



## RENOPROTECTIVE EFFECTS OF COMBINED ALISKIREN AND VALSARTAN IN PROGRESSIVE DIABETIC NEPHROPATHY IN RATS

Hemanth Kumar. Nyathani, Prashanth. Srirangam\*, Vidya Sagar Jinugu

Vaagdevi College of Pharmacy, Vishwambara Educational Society, Ramnagar, Hanmakonda, Warangal, A.P, India.

\*Corresponding author's E-mail: [prashanth\\_srirangam@yahoo.com](mailto:prashanth_srirangam@yahoo.com)

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### ABSTRACT

Renin angiotensin system plays a major role in the pathology of progressive Diabetic Nephropathy. In the present study, the renoprotective effects of low dose combined novel direct renin inhibitor-aliskiren and angiotensin II type 1 receptor blocker (ARB)-valsartan were estimated and compared with mono therapy in wistar rats. Rats were divided into 5 groups: control, diabetic control, diabetic rats treated with aliskiren (10 mg/kg/day), valsartan (20 mg/kg/day) and combination of aliskiren and valsartan (5 mg/kg/day, 10mg/kg/day respectively). Clinical characteristics like proteinuria, serum creatinine and renal histopathological studies like glomerulosclerotic index, tubulointerstitial fibrosis were estimated and compared between all diabetic rats. Results showed that combination therapy of aliskiren and valsartan was more effective than the mono therapy in progressive diabetic nephropathy.

**Keywords:** Renin angiotensin system, Diabetic Nephropathy, Aliskiren, Valsartan.

### INTRODUCTION

Diabetic Nephropathy (DN) is the major single cause of End Stage Renal Disease (ESRD) in developing countries<sup>1</sup> and extrapolations suggest that this number will multiply in the future<sup>2</sup>. End stage renal disease require dialysis and is becoming a staggering challenge to public health care systems due to the prohibitive costs of renal replacement therapy that could become unaffordable even for developed countries<sup>3</sup>. Advance diabetic nephropathy is also the leading cause of glomerulosclerosis and ESRD worldwide<sup>4, 5</sup>. DN manifests as a clinical syndrome that is composed of albuminuria, progressively declining glomerular filtration rate (GFR) and increased risk for cardiovascular disease<sup>6, 7</sup>. Persistent proteinuria is the hall-mark of diabetic nephropathy which is the leading cause of end-stage renal disease. The degree of proteinuria is closely associated with the rates of renal events. Furthermore, a reduction in proteinuria is associated with a slowing of both the decline in the GFR and the progression to ESRD. As a result, a reduction in proteinuria has been widely used as a surrogate end point for renoprotection.

The administration of angiotensin converting enzyme inhibitor (ACEI) results in a fall in plasma angiotensin II levels, the efficacy of ACEI is probably limited by their inability to completely block angiotensin converting enzyme (ACE) activity and the generation of angiotensin II through other enzymatic pathways. However, ACEI have other effects including interference with the breakdown of bradykinin. Long term ACEI use is associated with a return in circulating angiotensin II levels following a reactive rise in plasma renin and angiotensin I due to the interruption of angiotensin II feedback on renin release. On the other hand, angiotensin-receptor blockers (ARBs) do not affect bradykinin production and should

theoretically block the actions of angiotensin II chronically at the AT-1 receptor level<sup>8</sup>.

Clinical studies indicate that ARBs may be as effective as ACEI and have fewer side effects. ACEI can induce a very troubling cough in susceptible individuals as a result of the increase in kinins. ARBs serve as a very good substitute for such patients<sup>9</sup>. Valsartan has a higher affinity for the AT1 receptor than losartan, does not have an active metabolite, and has a slightly longer duration of action than losartan. Valsartan is the drug of choice if the patient has compromised liver function. With the exception of captopril and lisinopril, all of the available ACE inhibitors are prodrugs and require liver metabolism for elimination<sup>10</sup>.

Aliskiren is the first of novel direct renin inhibitor which is approved for the treatment of hypertension. Aliskiren, with its 40-hr half-life and ideal pharmacokinetics, can now address angiotensin production directly at its rate limiting step. Aliskiren binds to the active site of renin, preventing angiotensinogen from binding and being cleaved to form angiotensin I, thereby inhibiting the activation of renin system at the rate-limiting step.

