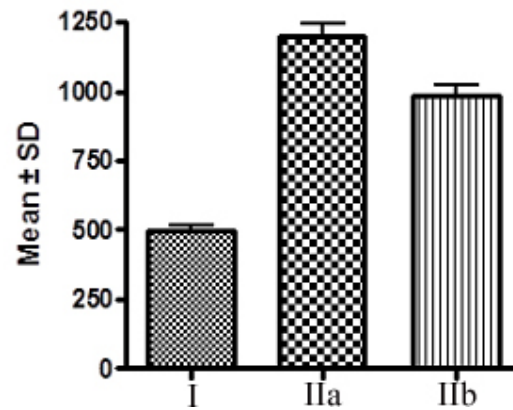
Histogram 1. Mean glomerular area (μm^2) in the
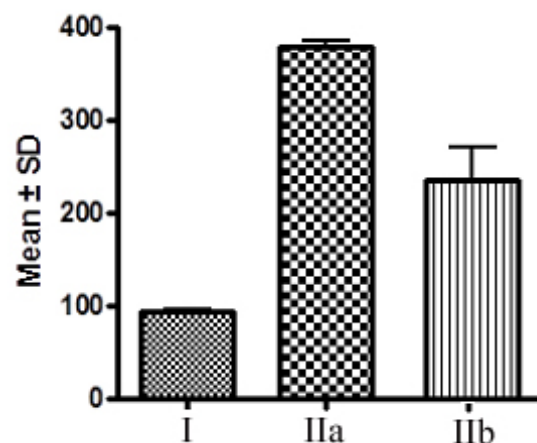
Histogram 2. Mean FBG (mg/dL) in the studied groups.

group IIb the mean area of the glomeruli was significantly decreased compared to group IIa (P < 0.05). It ranged from 554.1 - 1373 μm^2 (mean 983.8 ± 185.8 μm^2) (Histogram 1).

FBG Level

The level of FBG in group IIa (mean 380.0 ± 17.5 mg/dL) was significantly increased (P < 0.001) compared with group I (mean 94.15 ± 7.060 mg/dL) (Histogram 2). The level of FBG in group IIb (mean 235.7 ± 119.0 mg/dL) showed a significant improvement compared with group IIa (P < 0.001) (Histogram 2).

