

## Role of Angiotensin II and Renal Nerves on TGF- $\beta$ /Smad Pathway in Diabetic Nephropathy Rat Model

EMAN KOLIEB, M.D.; FATEN A. MOHAMMAD, M.D.; SHIMAA MOHAMMAD YOUSEF, M.D. and SALAH EL DIN KASSAB, M.D.

The Department of Physiology, Faculty of Medicine, Suez Canal University, Ismaillia, Egypt

### Abstract

**Background:** Renal nerves and Renin-Angiotensin Aldosterone System (RAAS) are involved in the early renal pathological changes occurring during the development of Diabetic Nephropathy (DN). Bilateral renal nerve denervation (BRD) and angiotensin II (AngII) blockade have renoprotective effects by retarding the progression of renal fibrosis through Transforming growth factor  $\beta$  1 (TGF- $\beta$  1) signaling. This study aimed to compare the role of AngII and renal nerves on TGF- $\beta$ /Smad pathway in diabetic nephropathy rat model.

**Material and Methods:** Eighty male Sprague-Dawley rats divided into 4 groups were used: Group I (normal control group), group II (untreated diabetic nephropathy group), group III (diabetic nephropathy treated by valsartan), group IV (diabetic nephropathy treated by renal denervation). Rats were assessed by measuring Blood Pressure (BP), blood glucose, body and kidney weights, fluid and food intake and urine volume/24h, renal function tests, mRNA expression and activity of TGF- $\beta$  1 and Smad3 and histopathological changes in all groups at the end of fourth week of valsartan administration and BRD operation.

**Results:** Valsartan and BRD treatment significantly improved renal function and reduced ABP, blood glucose level, histopathological score and TGF- $\beta$  1 and Smad3 and their mRNA expression with significant difference between them and the control and untreated groups.

**Conclusion:** Valsartan appeared to alleviate DN by suppressing TGF- $\beta$  1 and p-Smad3. This also occurs in case of renal denervation but to a lesser degree.

**Key Words:** Bilateral renal denervation – Diabetic nephropathy – Transforming growth factor  $\beta$ 1 and smad-3 – Valsartan.

### Introduction

**DIABETIC** Nephropathy (DN) is a lethal diabetic complication and account for approximately one-third of all cases of end-stage renal disease and it is expected to rise rapidly as a result of the growing incidence of diabetes and the aging population [1].

**Correspondence to:** Dr. Faten A. Mohammad, E-Mail: [drfaphysiology@gmail.com](mailto:drfaphysiology@gmail.com).

DN is manifested by albuminuria, basement membrane thickening, increase in mesangial matrix, and extracellular matrix accumulation, followed by development of glomerulosclerosis and tubulointerstitial fibrosis, and finally leading to irreversible renal damage [2]. The exact etiology of DN is unclear, but scientists have postulated some mechanisms to explain its pathogenesis as hyperglycemia, activation of cytokines and various growth factors and Renin-Angiotensin Aldosterone System (RAAS). Abnormalities in the signaling pathway may interact with the previous factors to produce DN [3,4].

In rat models of diabetes, bilateral renal nerve denervation (BRD) was found to abolish glomerular hyperfiltration and prevent glomerular hypertrophy at 14 days after diabetic onset. These findings suggested that renal nerves are involved in the early renal pathological changes occurring during the development of DN and the lack of sympathetic stimulation retards the progression of glomerulosclerosis [5,6].

Also, angiotensin II (AngII) blockade has been shown to significantly improve or reverse fibrosis in the skeletal muscle, heart, kidney, liver, and lung [7]. The fibrogenic effects of AngII have been linked to its activation of Transforming growth factor  $\beta$  1 (TGF- $\beta$ 1) signaling. Inhibition of the RAAS using an ACE inhibitor or an AT 1 receptor antagonist has been reported to be associated with reduced TGF- $\beta$ 1 expression in a number of renal disease models, including diabetes [8].

TGF- $\beta$ 1 is multifunctional cytokine critical to development and wound healing. It is known as a key mediator of sclerosing process in diseased glomeruli and is related to glomerulosclerosis and interstitial fibrosis in various renal diseases [9,10].

Following activation, TGF- $\beta$ 1 binds to TGF $\beta$ 1 type II receptor (T $\beta$ RII), which Transphosphorylates the TGF- $\beta$ 1 type I receptor (T $\beta$ RI), then active T $\beta$ RI in turn activates the intracellular Smad signaling cascade by phosphorylating Smad2 and Smad3 [11]. Smad2/3 form heteromeric complexes with Smad4 and translocate into the nucleus to regulate transcription of target genes which code for extracellular matrix proteins like type I collagen and fibronectin, leading to the accumulation of matrix which accumulate in podocytes resulting in glomerulosclerosis and interstitial fibrosis [12].

Up to our knowledge no previous studies have compared the effects of angiotensin II and renal denervation on this pathway. The aim of this study is to examine the underlying mechanisms of hyperglycemia associated renal changes and to understand the relationship between RAAS and sympathetic renal nerves and the TGF- $\beta$ /SMAD signaling pathway in DN by comparing the effect of Ang II receptor blocker and bilateral renal denervation as a therapeutic potential for DN for its application in clinical therapy.

### Material and Methods

This study is a comparative experimental design and was carried out in the Physiology and Biochemistry Departments, Faculty of Medicine, Suez Canal University (2014-2017).

#### Animals:

Eighty male Sprague-Dawley rats with average weight of 250-350g and normal random blood glucose level were included in the study. Animals were purchased from the ophthalmology research institute (Giza, Egypt); they were housed in plastic cages with 12:12h light-dark cycle and were fed with normal commercial rat chow and tap water. Animal care before and during the experimental procedures was done according to the guidelines of the Animal Ethics Committee, Faculty of Medicine, Suez Canal University and the study was approved by the Research Ethics Committee (protocol no 2586).

#### Study groups:

Rats were randomly divided into 4 groups (n=20/group); group I (GI normal control group), group II (GII untreated diabetic nephropathy group), group III (GIII diabetic nephropathy treated by Ang II receptor blocker, Valsartan (sigma Aldrich, Cat no: 137862-53-4): Rats have received intragastric valsartan at a dose of 30mg/kg per day for 2 weeks after diabetes induction and continued for 4 weeks [13], and group IV (GIV diabetic

treated by renal denervation group): Rats in this group had undergone renal denervation operation after 2 weeks of diabetes induction.

