Diabetic Nephropathy Progression in type 1 Diabetes: Temporal Renal Angiopoietin-Like Protein 2-Toll-Like Receptor 4 and Role of Early Renal Angiotensin II Inhibition by Valsartan

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Research Article

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Abstract

Diabetic nephropathy (DN) is a consequence of diabetes mellitus (DM). DM is associated temporal changes in renal angiotensin II (ANG II) release and multiple mediators leading to DN. These changes were evaluated using early ANG II blocker valsartan as a DN renoprotective drug. Adult male Wister rats were divided into (i) vehicle group; (ii) valsartan received oral 30 mg/Kg/day; (iii) diabetic received single 50 mg/Kg intraperitoneal streptozotocin injection; (iv) renoprotection, valsartan treated-diabetic rats after 7 days from DM. Other group of diabetic animals assigned to receive late valsartan intervention from week 9 to 12 of DM. The renoprotective effect evaluated at 4^{th}, 8^{th}, 12^{th} weeks. DN effects on urine albumin excretion, blood pressure and renal ANG II were measured. Urinary nephrin and kidney injury molecule-1 biomarkers, renal ANGPTL2, and toll-like receptor 4 (TLR 4) mRNA expression were tested. DN-initiated fibrotic markers integrin, α-smooth muscle expression and collagen IV and apoptotic protein caspase 3 were tested. DM induced changes starting from the 4^{th} week. At 12^{th} week, early valsartan intervention showed a significant reduction in ANG II, ANGPTL2 and TLR 4 expression and improvement in albuminuria, blood pressure, urinary biomarkers, fibrotic and apoptotic markers, more than the late intervention. Early inhibition of ANG II in diabetes is associated with decrease in ANGPTL2 and TLR 4 proteins and fibrotic changes. This observation helps in understanding DN pathophysiology and its therapeutic approaches.

1. Introduction

Diabetes mellitus (DM) has significant morbidity on patients; in which uncontrolled hyperglycemia induces diabetic nephropathy (DN) [1], renal fibrosis and end-stage renal disease (ESRD) [2]. Diabetes is associated with metabolic and hemodynamic alterations contributing to DN [3].

Hyperglycemia induces early glomerular hyperfiltration [4, 5], which occurs early in diabetes due to proximal tubular reabsorption of sodium chloride, leading to a reduction in sodium chloride reaching the macula densa and consequently release of renin and local production of angiotensin II (ANG II) [2, 4–6]. Hyperglycemia induces efferent arteriole constriction leading to intraglomerular hypertension with dysfunctional nephron injury[7].

Hyperglycemia-induced nephron dysfunction leads to inflammatory cytokines injury to podocytes and promote their apoptosis [2] which can be detected by nephrin, a podocyte marker [8, 9]. DN is correlated with albuminuria and kidney injury molecule-1 (KIM-1) [10] and inflammatory markers as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[3, 11]. Activation of NF-κB is associated with renal transforming growth factor-beta1 (TGF-β1) and collagen IV; leading to activation of toll-like receptor 4 (TLR4), extracellular matrix accumulation and fibrosis [2, 11, 12]. Hyperglycemia and ANG II-induced NF-κB activation with subsequent TLR4 expression modulate immune responses and the inflammatory cytokines release in DN [13, 14]. Diabetes-induced inflammatory processes is associated with integrin and alpha smooth muscle expression and glomerulosclerosis [15, 16]. Blocking ANG II showed to reduce DN

progression [17–19]. Valsartan blocks ANG II by antagonizing angiotensin subtype 1 (AT 1) receptor and relieve vasoconstriction [20, 21].

Angiopoietin-like protein 2 (ANGPTL2) is vascular growth factors with proinflammatory properties promoting vascular destabilization in diabetes [22]. *In vitro*, in hyperglycemia knockdown ANGPTL2 decreased collagen IV and fibrosis [23]. *In vivo*, ANGPTL2 knockdown ameliorated DN by inhibiting TLR4 expression [24]. Streptozotocin (STZ) injected rats, ANGPTL2 begins to increase with the disease progression, microalbuminuria denoting endothelial injury [21, 22, 25]. However, in DN ANG II blocker effect on ANGPTL2 is not clear yet.

Clinical trials have so far shown inconsistent results for using angiotensin blockade to decrease the risk of progression to microalbuminuria in normotensive patients [26, 27]. Clinical studies start when the disease is already started [28, 29] or with established DN[30] as therapeutic tool. Experimentally, valsartan administration could started after 8 weeks of diabetes and shows therapeutic effect[15]. Also experimentally, inhibiting ANG II early from 0 to 4 weeks post diabetes prevented early diabetic glomerular hypertension and ameliorated glomerulosclerosis (GS)[17, 18]. Hyperglycemia induces multiple factors leading to DN. DN-associated remodeling leads to renal structural changes via subcellular signaling pathways[31]. Therefore, the present study exploited the temporal progression of DN and the role of ANG II blocker, valsartan, on renal ANG II, ANGPTL2, TLR4, NF-κB and inflammatory cytokines on the progression of DN, fibrosis and albuminuria. This will differentially explore the role of these factors that contribute to deleterious mechanisms in the nature history of the disease and the therapeutic benefits of valsartan.

2. Materials And Methods

2.1. Experimental animal procedures

This study was carried using 20 weeks adult male Wister rats weighing 300 ± 30 g. The animal's care and handling were done in agreement with the guidelines of the National Institutes of Health (NIH), the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research, National Research Council, Washington, D.C., and the Suez Canal University, faculty of medicine animal care committee (Research #4302).
Animals were housed with free access to standard rat chow and water *ad libitum* and kept at a constant 12-hour light/dark cycle, room temperature and humidity. Animals were left for acclimatization for seven days before the start of experiments [32].

2.2. Drugs and chemicals

STZ was purchased from Sigma-Aldrich (MO, USA) and was prepared by dissolving in 0.1 M citrate buffer (pH = 4.5). Valsartan (30 mg/kg/day) [12] was provided from Novartis Chemical Co., Egypt as white powder which dissolved and given once daily by gastric tube. Flexible tube was used to decrease oesophageal trauma. The tube is gently inserted in the animal mouth and passed down to the stomach

without resistance. The dissolved drug was administered slowly to avoid stomach reflux. Observation to any signs of respiratory distress during the procedure was mandatory. The tube was then withdrawn slowly. The animals were observed for a while for any immediate adverse effect before returned to a holding cage.

2.3. Induction of T1D in rats

Rats were fasted overnight after which they got a single intraperitoneal injection (i.p.) of STZ (50 mg/kg) [33]. Sucrose (15 g/L) was added to the drinking water for 48 h to limit early mortality from the released insulin from the damaged pancreatic islets[34] and blood sugar monitored closely to avoid fatal hypoglycemia. To detect the successful animal model, after one-week, fasting blood glucose level[35] was measured using one Touch Ultra Mini glucometer (USA). Rats with fasting blood glucose of over 280 mg/dL were included in the study[34]. To prevent subsequent development of ketonuria and breakdown of body fat, diabetic rats received daily subcutaneous insulin of dual-acting insulin (Mixtard, 0.5 IU/Kg) (Novo Nordisk, Egypt) to maintain the blood glucose levels > 300 mg/dl and prevent rat death induced by excessively high blood glucose levels [34, 36, 37].

