

Role of Brain Angiotensin (1-7) In Chronic Hyperglycaemia Induced Nephropathy in Wistar Rats

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ABSTRACT

Objective: Ang (1-7) recognised as a biologically active component of renin angiotensin system (RAS). It has been documented that peripheral activity of Ang (1-7) gets reduced during diabetic nephropathy (DN) which is one of the most common cause of end stage renal disease. Peripheral activity of RAS is regulated by brain RAS. The purpose of present study is to investigate the role of brain angiotensin (1-7) in chronic hyperglycemia induced nephropathy in wistar rats. **Material and methods:** Diabetes mellitus (DM) is induced by single dose of alloxan (120 mg/kg; i.p.). The biochemical parameters related to DN was estimated using commercially available kits. **Results:** Diabetic rat, after 8 weeks of alloxan administration shows elevated serum creatinine, blood urea nitrogen, protein in urine, kidney weight/body weight and decreased level of serum nitrite. However, intracerebroventricular treatment with Ang (1-7) (4.8 µg/day) and valsartan (100 nmol/day) (which do not cross blood brain barrier) alone and combination of Ang (1-7) and valsartan for 2 weeks markedly attenuated these changes and increased serum nitrite in DN induced rats. **Conclusion:** The finding of this study suggests that brain Ang (1-7) play a vital role in controlling the peripheral activity of RAS in diabetic nephropathy which may be due to the decreased central sympathetic outflow and peripheral activity of Ang II.

Key words : Ang (1-7), diabetic nephropathy, alloxan, valsartan, brain renin angiotensin system

INTRODUCTION

Diabetes Mellitus (DM) has been considered as one of the major health complication in worldwide.¹ DM is a complex metabolic disorder which is characterized by absolute insulin deficiency or development of insulin resistance that leads to hyperglycaemia and an altered glucose, fat and protein metabolism.^{2,3} Long term complication of DM includes cardiomyopathy, retinopathy, neuropathy, and nephropathy.^{4,7} Diabetic nephropathy (DN) is one of the serious problem of diabetes and most common cause of end-stage renal disease, and it represents an increasing global public health problem.⁸ DN is characterized by persistent increase in level of albumin in urine, increase the glomerular filtration rate (GFR), excessive deposition of extracellular

matrix protein, glomerular hypotrophy, tubulointerstitial fibrosis, mesangial expansion and podocyte loss.⁹ Further, the blood urea nitrogen (BUN) and serum creatinine level are significantly increased,¹⁰ during DN followed by dyslipidemia.¹¹

The exact cause of DN is still unknown, but various mechanisms like hyperfiltration, renal injury, advanced glycation end products, and cytokines activation are involved in hyperglycaemia.¹² A wealth of evidence indicates that the renin-angiotensin system has a key role in the pathogenesis of DN.¹³ Peripheral renin-angiotensin-aldosterone system (RAAS) gets overactivated during the DN.¹⁴ The principle effector octapeptide angiotensin II (Ang II) expression get increased during DN, which

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leads to hyperfiltration and increased intraglomerular pressure and it also stimulates the mesangial cell proliferation.¹⁵ A galaxy of clinical trials has proven that angiotensin-converting enzyme inhibitors and type 1 angiotensin receptor antagonists are effective in attenuating the development of DN.¹³ However, the recent discovery of a new element of RAAS, angiotensin converting enzyme 2 (ACE 2), revealed a new pathway for angiotensin peptide metabolism.^{16,17} ACE 2 cleaves octapeptide (Ang II) to form fragment Ang (1-7), which has a renoprotective role.¹⁸⁻²⁰ The renoprotective effect of ACE 2 and its fragments diminished during DM.^{19,21} Moreover, prolonged hyperglycaemia is noted to decrease the renal concentration of ACE 2 and its fragments.²²⁻²⁴

The brain plays an important role in the regulation of peripheral activity of RAAS.²⁵ Brain RAS is involved in development of hypertension, heart failure and kidney failure.²⁶⁻²⁸ In brain, over expression of ACE-Ang II type 1 receptor was reported in cardiovascular diseases, which leads to increase the sympathetic outflow.²⁹ However, many studies from different laboratories revealed that intracerebroventricular (ICV) administration of ACE inhibitor and Ang II receptor blockers were beneficial to treat brain and cardiovascular diseases in animal experimental model.²⁵ Moreover, inhibition of ACE and Angiotensin type 1 receptors (AT1R) in the brain by ICV administration of ACE inhibitor or AT1 blocker is reported to increase formation of cerebral ACE 2 and Ang (1-7), and reduces the central sympathetic outflow and peripheral level of renin and Ang II.^{30,31,32} Therefore the present study has been designed to investigate the role of brain Ang (1-7) in chronic hyperglycemia induced nephropathy in wistar rats.

