Original Article

Possible renoprotective effect of valsartan/sacubitril versus valsartan and Metformin in rat model of diabetic nephropathy

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ABSTRACT

Diabetic nephropathy can be identified as diabetes mellitus's furthermost significant complications. Tne End-Stage Renal Disease is also a result of diabetic nephropathy. The first-line therapy for patients with diabetes accompanied by proteinuria is renin-angiotensin-aldosterone system inhibition. The possible renoprotective effect evaluation of valsartan/ sacubitril (ARNI) on diabetic nephropathy model induced in rats compared to valsartan, Metformin alone, or with their combination goal of this work was what this work aimed. Mature male rats are classified into 8 groups. Diabetes was instigated through once I.P streptozotocin injection. ARNI, valsartan, Metformin, along with their combination, was administered for 8 weeks starting from day 3 of the experiment. Blood pressure, biochemical parameters including serum levels of FBG, creatinine, Na^+, K^+, ANP and eGFR, urinary total protein excretion in 24 hours, Albumin – creatinine ratio, MDA content level in the kidney tissue, and histopathological examination were used to evaluate the effects of the tested drugs on model groups. Treatment with ARNI, valsartan, Metformin, as well as treatment with the combination of ARNI with Metformin and Metformin with valsartan, resulted in significant improvement of all measured parameters in the treated rats compared with untreated ones. (ARNI) has renoprotective effects nearly comparable to that produced by valsartan and Metformin so that it might be one of the new therapeutic targets for patients with diabetic nephropathy.

Keywords: Diabetic nephropathy, Sacubitril/valsartan, Streptozotocin, MDA, Ang II type 1 (AT1) receptor blocker

Introduction

Diabetic nephropathy (DN), which is categorized through hyperfiltration, hyperpermeability, albuminuria to macromolecules, and proteinuria resulting in end-stage kidney failure, can be described as the furthermost diabetes mellitus significant complications [1, 2].

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In type 1 diabetes (T1D), DN's main cause of mortality. The function of specific tissue abnormalities and organ impairment occurs early in T1D, while in the glomerulus, hyperglycemia is the main cause of abnormal homeostasis in vascular permeability and blood flow. The increased intra-capillary pressure and blood flow are assumed to reflect hyperglycemia-induced metabolic impairment as diminished NO, increased reactive oxygen species manufacture on the renal capillaries efferent side, and gradually an amplified sensitivity to angiotensin II [3]. DN's significant improvement is the chronic hyperglycemia-driven activation of the vasoactive hormonal pathways renin-angiotensin-aldosterone system (RAAS). The increased intra-glomerular pressure can be caused by the associated systemic hypertension [4]. Glomerular microvascular lesions develop secondary to increased vascular permeability and blood flow [5].

Subsequently, therapeutic regimens targeting RAAS blockage with angiotensin receptor blockers (ARBs) are advantageous in

stopping the DN advancement. Nonetheless, in delaying the DN progression, ACEI and ARBs are effective; but it stops the progression or cannot reverse diabetic nephropathy [6].

By increasing natriuretic peptide levels, leading to vasodilation and potent natriuresis in the kidney, neprilysin inhibitors (NI) induce beneficial effects; this vasodilatory effect reduces proteinuria intraglomerular pressure [7, 8]. However, these effects may be counteracted by ET-1 rise and simultaneous NI-induced angiotensin II. ARB [AT1] (angiotensin II type 1) receptor blockade could be related to NI (ARNI) to avoid this, and it was found that solitary RAS (Renin-Angiotensin System) blockade was less effective than ARNI (valsartan /sacubitril) in hypertension and heart failure [9]. Recently, it was concluded that in rats with subtotal nephrectomy, valsartan /sacubitril has the renoprotective effect, which was stronger than valsartan [10].

Metformin, the biguanide preparation, was widely used in diabetes mellitus treatment [11]. Metformin also reduces complications of diabetes by restoring blood glucose level, the body's response to insulin, and its antioxidant properties [2, 12].

The evaluation of the possible renoprotective effect of valsartan/ sacubitril (ARNI) on diabetic nephropathy model induced in rats compared to valsartan, Metformin alone, or with their combination was what this work aimed.

Materials and Methods

A 64 healthy matured male Wistar rats weighing 200-250 g matched for age and weight were used in the study. Animals were harbored on a12 hours light/dark cycle (lights on from 08:00 am). Normal rats chow together with H_2O obtainable ad libitum. Institutional Animal Care and Use Committee ratified the present research (IACUC) – Faculty of Medicine, Cairo University (approval No: CU/III/F/75/11)

Design of the experiment

The animals were arbitrarily assigned into 8 groups (8 rats each). The rats were grouped as follows: normal control group (I) normal rats injected once by 1 ml of saline intraperitoneally (I.P) at day one, and they received 1ml of distilled water daily (for 8 weeks starting from day3). Vehicle control group (II) injected once by comparable dimensions of 0.1 M Citrate Buffer I.P on day one, and they received1 ml of distilled water daily (for 8 weeks starting from day3). Streptozotocin treated Group (III) in which diabetes was induced by I.P injection of streptozotocin in a dose 60 mg/kg at day one-STZ was melted in 0.1 M citrate buffer (pH 4.5) immediately prior to use.

