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Histological and Immunohistochemical Study on Nitric Oxide Synthase and Effects of Angiotensin Receptor Blockade in Early Phase of Diabetes in Rat Kidney

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Hypothesis: Several studies demonstrated that the patho-physiologic and morphologic changes in early diabetic nephropathy were mediated by either an increase or decrease in the nitric oxide (NO) production and/or activity. There are few reports suggesting a relationship between NO and renin-angiotensin system.
Objectives: The present study was designed to determine the effects of early diabetic state on NO production and also to assess the protective effects of angiotensin receptor blockers (ARB) on these changes.
Material and Methods: Thirty adult male albino rats were included in this study. Twenty were injected with streptozotocin for induction of diabetes. The other ten were injected with the vehicle and served as control. Two days after injection, the diabetic animals were randomly divided into two groups of ten animals each. One group was given Valsartan as an ARB and the other group received no further treatment. Three weeks later, all animals were sacrificed and the kidneys were processed for paraffin sections. The sections were stained with H & E, Masson trichrome and PAS reaction. Also, the sections were stained with immunohistochemical stain against endothelium-derived nitric oxide synthase (eNOS).
Results: Diabetes induced histological changes in the form of glomerular hypertrophy,

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increased glomerular matrix, focal areas of tubular atrophy, medullary congestion and slight fibrosis. eNOS immunostaining was present in the control kidney in the golumeruli and the collecting tubules of the medulla. Diabetes induced positive reaction in the proximal and distal convoluted tubules and increased eNOS immunoreactivity in the collecting tubules. Treatment with Valsartan induced improvement of the morphology of the kidney and reduction in the intensity of eNOS immunostaining.
Conclusion: NO increases in early diabetic kidney and ARB as Valsartan could be recommended in the prevention of the development of diabetic nephropathy.

Keywords: Diabetic; Nephropathy; eNOS; Streptozotocin; Valsartan; Immunohistochemistry.

ABBREVIATIONS

AGE: Advanced Glycosylation End-product; ARB: Angiotensin receptor blocker; DN: Diabetic Nephropathy; ECM: Extracellular matrix; eNOS: Endothelium-derived Nitric Oxide Synthase; NO: Nitric Oxide; STZ: Streptozotocin.

1. INTRODUCTION

Diabetic nephropathy (DN) is one of the serious complications of diabetes and a common cause of end-stage renal failure worldwide. Recently, patients needing dialysis for DN have been increasing. So, the quality of life and prognosis of diabetic patients are influenced by nephropathy which is a severe and chronic disorder [1, 2]. Preventing the occurrence and slowing the development of DN has therefore become a very important issue because early stages DN were accompanied with glomerular hypertrophy and hyperfiltration leading to overt nephropathy [3]. Endothelium-derived Nitric Oxide (eNO) has been identified as one of the compounds responsible for the alterations in the glomerular filtration [4]. NO is a paracrine mediator with a wide spectrum of physiological actions, including the control of vascular tone, antithrombotic actions, cell cycle regulation, neurotransmission and inflammation. NO is synthesized during conversion of its physiological precursor L-arginine to L-citrulline. This reaction is catalyzed by an enzyme known as eNO synthase (eNOS) [5]. Meanwhile, evidence has suggested that there is an activation of intra-renal renin-angiotensin system in diabetes mellitus**.** Furthermore, although the renin release has been shown to be modified by eNO system, whether the interaction between the two systems in diabetes is altered has not been fully explored [6]. Angiotensin II type 1A receptor blockers (ARB) were shown to ameliorate endothelial-dependent vasodilatation in diabetic patients [7]. So, this study aimed at investigating changes in eNO in early experimental diabetes in rat kidney through immunolocalization of the enzyme endothelium-derived Nitric Oxide Synthase (eNOS) and also at evaluating the renoprotective effects of ARB and its interaction with eNOS.

2. MATERIALS AND METHODS

2.1 Drugs

2.1.1 Streptozotocin

(STZ; Sigma, St louis, Mo, USA) in a single intraperitoneal injection of a dose of 60 mg/kg dissolved in 0.5 ml of citrate buffer (0.1 mol/L) for induction of diabetes mellitus [8].

2.1.2 Valsartan

(Tareg Tablets 40 mg; Novartis, SAE, Cairo, Egypt); it is a selective Angiontensin II type 1A receptor blocker in a dose of 20 mg/kg/day given by gavage. Taking into consideration that Valsartan is a well-known antihypertensive drug. So the dose used is the least effective dose to minimize lowering blood pressure effect on the animals as much as possible [9].

2.2 Animals and Treatment Protocols

The present study included thirty adult male albino rats of 180 – 230 gm body weight; they were obtained from and housed in the animal house of Kasr- El-Aini Faculty of Medicine, Cairo University. This study has been approved by the ethics committee on animal research in the animal house of Kasr-El-Aini Faculty of Medicine, Cairo University, Egypt following international ethics and regulations for animal research in laboratory applications [10].

The animals received a standard diet for rodents and allowed free access to water. They were distributed into three groups each one contained ten rats.

Diabetes was induced in twenty animals by a single intraperitoneal injection of STZ. The remaining ten animals (control) were injected with the same volume of the vehicle (*Group 1*).

Diabetes was confirmed by measuring their tail blood glucose concentrations 48h after STZ injection. Rats with blood glucose over 300mg/dL were considered diabetic [11]. The diabetic animals were divided into two equal groups; *Group 2* did not receive any further treatment and *Group 3* received Valsartan once daily.

2.3 Evaluation Methods

Three weeks after induction of diabetes all animals were anesthetized using injection of thiopental sodium 50 mg/ kg subcutaneously and then sacrificed. Kidneys were cut, fixed in 10% formalin solution, embedded in paraffin and cut at 6 μm thickness. The sections were stained and studied as follows:

A) Histological Study

The sections were stained with H&E to examine the structural changes, PAS reaction to identify the changes in the mesangial matrix and the basement membranes Masson trichrome stain to demonstrate the collagen fibers.

B) Immunohistochemical Study

Immunohistochemical staining was carried out using primary antiserum to eNOS (*NeoMarkers, Cat.#* RB-1711). Tissue sections were boiled in 10mM citrate buffer, pH 6.0, (*NeoMarkers, Cat.#* AP-9003) for 20 min. followed by cooling at room temperature for 20 minutes and washing in Tris-bufferd saline. Then, the sections were incubated with the primary antibody overnight at 4°C. A standard labeled strept-avidin-biotin immunoenzymatic antigen detection procedure recommended by the manufacturer was followed using goat Anti-mouse IgG and counterstained with Mayer's hematoxylin. Cells positive for eNOS showed cytoplasmic brown deposits. Positive control tissue was performed by immunostaining a section of endothelial positive cells. Negative control sections were

performed by omission of incubation with the primary antibody (NeoMarkers, Lab Vision Corporation, California, United States) [12].

C) Morphometric Study

The data were obtained by using "Leica Qwin 500" image analyzer computer system (Hessen, Germany):

I) Glomerular Changes:

1) **Glomerular Matrix Index:** The glomerular matrix index presents the ratio of the mesangial matrix area to the glomerular tuft area in PAS stained sections. It has been used to determine the degree of increased glomerular matrix. It was measured as the percentage of the area positive for PAS reaction within the glomerular tuft in 10 randomly selected glomeruli per animal.
2) **Glomerular Fibrosis:** To evaluate fibrosis in the glomeruli, Masson trichrome stained sections were used. Percentage of the area stained blue was measured per unit tuft area in 10 randomly selected glomeruli per animal.

II) Cortical Interstitial Fibrosis: Cortical interstitium was defined as the peritubular space and included the tubular basement membrane and peritubular capillaries. Interstitial fibrosis was estimated by measuring the percentage of the area occupied by the Masson trichrome positive interstitium in 10 randomly selected non overlapping fields per animal**.**

III) Medullary Fibrosis: It was estimated by measuring the percentage of the trichrome positive area in 10 randomly selected non overlapping fields of the renal medulla per animal**.**

IV) Immuno-reactive Optical Density: The optical density was determined randomly in the cortex and medulla. Measurement was done in 10 randomly selected non overlapping fields at magnification x400 from each animal.

2.4 Statistical Analysis

Statistical analysis was performed with SPSS software. Data were presented as the Mean ± SD. Differences among the study groups were detected by one way analysis of variance (ANOVA) as global test to determine any differences in data prior to comparing pairs of groups then t- test to compare each two groups. P values < 0.05 were considered statistically significant [13].

3. RESULTS

All animals injected with STZ included in this study developed diabetes mellitus with blood glucose levels > 300mg\dL.

