EXPLORING THE ALTERED FUNCTIONS OF RENIN-ANGIOTENSIN SYSTEM (RAS) UNDER DIABETIC CONDITIONS

A Thesis Submitted to University of Hyderabad in Partial Fulfillment of the Requirements for the Award of the Degree of

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BIOMEDICAL SCIENCES

by

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This is to certify that the thesis entitled "**Exploring the Altered Functions of Renin-Angiotensin System (RAS) Under Diabetic Conditions**" submitted to the University of Hyderabad by **Mr. J. Ram Mukka Raju,** bearing the Reg. No: **17BMPH02** for the award of **Doctor of Philosophy in the Biomedical Sciences**, **School of Medical Sciences,** is based on the studies carried out by him under my supervision and guidance. The contents of this thesis in full or in part have not been submitted to any other institute or University for the award of any degree or diploma.

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This thesis is free from plagiarism and has not been submitted in part or in full to this or any other University or Institution for the award of any degree or diploma.

Parts of the thesis have been:

A. Published in the following publications:

Jogula RMR, Row AT, Siddiqui AH. The Effect of Treatment With Aminoguanidine, an Advanced Glycation End Product Inhibitor, on Streptozotocin-Induced Diabetic Rats and Its Effects on Physiological and Renal Functions. *Cureus*. Jul 2023;15(7):e42426. https://doi.org/10.7759/cureus.42426

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2. Presented poster titled "Effect of Aminoguanidine, an Advanced Glycation End Product

Inhibitor, on Rats with Streptozotocin-induced Diabetes: Assessment of Physiological and Renal Functions**. J. Ram Mukka Raju** [1], Anupama T. Row, and Athar H. Siddiqui.

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DECLARATION

I, J RAM MUKKA RAJU, hereby declare that this thesis titled **"Exploring the Altered Functions of Renin-Angiotensin System (RAS) Under Diabetic Conditions"** submitted under the guidance and supervision of **Dr. Athar Habib Siddiqui** is my bonafide research work and is free from plagiarism. I also declare that it has not been submitted in full or in part to this university or any other university or institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in Shodhganga/INFLIBNET. A report on plagiarism statistics from the University Librarian is enclosed. Reference, help, and materials obtained from other sources have been acknowledged.

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My Loving Parents

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ABSTRACT

Background/aim: Diabetes is a multifactorial disease that affects the functioning of the renin-angiotensin system (RAS). The role of advanced glycation end products (AGEs) in inducing diabetic complications is well known. In the present study, we hypothesized that the prevention of AGE accumulation or abrogation of AGE synthesis using an AGE inhibitor, aminoguanidine (AG), in streptozotocin (STZ)-induced diabetic animal models would affect the progression of diabetes and it may delay or prevent the onset of diabetes-related complications. We determined the effects of AG, in STZ-induced diabetic rats by determining various indices of RAS and renal functions. Additionally, we also investigated the effect of the drug, AG, on various hemodynamic and physiological functions in the body of the animals.

Methods: Male Sprague Dawley rats weighing 200-250 g were assigned to four groups (n = 4-6): Vehicle, Vehicle+AG, STZ-induced, and STZ-induced+AG treated rats. Diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (55 mg/kg) dissolved in sodium citrate buffer. The Vehicle rats were injected with only buffer. The blood glucose levels were measured after 48 hours, and animals with blood glucose levels > 300 mg/dL were included in the study. Blood glucose levels in the vehicle rats were also determined to ensure their non-diabetic state. After confirmation, AG was administrated at a dose of 1 g/L in drinking water for two weeks. Urine was collected to measure the urinary protein, urine flow rate, UFR, glomerular filtration rate (GFR), and also to determine the sodium excretion. Blood was collected to measure creatinine and sodium. The kidney tissue was harvested to determine the immune reactivity for renin, $AT_{1,}$ and AT_2 proteins by immunoblotting and to perform histological studies. Similarly, the heart tissue was collected to determine expression levels of AT_1 proteins by immunoblotting. Data were expressed as mean ± standard error of the mean (SEM), and a p-value < 0.05 was considered statistically significant.

Results: Diabetic rats had a significant drop in body weight, accompanied by increased food and water consumption. The diabetic rats exhibited significantly increased urine volume, urine flow rate, and GFR. These phenotypes were significantly or considerately reversed by AG treatment in the STZ+AG-treated diabetic rats. Aminoguanidine prevented the increase in blood sugar levels compared to STZ-induced diabetic rats alone (295.9 ± 50.69 mg/dl versus 462.3 ± 18.6 mg/dL ($p < 0.05$). However, it did not affect the glomerular filtration rate (GFR) and glomerular damage, as assessed by the renal histopathological studies. The STZ-induced diabetic rats had an increased sodium excretion (3.24 ± 0.40 mmol) and significantly increased expression of the AT_2 receptor and that of the AT_1 receptor, which was slightly reversed by the treatment with AG. Treatment with AG decreased sodium excretion (2.12 ± 0.63 mmol/L, as compared to the diabetic rats 3.24 ± 0.40 mmol/L. The STZ-induced+AG treated rats also had modestly decreased expression of the AT_2 receptor (0.99 ± 0.07 versus 1.12 ± 0.08, as compared to the STZ-induced diabetic rats, while the AT_1 receptors showed a slight increase in the STZ+AG-treated rats compared to the STZ-induced diabetic rats (1.1 ± 0.19 versus 1.08 ± 0.12).

Conclusion: This study highlights the action of the drug AG in not exacerbating any damage in diabetic rats. Employing AG as a pharmacological intervention to prevent an increase in blood sugar adds a new dimension to controlling increased blood sugar and preventing diabetic complications. The employability and pharmacological intervention of the drug AG, in diabetes, therefore, need a renewed and further investigation.

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AGEs	Advanced Glycated End Products
AMD	Age-Related Macular Degeneration
AD	Alzheimer's Disease
ACE	Angiotensin Converting Enzyme
AG	Aminoguanidine
AngII	Angiotensin II
AT_1	Angiotensin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
ARBs	Angiotensin Receptor Blockers
Ang-II	Angiotensin-II
AGT	Angiotensinogen
ADH	Antidiuretic Hormones
BCA	Bicinchoninic acid and assay
CVD	Cardiovascular Disease
dL	Deci litres
DM	Diabetic Mellitus
DN	Diabetic Nephropathy
GFR	Glomerular filtration rate
GLUT1	Glucose transporter type-1
GLUT4	Glucose transporter type-4
GOLD	Glyoxal-lysine Dimer
GPCRs	G-Protein – Coupled receptors
G	Grams
H&E	Hematoxylin and Eosin
HD	Huntington's disease
IDF	International Diabetes Federation
IOP	Intra-ocular Pressure
IP	Intraperitoneal
MAP	Mean Arterial Pressure

Jak/STAT	Janus kinase/signal transducers and activators of transcription
µg	Micrograms
µL	Microliters
mL	Milli litre
mMole	Milli mole
Min	Minutes
MAPKs	Mitogen-activated protein kinase
MS	Multiple Sclerosis
OK	Opossum Kidney
PD	Parkinson's Disease
PRA	Plasma Renin Activity
PAS	Periodic acid Schiff
PRR	Prorenin Activity
ROS	Reactive Oxigen Species
RAS	Renin Angiotensin System
SHR	Spontaneously hypertensive Rats
SD	Sprague Dawley
STZ	Streptozotocin
SNS	Sympathetic Nervous System
VSMCs	Vascular Smooth Muscle Cells
WKY	Wistar-Kyoto
WHO	World Health Organization

CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

1.1 Diabetes

Diabetes is a chronic metabolic disease with a high rate of morbidity and mortality on a global scale[1,2]. The well-known clinical symptoms of diabetes are polyuria, polyphagia, and polydipsia[3,4]. Additional symptoms include blurry vision, headache, fatigue, delayed wound healing, and itching of the skin[5]. Diabetes has further been classified into Type 1 and Type 2. The type I diabetes has an autoimmune etiology, whereby the β-cells are destroyed by the body's own antibodies and affects 5-10% of all cases[6,7]. The effective treatment is daily insulin injections to reduce blood glucose levels. Whereas Type II diabetes is caused by a combination of insulin resistance, most likely originating due to hyperinsulinemia[8]. This occurs primarily in the adipose tissue, but also in the liver, subsequently leading to decreased production by β-cells in the pancrease[9,10]. It is estimated that 90-95% of diabetes are type 2[7]. However, diabetes may also manifest during some conditions like pregnancy, drug toxicity, insulin receptor disorders, genetic disorders, endocrinopathies, and in association with pancreatic exocrine disease[11-14] .

The symptoms of diabetes may develop rapidly, typically, within weeks or months, mainly in type I diabetes, while they usually develop gradually and/or may be delayed or sometimes even missing in type 2 diabetes. The major risk factors for diabetes include genetic, environmental, and metabolic factors such as ethnicity, family history of diabetes, and gestational diabetes combined with older age, overweight and obesity, unhealthy diet, physical inactivity, smoking, and the intake of alcohol[15]. Several dietary practices have been linked to unhealthy body weight and/or high intake of saturated fatty acids, high total fat intake, and inadequate consumption of dietary fiber[16]. In addition, a high intake of sugar-sweetened beverages, which contain considerable amounts of free sugars, increases the likelihood of being overweight or obese, particularly among children[17]. Studies have shown an association between a high intake of sugar and an increased risk of the development of diabetes[18,19].

The economic burden of diabetes is high and bears a significant impact on the socio-economic progress and public health worldwide[20]. Although huge awareness has been created of the genesis of diabetes, the incidence of diabetes has increased immensely in developed and developing nations. According to current global estimates from the International Diabetes Federation (IDF), 537 million people worldwide are estimated to have diabetes, and that number is expected to rise to 643 million by 2030[7]. If no effective preventive measures are taken this number is expected to reach over 784 million by 2045, thereby acquiring an epidemic proportion. Approximately 50% of the individuals having diabetes remain undiagnosed, leading to the development of diabetic complications and avoidable or preventive economic burden to the patients. The global health expenditure for diabetes cases is estimated to reach approximately USD 1,027,600 billion by 2030[7].

Over the last three decades, diabetes prevalence has steadily increased in Asian countries, particularly India[21]. Currently, India leads the world with the largest number of cases and this is expected to increase further by 2045[7]. It is estimated that nearly one million children and adolescents under the age of 20 in India have type 1 diabetes. The total expenditure related to diabetes in the region is currently USD 10 billion, which could lead to be one of the considerable medical burdens on the health budget of this country[7].

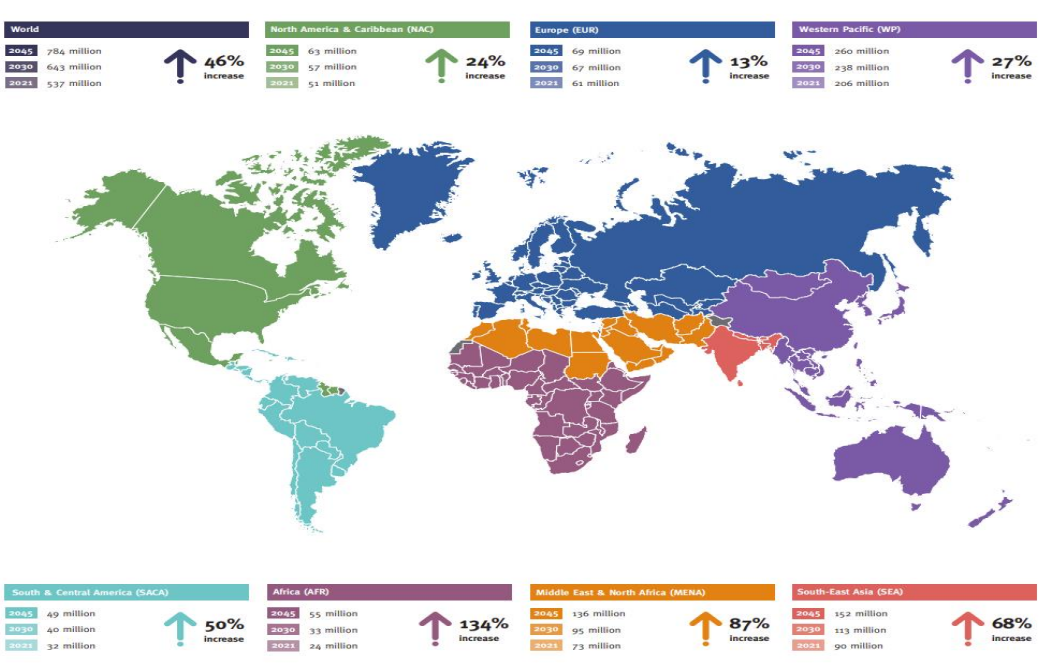

Figure 1.1: Diabetes estimated cases around the world including India

(The international and national statistics adapted from the International Diabetes Federation (IDF) Atlas. 2022)

1.1.1 Complications of Diabetes

Poor glucose control leads to hyperglycaemia coupled with insulin resistance thus initiating factors in the pathogenesis of diabetic complications[22,23]. The complications of diabetes are not limited to one organ and involve various organs of the body including the heart, kidneys, brain, nervous system, and eyes resulting, in various complications such as retinopathy[24], nephropathy [25], neuropathy [26], atherosclerosis, heart attack[27], and stroke[28], respectively. Among all vital organs, the most important ramification of diabetic complications is its effects on kidneys[29]. Various mechanisms and insights have been proposed for the pathophysiology of nephropathy in diabetic conditions[30]. However, the mechanisms by which hyperglycemia can affect renal disease have not been adequately addressed. As a consequence, their life expectancy decreases in comparison to that of the general population. Although the current diabetes treatment has been encouraging, the prevention and control of diabetes complications still remain a challenge. Various hyperglycemia-elicited metabolic and hemodynamic

derangements have been proposed to contribute to renal complications in diabetes[31]. The currently identified mechanisms include increased oxidative stress[32], polyol pathway, glycation end-product formation[33], activation of protein kinase C (PKC), chronic inflammation[34], mitochondrial dysfunction, and activation of the renin-angiotensin system (RAS)[35]. Among these, activation of the renin-angiotensin system and increased glycation end-product formation is considered to be the initial core mechanism leading to diabetic kidney diseases. Therefore, understanding the renin-angiotensin system in the context of renal complications could help develop effective therapeutic strategies against diabetes

1.2 Physiology of Renin-Angiotensin System

The renin-angiotensin system (RAS) is the very important hormonal system that regulates sodium balance/ fluid volume, and arterial pressure[36]. The RAS system comprises renin, angiotensinogen, angiotensin-converting enzyme I, II, Angiotensin, (1-10), II and their principal effector receptors AT_1 and AT_2 and prorenin/renin binding receptor (PRR)[36,37]. Components of the RAS and Angiotensin II receptors are found in the brain and many peripheral tissues such as the heart and kidney but also placenta, testis, adipose tissue, and eye[36].

Table 1.1: List of Renin-angiotensin system components and associated genes

S.no	Components	Genes and Chromosome number
1	Angiotensinogen	AGT-1q42-3
2	Renin	REN, Chromosome number 2 1q32
3	Angiotensin I	--------
4	ACE	ACE-17q23
5	Angiotensin II	--------
6	AT_1	AGTR13q 24
7	AT_2	AGTR2 XP 22
8	Prorenin receptor (PRR)	ATP6AP2 gene on the X-chromosome

Renin is an enzyme primarily produced and released by the juxtaglomerular cells (JG) of the kidney[38]. Renin cleaves the N-terminal portion of angiotensinogen, to form the biologically inert decapeptide Angiotensin I or Angiotensin (1-10)[39]. The amount of renin in the bloodstream is a key rate-limiting step in determining the level of Ang II and the activity of the RAS[39]. The kidney also releases unprocessed prorenin through a constitutive mechanism in addition to this controlled one. Hence, 70% - 90% of renin in human circulation is present as prorenin[37]. There are various mechanisms regulating the renin synthesis and release, namely (i) A renal baroreceptor mechanism (ii) changes in the Na^+ content and the distal tubular macula densa cells (iii) changes in the sympathetic nerve activity via beta-1 adrenergic receptors on JG cells, (iv) negative feedback by the direct action of Ang II via the AT_1 receptors, and (v) effect on renin secretion and synthesis by endothelial factors, such as prostaglandins, nitric oxide, and endothelin[40]. Renin and prorenin are the ligand for the pro renin receptor (PRR). Currently, this receptor has gained much attention since it plays an important role in conditions, such as, metabolic syndrome, diabetes, and hypertension[41]. The binding of prorenin/renin activates the angiotensin II-mediated or independent pathway. Thus, facilitate angiotensinogen generation and promote angiotensin II effects i.e induce the high blood pressure and increased heart rate. In addition, their binding triggers a range of cellular events for example inducing the up-regulation of the pro-fibrotic genes such as TGF-β1 (transforming growth factor-β1), PAI-1 (plasminogen activator inhibitor-1), collagens, fibronectin, as well as up-regulates COX2 (cyclo-oxygenase 2)[42,43]. The increased PRR synthesis could be linked in some way to altered cardiovascular and renal functions.

Angiotensinogen (AGT) prohormone with a molecular weight of 54-56 kDa[36]. The concentration of plasma angiotensinogen depends on angiotensin II levels[44]. Angiotensinogen is synthesized and released from the liver, but angiotensinogen mRNA expression has also

been noticed in many other tissues, including the kidney, brain, heart, vascular, adrenal gland, ovary, placenta, and adipose tissue[45,46].

Angiotensin I is an inactive decapeptide produced by renin from the substrate angiotensinogen[45]. Angiotensin (Ang I), a decapeptide is converted to the functional octapeptide Angiotensin II by the hydrolytic action of angiotensin-converting enzyme I (also known as ACE I)[47]. Although angiotensin-converting enzyme (ACE) is the major catalyst for the conversion of angiotensin I to angiotensin II, other enzymes including tissue plasminogen factor, cathepsin G, tonin, and chymase can also catalyze the conversion of angiotensin I into II. The known biological activity of the Angiotensin I peptide is still missing.

The exopeptidase enzyme Angiotensin-converting enzyme,) was identified and characterized in the 1950s by Skeggs and colleagues[48]. Angiotensin-converting enzyme (ACE) is a zinc metalloprotease[49]. It is localized on the plasma membranes of various cell types, such as vascular endothelial cells, microvillar brush border epithelial cells (e.g., renal proximal tubule cells), and neuroepithelial cells[50]. The main function of the Angiotensin-converting enzyme (ACE 1) is to remove the C-terminal dipeptide (His-Leu) to form the functional octapeptide Ang II[51].

Angiotensin II, (Ang II), is a powerful vasoconstrictor hormone or effector of this system[52], angiotensin (Ang) II, is produced by sequential cleavage of peptides derived from the substrate molecule angiotensinogen[36]. Angiotensin II binds to specific receptors (AT_1 and AT_2), triggering a broad range of biological actions impacting virtually every system in the body including the brain, heart, kidney, vasculature, and the immune system[53-55]. In addition, it stimulates the production of aldosterone by the zona glomerulosa of the adrenal cortex which helps in sodium reabsorption in the kidney, via the hormone Aldosterone[56]. Angiotensin II is also a potent growth modulator and proinflammatory peptide.[57] In addition, this peptide

degrades bradykinin, a vasodilator[58 59]. The heptapeptide angiotensin III may also stimulate aldosterone production[60]. Alteration in the activation of the Renin-Angiotensin System (RAS) can contribute to the development of hypertension, cardiac hypertrophy, and heart failure, via a number of pathways involving the kidneys, heart, and blood vessels[61,62].

Over the past few years, other angiotensin peptides, like Angiotensin IV, and especially angiotensin-(1-7), have been shown to selectively mediate different RAS effects[63]. With regards to the angiotensin-(1-7), this heptapeptide is formed from Angiotensin I by prolyl-endopeptidase or from Angiotensin II via prolyl endopeptidase, prolyl carboxy peptidase or mainly by ACE-2, an enzyme homologous to ACE-1[63]. Angiotensin-(1-7) binds to a G-protein coupled receptor, namely MAS receptor, and, in general, plays a counter-regulatory role to that of RAS by opposing the vascular and proliferative effects of angiotensin II[64]. Many experimental studies have provided ample support for the counter-regulatory effects of the RAS axis in diabetes[64]. A few studies have also provided insights into the role of MAS receptors in diabetes[65].

Angiotensin II is an octapeptide hormone that mediates its effects via various receptors, which include AT_1, AT_2, AT_3, and AT_4[36]. Among all the receptor types, the type 1 receptor (AT_1), and the type 2 receptor, (AT_2) are the most well-studied[66]. The Angiotensin II type I receptor (AT_1) and type II receptor (AT_2), belong to the family of G-protein-coupled receptors, (GPCRs)[67]. The AT_1 receptor can activate various signaling pathways. In the G-protein dependent pathway, it stimulates G-protein Gq/11, leading to the activation of phospholipases A2, C, and D. This results in the production of inositol trisphosphate (IP3), which triggers calcium signaling, and the activation of protein kinase C isoforms and MAPKs[68]. It also activates several tyrosine kinases, scaffold proteins, and the nuclear factor-kB pathway in smooth muscle[69]. In the G-protein independent signaling pathway, the AT_1 receptor can utilize arrestin-mediated signaling to activate MAPK and the Jak/STAT pathway[70]. Furthermore, the

AT_1 receptor can also signal through other G-proteins, such as Gi/o and G11/12[71]. These signaling pathways have various downstream effects, including the stimulation of various sodium transporters like the Na, H-exchanger, Na, K-ATPase, and Na/HCO3 co-transporter in the kidney and heart[72,73]. The receptor AT_1 is expressed prominently in vascular smooth muscle cells (VSMCs), endothelial cells, endometrium, kidney, liver, adrenal gland, ovary, brain, testis, lung, heart, and adipose tissue[36]. The AT_1 receptor is responsible for the classical actions of Angiotensin II such as vasoconstriction, aldosterone release from the adrenal zona glomerulosa, salt retention in the renal proximal tubules, heart contraction, cell growth in the cardiac left ventricle and in the arterial wall and stimulation of the sympathetic nervous system[36]. After exerting its effects, this receptor undergoes desensitization and internalization or phosphorylation, through its C-terminal region[74].

The Angiotensin II, type 2 receptor, the AT_2 receptor primarily acts through the Gi proteins and tyrosine phosphatases to exert inhibitory effects on cellular responses mediated by the AT_1 receptor and growth factor receptors[36]. Both (AT_1 and $AT_{2)}$ receptor subtypes have similar Angiotensin II-binding properties but differ in genomic structures, localization, tissue-specific expression, and regulation. The Angiotensin II, type 2 receptor (AT_2) is extensively expressed in the fetus, whereas its expression is hugely decreased after birth, being restricted to a few organs such as the brain, adrenal, heart, kidney, myometrium, and ovary[73]. Studies have shown that in the kidney the AT_2 receptor mRNA is mainly localized in the proximal tubules, glomeruli, juxtaglomerular apparatus, interlobular arteries, arcuate arteries, afferent arterioles, collecting ducts, and outer medullary descending vasa recta[75]. The AT_2 receptor plays an important role in embryonic development, growth, differentiation, regeneration of tissue, and cell death[36,75].

An angiotensin II receptor that is not blocked by either losartan (angiotensin II receptor antagonist that selectively blocks the AT_1 receptor subtype) or PD 123177 (a selective

antagonist for the AT_2 receptor subtype) has been classified as AT_3[76]. Evidence suggests that the AT_4 receptor activation, via angiotensin IV, is an important mediator of the expression of plasminogen activator inhibitor-1[77].

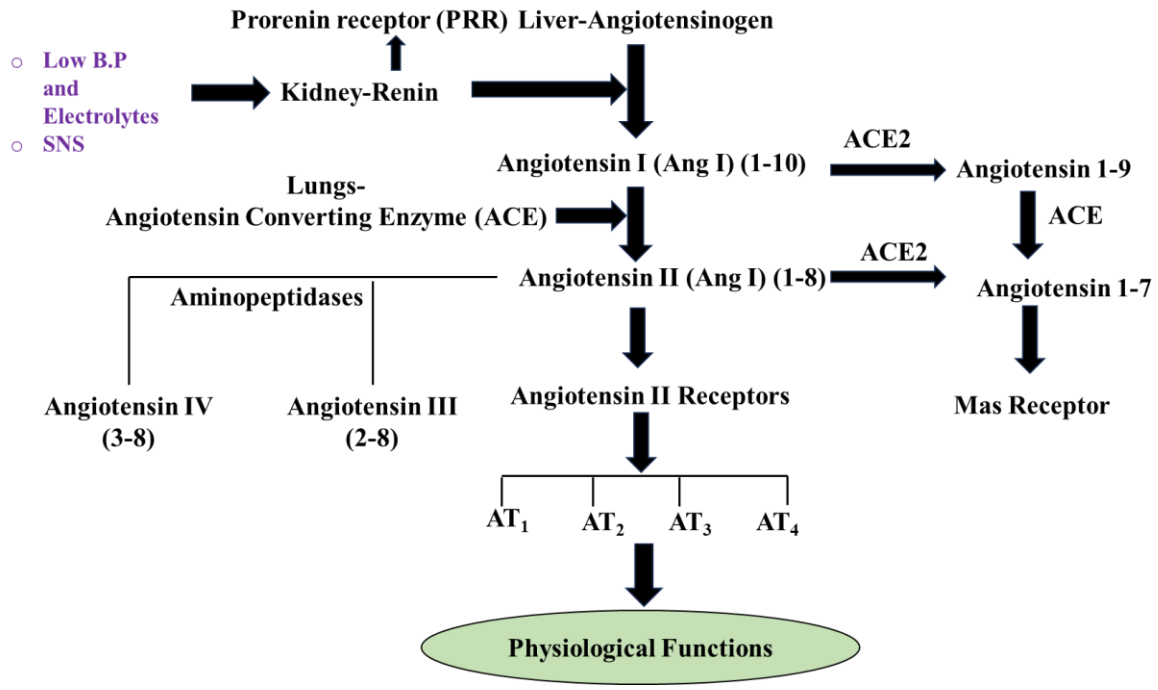

Figure 1.2: Renin-angiotensin system components synthesis, and their integrated function

1.3 Physiological Relevance of Renin-Angiotensin System, RAS, Biology/ The importance of RAS Cascade

The renin-angiotensin system (RAS) plays a significant role in various physiological processes apart from blood pressure regulation[36]. In response to decreased blood pressure or blood volume, angiotensin II causes the blood vessels to constrict, raising blood pressure, and also stimulates the release of aldosterone from the adrenal glands. Aldosterone acts on the kidneys to increase the reabsorption of sodium and water and promote the excretion of potassium. It also stimulates H^+ secretion and $HCO3^-$ reabsorption in both proximal and distal tubules thus regulating H^+-ATPase activity in the collecting tubule[78]. The activation of apical Na^+/H^+ exchanger and basolateral $Na^+/HCO3^-$ cotransporter, as well as Na+, K+-ATPase, and

apical H+-ATPase, are implicated in angiotensin II-induced transcellular sodium and bicarbonate reabsorption within the proximal tubule[79]. Whereas Na+/H+ exchange and H^+-ATPase contribute to the reabsorption of sodium and bicarbonate in distal tubules[79]. This mechanism is vital for regulating fluid balance and electrolyte concentrations. The hormone angiotensin II also constricts the efferent arterioles, to help maintain the glomerular filtration rate (GFR)[80]. The RAS promotes the release of another hormone called antidiuretic hormone (ADH) or vasopressin, which increases water reabsorption in the kidneys[81]. Angiotensin II has direct effects on the heart muscle, promoting cardiac hypertrophy (enlargement of the heart muscle cells), and fibrosis. In the brain angiotensin II stimulates the release of antidiuretic hormone (ADH) from the posterior pituitary gland[82]. The ADH reduces urine output and helps to maintain blood pressure[82]. It is important to note that angiotensin II is involved in a complex regulatory system, and its effects can vary depending on the physiological and pathological conditions of the body[55]. Collectively, these cascades of actions and reactions lead to increased blood pressure, with mediation by the heart, kidneys, blood vessels, brain, and adrenal glands [73].