MATERIALS AND METHODS

Animals

Wistar rats of either sex with an initial body weight of 180-250 g were used in these studies. The experimental protocol used in the present study was approved by Institutional Animal Ethics Committee (GLAIPAR/CPCSEA/IAEC/2015/P.Col) in accordance with the national guideline employed in use of laboratory animals. All animals were kept in standard light/dark cycle with free access to standard chow diet and water *ad libitum*. The animals were housed in metabolic cages. 24-h urine collection was obtained from each rat for laboratory investigation.

Drugs and chemicals

Angiotensin (1-7) and Valsartan (Sigma Aldrich [P] Ltd., Bangalore, India) was prepared in artificial cerebrospinal fluid and then infused by intracerebroventricular (ICV) administration for 2 week, 8 weeks after the administration of alloxan monohydrate (Sigma Aldrich [P] Ltd., Bangalore, India). All other reagents used in this study were of analytical grade and always freshly prepared before use.

Surgery and intracerebroventricular administration of valsartan and angiotensin (1-7)

Animal was anesthetized with thiopental sodium (30 mg/kg, i.p.)³³ and positioned in a stereotaxic apparatus. The head was positioned in a frame and a midline sagittal incision made in the scalp. Two holes were drilled in the skull for the placement of polypropylene cannula into both the lateral cerebral ventricles for smooth administration of drug with the help of Hamilton microsyringe. Co-ordinates for the ICV cannula implantation were 0.8 mm posterior to lambda, 1.8 mm lateral to the sagittal suture, and 3.6 mm beneath the cortical surface.³⁴ The scalp was then closed with a suture. Special care of the animals was taken during the postoperative period to provide food and water inside the cage of rats. Rats were infused ICV with artificial cerebrospinal fluid (ACSF; in mmol/l) 147 NaCl, 2.9 KCl, 1.6 MgCl₂, 1.7 CaCl₂, and 2.2 dextrose)³⁵ for two weeks. In the same way, the ICV administration of valsartan (100 nmol/day, 2 weeks)³⁶ and angiotensin (1-7) (4.8 µmol/day, 2 weeks)³⁷ in single or in combination were given in volume of 5 µl (ACSF) in both lateral ventricles by using a Hamilton microsyringe. Hamilton microsyringe positioned in the injection cannula and the syringe was kept in place for 2 minutes in order to allow for the diffusion of the injected volume and prevents pressure-induced damage.

Induction and assessment of diabetes

Experimental diabetes mellitus (DM) was induced by a single injection of alloxan monohydrate (120 mg/kg, i.p.).³⁸ Diabetic rats having a blood serum glucose level >200mg/dl were considered to be hyperglycaemic. Blood samples were obtained from retro orbital sinus and blood glucose levels were determined by glucose oxidase-peroxidase (GOD-POD) method by using commercially available kit (Span Diagnostics Ltd., Surat, India).

Assessment of diabetic nephropathy

Diabetes mellitus induced nephropathy was estimated biochemically by measuring the level of blood urea nitrogen, serum creatinine and protein in urine by using standard diagnostic kits (Span Diagnostics Ltd., Surat, India).

Estimation of serum nitrite

Unlike NO, nitrite can be measured easily and nitrite concentration can be used to infer level of NO production. Nitrite release in coronary effluent was measured. Greiss reagent 0.5 ml (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride in water) was added to 0.5 ml of serum. The optical density at 550 nm was measured using spectrophotometer. Nitrite concentration was calculated by comparison with spectrophotometer reading of standard sodium nitrite. Result was expressed as micromoles per litre ($\mu\text{M/L}$).

Estimation of serum cholesterol and triglyceride

The serum cholesterol and triglyceride were estimated by blood samples collected by retro orbital sinus by using commercially available kits (Span Diagnostics Ltd., Surat, India).