3.1 Histological Results

Control animals demonstrated normal appearance of the glomeruli composed of capillary tuft surrounded by Bowman's capsule enclosing space between the visceral layer adherent to capillary tuft and the parietal layer, proximal convoluted tubules with the characteristic narrow

lumen lined by few cuboidal epithelial cells and distal wider tubules lined by more low cuboidal epithelial cells and medulla containg the wider collecting tubules with wide lumen and thin wall lined by cubical cells. Glomeruli and tubules are crowded in the kidney with minimal interstitial tissue in between (Fig. 1). Diabetic animals demonstrated glomerular enlargement in most of the glomeruli. Some of them were markedly enlarged. Adhesions of the glomerular tuft with some areas of the Bowman's capsule were demonstrated. Some glomeruli demonstrated marked mesangial expansion so that Bowman's spaces were almost completely obliterated. Tubulu-interstitial changes were present. They were in the form of focal tubular dilatation, vacuolization of the lining cells with appearance of casts inside the tubules. The medullary region demonstrated vascular congestion among the collecting tubules (Fig. 2).

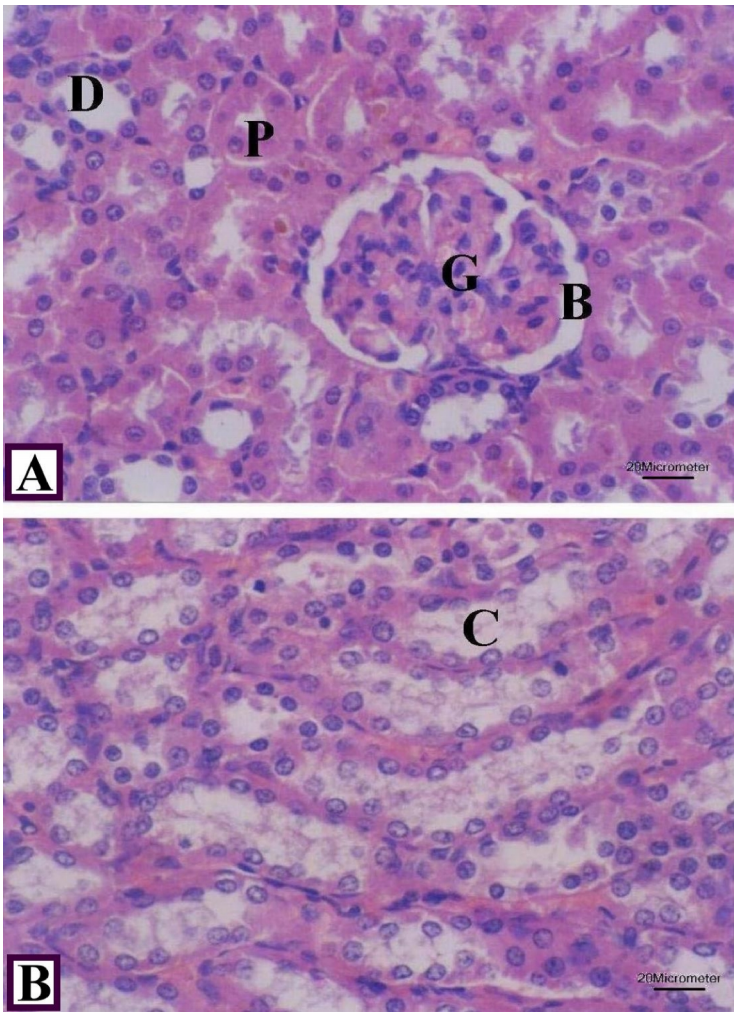

Fig. 1. Photomicrographs of sections from the control kidney demonstrating normal appearance of capillary tuft of glomerulus (G), Bowman's space (B), the proximal (P) and distal (D) convoluted tubules. In the medulla; collecting tubules appear wide lined with simple cuboidal epithelium (C).
(H&E; A, B x400)

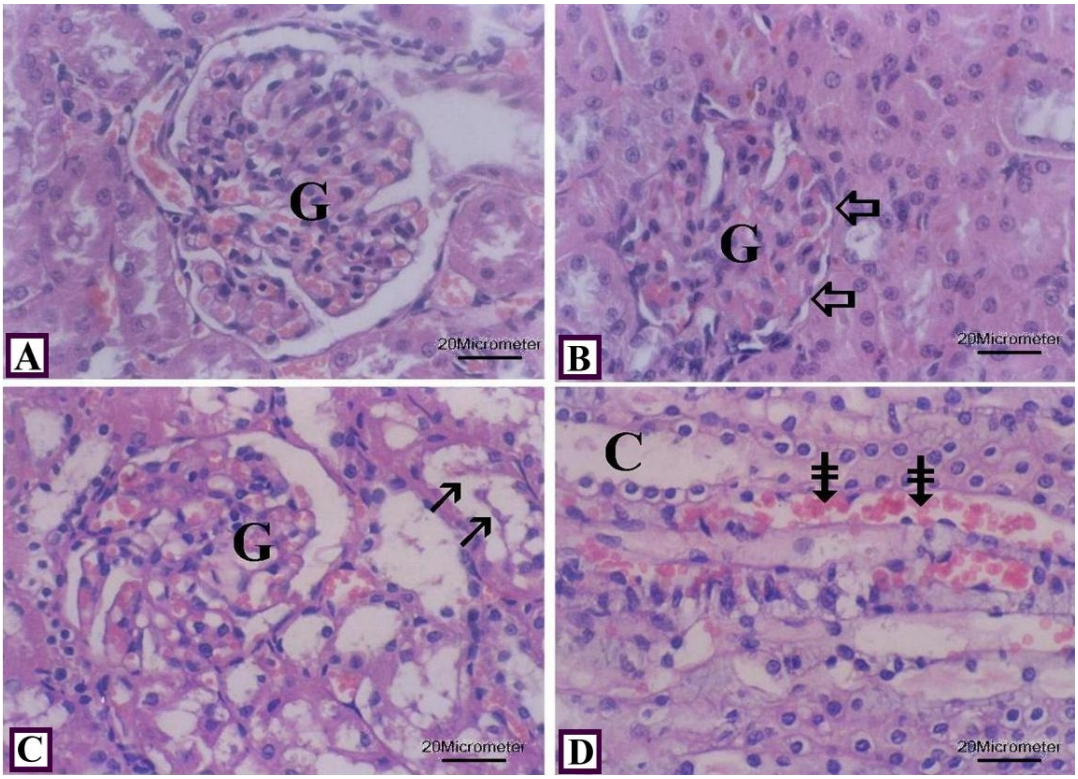

Fig. 2. Photomicrographs of sections from the kidney of diabetic group showing marked enlarged congested capillary tuft of glomeruli (G), almost obliterating the Bowman's space with tuft adhesion to the Bowman's capsule (thick arrows). Tubules appeared dilated with intratubular casts (thin arrows). While the medulla showed vascular congestion (crossed arrows) in between the collecting tubules (C).
(H&E; A, B, C, D x400)

Animals treated with Valsartan demonstrated less severe changes. Some glomeruli were enlarged while others appeared of normal size. Bowman's spaces were still narrow. Congestion was still present the in glomeruli. Most tubules appeared normal. No remarkable capillary congestion in the medulla (Fig. 3).

With PAS reaction, the control group showed staining reaction in mesangeal matrix in glomeruli and boundary of Bowman's capsule and staining of basement membrane of proximal, distal and collecting tubules (Fig.4), extracellular matrix of diabetic group appeared to be increased in the mesangium in the glomeruli with thickened parietal layer of Bowman's capsule in some areas. The basement membranes of the convoluted and collecting tubules appeared thickened also (Fig. 5). The group treated with Valsartan demonstrated less marked increase in the staining intensity of the glomerular tuft and apparently normal parietal layer of Bowman's capsule. The basement membranes of the tubules appeared normal (Fig. 6).

With Masson trichrome staining, the control group showed staining reaction in mesangeal matrix in glomeruli and boundary of Bowman's capsule and staining of basement membrane of proximal, distal and collecting tubules (Fig. 7) in diabetic group, connective tissue fibers

appeared increased in the glomeruli, with thickened parietal layer of Bowman's capsule in some areas. The basement membranes of the convoluted and collecting tubules appeared thickened also (Fig. 8). The group treated with Valsartan demonstrated slight increase in the connective tissue content compared to control but decreased compared to diabetic group (Fig. 9).