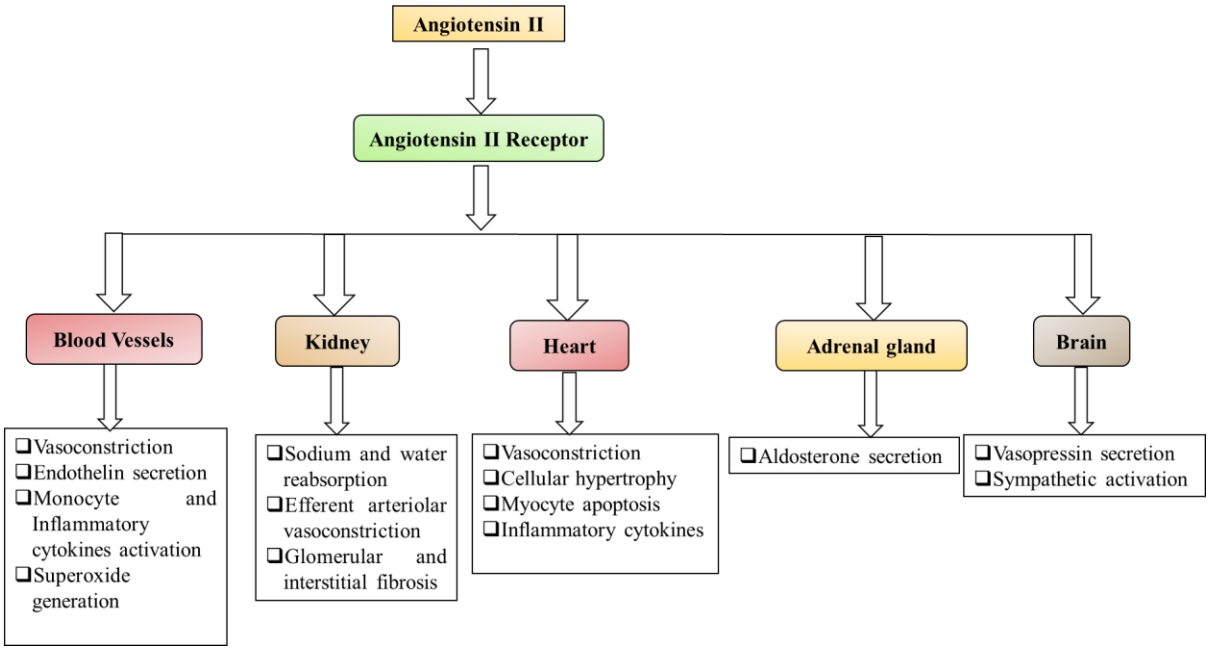

Figure 1.3: Functions of angiotensin II hormone on different organs/tissues

1.4 Alteration of Renin-Angiotensin System (RAS) in Various Pathological Conditions

The abnormal activation/ implication of (systemic or intra renal) renin-angiotensin system (RAS), can have pathological consequences like hypertension, heart failure, chronic kidney disease, eye disease (age-related macular degeneration (AMD), and glaucoma), and neurological disease (Alzheimer's disease (AD), Parkinson's disease (PD), stroke, multiple sclerosis (MS), and Huntington's disease (HD)[83-85]. Factors that contribute to RAS dysregulation include increased or decreased renin secretion, angiotensinogen production, angiotensin-converting enzyme (ACE) activity, or angiotensin receptor sensitivity[86,87].

1.4.1 Hypertension

The overactive or dysregulated renin-angiotensin system can lead to chronic elevation of angiotensin II levels. This chronic activation causes an increased action of Ang II via increased activation of the Ang II receptors, mainly the AT_1 receptors. This can result in hypertension, which is a major risk factor for cardiovascular diseases, including heart attacks, strokes, and heart failure

In addition, the alteration of the renin-angiotensin system (RAS) is closely associated with the development of preeclampsia, a hypertensive disorder that occurs during pregnancy[88]. Preeclampsia is characterized by high blood pressure and signs of organ damage, usually involving the kidneys and liver[88].

1.4.2 Chronic Renal Disease

The elevated angiotensin II production in the kidney causes inflammation, fibrosis[89], and modulation of Na+, K+, and ATPase pump[90]. Thus, dysregulated RAS contributes to renal damage, and impaired sodium and water balance, affecting the overall physiological balance of the ions and water, and consequently to the progression of kidney disease. The upregulation

of angiotensinogen (AGT), angiotensin II (Ang II), and AT_1 receptor expression within the podocytes of the kidney during DN has also been previously reported[91].

1.4.3 Eye Disease

1.4.3.1 Age-Related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) is characterized by the degeneration of the macula, the central part of the retina responsible for sharp vision[92]. Similarly, in AMD there is an upregulation of angiotensin-converting enzyme (ACE), which leads to increased production of angiotensin II. Thus, angiotensin II promotes inflammation and oxidative stress, contributing to retinal vascular damage[93] and age-related macular degeneration[92].

1.4.3.2 Glaucoma

Glaucoma is a group of eye conditions characterized by optic nerve damage, often associated with increased intraocular pressure (IOP). The effector hormone, angiotensin II has been shown to contribute to the regulation of IOP by modulating the resistance of aqueous humor outflow through the trabecular meshwork[94], thus leading to the build-up of the Aqueous humor, and subsequently to an increased IOP. Additionally, the RAS has been implicated in the pathogenesis of optic nerve damage and retinal ganglion cell death in glaucoma[95]. Thus, dysregulation in RAS is also a causative factor for Hypertensive Retinopathy.

1.4.4 Neurological Diseases

Activation or Dysregulation within the renin-angiotensin system (RAS) signaling has been implicated in the pathogenesis of various neurological diseases[96].

1.4.4.1 Alzheimer's Disease (AD)

Increased levels of angiotensin-converting enzyme (ACE) and angiotensin II (Ang II) have been observed in the brains of individuals with Alzheimer's Disease (AD). The increased action of angiotensin II can contribute to neuroinflammation, oxidative stress, and the accumulation

of amyloid-beta plaques, which are characteristic features of Alzheimer's Disease pathology[96]. Moreover, activation of the angiotensin type 1 receptor (AT_1 receptor) has been implicated in promoting cognitive decline and neuronal damage in Alzheimer's Disease[97]. Studies have also shown that increased expression of ACE, Ang II, and AT_1 receptors is associated with the loss of dopaminergic neurons in the brains of Parkinson's Disease patients[98,99].

1.4.4.2 Ischemic Stroke

During an ischemic stroke, the activation of the RAS can exacerbate brain damage. The effector hormone of the RAS system, angiotensin II, through AT_1 receptor activation, contributes to vasoconstriction, inflammation, oxidative stress, and neuronal cell death in the ischemic brain[100]. Therapies targeting the RAS, such as ACE inhibitors or AT_1 receptor blockers, have shown potential for reducing post-stroke brain injury[101].

1.4.4.3 Multiple Sclerosis (MS)

Altered RAS signaling has been implicated in Multiple Sclerosis (MS), an autoimmune neurodegenerative disease. Studies have reported increased expression of ACE and AT_1 receptors in the brains of Multiple Sclerosis patients. The hormone, Angiotensin II has been shown to promote neuroinflammation, blood-brain barrier dysfunction, and immune cell activation in Multiple Sclerosis[102]. Modulating the RAS components may represent a therapeutic approach in MS management.

1.4.4.4 Huntington's Disease (HD)

The RAS has been implicated in Huntington's Disease (HD), a genetic neurodegenerative disorder. Dysregulation of RAS components, including ACE and angiotensin II levels, has been observed in the brains of Huntington's Disease patients and mouse models. The hormone

angiotensin II is shown to contribute to neuronal dysfunction, oxidative stress, and neuroinflammation in Huntington's Disease[103].

Currently, ACE inhibitors angiotensin II type 1 receptor blockers (ARBs), and sympathetic inhibitors (α, and β adrenergic blockers) are extensively used to control the blood pressure and management of these conditions including renal diseases. However, further research is needed to fully understand the intricate mechanisms underlying the altered RAS and to develop effective therapeutic interventions. Therapeutic targeting of the renin-angiotensin system (RAS) is the most validated clinical strategy for slowing down or delaying the onset of the changes associated with these conditions.

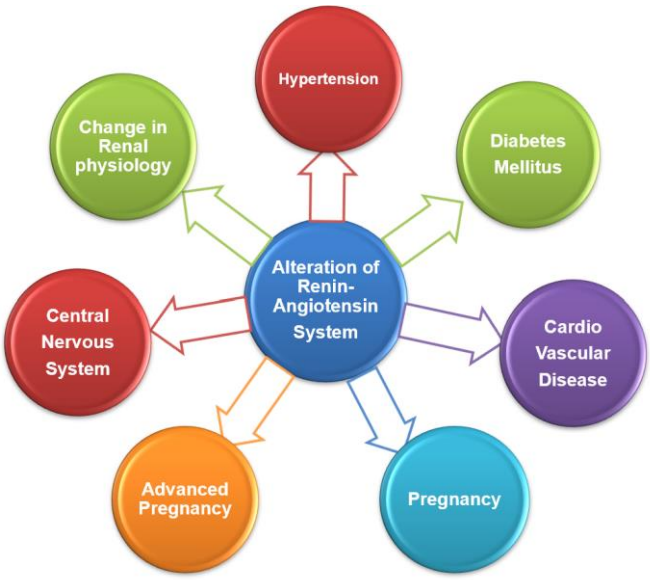

Figure 1.4: The central role of the renin-angiotensin system for various physiological functions and pathological conditions.

1.5 Renin-Angiotensin System Associated with Diabetic Complications

The most common cause of end-stage renal disease in the world is diabetes[22]. Along with various complications of diabetes, it is further associated with complications of the kidneys[104].

The most common complications of diabetes are DN (Diabetic nephropathy), Retinopathy, Neuropathy, and Cardiovascular disease (CVD)[105]. Many studies have reported that the alteration of RAS components in association with the endocrine system particularly angiotensin II (Ang II), and dysregulated signaling within the RAS have caused a change of renal hemodynamic and nonhemodynamic effects[106] resulting in not only the development of metabolic syndrome, hypertension, endothelium dysfunction[106], but also contributing to chronic renal disease[107].

1.5.1 Altered Renin-Angiotensin System Associated with Renal Disease in Diabetes

i) Altered Renin-Angiotensin System (angiotensin II) Effect on Renal Hemodynamic Parameters

The implication of angiotensin II contributes to various changes in various renal processes and functions[108]. Firstly, the upregulation of angiotensin II levels can cause vasoconstriction of the renal arterioles, thereby reducing renal blood flow, and hence impaired autoregulation of glomerular filtration rate (GFR)[109]. This is mostly prevalent in early diabetic conditions. Consequently, it can affect renal perfusion and glomerular filtration rate (GFR). These hemodynamic changes contribute to renal dysfunction and the development of diabetic nephropathy[110]. In addition, the changes in the aldosterone levels in the kidney, mediated and influenced by angiotensin II, can impact renal hemodynamics by altering fluid balance, including ionic and water balance and blood volume[111]. Furthermore, prolonged activation of the renin-angiotensin system (RAS) can lead to increased production of inflammatory mediators (MCP-1, IL-6, and TGF-β)[57,69,112]. These mediators induce and promote the deposition of extracellular matrix proteins which contribute to renal tissue damage and fibrosis[113]. The pathological changes can impair renal hemodynamics by reducing the

functional capacity of the kidneys[114]. Additionally, it has been reported that angiotensin II also reduces the glomerular filtration coefficient while increasing afferent and efferent arteriolar resistances, which contributes to a decrease in the GFR in chronic diabetic conditions[115,116].

Moreover, numerous experimental studies have also reported that acute infusion of angiotensin II, causes changes in renal hemodynamics, and thereby a sustained elevation of intrarenal angiotensin II induces proteinuria, which is accompanied by a progressive injury of the glomerular filtration barrier (composed of the glomerular endothelium, glomerular basement membrane, and podocytes), a hallmark of diabetic nephropathy[117]. A previous study from Mullier et al. showed that hyperglycaemia affects renal function by activating the RAS, via exerting an increase in plasma renin activity (PRA), mean arterial pressure (MAP), and renal vascular resistance[118]. In addition to these actions, angiotensin II is involved in the augmentation of tubulo–glomerular feedback sensitivity and inhibition of pressure-natriuresis, mediated by the AT_1 receptor[119].

ii) Altered Renin-Angiotensin System (angiotensin II)- Effect on Non-Hemodynamic Parameters:

Diabetes is associated with increased oxidative stress[120], characterized by an imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms[121]. The pro-oxidant effects contribute to oxidative stress in diabetes mediated by angiotensin II[122]. This oxidative stress plays an important role in the development of diabetic complications[123]. Dysregulated RAS signaling, particularly, angiotensin II contributes to endothelial dysfunction in diabetes[124]. This impairs endothelium-dependent vasodilation, promotes oxidative stress, and stimulates the release of endothelin-1, the most potent vasoconstrictor[124]. Endothelial dysfunction plays a crucial role in the development of cardiovascular complications associated with diabetes [125]. In diabetes, the implication of angiotensin II signaling can contribute to

abnormal cell growth and tissue remodeling, such as mesangial cell and mesangial and tubular epithelial cell hypertrophy[123,126]. These cellular changes are also implicated in the development of diabetic renal complications[127]. Furthermore, insulin resistance, which is caused by interference of the insulin-stimulated increase in insulin receptor substrate 1-associated PI3K activity in diabetes is also mediated by angiotensin II[128,129]. In addition, Giacchetti et al. in their studies have also shown that the renal RAS is clearly activated in DM, with increased tissue angiotensin II that leads to the development of diabetic nephropathy[130].

In diabetic nephropathy, alteration of RAS (angiotensin II) causes Na+K+ATPase modulation, inflammation, and fibrosis in the kidney proximal tubules[131]. Thus, dysregulated RAS contributes to the retention of sodium, hyperfiltration leading to renal damage, and progression of kidney diseases[131,132]. In addition, there is an upregulation of angiotensinogen (AGT), angiotensin II (Ang II), and AT_1 receptor expression within the podocytes of the kidney. Similarly, there is an upregulation of angiotensin-converting enzyme (ACE), which leads to increased production of angiotensin II.

Clinical trials on RAS component inhibitors like ACE inhibitors for example captopril, enalapril, fosinopril, and perindopril, and three common and angiotensin receptor blockers (ARBs)-for example irbesartan, losartan, and valsartan show a significantly decreased incidence of renovascular complications in diabetic patients[133]. The ACE inhibitors show their protective actions upon skeletal muscle, pancreatic islets, and enhanced insulin sensitivity associated with decreased adipocyte as well as increased transcapillary glucose transport[129].

Studies have reported that not only angiotensin II shows a significant influence on diabetes kidney disease, but other components within the RAS have also been found to play a significant role in renal complications[134]. A study conducted by Zimpelmann, J[135]., and colleagues reported that early-stage diabetes causes the stimulation of mRNA expression for renin in the

proximal tubule STZ-induced diabetic rats[135]. This finding suggests that diabetes has an impact on the regulation of renin production at the genetic level within the proximal tubule of the kidneys [135]. *In-vivo* study on transgenic mice overexpressing rat angiotensinogen (AGT) gene in the kidney, had significantly increased blood pressure, albuminuria, and renal injury; and administration of ARB or ACE inhibitor reversed these abnormalities in diabetes[136].

Previous studies have shown that angiotensin (1–7) serves a protective role by counteracting the effects of locally-generated angiotensin II in cardiovascular research, but on the contrary, a study has shown, that chronic angiotensin (1–7) accelerates renal injury and diabetic nephropathies in STZ induced rats[137,138].

1.5.2 Altered Renin-Angiotensin System Associated with Diabetes as well as Cardiovascular Disease

Cardiovascular complications are the primary cause of morbidity, mortality, and fatalities in individuals with diabetes[132]. These complications include conditions such as coronary artery disease, stroke, heart failure, peripheral artery disease, and others[139]. People with diabetes have a higher risk of developing these cardiovascular conditions compared to those without diabetes. Diabetes can lead to the development of atherosclerosis (hardening and narrowing of the arteries) vascular abnormalities, and organ damage. Proper management of diabetes, including control of blood sugar levels, blood pressure[132], and cholesterol, is crucial for reducing the incidence and severity of cardiovascular complications[140] and improving overall patient outcomes. According to the World Heart Federation and World Health Organization (WHO), risk factors for cardiovascular disease are mainly genetic, unhealthy diet, physical inactivity, use of tobacco, and use of alcohol[141]. Regular medical care, lifestyle modifications, and adherence to prescribed medications are important in reducing the impact of cardiovascular complications in individuals with diabetes[141].

The alteration of cardiac function in diabetics occurs through several different mechanisms, mainly, as, i) decreased glucose transport ii) carbohydrate oxidation, increase in free fatty acids (FFA) utilization, iii) decrease in sarcolemma calcium transport, and iv) alterations in myofibrillar regulatory contractile proteins[142]. Patients with diabetes often experience compromised cardiac glucose metabolism, impacting glucose uptake, glycolysis, and pyruvate oxidation[143]. This is primarily due to reduced glucose transport into heart cells via GLUT 1 and GLUT 4[144]. Hence, to manage cardiovascular dysfunction in diabetes, medications like beta-blockers, angiotensin-converting enzyme inhibitors, and angiotensin II receptor blockers are commonly clinically used[145]. These drugs help to improve overall cardiac function associated with diabetes[145].

The pathophysiological mechanisms of RAS's association with diabetes and cardiovascular disease (CVD) remain highly puzzling. In fact, studies reported that renin-angiotensin system (RAS) components, including renin, angiotensinogen, ACE, and Ang II receptors, are upregulated in various cardiovascular diseases (CVD) including cardiac injury, myocardial infarction, hypertension, and heart failure during diabetes[146]. For instance, angiotensin-converting enzyme (ACE) inhibitors, like perindopril, ramipril, and AT_1 receptor blockers improved cardiovascular morbidity and mortality in patients with diabetes[147]. The inhibition of angiotensin-converting enzyme (ACE) also prevented atherosclerosis and myocardial infarction in diabetic apolipoprotein E-deficient mice[148]. There is evidence that angiotensin II binding to its AT_1 receptors mediates cardiovascular damage by inducing reactive oxygen species generation, tissue inflammation, fibrosis, and apoptosis[69]. A study reported that prolonged hypertension could be manifested in rats with streptozotocin (STZ)-treatment to induce diabetes[149].

It has been reported that angiotensin II ($AT_{1)}$ receptor blockers reduce systemic inflammation and renal oxidative stress in diabetic patients and protect against diabetic nephropathy. A study

suggests that losartan, an angiotensin receptor blocker (ARB), could prevent proteinuria in type 1 diabetic patients[149]. The experimental results of Romero-Nava, R et al. suggest that diabetes with hypertension changes the mRNA and protein expression of angiotensin II receptors (AT_1 and AT_2)[86]. However, the overexpression of AT_2 could be associated with the reduction in the response to Ang II in the early stage of diabetes. In a study Musial, D. C. et al indicated that increased ACE activity leads to sympathetic dysfunction in streptozotocin (STZ)- induced diabetic rats[150]. The experiments reported by Kala P, et al., on nephrectomised rats showed that endothelin type A receptor blockade increases renoprotection in congestive heart failure combined with chronic kidney disease[151]. Similarly, the study conducted by Collett JA, et al. investigated the role of AT_1 receptor expression in the kidneys of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats in the development of hypertension and found that there was an elevated level of AT_1 receptor expression in the kidneys of SHR compared to WKY rats[152].[153] One of the major risk factors for hypertension related to chronic hyperinsulinemia and obesity is excess sodium retention by the kidneys[10]. Experiment study on opossum kidney (OK) cells, proximal tubule cell line) treated with insulin revealed that the AT_1 receptor is upregulated upon activation by ang II and it also produces greater stimulation of sodium transporters leading to an increased renal sodium reabsorption[154]. Thereby, indicating an increased action of the ang II receptors under hyperinsulinemia conditions, a typical manifestation of hyperglycemia[154]. In this regard, pharmacological inhibitors of the synthesis or activity of ang II, angiotensin-converting enzyme (ACE) inhibitors have proven immensely useful in cardio-vascular and renal therapeutics[147].

1.6 RAS Inhibitors in Treatment of Diabetes

The most commonly used drugs targeting the angiotensin II system in hypertension and diabetes are (i). Angiotensin-converting enzyme (ACE) inhibitors[155], (ii) Angiotensin receptor

blockers (ARBs), those that selectively block Angiotensin II type 1, ($AT_{1)}$ receptors,[156] and thereby block the various downstream actions of angiotensin II, and (iii) Renin inhibitors[157].

Angiotensin II receptor antagonists as well as ACE inhibitors have been reported and shown to slow down the progression of diabetic glomerular injury and decrease proteinuria in hypertensive patients with chronic renal failure[126]. A recent study suggested that captopril, an ACE inhibitor, offers protection against diabetic nephropathy (kidney damage) and neuropathy (nerve damage) by targeting multiple mechanisms[158]. There are mounting evidences that the combination therapy with both ACE inhibitors and ARBs is more beneficial compared to a single therapy[147]. Previous studies documented that synergic effect by using ACE inhibitors and AT_1-receptor antagonists in spontaneous hypertensive rats[159], and STZ-induced diabetic rats[160]. Treatment with captopril and olmesartan has been found to be beneficial in experimental models of diabetic rats containing albuminuria and podocyte injury[161]

A study Kohzuki et al. showed Cardiovascular and renal protective effects of losartan in spontaneously hypertensive rats with diabetes mellitus[162]. In addition, combination therapy of spironolactone (belongs to potassium-sparing diuretics), and moexipril also showed an improvement of renal structure and function in experimentally induced diabetic hypertensive nephropathy rats[163]. However, treatment with ACE inhibitors, angiotensin receptor blockers, and spironolactone are contraindicated in pregnancy as they may cause fetal damage[164]. Antihypertensive drugs known to be effective and safe in pregnancy include methyldopa, labetalol, and long-acting nifedipine, while hydralazine may be considered in the acute management of hypertension in pregnancy or severe preeclampsia[88].

Furthermore, though renin inhibitors (Aliskiren) have also emerged as a potential therapeutic strategy to block RAS and lower blood pressure during diabetes[157]. Although, the use of these agents is still not common. However, there is limited information on an important early link

between hyperglycaemia and complications and a consequence of pathogenic mechanisms in diabetes. Therefore, studying underlying mechanisms to control/ prevent/ manage blood glucose levels in the increasing diabetic population and its associated complications is the way forward for developing therapeutic strategies.

1.7 Advanced Glycation End Products

Glycosylation is an enzymatic post-translational modification that plays crucial roles in protein folding, trafficking, stabilization, cell-to-cell interaction, and function[165]. Proteins are also susceptible to post-translational modifications that could alter their structure, function, and half-life during normal aging and pathological conditions such as diabetes[166,167]. One such post-translational modification is non-enzymatic glycation and the formation of advanced glycation end products[167,168].

The advanced glycation end products (AGEs) were first described by Louis Camille Maillard in 1912. It was identified initially by the Maillard reaction–a process in which food proteins cross-link with monosaccharides and form a yellow-brown change in the colour of the food. The chemical reactions involved in the Maillard reaction were first recognized by Hodge, et al. in 1953[169]. The Advanced glycation end products (AGEs) are complex heterogenous substances. Although advanced glycation end-product formation occurs at a lower rate over a lifetime, it occurs more rapidly in clinical conditions such as diabetes[170]. When humans are exposed to exogenous AGEs, via tobacco, smoke, and diet, they accumulate in the circulating blood and various tissues, resulting in various disease complications[171]. Furthermore, food processing methods, such as prolonged heating, and microwave cooking can also accelerate the AGE formation[171].

Approximately a dozen forms of AGEs have been detected in tissues[172]and they have been classified into three categories[166] (i). Fluorescent cross-linking AGEs, such as

Vesperlysine, pentosidine, Argpyrimidine, and crossline (ii). Non-fluorescent cross-linking AGEs, such as glyoxal-lysine dimer (GOLD), deoxy glucose-lysine dimer (DOLD), and methylglyoxal-lysine dimer (MOLD), alkyl formyl glycosyl pyrrole (AFGP), imidazolium lysine, and arginine-lysine imidazole (ALI), and (iii) non-cross-linking AGEs based on their chemical structure such as pyrraline and N-carboxymethyl lysine (CML), and carboxyethyl lysine (CEL). Recently, scientists also discovered novel types of AGEs that exhibit cross-linking and their unique protein-bound properties[173]. In addition, recent findings also revealed that modern food habits add several critical AGE compounds which are formed during high-heat processing as well as heating of animal proteins and high-caloric dietary products[171].

The formation of advanced glycation end products (AGEs) is a progressively complex, and gradual process in the body[174]. The advanced glycation end products (AGEs) formation is divided into three major mechanisms: (i) the first is the Schiff base generation. The initial process of Schiff bases are early and unstable advanced glycation end products (AGEs), they are formed when the electrophilic carbonyl group of a sugar (aldose or ketose) reacts with an amine group, particularly those found in arginine or lysine residues of protein[174]. However, they can also undergo irreversible reactions with amino acid fragments of proteins, leading to the formation of new protein crosslinks.[175] The second stage is the formation of a more stable Amadori product [175]. Over a period of time, the reversible Schiff base or Amadori product can undergo rearrangement reactions through the process known as the Amadori rearrangement[175]. During this rearrangement, the carbon-nitrogen bond within the sugar-protein adduct undergoes shift and reorganization[174]. These reactions involve dehydration, condensation, and rearrangement of molecular structures. These rearrangements lead to the formation of reversible intermediates. The final stage of AGE formation involves the irreversible and complex modification of proteins, lipids, or nucleic acids. The rearranged

products from the Amadori rearrangement undergo additional chemical modifications, leading to the formation of diverse and heterogeneous AGEs[174,175].