Group (IV) in which sacubitril/valsartan (ARNI) was administrated orally in the diabetic rats (30 mg/kg/day). Group (V) diabetic rats have been treated valsartan dosage of 15mg/kg/ day orally. Group (VI) diabetic rats were treated using an oral metformin dosage of 100 mg/kg/ day. Group (VII) ARNI + Metformin group in which the diabetic rats received sacubitril/valsartan and Metformin orally in the previously mentioned daily doses. Group (VIII) Valsartan + Metformin treated group in which the diabetic rats received valsartan and Metformin orally in the previously mentioned daily doses. The rats (IV to group VIII) were treated for 8 weeks starting from day 3.

The animals of all groups were observed for 8 weeks after induction of diabetes and assessed for diabetic nephropathy.

Measurements

Measurement of arterial blood pressure

The measuring of Systolic blood pressure was done through a tail-cuff plethysmograph (Harvard Apparatus Ltd, Edenbridge, Kent, England) on day 3 of the experiment (i.e.at, 72 hours from induction of diabetes) and at week 8 of the experiment.

Biochemical study

Blood analysis

- Venous blood samples (tail blood samples) were attained from whole animals at 72h after STZ injection (day 3), 5 th week, and 8 th week for estimating fasting blood –glucose (mg/dl).
- Serum samples were analyzed for the serum level measurement of creatinine, Sodium, Potassium, ANP along with eGFR at the beginning (Day 3) together with the end of the experiment (8th week).

Urine analysis

Animals were accommodated in special cages (metabolic cages) for urine collection 72h, 5 th week, and 8 th week after STZ injection to determine urinary total protein excretion in 24 hours and Albumin –creatinine ratio. Urine was collected plus put in storage at -20°C in dark containers for biochemical analysis.

-When the experiment was done, using cervical dislocation, animals were sacrificed, then the kidney specimens were obtained, washed with ice-cold saline, and divided into two portions for the following assessment:

Measurement of oxidative stress marker in renal tissue (tissue malondialdehyde (MDA)

The first portion of the kidney was immediately frozen at -80 oC then a single minor segment (200 mg) of the kidney was precisely weighed. Subsequently, saline was added based on the tissue weight: Saline volume=1:9 (w/v). The homogenates were centrifuged at the following homogenization at 4°C by a DY89-I electric homogenate

(Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) 1,100xg for 15 min at room temperature.

Histopathological study

The other portion of kidney specimens was fixed in formalin 10%.

I) Histopathological examination: It was done using:

Hematoxylin and eosin (H&E) stain: for histological evaluation [13].

Periodic acid schiff (PAS) reaction: it was used to examine the glomerular basement membrane's optical density and the proximal convoluted tubules [14].

II) Morphometric study

Using Leica Qwin 500 C image analyzer computer system, Ltd., Cambridge, England, Morphometric data were obtained. The image analyzer comprised of a hard disc of IBM personal computer, a colored monitor, a colored video camera connected to the microscope along with controlled through Leica Qwin 500 C software.

The image analyzer was first calibrated automatically to convert the measurement unit (pixels) produced by the image analyzer program into actual micrometer units. The slides were observed underneath a light microscope.

The optical density of the basement membrane of the glomerular basement membrane along with the proximal convoluted tubule brush border was measured in PAS stained sections. The interactive measurement was used to measure the height of cells lining the PCT by measuring the distance between the basement membrane and the top of the brush border of the cells lining the proximal convoluted tubules in H&E stained sections with an objective magnification x 200 lens in 10 random fields in each section.

Morphometric analysis of optical density

The measure grey was used to detect the optical density of the basement membrane of the glomerular basement membrane together with the proximal convoluted tubule brush border. They were measured using magnification x 400 in 10 non-overlapping fields in PAS stained sections. The optical density (Grey measurements) (produced information about the grey levels) (degree of grey ranging from maximum to minimum grey).

The measured parameters were defined as

1. Pixels measured: The number of pixels measured, i.e., the area of the mask grey within the measuring frame.
2. Sum of grey: The sum of calibrated grey values from each pixel within the measuring frame and optionally masked by a binary image.
3. Mean grey: Sum of grey/number of pixels measured.
4. Maximum grey: The maximum grey calibrated grey values within the measuring frame and optionally masked by a binary image.
5. Minimum grey: The minimum grey calibrated grey values within the measuring frame and optionally masked by a binary image.

Statistical methods

The data were statistically analyzed and tabulated to determine the modification amid the groups underneath research regarding the countless parameters. Their correspondences were exasperated amid the indispensable premeditated parameters. The statistical investigation included analysis of variance (ANOVA) and the arithmetic means of the standard deviation (S.D.). The probability (p) value obtained from statistical tables was directly supplied by the computer using SPSS software version 16. Results were considered statistically significant when p was < 0.05 and highly significant when p was < 0.05 [15].

Results and Discussion

The effect of the tested drugs on systolic blood pressure

There was a substantial reduction in the mean systolic blood pressure in ARNI, ARB, Metformin, and their combination groups (from group IV to group VIII) compared to the corresponding values of the streptozotocin group (group III) at the 8th week of the experiment.

In the 8th week of the experiment, there was a significant reduction of mean systolic blood pressure in the ARNI and metformin combination-treated group (group VII) compared to the corresponding values in both the ARNI-treated groups (group IV) and metformin-treated group (group VI) **(Table 1)**.