#### Induction of diabetes:

Animals were acclimatized for 1 week, then fasted overnight and groups II, III, IV rendered diabetic by a single intraperitoneal injection of STZ (Sigma Chemical Co) in a dose of 60mg/kg dissolved in citrate buffer (10ml distilled water + 100mg citric acid + 180mg sodium citrate at pH=4.5) [14]. Non-diabetic rats (G=I) were injected only with citrate buffer. Three days following the STZ injection, non-fasting blood glucose was measured in blood samples from tail veins by blood glucose monitoring device (Kmeter and blood glucose test strips, Rbiotech, OK Biotech Co., Taiwan); rats with a blood glucose level of  $\geq 250$  mg/dl were confirmed as "diabetic," and were included in the study [15].

#### Renal denervation:

Rats in G IV were subjected to renal denervation at the second week after diabetes induction. The rats were anaesthetized with sodium pentobarbital (40mg/kg, i.p.i). Midline abdominal incision was done then both kidneys were exposed and the renal arteries and veins were isolated from connective tissue and renal fats and mechanical denervation was performed by carefully stripping all visible nerves, at 16 X magnification, along the renal arteries and veins from the aorta to the hilum of the kidney. Chemical denervation was performed by quickly painting the renal artery with 10% phenol in absolute ethanol for 2 minutes. Then, the artery was washed with isotonic saline. Sham denervation was done for the control and untreated diabetic group using identical procedures with renal nerves left intact [16].

#### Confirmation of successful BRD:

Renal cortical norepinephrine level was quantified at the end of the study using a commercial norepinephrine ELISA kit (BA E-5200, Labor Diagnostika Nord) according to the manufacturer's instructions. Completeness of denervation was established if the norepinephrine tissue content is  $< 10\%$  of the mean value in the control group [16].

*All groups were followed for 4 weeks and at the end of fourth week we measured the following tests:*

Body weight, blood glucose and blood pressure measurement:

Body weight, blood glucose and non-invasive rat tail blood pressure were monitored weekly

using the electronic animal weight scale, glucometer (Kmeter) and Biopac mp150 data acquisition system respectively. The average of each parameter was determined for each group at the end of fourth week.

*Urine and blood collection and renal function tests:*

All rats were placed in metabolic cage experiments (Tecniplast, Hohenpeissenberg, Germany) for 24 hours to measure urine volume and the food and fluid intake at the end of fourth week. Urine and blood samples were collected to measure urine creatinine concentration, urine albumin/urine creatinine (A/C) ratio, plasma creatinine and urea by an automatic biochemistry analyzer (Hitachi, Japan).

After 4 weeks of treatment, rats were sacrificed after anesthetizing with pentobarbital (40mg/kg, 1% concentration). Rt. Kidney weighed by sensitive weighing scale and its tissues were collected, excised and frozen instantly in liquid nitrogen for the PCR and ELISA examinations:

*TGF- $\beta$ 1 and Smad-3 mRNA expression:*

TGF- $\beta$ 1 and Smad-3 mRNA expression was measured by real time PCR [QuantiTect Reverse Transcription Qiagen kit, Primers for TGF- $\beta$ 1 (Cat no: QT00187320) and for Smad-3 (Cat no: QT00184961) and SYBR Green Master Mix] using RNeasyMini Kit (by Purification of Total RNA from (kidney tissue) according to manufacturer's instructions. The relative expression levels of genes were analyzed using the  $2^{-\Delta\Delta Ct}$  method by normalizing with  $\beta$ -actin house-keeping gene expression, and presented in fold increase relative to the control group [17].

*TGF- $\beta$ 1/Smad 3 signaling pathway activity:*

Quantitative measurement of phosphorylated Smad3 and TGF- $\beta$ 1 in protein homogenate of kidney tissue was done by ELISA kits [USCN, United Kingdom). The principle in this kit is Sandwich enzyme immunoassay done according to manufacturer's instruction.

*Renal histopathological examination:*

Assessment of glomerular damage was done blindly. Lf. kidney was fixed in 10% formaldehyde for 24 hours after perfusion with Hanks' buffer to eliminate the intravascular blood content then embedded in paraffin, and sectioned at 4 $\mu$ m thicknesses [15]. Renal sections were stained with Hematoxylin and Eosin (H & E) and Masson Trichrom staining. All images captured using calibrated standard digital microscope camera (Tucsen

ISH1000 digital microscope camera) using Olympus® CX21 microscope, with resolution of 10 MP (megapixels) (3656 X 2740 pixel each image). Collagen volume fraction was determined by the area stained blue in each field and expressed as a percentage of the total area within the field. About 10 to 15 fields were analyzed for glomerular fibrosis determination. Evaluation of renal glomerular and tubular damage was scored by a semi quantitative method [17]. The degree of renal tubular damage was scored as Follows: 0, no lesion, 1+, very mild focal dilatation, 2+, large number of dilated tubules with widening of the interstitium, 3+, fairly extensive dilatation of tubules with cystic formation and/or protein cast and widening of the interstitium, and 4+, complete atrophy of the tubules. Each animal was given a score (0 to 4+) and the individual scores were averaged for each group. Glomerular damage was scored from 0 to 4+; 0 no sclerosis, 1+ 01-25% mesangial expansion and sclerosing glomerulus, 2+ 25-50% mesangial expansion and sclerosing glomerulus, 3+ 50-75% mesangial expansion and sclerosing glomerulus, 4+ 75-100% mesangial expansion and sclerosing glomerulus [18].

*Statistical analysis:*

Data were analyzed using the statistical software package SPSS version 20 for Windows® (SPSS Inc., Chicago, IL, USA) and all data were presented as means  $\pm$  standard deviation. Analysis of variance (ANOVA) for data was used to elucidate differences between all groups. Post hoc range test (Bonferoni's) was used to test the difference between each pair of means. The level of significance was at ( $p < 0.05$ ).

## Results

*Confirmation of renal denervation:*

There was reduction in diabetic cortical norepinephrine content from (35.09 $\pm$ 4.1) in GII to (1.8 $\pm$ 0.4ng/mg) in GIV (renal denervation) ( $p=0.001$ ).

*Body and kidney weights:*

There was no statistically significant difference in body weight between groups ( $p=0.13$ ). There was statistically significant difference in kidney weight between groups ( $p=0.000$ ) with highest kidney weight at GII which was significantly different from GI ( $p=0.001$ ). GIII showed better improvement when compared to GIV ( $p=0.038$ ) (Table 1).