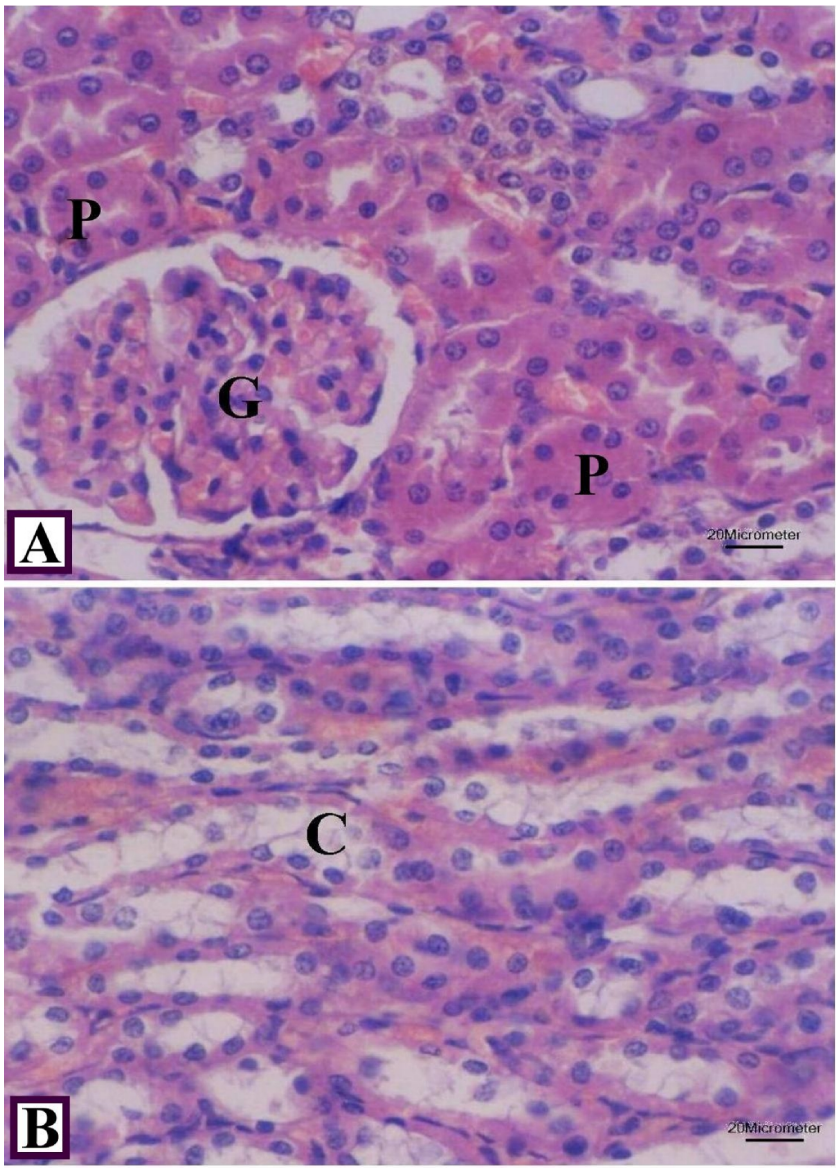

Fig. 3. Photomicrographs of sections from the kidney of diabetic rats treated with Valsartan (group 3) showing enlarged glomerulus (G) with tuft congestion. The cortical proximal convoluted tubules (P) appear normal. The collecting tubules in the medulla (C) also show normal appearance.
(H&E; A, B x400)

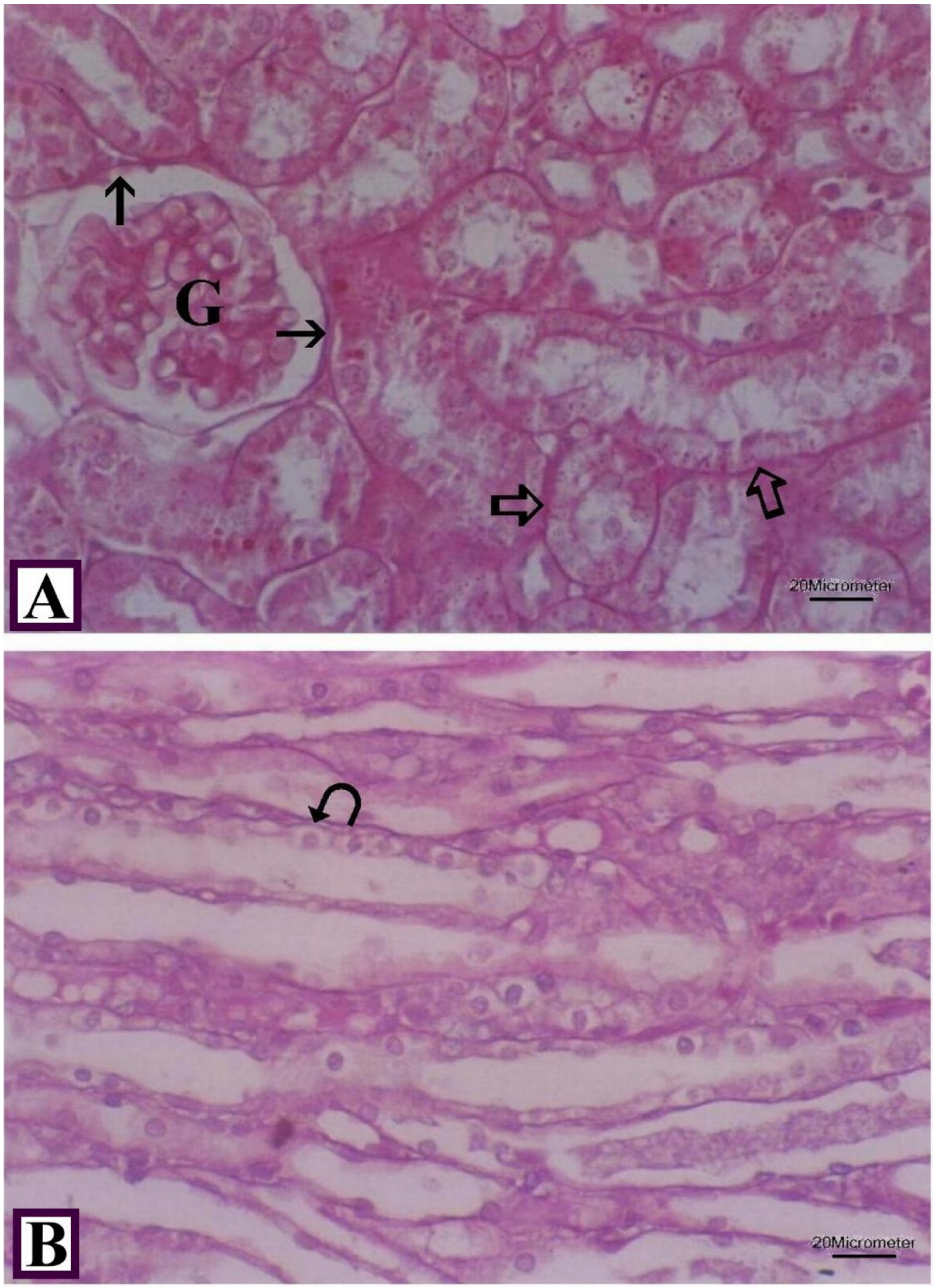

Fig. 4. Photomicrographs of sections from the control kidney demonstrating normal reaction of a glomerulus (G), boundaries of Bowman's capsule (thin arrows) and basement membranes of proximal and distal convoluted tubules (thick arrows) and collecting tubules in the medulla (curved arrows).
(PAS; A, B x400)