There is need for the studies of new renoprotective strategies include, dual blockade of the renin angiotensin aldosterone system with combined novel renin inhibitor Aliskiren and ARB (Valsartan) in progressive diabetic nephropathy. Hence, in the present study, the renoprotective effects of combined aliskiren and valsartan were estimated and compared with monotherapy in diabetic wistar rats.



## MATERIALS AND METHODS

**Animal Model:** Female Wistar rats (200-250g) were procured from Mahaveer enterprises (CPCSEA Reg No. 146/1999) Hyderabad registered by Government of India.

**Drugs:** Streptozotocin (STZ) 55mg/kg (Sigma, St Louis, MO, USA), Aliskiren (gift of Novartis NJ, USA), Valsartan (gift of Novartis NJ, USA), Insulin (2–4 U i.p, Humulin NPH, Eli Lilly, Indianapolis, IN, USA).

**Diagnostic Kits:** One Touch blood glucose monitoring systems (Johnson & Johnson Medical India Ltd, Mumbai, India), Uristix (SIEMENS), Serum Creatinine (Alkaline Picrate Method), (EXCEL DIAGNOSTICS Pvt. Ltd).

**Research work design:** Six-week-old female Wistar rats were used in the present study. Rats were assigned to receive either 55 mg/kg of STZ diluted in 0.1 mol/l citrate buffer, pH 4.5, or citrate buffer alone (non-diabetic) by tail vein injection following an overnight fast. Rats were then randomized into five groups (n=4), receiving one of the following treatments: Control, Diabetic control, Diabetic rats treated with Aliskiren (10 mg/kg per day), Valsartan (20 mg/kg per day), combination of Aliskiren and Valsartan (5 mg/kg per day + 10 mg/kg per day). Treatment commenced within 24 h of STZ or citrate buffer injection. All rats had free access to normal rat chow and drinking water. They were housed in a stable environment maintained at 22±1°C (12 h light–dark cycles with lights on at 06:00 hours).

**Clinical Characteristics of Animals:** Each week, rats were weighed and their blood glucose levels were measured by using One Touch blood glucose monitoring systems. Only STZ treated animals with blood glucose >250 mg/dl were considered diabetic. Every 4 weeks, Serum Creatinine (mg/dl), Body Weight (g), Urine Volume (ml/day) of each rat was serially monitored. Proteinuria (mg/dl/day), Kidney Weight (g) was monitored on the 8th week of the study. Diabetic rats received a daily injection of insulin (2–4 U i.p) to reduce mortality. Serum Creatinine was estimated by Alkaline Picrate Method. Proteinuria is estimated by Uristix. Twenty four hour urine samples were collected with the aid of metabolic cages. Rats were housed individually in metabolic cages equipped with drinking bottles and food cups outside the cages.

**Tissue preparation:** At 8 weeks post-STZ or control vehicle, rats were anaesthetized (Nembutal 60 mg/kg body weight i.p) and perfused via the abdominal aorta with 0.1 mol/l PBS, pH 7.4 (20 to 50 ml), to remove circulating blood. Simultaneously, the inferior vena cava adjacent to the renal vein was severed, allowing free flow of the perfusate. After clearance of circulating blood, 4% paraformaldehyde (wt/wt) in 0.1 mol/l phosphate buffer, pH 7.4, was perfused for a further 5 min (100 to 200 ml of fixative). Kidneys were excised and de-capsulated. The kidneys were sliced transversely, and post fixed overnight. After routine processing through graded alcohols, the kidneys were embedded in paraffin and sectioned at 3 µm.

**Histopathology:** Changes in kidney structure were assessed in a masked protocol in at least 25 randomly selected tissue sections from each group studied. Sections were stained with either periodic acid-Schiff's reagent or Masson's modified trichrome to assess glomerulosclerosis and demonstrate collagenous tubulointerstitial matrix, respectively.<sup>11</sup>

**Glomerulosclerotic index:** In 4µm kidney sections stained with periodic acid-Schiff's reagent, 150 glomeruli from each animal were examined in a masked protocol. The extent of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4, as previously described<sup>14</sup>, with the following grades: grade 0 normal, grade 1 sclerotic area <25% (minimal), grade 2 sclerotic area 25–50% (moderate), grade 3 sclerotic area 50–75% (moderate to severe) and grade 4 sclerotic area 75–100% (severe). A glomerulosclerotic index was then calculated using the formula:

$$GSI = \sum_{i=0}^4 Fi (i)$$

Where GSI is glomerulosclerotic index, Fi is the % of glomeruli in the rat with a given score (i).