Estimation of kidney weight / body weight ratio

Kidney weight / Body weight ratio was estimated by removal of both kidneys, renal fascias were removed and kidneys were weighed individually. The kidney weight / body weight (%) was calculated by following formula.³⁹

$$\text{Kidney weight / Body weight} = \frac{\text{Left kidney weight} - \text{Right kidney weight}}{\text{Body weight}} \times 100$$

Experimental protocol

Thirty six rats were allocated to following experimental groups, each group consisting of six animals.

Group I (Normal Control; n=6): Rats were fed with standard food and water *ad libitum* and no treatment is given.

Group II (Diabetic Control; n=6): Animals were administered with alloxan monohydrate (120 mg/kg, i.p.) to induce diabetes mellitus.

Group III (Vehicle control; n=6): Animals were treated with artificial cerebrospinal fluid (ACF) 5 μl in each hemisphere for two week after 8 weeks of alloxan administration.

Group IV (Angiotensin (1-7) ICV treated diabetic group; n=6): Animals were treated with Angiotensin (1-7) at dose of 4.8 $\mu\text{g/day}$ through ICV route for two weeks after 8 weeks of alloxan administration.

Group V (Valsartan ICV treated diabetic group; n=6): Animals were treated with valsartan at dose of 100 nmol/day through ICV route for 2 weeks after 8 weeks of administration of alloxan.

Group VI (Valsartan + Angiotensin (1-7) ICV treated group; n=6): Animals were treated with valsartan (100 nmol/day) and Ang (1-7) (4.8 $\mu\text{g/day}$) in combination through ICV route for 2 weeks after 8 weeks of administration of alloxan.

Statistical analysis

All values are expressed as mean \pm S.D (standard deviation). The data obtained from various groups were statistically analysed using one way analysis of variance (ANOVA) followed by Tukey's test multiple comparison test. P values of less than 0.05 were considered to be statistically significant.

RESULTS

Evaluations of different parameters were done at the starting of experimental research. All parameters were assessed up to 10th week after alloxan administration and results were compared between treatment group versus normal and diabetic control group. Diabetic nephropathy was confirmed at 8 week and treatment was continued from 8th week to 10th week.

Effect of pharmacological interventions on serum glucose

Single administration of alloxan monohydrate (120 mg/kg/*i.p.*) significantly ($p < 0.05$) increase the serum concentration of glucose in diabetic control group as compared to normal rats. Treatment with Ang (1-7) (4.8 $\mu\text{g/day}$; ICV) and valsartan (100 nmol/day; ICV) alone and in combination for 2 weeks after 8 week of alloxan administration were not affect the serum glucose level in diabetic rats (Figure 1).

Effect of pharmacological interventions on blood urea nitrogen (BUN), protein in urine, and urinary output

Administration of alloxan monohydrate (120 mg/kg/*i.p.*) significantly ($p < 0.05$) increase the blood urea nitrogen, protein in urine and volume of urinary output as compared to normal rats. However, administration of Ang (1-7) and valsartan alone and in combination for 2 weeks after 8 week of alloxan administration significantly ($p < 0.05$) reduces the elevated level of BUN, protein in urine and volume of urinary output as compared to diabetic rats. (Figure 2, Figure 3, Figure 4).

Effect of pharmacological interventions on serum creatinine

The concentration of serum creatinine was noted to be significantly increased ($p < 0.05$) in diabetic rats when compared with normal rats. Treatment with Ang (1-7) (4.8 $\mu\text{g/day}$; ICV) and valsartan (100 nmol/day; ICV)

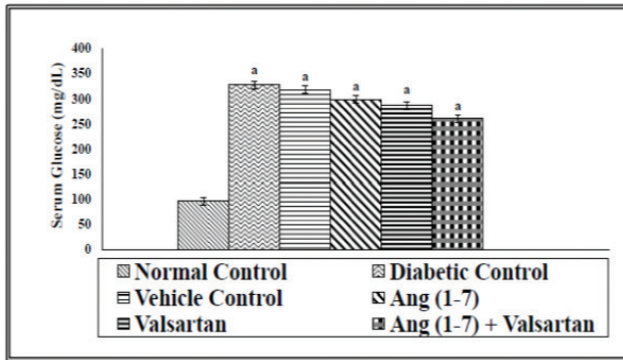


Figure 1: Effect of single administration of alloxan monohydrate on serum glucose in diabetic control group, effect of Ang (1-7) treatment on serum glucose in diabetic rats, effect of valsartan treatment on serum glucose in diabetic rats, effect of Ang (1-7) and valsartan treatment on serum glucose in diabetic rats. Graph represents the data of serum glucose of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control.