Also, there was a significant reduction of mean systolic blood pressure in the valsartan and metformin combination-treated group (group VIII) compared to the corresponding values in both valsartan treated group (group V) and metformin-treated group (group VI) at the 8th week **(Table 1)**.

It should be distinguished that not any substantial difference was detected in the mean systolic blood pressure reduction on comparing the values in the two combination groups (groups VII and VIII) to each other at the 8th week of the experiment.

The effect of the tested drugs on Fasting blood glucose level (FBG), albumin/creatinine (A/C) ratio, and total protein in 24 hours measurement

It was observed that a substantial reduction in mean FBG, A/C ratio, and total protein in 24 hours in valsartan /sacubitril (ARNI), valsartan, Metformin, and their combination groups (from group IV to group VIII) compared to the corresponding values of streptozotocin-induced diabetes (group III) at the 5th

week along with the end of the experiment (8th week) **(Table 2)**.

There was certainly not substantial variance noted in the reduction of the mean FBG, A/C ratio, and total protein in 24 hours resulted between valsartan (group V), ARNI (group IV), Metformin (group VI) treated groups or between the metformin combination groups (groups VII and VIII) at the 5 th and the end of experiment 8 th week **(Table 2)**.

The effect of the tested drugs on serum level of creatinine, ANP, Na, K, GFR, and tissue MDA measurement **(Table 2)**

It was observed that there was a significant decrease in the mean serum creatinine, ANP, Na, K, GFR, tissue MDA level in valsartan /sacubitril (ARNI), valsartan, Metformin, and their combination groups (from group IV to group VIII) compared to the corresponding values of streptozotocin-induced diabetes group III at the end of the experiment (8th week).

Definitely, no major change was noted in the mean serum creatinine reduction, ANP, Na, K, GFR, tissue MDA level resulted between valsartan (group V), ARNI (group IV), and Metformin (group VI) treated groups at the end of the experiment (8th week).

In the 8 th week of the experiment, there was a significant in Na level in the ARNI and Metformin combination-treated group (group VII) compared to the corresponding values of ARNI treated group (group IV) and metformin-treated group (group VI).

There was a substantial diminution in mean serum creatinine, ANP together with Na level in the valsartan and metformin combination group (group VIII) compared to corresponding values in the valsartan treated group (group V) **(Table 2)**.

Streptozotocin group (group III) showed a significant progressive decrease of the mean glomerular basement membrane optical density, the brush border also the optical height of the cells lining of the PCT compared to normal and vehicle control groups (group I & II) after the 8th week of the experiment.

It was observed that there was a major upsurge in the mean optical density of the glomerular basement membrane, the brush border as well as the optical height of the cells lining of the PCT in valsartan /sacubitril (ARNI), valsartan, Metformin, and their combination groups (from group IV to group VIII) compared to the corresponding values of streptozotocin group (group III) at 8th week.

Significant improvement was noted in the mean height of the cells lining of the PCT between ARNI (group IV) compared to the corresponding values in both valsartan (group V), and Metformin (group VI) treated groups at the 8th week **(Table 3 and Figures 1 and 2)**.

On the other hand, in the 8th week of the experiment, there was a major alteration in the mean optical density of the brush border along with the mean height of the cells lining of the PCT in the ARNI and Metformin combination-treated group (group VII) compared to the corresponding values in both ARNI treated group (group IV) and metformin-treated group (group VI) **(Table 3)**.

Also, there was a substantial modification in the mean optical height of the cells lining of the PCT in the valsartan and metformin combination-treated group (group VIII) compared to the corresponding values in both valsartan treated group (group V) and metformin-treated group (group VI) at the 8th week **(Figures 1 and 2)**.

It is to be noted that there was a noteworthy change in the mean optical density of the brush border of the PCT and mean height of the cells lining the proximal tubule comparing the values in the two combination groups (groups VII and VIII) to each other at the 8th week **(Table 3 and Figures 1 and 2)**.

Histological results

Table 1. The mean systolic blood pressure (Mean ± SD) of diverse groups as recorded at 8th week (n=8)

Groups	Arterial Blood Pressure at 8th week
Group I (Normal control)	125.75 ± 3.74
Group II (vehicle control)	125.25 ± 3.03
Group III (streptozotocin)	164.75 ± 5.19 Ω□
Group IV (ARNI treated group)	140.25 ± 3 *
Group V (Valsartan treated group)	139.75 ± 4.33 *
Group VI(Metformin treated group)	142.5 ± 2.81 *
Group VII (ARNI +Metformin-treated group)	120 ± 5.87 * ¥ #
Group VIII(Valsartan + Metformin-treated group)	125.5 ± 4.37 * @#

Values are presented as mean ± SD, Significant p-value = < 0.05

Statistically, significant sign compared to corresponding value to group I(normal control) Ω, to group II(vehicle control) □, to group III(streptozotocin) *, to group IV(ARNI treated) ¥, to group V (Valsartan) @, and to group VI (Metformin treated) #

Table 2. The mean fasting blood glucose, serum levels of creatinine (mg/ dl), Na+ (mmol/l) , K (mmol/l), total protein in 24 hours, Albumin- creatinine ratio , ANP (PG/ML)and eGFR level as recorded at 8 th week (n=8)