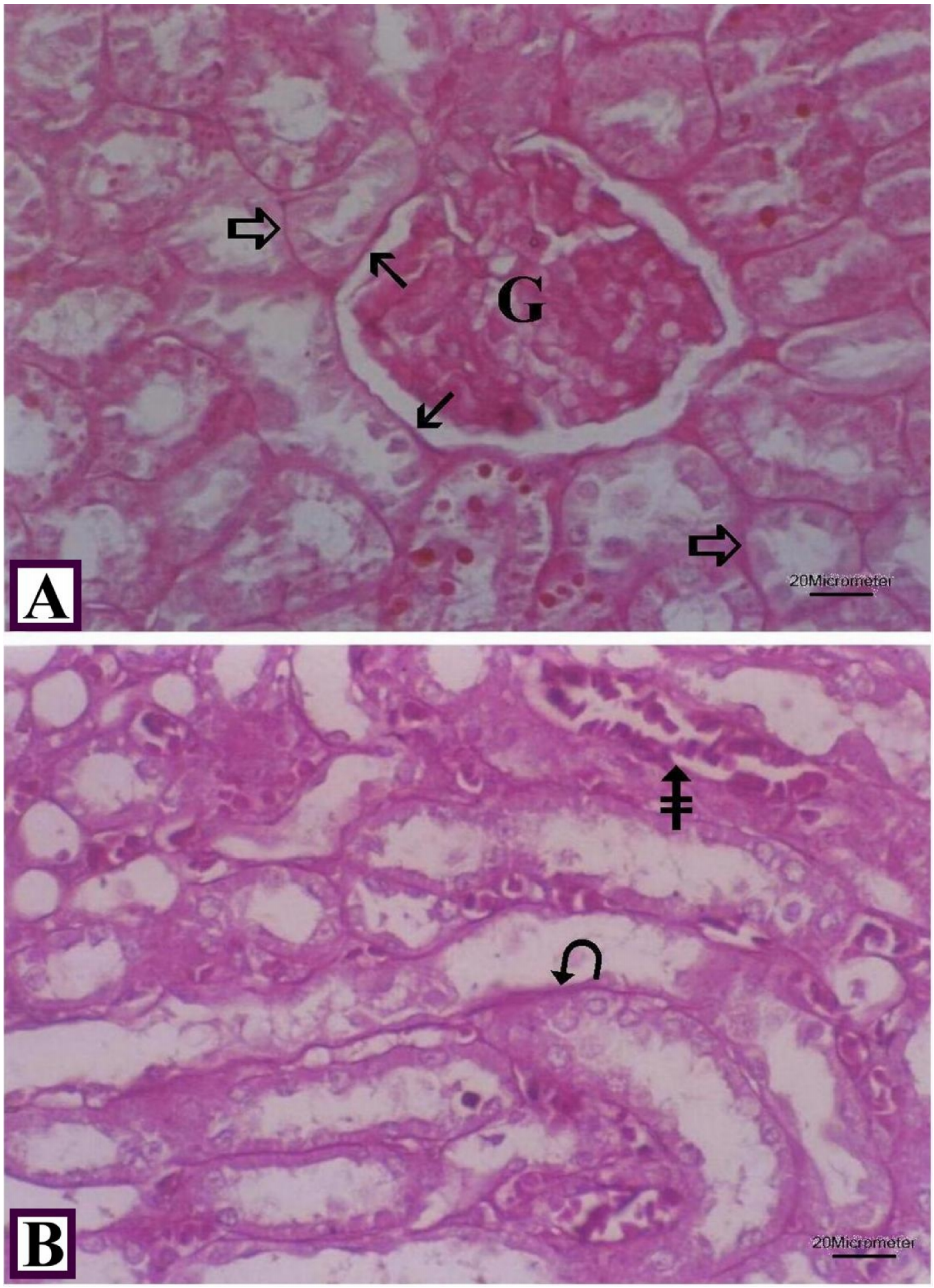

Fig. 5. Photomicrographs of sections from the kidney of diabetic rats demonstrating increased reaction of a glomerulus (G), boundaries of Bowman's capsule (thin arrows) and basement membranes around proximal and distal convoluted tubules (thick arrows) and collecting tubules (curved arrow) with the presence of vascular congestion (crossed arrows) in the medulla.
(PAS; A, B x400)

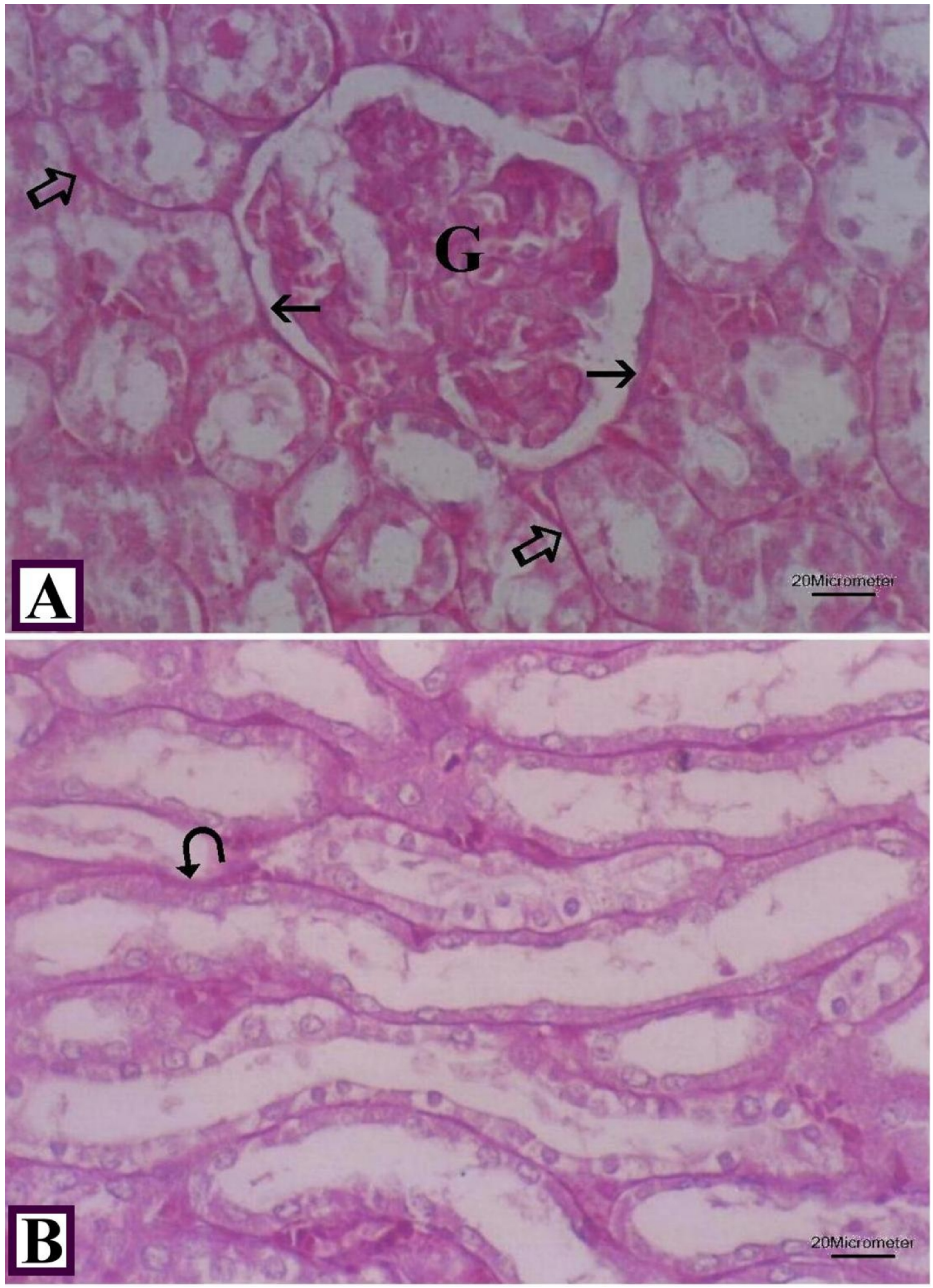

Fig. 6. Photomicrographs of sections from the kidney of diabetic rats treated with Valsartan demonstrating increased reaction of a glomerulus (G). The boundaries of Bowman's capsule (thin arrows). Basement membranes around proximal and distal convoluted tubules (thick arrows) are not thickened in addition to normal reaction around the collecting tubules (curved arrows).
(PAS; A, B x400)