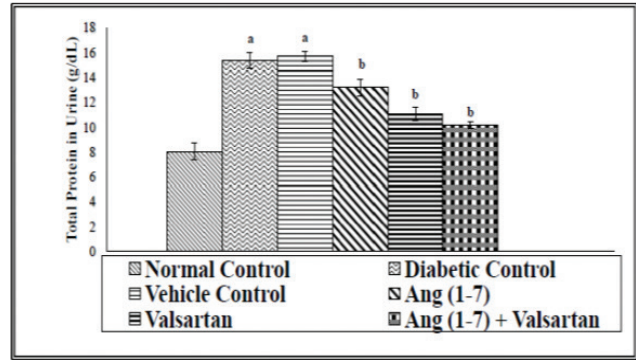


Figure 3: Effect of single administration of alloxan monohydrate on total protein in urine in diabetic control group, effect of Ang (1-7) treatment on total protein in urine in diabetic rats, effect of valsartan treatment on total protein in urine in diabetic rats, effect of Ang (1-7) and valsartan treatment on total protein in urine in diabetic rats. Graph represents the data of total protein in urine of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

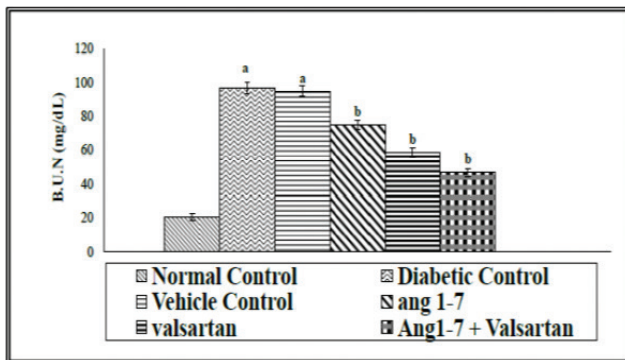


Figure 2: Effect of single administration of alloxan monohydrate on BUN in diabetic control group, effect of Ang (1-7) treatment on BUN in diabetic rats, effect of valsartan treatment on BUN in diabetic rats, effect of Ang (1-7) and valsartan treatment on BUN in diabetic rats. Graph represents the data of BUN of each group on 10th week. BUN denotes blood urea nitrogen; Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

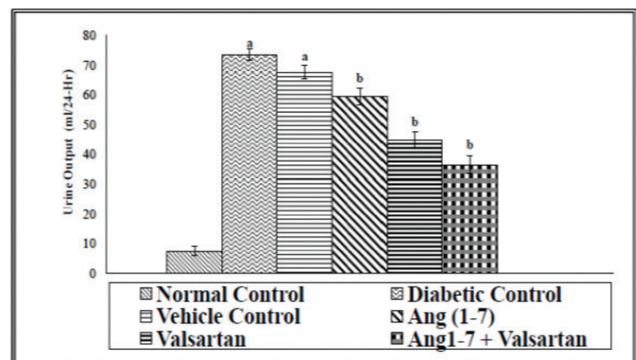


Figure 4: Effect of single administration of alloxan monohydrate on urine output in diabetic control group, effect of Ang (1-7) treatment on urine output in diabetic rats, effect of valsartan treatment on urine output in diabetic rats, effect of Ang (1-7) and valsartan treatment on urine output in diabetic rats. Graph represents the data of urine output of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

alone and in combination for 2 weeks after 8 week of alloxan administration significantly ($p<0.05$) reduced serum creatinine level when compared to diabetic control rats (Figure 5).

Effect of pharmacological interventions on serum nitrite

The concentration of nitrite in serum was noted to be significantly ($p<0.05$) reduced in diabetic rat when compared to normal rat. Treatment with Ang (1-7) (4.8 µg/day; ICV) and valsartan (100 nmol/day; ICV) alone and in combination for 2 weeks after 8 week of alloxan administration significantly ($p<0.05$) increased

the concentration of nitrite in serum of diabetic rat when compared to untreated diabetic rat (Figure 6).