*Fluid and food intake:*

There was significant difference between normal group and all diabetic groups, with highest

fluid and food intake in GII when compared to normal group GI ( $p=0.001$  and  $p<0.05$  respective-

ly). GIII showed significant lower value than GIV ( $p<0.05$  for both) (Table 1).

Table (1): Body and kidney weight and fluid and food intake in all groups.

Variables	GI	GII	GIII	GIV	<i>p</i> -value
Body weight (g)	278.4±70.2	248.05±54.61	266.75±44.79	223±43.29	0.13
Kidney weight (g)	0.49±0.11	1.03±0.12a	0.84±0.17ab	0.97±0.14abc	0.000*
Fluid intake (ml/24hr)	38±4	221±14.5a	66.8±5.6ab	200.13±15.6abc	0.001*
Food intake (g/24hr)	32±4	50.6±2a	39.2±3.4ab	44.8±3.5abc	0.001*

G1: Negative control.

G2: Diabetic nephropathy group.

G3: Diabetic nephropathy + valsartan group.

G4: Diabetic nephropathy with bilateral renal denervation group.

Results are expressed as mean  $\pm$  SD and analyzed using one-way ANOVA followed by Bonferroni' spost-hoc test at  $p<0.05$ .

05. \*: Represents a statistically significant difference between all groups.

a, b and c: Represents a statistically significant difference when compared to G1, GII and GIII respectively.

#### The blood glucose level:

There was highly statistically significant difference in blood glucose level between all groups at the end of the study ( $p=0.002$ ). GII showed highest blood glucose level and it was significantly different when compared to normal group GI ( $p=0.001$ ). GIII showed better improvement than GIV ( $p=0.017$ ) (Table 2).

#### The blood pressure:

Systolic and diastolic blood pressures were significantly higher in GII, III and IV than GI ( $p<0.01$  for both). Valsartan markedly lowered systolic and diastolic blood pressures than BRD group ( $p=0.000$  and  $p<0.02$  respectively) (Table 2).

Table (2): Systolic blood pressure (SBP), diastolic blood pressure (DBP) and blood glucose level in all groups.

Variable	GI	GII	GIII	GIV	<i>p</i> -value
SBP (mmHg)	119.57±17.06	175.0±23.90a	125.5±11.9ab	139.0±14.28abc	0.001*
DBP (mmHg)	77.41±14.2	102.9±20.2a	83.61±11.25ab	84.80±5.09abc	0.001*
Blood glucose (mg/dl)	99.25±17.29	382.15±83.22a	209.30±51.27ab	289.35±130.6abc	0.000*

G1: Negative control.

G2: Diabetic nephropathy group.

G3: Diabetic nephropathy + valsartan group.

G4: Diabetic nephropathy with bilateral renal denervation group.

Results are expressed as mean  $\pm$  SD and analyzed using one-way ANOVA followed by Bonferroni' spost-hoc test at  $p<0.05$ .

05. \*: Represents a statistically significant difference between all groups.

a, b and c: Represents a statistically significant difference when compared to G1, GII and GIII respectively.

#### Renal function tests:

Table (3) and Fig. (1) showed renal function tests, rats subjected to DN (GII) showed marked deterioration when compared to the control group (GI) not subjected to the diabetic nephropathy. Regarding serum urea level; GII showed marked elevation in serum urea levels when compared to normal GI ( $p=0.001$ ), there was marked improvement in GIII than GIV ( $p=0.003$ ), urinary creatinine level: GII showed marked reduction in urinary creatinine when compared to GI ( $p=0.000$ ). Meanwhile GIII showed better improvement than GIV ( $p=0.006$ ). Microalbuminuria level: GII showed marked elevation in microalbuminuria when compared to GI ( $p=0.001$ ). Meanwhile GIII (7.59±5.34) showed better improvement than GIV (14.23±6.36) with non-significant difference ( $p=1$ ).

A/C ratio: GII showed marked elevation in A/C ratio when compared to GI ( $p=0.001$ ). Meanwhile GIII (12.27±10.34) also showed better improvement than GIV (44.4±0.93) with significant difference ( $p=0.000$ ). Serum creatinine: GII showed significant increase in creatinine level when compared to GI ( $p=0.02$ ). GIII showed better improvement than GIV with insignificant difference ( $p=0.6$ ). Urine volume/24hrs: GII showed significant higher urine volume compared to GI ( $p=0.000$ ). GIII had significant decrease in urine volume compared to GIV ( $p=0.000$ ). Creatinine clearance: GII showed significant decrease in creatinine clearance when compared to GI ( $p=0.000$ ). Meanwhile GIII also showed better improvement than GIV with non-significant difference ( $p=0.05$ ).

Table (3): Renal function tests in all groups.

Variable	Groups	GI	GII	GIII	GIV	p-value
Serum urea mg/dl		17.89±6.74	81.86±47.4a	25.4±8.8ab	56.18±21.55abc	0.000*
Urinary creatinine mg/dl		120.97±56.98	32.52±42.72a	76.49±36.54ab	35.98±15.08abc	0.000*
Serum creatinine mg/dl		0.496±0.136	2.53±3.38a	0.52±0.16ab	1.86±2.51 ab	0.006*
Creatinine clearance ml/min		1.06±0.58	0.17±0.12a	0.77±0.42ab	0.37±0.57ab	0.000*
Microalbuminuria mg/dl		1.07±0.57	24±30.6a	7.59±5.34ab	14.23±6.36ab	0.000*
A/C ratio		0.93±0.52	75.1±17.88a	12.27±10.34ab	44.4±0.93 abc	0.000*
Urine volume ml/24hr		6.31±3.09	13.93±1.20a	7.10±1.36ab	9.99±2.25abc	0.000*

G1: Negative control.

G2: Diabetic nephropathy group.

G3: Diabetic nephropathy + valsartan group.

G4: Diabetic nephropathy with bilateral renal denervation group.

Results are expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni' spost-hoc test at  $p < 0.05$ .

\*: Represents a statistically significant difference between all groups.

a, b and c: Represents a statistically significant difference when compared to G1, GII and GIII respectively.