2.4. Study Protocol

Rats were assigned into different durations of the early valsartan protective effect, renoprotecive (Fig. 1, A, B, C) to test the duration effect of valsartan (4, 8, 12 weeks), and renotherapeutic, testing late valsartan effect after DM development from week 9 to 12 of diabetes induction (Fig. 1, supplementary), in STZ-induced DN. The renoprotective effect was assessed by allocating the rats into four groups. Group 1: vehicle control group: normal rats injected with single i.p. injection of citrate buffer, then treated with normal oral saline from the 2nd week. Group 2: valsartan control group: normal rats injected with single i.p. injection of citrate buffer, then treated with valsartan from the beginning of 2nd week. Group 3: diabetic control group: normal rats injected with single i.p. injection of STZ, then treated with normal saline from the 2nd week. Group 4: valsartan treated group: diabetic rats treated with valsartan from the beginning of the 2nd week. For, the renotherapeutic effect, valsartan effect tested from the beginning of the 9th week to 12th week of diabetes (Fig. 1, Fig. 1 supplementary).

2.4.1. Experiments

Early, valsartan administration began from the beginning of 2nd week, and rats were sacrificed after the end of the week 4, 8 and 12 of treatment (Fig. 1). Testing the renoprotective effect of valsartan in STZ-induced DN was carried out on four groups (vechicle, valsartan control, diabetic control, valsartan treated) including three separate sets per group (4th, 8th, 12th weeks; n = 8/set). Late valsartan administration were done on a separate diabetic animals which valsartan began from week 9–12 (Fig. 1, supplementary). Late valsartan treatment was considered as renotherapeutic to DN. All rats were sacrificed after the end of the 12th weeks

2.4.2. Non-invasive blood pressure meaurement and urine samples collection

. To evalaute ANG II blocking effect in all groups using a BIOPAC non-invasive tail-cuff system (BIOPAC Systems, CA, USA) [12]. After a period of acclimatization of 14 days, systolic and diastolic blood pressure (SBP and DBP, respectvely) were mearsured by single trained personale away from any disturbing enivironment causing stress[38]. Urine samples were collected using the metabolic cages for 24 hours [39]

2.4.3. Renal tissues and blood samples collection

At experiments end, blood samples were collected via cardiac puncture [40] and centrifuged (1600g, 20 min, 4°C) for 10 min to obtain the serum and stored at -80°C until use for various biochemical analyses, and then rats were sacrificed. Both kidneys were removed. The left kidney was immediately frozen at -80°C for the different biochemical determinations, while the right one was processed to perform the histopathological and immunohistochemical assays.

2.5. Biochemical analysis

2.5.1. Urine biochemical analysis: Determination of nephrin and KIM-1 levels

Nephrin and KIM-1 levels as markers of glomerular filtration barrier integrity and renal injury were measured in urine using ELISA (BioSource Europe S.A., Brussels, Belgium) [10].

2.5.2. Measurement of urine albumin excretion

Twenty four hours urine albumin excretion (UAE) as a marker of DN was measured by using microalbumin kits strip (Nephelometry BN ProSpec, Siemens Health Care Diagnostic Products, Germany) [41].

2.5.3. Renal NF-κB, ANGPTL2, integrin and TLR 4 mRNA expression by RT-PCR

. Renal NF-κB, ANGPTL2, and TLR 4 were assessed to determine DM inflammatory effect on the kidney [42, 43]. Renal tissue was homogenized by SV total RNA isolation System (Promega, Madison, WI, USA) to extract RNA. Then, an ultraviolet spectrophotometer was used to measure RNA concentration and purity. We used 1µg extracted RNA to make cDNA using SuperScript III First-Strand Synthesis System (#K1621, Fermentas, Waltham, MA, USA). Then, cDNA was amplified by real-time PCR (RT-PCR) and analyzed by Applied Biosystems software version 3.1 (StepOne™, USA). The PCR reaction used SYBR Green Master Mix (Applied Biosystems). Gene Runner Software (Hasting Software, Inc., Hasting, NY) was used in primers design using RNA sequences gene bank. RT-PCR amplification cycles were: 2 min at 50°, 10 min at 95° and 15 seconds 40 cycles of denaturation and 10 min annealing/extension at 60°. RT-PCR data were estimated with the v1·7 sequencing program (PE Biosystems, Foster City, CA). Relative expression of studied gene mRNA was measured by the comparative (Ct) method. Results were normalized to the β-actin which was used as the control housekeeping gene and reported as fold change over background levels detected in the studied groups. The gene-specific primer pairs for (NF-κB), forward

primer 5’-CATTGAGGTGTATTTCACGG – 3, reverse primer 5’-GGCAAGTGGCCATTGTGTTC – 3. The (ANGPTL2) forward primer 5'-GGAGGTTGGACTGTCATCCAGAG-3', the reverse primer 5'-GCCTTGGTTCGTCAGCCAGTA-3'.The (TLR 4)forward primer AATCCCTGCATAGAGGTACTTCCTAAT-3, the reverse primer CTCAGATCTAGGTTCTTGGTTGAATAAG-3. The integrin forward primer 5'-AGGAGACTGAGAGCGAGCTG-3', the reverse primer 5'-TCAAAGCAGGCAAACAGATG-3'. The (β-actin) forward primer 5'-TGTTTGAGACCTTCAACACC-3', the reverse primer 5'-CGCTCATTGCCGATAGTGAT-3'.

2.5.4. Renal contents of IL-1β, IL-6, TNFα, MCP-1 inflammatory levels using ELISA kits

Renal tissue content of inflammatory cytokines produced during diabetes were estimated. Tissue samples were assessed for IL-1β, IL-6, TNF-α, and MPC-1 concentrations using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer’s instructions [35].

2.5.5. Renal contents of angiotensin II, TGF-β1 and collagen IV *using ELISA kits*

Angiotensin II, TGF-β and collagen IV contents were measured in renal homogenates by ELISA to detect the diabetic and ANG II blocking effect on renal tissue; using Cusabio rat ANG II, and the BioVendor rat TGF-β1 and collagen IV [12].

2.5.6. Determination of ASMA and collagen IV gene expression by western blot

To further explore the diabetes-induced fibrotic activity renal α-Smooth muscle (ASMA) and collagen IV proteins were assessed to evaluate the renal reaction to angiotensin II blocking effect in the renoprotective and renotherapeutic experiments. extracted from tissue homogenates using ice-cold radioimmunoprecipitation assay (RIPA) and buffer supplemented kit, usingV3 Western Workflow™ Complete System, Bio-Rad® Hercules, (CA, USA). The tissue buffer extraction was centrifuged at 4000 × g for 20 min. Extracted protein was assessed using Bradford assay. Equal amounts of protein were loaded (20–30 µg of total protein) and separated by SDS/polyacrylamide gel electrophoresis (10% acrylamide gel) using a Bio-Rad Mini-Protein II system. Then, transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA) with a Bio-Rad Trans-Blot system. The membrane was washed with PBS and blocked for 1 h at room temperature. After transfer, the membranes were washed with PBS and were blocked with 5% (w/v) skimmed milk powder in PBS for 1 h at room temperature. Following blocking, the primary antibodies for ASMA and collagen IV and beta actin (Thermoscientific, Rockford, Illinois, USA) were incubated overnight at pH 7.6 at 4°C with gentle shaking. Then the secondary antibodies were applied after washing the primary antibodies, and were incubated at 37°C for 1h. Band intensity was analyzed by ChemiDocTM imaging system with Image LabTM software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA).The results were expressed as arbitrary units after normalization for β-actin protein expression[44].