**Quantitation of matrix deposition:** The accumulation of matrix within the tubulointerstitial was assessed on Masson's trichrome stained sections using computer-assisted image analysis<sup>22, 23</sup>. Briefly, ten random non-overlapping fields of each section from six rats per group were captured and digitized using a BX50 microscope (Olympus Optical, Shibuya-Ku, Tokyo, Japan) attached to a Fujix HC5000 digital camera (Fuji Photo Film, Minato-Ku, Tokyo, Japan). Digital images were then loaded onto a Pentium IV IBM computer. An area of blue on a trichrome-stained section was selected for its color range and the proportional area of tissue with this range of color was then quantified. Calculation of the proportional area stained blue (matrix) was then determined using image analysis (Analytical Imaging Station, Indian Institute of Chemical Technology, Hyderabad, India).

**Statistical Analysis:** All data are expressed as the mean ± S.D. The differences in all parameters, except blood glucose levels, proteinuria between diabetic and non-diabetic rats were analyzed by a one-way analysis of variance (ANOVA) followed by a Newman-Keuls Multiple Comparison Test using Graph Pad Prism version-5. A change was considered statistically significant if P<0.05.

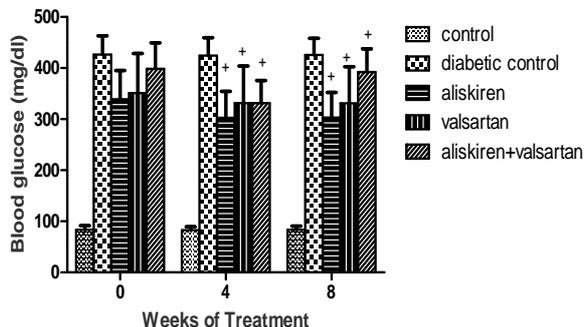
## RESULTS

### Clinical Characteristics of Animals

**Blood glucose levels (mg/dl):** There was significant difference in blood glucose levels (80-426 mg/dl) observed between control and diabetic rats and also significant difference in blood glucose levels (426-398.4 mg/dl) observed between the diabetic control and drug treatment groups on progression of the study from 1<sup>st</sup>

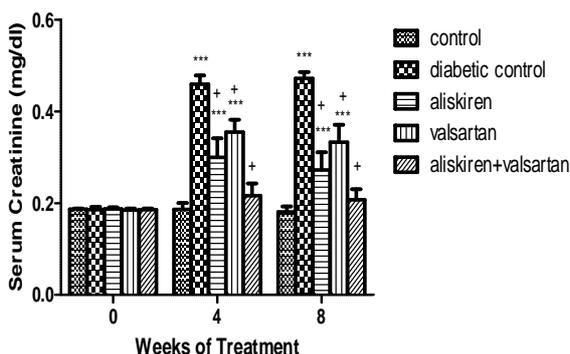


week to 8<sup>th</sup> week (Fig. 1). But there was no significant difference between the blood glucose levels of diabetic rats treated with mono therapy and combination of Aliskiren and Valsartan therapy.



**Figure 1:** Blood glucose levels (mg/dl) in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan. + P<0.001 vs diabetic control. Values are expressed as means ± S.D. N=4 rats/group.

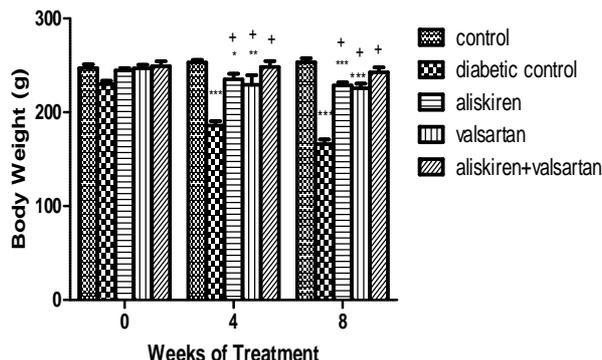
**Serum creatinine (mg/dl):** After 4<sup>th</sup> and 8<sup>th</sup> week, in comparison with control animals (0.1863, 0.1808 mg/dl), diabetic control rats had elevated serum creatinine (0.4595, 0.4718 mg/dl), which was extremely significantly reduced in drug treatment groups with Aliskiren, Valsartan and combination of Aliskiren and Valsartan. But after 4<sup>th</sup> week, combination of Aliskiren and Valsartan has extremely significantly reduced serum creatinine (0.2163 mg/dl) compared to Aliskiren (0.2998 mg/dl) and Valsartan (0.3550 mg/dl) mono therapy. Whereas after 8<sup>th</sup> week, combination of Aliskiren and Valsartan has very significantly reduced serum creatinine (0.2073 mg/dl) compared to Aliskiren mono therapy (0.2720 mg/dl), but extremely significantly reduced compared to Valsartan mono therapy (0.3330 mg/dl) (Fig. 2).