Groups	Fasting blood - glucose level	serum level of creatinine	serum level of Na+	serum level of K	Total protein in 24 hours	Albumin-creatinine ratio	serum level of ANP	eGFR level	Tissue malondyaldehyde (MDA)
Group I (Normal control)	90.25 ± 9.04	0.19 ± 0.03	139.83 ± 6.66	4.7 ± 0.26	0.81 ± 0.11	0.82 ± 0.4	15.1 ± 3.69	21.07 ± 3.94	35 ± 9.14
Group II (vehicle control)	86.75 ± 10.43	0.16 ± 0.02	139.18 ± 2.38	4.87 ± 0.26	0.87 ± 0.03	1.28 ± 0.14	15.3 ± 3.34	22.73 ± 2.62	27.35 ± 6.15
Group III (streptozotocin)	311.75 ± 30.24 Ω☐	1.49 ± 0.4 Ω☐	189.15 ± 8.08 Ω☐	3.85 ± 0.11 Ω☐	1.45 ± 0.21 Ω☐	64.39 ± 24.9 Ω☐	88.7 ± 10.38 Ω☐	0.25 ± 0.12 Ω☐	99.7 ± 12.7 Ω☐
Group IV (ARNI treated group)	146.75 ± 9.68 *	0.78 ± 0.1 *	167.35 ± 11.37 *	4.23 ± 0.22	0.9 ± 0.03 *	9.58 ± 2.81 *	33.35 ± 9.12 *	2.82 ± 1.13	68.5 ± 6.51 *
Group V (Valsartan treated group)	149 ± 13.38 *	0.85 ± 0.09 *	169.88 ± 5.36 *	4.24 ± 0.16	1.04 ± 0.11 *	9.58 ± 2.74 *	46.9 ± 9.91 *¥	2.88 ± 0.39	63.88 ± 6.02 *
Group VI (Metformin treated group)	148.25 ± 12.3 *	0.63 ± 0.13 *	159.8 ± 4.05 *	4.33 ± 0.32 *	1.11 ± 0.23 *	7.96 ± 1.73 *	38.43 ± 3.27 *	4.52 ± 0.93 *	60 ± 5.6 *
Group VII (ARNI +Metformin-treated group)	139.25 ± 6.57 *	0.5 ± 0.07 *	146.9 ± 1.9 *¥#	4.4 ± 0.29 *	0.96 ± 0.04 *	6.06 ± 1.05 *	29.3 ± 3.86 *	4.61 ± 0.49 *	54.2 ± 7.75 *
Group VIII (Valsartan + Metformin-treated group)	141 ± 8.56 *	0.51 ± 0.12 *@	149 ± 5.86 *@	4.6 ± 0.24 *	0.96 ± 0.07 *	3.84 ± 0.81 *	26.42 ± 8.26 *@	5.95 ± 1.48 *	

Values are presented as mean ± SD. Significant p value = < 0.05

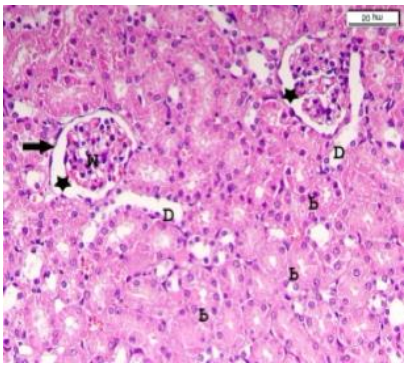
Statistically, significant sign compared to corresponding value to group I (normal control) Ω, to group II(vehicle control) ☐, to group III(streptozotocin) *, to group IV(ARNI treated) ¥, to group V (Valsartan) @, and to group VI (Metformin treated group) #.

Table 3. Mean optical density of the glomerular basement membrane, brush border, and height of cells lining the PCT of all studied groups

Group	Mean optical density of the glomerular basement membrane	Mean optical density of the brush border	Mean height of the cells lining the proximal tubule
Group I (Normal control)	0.376 ± 0.017	0.283 ± 0.031	16.47 ± 1.784
Group II (vehicle control)	0.375 ± 0.013	0.289 ± 0.033	16.47 ± 1.784
Group III (streptozotocin)	0.256 ± 0.006 Ω☐	0.215 ± 0.010 Ω☐	8.151 ± 1.191 Ω☐
Group IV (ARNI treated group)	0.307 ± 0.009*	0.243 ± 0.010*	16.472 ± 1.024*¥@#
Group V (Valsartan treated group)	0.295 ± 0.011*	0.266 ± 0.018*	13.218 ± 2.813*
Group VI(Metformin treated group)	0.309 ± 0.017*	0.25 ± 0.009*	13.95 ± 1.841*
Group VII (ARNI +Metformin-treated group)	0.325 ± 0.018*	0.281 ± 0.030*¥#	13.889 ± 1.843*
Group VIII(Valsartan + Metformin-treated group)	0.3 ± 0.027*	0.265 ± 0.00*	16.028 ± 1.858*$@

Values are presented as mean ± SD, Significant p-value = < 0.05

Statistically, significant sign compared to corresponding value to group I(normal control) Ω, to group II(vehicle control) ☐, to group III(streptozotocin) *, to group IV(ARNI treated) ¥, to group V (Valsartan) @, to group VI (Metformin treated) #, to group VII (ARNI+ Metformin treated) $.