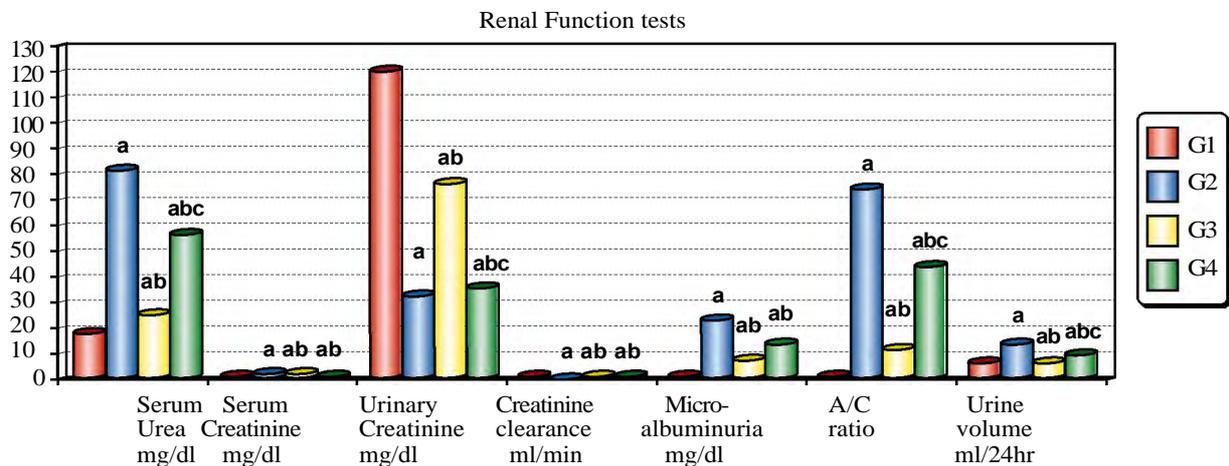


Fig. (1): Renal function results in all groups.

**TGF-β1 and Smad3 mRNA expression:**

TGF-β 1 and Smad3 mRNA expression in GII were significantly increased by 73 folds and 42 respectively when compared with the GI group

( $p=0.005$ ). GIII and GIV showed marked decrease in the expression level for both when compared to GII ( $p=0.000$ ). GIII had insignificant lower expression level than GIV (Table 4) & Fig. (2).

Table (4): TGF-β1 and Smad3 measurement by PCR and ELIZA.

Variable	Groups	GI	GII	GIII	GIV	p-value
PCR-TGF-β1		1±0	73.51±111a	13.51±26.05ab	19.14±24.22ab	0.007*
PCR-Smad3		1±0	42.43±29.94a	10.14±18.55ab	21.98±13.66ab	0.001*
ELISA-TGF-β1		19.20±5.50	93.70±48.90a	31.52±9.92ab	71.21±110.50ab	0.001*
ELISA-Smad3		0.45±0.33	0.94±0.59a	0.36±0.25ab	1.51±2.11 abc	0.001*

G1: Negative control.

G2: Diabetic nephropathy group.

G3: Diabetic nephropathy + valsartan group.

G4: Diabetic nephropathy with bilateral renal denervation group.

Results are expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni' spost-hoc test at  $p < 0.05$ .

\*: Represents a statistically significant difference between all groups.

a, b and c: Represents a statistically significant difference when compared to G1, GII and GIII respectively.

**TGF-β1 and Smad3 proteins:**

Table (4) showed that GII had marked elevation in TGF-β1 expression than GI ( $p=0.002$ ). There was significant reduction in TGF-β 1 expression in GIII than GII ( $p=0.011$ ), and GIII was better improved than GIV but not significant ( $p=0.2$ ).

Meanwhile in Smad3, there was significant difference between groups ( $p=0.001$ ). There was significant difference between GI and GII ( $p=0.01$ ). GIII showed marked improvement than GIV ( $p=0.008$ ) (Table 4).

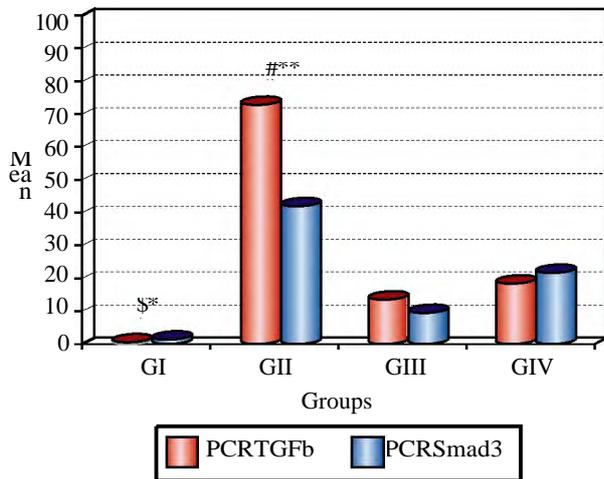


Fig. (2): TGFβ 1 and Smad3 expression by PCR.

**Histopathological results:**

Figs. (3,4) showed the histopathological results in all groups. GI had normal kidney morphology and zero score for tubular and glomerular damage. GII had scored 3+ and 4+ for tubular and glomerular damage respectively and interstitial fibrosis. GIII had scored 1+ for both type of damage without fibrosis. GIV had scored 2+ for both type of damage with minimal interstitial fibrosis. There was statistically significant difference between groups ( $p=0.001$ ) regarding the degree of tubule-glomerular damage and fibrosis.

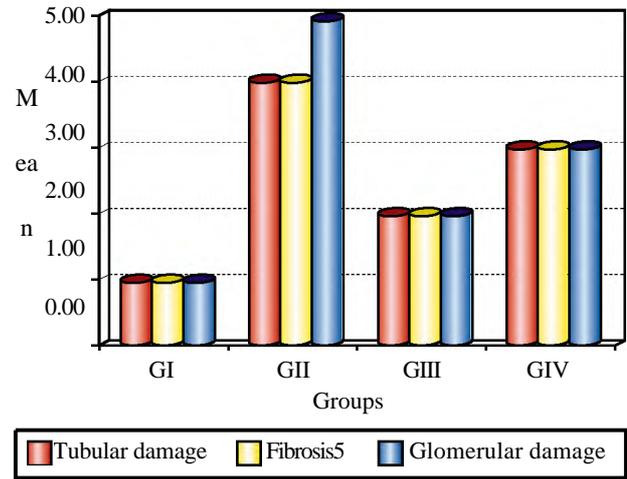


Fig. (3): Tubular, glomerular damage and degree of fibrosis.