**Figure 2:** Serum creatinine (mg/dl) in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan. \*\*\*P<0.001 vs combination of aliskiren and valsartan. + P<0.001 vs diabetic control. Values are expressed as means ± S.D. N=4 rats/group.

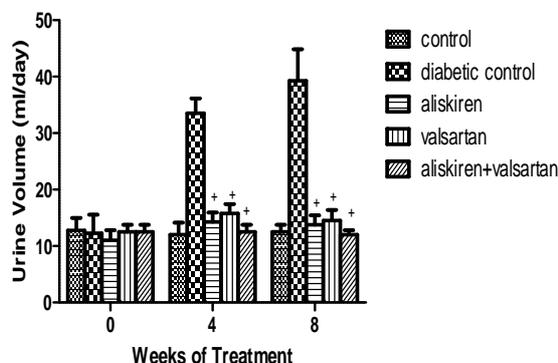
**Body weight (g):** After 4<sup>th</sup> and 8<sup>th</sup> week, in comparison with control animals (253, 253.5 g), diabetic control rats had reduced body weight (185.8, 166 g), which was very significantly increased in drug treatment groups with Aliskiren, Valsartan and combination of Aliskiren and

Valsartan. After 4<sup>th</sup> week there was significant increase in body weight of diabetic rats treated with combination of Aliskiren and Valsartan (248.3 g) compared with diabetic rats treated with Aliskiren mono therapy (235 g) and very significant increase in diabetic rats treated with combination of Aliskiren and Valsartan (248.3 g) compared with Valsartan mono therapy (229.3 g). But after 8<sup>th</sup> week there was extremely significant increase in body weight of diabetic rats treated with combination of Aliskiren and Valsartan (242.5 g) compared with diabetic rats treated with Aliskiren (228.5 g) and Valsartan (225.5 g) mono therapy (Fig. 3).



**Figure 3:** Body weight (g) in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 vs combination of aliskiren and valsartan. + P<0.001 vs diabetic control. Values are expressed as means ± S.D. N=4 rats/group.

**Urine volume (ml/day):** After 4<sup>th</sup> and 8<sup>th</sup> week, in comparison with control animals, diabetic control rats had elevated urine volume (33.50, 39.25 ml/day), which was extremely significantly reduced in drug treatment groups with Aliskiren (14.25, 13.75 ml/day), Valsartan (15.75, 14.50 ml/day) and combination of Aliskiren and Valsartan (12.50, 12.00 ml/day). But there was no significant reduction in urine volume of diabetic rats treated with combination of Aliskiren and Valsartan compared with diabetic rats treated with Aliskiren and Valsartan mono therapy (Fig. 4).

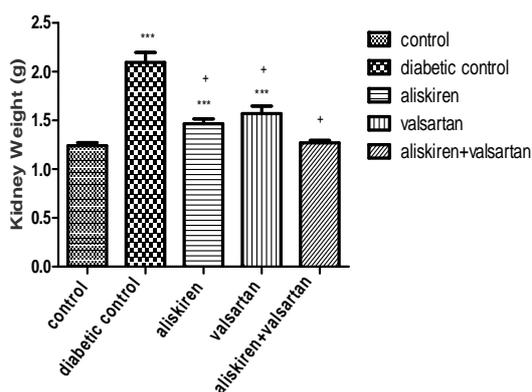


**Figure 4:** Urine Volume (ml/day) in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan. + P<0.001 vs diabetic control. Values are expressed as means ± S.D. N=4 rats/group.