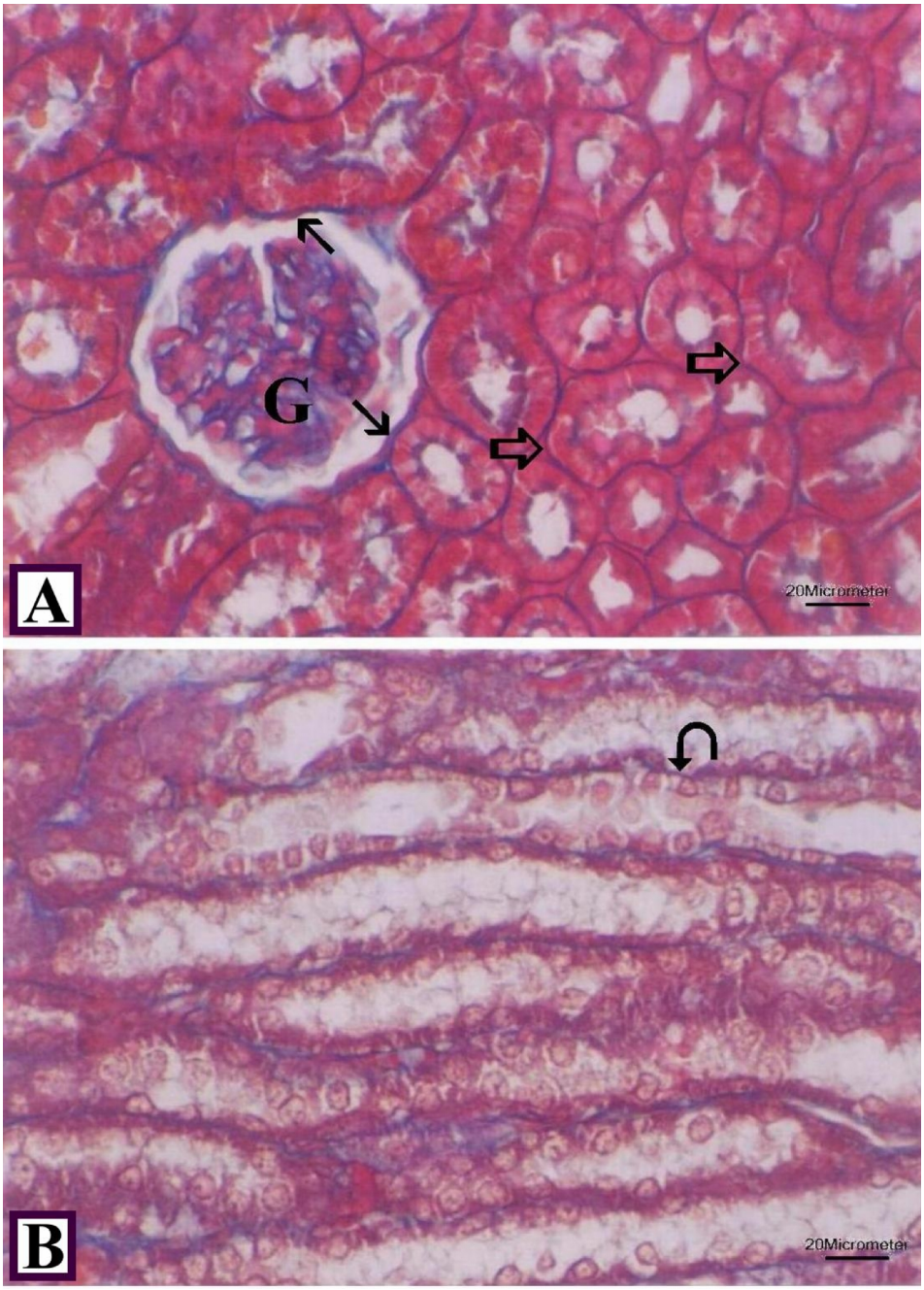

Fig. 7. Photomicrographs of sections from the control kidney demonstrating normal distribution of the collagen fibers in a glomerulus (G), boundaries of Bowman's capsule (thin arrows) and basement membranes of proximal and distal convoluted tubules (thick arrows) and collecting tubules in the medulla (curved arrows).
(Masson trichrome stain; A, Bx400)

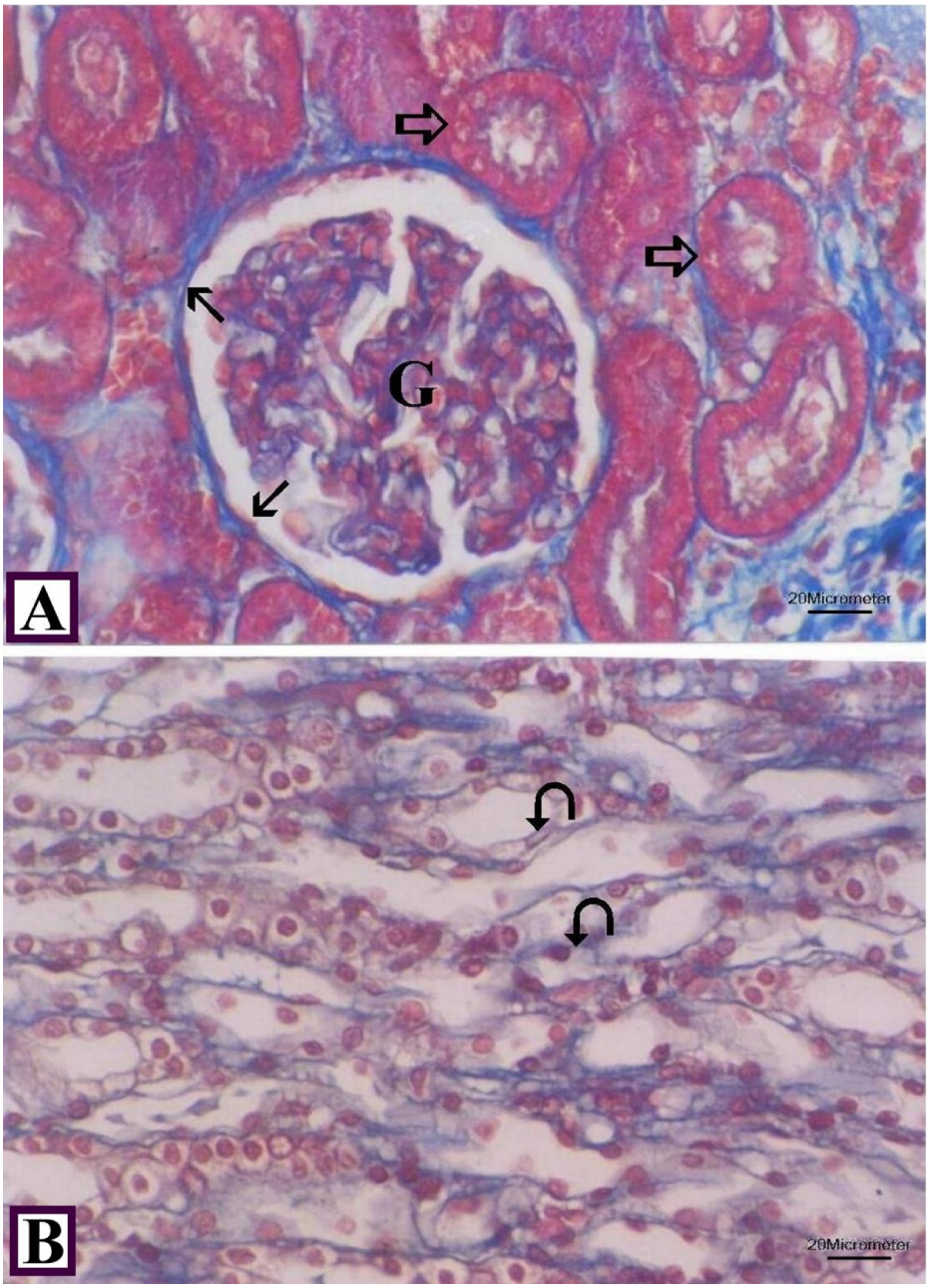

Fig. 8. Photomicrographs of sections from the kidney of diabetic rats demonstrating increased collagen fibers (blue color) in a glomerulus (G) with thickening of the glomerular basement membrane (thin arrows) and basement membranes of proximal and distal convoluted tubules (thick arrows) and collecting tubules in the medulla (curved arrows).

(Masson trichrome stain; A, Bx400)