The advanced glycation end products are not only produced from glucose but also from dicarbonyl compounds produced from auto-oxidation and the degradation products of glucose such as glyoxal, methylglyoxal, and 3-deoxyglucosone or α-hydroxy aldehydes such as glyceraldehyde's and glycolaldehyde[175].

The formation of stable advanced glycation end products (AGEs) shows numerous consequences including their action as cross-linkers between proteins, resulting in the production of protein-resistant aggregates[176]. These products show deleterious effects by modifying the biological properties of extracellular matrix (ECM) proteins such as elastin, collagen, and laminin via crosslinking and altering the functional and mechanical properties of the target tissues[177]. The intracellular accumulation of AGEs in the endoplasmic reticulum (ER) impairs the folding of proteins and induces ER-mediated stress, leading to inflammation or cellular apoptosis[178]. In addition, advanced glycation end products (AGEs) also crosslink mitochondrial protein leading to mitochondrial protein dysfunction and thereby contributing to a disturbance in the electron transport chain thus, reducing ATP synthesis and enhancing free radical generation[179]. The formation of AGEs is influenced by various factors which include hyperglycemia[180], oxidative stress[181], lipid oxidation[182], and increasing age[183]. The accumulated advanced glycation end products are metabolized and eliminated by the liver and kidney[184]. Dysfunction of these organs in the body can, therefore contribute to the development and progression of various complications[184].

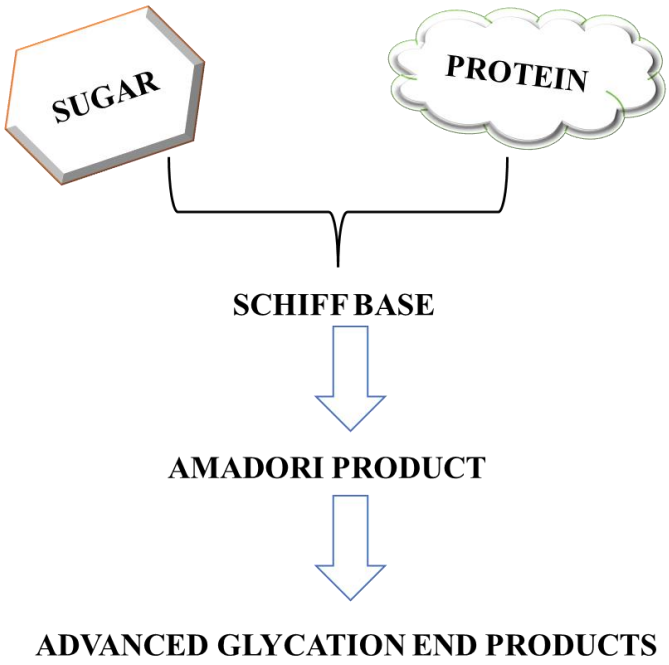

Figure 1.5: Generation of Advanced glycation end-product formation

1.7.1 Receptors For The Advanced Glycation End Products

The receptor for advanced glycation end products (RAGE) is a group of cell surface receptors belonging to the immunoglobulin superfamily that play a significant role in various physiological and pathological processes[185,186]. The binding of advanced glycation end products (AGEs) to the receptors for advanced glycation end products (RAGE) activation triggers many intracellular signaling pathways[186]. This receptor was expressed in different cell types, including endothelial cells[187], immune cells[188], neurons[189], and renal cells[190]. They have been implicated in the pathogenesis of various diseases, including diabetes[190], cardiovascular diseases[191], neurodegenerative disorders[192], and inflammatory conditions[188]. The receptor (RAGE)-mediated signaling can promote chronic inflammation, endothelial dysfunction, and tissue fibrosis, leading to the development and progression of these conditions[193].

Furthermore, RAGE signaling has been associated with the activation of nuclear factor kappa B (NF-κB), a transcription factor, and the production of pro-inflammatory cytokines, such as

interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)[194,195]. The interaction between AGEs and RAGE also contributes to oxidative stress through the generation of reactive oxygen species (ROS) and the impairment of antioxidant defense mechanisms[196].

The receptors for AGEs (RAGE) have other isoforms including soluble RAGE (sRAGE), endogenous secretory RAGE (esRAGE), and human RAGE secreted (hRAGEsec) and they play an important role in AGE clearance[197]. The S100-calgranulins (pro-inflammatory cytokines), amphoterin, amyloid-beta, and fibrillar are a few other ligands that bind to RAGE Targeting the RAGE receptors and the RAGE-AGE interaction has emerged as a potential therapeutic strategy for managing various diseases associated with AGE accumulation and RAGE-mediated pathologies, including diabetes[198]. Inhibition of RAGE signaling or blockade of AGE-RAGE interactions may help attenuate inflammation[199] oxidative stress,[199] and tissue damage,[199] offering potential benefits in the prevention and treatment of conditions such as diabetic complications[194], cardiovascular diseases[200], and neurodegenerative disorders[201].

The accumulated advanced glycation end products (AGEs) also elicit downstream effects through their interaction with other cellular receptors which including AGE-R1, AGE-R2, and AGE-R3/galactin-3, the ezrin, radixin, and moesin (ERM) family of receptors[202]. These receptors are found in many cell types such as macrophages[203], epithelial cells[204], podocytes[205], mesangial cells[206], and endothelial cells[207].

The receptor RAGE serves as a key receptor for AGE and plays a crucial role in mediating the cellular effects of AGEs. This increased response to AGEs, as indicated by increased RAGE expression in diabetic blood vessels[208] and kidneys[190], suggests that RAGE may contribute to the development and progression of diabetic vascular[209] and renal complications[208,210].

Advanced glycation end products AGEs/ Receptor of AGE (RAGE) axis in endothelial cells activates and triggers the activation of various pathways[211]. One of the important signalling

mechanisms associated with diabetes is the NFKB pathway[194,209], which results in the expression of cytoskeleton cell adhesion proteins (Vascular Cell Adhesion Molecule (VCAM), intercellular Adhesion Molecule (ICAM), and adhesion molecule-1) and proinflammatory cytokines (Interleukine-6 (IL-6), IL-1β, and Tumor Necrosis Factor-alpha (TNF-α), indicating AGEs enhance their permeability and thereby leads to increased inflammation[195,212,213]. Similarly, AGEs/RAGE signaling in vascular smooth muscle cells (VSMCs) activates metalloproteases (MMP-2/9), inflammatory cytokines and chemokines, and endoplasmic reticulum stress pathways resulting in proliferation and extracellular matrix degradation, along with impaired autophagy[214] and lysosomal degradation[211].

1.7.2 Advanced Glycation End Products (AGEs) In The Pathophysiology of Diabetes

In diabetes, there is a persistent elevation of blood glucose levels, known as hyperglycaemia, which provides an increased substrate for the non-enzymatic glycation process [215]. The increased glycation affects the structure and function of proteins, thus, disrupting their molecular conformation and altering enzymatic activity[216]. This process also interferes with ligand-binding interactions[215,217]. Advanced glycation end products (AGEs) form cross-links with both intra- and extracellular structural proteins through non-receptor-mediated or receptor-mediated pathways leading to resistance to proteolytic cleavage and increased stiffness[218]. For example, AGE cross-linking on type I collagen and elastin[218]. A previous study suggested that intracellular AGEs interact with their receptor (RAGE) and alter the intracellular metabolic pathway[217] as well as gene expression of pro-inflammatory molecules,[195] and induce the release of free radicals[212]. The accumulated advanced glycation end products, therefore interfere with the normal functioning of the cells through multiple actions like apoptosis, inflammation, protein dysfunction, mitochondrial dysfunction[219], and oxidative stress[195]. The researchers, Brownlee et al in the 1980s first described the deleterious

effects of AGE formation on the renal and cardiovascular systems in humans and showed how aminoguanidine (AG), (an inhibitor of advanced glycation end product formation), prevents diabetes-induced arterial protein cross-linking in rats[220]. Accumulation of AGEs generates oxidative species/radicals that lead to the generation of oxidative stress further hastening up the generation and accumulation of AGEs[195].

In diabetes, the impaired function of the kidneys can contribute to the accumulation of AGEs because the kidney is the major site of clearance of AGEs[221]. The accumulation of these modified proteins can impair normal protein turnover and degradation processes[221]. An *in vivo* study on Streptozotocin-induced diabetic mice on a high-AGE diet exhibited increased serum levels of inflammatory markers (TNF-α and IL-6) along with significant injury to organs like the kidney and heart[214]. The advanced glycation end products (AGEs) activate an autophagy signaling pathway in β-cells, thereby resulting in increased apoptosis and decreased insulin secretion due to β-cells destruction[222]. The accumulated advanced glycation end products (AGE) and their cross-linking with collagen lead to altered arterial and myocardial compliance and increased vascular stiffness, often leading to diastolic dysfunction and systolic hypertension seen in diabetic patients[223]. Accumulating evidence suggests that CML (Nε-(carboxymethyl) lysine, which is a specific advanced glycation end product (AGE) - is highly prevalent in diabetes[224]. Dicarbonyl stress caused by the formation and accumulation of methyl glyoxal (MGO) (a reactive glucose metabolite) and its reduced detoxification by glyoxalase 1 (GLO-1), during diabetes results in the development of diabetic nephropathy[225]. In mature podocytes, AGEs activate Notch 1 signaling, which could lead to proteinuria or glomerular disease during diabetes[226].

1.7.3 Advanced Glycation End Products in Various Disease as well as Diabetic Complications

Elevated level of AGEs is one of the key risk factors that has been associated with the pathology of diabetic complications such as diabetic nephropathy[180], neuropathy[227], retinopathy[228], and cardiovascular disease[229] and others such as arthritis[230], liver[231], and neurodegenerative disease[232]. Extensive studies demonstrated that AGEs are associated with a disease incidence in several organs through a common mechanism of oxidative stress inflammation[233], cross-linking with extracellular matrix proteins[234], and neovascularization[235]. In diabetic nephropathy, AGEs accumulate within the renal tissue[236], including the glomeruli and tubules of the kidneys rendering the development and progression of kidney damage[236]. They can also crosslink with proteins in the glomerular basement membrane, impairing its permeability and hence the filtration function[237]. This results in the leakage of proteins, including albumin, into the urine, a condition known as proteinuria[237]. The binding of accelerated advanced glycation end products (AGEs) with RAGE promotes inflammation within the kidneys and stimulates the production of pro-inflammatory cytokines and chemokines, via activating inflammatory pathways[190]. This chronic inflammation contributes to the progressive damage to the renal tissue[190]. In addition, the formation of advanced glycation end products (AGEs) increases oxidative stress within the kidneys, leading to an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms[238]. This imbalance therefore an increased oxidative stress, which damages the renal cells, exacerbating the progression of diabetic nephropathy[238,239]. The excessive accumulation of extracellular matrix proteins due to AGEs also contributes to renal fibrosis[240]. Renal fibrosis results in the scarring of the kidney tissue and the loss of normal kidney function [240]. Impaired autophagy due to AGE accumulation can lead to the build-up of dysfunctional cellular components and contribute to the progression of renal damage[241]. Therefore,

accumulation of AGEs and managing blood sugar levels, are crucial strategies for preventing and slowing the progression of diabetic nephropathy[241]. Recent studies have also shown that AGEs also contribute to the micro and macrovascular disease associated with diabetes[242]. The presence of advanced glycation end products (AGEs), can directly affect the structure and function of blood vessels, thus rendering thickening and stiffening of blood vessel walls[243]. This process, known as vascular fibrosis, leads to impaired blood flow and can result in conditions like diabetic vascular and peripheral artery disease[243].

The activation of the receptor for advanced glycation end-products (RAGE), leads to alterations in cell signaling pathways, and endothelial cell dysfunction which in turn triggers inflammation, atherogenesis, and vasoconstriction, thereby leading to thrombosis and coronary dysfunction in diabetic cardiovascular disease[244].

In atherosclerosis, a condition characterized by the formation of plaques in the arterial walls, advanced glycation end products (AGEs) promote the oxidation of low-density lipoproteins (LDL), enhancing foam cell formation, and facilitating the migration and proliferation of smooth muscle cells in the arterial walls. These processes lead to the formation of fatty plaques that narrow the arteries and increase the resistance to blood flow, thereby contributing to hypertension[245]. In addition, the higher level of AGEs can impair HDL function and metabolism, leading to decreased levels of this protective lipoprotein[246]. This disruption in HDL concentration can have negative implications for cardiovascular health and contribute to the development of cardiovascular complications[247]. This also highlights the increasing incidence of hypertension in diabetic patients, over a period of time. It is very well known that diabetics end up with hypertension at some point of time, as a consequence of diabetes[247].

Advanced glycation end products (AGEs), can also directly impact cardiac tissue[248]. They accumulate in the myocardium and effects the cardiomyocytes, leading to impaired

contractibility and increased stiffness of the heart muscle[248]. This can result in the development of diabetic cardiomyopathy, a condition characterized by structural and functional abnormalities of the heart [248].

Research findings have indicated that AGEs can disrupt the normal functioning of retinal pericytes, which are cells that play a crucial role in maintaining the integrity of blood vessels in the retina[228]. Specifically, advanced glycation end products (AGEs) have been found to interfere with two key processes: autophagy and migration of retinal pericytes in diabetic retinopathy[249].

Diabetic condition has an increase in the glycation process affecting myelin and cytoskeleton proteins[250]. The presence of AGEs on myelin has the potential to trap immunoglobulin proteins such as IgG and IgM[250]. This trapping effect contributes to the demyelination of both neurons and nerves, which is a characteristic feature of diabetic neuropathy[250].

The increased liver AGE levels induce hepatic injury and acute liver failure due to the activation of the RAGE receptor. Additionally, studies have reported the presence of glycated tau protein in the brains of individuals with Alzheimer's disease. This protein is responsible for the production of oxidative stress and is associated with the formation of neurofibrillary tangles, which are characteristic of the disease.

In patients with rheumatoid arthritis, the binding of high concentrations of the potential proinflammatory ligand, namely S100, to the receptor for advanced glycation end products (RAGE), triggers the production of autoantibodies and leads to inflammation[251]. Furthermore, glycation and AGE formation have been identified to occur at the gene level, specifically affecting DNA and histones[252,253]. This process can result in errors during DNA replication and transcription, ultimately promoting mutations that are responsible for diabetic embryopathy[252].

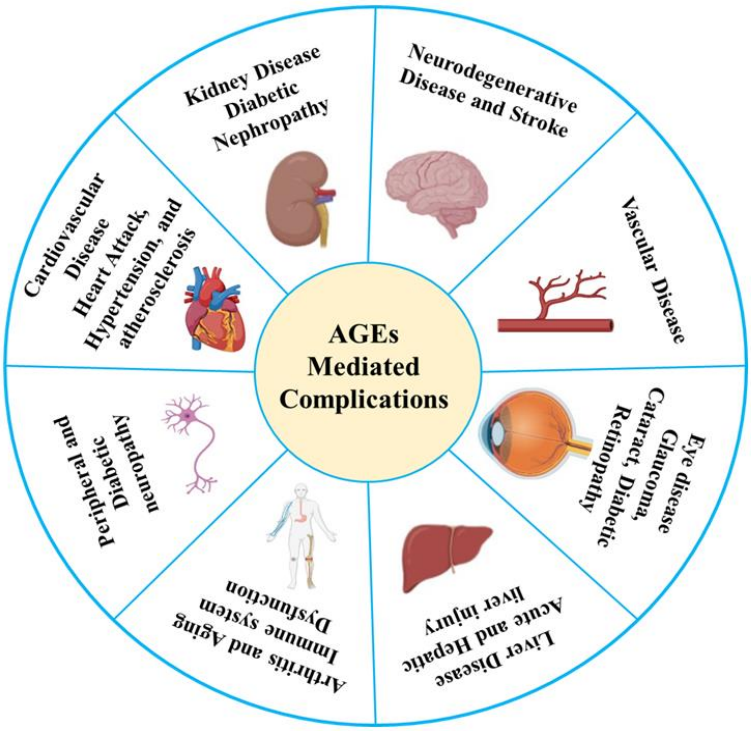

Figure 1.6: Advanced glycation end products mediate the various metabolic disorders

1.7.4 Therapeutic Inhibitors of AGEs in Diabetes

According to many reports, advanced glycation end products (AGEs) formation has been linked to diabetes and various other disorders and their complications[254,255]. Traditionally, various drugs are available for the management of diabetes[256]. However, current therapeutic options are not adequate, and intensive therapy for diabetic have their own limitations. Therefore, prevention of AGE formation could also be a way forward for the effective management of diabetes and its related complications. Currently, few drugs reduce the damage induced by AGEs in diabetes[257]. These agents include i) AGEs breakers (alagebrium and TRC4186) or inhibitors (Aminoguanidine, (AG)) ii) angiotensin II receptor antagonists (losartan), and iii) angiotensin-converting enzyme inhibitors (Ramipril), iv) natural flavonoids (ascorbic acid, *Psoralea corylifolia L*. Seed Extract, alpha-lipoic acid, forskolin, carnosine, and quercetin)[257].

The drug, Aminoguanidine, (AG) was the first AGEs inhibitor employed in diabetic complications that prevented the accelerated formation of AGEs *in vitro and in-vivo* experimental STZ-induced diabetic rat model[258,259]. Clinical trials have determined AGE-modified lipids or proteins to assess the impact of therapeutic options against AGEs, but the effects of these approaches on AGEs still remain unclear. Future pharmacological interventions employing these therapeutic approaches against AGEs formation and/or preventing its binding to RAGE are vital to evaluate the benefits of an anti-AGE and RAGE-antagonist treatment in diabetic patients.

GAPS IN EXISTING RESEARCH

There are various therapeutic options available for diabetes control, but the major challenges are their efficacy, adverse effects, other complications, and more importantly not knowing the exact mechanism by which the treatments work, which is proving to be disadvantageous. Although the treatments are available, the frequency of patients reaching till End Stage Renal Disease (ESRD) stage is increasing. The major mechanism leading to diabetic complications is the alteration and differential regulation of the renin-angiotensin system, elevated blood sugar levels, and resultant AGEs that may activate multiple pathways upon interaction with their plasma membrane receptor (RAGE). The inhibition of RAS by angiotensin-converting enzyme (ACE) inhibitor or angiotensin II (Ang II) type-1 receptor (AT_1R) blocker has been shown to suppress the development and progression of nephropathy in both type-1 and type-2 diabetic patients. Moreover, previously Matsui T, and colleagues, found that RAS blockers could inhibit the AGE-elicited mesangial cell hypertrophy and proximal tubular cell injury. Similarly, Fukami K, and colleagues, found that RAS blockers inhibit DNA damage and detachment of podocytes *in vitro*. In addition, studies have also revealed that RAS inhibition by ramipril suppressed diabetes or AGE-induced MMP-2 activation *in vivo and in vitro*. Hence, we, therefore, hypothesize that the inhibition of the early formation of AGEs and their subsequent accumulation could not only prevent diabetes-associated complications but also can contribute to protection against various complications related to diabetes.

HYPOTHESIS

The altered function of the renin-angiotensin-system (RAS) is a very common ramification of diabetes, leading to renal (acute or chronic diseases) and cardiovascular complications (angina, myocardial infarction, and stroke). The dysregulation of the renin-angiotensin system (RAS) and the formation of Advanced Glycation End Products (AGEs) are the major factors that contribute to Diabetic complications. Therefore, we hypothesized whether treatment with Aminoguanidine (AG), an AGE inhibitor could attenuate the diabetic complications in diabetes. Because accumulation of AGEs has been known to progress at an accelerated rate in Diabetes, leading to increased complications. Hence, we proposed to employ various kidney functions and markers as our tool to monitor their role in Diabetic and Non-Diabetic conditions. Additionally, we have used the heart to monitor cardiovascular functions. These two parameters, majorly, serve as an index of diabetic complications, including hypertension. Angiotensin II acts via its receptors (AT_1 and AT_2) which has a potent effect that regulates the renal excretory mechanism, especially Na^+ levels by the kidney. The hormone Ang II also regulates vasoconstriction that controls blood flow, hence regulating Blood Pressure. Therefore, the levels of the Ang II receptors (AT_1 and AT_2), were determined in the diabetic animals that could reflect on the functionality of these receptors under diabetic conditions and *vis a vis* hypertension.

OBJECTIVES

1. ESTABLISHMENT OF ANIMAL MODEL FOR DIABETES IN SD (SPRAGUE DAWLEY) RATS

a) Developing the STZ- induced Diabetic Rat model

b) Developing the STZ-induced diabetic rats and the Employability of AG in STZ- STZ-induced rats- to study the complication of diabetes in the presence of AG (Aminoguanidine), an inhibitor of Advanced Glycated End Products.

2. TO INVESTIGATE THE EFFECT OF AMINOGUANIDINE (AG) IN STREPTOZOTOCIN-INDUCED (STZ-induced) DIABETICS RATS

a) Determination of various parameters as indicators of renal injury

b) Determination of various parameters as indicators of renal functions

c) Renal histopathology, assessed as a marker for renal damage

3. PROTEIN EXPRESSION ANALYSIS OF ANGIOTENSIN II RECEPTORS

1) Protein expression analysis of Angiotensin II receptors (AT_1, & AT_2) and Renin from the kidney

a) AT_1, AT_2 Renin in the Kidney (IB)

2) Protein expression analysis of Angiotensin II Receptor (AT_1) from Heart

b) AT_1 Heart (IB)

CHAPTER 2: MATERIAL AND METHODS

2.1 Objective 1: Establishment of a Diabetic Animal Model

2.1.1 Introduction

Animal models provide a good association between basic and translational research by providing *in-vivo* conditions to study the disease pathobiology[260]. They are also the most readily available tools to test therapeutic drugs, their safety, and efficacy before they can be used in clinical trials[261,262]. However, it is important to use them ethically and responsibly to recognize their limitations and differences from humans and to properly acknowledge their contributions and sacrifices made to the research for the betterment of humankind[263]. Even though a number of *in vitro* [264,265]and *insilico* [266]studies are available and have improved consistently over the last few decades, animal models still remain an effective method for understanding the complex etiology[267], pathogenesis[268], and multi-systemic interactions[269] under diabetic conditions. A number of animal models, such as rats[270], mice [271], and non-rodent animals for example Invertebrate animal model-Bombyx mori, Primate model-obese Rh monkey, zebra fish, hamsters, and pigs[272]. Other species with inherited diabetes symptoms for example Chinese hamster, TUCO-TUCO[272], etc. are used for to understand diabetes pathogenesis since they share many similarities with humans in terms of physiology, anatomy, and genetics[273]. These animal models have been developed using different strategies such as chemical induction, (Alloxane[274]or Streptozotocin[275]), surgical (pancreatectomy model)[276], virus-induced (Coxsackie B virus-induced model[277], and EMC virus-induced model[278]), genetically engineered and induced (KK mouse[279], Obese hyperglycemic mice[280], AKITA mice[267], Zucker diabetic fatty rats, db/db[281], GK rats[282]), spontaneous autoimmune (NOD) mouse and the Bio-Breeding (BB) rat[267], KDP rats[283], LETL[284], and LEW-iddm[267]). Since diabetes is a metabolic syndrome that reflects the complex integration of body systems, careful consideration is needed in choosing the correct animal model to be used in different *in vivo* experiments. This allows researchers to study the various aspects of the disease and the effects

of diabetes on various organs and tissues, such as the pancreas, liver, kidney, heart, brain, and blood vessels. They have also been used to study the effects of diabetes on various functions, including cognitive, cardiovascular, renal, circulatory, and ocular (Rats, and Mice). By using these diabetic animal models researchers established specific animal model by monitoring the changes in blood glucose levels, insulin production, and other physiological and clinical parameters related to diabetes.

Table 2.1: Various animal models for diabetic complications (Adapted from Kottaisamy, C. et al.)

s.no	Animal Models	Complication of Diabetes	Characterization
1	NOD mice, C57BL/6, GK rat, Zucker diabetic fatty rat, Zebrafish	Diabetic Nephropathy	Enlarged glomeruli and mesangial sclerosis, Albuminuria and reduced renal functions, thickening of glomeruli leading to glomerular hypertrophy, Glomerulosclerosis, Tubulointerstitial fibrosis, and renal hypertrophy, Overexpression of CIN85/RukL causing edema
2	Alloxan-induced model, BB rats, OLETF rats, STZ-induced model, GK rats	Diabetic Cardiomyopathy	Formation of advanced glycation end products leading to oxidative stress, Reduced calcium—stimulated ATPase activity and cardiac contractility, Alteration in left ventricular diastolic function, Fibrosis, and apoptosis leading to myocardial damage, Hyperglycemia, hyperlipidemia, and cardiac cell death
3	Alloxan-induced model, Akita mice, db/db mouse, Surgical model, Zebrafish	Diabetic Retinopathy	Microaneurysms with increased acellular capillaries, Decreased number of amacrine and ganglion cells, Reduced number of Retinal ganglion cells with thickened retina, Formation of proliferative and contractile cellular membranes in the retina, Degradation, and thinning of the retina
4	STZ-induced model, C57BL/KS (db/db) mice, Ischemic reperfusion injury	Diabetic Neuropathy	Reduced fiber size of the peroneal nerve and axon than that of the myelin sheath with impaired motor function, Decreased sensory nerve conduction velocity and

	model, Chinese Hamster, Obese Rh Monkey		density of intraepidermal nerve fibers (IENF), Decreased serum IL-10 level and nerve conduction velocity and nerve fiber density, Reduced conduction velocity, Reduced conduction velocity and prolonged duration of F-wave latencies

Despite the animals models mentioned above, streptozotocin-induced Sprague Dawley(SD) rats extensively used in diabetic research since 1963 due to their easy availability and short generation interval, more biological similarity to human diabetes[267]. Moreover, this model mimics the Type 1 diabetes in humans, making them the first choice of animal models in diabetes research[285]

Feeding of a high-fat diet, and unrestricted Calories intake, have been extensively used in diabetic research[286], however, there are some limitations, when such animal models have not resulted in the development of diabetes in the animals[287]. With the STZ treatment, the chances of developing diabetes have a higher success rate, which requires just a single IP injection and a sustained high level of glucose in the blood[288]. Hence, STZ that has been considered advantageous over other methods

Streptozotocin (STZ) is an antibiotic isolated from *Streptomyces achromogenes* in 1960, that causes pancreatic islet β-cell destruction[289]. Several animal species, including the mouse, rat, and monkey, are sensitive to the pancreatic β-cell cytotoxic effects of STZ[289], with the rabbit being the least. Therefore, chemical induction with STZ is most often used to induce diabetes and related complications in rats and mice[290].