2.7. Renal histopathological examination

Renal damage was evaluated and scored according to [45]. Tubular and interstitial changes including, desquamation and cytoplasmic changes examined by: H&E: showed hydropic degeneration (swelling/vacuolization); PAS: showed brush border loss, hyaline globules, and peritubular& interstitial inflammatory infiltration, Masson: showed interstitial fibrosis and interstitial vascular wall thickening or congestion. Sections were examined blindly and randomly in 5 locations. To accurately compare and analyze the damage a graded score was followed: 0: (normal), 1: mild, 2: moderate, and 3: severe, where minimum score 0, and maximum score of 15 [max. score 3 in 5 locations]. Glomerulo-sclerotic injury was semi-quantitatively graded as follows:0: intact glomeruli (normal), 1: lesions affecting ≤ 25% of the glomerular area (mild sclerosis),2: lesions affecting 25–50% of the glomerular area (moderate sclerosis), and 3: lesions affecting ≥ 75%of the glomerular area (severe sclerosis). Renal damage was calculated as the sum of both tubular and glomerular damage[36].

2.8. Renal Immunohistochemistry and image analysis

Renal tissue specimens were deparaffinized, rehydrated and prepared for immunohistochemical staining. Rat monoclonal antibodies against apoptotic markers (caspase-3) (Abcam, Cambridge, UK) was used. Then slides were examined using a light microscope (Olympus cx21, Japan). The percentage of immunopositive areas was determined using Image J 1.45 F (National Institute of Health, USA) [43].

2.9. Statistical Analysis:

Results were expressed as Mean ± SD using statistical software for the social sciences (SPSS), version 17. The difference between variables was analysed using one-way analysis of variance (ANOVA) for quantitative variables and Kruskal–Wallis for parameters with non-Gaussian distribution, followed by Tukey's post-hoc test for multiple comparisons. Unpaired Student's T-test was used to compare two individual groups. The difference was considered significant when P value < 0.05.

3. Results

3.1. Pattern of urine albumin excretion response to valsartan in STZ- induced diabetic nephropathy: Renoprotective versus renotherapeutic effect

To evaluate the establishment of the DN rat model, the UAE was assessed and showed albuminuria in diabetic rats (Table 1) which proves the establishment of the model. Suppressing ANG II in diabetic rats effect on UAE was assessed to determine the renoprotection and renotherapeutic (Table 1, Table 1 supplementary) effect of valsartan on DN. Starting valsartan treatment at the beginning of the 2nd week of diabetes to the 4th, 8th, 12th weeks prevented the chronological increment of UAE (normal < 30 mg/24 hr) compared to non-treated diabetic animals ($p < 0.05$, Table 1). The diabetic valsartan-treated started at the beginning of the 9th week, renotheraeutic groups, showed normalized UAE compared to diabetic group ($p < 0.05$, Tables 1 supplementary).

Table 1
Urine albumin excretion response to valsartan in STZ- induced diabetic nephropathy

	Vehicle	Valsartan control	Diabetic control	Valsartan treated
UAE (mg /day),	0.90 ± 0.16	0.94 ± 0.12*	4.51 ± 1.28¶	2.45 ± 1.03¶*
Valsartan (*Reno-protective)*	1.04 ± 0.17	0.91 ± 0.15*	17.55 ± 3.04¶#	4.50 ± 1.25¶*#
treatment to	0.99 ± 0.12	0.90 ± 0.11*	31.73 ± 3.27¶#$	7.15 ± 1.18¶*#$
-4 weeks				
-8 weeks				
-12 weeks				

UAE, urine albumin excretion, values are means ± SD (n = 6–8) and analyzed using one-way ANOVA followed by Tukey's *post-hoc* test at P < 0.05. Valsartan treatment started at day 7 of diabetes. ¶ compared with vehicle group, * compared with diabetic control group at the same time point, # compared with week 4, $ compared with week 8.

3.2. Pattern of blood pressure response to valsartan in STZ- induced diabetic nephropathy: Renoprotective versus renotherapeutic effect

The effect of suppressing ANG II on BP was evaluated to assess the hemodynamic changes in treated and untreated diabetic rats (diabetic control group, Tables 2–3). Diabetic animals started valsartan at the beginning of 2nd week (valsartan treated group) did not show significant increase in SBP and DBP at the 12th week when compared to non-treated diabetic group (diabetic control group; $p < 0.05$, Tables 2–3). The diabetic valsartan-treated renotheraeutic groups showed normalized SBP and DBP compared to diabetic group ($p < 0.05$, Tables 1 supplementary).

Table 2

Systolic blood pressure response to valsartan in STZ- induced diabetic nephropathy

	Vehicle	Valsartan Control	Diabetic control	Valsartan treated
SBP (mgHg),	129.3 ± 5.80	123.1 ± 6.31*	134.4 ± 6.09	131.8 ± 5.06
valsartan (*Reno-protective)*	127.4 ± 4.57	127.3 ± 3.85*	154.6 ± 5.48¶#	132.1 ± 5.89*
treatment to	130.9 ± 5.17	123.4 ± 9.29*	157.9 ± 20.63¶#	139.0 ± 12.12*
-4 weeks				
-8 weeks				
-12 weeks				

SBP, systolic blood pressure, values are means ± SD (n = 6–8) and analyzed using one-way ANOVA followed by Tukey's *post-hoc* test at P < 0.05. Valsartan treatment started at day 7 of diabetes. ¶ compared with vehicle group, * compared with diabetic control group at the same time point, # compared with week 4.

Table 3

Diastolic blood pressure response to valsartan on renal function in STZ- induced diabetic nephropathy

	Vehicle	Valsartan Control	Diabetic control	Valsartan treated
DBP (mgHg),	80.9 ± 11.56	71.25 ± 8.63*	86.50 ± 7.82	78.75 ± 4.74
Valsartan (*Reno-protective)*	78.38 ± 6.55	69.25 ± 6.41*	92.13 ± 5.14¶	78.63 ± 9.18*
treatment to	79.25 ± 8.92	70.63 ± 7.25*	98.50 ± 3.34¶#	77.75 ± 7.92*
-4 weeks				
-8 weeks				
-12 weeks				

DBP, diastolic blood pressure, values are means ± SD (n = 6–8) and analyzed using one-way ANOVA followed by Tukey's *post-hoc* test at P < 0.05. Valsartan treatment started at day 7 of diabetes. ¶ compared with vehicle group, * compared with diabetic control group at the same time point, # compared with diabetic control group at week 4.