**Proteinuria (mg/ml/day) and Kidney weight (g)** : After 8<sup>th</sup> week, in comparison with control animals (<30 mg/dl/day, 1.240 g), diabetic control rats had elevated proteinuria (>30 mg/dl/day), kidney weight (2.095 g), which was extremely significantly reduced in drug treatment groups with Aliskiren, Valsartan and combination of Aliskiren and Valsartan (Table1). Combination of Aliskiren and Valsartan extremely significantly reduced kidney weight (1.27 g) compared to Aliskiren (1.465 g) and Valsartan (1.570 g) mono therapy (Fig. 5).

**Table 1:** Proteinuria (mg/dl/day) in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan.

Group	< 30 mg/dl	> 30 mg/dl	Observation
Control	Yellow		Negative
Diabetic control		Yellow – Green	Positive
Aliskiren	Yellow		Negative
Valsartan	Yellow		Negative
Aliskiren + Valsartan	Yellow		Negative

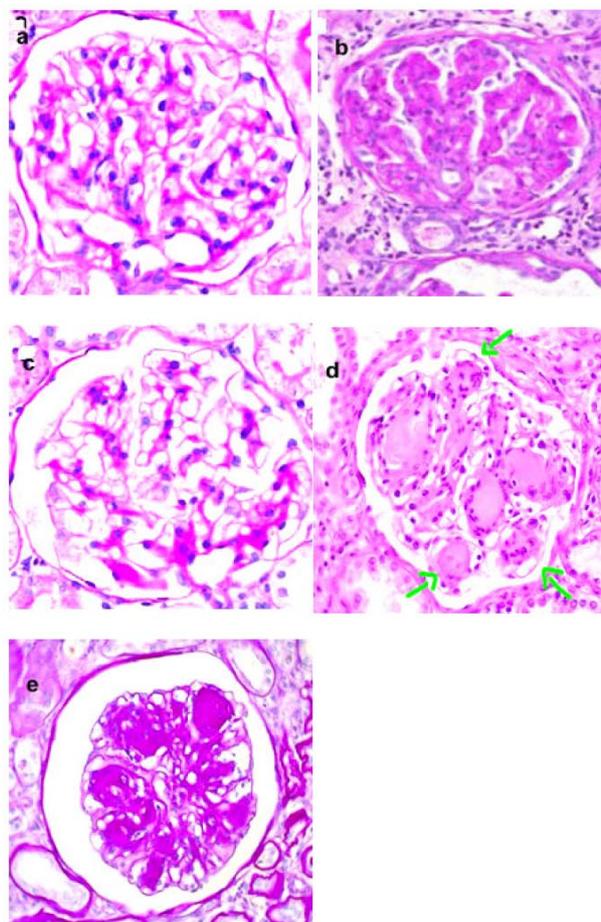


**Figure 5:** Kidney weight (g) in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan. \*\*\*P<0.001 vs combination of aliskiren and valsartan. + P<0.001 vs diabetic control. Values are expressed as means ± S.D. N=4 rats/group.

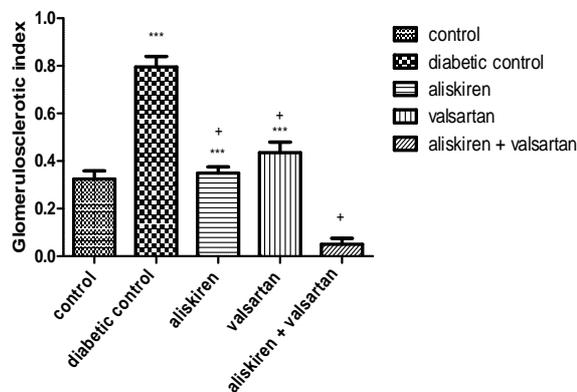
**Histopathology:** In control rats, the kidney cortex appeared normal with only some glomeruli displaying thickened glomerular basement membranes (GBM) (Fig. 6, Fig. 7). In contrast, in diabetic control rats most glomeruli exhibited thickened GBM, capillary occlusion, and mesangial expansion; In addition, many cortical tubules were vacuolated. In diabetic rats treated with Aliskiren, Valsartan, or a combination of Aliskiren and Valsartan, glomerular pathology was improved (Fig. 8, Fig. 9).

**Glomerulosclerotic index (GSI):** Diabetes was associated with an increase in GSI. Compared to diabetic control rats, GSI was lower in diabetic rats treated with Aliskiren and Valsartan mono therapy and the combination of Aliskiren and Valsartan. There is extremely significant reduction in

the GSI of diabetic rats treated with combination of Aliskiren and Valsartan (0.05) compared with Aliskiren (0.35), Valsartan (0.435) mono therapy (Fig. 6).