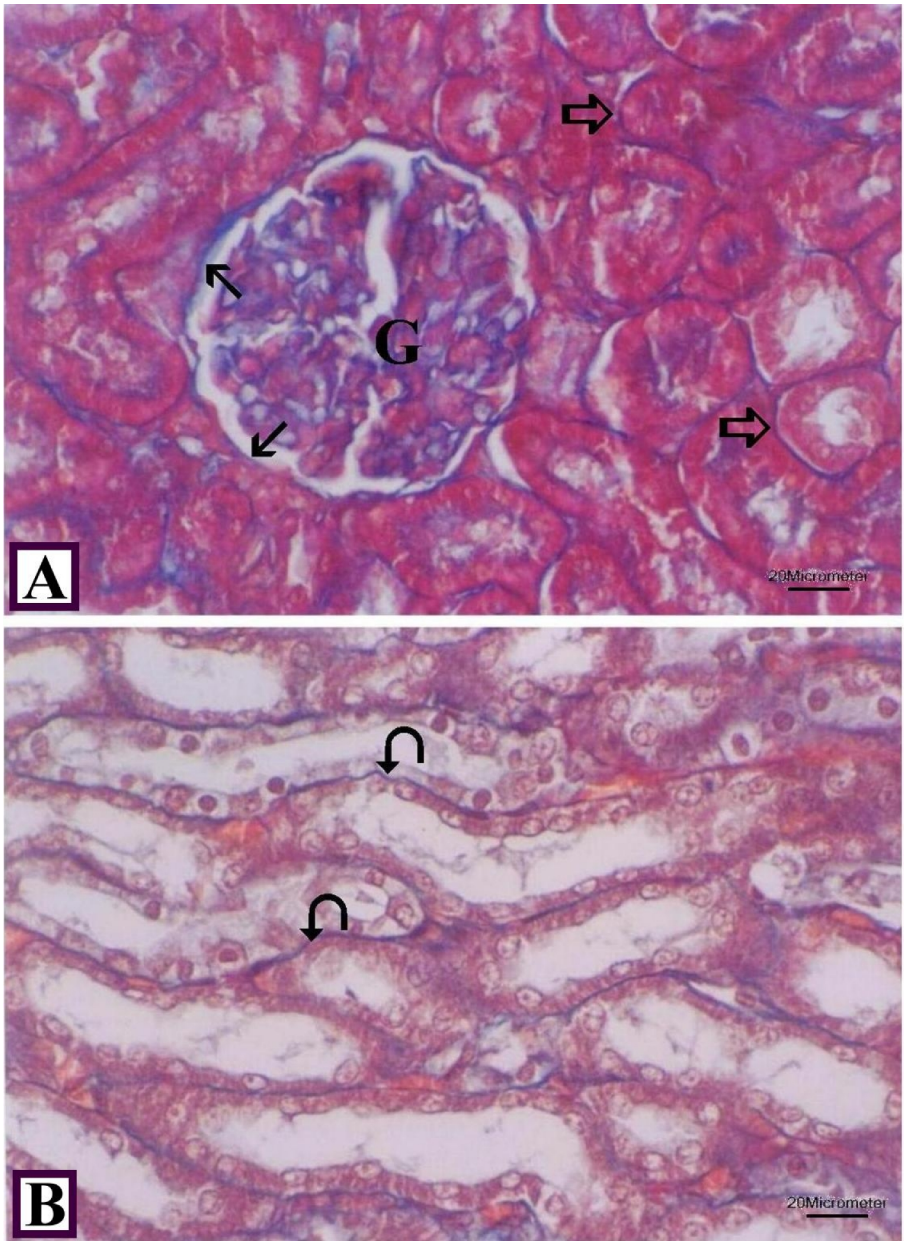

Fig. 9. Photomicrographs of sections from the kidney of diabetic rats treated with Valsartan demonstrating increased collagen fibers (blue color) in a glomerulus (G) with no remarkable change of the glomerular basement membrane thickness (thin arrows) compared to control group and also no remarkable change in basement membranes of proximal and distal convoluted tubules (thick arrows) and collecting tubules in the medulla (curved arrows) compared to control group.
(Masson trichrome stain; A, Bx400)

3.2 Immunohistochemical Staining and Intensity

The control group demonstrated absent reaction in the proximal and distal convoluted tubules. The capillary tufts of most glomeruli were imunoreactive. The collecting tubules in the medullary region demonstrated weak reaction in the most of the collecting tubules (Fig. 10).

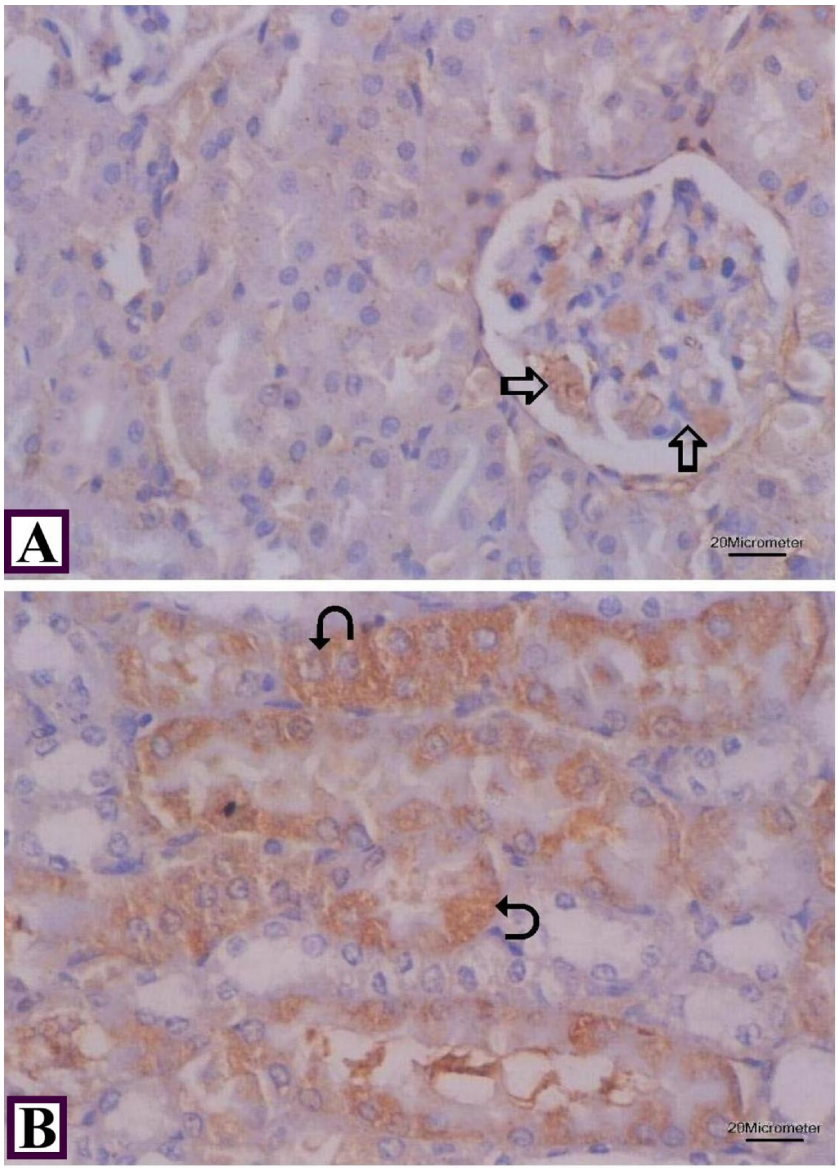

Fig. 10. Photomicrographs of sections from the control kidney showing positive eNOS immunoreaction in the capillary tuft (thick arrows) while the proximal and distal convoluted tubules are negative. There is positive eNOS immunoreaction in some collecting tubules (curved arrows) in the medulla.
(eNOS immunostaining; A, B x400)

The diabetic group demonstrated positive eNOS immunostaining in the glomerular tufts. There was strong reaction in most tubules occupying the whole cytoplasm in the proximal and distal convoluted tubules. Intense reaction was demonstrated in most collecting tubules (Fig. 11).