3.3. Renoprotective and renotherapeutic effect pattern of valsartan on urinary nephrin and KIM-1 concentrations in STZ- induced diabetic nephropathy

The pattern of urinary nephrin and KIM-1 were used to assess the impact of ANG II suppression in diabetic treated and untreated animals. It was evident that the urinary concentration of both nephrin and KIM-1 were higher in the diabetic control group compared to the vehicle group, with a significant difference between their levels at the 12th week and the 4th and 8th weeks ($p < 0.05$, Fig. 2). Treatment with valsartan reduced $(p < 0.05)$ both nephrin and KIM-1 concentrations in comparison with the diabetic control group. Implementing the valsartan "renoprotective" effect significantly attenuated KIM-1 concentration $(p < 0.05)$ in comparison with the "renotherapeutic" regime (Fig. 2, supplementary).

3.4. Renoprotective and renotherapeutic effect pattern of valsartan on renal expression of NF-κB, ANGPTL2, TLR 4 and integrin in STZ- induced diabetic nephropathy

Then the pattern of ANGPTL2 as a marker of endothelial integrity, inflammatory marker, and the anchoring protein integrin response to valsartan was assessed in addition to inflammatory markers NF-κB and TLR 4 (Fig. 3). STZ-induced DN was associated with an increase ($p < 0.05$) in mRNA expression of NF-κB, ANGPTL2, TLR 4 and integrin compared to the vehicle group, with a significant difference in NF-κB expression between the 4th and the 12th weeks. The renotherapeutic regime resulted in downregulation of the high mRNA expression of these markers in comparison with the diabetic control group. Implementing the "renoprotective" regime significantly attenuated ANGPTL2, TLR 4 and integrin expression $(p < 0.05)$ as compared to the the "renotherapeutic" one ($p < 0.05$, Fig. 3-supplementary).

3.5. Renoprotective and renotherapeutic effect pattern of valsartan on renal expression of IL-1β, IL-6, TNFα, MCP-1 inflammatory cytokines in STZ- induced diabetic nephropathy

The current study showed valsartan affecting NFκB expression which regulates inflammatory cytokine expression. Renal inflammatory cytokines were further examined using ELISA kit. The results in Table 4 show that IL-1β, IL6, TFNα and MCP1 inflammatory cytokines increased significantly in the control diabetic group ($P < 0.05$) and early valsartan administration was able to inhibit these effects in the renoprotective regime compared to diabetic group (valsartan treated, $P < 0.05$; Table 4). The decrease in the cytokines were not significant from the vehicle group. Late administration of valsartan in renothereapeutic regime, showed decrease of the renal inflammatory cytokines (supplementary Table 4). The decrease in IL-1β, IL6, and MCP1 was significantly different from vehicle group ($P < 0.05$). An increasing trend of the cytokines in renotherepeutic compared with the renoprotective regime.

Table 4
Renoprotective effect of valsartan by downregulating renal IL-1β, IL-6, TNFα, MCP-1 inflammatory levels

	Vehicle	Valsartan control	Diabetic control	Valsartan treated
IL-1β (pg/mg protein)				
Reno-protective	398.1 ± 29.38	361.4 ± 54.11	1473 ± 172.50¶	548.7 ± 127.70*#
IL-6 (pg/mg protein)				
Reno-protective	140.7 ± 7.61	142.7 ± 33.70	567.0 ± 69.39¶	208.9 ± 53.65*
TNFα (pg/mg protein)				
Reno-protective	86.88 ± 6.65	77.50 ± 15.59	531.3 ± 47.68¶	166.8 ± 61.03*#
MCP-1 (pg/mg protein)				
Reno-protective	32.63 ± 8.37	36.16 ± 6.24	155.2 ± 12.92¶	52.38 ± 13.11*

Interleukin (IL), Tumour Necrosis Factor alpha (TNFα), monocyte chemoattractant protein-1 (MPC-1). values are means ± SD (n = 6–8) and analyzed using one-way ANOVA followed by Tukey's *post-hoc* test at P < 0.05. Comparison within the same group, ¶ compared with vehicle group, * compared with diabetic control group,# compared with valsartan group.

3.6. Renoprotective and renotherapeutic effect pattern of valsartan on renal content of angiotensin II, TGF-β and collagen IV in STZ- induced diabetic nephropathy

Renal ANG II and profibrotic markers, TGF-β and collagen IV, were assessed to explore the pattern valsartan effect over time (Fig. 4). The results showed that the mean renal ANG II, TGF-β and collagen IV in the diabetic control group were higher *($p < 0.05$)* than that in the vehicle group. It was obvious that TGF-β and collagen IV contents were higher ($p < 0.05$) during the course of diabetic nephropathy with a significant (*$p < 0.05$*) difference in their levels at different time points. The implemented angiotensin II blockade reduced these parameters in comparison with the diabetic control group. Valsartan "renoprotective" treated group at 12th week displayed a decline in the high concentrations of these markers in diabetic control group as compared with the valsartan "renotherapeutic" treated group (*$p < 0.05$*, Fig. 4-supplementary).

3.7. Renoprotective and renotherapeutic effect of valsartan on the relative renal expression of ASMA and Collagen IV in STZ- induced diabetic nephropathy

Diabetic groups received the renoprotective regime showed less ANGPTL2, inflammatory, proliferative and DN features as compared to renothereapeutic regime (Figs. 2, 3, 4 supplementary). Therefore, Fig. 5 illustrates the comparison between the two regimes regarding the alpha-smooth muscle actin, ASMA, as a marker of cellular proliferation and collagen IV expression, as a marker of increased mesangial matrix and glomerular injury. Diabetes enhanced significantly both marker expression (P < 0.05) in comparison

to normal rats. It was evident that valsartan “renoprotective” treated group displayed a significant reduction in the relative expression of ASMA and collagen IV as compared to the valsartan “renotherapeutic” treated group ($p < 0.05$, Fig. 5).

3.8. Renoprotective and renotherapeutic effect pattern of valsartan on the renal histopathological picture in STZ- induced diabetic nephropathy

Then, renal tissues were examined to detect the chronological pattern of fibrotic changes associated with DN in renoprotective and renotherepeutic regimes using H&E, Masson and PAS staining. The histopathological examination of renal tissues revealed that the vehicle group showed preservation of the normal architecture of renal glomeruli and tubules. However, kidney tissues in the diabetic control group exhibited increment pattern of pathological changes (4th, 8th, 12th week) with different extent at different time points in the term of interstitial inflammatory infiltrate with congested thick-walled vessels. The tubular epithelial cells showed desquamation and hydropic degeneration with focal sclerosis in the glomeruli. Masson showed interstitial and peritubular fibrosis (stained blue) and PAS showed thickening of basement membrane with loss of brush border and vacuolation (Figs. 6,7-I). It was evident that diabetes was associated with an increase in the mean histopathological score in renal tissues in both renouprotective and renotherepeutic regimes compared to the vehicle group ($p < 0.05$, Figs. 6,7-II), with a significant difference between its score in the 12th week and the 4th and 8th weeks (Fig. 7-II).

In both renouprotective and renotherepeutic regimes (Figs. 6,7-I), it was evident that valsartan treatment duration showed chronological effectiveness with varying degrees in ameliorating the mean histopathological score in comparison with the diabetic control group ($p < 0.05$; Figs. 6,7-II). Notably, the renoprotective role of valsartan was more prominent in attenuating the severity of diabetic nephropathy and improving the renal histopathological score compared to valsartan “renotherapeutic” treated group ($p < 0.05$; Fig. 7-II).