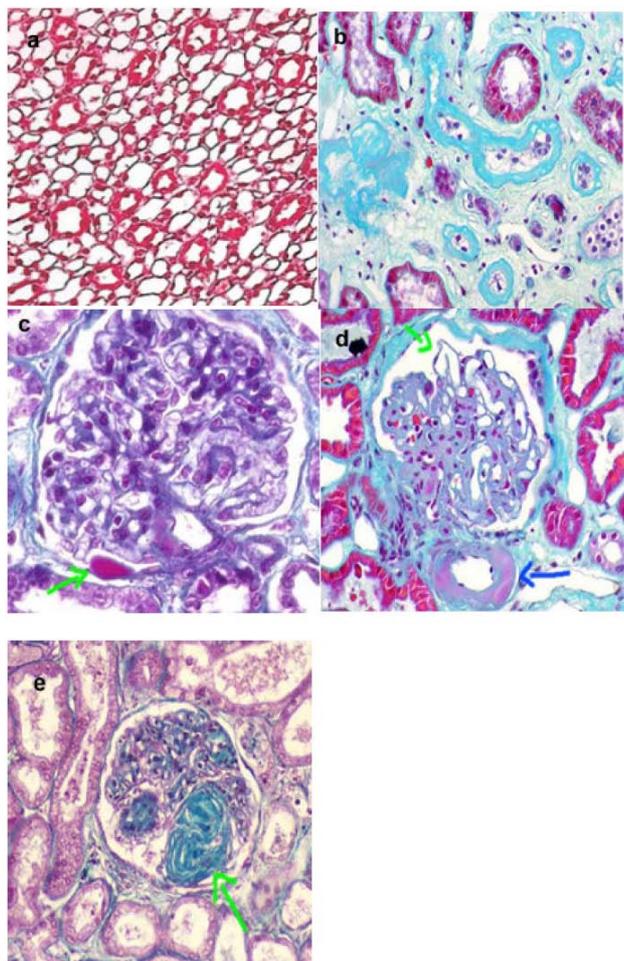


**Figure 6:** Representative periodic acid-Schiff's reagent-stained section from a. control rat, only minimal glomerulosclerosis was noted, b. diabetic control rat was associated with marked glomerulosclerosis, c. Aliskiren, d. Valsartan treated diabetic rats were associated with a significant reduction in the glomerulosclerosis, e. combination of aliskiren and valsartan treated diabetic rat was associated with extremely significant reduction in the glomerulosclerosis compared with mono therapy of either.

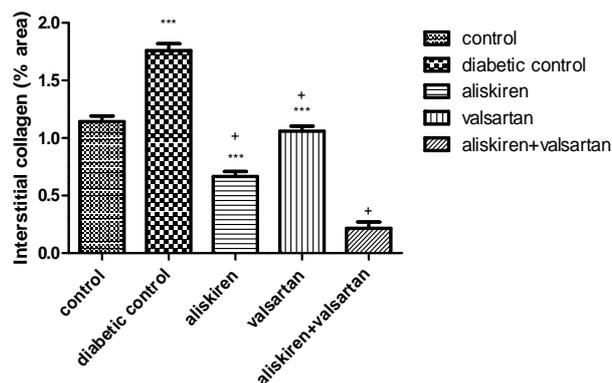


**Figure 7:** Glomerulosclerotic index in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan. \*\*\*P<0.001 vs combination of aliskiren and valsartan. + P<0.001 vs diabetic control. Values are expressed as means ± S.D. N=4 rats/group.

**Quantitation of matrix deposition:** In the tubulointerstitium of kidney cortex or medulla, increased collagen and inflammatory cells were observed in diabetic control rats compared to control rats. Compared to diabetic control rats, interstitial fibrosis was lower in diabetic rats treated with Aliskiren and Valsartan mono therapy and the combination of Aliskiren and Valsartan. There was extremely significant reduction in the interstitial fibrosis of diabetic rats treated with combination of Aliskiren and Valsartan (0.215) compared with Aliskiren (0.665) and Valsartan (1.06) mono therapy. (Fig. 8, Fig. 9)



**Figure 8:** Representative Masson's trichrome-stained section from a. control rat, very little collagen (blue staining) is present within the interstitium, b. diabetic control rat, extensive collagen (blue staining) is present within the interstitium indicating extensive interstitial fibrosis, c. Aliskiren, d. Valsartan treated diabetic rats, interstitial fibrosis was significantly reduced, e. Combined aliskiren and valsartan treated diabetic rat, interstitial fibrosis was extremely significantly reduced compared with monotherapy of either.