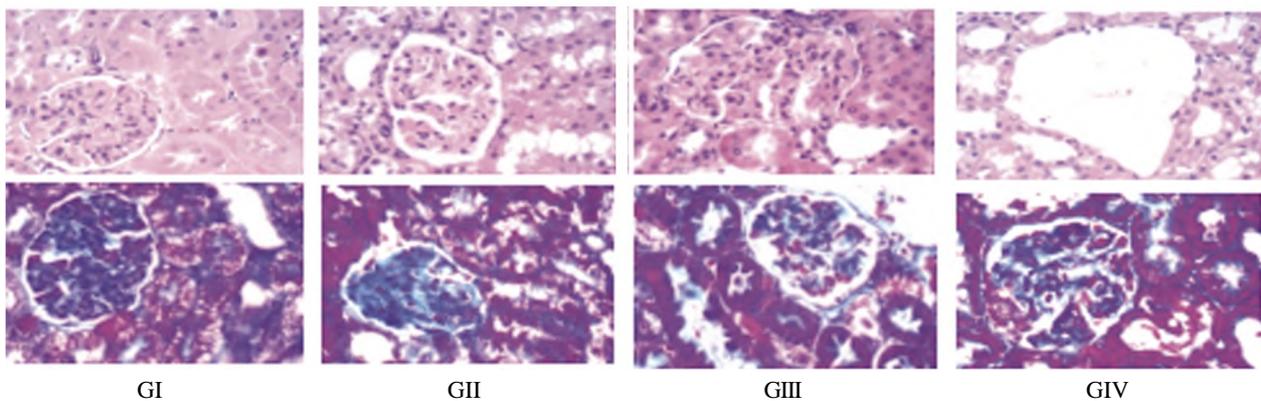


Fig. (4): Histopathological results in all groups. Hematoxylin and Eosin (H & E) and Masson Trichrom staining (X400). GI had normal kidney morphology; GII had extensive dilatation of the tubules and 75% mesangial expansion and sclerosing glomerulus and interstitial fibrosis; GIII had mild focal dilatation and 25% mesangial expansion and sclerosing glomerulus without fibrosis; GIV had large number of diluted tubules with wide interstitium and 25% mesangial expansion and sclerosing glomerulus with minimal interstitial fibrosis.

**Discussion**

The present study tried to answer the question of whether the functional contribution of angiotensin II and RSN (renal sympathetic nerves) in mediating increased renal vasculature resistance and renal fibrosis are achieved by the activation of TGFβ1/Smad-3 signaling pathway in Diabetes Mellitus

(DM). This issue was studied by evaluating the magnitude of changes in renal function tests and the level of expression of mRNA of TGF-β 1/ Smad3 by real-time PCR and then detecting their protein level by ELISA to determine how these responses were altered in the presence of angiotensin II type I receptor blocker AT1-receptor blocker (valsartan) and in bilateral renal

In the setting of diabetic rats, previous studies have emphasized that prominent elevations in blood glucose level, polyuria, polydipsia and massive reduction in the body weight are the most significant markers of the likely occurrence of DM [19]. The presence of these manifestations in this study obviously confirmed the accuracy of the experimental model of Streptozotocin (STZ)-induced DM.

In general, findings from the metabolic cage experiments in STZ-induced DN rats showed that elevated blood glucose level caused considerable increase in kidney weight, fluid and food intake, serum urea, plasma creatinine, marked increase in A/C ratio and marked albuminuria when compared to control group. This was also previously reported by Cao et al., [8].

The angiotensin receptor blocker (valsartan) is an effective medical prescription used clinically for the treatment of DN [17]. In the current study, valsartan for 4 weeks produced a mild hypoglycemic effect as compared to DN model group; this finding were in agreement with Top C et al., [20] who found improvement in insulin sensitivity followed by improvement in blood glucose with valsartan in hypertension model. But, this finding is incompatible with Hartnera et al., [17] who reported that blood glucose values were significantly higher in diabetic animals and even tended to be increased more prominently after irbesartan treatment. The different effects of various AT 1-receptor blockers on blood glucose level in the experimental model of DN may be due to their different pharmaceutical characteristics, such as duration of actions and lipophilicity. The lower lipophilicity of certain AT1-receptor blocker is likely to be an important determinant of tissue penetration, which would affect their efficiency to reduce local angiotensin II production. Also, Arnoni et al., [21] had reported that Losartan treatment initiated after diabetes induction did not alter the hyperglycemia level.

On the other hand, BRD had lowered blood glucose level with significant difference between the model group and BRD group; these findings were in agreement with Luippold et al., [14]. However, Yao et al., [16] did not document this reduction in blood glucose level. In this current study the valsartan significantly lowered blood glucose level than BRD.

Systolic blood pressure measurements confirmed hypertension in diabetic model group, and it was significantly improved with AT-1 receptor

blocker (valsartan) which was in agreement with Arnoni et al., [21] who reported that losartan had significantly lowered systolic and diastolic blood pressure even to levels below control group. On the other side, the study findings were incompatible with Hartnera et al., [17] where AT-1 receptor blocker treated group with irbesartan showed higher systolic blood pressure using tail cuff plethysmography but lower mean arterial blood pressure than model untreated group measured intra-arterially.

BRD group showed lower systolic and diastolic blood pressure, which was comparable with Yao et al., [16] in which BRD intervention in diabetic animals produced progressive, significant decrease in SBP compared with the sham-operated group and This supports the suggestion that renal nerves are functionally involved in intrarenal hemodynamic abnormalities observed in the early state of experimental DM; however this reduction in blood pressure was less than valsartan group.

These conflicting results when compared with the findings of the present study, in which a beneficial effect of renal denervation was proposed, might be caused by various study designs, the evaluation of different study parameters (albuminuria vs glomerular hyperfiltration), the application of insulin or, even more important, the problem of renal reinnervation. Functional reinnervation has been reported to begin 3 weeks post-denervation with a complete return of function by 8 weeks [22].

Another study explored the effect of bilateral renal denervation on DN in non-insulin dependent diabetes mellitus (NIDDM) of rat model that had showed significant decrease in microalbuminuria and improvement in renal function in rats with denervated kidneys than in sham operated controls and the glomerular matrix score, was reduced in rats 4 weeks after renal denervation, suggesting that the lack of sympathetic stimulation retards the progression of glomerulosclerosis in NIDDM rats [5]. Taken together, the role of renal nerves in the development of DN shows debate and has to be further investigated.