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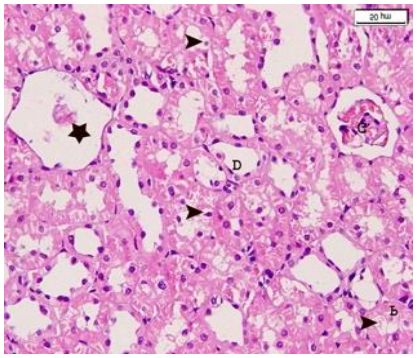

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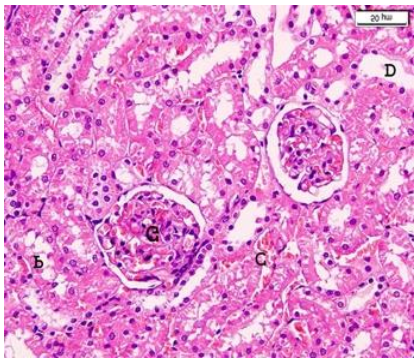

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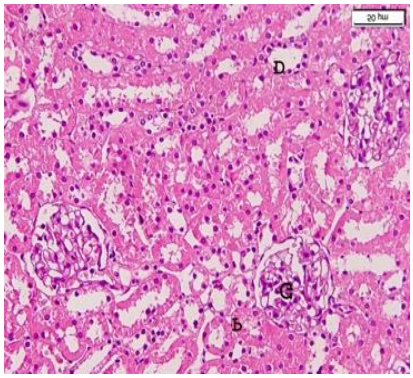
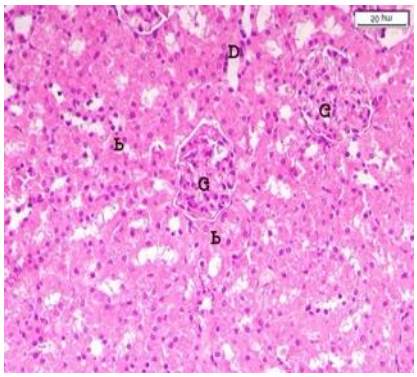
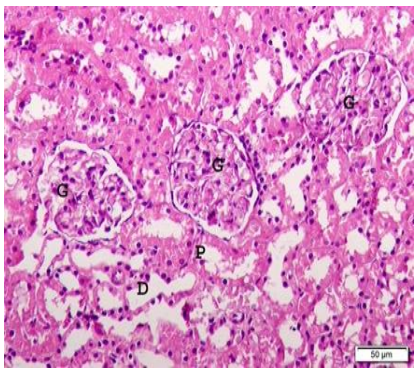


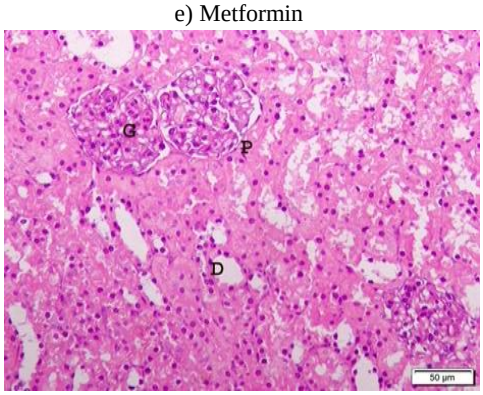
d) Valsartan



e) Metformin

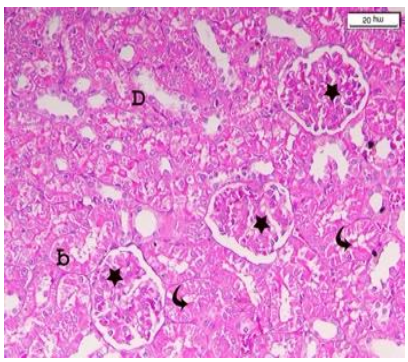


f) ARNI+ Metformin



g) Valsartan + metformin

Figure 1. The histological morphology in the cortex of kidneys harvested from rats with diabetic nephropathy, as detected by hematoxylin and eosin staining. (a) Normal rats, (b) Model rats, (c) ARNI–treated rats, (d) Valsartan-treated rats, (e) metformin-treated rats, (f) ARNI + Metformin – treated rats, and (g) Valsartan+ metformin-treated rats. (Magnification, x200).



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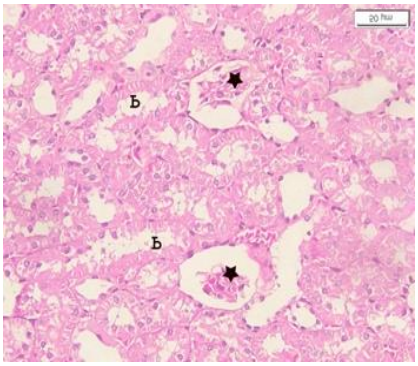

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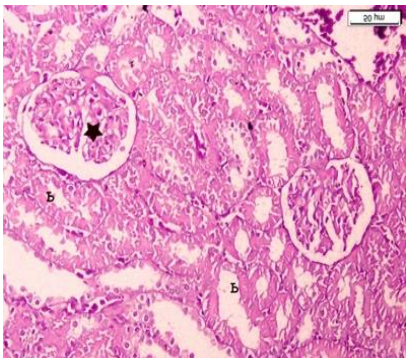