Effect of pharmacological interventions on serum cholesterol and triglyceride

A significant ($p<0.05$) increase in serum cholesterol and triglyceride was noted in diabetic rats when compared to normal rats. Treatment with Ang (1-7) (4.8 µg/day; ICV) and valsartan (100 nmol/day; ICV) alone and in combination for 2 weeks after 8 week of alloxan administration significantly ($p<0.05$) reduces the elevated level of serum cholesterol and triglyceride as compared to diabetic rats. (Figure 7, Figure 8).

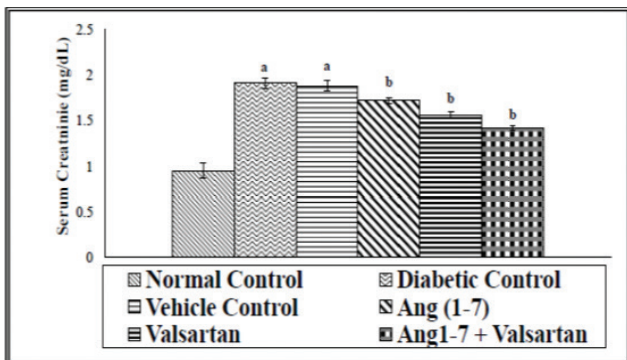


Figure 5: Effect of single administration of alloxan monohydrate on serum creatinine level in diabetic control group, effect of Ang (1-7) treatment on serum creatinine level in diabetic rats, effect of valsartan treatment on serum creatinine level in diabetic rats, effect of Ang (1-7) and valsartan treatment on serum creatinine level in diabetic rats. Graph represents the data of serum creatinine level of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

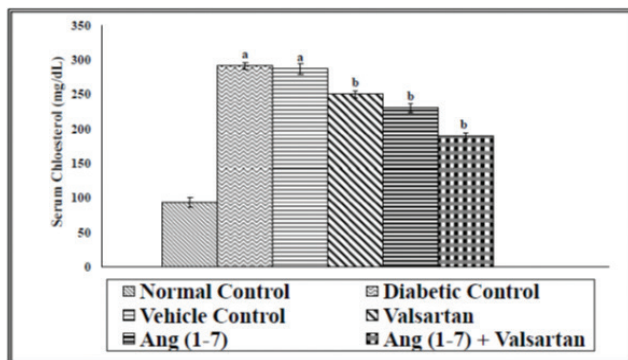


Figure 7: Effect of single administration of alloxan monohydrate on serum cholesterol level in diabetic control group, effect of Ang (1-7) treatment on serum cholesterol level in diabetic rats, effect of valsartan treatment on serum cholesterol level in diabetic rats, effect of Ang (1-7) and valsartan treatment on serum cholesterol level in diabetic rats. Graph represents the data of serum cholesterol level of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

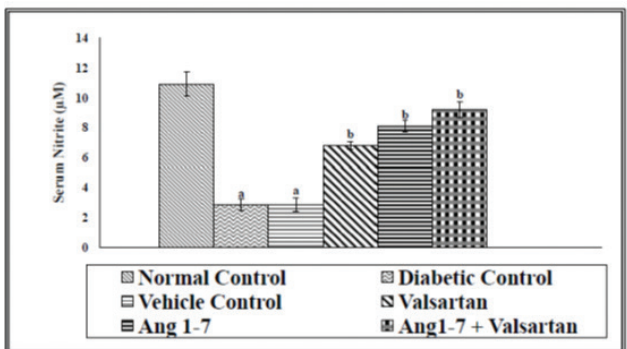


Figure 6: Effect of single administration of alloxan monohydrate on serum nitrite level in diabetic control group, effect of Ang (1-7) treatment on serum nitrite level in diabetic rats, effect of valsartan treatment on serum nitrite level in diabetic rats, effect of Ang (1-7) and valsartan treatment on serum nitrite level in diabetic rats. Graph represents the data of serum nitrite level of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

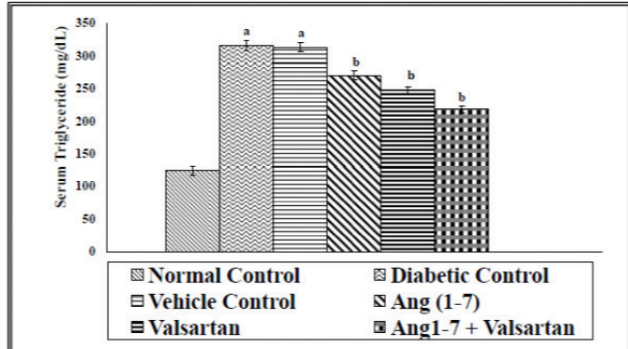


Figure 8: Effect of single administration of alloxan monohydrate on serum triglyceride level in diabetic control group, effect of Ang (1-7) treatment on serum triglyceride level in diabetic rats, effect of valsartan treatment on serum triglyceride level in diabetic rats, effect of Ang (1-7) and valsartan treatment on serum triglyceride level in diabetic rats. Graph represents the data of serum triglyceride level of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