The results of the present study obtained evidence indicating that renal sympathetic nerve integrity in rat models of DN play a major contributor role in the deterioration of the renal hemodynamic and signaling pathway accompanying these disease states. These findings are compatible with Salman et al., [23] who documented that renal sympathetic excitation is involved in the pathogenesis of renal impairment accompanying DM, and may even precede the establishment of an observable renal

injury. However, these findings were incompatible with Matsuoka [24] who documented that renal denervation appears to exacerbate the progress of DN in STZ treated rats as judged through assessment of urinary albumin excretion. This suggests that the renal nerves may provide some protection against the progression of DN and improvement of renal function. Loss of this protective role may be one mechanism by which diabetic autonomic dysfunction may aggravate the progression of DN.

In the current study, diabetic model group was associated with an increase in the glomerulosclerosis score and in the degree of tubulo-interstitial injury when compared with non-diabetic control group. This evident increase in glomerulosclerosis and tubulointerstitial injury was reduced in rats treated with valsartan which is compatible with Hartnera et al., [17]. However, BRD group showed less improvement regarding histopathological score which was incompatible with Yao et al., [16] where in BRD kidneys, the occurrence of glomeruli with a thickened glomerular basement membrane and mesangial hypertrophy was reduced to normoglycemic levels. This could be attributed to the different method of denervation where BRD or sham surgeries were performed, repetitively, at 9, 12, and 15 weeks of age.

Regarding the renal expression of TGF- $\beta$ 1/Smad-3, there was significant increase in their expression in diabetic group when compared with control group, also there was significant reduction in their expression in valsartan treated group which is in accordance with Matsubara et al., [25] and Zhou et al., [26].

Surgical renal denervation produced significant drop in the expression of TGF- $\beta$  1/Smad-3 pathway. These findings indicated the effectiveness of this procedure in establishing a sufficient level of peripheral sympathoinhibition that can sufficiently improve the prognosis of diabetic renal disease, and protecting the kidney from future complications. This agreed with Yao et al., [16] where TGF $\beta$  1 was elevated in both the cortex and medulla of the diabetic kidneys compared with normoglycemic by Western blot analysis. This expression was reduced in the diabetic kidneys subjected to BRD.

This study is the first to compare the effects of valsartan administration and BRD as a treatment for DN by focusing on the TGF- $\beta$  1/Smad3 pathway, administration of valsartan may be appropriate for controlling blood glucose levels and body weight. It also can reverse changes in renal histopathology and attenuate albuminuria which could lead to

improvement in renal function. However, there was lesser improvement in these parameters in the BRD group.

By detecting Smad-3 mRNA expression by real time PCR, the results revealed that phosphorylated Smad3ph-Smad-3 levels were elevated in diabetic kidneys compared to control group and that valsartan had lowered their level of expression better than BRD, which was in accordance with previously reported findings [27]. This suggests that valsartan may play a role in the down-regulation of TGF- $\beta$ 1/Smad3 signaling pathway at the transcriptional level.

ELISA analysis confirmed that TGF- $\beta$ 1 and Smad-3 proteins were also upregulated in the model group; Smad3 is a critical mediator responsible for renal fibrosis that has been shown to function in the diabetes-induced up-regulation of fibronectin and collagen, and that may play a critical role in the early phase of DN. These findings are compatible with Wang et al., [28]. Inazaki et al., [29] also had documented that Smad3 deficient mice are protected from renal fibrosis by reduction in Epithelial Mesenchymal Transition (EMT), collagen deposition, and the expression of profibrotic TGF $\beta$  1 target genes.

As indicated by the study results, the expression of Smad3 was increased in the kidneys of diabetic rats. Furthermore, the fact that the administration of valsartan suppressed its expression, which resulted in improved renal conditions, provides further evidence for the effectiveness of valsartan in the treatment of DN [28].

#### *Conclusion:*

In conclusion, DN is caused by an imbalance in the TGF $\beta$ /Smad pathway, which in turn leads to glomerular sclerosis and interstitial fibrosis. Consistent with our theory, the valsartan injection appeared to alleviate DN by suppressing TGF- $\beta$  and ph-Smad3. This also occurs in case of renal denervation but to a lesser degree. Collectively, these results demonstrated that valsartan administration could be a better potential treatment for DN, and that it could ameliorate the outcomes associated with DN by hindering the TGF $\beta$ /Smad pathway. Further studies are needed to investigate how to improve the therapeutic effects of BRD.

#### *Acknowledgement:*

The authors wish to thank Histopathology Departments, Faculty of Medicine, Suez Canal Uni-