Sprague Dawley (SD) rats are a strain of albino rats that have been bred specifically for use in medical research [290]. They are popular due to their easy handling, disease-free nature, cost-effective, docile nature, and good adaptability to laboratory environments[290]. The SD rats share many characteristics with humans, and very useful model for studying diabetes[290]. Based

on dosage concentrations of streptozotocin these rats sooner developed insulin resistance, hyperglycaemia, and metabolic abnormalities similar to those found in human diabetes[291]. These rats are larger than other commonly used diabetic animal models, such as mice. In addition, rats make them easier to monitor, handle, and collect samples[292]. In addition, these rats have larger organs and tissues, making them easier to use for histological, biochemical, and molecular analysis [292]. Therefore, we have employed the SD rats in our study, and induced by STZ to studying diabetes-related renal complications.

There are numerous published research papers that have used streptozotocin (STZ) to induce diabetes in SD rats to study the development of diabetes and the progression of their associated complications. The below-mentioned table below shows that such studies

Table 2.2: List of literature used STZ-induced SD rats in various diabetic complication

S.No	Title of the study	Description	References
1	Downregulation of the renin-angiotensin system in 4-wk STZ-diabetic rats restored by insulin therapy	Results from this study suggested a downregulation of the renin-angiotensin system (RAS) at the mRNA expression level in 4-week STZ-diabetic rats, which was restored by insulin replacement therapy. It indicated that insulin may directly or indirectly regulate the RAS.	293
2	Reduction of the accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy	The findings of the present study suggest an interaction between the RAS and advanced glycation in experimental diabetic nephropathy	294
3	The breakdown of preformed advanced glycation end products reverses erectile dysfunction in	The AGE breaker was found to improve erectile function, increase smooth muscle content, and decrease collagen content in the penile tissues of diabetic rats	295

	streptozotocin-induced diabetic rats: Preventive versus curative treatment		
4	Renal angiotensin II AT_2 receptors promote natriuresis in streptozotocin-induced diabetic rats	Identified that increased expression of the tubular AT2 receptors contributes to enhanced urinary sodium excretion in STZ-treated rats.	[296]
5	Impaired angiotensin II AT1 receptor function and enhanced Na, K-ATPase affinity for sodium in the proximal tubule of streptozotocin-treated diabetic	This data suggests that the higher basal NKA affinity for Na, possibly due to lower Ser-phosphorylation of α1-subunit and not the AT_1 receptor function, in the PTs may be responsible for increased renal Na reabsorption associated with early stage of streptozotocin-induced diabetes	[297]
6	Protective effects of angiotensin-converting enzyme inhibitors on diabetic retinopathy	This study investigated the protective effects of the angiotensin-converting enzyme (ACE) inhibitor captopril on diabetic retinopathy in STZ-induced diabetic rats. Captopril was shown to reduce retinal vascular leakage and inhibit neovascularization, suggesting its usefulness in preventing diabetic retinopathy	[298]
7	Corneal Complications in Streptozotocin-Induced Type I Diabetic Rats	This study seeks to characterize corneal functions and complications in a streptozocin (STZ)-induced rat model of type I diabetes mellitus (DM) and to understand the pathogenesis of diabetic keratopathy	[299]
8	Renal sympathetic nervous system hyperactivity in early streptozotocin-induced diabetic kidney disease	This data demonstrates an early role for the renal sympathetic innervation in the pathogenesis of DKD	[300]

9	The role of spironolactone on myocardial oxidative stress in rat model of streptozotocin-induced diabetes	The use of spironolactone reduced nitrite generation and improved vitamin E levels independent of blood pressure	301

There are growing body of evidence showing that during diabetes high glucose levels in the systemic circulation contribute to the acceleration of glycation of proteins and their accumulation in the blood or tissues[242]. This allows the slowly formation of the advanced glycation end products (AGEs),[302] and their accumulation triggers the insulin resistance[254,303], ROS production[255], oxidative stress[195], and inflammation[180]. Thus, causing deterioration of tissues, and progression of pathological complications of diabetes such as cardiomyopathy[304], atherosclerosis[305], nephropathy[306], and neurovascular diseases[307]. Many studies have been found a relationship between the severity of vascular complications and the level of accumulation of AGEs[255,305,308]. Recent studies have shown that the accumulation of AGEs contributes to the activation of nuclear NF-κB polymorphisms[213] leading to the oxidative stress[195] and inflammation[180].

The drug, Aminoguanidine also known as Pimagedine is an inhibitor of advanced glycation end-product formation and has been studied for its potential effects on diabetes treatment in various animal models[309]. Specifically, it has been investigated in the context of streptozotocin (STZ)-induced SD diabetic rats[258]. The *invitro* and *invivo* studies have shown the beneficial effects of aminoguanidine (AG) in ameliorating or preventing complications caused in experimental diabetic models[220,258]. In the STZ-induced diabetic rats, the administration of aminoguanidine has been shown to have several effects, viz. reduction of nitric oxide production [310] and to preserve the function viability of pancreatic beta cells[258] [220,310]. These effects help to maintain insulin secretion and hence improve glucose control in

diabetic models[311]. Despite the demonstrable inhibition of AGE formation by AG under *in vitro* and *in vivo* conditions, its translation to clinical practice in humans is still not practiced. Further research is needed to determine its efficacy, safety, and optimal dosage in human subjects.

The contents of this chapter discuss our attempts to establish the Streptozotocin-induced SD diabetic model and evaluate the effect of aminoguanidine, an inhibitor of advanced glycation end product, on the phenotypic changes in the early diabetic condition.

2.1.2 Materials

Plastic wares, Drugs, and Reagents

Our experiments involved the use of Sprague-Dawley rats weighing between 200-225 g, aged 10 to 11 weeks. The rats were fed a standard chow diet and provided with tap water, *ad libitum*. The chemicals Streptozotocin (S0130) and aminoguanidine hydrochloric acid (CAS 396494) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Additionally, Neosporin antibiotic, hydrogen peroxide, one-touch ultra-soft lancets (Life Scan), spirit, and 70% isopropanol were used for the experimental procedures. The laboratory apparatuses used included measuring cylinders, vacutainers, gloves, masks, and cotton. The solution, Betadine (Povidone-Iodine solution IP 10% w/v) was used for disinfection purposes. Rat cages and metabolic cages were utilized for housing the rats in a controlled environment with regulated temperature, humidity, and lighting conditions. Various laboratory supplies such as 1.5-ml microcentrifuge tubes, tube stands (tarsons), tips, 15 ml Falcon tubes, aluminum foil, 1 ml syringes, and 26-G needles, were employed throughout the experiment.

Instruments:

Major instruments used to conduct present experiments methods are enlisted below (Table 2)

Table 2.3: List of instruments used in the study

Name of instruments	Company
Glucometer	One Touch, ONECARE
Weighing Balance	Sartorius
Metabolic Cages	B.I.K industries
Desiccator (WT-130,)	United Scientific Supplies DSGL150 Glass Desiccator
Ice machine, and water bath	Blue star
Pipettes	Pipetman (Gilson)
Seizers and forceps	Standard local commercial suppliers

2.1.3 Methods

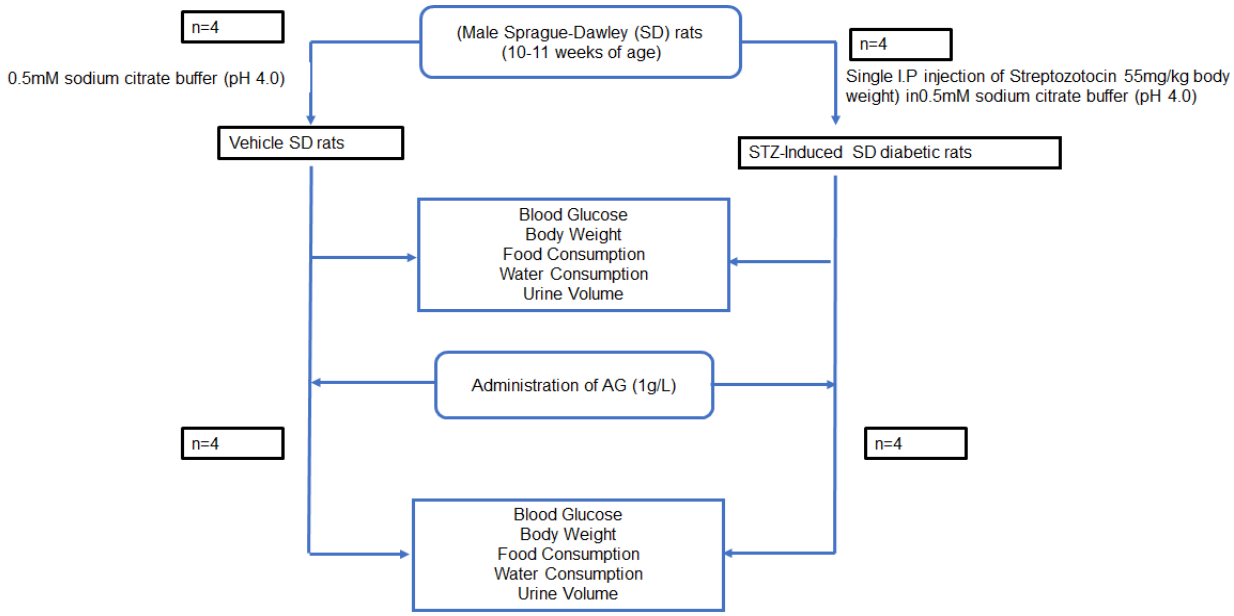

Figure 2.1: Schematic representation of the study design

2.1.3.1 Animals

All the experimental protocol was approved by the Institutional Animal Use and Care Committee of the University of Hyderabad (Approval number: UH/IAEC/AHS/2021-22/07). Age-matched male Sprague-Dawley rats (10–11-week-old), weighing approximately 200–225 g, were procured from the Indian Council Medical Research Animal Facility at the National Institute of Nutrition, Hyderabad. The animals were housed at the University of Hyderabad Animal Facility and had free access to standard rat chow and tap water.

2.1.3.2 Induction of Diabetes in SD Rats

Male Sprague-Dawley rats (10-11 weeks of age), were procured and housed at the University of Hyderabad animal facility and fed with the standard rat chow diet and water for two weeks. Briefly, animals were divided into two groups: Vehicle (n=4), and STZ-induced SD rats. A single IP (intra-peritoneal) injection of Streptozotocin (55mg/kg body weight), dissolved in 0.5mM sodium citrate (pH 4.0) was used to induce diabetes[312]. Vehicle animals were injected with (0.5mM sodium citrate (pH 4.0). Forty-eight hours after the injection, blood glucose was measured, from the tail, to test whether these animals non diabetic or not. The animals having plasma glucose > 300mg/dl were included in our study. The rats were held in the diabetic state for two weeks and placed in metabolic cages 2-3 days before sacrifice, to collect urine, for 24-hour urine volume measurement and urinalysis.

2.1.3.3. Measurement of Blood Glucose Levels and Physiological Parameters

During the STZ treatment period, the animal's change in body weight, food, and water intake was monitored for two weeks for each experimental group. Blood glucose levels of experimental animals were measured by taking one drop of blood from the tail vein onto the test strip forty-eight hours after the injection with STZ to confirm their diabetic state.

2.1.3.4. Treatment with Aminoguanidine-Experimental Design

Animals for this treatment were assigned to four groups (n = 4–6): Vehicle, Vehicle+AG, STZ-induced, and STZ-induced+AG rats. After the acclimatization period, a single intraperitoneal (IP) injection of STZ (55 mg/kg) dissolved in 0.5 mM sodium citrate buffer (pH 4.0) was used to induce type 1 diabetes, as described previously. The animals in the control group (Vehicle) were injected with 0.5 mM sodium citrate buffer (pH 4.0). After 48 h of the injection, glucose levels in the blood drawn from the tail vein were measured and animals having blood glucose levels >300 mg/dL were included in this study. The blood glucose levels in the Vehicle rats were also determined to ensure that they had not developed diabetes. Rats in the STZ-induced and Vehicle groups were administered AG at a dose of 1 g/L in drinking water for 2 weeks after the confirmation of their nondiabetic and diabetic state.

2.1.3.5. Measurement of Blood Glucose Levels

Blood glucose levels were measured using a OneTouch Select Plus Glucometer (ONECARE, Bengaluru, India).

2.1.3.6. Determination of Body weight, and Food and Water Intake

The body weight and food and water intake were measured over the course of treatments and also after transferring the rats to metabolic cages for 2–3 days, prior to their sacrifice. The food intake was measured by providing the rats in individual cages with a weighed amount of food and determining the weight of the leftover food in each cage on the next day. Similarly, water intake was measured by determining the average amount of water, over a period of time, consumed by the rats from a calibrated water bottle.

2.1.3.7. Measurement of Urine Volume

The rats in all the four groups were placed in metabolic cages 2-3 days before their sacrifice to collect urine. This was followed up to 24 hours to measure the 24-hour urine volume.

2.1.3.8 Statistical Analysis

All Results are expressed as mean ± SEM. The data were analyzed using GraphPad Prism 6. The student's t-test was used to compare between the groups. A *p-value <0.05* was considered to be statistically significant.

2.2 Objective 2: The Effect of Aminoguanidine on Streptozotocin-induced Diabetics Rats: Renal Function

2.2.1 Introduction

There are numerous evidences reporting that enhanced AGEs formation and accumulation of AGEs most prevalence in various conditions such as (i) diabetes[254], (ii) ageing[215], (iii) renal failure[313], and (iv) chronic inflammation[209]. The AGEs in the kidney accumulate in the renal compartment, and cross-linking with matrix proteins (long-lived proteins such as collagen, and elastin) leads to stiffness [314] and altered structural function at renal sites such as the glomerulus, peritubular vasculature, and arterioles of the kidney, thus promoting glomerulosclerosis and thickening of the basement membrane, and induces kidney damage[315]. The accumulation of AGEs in the glomerulus is associated with podocyte epithelial-mesenchymal transition[316]. Similarly, *in vitro* exposure to high concentrations of AGEs induces tubular-epithelial-myofibroblast transition via RAGE-dependent pathways, contributing to tubulointerstitial fibrosis[317]. There is evidence showing that AGEs accumulate in the circulation of both diabetic and non-diabetic patients with renal failure[318]. In addition, it has been suggested that a link exists between advanced glycation, the development of diabetic complications[242], and the presence of the quantum of the AGE products in diabetic tissues[242]. This relationship has been

identified in studies in both humans[319] and rodents[320]. Previous experimental studies on the rat kidney after 32 weeks of Streptozocin diabetes showed increased levels of AGEs associated with alterations in renal function as well as structural parameters of the kidneys, as manifested by albuminuria and mesangial expansion[258]. It has also been shown that excess formation of AGEs in diabetes results in crosslinking of collagen and distortion of subcellular structures, resulting in irreversible tissue damage of the macro- and microvasculature in the kidney[321]. Furthermore, experimental diabetes rats showed impaired kidney function and kidney anatomical abnormalities, damaging the small blood vessels and filtering units (glomeruli) in the kidneys, impairing their ability to filter waste products and excess fluids from the blood. This leads to a gradual decline in the kidney functions[321]. However, it is not known and clear as to upto what extent of glycosylation could be related to causing functional and structural abnormalities in the kidney. Certain reports have shown that the accumulated AGEs, act as signaling molecules and interact with membrane-bound forms of RAGE, thereby activating multiple inflammatory pathways, including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) under diabetic conditions[209]. These pathways induce the expression of pro-inflammatory cytokines and adhesion molecules, contributing to chronic inflammation and tissue injury[209]. The aforementioned pathways are well known and mechanistically attributed to the development of fibrosis, glomerulosclerosis, apoptosis, and cell death and are classic contributors to the progression of diabetic kidney disease in both humans and experimental animal models (mice and rats)[322,323]. There is evidence to showed that accumulation of AGEs, stimulates increased RAGE expression on podocytes in diabetic nephropathy patients[190] and rodent models[324]. A Study have shown that RAGE knock-out in diabetic mice showed reduced renal fibrosis[325]. Therefore, management of diabetes is very critical and important. If the condition is not managed effectively, elevated glycation can cause damage to the kidneys over a period of time, leading to diabetic nephropathy. Diabetic

nephropathy is a major cause of end-stage renal disease (ESRD) and a major contributor of other cardiovascular disease[242].

Ample evidence from the literature shows that aminoguanidine reduces the AGEs in in STZ-induced animal models of diabetic nephropathy[294], retinopathy in spontaneous hypertensive rats[326], cardiac hypertrophy, and aortic lesions in STZ-induced animal models[133]. In diabetic nephropathy, AG attenuated the rise in albuminuria and prevented mesangial expansion in the kidney of experimental diabetic rats[258]. Furthermore, it also prevented diabetes-related increases in collagen-cross linking in the atrial wall[220], the renal glomeruli, and tubules[327]. A previous experimental report by Ellis and Good et al; showed that AG prevented glomerular basement membrane thickening in diabetic nephropathy[328]. However, no such effects of AG were not observed in another study[329]. Therefore, the effects of AG on the kidney in experimental diabetes remain debatable. While studies have demonstrated that aminoguanidine inhibits the accumulation of renal AGEs and thereby reduces the development of experimental diabetic nephropathy, more clarity on this aspect needs to be established[294]. Previous studies have also assessed the accumulation of AGEs in the aorta and kidney, as well as renal functional and structural parameters over 32 weeks of experimental diabetes in the absence and presence of aminoguanidine[327].

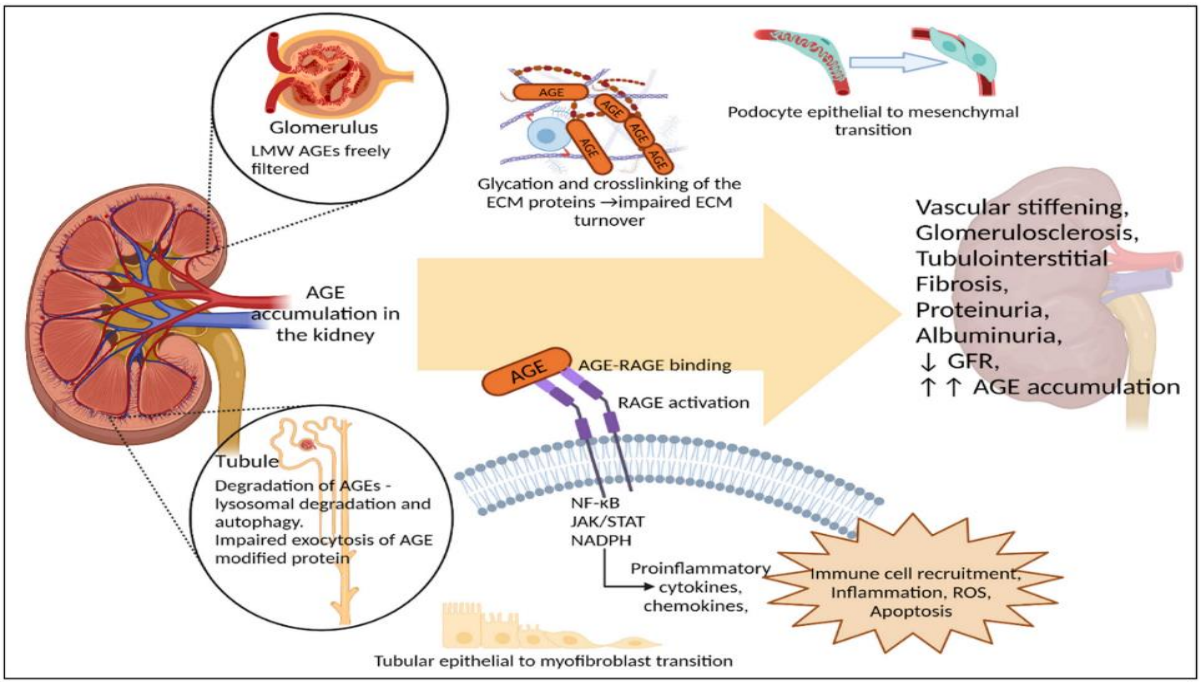

Figure 2.2: Mechanisms by which AGEs Damage the Kidney (Adapted from Fotheringham, A. K et al. 2022)[330]

For diagnosis of diabetic complications, and management of diabetes, both urine and blood analysis are frequently employed to evaluate renal function. This assessment contains variables like urine protein, creatinine glomerular filtration rate (GFR), and electrolyte (mainly sodium[331]. Monitoring these parameters is very important to predicting the complications, and designing the therapeutic plan.

2.2.1.1 Clinical and Kidney Function Parameters

2.2.1.1.1 Urine Protein

In a healthy individual, the kidneys act as filters, removing waste products and excess substances from the blood, while retaining essential proteins and other beneficial compounds. However, in diabetes, particularly if blood sugar levels are consistently high, the blood vessels in the kidneys can become damaged over time. This can lead to a condition called diabetic nephropathy[332], where the kidney's filtering function is impaired, and protein starts to leak into the urine known as proteinuria or albuminuria, which can be an early sign of diabetic kidney

disease (diabetic nephropathy)[333]. Proteinuria is usually detected through a simple urine test. Microalbuminuria is a specific type of proteinuria that refers to the presence of small amounts of albumin (a specific type of protein) in the urine[334]. It is often considered an early marker of diabetic kidney disease, a disease associated with diabetes, and an increased risk of more severe kidney and heart problems in the future[334].

2.2.1.1.2 Creatinine

Creatinine is a waste product that is produced in the muscles through the breakdown of creatine, an energy molecule. It is filtered out of the blood by the kidneys and excreted in urine. Measurement of creatinine levels in the blood and urine is an essential tool in assessing kidney function, as the kidneys play a vital role in clearing creatinine from the body[335,336].

In the context of diabetes, monitoring creatinine levels is important because diabetes can have significant effects on kidney function[335]. Prolonged high blood sugar levels can lead to damage to the blood vessels in the kidneys, a condition known as diabetic nephropathy[335]. As diabetic nephropathy progresses, the kidneys' ability to filter waste products, including creatinine, can be impaired[335].

2.2.1.1.3 Glomerular Filtration Rate (GFR)

The GFR is calculated based on the urine and serum creatinine levels[312,336]. The level of creatinine in the blood is influenced by the filtration capacity of the kidneys, and therefore, serves as a marker to estimate GFR[297].

2.2.1.1.4 Electrolyte (Sodium)

Electrolytes play critical roles in maintaining various bodily functions, including nerve function, muscle contraction, and fluid balance[337]. In diabetes, imbalances in electrolyte concentration, mainly sodium can occur due to various factors, including changes in insulin levels, medication use, and complications related to the disease[312,338]. The maintenance of sodium is altered under the diabetic condition and any imbalance in the ion levels can cause a

significant physiological and homeostatic shift in the body[337]. A damaged kidney either cannot filter out sodium or cannot selectively reabsorb sodium from the filtered load, thereby serving as a precursor for Na^+ dependent hypertension, under diabetic conditions[297].

High blood sugar levels can lead to increased thirst and frequent urination, which may cause dehydration. Dehydration can affect sodium levels in the blood, leading to hypernatremia (high sodium levels)[337].

While performing the assay to determine renal damage, renal histology can provide insights into the mechanisms underlying diabetes[339]. Diabetes can cause changes in the structure of the kidneys, such as thickening of the glomerular basement membrane and expansion of the mesangium. These changes can lead to decreased filtration capacity and increased proteinuria, hallmarks of renal damage[340]. Assessment of renal histology in diabetic rats, therefore can help researchers understand the ways these changes occur and possibly could identify potential therapeutic targets.

Aminoguanidine, a hydrazine derivate that acts as an inhibitor of an enzyme called tissue transglutaminase, is involved in the formation of AGEs and reduces glycation. Aminoguanidine (AG) has been investigated for its potential role in diabetes and its complications[294]. Studies showed that treatment with AG has resulted in the improvement of kidney functions [327] and decreased retinal pericyte loss, abnormal endothelial proliferation[341], and peripheral nerve impairment in streptozotocin-induced diabetic (STZ-D) rats[342]. Also, it is reported that AG treatment reduces lipid peroxidation in vascular complications in streptozotocin-induced diabetic (STZ-D) rats, and hence reduces the free radical generation[343]. Hence, in this study, we determine the effect of AG's effect on renal functions and renal structure in early diabetes conditions.

2.2.2 Material

Haematoxylin**,** eosin, xylene, ethanol, glass slides, coverslips, all other chemicals used were purchased from standard local commercial suppliers and were of analytical grade

2.2.3 Methods

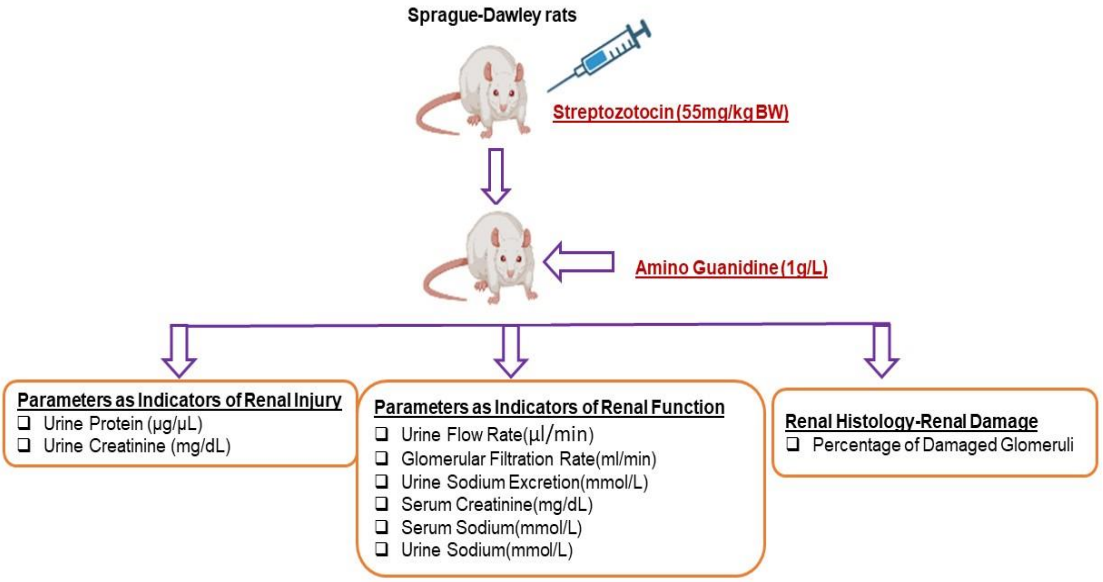

Figure 2.3: Schematic representation of the objective to study renal functions/injury/damage

2.2.3.1 Determination of Renal Functions

The renal function parameters were determined in partial modifications of the methods as described previously[297]. Briefly, rats were transferred to metabolic cages for 2–3 days to collect urine samples, for determining the urine flow rate (UFR), glomerular filtration rate (GFR), and urinary sodium and protein levels. A clinical biochemistry analyzer (Beckman Coulter and Olympus) was used to determine the levels of creatinine and electrolytes in the urine. The urinary protein levels were quantitated spectrophotometrically using the BCA method.