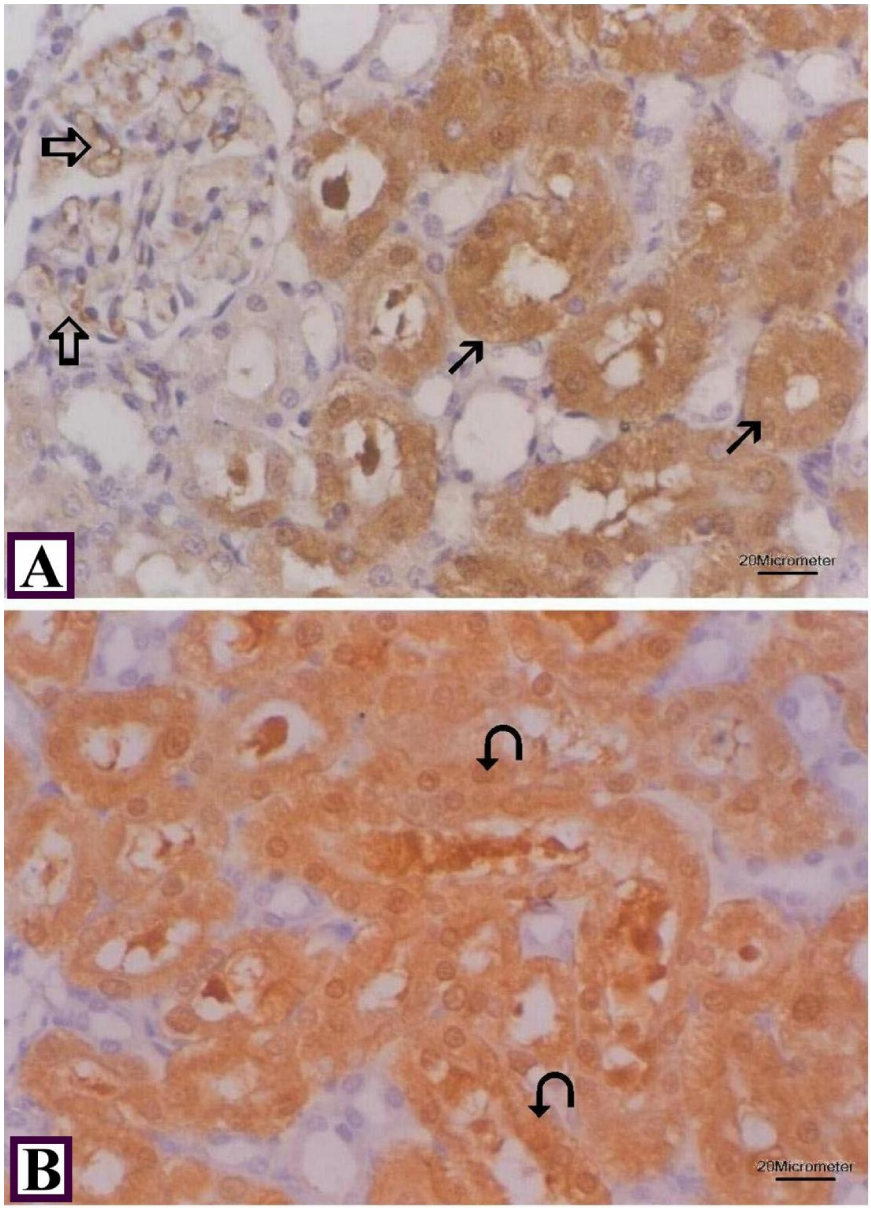

Fig. 11. Photomicrographs of sections from the kidney of diabetic rats showing positive eNOS immunostaining in the capillary tuft (thick arrows). The proximal convoluted tubules (thin arrows) demonstrate intense reaction. There is positive intense eNOS immunostaining in the collecting tubules (curved arrows) in the medulla.

(eNOS immunostaining; A, B x400)

In the group treated with Valsartan, most glomeruli were imunoreactive. Both the proximal and distal convoluted tubules showed variable degrees of reaction. Some tubules demonstrated weak reaction in the basal parts of the cytoplasm. Other tubules demonstrated moderate reaction. Few tubules were intensely stained. Non reactive tubules could also be noticed. The collecting tubules of the treated group demonstrated moderate reaction (Fig. 12).

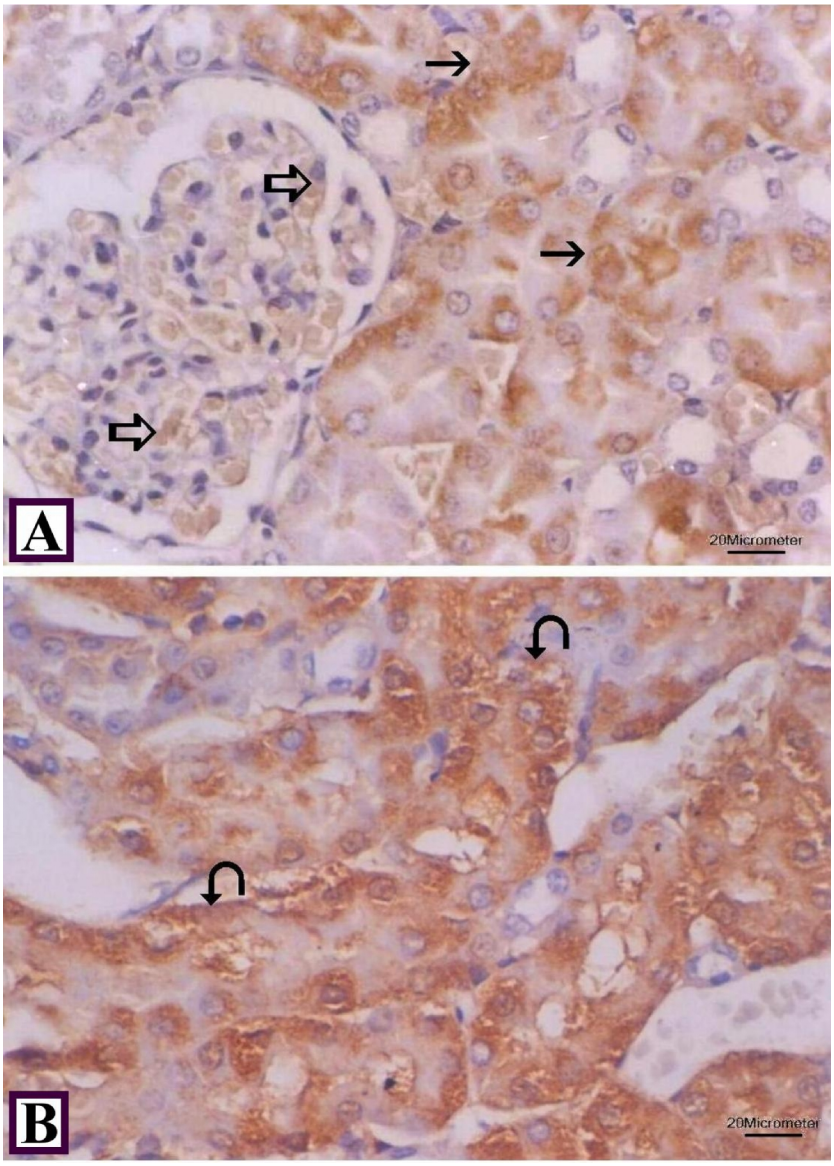

Fig. 12. Photomicrographs of sections from the kidney of diabetic group treated with Valsartan showing weak eNOS immunoreactivity in the capillary tuft (thick arrows). Some proximal convoluted tubules (thin arrows) and collecting tubules (curved arrows) demonstrate moderate eNOS immunoreactivity.
(eNOS immunostaining; A, B x400)

3.3 Morphometric Results

Analysis of variance (ANOVA) showed statistically significant difference among groups that need further clarification using t-test between each two groups. So, diabetic group demonstrated significant increase in the glomerular matrix index, glomerular collagen fibers, interstitial fibrosis and medullary fibrosis and optical density of eNOS immunostaining in both cortex and medulla when compared to the control group. Treated group demonstrated significant reduction of these parameters when compared to the diabetic group but still significantly higher than those of the control group (Charts: A-B; Tables 1-2).

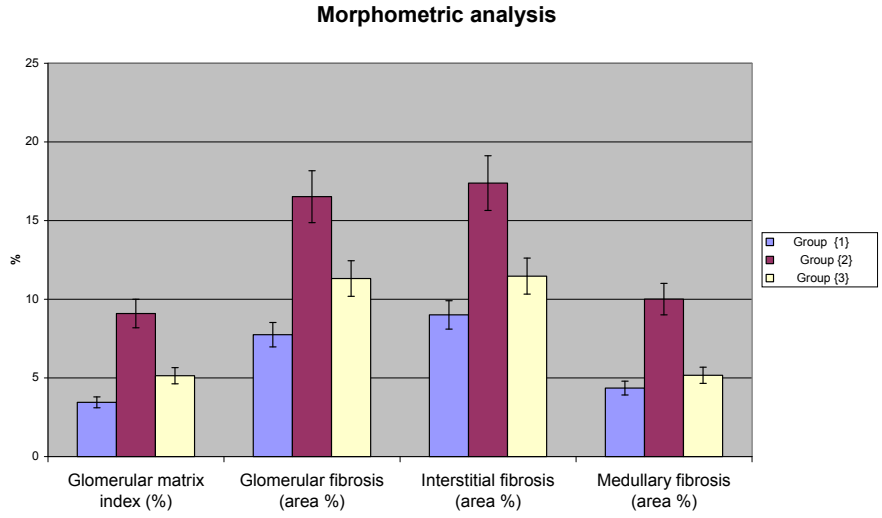

Chart (A): morphometric analysis of mean percent of Glomerular matrix index, Glomerular fibrosis, Interstitial fibrosis, Medullary fibrosis