## References

- 1- SHUMWAY J.T. and GAMBERT S.R.: Diabetic nephropathy-pathophysiology and management. *International Urology and Nephrology*, 34 (2): 257-64, 2015.
- 2- WILKINSON BERKA J.L., GIBBS N.J., COOPER M.E., SKINNER S.L. and KELLY D.J.: Renoprotective and anti-hypertensive effects of combined valsartan and perindopril in progressive diabetic nephropathy in the transgenic (mRen<sub>2</sub>)<sup>27</sup> rat. *Oxford Journals Medicine & Health Nephrology Dialysis Transplantation*, 16 (7): 343-1349, 2001.
- 3- FORBES J.M., FUKAMI K. and COOPER M.E.: Diabetic nephropathy: Where hemodynamics meets metabolism. *Exp. Clin. Endocrinol. Diabetes*, Feb., 115 (2): 69-84, 2007.
- 4- NI W.J., TANG L.Q. and WEI W.: Research progress in signalling pathway in diabetic nephropathy. *Diabetes Metab. Res. Rev.*, 31: 221-33, 2015.
- 5- SUGAWARA H., IZUMINO K., FUTAMURA A., NAKAGAWA Y., TAKATA M. and INOUE H.: Renal denervation retards glomerular sclerosis in non-insulin dependent diabetes (NIDDM) rats. *J. Am. Soc. Nephrol.*, 9: 642A, 1998.
- 6- MAHFOUD F., SCHLAICH M., KINDERMANN I., UKENA C., CREMERS B., BRANDT M.C., HOPPE U.C., VONEND O., RUMP L.C., SOBOTKA P.A., KRUM H., ESLER M. and BÖHM M.: Effect of renal sympathetic denervation on glucose metabolism in patients with resistant hypertension. *Circulation*, 123: 1940-6, 2011.
- 7- ELBAZ M., YANAY N., AGA-MIZRACHI S., BRUNSCHWIG Z., KASSIS I. and ETTINGER K.: Losartan, a therapeutic candidate in congenital muscular dystrophy: Studies in the dy (2J)/dy (2J) mouse. *Ann. Neurol.*, 71: 699-708, 2012.
- 8- CAO Z., BONNET F., DAVIS B., ALLEN T.J. and COOPER M.E.: Additive hypotensive and anti-albuminuric effects of angiotensin-converting enzyme inhibition and angiotensin receptor antagonism in diabetic spontaneously hypertensive rats. *Clinical Science*, 100: 591-9, 2001.
- 9- AUGUST P. and SUTHANTHIRAN M.: Transforming growth factor  $\beta$  signaling, vascular remodeling, and hypertension. *N. Engl. J. Med.*, 354: 2721-3, 2006.
- 10- DOBACZEWSKI M., CHEN W. and FRANGOGIANNIS N.: Transforming growth factor (TGF)- $\beta$  signaling in cardiac remodeling. *J. Mol. Cell. Cardiol.*, 51: 600-6, 2011.
- 11- MASSAGUÉ J.: How cells read TGF- $\beta$  signals. *Nat. Rev. Mol. Cell Biol.*, 1: 169-78, 2000.
- 12- CHENG X., GAO W., DANG Y., LIU X., LI Y., PENG X. and YE X.: Both ERK/MAPK and TGF- $\beta$ /Smad signaling pathways play a role in the kidney fibrosis of diabetic mice accelerated by blood glucose fluctuation. *Journal of Diabetes Research*. Vol. 2013, Article ID 463740, 2013.
- 13- ZHANG K., MENG X., DONGMEI LI, YANG J., KONG J., HAOP., GUO T., ZHANG M., ZHANG Y. and ZHANG C.: Angiotensin (1-7) attenuates the progression of streptozotocin-induced diabetic renal injury better than angiotensin receptor blockade. *Kidney International*, 87: 359-69, 2015.
- 14- LUIPPOLD G., BEILHARZ M. and MÜHLBAUER B.: Chronic renal denervation prevents glomerular hyperfiltration in diabetic rats. *Nephrol. Dial. Transplant.*, 19: 342-7, 2004.
- 15- GAO C., AQIE K., ZHU J., CHEN G., XU L., JIANG L. and XU Y.: MG132 ameliorates kidney lesions by inhibiting the degradation of Smad7 in streptozotocin-induced diabetic nephropathy. *Journal of Diabetes Research*. Vol. 2014, Article ID 918396, 8 pages, 2014.
- 16- YAO Y., FOMISON-NURSE I.C., HARRISON J.C., WALKER R.J., DAVIS G. and SAMMUT I.A.: Chronic bilateral renal denervation attenuates renal injury in a transgenic rat model of diabetic nephropathy. *Am. J. Physiol. Renal. Physiol.*, 307: F251-F262, 2014.
- 17- HARTNERA A., CORDASICB N., KLANKEB B., MENENDEZ-CASTROA C., VEELKENB R., SCHMIEDERB R.E. and HILGERS K.F.: Renal protection by low dose irbesartan in diabetic nephropathy is paralleled by a reduction of inflammation, not of endoplasmic reticulum stress. *Biochim. Bio. Phys. Acta*, Pages 558-65, 2014.
- 18- CAO Z., COOPER M., WU L., COX A., JANDELEIT-DAHM K., KELLY D. and GILBERT R.: Blockade of the renin-angiotensin and endothelin systems on progressive renal injury. *Hypertension*, 36: 561-8, 2000.
- 19- CASEY R.G., JOYCE M., ROCHE-NAGLE G., CHEN G. and BOUCHER-HAYES D.: Pravastatin modulates early diabetic nephropathy in an experimental model of diabetic renal disease. *J. Surg. Res.*, 123: 176-81, 2005.
- 20- TOP C., CIMQOZBAY B.Y. and TAREKECHI H.: The effect of valsartan on blood glucose level in hypertension model. *J. Int. Res.*, 30 (1): 15-20, 2011.
- 21- ARNONI C.P., MAQUIGUSSA E., PASSOS C.S., PEREIRA L.G. and BOIM M.A.: Inhibition of cellular transdifferentiation by losartan minimizes but does not reverse type 2 diabetes-induced renal fibrosis. *Journal of the Renin-Angiotensin-Aldosterone System*, 16 (3): 469-80, 2015.
- 22- KLINE R.L. and MERCER P.F.: Functional reinnervation and development of supersensitivity to NE after renal denervation in rats. *Am. J. Physiol.*, 238: R353-R35, 1980.
- 23- SALMAN I.M., AMEER O.Z., SATTAR M.A. and ABDULLAH N.A.: Characterization of renal hemodynamic and structural alterations in rat models of renal impairment: Role of renal sympathoexcitation. *J. Nephrol.*, 24 (01): 68-77, 2011.
- 24- MATSUOKA H.: Protective role of renal nerves in the development of diabetic nephropathy. *Diabetes Res.*, 23: 19-29, 1993.
- 25- MATSUBARA T., ABE H., ARAI H., NAGAI K., MIMA A., KANAMORI H. and SUMI E.: Expression of Smad1 is directly associated with mesangial matrix expansion in rat diabetic nephropathy. *Laboratory Investigation*, 86: 357-68, 2006.
- 26- ZHOU G., CHEUNG A.K., LIU X. and HUANG Y.: Valsartan slows the progression of diabetic nephropathy in db/db mice via a reduction in podocyte injury, and renal oxidative stress and inflammation. *Clinical Science*, 126 (10): 707-20, 2014.
- 27- HONG S.W., ISONO M., CHEN S. and ZIYADEH F.N.:

- its type II receptor, and activation of the Smad signaling pathway in the db/db mouse. *Am. J. Pathol.*, 158: 1653-63, 2001.
- 28- WANG A., ZIYADEH F.N., LEE E.Y., PYAGAY P.E., SUNG S.H., SHEARDOWN S.A., LAPING N.J. and CHEN S.: Interference with TGF-beta signaling by Smad3-knockout in mice limits diabetic glomerulosclerosis without affecting albuminuria. *Am. J. Physiol. Renal. Physiol.*, 293: F1657-1665, 2007.
- 29- INAZAKI K., KANAMARU Y., KOJIMA Y., SUEY-OSHI N., OKUMURA K., KANEKO K., YAMASHIRO Y., OGAWA H. and NAKAO A.: Smad3 deficiency attenuates renal fibrosis, inflammation, and apoptosis after unilateral ureteral obstruction. *Kidney Int.*, 66: 597-604, 2004.