3.9. Reno-protective and reno-therapeutic effect pattern of valsartan on the apoptotic marker (caspase-3) immunostaining in STZ- induced diabetic nephropathy

Figure 8 highlighted the pattern of DN-associated apoptosis ($p < 0.05$) in diabetic renal tissues as revealed by the elevated pro-apoptotic activators “caspase-3” percentage compared to vehicle group with a significant difference between its level in the 8th and 12th weeks in comparison with the 4th week ($p < 0.05$, Fig. 8). It was apparent that valsartan inhibits apoptosis, as evidenced by the reduction in the pro-apoptotic caspase-3 compared to the diabetic control group (Fig. 8). However, valsartan “renoprotective” treated group at 12th week displayed a significant reduction in the pro-apoptotic activators as compared to the valsartan “renotherapeutic” treated group ($p < 0.05$, Fig. 8-II).

Discussion

The current study findings highlighted the temporal pattern of ANG II inhibitor (valsartan) in preventing diabetes-induced angiotensin vasoconstrictor effect on renal arterioles [17] and improving the associated

increase of UAE, ANGPTL2, TLR4, integrin, NF-κB, inflammatory cytokines, TGF-β1 and GS [23, 24, 46]. Chronic kidney disease endpoint is fibrosis which is associated with high ANGPTL2- TGF-β1-integrin expression[47, 48]. Integrin is a cell-matrix interactions protein activates intracellular signalling[49]. ANGPTL2 increases TGF-β1 expression through integrin-mediated activation[48] leading to renal fibrosis[47]. Diabetes enhanced renal TGF-β1, integrin kinase and ASMA expression which is under ANG II regulation[50] as observed in this study. Therefore, decreasing the early diabetes-induced hyperfiltration-induced renal hypertension[5] effect is the cornerstone in improving its consequences on renal functional deterioration, inflammation, angiogenesis and fibrosis[51]. The consensus of previous studies comes in agreement with the current study findings regarding the protective and therapeutic effect of valsartan by disrupting diabetes-induced local ANG II release in DN. However, this study showed the chronological pattern of blocking ANG II effect on mitigating DN-associated cofactors.

DN has early glomerular barrier and tubular dysfunction component [4, 52]. Early in diabetes, the hemodynamic changes involve efferent arteriolar vasoconstriction in contrast to afferent vasodilation that ultimately increases glomerular hydrostatic pressure [17], leading to glomerular hyperfiltration in diabetes[53]. Afferent arteriolar dilation per se and subsequent glomerular capillary hypertension lead to proteinuria independent of systemic arterial pressure [7]. Throughout the study period from the 4th -12th weeks post STZ, the study findings showed the influence of ANG II inhibition on decreasing UAE as features of DN[54]. The DN model shares the significant hallmarks of most forms of kidney injury seen in humans, developing hypertension and proteinuria [8, 54] which were observed in the current study. Diabetes increases glomerular capillary hydrostatic pressure and systemic pressure, and both contribute to glomerular protein loss [1]. The increase in blood pressure was observed in STZ -injected rats from the 2–7 weeks [55], which was due to the increase vasopressors in rats injected with the same STZ dose (50 mg/Kg) as used in the current study. The high plasma glucose level increases blood osmolarity inducing osmoreceptor vasopressin release [56]. A modest increase in systemic blood pressure results in vasodilated preglomerular microvasculature which is transmitted to the glomerulus with exacerbation of glomerulosclerosis processes[53]. Therefore, reductions in systemic arterial and glomerular pressures by valsartan reduced the glomerular damage in DM.

Urinary nephrin assesses the kidney's podocyte proteins that reflect the filtration barrier integrity[9]. Diabetes-induced ANG II promotes podocyte injury and promotes the progression to DN via persistent activation of Notch1 and Snail signalling in podocytes, and eventually down-regulation of nephrin expression [57]. Transient increase in nephrin expression occurs in the first eight weeks [58]as observed in the current study. Valsartan showed to ameliorate DN by increasing glomerular nephrin expressions and consequently lowering urinary albumin, collagen type IV, and improved renal function [15]. Urinary KIM-1 and nephrin as early DN markers were also proven to increase with the progression of the disease [10, 15]. The current results showed improvement of DN markers from progression as the therapeutic effect of ANG II blockade by valsartan. Early inhibition of renal ANG II halts the progression of DN which is an essential step in the pathophysiology and treatment of DN[59].

In STZ injected rats, antagonizing AT1 receptor by valsartan attenuated renal ANG II and other cytokines which modulated the diabetes-associated hemodynamic changes and DN[21]. Whole glomeruli or glomerular endothelial cells showed a persistent increase in ANGPTL2 expression at the 4th week of the STZ injected rats [22]; and human patients[42] indicative of disrupting renal vascular integrity. In patients with T1D, serum ANGPTL2 showed a higher level than nondiabetic and associated with microalbuminuria. Consequently, blocking the ANG II pathway in the current study resulted in preservation of endothelial integrity and pointed to the initiation of disturbed angiogenesis and vascular inflammation-induced DN[25]. Therefore, it is good reno-therapeutic treatment in DN as the current results showed.

Persistent hyperglycemia and ANG II activate renal NFκB and trigger inflammatory cytokines and profibrotic factors [11]. In diabetic animals, blocking ANG II by valsartan attenuated the NFκB and the inflammatory pathway [12] leading to DN, as the current results show valsartan as a renal therapeutic and protective agent. NFκB was proven to be associated with renal macrophage infiltration as a response to hyperglycaemia[14]. The downstream of NF-κB signalling of inflammatory cytokines were investigated in diabetic animals in the present study and increase in IL-1β, IL-6, TNFα, MCP-1 inflammatory cytokines were detected as previously reported[24] that was ameliorated by valsartan. Knocking down ANGRTL2 resulted in decrease TLR4 and consequently inflammatory cytokines level [24]. TLR4, an immune modulator [43], expression in renal tissue increased with DN and correlated with macrophage infiltration, poor glycaemic control and deterioration of renal function in diabetic patients[14]. Activated TLR4 receptor initiates NFκB which in turn induces multiple various pro-inflammatory cytokines participates in diabetes-induced inflammatory response and apoptosis leading to DN [43, 60]. Previous study also showed that ANG II induces TLR4 expression in renal tissue[13]. In the current study, results showed increased expression of TLR4 as well as NFκB which were attenuated by valsartan administered early in the disease which confirms their implication in the DN pathophysiology. Valsartan administration late in the disease improved both markers which show its therapeutic role in decreasing DN processes by lowering the expression of these two inflammatory markers. It also improved the histopathological score and apoptosis (Caspase-3) either administered early or late in the disease. Hyperglycaemia-induced NFκB activation induces inflammatory, TNFα and IL-1β, and oxidative stress, superoxide dismutase, release with the initiation of profibrotic markers TGF-β1 and collagen IV in diabetic animals as observed in the current study[12, 61]. Thus, the role of ANG II with TLR4 and NFκB are associated with downstream DN proinflammatory and apoptotic sequences. Thus, rregulation of intrarenal ANG II activation is essential in developing DN and its control improves the fate of DN patients.