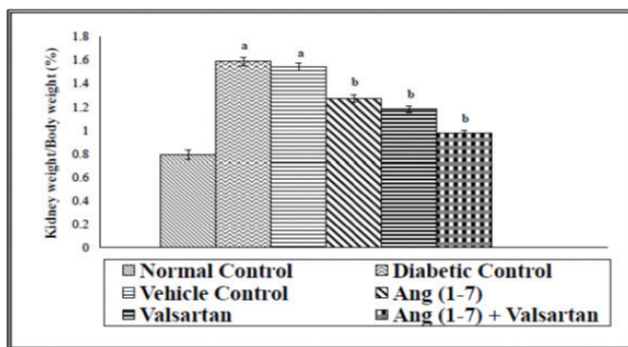


Figure 9: Effect of single administration of alloxan monohydrate on kidney weight / body weight ratio in diabetic control group, effect of Ang (1-7) treatment on kidney weight/body weight ratio in diabetic rats, effect of valsartan treatment on kidney weight/body weight ratio in diabetic rats, effect of Ang (1-7) and valsartan treatment on kidney weight/body weight ratio in diabetic rats. Graph represents the data of kidney weight/body weight ratio of each group on 10th week. Ang (1-7) denotes Angiotensin (1-7). Values are expressed as mean ± S.D, a = p<0.05 vs. Normal control; b = p<0.05 vs. Diabetic control.

Effect of pharmacological interventions on kidney weight and kidney weight / body weight ratio

A significant increase in kidney weight/body weight ratio was noted in diabetic rats when compared to normal rats. Treatment with Ang (1-7) (4.8 µg/day; ICV) and valsartan (100 nmol/day; ICV) alone and in combination for 2 weeks after 8 week of alloxan administration significantly ($p < 0.05$) reduced elevated kidney weight/body weight ratio as compared to diabetic rats. (Figure 9)

DISCUSSION

Single dose administration of alloxan monohydrate (120 mg/kg, *ip*) significantly increased the serum glucose level.³⁸ The increase in serum creatinine, blood urea nitrogen, and protein in urine has been documented to be index of nephropathy.⁴⁰ In the present study, administration of alloxan monohydrate significantly increased the serum creatinine, blood urea⁴¹ and concentration of protein in urine.⁴² A decrease in serum nitrite concentration and increase in ratio of kidney weight/body weight has been reported to be a marker of DN.⁴³ Nephropathy is late complication of DM and renal eNOS production has been reported to be decreased in prolong diabetes mellitus.⁴⁴ In the present study, the ratio of kidney weight/bodyweight was noted to increase while the serum nitrite concentration was decreased in diabetic group as compared to normal rats.

The peripheral RAAS gets overactivated during DN.¹⁴ The principal effect of RAAS is generation of angiotensin II (Ang II) by angiotensin converting enzyme (ACE), which is a potent vasoconstrictor.¹⁹ The increased level of Ang-II further exacerbates the renin release from kidney by decreasing its blood supply, glomerular injury by increasing the intraglomerular pressure, which leads to nephropathy.¹⁵ Angiotensin converting enzyme 2 (ACE 2) which is one of the new element of the RAAS¹⁷ cleaves decapeptide Angiotensin-I (Ang I) and octapeptide (Ang II) to form fragments Ang (1-7) and Ang (1-9)⁴⁵ acts as a major regulator of renin release from juxtaglomerular cells.⁴⁶ The release of renin is increased by the decreased level of ACE 2 and its fragments in the renal cortex.⁴⁷ In the studies from different laboratories, it has been reported that renal release of ACE 2 diminished during diabetes mellitus. Moreover, prolonged hyperglycaemia is noted to decrease the renal concentration of ACE 2 and its fragments.^{24,48,49}