2.2.3.2 Calculation of GFR

The glomerular filtration rate in the various groups of rats was calculated using the following formula:

GFR = Urine creatinine × Urine flow rate (µL/min)/Serum creatinine

2.2.3.3 Calculation of Urinary Sodium Excretion Rate

The urinary sodium excretion rate, as an index of renal function and natriuresis, was calculated using the following formula:

Urinary sodium excretion rate = Urine sodium concentration (mmol/L) × 24 hour-urine volume/mMoles of Sodium

2.2.3.4 Blood Sample Collection

Blood samples were collected via cardiac puncture into plain sample vacutainer tubes after anesthetizing the animals with diethyl ether. The blood samples were centrifuged at 2500 rpm for 10 min to obtain clear serum for biochemical analysis of electrolytes, and serum creatinine

2.2.3.5 Histopathological Analysis of the Kidney

For histopathological analysis, the kidneys from each animal were excised at the time of sacrifice and placed in 10% formalin, as described previously[88,312]. They were then dehydrated and embedded in paraffin. The paraffin-embedded kidney tissues were sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E)[312]. The sections were examined by a pathologist. At least 4–10 random fields were examined per section, comparing 10 or more glomeruli in each field.

2.2.3.6 Statistical Analysis

Results are expressed as mean ± SEM. The data were analyzed using GraphPad Prism 6. Student's t-test was used to compare the groups. A p-value<0.05 was considered to be statistically significant.

2.3 Objective 3: Change in Protein Expression of Renin, Angiotensin II Type I and Type II Receptors (AT_1 and AT_2)

2.3.1 Introduction

The renin-angiotensin system plays a crucial in blood pressure regulation, fluid volume, and electrolyte homeostasis[62]. It contains several key components i) ii) renin, iii) angiotensin-converting enzyme, ACE iv) angiotensin II, and v) the angiotensin II receptor. The binding of the angiotensin II with two subtype receptors (AT_1 and AT_2) mediates the various biological effects including vasoconstriction, aldosterone secretion, catecholamine release as well as drinking, secretion of prolactin and adrenocorticotrophic hormone, and glycogenolysis, whereas AT_2 show the opposite effects of that of the AT_1 receptors[36,67,344].

The AT_1 and AT_2 receptors are localized in various tissues including kidney [73]. A number of studies have established the contribution of these receptors to the development of kidney disease in diabetes[67,344].

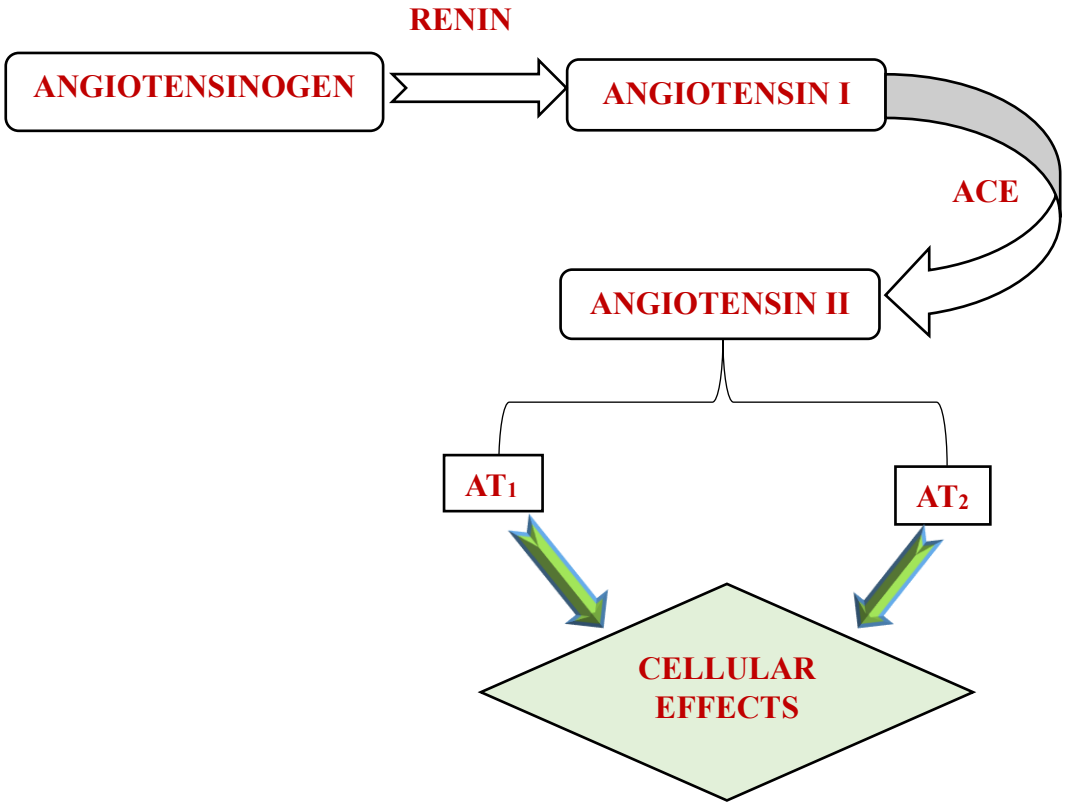

Figure 2.4: Schematic representation of the major component of the renin-angiotensin system

It has been reported that altered RAS components such as angiotensin II, renin, ACE as well as the angiotensin II receptor subtypes are frequently associated with the development of hypertension and metabolic diseases such as atherosclerosis[345], stroke, coronary artery[346], obesity[347] disease under diabetes conditions. Moreover, there are reports that chronic high blood glucose levels in diabetes contribute to long-term kidney disease, such as end-stage renal disease (ESRD)[348].

Hyperactivity of RAS via angiotensin II receptor subtypes, (AT_1, and AT_2) has been studied in the context of diabetes[296,297]. However, the changes in the expression of AT_1 and AT_2 receptors are tissue-dependent, for example, the overexpression of AT_1 receptors has been shown in the kidney, heart, lungs, aorta, and brain, while the overexpression of AT_2 receptors has been reported in mesenteric arteries, kidney, and endothelium[73,86,349]. It has been shown that there is an upregulation of AT_1 receptors in the glomeruli and tubules in the kidneys in

diabetic nephropathy[350]. Increased AT_1 receptor expression has also been observed in the retina of patients with diabetic retinopathy[351] and diabetic cardiovascular complications[86]. On the other hand, The AT_2 receptors have been shown to have anti-inflammatory, vasodilatory, and tissue-protective effects [352]. There is evidence that the expression and function of the AT_2 receptors become relevant under pathophysiological conditions such as hypertension and diabetes[36,86]. A study, a model of type 2 diabetes obese Zucker rats, showed that the tubular AT_2 receptor is upregulated and mediates the natriuretic effects mediated by an AT_1 receptor antagonist[353]. Similarly, increased expression of the tubular AT_2 receptors contributes to enhanced urinary sodium excretion in STZ-treated rats[296]. The upregulated AT_2 receptors via the NO/cGMP pathways mediate tubular sodium transport inhibition in STZ-treated rats, leading to enhanced urinary sodium excretion[296]. Some studies showed that early streptozotocin-diabetes mellitus downregulates rat kidney AT_2 receptors[135]. However, the exact mechanisms and significance of AT_2 receptor function in diabetic complications are still under investigation, and further research is needed to fully understand their role.

The first and highly regulated rate-limiting step of the renin-angiotensin system is mediated by renin and its inhibition has been a target for nearly 60 years[118]. The study by Miller et al.; demonstrated that short-term moderate hyperglycemia without glycosuria during the early stages of diabetes has been linked to an increase in plasma renin activity, mean arterial pressure, and renal vascular resistance[118]. Moreover, the presence of immunoreactive renin has been demonstrated in the human end-stage diabetic kidney[354]. Early diabetes mellitus stimulates proximal tubule renin mRNA expression in the rat[135]. Therefore, the medications that target the RAS cascade, such as ACE inhibitors, and angiotensin receptor blockers (ARBs), are solely used to control/slow down the progression of diabetes complications including hypertension[355], nephropathy[356], retinopathy[357], and neuropathy[358].

The most commonly used ARBs, such as losartan, telmisartan, and valsartan, are the AT_1 receptor antagonists, and they block the AT_1 receptor and subsequently prevent the binding of Angiotensin II to this receptor subtype[359,360]. These drugs are known to reduce vasoconstriction, inflammation, and oxidative stress, among other functions [359]. In the STZ-induced diabetic rats, treatment with ARBs has been shown to ameliorate diabetic nephropathy, retinopathy, and cardiovascular complications, thereby implying the role of AT_1 receptors in aggravating diabetic complications[361,362].

The inhibitors of ACE, such as enalapril, lisinopril, and ramipril work by inhibiting the enzyme ACE, which converts Angiotensin I to Angiotensin II[147]. By inhibiting this conversion, ACE inhibitors indirectly reduce the activation of AT_1 receptors, due to a decreased Ang II availability[147]. These inhibitors have been demonstrated to improve renal function, decrease proteinuria, and attenuate diabetic complications in STZ-induced diabetic rats[363]. Either the reduction in the levels of Ang II or the decreased activation of the AT_1 receptors by Ang II has shown a significant reduction in the various complications as mediated by the RAS[363]. In addition to the above therapy, a combination of therapeutic approaches is also used in the treatment of diabetes to manage its complications specifically hypertension[364].

Recently, emerging evidence showed that the reduction of accelerated formation of advanced glycation end products (AGEs), provides an intensive management of diabetic complications[294]. The drug, Aminoguanidine, AG, an inhibitor of Advanced Glycation Products, has been studied for its potential effects on diabetic complications[294]. There is a report that AG has been shown to impact the renin-angiotensin system (RAS) indirectly reducing oxidative stress, and hemodynamic renal changes and improving endothelial function in diabetic rats[327,365]. Additionally, aminoguanidine has been shown to reduce the prevent diabetes-induced increases in protein kinase C activity in glomeruli, retina, and mesenteric

artery. Thus, by reducing AT_1 and AT_2 receptor expression, aminoguanidine helps attenuate diabetic complications[106].

The change in expression of AT_1 and AT_2 receptor in the diabetic kidney have been reported [67,366] but the impact of AG on AT_1 and AT_2 receptor AT_2 receptor expression and functional changes is not known in the kidney of diabetes condition. The AT_2 receptor activation is generally associated with vasodilation, anti-inflammatory responses, and tissue repair[366] processes, such effects that are opposite to the effects of the AT_1 receptors[349].

It is important to note that while aminoguanidine has shown promise in preclinical studies, its clinical effectiveness and safety for treating diabetic complications in humans are still under investigation. Additionally, there may be other factors and pathways involved in the effects of aminoguanidine beyond the RAS and the AT_1/AT_2 receptors. Therefore, further research is needed to fully understand the extent and mechanisms of aminoguanidine under diabetic conditions.

This work aimed to evaluate the protein expression of, renin, angiotensin II receptors, AT_1, and AT_2 in the kidney, and heart of diabetic rats before and after administration of AG.

2.3.2 Materials

Chemicals and Reagents: Tri- sodium citrate (Fisher scientific) Sodium Chloride, Potassium chloride, Calcium Chloride, Magnesium Chloride, Sodium Bicarbonate (Sigma), Potassium Dihydrogen orthophosphate, D-Glucose anhydrous, 4-(2- Hydroxyehyl)piperazine-1-ehanesulfonic acid sodium (HEPES Sodium), β-Glycerophosphate di sodium salt hydrate (sigma), Magnesium Chloride (Fisher Scientific), Ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N' tetra acetic acid tetrasodium salt (EGTA/Sigma-Aldrich), Triton X-100 (Sigma), Dithiothreitol (Biorad), Phenyl methane sulfonyl fluoride (PMSF) (Sigma), Protease inhibitor cocktail (Roche), Sodium dodecyl sulphate (Fisher scientific) N,N,N',N'

Tetramethylenediamine (TEMED) (Biorad), Ammonium per sulfate (Biorad), Tween-20 (Biorad), 30%Acrylamide/ Bis-acrylamide (29:1)(Bio rad), Ponceau S dye, Tris free base (Biorad), Nitrocellulose membrane (0.45 µM (Biorad), Bovine serum albumin fraction V (BSA) **(**Roche), diethyl ether (Thermoscientific), 70% isopropanol, Formaldehyde (Himedia), Ethanol, (Himedia), hematoxylin and eosin

Kit: Pierce BCA protein estimation kit (Bicinchonic acid reagent) (Thermo Fischer), femto LUCENT TM PLUS HRP chemiluminescent (G Bioscience),

Proteins and Antibodies: Bovine serum albumin fraction V (BSA) **(**Roche), Anti-Rabit IgG HRP, Anti-mouse IgG HRP (Cell Signaling). The antibodies for AT_1 and AT_2 receptors (PA5-20812 and PA5-20813, respectively) were purchased from ThermoFisher Scientific, and the β-actin antibody was obtained from Cell Signaling (Danvers, MA, USA; Cat. # 3700S). The Femto chemiluminescence substrate was purchased from G- G-biosciences. All other chemicals used for immunoblotting were purchased from Bio-Rad, Hercules, CA, USA.

Composition of Buffers and other reagents:

Resolving buffer pH 8.8 (40 ml): 1.5 M Tris base 7.26g volume made up of double distilled water (DDW)

Stacking buffer pH 6.8 (40 ml): 0.5 M Tris base 2.43 g, volume made up of double distilled water (DDW)

4X Laemmli sample buffer: 30% Glycerol, β-Mercaptoethanol (sigma), 20% SDS, 0.5M Tris PH-6.8, 1% Bromophenol blue 5X Reservoir buffer (500 ml): Tris base 7.5 g, Glycine 36 g, volume made up with double distilled water (DDW)

Separating buffer (1 Liter): 5X Reservoir buffer (200ml), 10% SDS (10ml) volume made up of double distilled water (DDW)

Transfer buffer (1 Liter): 5X Reservoir buffer (200ml), Methanol 200 ml, volume made up with DDW

1X Phosphate Buffer Saline: Sodium chloride, potassium chloride, disodium hydrogen orthophosphate anhydrous, Potassium dihydrogen orthophosphate

PBST: 1X PBS+ 0.1% Tween 20, Blocking buffer: 5% BSA in 1XPBS (pH-7.4), Washing buffer (PBST): 0.1% Tween20 in 1XPBS (pH-7.4), Antibody dilution (1:1000) buffer: 5% BSA in 1X PBS+ 0.1% Tween 20, for Beta-Actin (1:3000)

Laboratory Glassware, Materials, and Plates: 96 well plates (Corning), falcon tubes (15ml, and 50ml), test tubes, desiccator, sterile surgical blades, needles, syringes (Dispo van), Measuring cylinders, Rat restrainer, cotton, Sterial combine dressing pad, Butter papers, blotting papers, 10 µl, 200µl (Axygen), and 1ml tips (tarsons), 1.5 ml tubes (Axygen) Petri dish (corning), nitrile powder free gloves (Lab serve).

Table 2.4: List of instruments used in the study

Name of instrument/ Apparatus	**Company**
Tecan plate reader	Thermo Scientific
Tissue homogenizer	Unigenetics
chemo doc XRS+ imaging system	Biorad
PH meter	Oakion
Spinvin	Tarson
western blot cassettes	Biorad
-80°c freezer	Thermo Scientific
-20°c freezer	Blue star
Vortex	Tarson
Orbital Shaker	Tarson
Magnetic stirrer, and beads	Tarson
Protein electrophoresis Apparatus	Bio-Rad
Mini Trans-Blot Cell	Bio-Rad

2.3.3 Methods

2.3.3.1 Protein Extraction

The kidneys were dissected from the sacrificed rats and rinsed with the Krebs- Henseleit buffer (118 mM NaCl, 4 mM KCl, 1.25 mM CaCl 2, 1.2 mM MgCl 2, 27.2 mM NaHCO 3, 1 mM KH 2 PO 4, 5 mM glucose, and 10 mM HEPES; pH 7.4)[296,297]. The kidney cortex was minced and homogenized in a lysis buffer (50 mM β- glycerophosphate, 2 mM MgCl 2, 1 mM EGTA, 0.5% Triton X-100, 1 mM dl-dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing a cocktail of protease inhibitors with a broad inhibition specificity for serine and cysteine proteases, metalloproteases, and calpains. The kidney homogenates were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatants were recovered. Total protein was quantitated using the BCA method by measuring the absorbance at 562 nm, according to the manufacturer's protocol

2.3.3.2 Immunoblot Analysis

For immunoblot analysis, the supernatant of the kidney homogenate was dissolved in 4X loading sample buffer, containing β-mercaptoethanol, and boiled for 5 min at 95 °C. The proteins (35 µg of renin, 35 µg protein for AT_1, and 40 µg protein for AT_2) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin in PBS with 0.1% Tween-20. The blots were incubated with the primary antibodies (polyclonal Renin ((1:1000), AT_1(1:1000) and AT_2 (1:1000) and subsequently with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:5000). The signal was detected using a chemiluminescence reagent, and bands were visualized using the Chemidoc XRS instrument (Bio-Rad). The blots were stripped off the antibodies and reprobed for β-actin (1:3000, Cell Signaling, cat. 3700S) as a loading control. Densitometry analysis of

the bands was performed using the ImageJ software (NIH, USA), and renin, AT_1, and AT_2 levels relative to those of β-actin were determined for all the various groups

2.3.3.3 Statistical Analysis

Results are expressed as mean ± SEM. The data were analyzed using GraphPad Prism 6. Student's t-test was used to compare the groups. A p-value<0.05 was considered to be statistically significant

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Objective 1

3.1.1 Blood Glucose Levels after STZ treatment (mg/dL)

Two weeks after the induction of diabetes, STZ-treated rats had significantly increased blood glucose levels, compared to the corresponding parameter in the Vehicle group (381.8± 15 vs. 108.3± 2.13 mg/dL ($p<0.05$, n = 4; **Figure 3.1**).

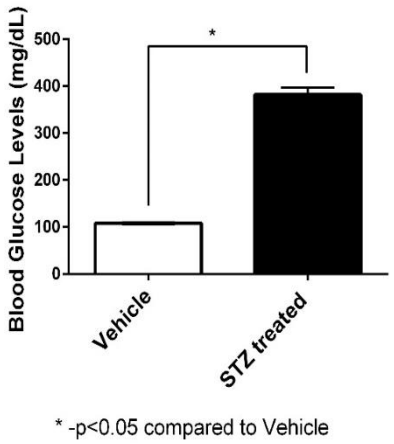

Figure 3.1: Streptozotocin treatment on SD rats, measurement of blood glucose level in vehicle and streptozotocin (STZ)-induced diabetic rat groups. Values are means ± SEM, *$p<0.05$ compared to vehicle rat groups, (Student's t-test). All experiments were performed in triplicate

3.1.2 Body Weight after STZ treatment (g)

Two weeks after the induction of diabetes, STZ-treated rats a had significantly decreased body weight (expressed as percent change in body weight) compared to the corresponding parameter in the Vehicle group (−9.70± 5.47 vs. 22.82 ± 2.85 g ($p<0.05$, n=6-8; **Figure 3.2**)

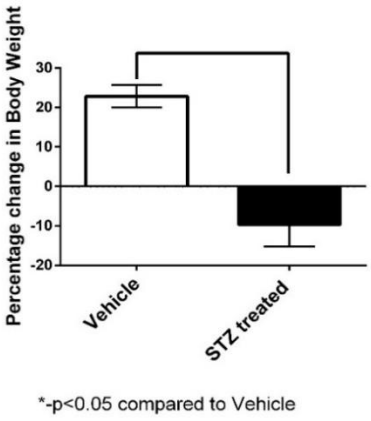

Figure 3.2: Streptozotocin treatment on SD rats, monitoring body weight in vehicle and streptozotocin (STZ)-induced diabetic rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rat groups, (Student's t-test). All experiments were performed in triplicate

3.1.3 Food Consumption after STZ Treatment (g)

A significant change in food consumption was observed between rats in the STZ-induced diabetic and Vehicle groups of rats, with the STZ-treated rats consuming an increased amount of food compared to the Vehicle rat groups (23.20± 1.73 vs. 17.18 ± 0.11 g (p<0.05, n=6-8; **Figure 3.3**)

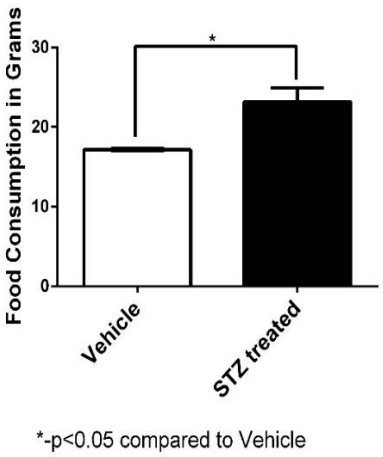

Figure 3.3: Streptozotocin treatment on SD rats, measurement of food consumption in vehicle and streptozotocin (STZ)-induced diabetic rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rat groups (Student's t-test). All experiments were performed in triplicate.

3.1.4 Water Consumption after STZ treatment (mL)

Two weeks after the induction of diabetes, STZ-treated rats showed a significantly increased water consumption compared to the corresponding parameter in the Vehicle group (98.59 ± 9.49 mL vs. 29.09 ± 0.19 mL ($p < 0.05$), n=6-8; **Figure 3.4**)

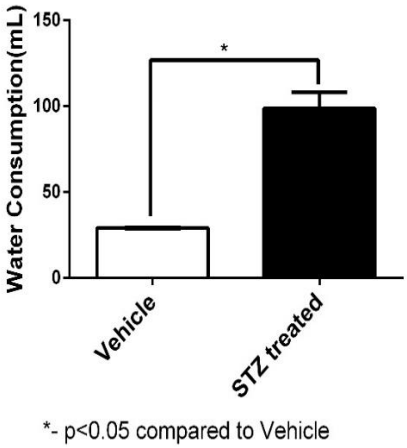

Figure 3.4: Streptozotocin treatment on SD rats, measurement of water consumption in vehicle and streptozotocin (STZ)-induced diabetic rat groups. Values are means ± SEM, *$p<0.05$ compared to vehicle rat groups, (Student's t-test). All experiments were performed in triplicate.

3.1.5 Urine Volume after STZ treatment (mL)

Two weeks after the induction of diabetes, STZ-treated rats had a significantly increased 24-hour urine output (mL) compared to the Vehicle rats (32.20±8.29 vs. 10.22±0.29 mL ($p<0.05$), n=6-8; **Figure 3.5**).

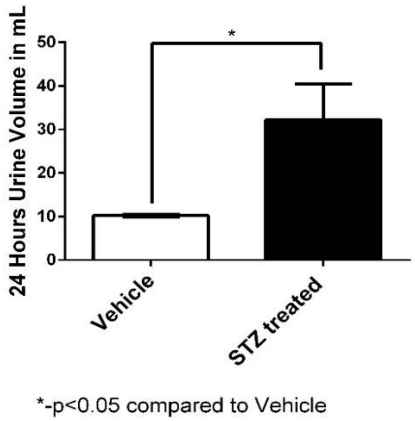

Figure 3.5: Streptozotocin treatment on SD rats, measurement of 24-hour urine volume in vehicle and streptozotocin (STZ)-induced diabetic rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rat groups, (Student's t-test). All experiments were performed in triplicate.

3.1.6 AG Treatment in STZ-induced+AG treated Diabetic Rat Groups Prevented an Increase in Blood Sugar Levels (mg/dL)

Two weeks after the induction of diabetes, STZ-treated rat groups had significantly increased blood glucose levels compared to the corresponding parameters in the Vehicle rat group (462.3 ± 18.6 vs. 109.7 ± 1.25 mg/dL ($p<0.05$), n=6-8; **Figure 3.6**). Administration of AG (1 g/L) to the STZ-induced+AG treated diabetic rat groups had significantly decreased blood glucose levels, compared with that in the STZ-treated rat groups (295.9 ± 50.69 vs. 462.3 ± 18.6 mg/dL ($p<0.05$); **Figure 3.6**). There was a significant change in the blood sugar levels, between the STZ-induced+AG treated rat groups and Vehicle+AG treated rat groups too (295.9 ± 50.69 vs. 111.6±1.49 mg/dL ($p<0.05$); **Figure 3.6**). No differences in blood glucose levels were noted between the Vehicle and Vehicle+AG treated rat groups, (109.7 ± 1.25 ± 1.8 vs. 111.6 ± 1.49 mg/dL); **Figure 3.6)**.

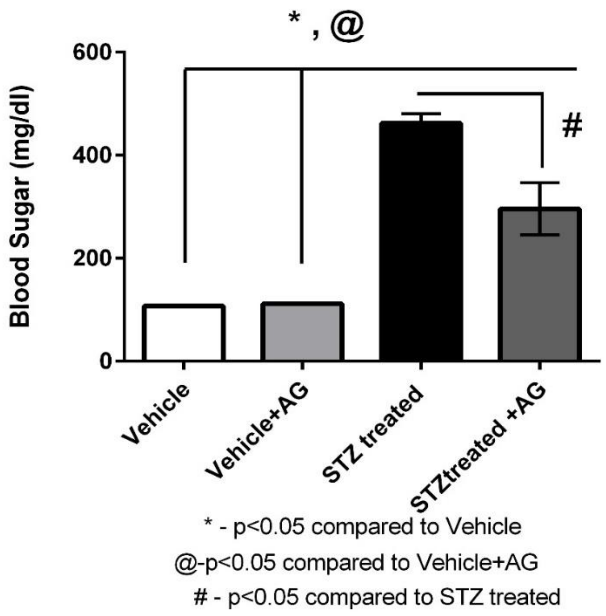

Figure 3.6: Effect of aminoguanidine (AG) treatment (1 g/L) on blood glucose level in vehicle and streptozotocin (STZ)-treated rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rats, @ p<0.05 compared to Vehicle+AG treated rat groups, #p<0.05 compared to STZ-treated rat groups (Student's t-test). n = (6–8).