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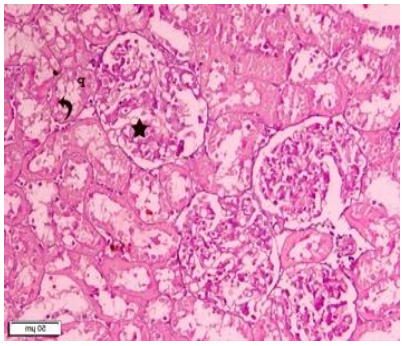
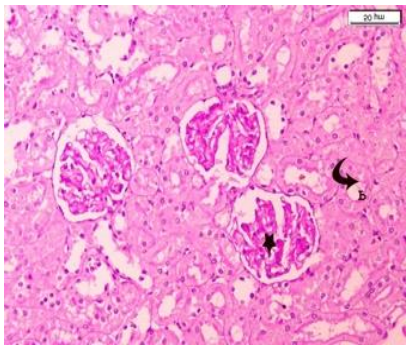
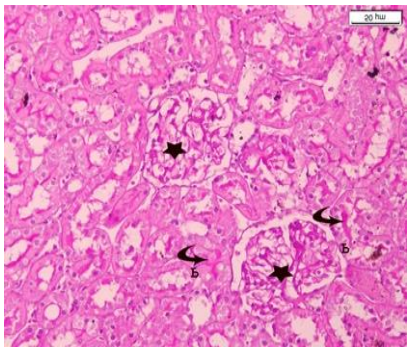


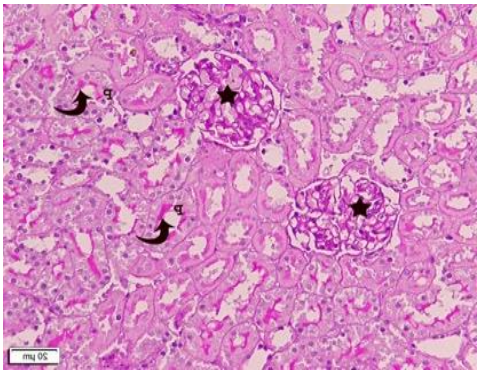
d) Valsartan



e) Metformin



f) ARNI + Metformin



g) Valsartan + Metformin

Figure 2. The histological morphology in the cortex of kidneys harvested from rats with diabetic nephropathy, as detected by Periodic Acid Schiff's staining. (a) Normal rats, (b) Model rats, (c) ARNI–treated rats, (d) Valsartan treated rats, (e) metformin-treated rats, (f) ARNI + Metformin – treated rats, and (g) Valsartan+ Metformin -treated rats. (Magnification, x200).

In the present study, diabetic nephropathy (DN) induced in rats by streptozotocin (STZ) in a single intraperitoneal injection (group III) resulted in significantly elevated blood pressure, an increase in serum Na, K, creatinine, and ANP. Also, a significant increase in 24 hrs total protein, albumin /creatinine ratio in urine, and increased MDA level in renal tissues was recorded. In addition, there was a significant decrease in eGFR at the end of the experiment.

The results from the current research were following the results described by Nobrega *et al.*, 2004, who used STZ to induce diabetes in the rat model, showed combined hypertension and prolonged severe hyperglycemia with a similar degree of albuminuria [16]. Also, Ahad *et al.*, 2018 observed that the STZ-induced DN model resulted in increased serum creatinine (SCR) and declined creatinine clearance (CCr) Levels [17]. In addition, Maheshwari, Balaraman, Sen, and Seth, 2014 observed that STZ single intraperitoneal injection in a dosage of 65 mg/kg in rats induced a significant increase in the same biochemical serum and urinary parameters measured in the present study [18].

Zhang, Xu, Yu, Wu, and Sui, 2017 study [13]–revealed that elevated glucose levels after a single injection of STZ effectively prompted kidney lacerations that were correspondingly existing in DN human patients, hyperglycemia, elevated MDA levels together with kidney injury, which was in agreement with the present study.

In addition, Pilmore, 2010 reported alterations in the Glomerular Basement Membrane (GBM) composition, hyperglycemia, hemodynamic alterations, reactive oxygen species, altered podocyte biology, and glycation of proteins are common mediators of the pathophysiological effects of hyperglycemia and subsequent diabetic nephropathy [19].

Regarding the elevated plasma ANP level of group III in the present study, Chattington *et al.*, 1998, reported that ANP levels were outlined to be lesser in comparison with non-diabetic patients in patients with diabetes mellitus, although the absence of change established in extracellular fluid volume [20]. However, as the kidney role deteriorated, plasma ANP levels rose, which increased glomerular filtration pressure thru effects on arteriolar resistance. In this way, ANP could provide proteinuria progression and hyperfiltration in diabetic nephropathy. Also, McFarlane *et al.*, 2003 stated that diabetes mellitus combined with hypertension had been described to be concomitant with higher ANP [21].

The results of the present work showed that valsartan/sacubitril (ARNI) and valsartan administration in group IV and group V respectively for nephropathic rats for 8 weeks resulted in improvement in the serum glucose levels after 5 and 8 weeks and significant improvement in the levels of the systolic blood pressure, electrolytes, creatinine, eGFR and total protein in the urine. Also, significant reduction of albuminuria, urinary A/C ratio, ANP, and tissue Malondialdehyde (MDA) level were detected in the diabetic nephropathic rats after 8 weeks of therapy.