Apart from the peripheral system, a local independent RAS is also present in the brain.⁵⁰ Interestingly, in our study we demonstrated that peripheral renin angiotensin system is controlled by brain renin angiotensin system. This contention is supported by the research of other

laboratories that brain renin angiotensin system is involved in development of hypertension, kidney failure and heart failure.^{26-28,51,52} Central infusion of renin inhibitor i.e. aliskerin significantly decreases the blood pressure and inhibit sympathetic overactivity.⁵³ The ACE 2 and its fragments of the brain play an important role in the regulation of peripheral activity of RAAS.^{13,31} It has been observed that ACE 2 and Ang (1-7) over-expression of the brain reduces sympathetic outflow and decreases the peripheral activity of angiotensin-II.³⁷ Ang (1-7) is generally regarded as an antagonist of Ang II mediated renal injury.⁵⁴ Peripheral renin angiotensin system get disturbed in DN due to increased level of glomerular angiotensin converting enzyme (ACE) and Ang II¹⁴ whereas the level of ACE2 get decreased in diabetic condition.^{22,24} However, it has been documented that inhibition of Angiotensin type 1 receptors (AT1R) in the brain by ICV administration of ACE inhibitor or AT1 blocker is reported to increase formation of cerebral ACE 2 and Ang (1-7) and reduces the central sympathetic outflow and plasma levels of rennin and Ang II.^{30-32,50,55,56}

In our study, 2 week ICV administration of Ang (1-7) in a rat model of DN attenuated the progression of DN, as exhibits by reduced in level of serum creatinine, ratio of kidney weight to body weight, 24-hr urinary volume, blood urea nitrogen, protein in urine, serum cholesterol, serum triglyceride and increased in level of serum nitrite. We found that the valsartan (AT1R blocker) treatment was more effective in ameliorating renal function than Ang (1-7). Valsartan acts by blocking the binding of Ang II to the AT1 receptor thereby prevent the activation of RAS.^{57,58} Combined treatment with valsartan and Ang (1-7) provided better effect as comparison to both drugs treatment alone. These observed renoprotective effects may be due to the decreased sympathetic outflow and peripheral activity of Ang II.

Ang (1-7) and valsartan treatment on diabetic rats did not produce any effects on elevated level of serum glucose, this result propose that the renoprotective effect of valsartan and Ang (1-7) were possibly independent.¹³ Experimental characteristics of the study clearly suggested that ICV administration of Ang (1-7) and valsartan monotherapy and in combination produce beneficial effects in DN by controlling peripheral activity of RAAS noted in terms of biochemical parameters detected in this study.

CONCLUSION

On the basis of above discussion, it may be concluded that ICV administration of Ang (1-7) and valsartan which do not cross blood brain barrier attenuate the

diabetic nephropathy induced increase in level of serum creatinine, ratio of kidney weight to body weight, 24-hr urinary volume, blood urea nitrogen, protein in urine, serum cholesterol, serum triglyceride and decrease in level of serum nitrite. These observed renoprotective effects may be due to the decreased central sympathetic outflow and peripheral activity of Ang II.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

ABBREVIATIONS USED

ACE-2: Angiotensin converting enzyme-2; ACSF: Artificial cerebrospinal fluid; Ang (1-7): Angiotensin (1-7), Ang II: Angiotensin II; AT1 R: Angiotensin type 1 receptors; BUN: Blood urea nitrogen; DM: Diabetes mellitus; DN: Diabetic nephropathy; eNOS: Endothelial nitric oxide synthase; GFR: Glomerular filtration rate; GOD – POD: Glucose oxidase peroxidase; ICV: Intracerebroventricular; NO: Nitric oxide; RAAS: Renin angiotensin aldosterone system; RAS: Renin angiotensin system.

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SUMMARY

- Diabetic nephropathy (DN) is one of the serious problem of diabetes and most common cause of end-stage renal disease.
- Peripheral renin-angiotensin-aldosterone system (RAAS) gets overactivated during the DN.
- The brain plays an important role in the regulation of peripheral activity of RAAS.
- It has been observed that inhibition of angiotensin type 1 receptors and over-expression of ACE 2 and Ang (1-7) in brain reduces sympathetic outflow and decreases the peripheral activity of angiotensin-II.

- In our study, 2 week ICV administration of Ang (1-7) and valsartan (AT1R blocker) in a rat model of DN attenuated the progression of DN.
- The results obtained are indicative of appreciable renoprotective effects which may be due to the decreased central sympathetic outflow and peripheral activity of Ang II.

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