3.1.7 AG Treatment of STZ-induced+AG treated Diabetic Rat Groups Demonstrates a Slight Restoration in The Body Weight (g)

Two weeks after the induction of diabetes, STZ-treated rat groups had significantly decreased percent change in body weight compared to the corresponding parameter in the Vehicle rat groups (−7.35 ± 5.97 vs. 23.87 ± 4.1 g (p<0.05), n=8; **Figure 3.7**). Administration of AG (1 g/L) to the STZ-induced+AG treated diabetic rat groups caused a slight restoration in the percent change in body weight, compared with that in the STZ-treated rat groups (1.6. ± 5.8 vs.−7.35 ± 5.97 g). A significant difference was also noted in the percent change in body weight between the STZ-induced+AG treated rat groups and Vehicle+AG treated rat groups (1.6. ±

5.8 vs. 18.8 ± 5.9, p<0.05; **Figure 3.7**). No differences in these parameters were noted between the Vehicle and Vehicle+AG treated group of rats (23.87 ± 4.1 vs. 18.8 ± 5.9 g); **Figure 3.7)**.

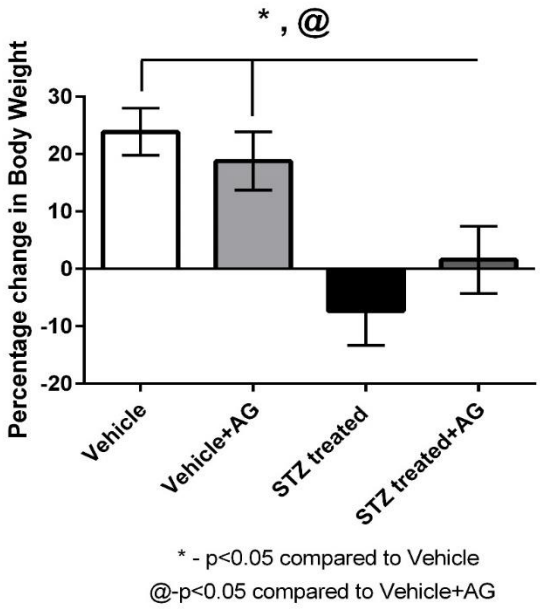

Figure 3.7: Effect of aminoguanidine (AG) treatment (1 g/L) on body weight (g) in vehicle and streptozotocin (STZ)-treated rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rats, @ p<0.05 compared to Vehicle+AG treated rats, #p< 0.05 compared to STZ-treated rat groups (Student's t-test). n =8.

3.1.8. AG Treatment of STZ-induced+AG treated Diabetic Rat Groups Showed no Change in Food Consumption (g)

Two weeks after the induction of diabetes, the STZ-treated rat group had a significant change in food consumption compared with the Vehicle groups of rats (19.51 ± 1.61 vs. 16.44 ± 0.29 g (p<0.05), n=6-8; **Figure 3.8**). Administration of AG (1 g/L) no difference was recorded between the food consumption in the STZ-treated rat groups and STZ-induced AG-treated rat groups (19.51 ± 1.61 vs. 19.58 ± 1.08 g; **Figure 3.8**). No differences in these parameters were noted between the Vehicle and Vehicle+AG treated rat groups (16.44 ± 0.3 vs. 16.70 ± 0.5g).

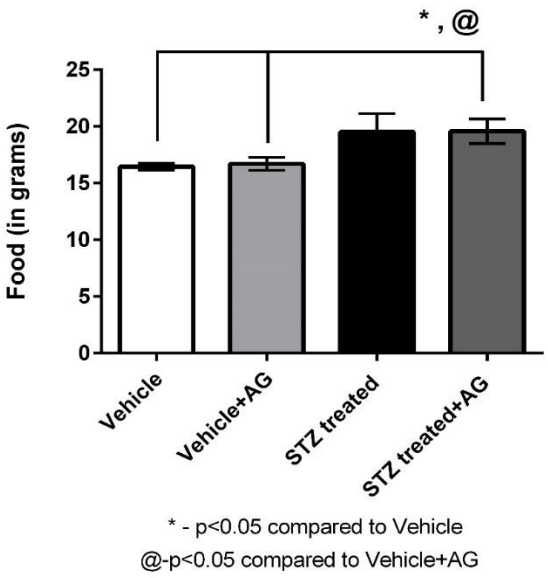

Figure 3.8: Effect of aminoguanidine (AG) treatment (1 g/L) on food consumption in vehicle and streptozotocin (STZ)-treated diabetic rats, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rats, @ p<0.05 compared to Vehicle+AG treated rat groups, #p< 0.05 compared to STZ-treated diabetic rat groups (Student's t-test). n = (6–8).

3.1.9 AG Treatment of STZ-induced+AG treated Diabetic Rat Groups Moderately Decrease the Water Consumption (mL)

Two weeks after the induction of diabetes, STZ-treated rat groups showed significantly increased water consumption compared to the corresponding parameter in the Vehicle rat groups (67.13 ± 8.78 vs. 30.06 ± 1.56 mL (p<0.05); n= 6-8, **Figure 3.9**). Administration of AG (1 g/L) to the STZ-induced+AG treated rat groups modestly decreased water consumption (62.44 ± 10.93 vs. 67.13 ± 8.7 mL). There was a significant change in the water consumption between the STZ-induced+AG treated rat groups and Vehicle+AG treated rat groups (62.44 ± 10.93 vs. 28.40 ± 0.93 ml; p<0.05); **Figure 3.9**). No differences in these parameters were noted between the Vehicle and Vehicle+AG treated rat groups, 30.06 ± 1.56 vs. 28.40 ± 0.93 mL)

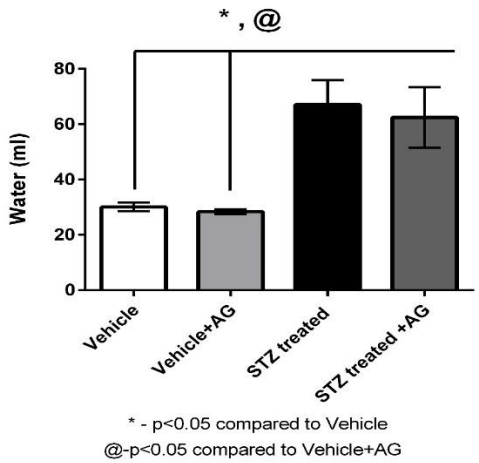

Figure 3.9: Effect of aminoguanidine (AG) treatment (1 g/L) on water consumption in vehicle and streptozotocin (STZ)-treated diabetic rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rats, @ p<0.05 compared to Vehicle+AG treated rat groups, #p< 0.05 compared to STZ-treated diabetic rat groups (Student's t-test). n = (6–8).

3.1.10 AG Treatment of STZ-induced+AG treated Diabetic Rat Groups Showed Decreased Urine Volume (ml)

Two weeks after the induction of diabetes, STZ-treated rats shown significantly increased 24-hour urine volume (mL) in comparison to the Vehicle rat groups (29.59±4.21 vs. 6.70±0.82 mL (p<0.05); n=11-15; **Figure 3.10**). Treatment with AG have resulted in a decreased urine volume in the STZ-induced +AG treated rat groups, compared to the STZ-treated diabetic rat groups (17.47±5.8 vs. 29.59±4.21 mL **Figure 3.10**). Significant changes in urine volume were observed between the STZ induced+AG treated rat groups and Vehicle+AG treated rat groups (17.47±5.81 vs. 5.63±0.69), **Figure 3.10**). No differences in these parameters were noted between the Vehicle and Vehicle+AG treated rat groups (6.70±0.82 vs. 5.63±0.69 **Figure 3.10**). Although the Urine volume is significantly higher in STZ+AG-treated rat groups compared to the Vehicle rat groups, (17.47±5.81vs. 6.70±0.82). It is significantly /

substantially decreased in Vehicle+AG treated rat groups compared to STZ-induced diabetic rats compared with that in the STZ-treated rats (5.63±0.69 vs. 29.59±4.21).

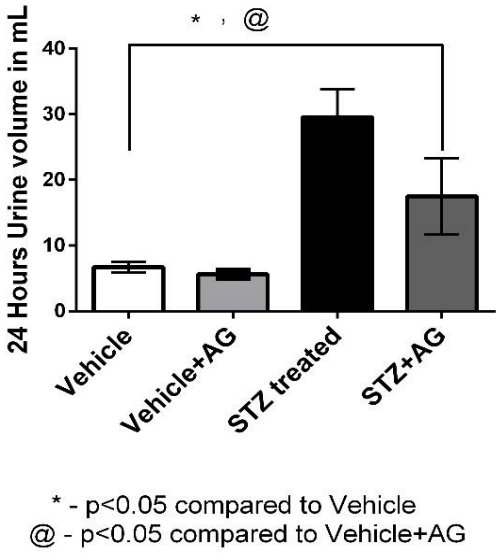

Figure 3.10: Effect of aminoguanidine (AG) treatment (1 g/L) on urine volume in vehicle and streptozotocin (STZ)-treated diabetic rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM, *p<0.05 compared to vehicle rats, @ p<0.05 compared to Vehicle+AG treated rat groups, #p<0.05 compared to STZ-treated rat groups (Student's t-test). n = (11-15).

3.1.11 Discussion

Diabetes accounts for 1.51 million affected persons from 2000-2023 and it can be fatal if not managed. Hence, several well-designed *in vitro* and animal model systems have been explored to study the underlying mechanism of diabetes and related complications. Investigating the advanced glycation end-product formation (AGEs) and its effect on the regulation of the RAS system in the progression of diabetic complications is a challenge. This work demonstrated that the STZ-induced SD rat model is cost-effective, which is economically viable, and is a suitable model for studying renal function and diabetes progression that could pave the way for evaluating the effective targeted therapy. In this chapter, we demonstrated the establishment of a Streptozotocin (STZ)-induced SD rat model for diabetes and its associated

complications and the reduction/ and or prevention of renal complications with aminoguanidine administration.

The diabetogenic effect of streptozotocin (STZ) is related to its cytotoxic effect on beta cells. When this compound enters the pancreatic beta-cell through GLUT-2 transporters, it produces alkylation and DNA fragmentation, which leads to the destruction of these cells. This destruction leads to increased glucose levels as a result of their inability to produce insulin, which is the hormone responsible for the regulation of carbohydrates. Depending on the STZ dose, it can produce a type I diabetes model or a type II diabetes model. This effect is mainly due to the complete or partial destruction of pancreatic beta cells[274].

The results of our study documented that after 48 hours of streptozotocin treatment, STZ-induced SD diabetic rats had high blood glucose levels (hyperglycaemia). This increase in blood sugar levels in the STZ-induced diabetic rats was accompanied by a decrease in body weight (% weight loss) and an increase in water intake (polydipsia), urine volume (polyuria), and polyphagia, compared to that of the vehicle rat groups. The observed changes in these parameters are established phenotypes of diabetes[367], [368]and these features are consistent with type I diabetes criteria established by different associations such as the American Diabetes Association (ADA)[369], International Diabetes Federation (IDF)[370], and other[371]. This evidence that we could develop a good animal model of diabetes and successfully induce the various complications of diabetes concerning the clinical phenotypes and physiological determinants. We also documented that, after, administration of AG (1g/L water) in the STZ-induced diabetic rats, the drug prevented the increase in blood glucose levels compared with that in the STZ-induced diabetic rats. The AG treatment was also helpful in restoring the weight loss *vis-à-vis* and a partial improvement in the other parameters after two weeks in the STZ-induced diabetic rats. Although we did not evaluate the mechanism in this study, the most plausible explanation for this could be the fact that AG prevents the formation of AGEs by reacting with

fragmentation products of sugar-derived ketamine[372]. There are also reports that AG helps improve insulin sensitivity and enhance glucose uptake by cells, leading to a reduction in blood glucose levels[373,374]. Other studies have also shown that aminoguanidine had been shown to protect pancreatic beta cells[375], which are responsible for producing insulin, thereby improving glucose control, reducing blood glucose levels, and preventing excessive weight loss. In addition, aminoguanidine has been reported to improve insulin sensitivity in certain studies [373]. Taken together, these factors have most likely lead to a prevention of the increase in the blood sugar levels in the STZ+AG treated rat groups. This is the most plausible explanation that we could offer. Measuring the serum insulin levels could provide more insights into this mechanism and this definitely warrants further study.

Diabetes generally, leads to altered appetite regulation due to an altered metabolic function and could result in reduced/increased food intake[127]. It has been shown diabetes influences the release of hormones involved in appetite control, such as leptin[376] and ghrelin[376]. By modulating these hormones, aminoguanidine may also help regulate appetite and prevent excessive weight loss or weight gain. The specific effects of aminoguanidine on weight loss in diabetic rats may vary depending on the experimental conditions, dosage, and duration of treatment [259,327]. Further research is needed to fully understand the effects of aminoguanidine on body weight regulation in the context of diabetes. Our studies have also documented that there is an appreciable restoration of body weight in the STZ+AG-treated rats, with food consumption remaining almost the same**.** This suggests a better utilization of the nutrients in the STZ+AG-treated rats, compared to the STZ-induced diabetic rats. As evidenced, the STZ-induced rats are often associated with increased thirst and as a consequence excreted a higher volume of urine due to hyperglycaemia-induced hyperfiltration by the kidneys[297]. Administration of AG resulted in a partial decrease in water consumption and as a result, decreased urine excretion as that exhibited by STZ+AG-treated - diabetic rats.

Various studies have shown previously that aminoguanidine has been shown to have some potential effects on water consumption in streptozotocin (STZ)-induced diabetic rats, as observed here in our studies[342].

In diabetes, the kidneys can be damaged due to excess glucose filtration, leading to hyperfiltration, impaired fluid reabsorption, and increased urine production leading to an altered urinary excretion of water and ions[258]. It is postulated hence that treatment of AG may improve kidney function, and thus help in reducing excessive urine production and subsequent thirst, a normal physiological response. Diabetes also affects the production and release of hormones involved in fluid balance and thirst regulation. Additionally, AG has also been shown to influence the release of certain hormones indirectly such as vasopressin[327] and atrial natriuretic peptide[106]. By modulating these hormones, aminoguanidine may actually help regulate fluid balance and could possibly reduce excessive thirst and water consumption, and hence affect the renal functions.

Diabetes causes oxidative stress, which can damage cells and tissues throughout the body, including the kidneys[377]. Evidence from the literature, suggests that aminoguanidine also possesses antioxidant effects, which may help in protection against oxidative stress-induced damage [378].

In conclusion, the acceleration of advanced glycation is the major challenge in clinical diabetes complications. We have established an STZ-induced diabetic model to evaluate the phenotypic characteristics after AG treatment. Our study provides promising results in preventing the increase in blood glucose levels by AG treatment. Furthermore, the study demonstrated an anomalous or selective action of AG, on body weight, food, and water consumption, and urine volume thus, it provides a new dimension in strategies to treat diabetic complications.

3.2 Objective 2

3.2.1 AG Treatment of STZ-induced+AG-treated Diabetic Rat Groups Not Significantly, Increased the Urine Protein Levels (µg/µL)

Diabetic rats had significantly higher urinary protein levels, compared with that in the Vehicle group rat groups (52.23 ± 7.16 vs. 15.14 ± 1.94 µg/µL ($p<0.05$); n=(3-5). Administration of AG (1 g/L) slightly, albeit not significantly, increased the urine protein levels in the STZ-induced+AG-treated rat groups compared with that in STZ-treated rats (61.95 ± 7.30 vs. 52.23 ± 7.16 µg/µL; **Figure 3.11**). Surprisingly, urine protein levels in vehicle-treated rats that were administered AG were significantly increased compared with those in vehicle+AG-treated rat groups (42.35 ± 7.45 vs. 15.14 ± 1.94 µg/µL ($p<0.05$)). The urine protein levels were substantially increased in the STZ-treated rat groups that were administered AG compared with that in vehicle+AG-treated rat groups that were administered AG, although not significantly (61.95 ± 7.30 vs. 42.35 ±7.45 µg/µL; **Figure 3.11).**

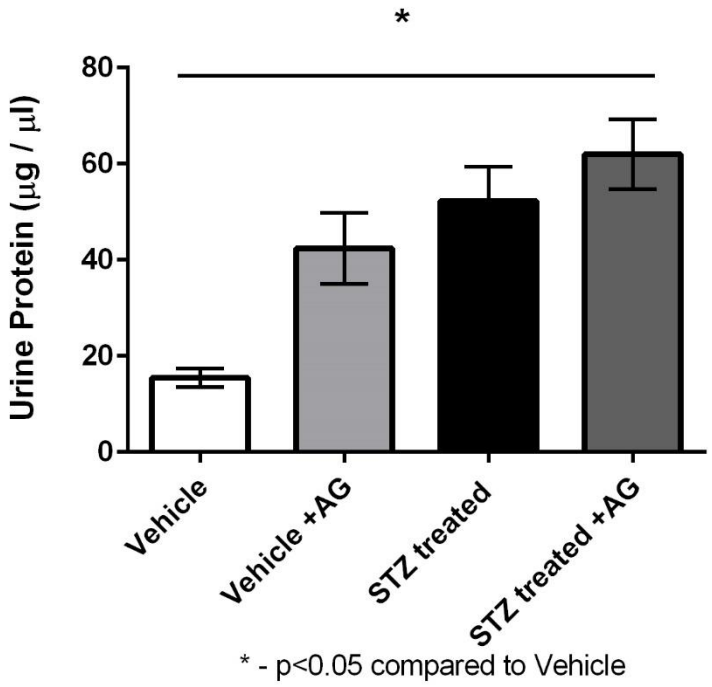

Figure 3.11: Effect of aminoguanidine (AG) treatment (1 g/L) on the urinary protein level of STZ-induced rat groups compared to vehicle rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM. *p<0.05 compared to vehicle rat groups, @ p<0.05 compared to Vehicle+AG treated rat groups (Student's t-test). n = (3–5).

3.2.2 AG Treatment of STZ-induced+AG-treated Diabetic Rat Groups Had Significantly Decreased Urine Flow Rate (UFR) (µL/min)

STZ-treated rat groups had significantly higher urine flow rates compared with that in the Vehicle group rats (20.55 ± 1.94 vs. 4.6 ± 0.57 µL/min (p<0.05); n=(3-5). The administration of AG significantly decreased Urine flow rate (UFR) **(Figure 3.12)** in STZ-induced+AG treated rat groups compared with that in STZ-induced rat groups (12.14 ± 4.043 vs. 20.55 ± 1.94 µL/min, (p<0.05). There was no effect on the UFR in the Vehicle+AG treated rat groups and Vehicle rat groups (3.90 ± 0.48 vs. 4.6 ± 0.57 µL/min). However, the UFR was significantly increased in STZ-induced+AG treated rat groups compared with that in the Vehicle+AG treated rat groups (12.14 ± 4.04 vs. 3.90 ± 0.48 µL/min (p<0.05); **(Figure 3.12)**.

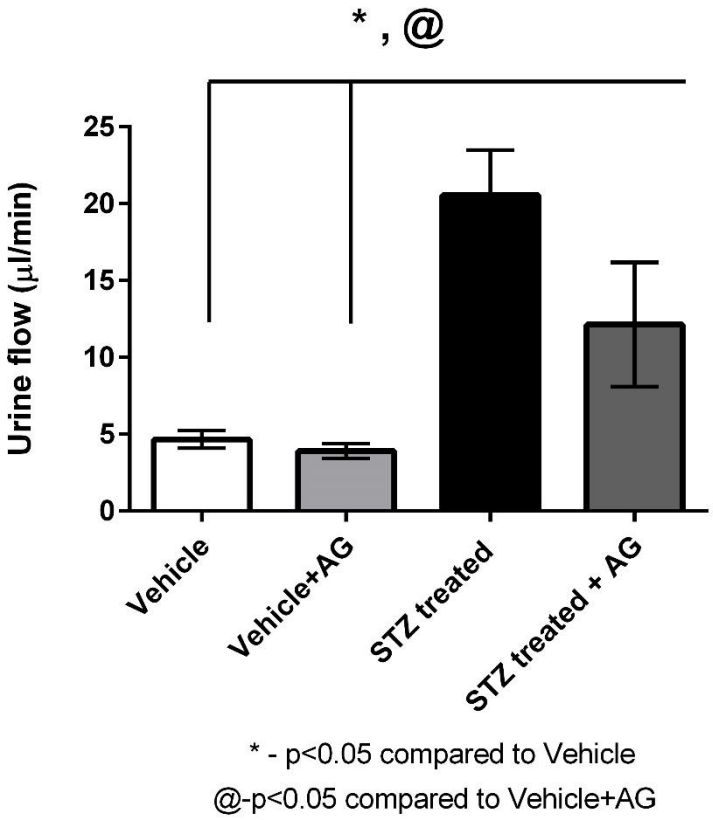

Figure 3.12: Effect of aminoguanidine (AG) treatment (1 g/L) on urine flow rate (µL/min) of STZ-induced rat groups compared to vehicle rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM. *p<0.05 compared to vehicle rat groups @ p<0.05 compared to Vehicle+AG treated rats (Student's t-test). n = (3–5).

3.2.3 AG Treatment of STZ-induced+AG-treated Diabetic Rat Groups Did Not Affect Glomerular Filtration Rate (GFR) (mL/min)

STZ treated diabetic rat groups had a significantly higher glomerular filtration rate (GFR) compared with that in the Vehicle group rats (1.1 ± 0.08 vs. 0.27 ± 0.01 mL/min ($p<0.05$); (n=3-5). The AG administration did not affect the glomerular filtration rate (GFR) in STZ-induced+AG treated rat groups compared with that in STZ-induced rat groups (1.1 ± 0.07 vs. 1.1 ± 0.08 mL/min; **Figure: 3.13)** and the GFR was also found to be similar in Vehicle+AG treated rat groups and Vehicle rat rat groups (0.29 ± 0.02 vs. 0.27 ± 0.01 mL/min). The GFR

was, however, significantly increased in the STZ-treated diabetic rats and STZ-induced+AG treated rat groups compared to the Vehicle and Vehicle+AG-treated rat groups **(Figure 3.13)**

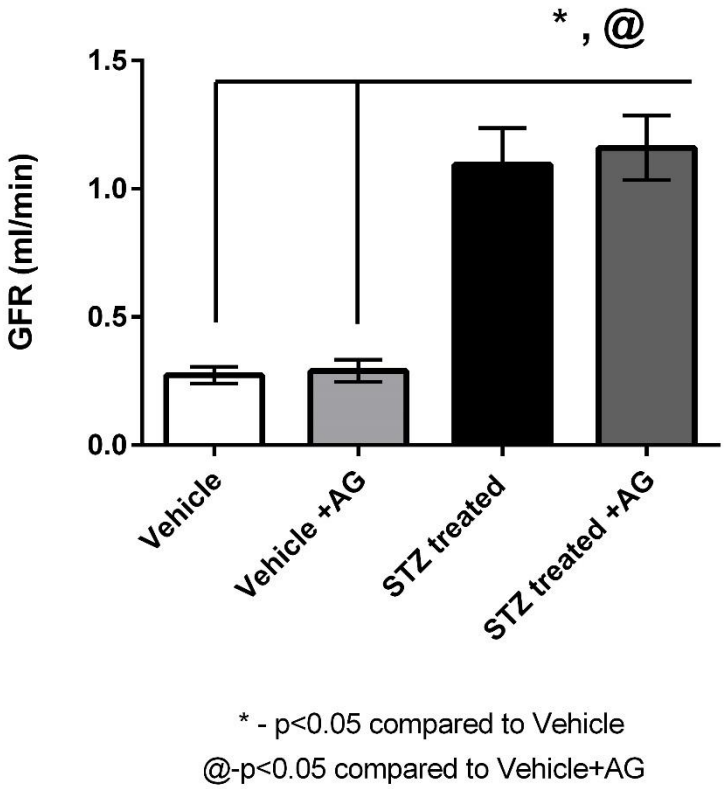

Figure 3.13: Effect of aminoguanidine (AG) treatment (1 g/L) on glomerular filtration rate (ml/min) of STZ-induced diabetic rat groups compared to vehicle rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM. *p<0.05 compared to vehicle rat groups, @ p<0.05 compared to Vehicle+AG treated rat groups (Student's t-test). n = (3–5).

3.2.4 AG Treatment of STZ-induced+AG treated Diabetic Rat Groups Considerably Decreased Urine Sodium Excretion (mmol/L)

The STZ-treated diabetic rat groups had significantly increased urine sodium excretion compared with that in the Vehicle rat groups (3.24 ± 0.40 vs. 1.35 ± 0.21 mmol/L, p<0.05; **Figure 3.14).** The administration of AG resulted in a considerably decreased urine sodium

excretion, although not significant, in the STZ-induced+AG treated rat groups compared with that in STZ-induced rat groups (2.12 ± 0.63 vs. 3.24 ± 0.40 mmol/L; **Figure 3.14**). The urine sodium excretion in Vehicle+AG treated rat groups was similar to those in Vehicle rats (0.84 ± 0.06 vs. 1.35 ± 0.21 mmol/L). However, urine sodium excretion in the STZ-induced+AG treated rat groups were increased compared with those in Vehicle+AG treated rat groups (2.12 ± 0.63 vs. 0.84 ± 0.06 mmol/L, $p<0.05$) **(Figure 3.14).** Moreover, the urine sodium excretion were significantly increased in the STZ-induced diabetic rat groups compared with that in the Vehicle +AG treated group of rats (3.24 ± 0.40 vs. 0.84 ± 0.06 m mol/L, ($p<0.05$); **Figure 3.14)**. No difference was recorded in the urinary sodium excretion between the Vehicle rat groups and the Vehicle+AG-treated rat groups (1.35 ± 0.21 vs. 0.84 ± 0.06 m mol/L; **Figure 3.14)**.

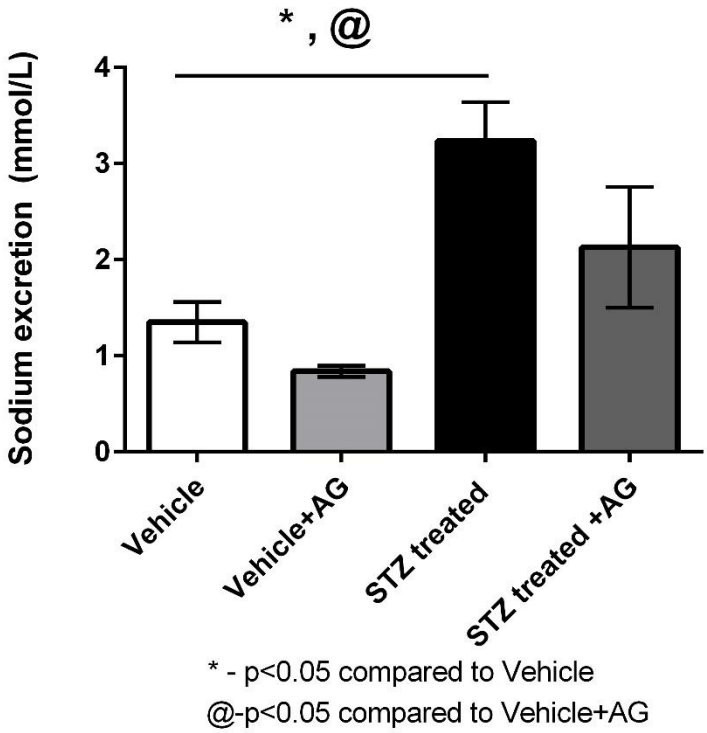

Figure 3.14: Effect of aminoguanidine (AG) treatment (1 g/L) on urinary sodium excretion in the streptozotocin (STZ)-induced diabetic rat groups compared to vehicle rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM. *p<0.05 compared to vehicle rat groups, @ p<0.05 compared to Vehicle+AG treated rat groups (Student's t-test). n = (3–5).