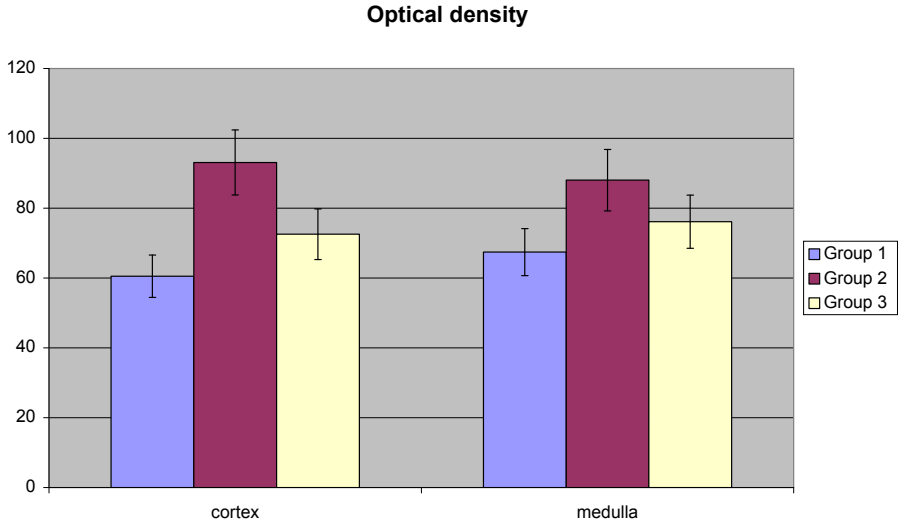

Chart (B): Mean optical density eNOS immunostaining in cortex and medulla

Table 1. Morphometric analysis in the studied groups:

Mean±SD	Group {1}	Group {2}	Group {3}
Glomerular matrix index (%)	3.4526±.047[a]	9.093±0.073021[b]	5.143±0.095[c]
Glomerular fibrosis (area %)	7.746±0.195[a]	16.519±0.449337[b]	11.315±0.492[c]
Interstitial fibrosis (area %)	9.008±0.127[a]	17.384±0.320846[b]	11.470±0.249[c]
Medullary fibrosis (area %)	4.355±0.228[a]	10.012±1.546419[b]	5.168±0.444[c]

Different superscripts indicate statistically significant difference (P < 0.05) compared to other groups.

Table 2. Mean optical density eNOS immunostaining in cortex and medulla

MEAN±SD	Group 1	Group 2	Group 3
Cortex	60.52±6.11[a]	93.09±2.573[b]	72.55±2.668[c]
Medulla	67.42±8.41[a]	88.029±4.468 [b]	76.135±6.509 [c]

Different superscripts indicate statistically significant difference (P < 0.05) compared to other groups.

4. DISCUSSION

Diabetic nephropathy is one of the major causes of end-stage renal failure. The mechanism of development of abnormal hemodynamics of this disorder is unclear. Clarification of the pathogenesis of DN and the development of novel and effective therapeutic strategies are therefore very important [14]. This study aimed at studying the role of NO in early diabetic changes in the kidney and also at evaluating the efficacy of ABR using Valsartan in the prevention of these changes.

In the present study, diabetic rats exhibited diffuse glomerular enlargement, mesangial expansion and congestion, obliteration of Bowman's spaces, increased glomerular matrix and thickened basement membrane of parietal layer of Bowman's capsule. Similar findings were reported in many previous researches. These changes were defined as glomerulosclerosis and may be attenuated by Valsartan [15].

Consistent to the present findings, glomerulosclerosis has been reported to be a characteristic of experimental diabetic animals and humans with diabetes mellitus. Recent advances in renal patho-physiology strongly suggest that the diffuse expansion of the mesangial region may play a critical role in the obliteration of the capillary lumen, leading to reduction of the surface area available for filtration and ultimate cessation of glomerular function in various forms of glomerulopathy including diabetic glomerulosclerosis [2, 14]. These alterations appear to be homologous to early features found in the human diabetic kidney with increased NO level in which glomerular hypertrophy with mesangial expansion is claimed to be a central pathology [5].

The present study demonstrated that diabetes induced tubular changes in the form of tubular dilatation, vacuolization of the lining cells, tubular casts in addition to vascular congestion and slight fibrosis. Previous reports demonstrated similar findings [16].

The present study demonstrated eNOS expression both in the glomerular tuft and the collecting tubules of the medulla of the control kidney. Previous reports demonstrated similar findings showing that NO were mainly present in the renal medulla [4]. While others reported that NO, a free radical in the form of a highly diffusible gas, exerts a wide spectrum of physiological actions, including the control of vascular tone, antithrombotic actions, cell cycle regulation, neurotransmission, signal transduction, and inflammation. There is now abundant evidence that physiological levels of NO have a crucial role in the maintenance of renal hemodynamics, renal perfusion and glomerular filtration in normal kidney [17].

In the present study, immunostaining intensity for eNOS was higher in diabetic rats than in the control animals in the renal medulla. Moreover, the proximal and distal convoluted tubules developed positive eNOS immunoreactivity which was decreased after Valsartan treatment. Similar findings were demonstrated by other authors [18].

So that NO produced by eNOS of the kidney could induce hyperfiltration and an increase in the glomerular volume. NO was considered as a potential candidate for mediating the early diabetes and vascular permeability [19].

Increased glomerular blood flow cannot necessarily increase intraglomerular pressure so diabetic kidney abnormally regulates intraglomerular pressure with imbalance between afferent and efferent arteriolar vasodilatation leading to increase in glomerular pressure and allowing systemic hypertension to be transmitted to the glomerulus [20].

Increased renal NO production could be explained by disturbance in amino acid metabolism in diabetes. Elevated glucose induces increased intracellular levels of diacylglycerol, leading to activation of protein kinase C, which has been shown to activate NOS. This pathway may represent the missing link between hyperglycemia and hyperfiltration [21].

Oxidative and nitrosative stresses are involved in a nonenzymatic reaction between sugar and other carbonyl compounds with long-lived matrix proteins leading to formation of a group of molecules known as advanced glycosylation end-products (AGEs). Each AGE structure has its own formation mechanism and thus its own dependence on oxidative stress [22].

Hyperglycemia, AGE and diabetes create environments favoring ECM deposition. Increased tubular mRNA expression for type IV collagen has also been demonstrated within hours to days after induction of diabetes leading to collagen fiber deposition in the interstitial tissue of the cortex and around the collecting tubules [23].

Large amounts of NO have been also implicated in the renal abnormal vasodilatation with inflammation. NO produced in large amounts has been suggested to cause direct cytotoxic effects on the endothelium. Another effect of NO may be through DNA damage. The interaction of NO and the superoxide anion generates peroxinitrite, which induces lipid peroxidation and cytotoxicity [24].

This can explain the present results of tubulointerstitial affection and vascular congestion. The present results are in agreement with previous studies showing reduced inflammatory interstitial cells as a result of NO production in diabetic nephropathy [25].

In contrast to the implication of the present observations of reactive increase in NOS in diabetic rat kidney, many investigators have reported that endothelium-dependent, NO-

mediated relaxation of the arteries has been decreased in diabetic animals and inferred that NO production is decreased [26,27].

In the present study, we investigated the efficacy of early intervention with ARB as Valsartan in preventing the development of diabetic renal changes in rats, treatment was commenced immediately after diabetes induction and animals were followed for three weeks. ARB slowed down mesangial expansion and reduced the development of glomerulosclerosis. Also, tubulointerstitial and medullary affection was minimized.

The present results are consistent with previous studies in diabetic and nondiabetic nephropathies. The studies of diabetic and non diabetic renal disease have indicated that an initial reduction in proteinuria after the onset of antihypertensive medication predicts the long term preservation of kidney function and that ARB may have long term reno-protective effect [28].

In agreement, ARB significantly retarded the rate of loss of renal function in a group of patients with DN. This could be explained by that ARB may interfere with trophic properties of Angiotensin II to promote cellular and glomerular hypertrophy or diminish the accumulation of ECM. Either of these processes could be an important initial step leading to glomerular scarring [29].

The present study demonstrated that treatment with Valsartan led to decreased eNOS immunoreactivity. This result was consistent with previous studies showing increased expression of eNOS after two weeks of STZ-induced diabetes and this was attenuated by ARB [11].

Angiotensin-II has been reported to upregulate the *in vitro* synthesis of NOS in various cell types, including glomerular endothelial cells, mesangial cells and tubular epithelial cells. Therefore, blockade of angiotensin II receptors by ARB may decrease the generation of nitric oxide [30,31].

In summary, this study indicates that the increase in the eNOS expression in the kidney possibly participate in the regulatory pathways activated by diabetes mellitus. Intervention studies to block eNOS are needed to address this hypothesis directly. Also, the present results suggest that ARBs represent valuable drugs in the treatment of diabetic nephropathy. More investigations are needed to clarify the complex pathophysiology for further therapeutic intervention.

CONSENT

Not applicable.

ETHICAL APPROVAL

This study has been approved by the ethics committee on animal research in the animal house of Kasr-El-Aini Faculty of Medicine, Cairo University, Egypt following NIH international ethics and regulations for animal research in laboratory applications [10].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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