**Figure 9:** Interstitial fibrosis in control and STZ induced diabetic rats treated with mono- and combination therapies of aliskiren and valsartan. \*\*\* $P < 0.001$  vs combination of aliskiren and valsartan. +  $P < 0.001$  vs diabetic control. Values are expressed as means  $\pm$  S.D. N=4 rats/group.

## DISCUSSION

In the present study, the renoprotective effects of Aliskiren, Valsartan and combination of Aliskiren and Valsartan were compared in diabetic nephropathy in rats. Clinical characteristics like blood glucose levels, serum creatinine, urine volume, proteinuria, body weight and kidney weight were estimated in all experimental rats. In addition kidney histopathological parameters like Glomerulosclerotic index and tubulointerstitial fibrosis were also estimated and compared.

In the present study with regard to the attenuation of the decline in renal function, combined Aliskiren and Valsartan treatment significantly reduced serum creatinine, urine volume and kidney weight in comparison with mono therapy of Aliskiren and Valsartan. Body weight of diabetic rats treated with Aliskiren and Valsartan mono therapy and combination of Aliskiren and Valsartan was significantly increased compared with diabetic control rats. By the dual blockade of RAS, with combination of Aliskiren and Valsartan at the two different important steps, the renin activation might effectively prevented progression of diabetic nephropathy to end stage renal disease.

Aliskiren acts through the direct renin inhibition which is the rate limiting step for the activation of RAS, It binds to the active sites of renin, preventing angiotensinogen from binding and being cleaved to form angiotensin I, thereby inhibiting the activation of renin system at the rate limiting step. It is possible that Aliskiren also differs from ACEIs or ARBs in other ways that might explain these results. In 2002, Nguyen et.al discovered a (pro) renin receptor that was detected in the brain, heart, liver, and kidney. Prorenin, when bound to the (pro) renin receptor, displayed enzymatic activity and activation of intracellular signaling pathways without proteolytic removal of the pro segment. Recent studies in animals with diabetes and in in-vitro conditions with high glucose have shown that aliskiren reduces the number of (pro) renin receptors in

the kidney, mitigates profibrotic activity in the kidney, and nearly abolishes the apoptotic effects on cultured podocytes. Furthermore, data from transgenic (mRen-2)27 rats with diabetes suggest that aliskiren has a greater renoprotective capacity than ACE inhibitors<sup>12</sup>.

Valsartan acts by blocking the binding of angiotensin II to the AT1 receptor, thereby preventing the activation of RAS. Clinical characteristics of the diabetic control rats and diabetic rats treated with Aliskiren and Valsartan mono therapy and combination therapy clearly suggests that combination therapy is more effective than the mono therapy.

In diabetic rats, high glucose activates the RAS providing a further mechanism for a diabetic induced amplification of angiotensin II-mediated tissue injury. RAS plays a central role in mesangial cell proliferative signaling, angiotensin II is a major contributor to the apoptosis of proximal tubular cells either directly or via other injurious cytokines, such as transforming growth factor-beta and platelet-derived growth factor. In diabetic rats, glomerular angiotensin II levels are increased due to increase in angiotensinogen. Angiotensin II also produces deleterious effects on kidney by affecting the blood pressure and renal haemodynamics, renal tubular and glomerular hypertrophy and oxidative stress in kidney. Therefore, blockade of angiotensin II is very important factor for preventing the progress of DN. Hence, activation of RAS leads to the increased glomerular basement membrane thickness, glomerulosclerotic index and mesangial expansion and tubulointerstitial fibrosis in diabetic rat kidney. Further these histopathological changes lead to increased kidney weight, protein synthesis and proteinuria and serum creatinine and urine volume.

In the present study, after 8<sup>th</sup> week, fewer numbers of rats were studied for renal structural damage. Combination of Aliskiren and Valsartan exerted a significant additive effect in reducing the development of severe glomerulosclerosis, tubulointerstitial fibrosis compared to Aliskiren and Valsartan mono therapy. Tubulointerstitial injury is a major feature of diabetic nephropathy and also an important predictor of renal dysfunction and the response to therapeutic interventions in both the experimental and human settings<sup>13</sup>.