3.2.5 AG Treatment of STZ-induced+AG-treated Diabetic Rat Groups Shows Modest Reversal in Serum Creatinine (mg/dL)

STZ treated diabetic rat groups had significantly decreased serum creatinine levels, compared with that in the Vehicle group rat groups (0.77 ± 0.02 vs. 1.12± 0.06 mg/dL ($p<0.05$, n=4). Administration of AG (1 g/L) resulted in a modest reversal in serum creatinine in the STZ+AG-treated rat groups compared with that in STZ-treated rat groups (0.97±0.08 vs. 0.77±0.02 mg/dL). The levels of Creatinine in the Vehicle +AG treated rat groups had no significant changes compared with those in vehicle rat groups (1.37±0.14 vs. 1.12±0.06 mg/dL). There

was no significant difference between the levels of serum creatinine in the STZ-treated rat groups that were administered AG compared with that in Vehicle+AG treated rat groups (0.97±0.08 vs. 1.37±0.14 mg/dL). There is no significant difference between serum creatinine in STZ-induced +AG treated rat groups compared with that in Vehicle rat groups (0.97±0.08 vs. 1.12±0.06 mg/dL, n=4; **Table 3.1**).

Table 3.1 Serum Creatinine(mg/dL) levels in the various animal groups

Animal Groups	**Mean ± SEM values**
Vehicle rats	1.12± 0.06
Vehicle + AG treated	1.37±0.14
STZ treated rats	0.77 ± 0.02*
STZ + AG treated rats	0.97±0.08@

Values are expressed means ± SEM. *p<0.05 compared to Vehicle rats, @ p<0.05 compared to Vehicle+AG rats (Student's t-test). n = (3–5).

3.2.6 AG Treatment of STZ-induced+AG-treated Diabetic Rat Groups Shows a Considerable Decrease in the Urine Creatinine (mg/dL)

STZ treated diabetic rat groups showed a significantly decreased urine creatinine concentration, compared with that in the Vehicle group rat groups (43.38± 13.69 vs. 115.2 ± 17.56 mg/dL) (p<0.05, n=3-4 Table 3.2). Administration of AG (1 g/L) showed a considerable decrease in the urine creatinine in STZ+AG treated rat groups, as that of the STZ-treated rat groups ((36.87± 1.59 vs. 43.38±13.69 mg/dL; n=3-4, **Table 3.2**). The levels of urine creatinine, in the Vehicle+AG treated rat groups had no significant changes compared with those in Vehicle rat groups (95.97± 14.08 vs. 115.2 ± 17.56 mg/dL n=3. The urine creatinine significantly decreased in the STZ+AG treated rat groups compared with that in Vehicle+AG

treated rat groups (36.87±59 vs. 95.97± 14.08 mg/dL, n=3, ($p<0.05$); **Table 3.2.** The urine creatinine was also significantly decreased in STZ-induced rat groups compared with that in the Vehicle+AG treated rat groups (43.38 ± 13.69 vs. 95.97±14.08 mg/dL, n=3. The urine creatinine also shows a significant decrease in the STZ-induced+AG treated rat groups compared with that in Vehicle rat groups (36.87±1.59 vs. 115.2±17.56 mg/dL, n=3 ($p<0.05$); **Table 3.2**

Table 3.2 Urine Creatinine (mg/dL) levels in the various animal groups

Animal Groups	**Mean ± SEM values**
Vehicle rats	115.2 ± 17.56
Vehicle + AG treated rats	95.97± 14.08
STZ treated rats	43.38± 13.69*
STZ + AG treated rats	36.87± 1.59@

Values are expressed means ± SEM. *$p<0.05$ compared to vehicle rats, @ $p<0.05$ compared to Vehicle+AG rats (Student's t-test). n = (3–5).

3.2.7 AG Treatment of STZ-induced+AG-treated Diabetic Rats Shows No Change in Serum Sodium (mmol/L)

The STZ treated diabetic rat groups had significantly increased Serum sodium levels compared with that in the Vehicle group rat groups (145.4±2.98 vs. 135.7±2.47 mmol/L ($p<0.05$); n=4, **Table 3.3).** Administration of AG (1 g/L) resulted in no change in the serum sodium in the STZ- induced+AG-treated rat groups compared with that in STZ-treated rat groups (143.4± 1.84 vs. 145.4 ± 2.98 mmol/L, n=4, **Table 3.3).** The levels of serum sodium, in Vehicle+ AG treated rat groups, had no significant changes compared with those in Vehicle rat groups (135.8±2.03 vs. 135.7±2.47 mmol/L n=4, **Table 3.3).** The serum sodium was significantly

increased in the STZ-induced+AG treated rat groups compared with that in Vehicle+AG treated rat groups (143.4±1.84 vs. 135.8±2.03 mmol/L, n=4, ($p<0.05$); **Table 3.3).** The serum sodium levels were, however, significantly increased in the STZ-treated diabetic rat groups compared to the Vehicle+AG-treated rat groups (145.4 ± 2.98 vs. 135.8± 2.03 mmol/L, n=4, ($p<0.05$) :(**Table 3.3).** The serum sodium level was significantly increased in the STZ-induced+AG treated rat groups compared with that in Vehicle rat groups (143.4±1.84 vs. 135.7±2.47 mmol/L, n=4 ($p<0.05$); **Table 3.3).**

Table 3.3 Serum sodium(mmol/L) levels in the various animal groups

Animal Groups	**Mean ± SEM values**
Vehicle	135.7±2.47
Vehicle + AG treated rats	135.8±2.03
STZ treated rats	145.4±2.98*
STZ induced+ AG treated rats	143.4± 1.84@

Values are expressed means ± SEM. *$p<0.05$ compared to vehicle rats, @ $p<0.05$ compared to Vehicle+AG rats (Student's t-test). n = (3–5).

3.2.8 AG Treatment of STZ-induced+AG-treated Diabetic Rat Groups Show Reversal of the Decrease in The Urine Sodium (mmol/L)

Urine sodium level was significantly decreased in the STZ-induced diabetic rat groups, compared with that in the Vehicle group rat groups (77.50 ±3.428 vs.109.7±8.41 mmol/L ($p<0.05$); n=4, **Table 3.4).** Administration of AG (1 g/L) resulted in a considerably reversal of the decrease in the urine sodium levels in the STZ- induced+AG-treated rat groups compared with that in STZ-treated rat groups (104.7±29.81 vs. 77.50 ±3.428 mmol/L, n=4, **Table 3.4).** The levels of urine sodium, in Vehicle+AG treated rat groups, showed a moderate change

compared with those of the Vehicle rat groups (122.0± 18.00 vs. 109.7±8.41 mmol/L n=4, **Table 3.4).** The Urine sodium was moderately decreased in the STZ-induced+AG treated rat groups compared with that in vehicle+ AG treated rat groups (104.7±29.81 vs. 122.0± 18.00 mmol/L, n=4, ($p<0.05$); **Table 3.4).** The urine sodium was, however, significantly decreased in the STZ-treated diabetic rat groups compared to the Vehicle+AG-treated rat groups (77.50± 3.428 vs. 122.0±18.00 mmol/L, n=4, ($p<0.05$); **Table 3.4).** The urine sodium level was almost similar in the STZ-induced+AG treated rat groups compared with that in Vehicle rat groups (104.7±29.81 vs. 109.7±8.41mmol/L, n=4 ($p<0.05$); **Table 3.4.**

Table 3.4 Urine Sodium (mmol/L) levels in the various animal groups

Animal Groups	**Mean ± SEM values**
Vehicle	109.7 ± 8.41
Vehicle + AG treated rats	122.0 ± 18.00
STZ treated rats	77.50 ± 3.428*
STZ induced+ AG treated rats	104.7 ± 29.81

Values are expressed means ± SEM. *$p<0.05$ compared to vehicle rats, @ $p<0.05$ compared to Vehicle+AG rats (Student's t-test). n = (3–5)

3.2.9 AG Treatment of STZ-Induced Diabetic Rats Did Not Affect The Changes in The Glomerular Damage (%) in The STZ-induced+AG-treated Rat Groups

Significant changes were noted in the glomerular structures between the STZ-induced diabetic rat groups and Vehicle rat groups **(Figure 3.15)**. The extent of glomerular damage (%) was recorded by the visualization of the damaged glomerulus in various fields, as observed under the microscope. The glomerular damage was recorded as a percent of glomerular damage, compared to the Vehicle groups of rats by the H&E staining **(Figure. 3.15)**. While the Vehicle

rats had a normal glomerulus showing normal Bowman's space and capillaries, the STZ-induced diabetic rats showed obliteration of the Bowman's space, collapsed capillaries, mesangial cell proliferation, and deposition of hyaline material. **(Figure. 3.2.5)**. The STZ-induced diabetic rat groups and STZ-induced+AG-treated rats groups both showed diffused deposition of hyaline material with collapsed capillaries. These are the classical features of diabetic nephropathy that were visualized in the kidneys of diabetic rats using H&E staining.

A greater deposition of PAS-positive material was observed in STZ-indued diabetic rats compared with that in Vehicle rats. The mesangial cell proliferation was increased in STZ treated rat groups and STZ-induced+AG treated rat groups compared with that in Vehicle and Vehicle+AG) **(Figure 3.16)**.

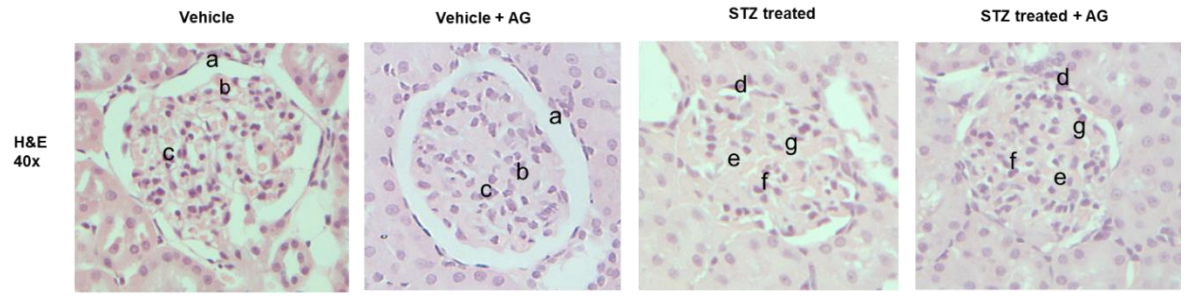

Figure 3.15 Effect of aminoguanidine (AG) treatment (1 g/L) on the renal damage as visualized with hematoxylin and eosin (H&E) staining of all four groups. a) Bowman's space b) Normal Glomerular capillaries c) Mesangial cells d) Obliteration of Bowman's Space e) Collapsed capillaries f) Mesangial Cells proliferation.

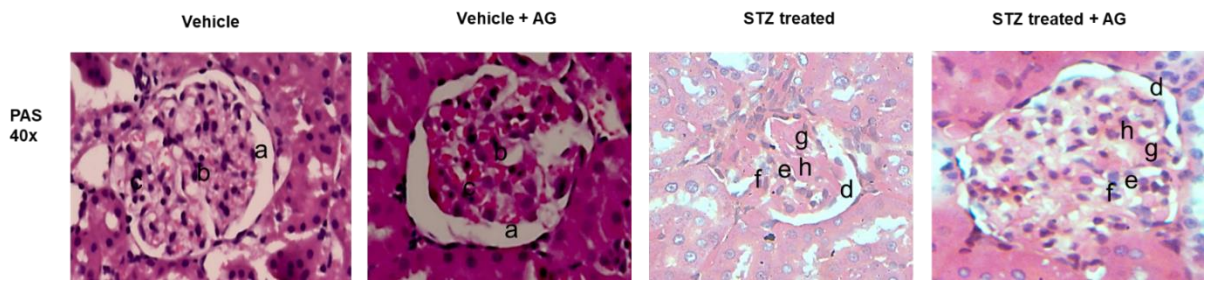

Figure 3.16 Effect of aminoguanidine (AG) treatment (1 g/L) on the renal damage as visualized with hematoxylin and eosin PAS staining of all four groups. Changes in glomerular histology visualized included. a) Bowman's space b) Normal Glomerular capillaries c) Mesangial cells d) Obliteration of Bowman's Space e) Collapsed capillaries f) Mesangial Cells proliferation g) Hyaline deposition in matrix h) PAS-positive material deposition in matrix

3.2.10 Effect of Aminoguanidine (AG) Treatment (1 g/L) on the Renal Damage as Visualized with Hematoxylin and Eosin (H&E) and PAS Staining in the STZ-induced+AG-treated Rat Groups

The extent of glomerular damage, expressed as percent damage, as quantified by the field analysis, having 10 or more glomeruli in a field, was significantly increased in the STZ-induced diabetic rat groups compared with that in the Vehicle rat groups (43.34 ± 7.60 vs. 7.79 ± 4.60 ($p<0.05$); **Figure 3.17**). Administration of AG (1 g/L) did not affect the changes in the glomerular damage, as observed for the STZ-induced diabetic rat groups (41.50 ± 2.06% vs. 43.34 ± 7.60 %). No significant difference in the glomerular damage was recorded between the Vehicle+AG treated rat groups and Vehicle rat groups (10.67 ± 1.76 vs. 7.79 ± 4.60). However, significantly increased glomerular damage was observed in STZ-induced+AG treated rat groups compared with that in Vehicle+AG treeated rat groups (41.50 ± 2.06% vs. 10.67 ± 1.76, $p<0.05$).

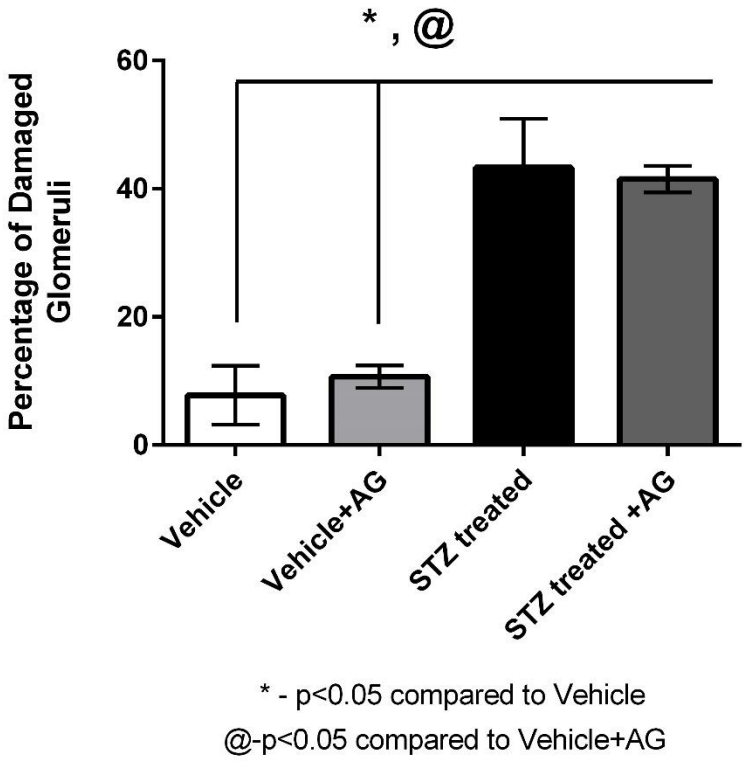

Figure 3.17: Effect of AG treatment (1 g/L) on (3 B) glomerular damage in vehicle and streptozotocin (STZ)-induced diabetic rat groups and Vehicle+AG treated rat groups and STZ+AG treated rat groups. Values are means ± SEM. *p<0.05 compared to vehicle rats, @ p<0.05 compared to Vehicle+AG rats (Student's t-test) n = (3–5).

3.2.11 Discussion

Diabetes leads particularly to a filtration process overload by the kidneys. This excess workload makes the kidneys susceptible to various regulatory mechanism that affects the functions of the kidneys leading to a decline in the kidney function over a period of time. Among the notable factors that affect kidneys are the increased glycation and accumulation of glycated plasma proteins[321]. These glycated products also affects the functioning of various organs including , blood vessels[305], eyes[228], and nerves[250] play an essential role in the pathogenesis of diabetic complications.

A previous study by Thomas MC et al. have shown that there is an interaction between the renin-angiotensin system and advanced glycation in the kidney[379] and AG showed a beneficial effect in the prevention of diabetic nephropathy due to its ability to reduce urine volume, GFR, and serum AGEs[327]. Therefore, treatment with AG has been reported to be beneficial for the prevention of diabetic complications in STZ-induced diabetic rats. Hence, in the current study, we also investigated the effect of AG on STZ-induced rats and indices of renal functions. Our results demonstrated that AG treatment did not cause an increase in urine protein or an index of renal injury in STZ treated AG rats, but it did lead to decreased creatinine and urine sodium. Furthermore, it did cause significant reversion of the UFR and did not further increase the GFR, in early diabetic conditions. Additionally, AG did not increase serum sodium and potassium. Therefore, treatment with AG indicated the importance of controlling and regulating the renal functions in the early diabetic condition.

In the present most promising results, we observed that alteration of kidney function parameters, including the presence of protein in the urine, elevated urine flow rate, GFR, and urine sodium excretion, as well as serum sodium levels in STZ-induced diabetic model. Although we did not perform experiment on the underlying mechanism, but we speculating that alteration in the RAS system in diabetes, and this observation is supported by Anderson et al[380] and other [381]. We observed renal injuries as observed by - collapsed capillaries in the Bowman's capsule, mesangial cell proliferation, deposition of the hyaline material, etc.—in STZ-treated rats, which are characteristics of diabetic lesions. This is also manifested by increased protein levels in the urine[380]. Surprisingly, the Vehicle+AG rats also showed slightly increased urine protein levels compared with that in Vehicle rats, which may be attributable to an acute effect, most likely induced by AG, and this observation is supported by Kostic T[382]. However, we did not see any noticeable changes in the glomerular structure (V+AG), which implies an acute effect only, thereby causing proteinuria, and most likely not affecting the

glomerular structure, greatly. This notion is apparently more credible considering the fact that the urinary protein levels were also enhanced in the STZ-induced+AG rats compared with that in the STZ-induced rats. This is more likely an acute phenomenon that is induced by AG treatment, which warrants further investigation.

Creatinine is a breakdown product of creatine phosphate in muscle and its clearance rate from blood to urine correlates with the glomerular filtration rate**.** During this phase, creatinine levels may not be significantly elevated, as the kidneys are still capable of maintaining normal to slightly increased filtration rate, as the integrity of the glomerulus is not damaged to that of the level of end state damage. These are classical characteristics of an early stage of diabetic complications[297]. In the present study highlights the significantly increased sodium excretion STZ-induced diabetic rats showed compared to vehicle rats as previously reported by Hakem et al.[297]. This might be due to the underlying mechanisms associated with renal sodium excretion affected by the sodium transporters, which include sodium-glucose cotransporter 2 (SGLT2) and the sodium-hydrogen exchanger 3 (NHE3), and Na, K ATPase pump[297,383]. Treatment with AG slightly decreased the sodium excretion in STZ+AG rats but had no change in serum sodium levels in STZ+AG rats. This indicates that the AG is helping in the attenuation of the loss of excess sodium and balances electrolytes, contrary to what happens in the early diabetic condition. Treatment with AG partially or modestly reverses kidney dysfunctions, such as UFR and GFR as reported by Soulis et al[327]. It has, however, not affected a few other parameters. We, therefore, see an anomalous and/ or selective action of the action of AGE

3.3. Objective 3

3.3.1 Effect of Aminoguanidine (AG) Treatment (1 g/L) on STZ-induced diabetic rats had Moderate Increased Expression of Renin in The Kidney of STZ-induced+AG treated Rat Groups

The expression of the renin protein in the kidney tissue was determined using western blot analysis. Renin antibodies detected a band of approximately 45 kDa. Densitometry analysis showed that the levels of the Renin protein were not changed in STZ-induced diabetic rat groups compared with that in Vehicle rat groups (1.39± 0.23 vs. 1.38± 0.33, n=4, **Figure 3.18**). However, upon treatment with AG, the STZ-induced+AG treated rat groups showed a moderate increased, but not significant, expression levels of the renin protein (1.65± 0.21 vs. 1.39± 0.23, (**Figure: 3.18**).

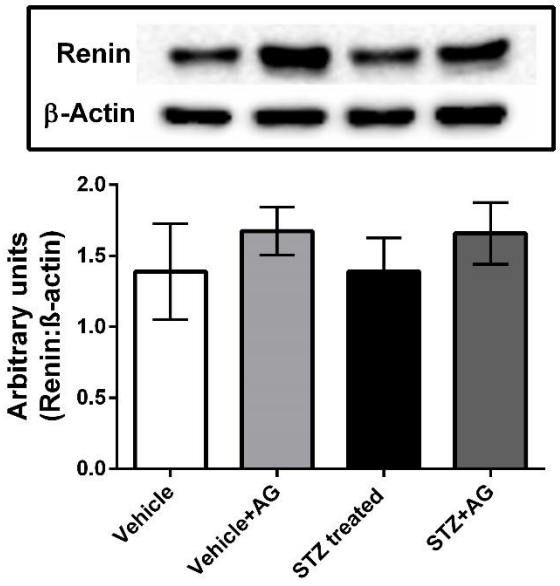

Figure 3.18: Effect of aminoguanidine (AG) treatment (1 g/L) on the expression of renin in the kidney of the vehicle and streptozotocin (STZ)-induced diabetic rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. The renin protein normalized against β-actin. Values are means ±SEM.

3.3.2 Effect of Aminoguanidine (AG) Treatment (1 g/L) on STZ-induced diabetic rats had No Change in Expression of Ang II, Type 1, AT_1, and a Modest Decrease in Type 2 receptors, AT_2 in STZ-induced+AG treated Rat Groups

The expression of the AT_1 and AT_2 receptor proteins in the kidney tissue was determined using western blot analysis. The AT_1, and AT_2 receptor antibodies detected a band of approximately 45 kDa. Densitometry analysis showed that the levels of the AT_1 receptors were significantly increased in STZ-induced rat groups compared with that in Vehicle rats (1.08 ± 0.12 vs. 0.55 ± 0.04, almost 1.9 fold ($p<0.05$); **Figure 3.19**). The AT_2 receptor levels were also significantly increased by almost 2.6-fold in the STZ-induced diabetic rats compared with those in the Vehicle rats (1.12 ± 0.08 vs. 0.46 ± 0.07 ($p<0.05$); **Figure 3.19**). However, upon treatment with AG, the STZ-induced+AG treated rat groups showed a modest decrease (0.99 ± 0.07 vs. 1.12 ± 0.08) in AT_2 receptor levels (**Figure 3.19**). On the contrary, the AT_1 receptor levels did not change in STZ-induced+AG treated rat groups compared with that in the STZ-induced diabetic rat groups (1.1 ± 0.19 vs. 1.08 ± 0.12; **Figure 3.19**). No significant difference was observed in the expression of AT_1 and AT_2 between the Vehicle and the Vehicle+AG-treated rats.

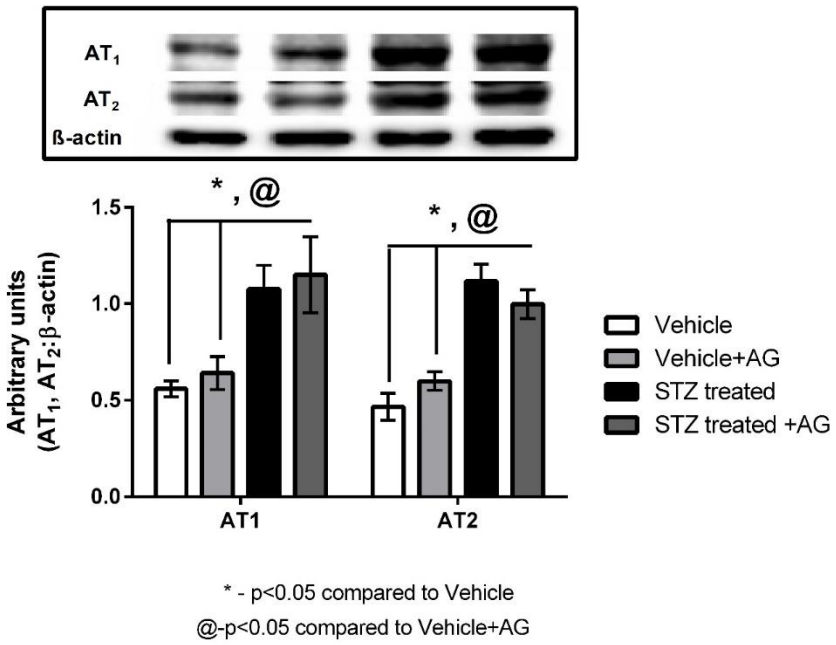

Figure 3.19: Effect of aminoguanidine (AG) treatment (1 g/L) on the expression of AT_1 and AT_2 receptors in the kidney of the vehicle and streptozotocin (STZ)-induced diabetic rats and Vehicle+AG treated rat groups and STZ+AG treated rat groups. The AT_1 and AT_2 proteins normalized against β-actin. Values are means ±SEM. *p<0.05 compared to vehicle rat groups, @ p<0.05 compared to Vehicle+AG rat groups (Student's t-test) n = (3–5).

3.3.3 Effect of Aminoguanidine (AG) Treatment (1 g/L) on STZ-induced diabetic rats No Change in Expression of Ang II, Type 1, AT_1 receptor in the heart of in STZ-induced+AG treated Rat Groups

The expression of the AT_1 receptor proteins in the heart tissue was determined using western blot analysis. The AT_1, receptor antibodies detected a band of approximately 45 kDa. Densitometry analysis showed that the levels of the AT_1 receptors were significantly increased in STZ-induced rat groups compared with that in Vehicle rat groups (1.64± 0.19 vs. 0.71 ± 0.08, n=4, almost 2 fold (p<0.05); **Figure 3.20**). Upon treatment with AG, the AT_1 receptor levels did not change further in STZ-induced+AG treated rat groups compared with that in the

STZ-induced diabetic rat groups and were almost similar to that observed in the STZ-induced diabetic rats (1.71 ± 0.26 vs. 1.64± 0.19; **Figure 3.20**). No significant difference was observed in the expression of AT_1 between the Vehicle and the Vehicle+AG-treated rat groups.