In addition, sacubitril /valsartan (ARNI) and valsartan treatment improved the histopathological finding in the kidneys of diabetic nephropathic rats.

It is to be noted that the magnitude of suppression of proteinuria by sacubitril /valsartan (ARNI) management was marginally larger compared to only valsartan, which shows that the adding

of NEPi (sacubitril) on top of an ARB (valsartan) elicits additional renoprotective properties. This discovery is in arrangement with the outcomes of Kim *et al*. (2014) [22]. Also, Roksnoer *et al.*, 2015 reported that ARNI utilizes advantageous effects on kidney function and blood pressure in heart failure, related to only ARB [23].

Chronic hyperglycemia-driven activation of the conventional renin-angiotensin system (RAS) has an important role in DN growth. Subsequently, in halting the DN progression, therapeutic regimens aiming at RAS blockage by angiotensin receptor blockers (ARBs) are beneficial [24].

As regards the effect of sacubitril /valsartan on serum glucose levels, the outcomes of the current work are in agreement using the outcomes of Gallagher and Suckling, 2016 and Malek, Sharma, Sankrityayan and Gaikwad, 2019, who pragmatic that diabetic rats exposed to NEPi/ARB blend treatment indicated a substantial decline in plasma glucose together with urinary glucose excretion [25, 26].

Regarding the reduction of blood glucose level with valsartan /sacubitril and valsartan treatment, Iwai *et al*., 2011 found that the insulin receptors expression was amplified through irbesartan therapy, saying that the insulin-sensitizing actions of Angiotensin Receptor Blockers (ARB) like irbesartan, telmisartan with Peroxidase Proliferators Activated Receptor y (PPARY) agonist activity eased glucose uptake, interference with ANG II actions on insulin receptor and insulin receptor substrates improves insulin sensitivity [27]. Jordan *et al*., 2017 reported that ARNI augmented insulin sensitivity in obese patients with hypertension [28].

As regards the effect of ARB–neprilysin inhibitor and ARB treatment on the level of ANP in the plasma, Roksnoer, 2016, reported that in the diabetic rat, ARB condensed plasma ANP, at both 5 along with 12 weeks, in addition to accumulating the neprilysin inhibitor, thiorphan inverted this effect [29]. Nevertheless, this change was insignificant. Roksnoer, 2016 explained that the ANP levels during ARNI treatment reflected the net result of its synthesis and its metabolism [29]. Its synthesis in the heart diminished because of the drop in the blood pressure, causing the diminished atrial stretch. This was apparent thru the abridged cardiac ANP expression at 12 weeks.

In agreement with the results of the present study, Seferovic *et al.*, 2017 demonstrated that valsartan /sacubitril treatment resulted in improvement in HbA1c levels, with significant improvement in electrolytes, plasma creatinine, total protein, biochemical parameters, GFR, and glomerular and tubular remodeling, and lowering of MAP(Mean arterial pressure) in patients with diabetes and HFrEF [30].

Also, Uijl *et al.*, 2020 showed that (sacubitril/valsartan) had renoprotection in DN patients related to HFpEF [31]. Sacubitril/Valsartan may perhaps utilize advantageous effects on renal impairment away from its antihypertensive properties. When paralleled by conventional RAS inhibitors, sacubitril/valsartan decelerated the rate at which kidney role deteriorated in CKD patients [32].

Whaley-Connell *et al*., 2008 suggested the role of oxidative stress in kidney fibrosis [33]. In the metabolic diabetes setting, syndrome showed higher tissue Ang-II induced nitrous oxide (NOX) nephrin interceded loss, podocin along with oxidative stress, which leads to loss of slit pores diaphragms and podocyte effacement, and that these protein insufficiencies are also convoying ultrastructural anomalies are prerequisite to proteinuria/albuminuria.

Nistala *et al.*, 2014 showed that valsartan /sacubitril and valsartan lessened kidney fibrosis, and this reticence followed with comparable oxidative stress suppression, Angiotensin II receptor type 1 (AT1R) expression, and NOX2 [34]. These results recommend that AT1R antagonism as well as decreased oxidative stress, either through ARB's usage or ARNI. Hemodynamic effects might have contributed to the suppression of kidney fibrosis.

In addition, Goru *et al.*, 2017 and Malek and Gaikwad., 2019 found that sacubitril/valsartan (ARNI) combined rehabilitations prevent diabetes-induced renal cell apoptosis as established thru the substantial decrease in Poly (ADP-ribose) polymerase (c-PARP) and c- Caspase-3 expression alongside the improvement streptozotocin-induced DN in male Wistar rats [24, 26].

Uijl *et al.*, 2020 reported that valsartan sacubitril/valsartan repressed the TRPC6-NFATc-Rcan1 pathway, thus preserving glomerular function and structure and potentially improving podocyte integrity [31]. As sacubitril/valsartan appears mostly to inhibit disease development associated with glomerulosclerosis and podocyte loss, this may be the additional mechanism for the renoprotective effect.