This difference may be due to the combined blockade of RAS, direct inhibition of renin with aliskiren and blockade of angiotensin II at its receptor level which more effectively prevents the formation and activation of angiotensin II in diabetic kidney. In Valsartan mono therapy, there are chances, for the binding of angiotensin II to AT1 receptor, when it is over expressed. In Aliskiren mono therapy, the binding of circulating angiotensin II to AT1 receptor may occur, which leads to further progression of the diabetic nephropathy. Hence, the combination of Aliskiren and Valsartan is more effective than the mono therapy of either drug in prevention of

progression of diabetic nephropathy to end stage renal disease.

The present study reports that in a model of severe renal pathology in diabetic wistar rat, low dose combination therapy of Aliskiren and Valsartan was more efficacious than either mono therapy in improving severe diabetic glomerulosclerosis and effective at ameliorating the decline in renal function and increase in proteinuria.

## CONCLUSION

In conclusion, Proteinuria, serum creatinine, kidney weight and urine volume were significantly reduced and body weight was significantly increased in diabetic rats treated with combination of Aliskiren and Valsartan compared with Aliskiren and Valsartan monotherapy. Glomerulosclerotic index and tubulointerstitial fibrosis were significantly decreased in diabetic rats treated with combination of Aliskiren and Valsartan compared with Aliskiren and Valsartan mono therapy. Hence, from the results of present study, combination of novel direct renin inhibitor-Aliskiren and ARB-Valsartan is more renoprotective compared to Aliskiren and Valsartan mono therapy in progressive diabetic nephropathy.

## REFERENCES

1. Stern M. Diabetes and cardiovascular disease. The "common soil" hypothesis. *Diabetes* 1995; 44:369-374.
2. Eastman R, Siebert C, Harris M, Gorden P. Clinical review: implications of the Diabetes Control and complications trial. *J Clin Endocrinol Metab* 1993; 77: 1105-1107.
3. Turner R. The U.K. Prospective Diabetes Study. A review. *Diabetes Care* 1998; 21 (Suppl 3): C35-C38.
4. Clark CJ, Lee D. Prevention and treatment of the complications of diabetes mellitus. *N Engl J Med* 1995; 332: 1210-1217.
5. Fore W. Noninsulin-dependent diabetes mellitus. The prevention of complications. *Med Clin North Am* 1995; 79: 287-298.
6. Remuzzi G, Ruggenenti P, Benigni A. Understanding the nature of renal disease progression. *Kidney Int* 1997; 51: 2-15
7. Parving H.H, Osterby R, Ritz E: Diabetic Nephropathy. *The Kidney*, edited by Brenner BM, Philadelphia, WB Saunders, 2000, P.1731.
8. Jennifer L. Wilkinson-Berka. Renoprotective and anti-hypertensive effects of combined valsartan and perindopril in progressive diabetic nephropathy in the transgenic (mRen-2) 27 rat. *Nephrol Dial Transplant* 2001; 16: 1343-1349.
9. Michel Azizi, MD, PhD; Joël Ménard, MD. Combined Blockade of the Renin-Angiotensin System With Angiotensin-Converting Enzyme Inhibitors and



- Angiotensin II Type 1 Receptor Antagonists. *Circulation* 2004; 109:2492-2499.
10. Charles R. Craig, Robert E. Stitzel, *Modern Pharmacology with Clinical Applications*. Fifth Edition: 156, 213, 217.
  11. J Kelly. Aliskiren, a novel renin inhibitor, is renoprotective in a model of advanced diabetic nephropathy in rats. *Diabetologia* 2007; 50: 2398-2404.
  12. Kelly DJ, Hepper C, Zhang Y, Jaworski K, Wilkinson-Berka JL, Gilbert RE., Protein kinase C  $\beta$  inhibition attenuates the progression of experimental diabetic nephropathy in the presence of continued hypertension. *Diabetes* 2003; 52: 512–518.
  13. Lehr HA, Mankoff DA, Corwin D, Santeusanio G, Gown AM. Application of Photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem* 1997; 45: 1559–1565.
  14. Lehr HA, van der Loos CM, Teeling P, Gown AM. Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-based image analysis. *J Histochem Cytochem* 1999; 47: 119–126.

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