DISCUSSION

The current study revealed that N-thioacetylbigaunidine administration markedly improved the renal diabetic histological changes, in spite of chronic hyperglycemia, which included damage to the glomeruli, thickening of the basement membranes and expansion in the mesangial matrix and edematous PCTs with expansion of their basal infoldings. This expansion proves that the ion-transport between cells and peritubular areas is highly affected, as hyperglycemia enhances the amounts of glucose filtered by the glomeruli, and thereby increases the tubular glucose load, exposure, and reabsorption (Gurel et al., 2012).
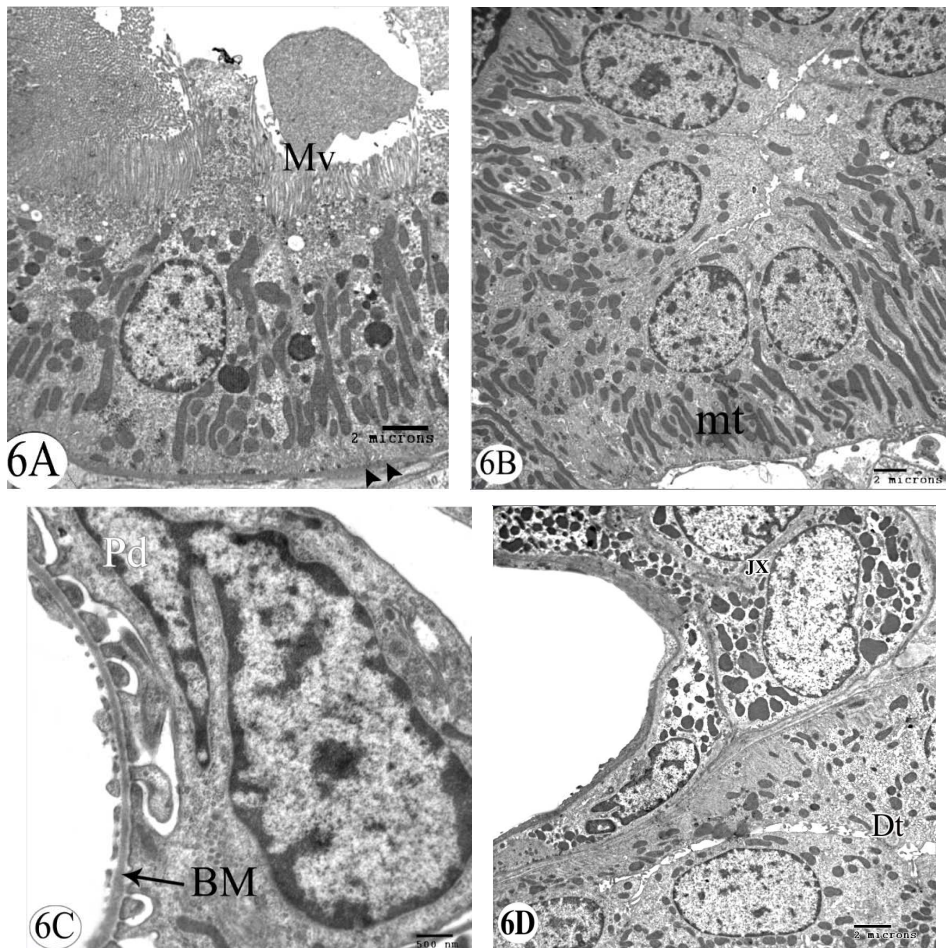
Fig. 6. Electron micrographs, renal cortex, group IIb. **(A)** PCT. Microvilli (MV), basement membrane (▲). **(B)** DCT. Regularly arranged mitochondria (mt) along the basal infoldings. **(C)** Podocyte (Pd), glomerular capillary basement membrane (BM). **(D)** Juxtaglomerular cells (Jx) contain numerous intra-cytoplasmic dark granules. Dt (distal convoluted tubule).

The immune-reactivity for the enzyme iNOS in the normal kidneys was low or undetectable, whereas a strong expression of iNOS in diabetic rat-proximal tubules is in accordance with several studies (Bank et al., 1996; Heeringa et al., 1998; Chou et al., 2002), although some studies denied its expression in diabetic kidney Veelken et al., 2000; Chen et al., 2005). In support of our findings, a critical role of iNOS is suggested in the progressive renal dysfunction and structural injury. Gurel et al. (2012) reported that increased iNOS caused kidney tissue degeneration, whereas inhibition of iNOS ameliorated renal damage.

The use of N-thioacetylbiguanidine suppressed iNOS immune-reaction in the renal tissue. Use of N-thioacetylbiguanidine demonstrated marked antiglycation activity in an in vitro glycation reactions (Mohamed et al., in progress). It is a nucleophilic hydrazine compound like AG, which possesses multiple biological effects including prevention of AGEs formation (Vlassara, 1994), inhibition of diamino oxidase (Sessa and Perin, 1994) and selec-

tive inhibition of iNOS (Corbett et al., 1992; Misko et al., 1993). In support of our hypothesis, some investigators suggested that AG inhibits a pathophysiologic function of iNOS independent of free radical-mediated lipid peroxidation or significant effects on AGEs deposition (Reckelhoff et al., 1999).

Suppression of renin in JGCs of a diabetic kidney, detected in his work, was frequently observed (Christlieb et al., 1976; Perez et al., 1977). The low renin state has been attributed to sclerotic renal arterioles and glomeruli, possibly amplified by functional factors such as autonomic neuropathy and sodium retention. The use of the substituted biguanidin produced a remarkable decrease in renin release reflected by the presence of numerous granules in JGCs.

A permissive role for nitric oxide in renin release has been suggested (Beierwaltes, 1994, 1995; Reid and Chou, 1995). According to Kurtz (2011), nitric oxide appears as a tonic enhancer of renin secretion, acting via inhibition of cAMP degradation through the action of cGMP.

Conclusion

In this study we report that N-thioacetylbiguanidine attenuated the histological changes of diabetic nephropathy reaching healing features, which resembled that of a normal kidney, reduced iNOS tissue reactivity and increased renin secretory granules in the JGCs.

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