In the present work, animals with streptozotocin-induced diabetes, which received Metformin for 8 weeks starting from day 3 of induction of diabetes (group VI), showed significant amelioration in systolic blood pressure, significant reduction of the elevated serum levels of fasting glucose level FGL, creatinine, with significant improvement in GFR, Na, K electrolytes levels and reduction of urinary total protein excretion, urinary A/C ratio, ANP, MDA in comparison to the diabetic non-treated group. Moreover, there was a significant improvement in the histopathological picture of the kidney tissue compared to the diabetic non-treated group.

As regards the effect of Metformin on albuminuria, the present work is in agreement with Pilmore., 2010, who reported that Metformin decreases microalbuminuria in diabetic patients [19]. Also, This result was following Louro, Matafome, Nunes, Da Cunha, and Seica, 2011, who showed that Metformin lower systemic markers of renal function, total proteins, albumin, and creatinine to normal range in diabetic rats [35].

Also, the result of the present study agree with Emam, 2015 and Louro *et al.*, 2011, who showed that the use of Metformin produced a major reduction in FBG, serum creatinine, urinary protein excretion along kidney tissue Malondialdehyde (MDA) level together with the improvement of the histopathological picture of kidney tissue in comparison to the diabetic non-treated group [35, 36].

Zhang *et al.*, 2017 showed that metformin administration considerably reduced the serum creatinine levels, fasting blood glucose, urinary albumin excretion, and creatinine clearance in rats with DN and with significantly decreased the MDA level in

diabetic rats, as related to the model group. This finding is in agreement using the results of the present work [13].

Alhaider *et al.*, 2011 found that the renoprotective metformin effect could be because it influences ROS production together with Advanced Glycation End-products (AGEs) formation, as well as its antihyperglycemic effect [37]. These interpretations suggested a possible clinical usage of Metformin in preventing diabetic nephropathy through enhancing the free-radical defense system and AGEs inhibition.

Also, Metformin has been found to activate AMPK, a most important glucose cellular regulation along with lipid metabolism, and is an inhibitor of complex I of the mitochondrial respiratory chain independent of the pathway of AMPK and a potent inhibitor of the formation of AGE [38].

Also, Sohn *et al.*, 2013 showed that Metformin considerably declines the urine albumin excretion in Zucker Diabetic Fatty (ZDF) rats with diabetes accompanied by the tubular injury amelioration, and revealed that the treatment of chronic Metformin significantly decreased tubule-interstitial injury and improved microalbuminuria in ZDF rats and protecting from hypoxia-induced renal fibrosis [39].

Shishavan *et al.*, 2017 reported that Metformin reduced blood pressure and improved endothelium-dependent relaxation (EDR) in Spontaneously-hypertensive-rats (SHR) aorta via up-regulation of endothelial-vasodilator mediators (NO and EDHF), an effect that was independent of glycemic control and maintained during DM induced with streptozotocin (STZ) in rats [40].

The interesting observation in this work was that combined administration of Sacubitril /Valsartan and Metformin (group VII) produced a prominent renoprotective effect than each drug alone in all biochemical parameters and histopathological picture.

As regards this combination therapy in such a situation, no available studies were found. Synergistic beneficial effects of both drugs on the different mechanisms involved in the pathogenesis of diabetic nephropathy may be the probable explanation of these prominent improvements of biochemical parameters and histopathological features.

Also, in the present work, it was found that the addition of valsartan to Metformin (group VIII) gives a better improvement of not only antioxidant status but also kidney functions and morphology than with each of them alone.

In agreement with this result, Emam, 2015 found that the addition of Metformin to irbesartan (ARB) showed a significant decrease of kidney tissue Malondialdehyde (MDA) level in comparison to the diabetic non-treated group [36]. Also, a significant decrease of FBG, serum creatinine, and urinary protein excretion, together with the improvement of the histopathological picture of kidney tissue, was recorded compared to the diabetic non-treated group.

Ishibashi, Matsui, Takeuchi, and Yam-Agishi., 2012 reported that combined treatments with irbesartan together with Metformin could have a tonic advantage in diabetic nephropathy [41]; it may possibly have a defensive function contrary to tubular injury in diabetes by preventing the formation of AGEs and also mitigating the damaging effects of AGEs via the down-regulating receptor for advanced Glycation End (RAGE) expression also consequently suppressing ROS generation.

Conclusion

The present study showed that valsartan /sacubitril (ARNI) has Renoprotective effects nearly comparable to that produced by both valsartan and Metformin when applied in a streptozotocin-induced diabetic nephropathy model. The best nephroprotective effect was recorded with the concomitant use of Metformin with either valsartan /sacubitril (ARNI) or with valsartan. This nephroprotective effect was produced by restoring the systolic blood pressure, blood glucose, biochemical alterations, serum ANP level, and regulation of oxidative stress in renal tubules, also by protecting against glomerulosclerosis in the nephropathy of diabetes.

It is to be noted that ARNI reduces proteinuria and improves renal histological findings a little higher compared to only valsartan, which shows that the NEPi addition (sacubitril) on top of an ARB (valsartan) may elicit supplementary nephroprotective properties. These answers can further firmly intensify the clinical usage of a combination of valsartan /sacubitril and Metformin in the nephropathy inhibition of diabetes. Certainly, more experimental rat models together with clinical research are needed to understand the nephroprotective properties of this combination better.

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