Clinical consideration

The present study demonstrates long-term ARB inhibition in an animal model of T1D, which can delay and decrease the development of diabetic glomerulopathy, by modulating glomerular diabetes-associated hemodynamic changes. Further studies are needed to weight hemodynamic versus non-hemodynamic [12, 52] factors that predominate in the pathophysiology and pathogenesis of DN in clinical practice and the starting time of ARB initiation.

Potential Limitations

The successful time point of ANG II inhibition of ANGPTL2/TLR4 [24] will require further investigation. DN will eventually end up by fibrosis due to ANGPTL2/TGF-β1/integrin activation[47, 48]. However, it remains to be determined whether ANG II inhibition affect which part of the nephron, the interstitium and interstitial macrophages or podocyte, to stop the fibrotic cascade [24, 42, 48].

In the present study, ANG II inhibition was associated with decrease ANGPTL2 expression that was tested *in vivo* conditions that are different from those *in vitro*. Cultured 3-day-old Wistar rat cardiomyocytes shown that ANG II suppresses ANGPTL2 expression whereas ARB could significantly reverse this decrease by inhibiting AT1 receptor [62]. The authors argued that ANG II could enhance ANGPTL2 expression or be without effect due to the difference in the used techniques, various exposure times and concentrations. Therefore, isolated renal tissues need further investigation to study subcellular molecular localization under ANG II inhibition during the DN progression.

Conclusion

These results suggest that early inhibition (renoprotective) of ARB (valsartan) is better than late (renotherapeutic) that affect the initiation or progression of DN by blood glucose-independent mechanisms. Early inhibition of ANG II in diabetes showed to decrease renal injury tested by decrease urinary nephrin and KIM-1. The decrease in renal ANG II is accompanied by decrease in ANGPTL2, TLR4, ASMA, integrin expression and inflammatory state leading to fibrosis and renal injury as indicated by decrease in UAE and BP.

Declarations

Conflict of interest

The authors declare that there are no conflicts of interest associated with this study

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Figures

Figure 1

Scheme showing experimental protocols employed. Diabetes mellitus induction by streptozotocin (STZ) was injected intraperitoneal as a single dose (50 mg/kg) on day (0). Valsartan was given orally daily at dose of 30 mg/Kg. Pharmacological regimen was administered from the 2nd week, and rats were sacrificed after 4th, 8th and 12th weeks after treatment.

Figure 2

Renoprotective and renotherapeutic effect of valsartan on urinary (a: nephrin “ng/ml”, b: KIM-1“pg/ml”) in STZ- induced diabetic nephropathy. Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ¶,*,#, $ P< 0.05; ¶ compared with vehicle group, * compared with diabetic control group at the same time point,# compared with diabetic control group at week 4, $ compared with diabetic control group at week 8.

Figure 3

Renoprotective effect of valsartan on renal gene expression of (RT-PCR; a: NF-κB, b: ANGPTL2, c: TLR 4, d: integrin) in STZ- induced diabetic nephropathy. Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ¶,*,#, P< 0.05; ¶ compared with vehicle group, * compared with diabetic control group at the same time point,# compared with diabetic control group at week 4. Angiopoietin-like protein 2 (ANGPTL2), and toll-like receptor 4 (TLR 4), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells).

Figure 4

Renoprotective effect of valsartan on renal protein expression (ELISA; a: angiotensin II ”pg/ml protein“, b: TGF-β “ng/ml protein”, c: collagen IV “ng/ml protein”) in STZ- induced diabetic nephropathy. Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ¶,*,#, $

P< 0.05; ¶ compared with vehicle group,* compared with diabetic control group at the same time point,# compared with diabetic control group at week 4, $ compared with diabetic control group at week 8.

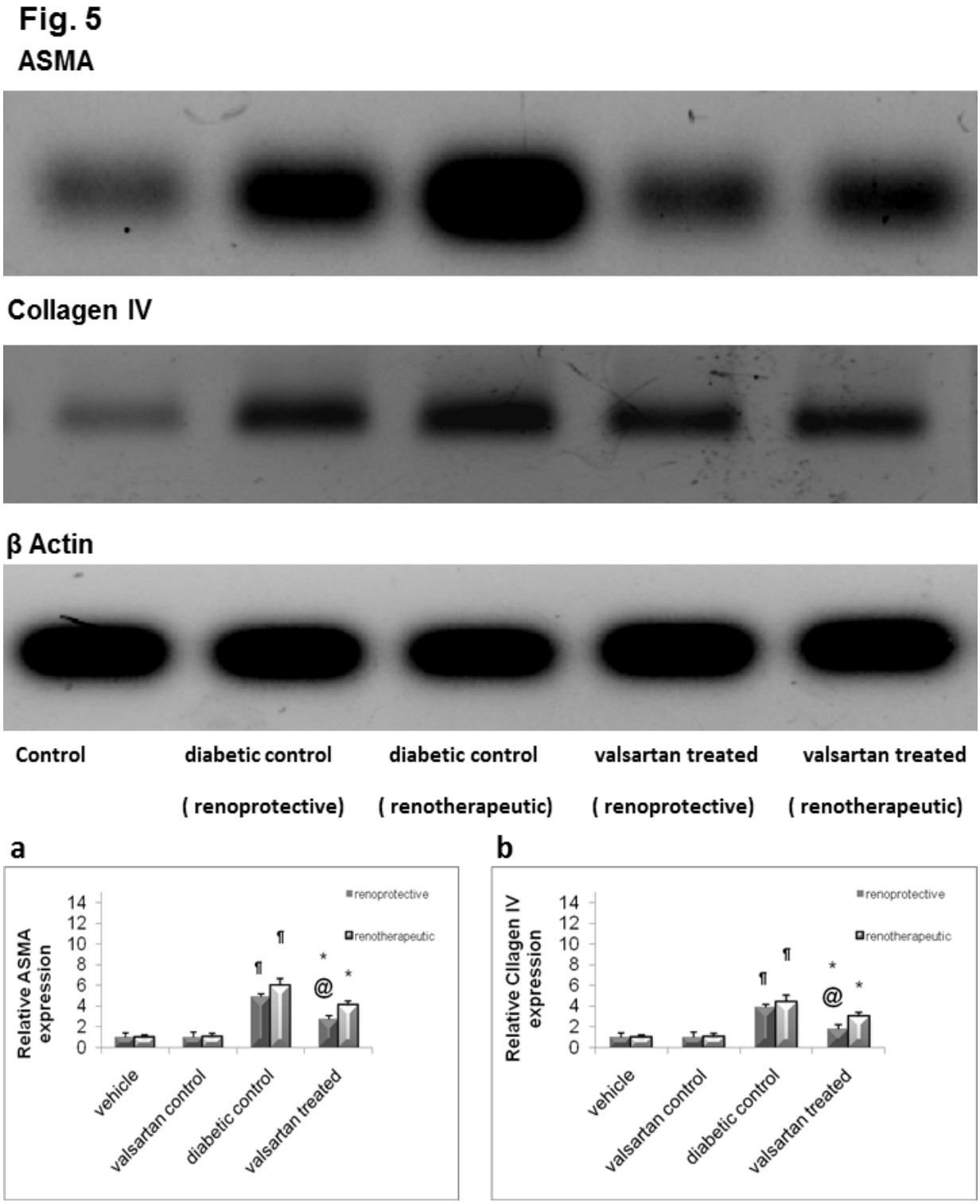

Figure 5

Renoprotective and renotherapeutic effect of valsartan on the relative renal expression of (western blot; a: ASMA, b: Collagen IV) in STZ- induced diabetic nephropathy. Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ¶,*, @ P< 0.05; ¶ compared with vehicle

group, * compared with diabetic control group at the same time point, @ compared with valsartan treated group “renotherapeutic”. Alpha smooth muscle actin (ASMA).