## دور أنجيوتنسين-2 والأعصاب الكلوية على مسار عامل تحويل النمو-بيتا/بروتين الإشارات الخلوية (سماد) في إعتلال الكلى السكري في الجرذان

مقدمة: يعتبر مرض السكري أحد أخطر الإضطرابات الأيضية والذي ينتج من إضطراب عمليات الأيض للكربوهيدرات والدهون والبروتين نتيجة نقص إفراز الأنسولين في الجسم أو خلل في وظيفته أو لكلا السببين > يمثل مرضى السكر حوالي ثلث مرضى الفشل الكلوي من المرحلة الأخيرة، والتي تعد من المضاعفات الخطيرة لمرض السكر التي تنتج بسبب تدمير الأوعية الدموية الصغيرة في الكلى حيث تكون غير قادرة على العمل وتؤدي إلى فشل كلوي. وقد لوحظ من التجارب على داء السكر في الجرذان أن إزالة العصب الكلوي يمنع توسيع الكبيبي الناجم عن داء السكر. كما تلعب الأعصاب الكلوية دوراً هاماً في السيطرة على النشاط العصبي السيمبثاوي (SNA) وتحسن أداء الكلى المريضة في المرضى الذين يعانون من الفشل الكلوي المزمن. يعتبر أنجيوتنسين-2 (AngII) وسيطاً فعالاً في الأوعية الدموية حيث يشارك في عمل نظاماً لرينين أنجيوتنسين (RAS) وأصبح من الواضح أن آثار (AngII) تتجاوز القلب والأوعية الدموية النظامية. إنحجب (RAS) بواسطة أيمن مثبط لإنزيم المحول للأنجيوتنسين (ACE) أو مستقبلات نوع 1 (AT1) anangiotensin 1 يؤخر تطور الإصابة الكلوية المرتبطة بداء السكر. وقد وجد إمكانية مساهمة TGF $\beta$ 1 في إعتلال الكلى السكر حيث وجد أن TGF $\beta$ 1 يزيد بارتفاع مستويات السكر، كما تظهر الآثار المرضية للأنجيوتنسين، من خلال مسارات التي تعتمد على TGF-b1 ولذلك فإن تثبيط RAS باستخدام مثبطات ACE أو مستقبلات AT1 يؤدي إلى إنخفاض التعبير عن TGF-b1 في عدد من النماذج مرض الكلى وبالتالي تقليل الضرر في الكلى والتليف.

الهدف من هذه الدراسة: هو دراسة دور كلا من الأعصاب الكلوية والأنجيوتنسين-2 في تطور إعتلال الكلى السكري من خلال حجب مستقبلات الأنجيوتنسين-2 وإزالة العصب الكلوي في نموذج الجرذان من إعتلال الكلى السكري ودراسة تأثير كلاهما على مسار والذي يعتبر أحد آليات حدوث الإصابات الكلوية في مرضى السكر.

الطرق: دراسة عشوائية مقارنة في معامل قسم الفسيولوجي والكيمياء الحيوية ووحدة التميز الخلوية بكلية الطب - جامعة قناة السويس في الفترة ما بين 2014-2017. تم تقسيم الجرذان عشوائياً إلى 4 مجموعات كل منها تحتوي على 20 جرذ: مجموعة (أ): الجرذان الطبيعية، مجموعة (ب): تحتوي الجرذان التي تم حقنها بمادة الستيروئيدوسين (35مجم/كجم) لعمل نموذج إعتلال الكلى السكري، مجموعة (ج): تم حقنها بواسطة مادة الستيروئيدوسين داخل الغشاء البريتوني ثم بعد إسبوعين تم علاجها بواسطة الفالسارتان (30مجم/كجم) تم إعطاؤه بواسطة الأنوية المعدية وأخيراً مجموعة (د): التي تم حقنها بواسطة مادة الستيروئيدوسين ثم عمل إزالة للعصب الكلوي، وقد تم متابعة المجموعات الأربعة لمدة 4 أسابيع بعد العلاج بواسطة الفالسارتان وإزالة العصب الكلوي. تم متابعة الجرذان لمدة 4 أسابيع وقياس وزن الجسم إسبوعياً ومعدل السكر في الدم، وكذلك قمنا بتجميع عينات البول في 24 ساعة عن طريق الأقفاس الأيضية وعينات الدم قبل ذبح الجرذان لعمل تحاليل لوظائف الكلى. تم ذبح الجرذان وعمل قياس كمي لنسبة (mRNA) لجين (TGF-b and Smad-3) عن طريق تقنية البيولوجيا الجزيئية (PCR).

النتائج: أظهرت نتائج تحاليل وظائف الكلى للجرذان وجود إرتفاع ملحوظ في مجموعة (ب) والذي تحسن بعد العلاج بواسطة الفالسارتان أكثر من تحسنه بعد إزالة العصب الكلوي. وقياس نسبة ظهور (mRNA of TGF-b & Smad-) فقد وجد إرتفاع ملحوظ في نسبة ظهورهم في نسيج الكلى للجرذان في مجموعة (ب) والذي قل بنسبة كبيرة في مجموعة (ج) عن مجموعة (د). تم قياس معدل ضغط الدم ووجد إرتفاعه في مجموعة (ب) (نموذج إعتلال الكلى السكري) والذي تحسن في مجموعة العلاج بواسطة الفالسارتان (ج) عن مجموعة إزالة العصب الكلوي (د)، وقد وجد علاقة طردية إيجابية بين معدل ضغط الدم ونسبة السكر في الدم. أظهرت دراسة نسيج الكلى بواسطة الهستوباثولوجي تغيرات واضحة في الشكل الطبيعي للكلى في مجموعة (ب) مثل زيادة عدد خلايا ميسانجيا وزيادة حجم أنابيب الكلى والتليف وإحتقان الأوعية الدموية، جميع هذه التغيرات تحسنت في مجموعة (ج) بشكل أوضح من مجموعة (د).

الإستنتاجات: يمكن أن يحدث إعتلال الكلى السكري بواسطة إختلال توازن مسار الإشارات الخلوية (TGF-b & Smad-3) والذي يؤدي في النهاية إلى تليف الكلى، وقد أظهرت الدراسة الحالية نتائج متوافقة مع فرضيتنا حيث أن الفالسارتان قد حسن إعتلال الكلى من خلال تثبيط هذا المسار بشكل أفضل من إزالة العصب الكلوي. إجمالاً فإن فالسارتان كمثل على حجب مستقبلات الأنجيوتنسين-2 يعتبر إختياراً علاجياً أفضل حيث أنه يحسن إعتلال الكلى السكري من خلال تثبيط مسار الإشارات الخلوية (TGF-b & Smad-3) بنسبة أفضل من إزالة العصب الكلوي.