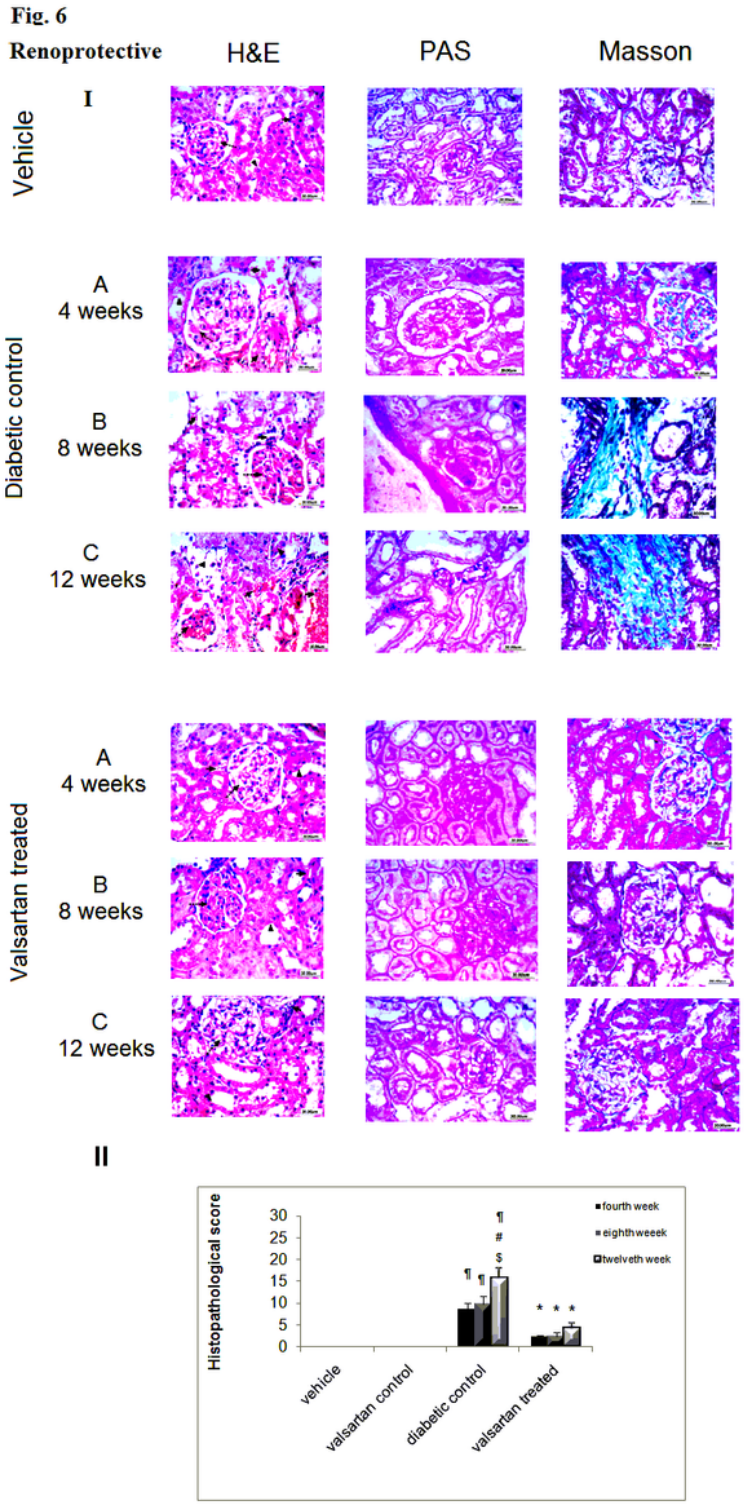

Figure 6

(I): Photomicrographs of renal tissue using renoprotective regime. Vehicle group showed normal appearance in hematoxylin–eosin (H&E, left); periodic acid Schiff (PAS, middle); Masson (right), glomeruli (dashed arrow), tubules (arrowhead), intesrtitium (arrow). Diabetic control group after four weeks (A) H&E

showed marked congested thick-walled vessels, PAS showed thickening of basement membrane. At eight weeks (B) H&E showed moderate glomerular congestion, PAS showed glomerular sclerosis. At twelve weeks (C) PAS shows markedly glomeruli with pronounced focal sclerosis affecting ≥75% of surface. The renoprotective effect of valsartan after 4, 8, 12 weeks showed restored brush border improving fibrosis and minimal glomerular sclerosis (H&E, PAS, Masson 400 ×). (II): Renoprotective effect of valsartan on the renal histopathological score in STZ- induced diabetic nephropathy. Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ¶,*,#, $ P< 0.05; ¶ compared with vehicle group, * compared with diabetic control group at the same time point,# compared with diabetic control group at week 4, $ compared with diabetic control group at week 8.

Fig. 7
Renotherapeutic

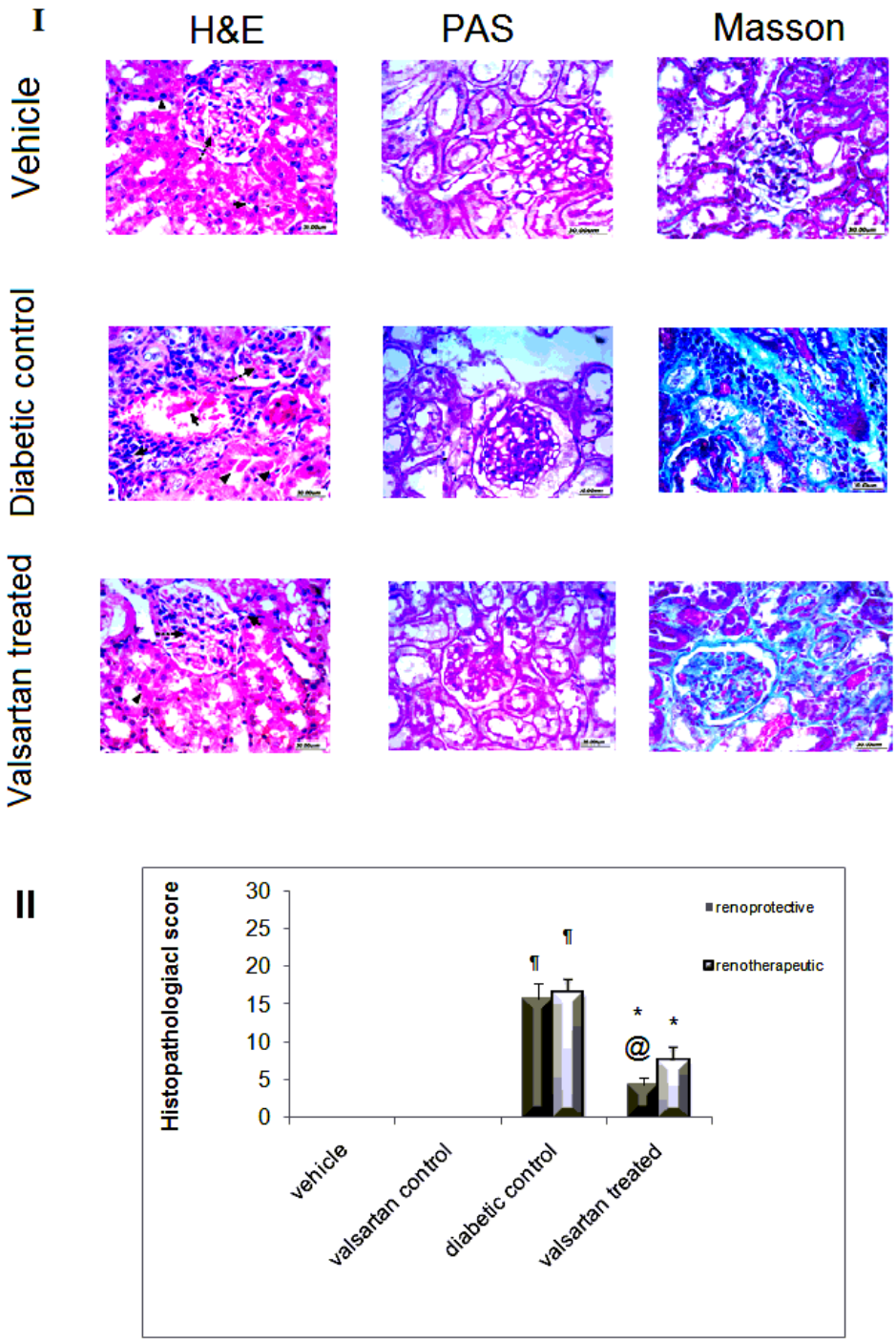

Figure 7

(I): Photomicrographs comparing renoprotective and renotherepeutic valsartan effect, in which vehicle group showed normal architecture. Renal tissues in the diabetic control group showed congested thick-walled, tubular epithelial cells showed desquamation, glomeruli focal sclerosis affecting ≥75% of surface. Masson showed marked peritubular fibrosis. PAS showed loss of brush border and glomerular sclerosis. Valsartan treated group showed moderate decrease of fibrosis, vascular changes and the

glomeruli sclerosis. PAS showed residual focal brush border loss with minimal vacuolation. Masson showed improved fibrosis(H&E, PAS, Masson 400 ×). (II): Renotherapeutic effect of valsartan on the renal histopathological score in STZ- induced diabetic nephropathy. Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ¶,*, @ P< 0.05; ¶ compared with vehicle group,* compared with diabetic control group, @ compared with valsartan treated group “renotherapeutic”.

Fig. 8

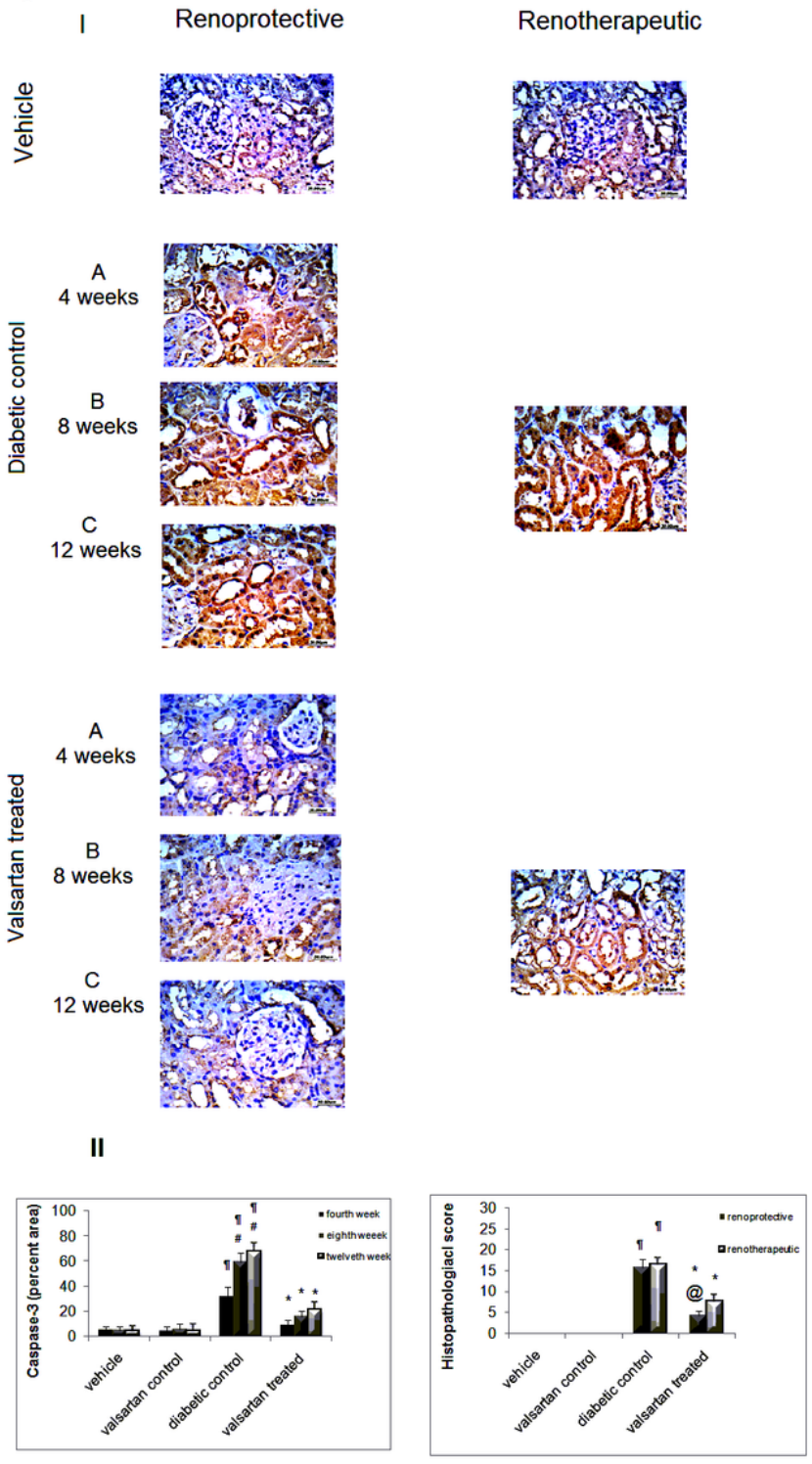

Figure 8

(I): Photomicrographs sections in renal tissue represent the intensity of caspase-3 protein expression in different groups in both renoprotective and renptherepeutic regimes (400 ×). (II): Renoprotective and renotherapeutic effect of valsartan on the renal expression percentage of the caspase-3 protein in STZ-induced diabetic nephropathy. Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ¶,*,#, @ P< 0.05; ¶ compared with vehicle group, * compared with diabetic control group at the same time point,# compared with diabetic control group at week 4, @ compared with valsartan treated group “renotherapeutic”.

Supplementary Files

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- Supplementary.docx