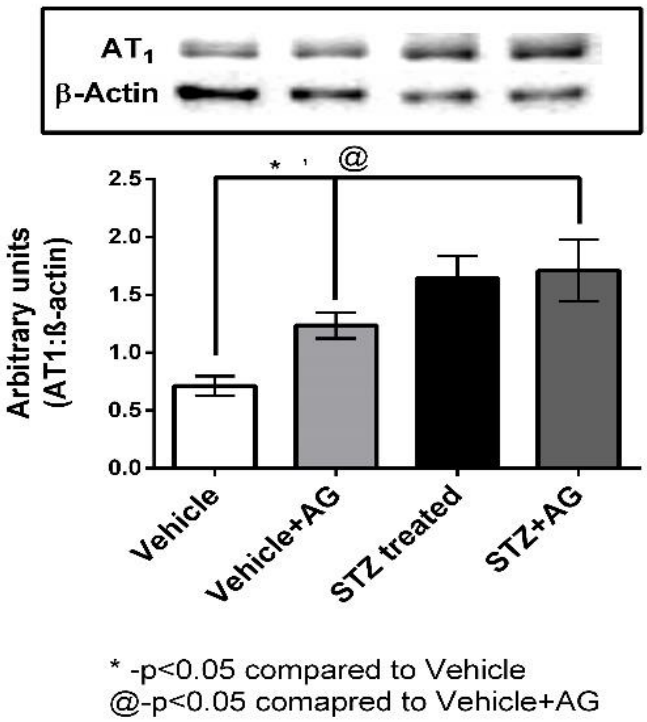

Figure 3.20: Effect of aminoguanidine (AG) treatment (1 g/L) on the expression of AT_1 receptors in the heart of the vehicle rat groups and streptozotocin (STZ)-induced diabetic rat groups, and Vehicle+AG treated rat groups and STZ+AG treated rat groups. The AT_1 protein of the heart normalized against β-actin. Values are means ±SEM. *p<0.05 compared to vehicle rats, @ p<0.05 compared to Vehicle+AG rats (Student's t-test) n = (3–5).

3.3.4 Discussion

There is an evidence showed that diabetic complications have been associated with hyperglycemia[384] and hypertension[132]. A study explored early diabetes rats stimulating the proximal tubules renin and angiotensin II and receptors in diabetic kidney [135,385,386]. Our study demonstrates the upregulation of renal AT_1 and AT_2 protein expression, and no change in renin in STZ-diabetic rats by the immunoblotting experiment as previously reported [135,296,297,387]. Despite the upregulation of renal AT_1 and AT_2, the most robustic finding in this study is that the treatment with AG causes a considerable decrease in AT_2 expression in STZ induced+AG treated rat groups, which is a most desirable effect from a clinical point of view. Although we did not study functional significance, we speculated that angiotensin II receptors to specific kidney sites may suggest their involvement in the physiological regulation of renal haemodynamic [386], excretory functions (natriuresis) [296], and their possible contribution to renal diseases. It is important to note that the fold increases in the AT_2 receptors in the STZ treated diabetic rats was more (2.6) compared with that of the AT_1 receptors (1.9), which is in consonance with previous studies by Romero-Nava, R. et al and Athar H Siddiqui [86,388]. The overexpression of AT_2 could be associated with the reduction in the response to Ang II in the early stage of diabetes. Numerious studies showed that significantly increased AT_1 receptors was associated with an increased stimulation of Na+/K+ ATPase pump in obese Zucker rats and OK cells [154,389]. There is also a report that shows that the renal angiotensin II AT_2 receptors promote natriuresis in streptozotocin-induced diabetic rats and obese Zucker rats [388,390]. We, very positively speculate that the natriuretic effects of the AT_2 receptors overcome the anti-natriuretic effects of the AT_1 receptors, in our current study. As a consequence of which, we see increased sodium excretion levels in the STZ-treated diabetic rats. This is a very common feature in early-stage diabetes where hyperfiltration could lead to increased sodium excretion. We also demonstrate increased sodium excretion in STZ-induced diabetic rats, which implies

an increase in natriuretic functions in the STZ-induced diabetic rats[312]. The administration of AG led to decreased renal levels of the AT_2 receptor and did not observe any change in the renal levels of the AT_1 receptor in STZ+AG rats as compared to STZ treated diabetic rats. We, therefore speculate that no major difference exists in the anti-natriuretic function AT_2 receptor in STZ +AG induced rats.

Our data also show no change in renal renin protein expression in STZ treated rats and moderately increased renin protein expression after AG administration in STZ+AG treated diabetic rats as compared to STZ treated diabetic rats. The AT_1 receptor regulates renin production through the negative feedback mechanism. Thus, it is possible that increased AT_1 expression in STZ treated rats may have contributed change in renin protein expression, as increased renin would have further increased the signalling cascade of Ang II and an increased Ang II would have led to an increase in the anti-natriuretic function[385]. We, are therefore, inclined to say here that the increase in renin is prevented or inhibited, as a compensatory mechanism to regulate the anti-natriuretic functions in our model of rats, in the present study.

The heart is a muscular organ containing a network of blood vessels and Angiotensin II receptors (AT_1 and AT_2). The activation of the AT_1 receptor causes vasoconstriction leading to increased blood pressure[86]. Our results showed increased protein expression in AT_1 expression in the heart of STZ-induced rats compared to vehicle rats similar to other reports [86]. However, we did not see any significant difference after AG treatment in STZ-induced rats, meaning thereby a minimal effect of AG treatment in affecting the vasoconstriction of the arteries in the heart.

In conclusion, this study demonstrated the upregulation of Angiotensin II (AT_1 and AT_2) and no change in renin protein expression in diabetic conditions. After AG treatment a

slight decrease in AT_2 receptor may have contributed to the enhancement of renin expression levels.

CHAPTER 4: SUMMARY AND CONCLUSIONS

SUMMARY

Diabetes is a multifactorial disease that affects millions of people worldwide. It leads to various complications, affecting most of the organs in the body, including kidneys, heart, eyes, nerves, and vasculature. The role of advanced glycation end products (AGEs) in the pathogenesis of diabetic complications is well known. This study was aimed at investigating the efficacy of aminoguanidine (AG), a drug that inhibits the production of AGEs, to mitigate the complications of diabetes, with a focus on renal functions.

This study was mainly aimed at two aspects (i) rethinking the roles of AGEs in diabetes and diabetes-induced hypertension, in light of the background information collected from a thorough and detailed literature survey and (ii) the regulation of RAS and its effect on the renal functions in diabetic rats treated with AG.

Although the roles of AGEs in diabetes and related complications have been convincingly demonstrated, the accumulation of AGEs and their specific roles in the regulation of hypertension under the diabetic conditions remain unclear. Accumulating evidence indicates the roles of AGEs in insulin signaling and insulin resistance[128,391,392], and hence in diabetes. As the accumulation or production of AGEs is inhibited in the presence of AG, in this study, it was hypothesized that AG treatment of STZ-induced diabetic rats would result in the reversion of the increased blood glucose levels or at least prevent hyperglycaemia.

The renin-angiotensin system (RAS) is critical in maintaining water and electrolyte homeostasis. It regulates many physiological functions of the body, especially the function of the kidneys. Because altered regulation of RAS under diabetes leads to diabetic complications, including hypertension and renal diseases, an attempt was made in this study to examine these complications under diabetic conditions.

Various animal models have been used to study diabetic complications. The streptozotocin (STZ)-induced diabetes model is a very good animal model to study the effect of diabetes on RAS. In the present study, the STZ-induced diabetes rat model was used to determine the effects of AG on various indices of RAS and renal function. Additionally, the effects of AG on various hemodynamic parameters and physiological functions in rats were also investigated. This study focused on observing various renal functions, such as glomerular filtration rate (GFR) and urine flow rate (UFR), as well as, on determining the glomerular damage and natriuretic functions in diabetic and AG-treated rats. The action of the most important hormone in the pressor arm of RAS, angiotensin II (Ang II), is mediated via angiotensin II receptor subtypes 1 (AT_1) and 2 (AT_2). The expression levels of these receptors were determined in the kidneys of diabetic rats to assess the effect of Ang II on renal functions mediated by these receptors.

This study was further aimed at understanding the effect of AG treatment on RAS. The alterations in RAS under diabetic conditions are well known[293,380]. This study was focused on evaluating the action of the most important hormone in the pressor arm of RAS, Ang II, and on the receptors that mediate the actions of Ang II, AT_1, and AT_2. The expression levels of these receptors in the kidneys were determined. Moreover, another important index of RAS function—the sodium homeostasis—which is a major contributor to hypertension, was also investigated. This study was conducted with the following specific objectives:

1. To establish an animal model of diabetes in Sprague-Dawley (SD) rats and study the effects of AG treatment on clinical phenotypes and hemodynamic parameters in STZ-induced diabetic rats.
2. To investigate the effects of AG on various aspects of RAS functions, renal functions, and renal damage, viz., urine flow, urinary protein, glomerular flow rate, sodium excretion, and glomerular damage, in STZ-induced diabetics rats.

3. To perform expression analysis of the angiotensin II receptors, AT_1 and AT_2.

An STZ-induced diabetic animal model was employed in the present study. Four groups of rats, which included the vehicle-treated rats, vehicle+AG treated rats, STZ-induced diabetic rats, and AG-treated STZ-induced diabetic rats, were used in this study.

Objective 1: An STZ-induced diabetic rat model was established and used to determine the therapeutic effects of AG by evaluating the phenotypic changes. It was ensured that the vehicle-treated rats were not diabetic. AG treatment prevented the increase in blood glucose levels in the STZ-induced diabetic rats. AG treatment also affected the body weight, food and water consumption, and urine volume and should provide a new dimension to strategies for the prevention or delay of the onset of diabetic complications.

STZ destroys the islet cells of the pancreas resulting in the impairment of insulin production, which leads to an increase in the blood sugar levels. Considering the prevention of the increase in blood sugar levels by AG, it was postulated that the AG treatment might result in enhancing the sensitivity of rats to the small amounts of insulin present after STZ treatment. Thus, we could see some insulin action, evident by improved glucose utilization, and thereby, a significant decrease in the blood glucose levels in the STZ+AG treated rats compared with that in the STZ-induced diabetic rats. Determining the insulin levels in the STZ+AG treated rats will be one of the aims of a future study, which will help in providing us with a clear answer to this notion.

Objective 2: The notion that AG treatment affects the functions of the kidney was analyzed. AG had an anomalous and/or selective action. The changes in renal function, including UFR, GFR, sodium excretion (natriuresis), urine protein, and glomerular damage, were determined. AG partially or moderately reversed the alterations in kidney function parameters, such as UFR, GFR, and natriuresis, indicating that, in diabetes, renal function is either reversed or

remains unaffected, but is not exacerbated, by AG treatment. However, AG did not affect such as urinary protein and glomerular damage.

A few of the physiological functions were reversed upon AG treatment and were not as severe as in the untreated diabetic animals. Moreover, glomerular damage, which is associated with diabetic lesions and is a very important manifestation of diabetic nephropathy, was clearly evident in diabetic rats. Treatment with AG did not prevent the glomerular damage, and it was almost the same as that in STZ-induced diabetic animals. However, other renal functions, such as natriuresis (sodium excretion), GFR, and UFR, were reversed or partially restored/improved upon treatment with AG compared with that of the STZ-induced diabetic rats. Natriuresis is an important physiological indicator of sodium regulation in the body, and hence, a critical determinant of blood pressure. Sodium excretion was noted to increase in the diabetic rats. This phenomenon is noticed in early-stage diabetic subjects, who have dysregulated sodium homeostasis due to hyperfiltration by the kidneys. Treatment with AG considerably reversed the natriuresis in the STZ+AG-treated diabetic rats. These results suggest that AG treatment prevents deterioration of the renal functions that are mostly of physiological and homeostatic origin. It also highlights the important role of AGEs in the pathogenesis of diabetic complications, especially in the kidneys.

Objective 3: The expression levels of AT_1 and AT_2 receptors and renin in the kidney and that of AT_1 receptors in the heart were determined. The expression of AT_1 and AT_2 receptors was altered under diabetes conditions. On AG treatment, a decrease in the levels of the AT_2 receptor was observed. The decreased glucose levels in STZ+AG-treated rats, coupled with decreased expression of AT_2 receptors, reversed the natriuresis. These results provide insights into the role of RAS in diabetic complications. However, further studies on the mechanistic pathways facilitating the increased natriuresis in diabetic rats that caused reversion upon AG treatment, need to be done.

We intend to mention here that AG treatment prevents the increase in blood sugar levels. However, in the STZ-induced diabetic rats, an increase in AT_2 receptors was observed, which is directly responsible for an increased natriuretic function[296,312]. Corroborating these findings, an increased expression of AT_2 receptor and natriuresis in diabetic rats was demonstrated in this study. Because a direct link between the blood sugar and AT_2 receptor levels has been reported[296 312], we emphasize that decreased sugar levels in STZ+AG-treated rats might also lead to decreased AT_2 receptor levels in these rats. A decrease in the expression of these receptors will lead to a decrease in natriuretic function, as observed, in our study. Because natriuresis was reversed by AG treatment, and although they exhibited significantly high levels of glucose, the sodium excretion was not as high as that in the STZ-induced diabetic rats. Therefore, it is speculated that although the levels of AT_2 receptors were modestly decreased, sodium excretion decreased considerably indicating that these receptors may not be playing a prominent role in STZ+AG-treated rats.

CONCLUSIONS

1. The animal model developed in the present study can be used for studying diabetic complications. This is due to the important ramifications of diabetes as mimicked in the diabetic animal model, with regards to :

(i) Clinical phenotypes: Elevated blood glucose levels, and decreased body weight

(ii) Physiological determinants: Increased food and water consumption, increased urine output, and increased urine flow rate

(iii) Altered renal functions: The effect on glomerular filtration rate, presence of urine protein, dysregulation of urine and serum creatinine as well as serum sodium, urinary sodium excretion, and glomeruli damage

2. Aminoguanidine (AG), an inhibitor of advanced glycation end products (AGEs) has been shown to be effective in significantly preventing the increase in blood glucose levels in the STZ+AG-treated rats. Additionally, it has been shown to be responsible for partially or modestly reversing the conditions noted in STZ-induced diabetic rats, namely urine flow and body weight. However, it did not affect a few other parameters. Therefore, an anomalous and/or selective action of AG was observed in diabetic rats.

3. The breakdown of AGEs is being utilized to treat hypertensive conditions in diabetes. With the information obtained from these studies, with regard to the prevention of an increase in blood glucose levels by AG, a new dimension is provided to the biology of diabetes and AGEs involving RAS.

4. The increased expression of AT_1 and AT_2 receptors in the kidney of STZ-induced diabetic rats has been shown previously (Ref.) and our results are consistent with those findings. It is plausible, therefore, to comment on the anti-natriuretic and natriuretic functions of these

receptors, which regulate the sodium levels in the body. This is a very common feature, in the regulation of sodium homeostasis in diabetes. This is primarily attributed to the altered regulation and functions of RAS.

LIMITATIONS OF THE STUDY

The present study aimed at investigating the effects of AG on STZ-induced diabetic rats has several limitations as listed below:

1. Diabetic animals are prone to frequent urination and diarrhea, which leads to the development of infection. Maintaining the animals in a proper disease-free state for a longer period of time is very important, but challenging.

2. To study the long-term effects of AGE inhibitors on diabetic rats, they need to be maintained for a longer duration of time. The titration between time and the dose of STZ to be employed that can cause the disease and have all the complications of diabetes is a challenge.

3. The early-stage diabetic condition may not be associated with AGEs formation. Hence, the induction of diabetes and subsequent accumulation of AGEs requires time-based studies because no particular time frame can be guessed for such accumulation to occur.

4. In physiological studies, conditions, factors, and homeostatic balance vary from animal to animal. Achieving statistically reliable numbers for a desired parameter sometimes becomes difficult.

5. For volumetric-based measurements, handling has to be done very carefully, and sometimes only approximate measurements are possible.

FUTURE SCOPE

In the future studies, the activity of various other RAS components, including various enzymes and other components, will be looked into. These include:

1. Understanding the activity of prorenin

2. Understanding the activity of prorenin receptor

3. Understanding plasma renin activity

4. Detection of the enzyme activity of ACE1 will give an idea of how much Ang II is being produced.

5. Analysis of the aldosterone assay will predict the role this hormone plays under in a diabetic state.

Functional studies of the heart (thoracic aorta) blood vessels can be carried out. Although the blood vessels are hard and contain a high amount of connective tissue, the study can be attempted. This will give an idea on how the blood vessels are affected in diabetic conditions, providing an understanding of the blood flow in the vessels. This would be a reflection on the arterial flow and subsequent hypertension.

AG was able to ameliorate certain deleterious effects of diabetes in the short-term treatment, as presented in this study. However, further studies are warranted to evaluate the effect of AG in STZ-induced diabetic rats in a long-term duration model, which is of more than four weeks. This will be helpful in obtaining a detailed and better understanding of the functions of AGEs, AG, and its involvement with the blood sugar levels, as well as the factors that regulate the blood sugar levels. Additionally, the effects of AGEs and AG need a more detailed study of the action of these components, with specific reference to RAS.

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THESIS PUBLICATIONS

Cureus

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The Effect of Treatment With Aminoguanidine, an Advanced Glycation End Product Inhibitor, on Streptozotocin-Induced Diabetic Rats and Its Effects on Physiological and Renal Functions

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Abstract

Background/aim: Diabetes is a multifactorial syndrome that affects the functioning of the renin-angiotensin system (RAS). The role of advanced glycation end products (AGEs) in diabetes is well known. In the present study, we hypothesized that the prevention of AGE accumulation or abrogation of AGE synthesis using an AGE inhibitor, aminoguanidine (AG), in streptozotocin (STZ)-induced diabetic animal models would affect the progression of diabetes and its related complications. We determined the effects of aminoguanidine (AG), an AGE inhibitor, in STZ-induced diabetic rats by determining various indices of RAS and renal functions. Additionally, we also investigated the effect of the drug, AG, on various hemodynamic and physiological functions in the body of the animals.

Methods: Male Sprague Dawley rats weighing 200-250 g were assigned to four groups (n = 4-6): Vehicle, Vehicle+AG, STZ-induced, and STZ-induced+AG rats. Type 1 diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (55 mg/kg) dissolved in sodium citrate buffer. The control groups (Vehicle) were injected with buffer. The blood glucose levels were measured after 48 hours, and animals with blood glucose levels > 300 mg/dL were included in the study. Blood glucose levels in the vehicle rats were also determined to ensure non-diabetic conditions. After confirmation, AG was administrated at a dose of 1 g/L in drinking water for two weeks. Urine was collected to measure the glomerular filtration rate (GFR), and the immune reactivity for AT_1 and AT_2 proteins was analyzed by immunoblotting. Data were expressed as mean ± standard error of the mean (SEM), and a p-value < 0.05 was considered statistically significant.

Results: Diabetic rats had a significant drop in body weight, accompanied by increased food and water consumption. The diabetic rats exhibited significantly increased urine flow and GFR. These phenotypes were significantly or considerately reversed by AG treatment in the STZ+AG-treated diabetic rats. Aminoguanidine prevented the increase in blood sugar levels compared to STZ-induced diabetic rats alone (295.9 ± 50.69 versus 462.3 ± 18.6 mg/dL ($p < 0.05$)). However, it did not affect the glomerular filtration rate (GFR) and glomerular damage, as assessed by the renal histopathological studies. The STZ-induced diabetic rats had an increased sodium excretion (3.24 ± 0.40 mmol) and significantly increased expression of the AT_2 receptor and that of the AT_1 receptor, which was slightly reversed by the treatment with AG. Treatment with AG decreased sodium excretion (2.12 ± 0.63, as compared to the diabetic rats). These rats also had modestly decreased expression of the AT_2 receptor (0.99 ± 0.07 versus 1.12 ± 0.08, as compared to the STZ-induced diabetic rats), while the AT_1 receptors showed a slight increase in the STZ+AG-treated rats compared to the STZ-induced diabetic rats (1.1 ± 0.19 versus 1.08 ± 0.12).

Conclusion: This study highlights the action of the drug AG in not exacerbating any damage in diabetic rats. Employing AG as a pharmacological intervention to prevent an increase in blood sugar adds a new dimension to controlling increased blood sugar and preventing diabetic complications. The employability and pharmacological intervention of the drug AG, in diabetes, therefore, need a renewed and further investigation.

Categories: Cardiology, Endocrinology/Diabetes/Metabolism, Nephrology
Keywords: renin-angiotensin system, aminoguanidine, advanced glycation end products, renal functions, angiotensin ii receptors, diabetes

Introduction

Diabetes is a multifactorial disease caused by a lack of insulin or insulin resistance [1]. It is mainly characterized by increased blood sugar levels. Epidemiological studies have revealed that more than one in 10 adults are now living with diabetes, and the global diabetic population is estimated to reach 637 million by 2030 [2].

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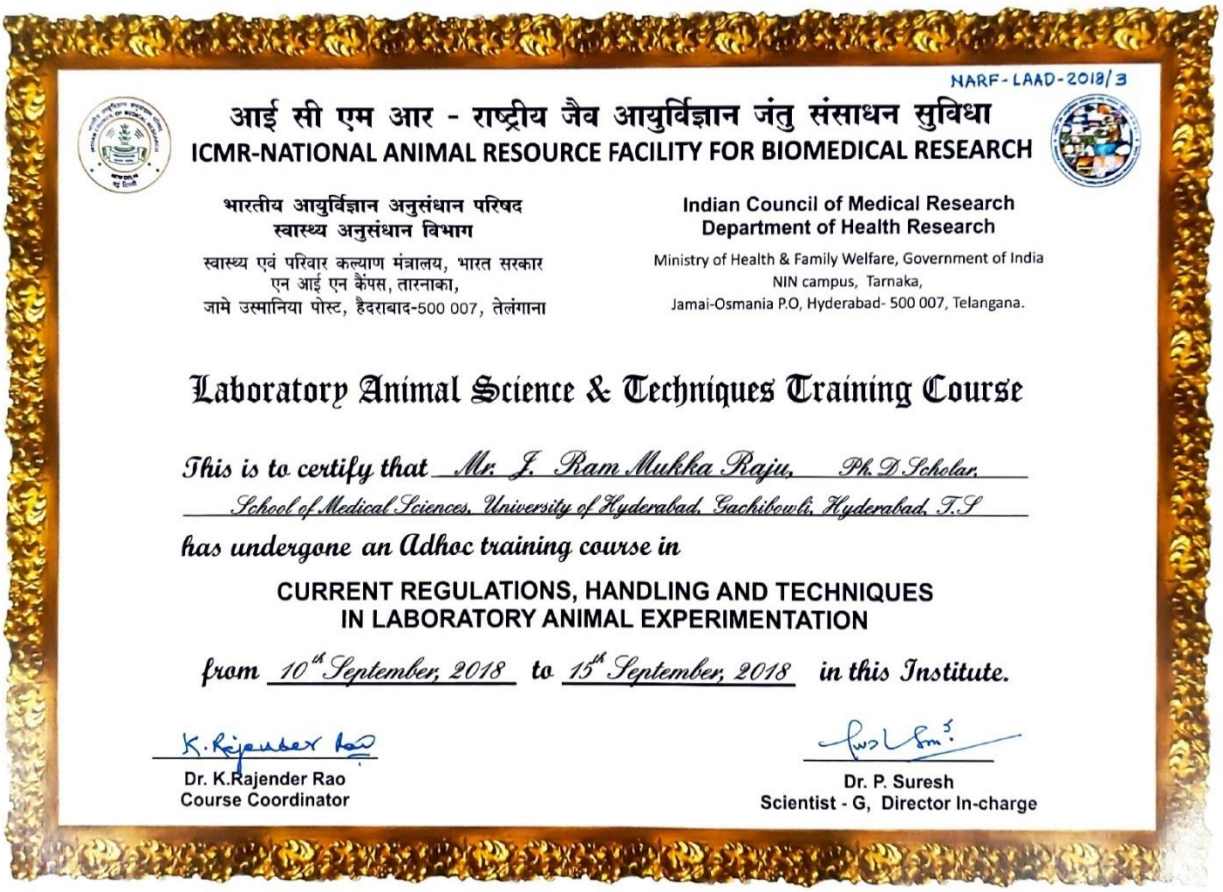

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K. Rajender Rao
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Course Coordinator

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INTERNATIONAL CONFERENCE ON FRONTIER AREAS OF SCIENCE AND TECHNOLOGY
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ISNCON PUNE 2022

01- 04 December, 2022
J W Marriott Pune, India

This is to certify that

Dr. Ram Mukka Raju J

has attended and participated as a **Delegate** during
52nd Annual National Conference of Indian Society of Nephrology

ISNCON 2022

CME held on 1st December **(CPD Code : MMC/MAC/2022/C-016660)**
& Conference held from 2nd - 4th December **(CPD Code - MMC/MAC/2022/C-016663)**
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Dr. Santosh K. Padhi
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BioQUEST
12th & 13th October, 2017

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University of Hyderabad
Hyderabad – 500 046, INDIA

Certificate of Participation

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Workshop on Animal Models in Cancer Research

Organized by

School of Medical Sciences, University of Hyderabad, India

24th - 25th October 2017

CERTIFICATE

This is to certify that ~~Dr.~~/Mr./~~Ms.~~/~~Shri.~~ J. Ram Mukka Raju has participated in the workshop on **Animal Models in Cancer Research** conducted by School of Medical Sciences, University of Hyderabad.

Prof. Geeta Vemuganti,
Dean, School of Medical Science,
University of Hyderabad

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Dean
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This is to certify that Mrs/Ms/Dr J. RAM MUKKA RAJU **Department of** _____ **Participated in 5K "*Fit India Freedom Run 2.0*", in the University Campus on Sunday, the 15th August, 2021 organized jointly by the office of Dean, Student Welfare, Unnat Bharat Abhiyan and Department of Physical Education, University of Hyderabad.**

(Prof.B. Nagarjuna)
Dean, Student Welfare

(Dr.K.V. Rajasekhar)
Director of Physical Education

(Dr.Ravula Krishnaiah)
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LV Prasad Eye Institute

CERTIFICATE OF PARTICIPATION

This is to certify that

RAM MURRA RAJU.T

has participated in two days hands-on workshop on

CHICK EMBRYO:
AN EMERGING PRECLINICAL ANIMAL MODEL FOR CANCER RESEARCH

Organized by School of Medical Sciences, University of Hyderabad in association with Institute of Eminence, Federation of Asian Biotech Associations and LVPEI on 8th & 9th December 2022

Prof. Geeta K Vemuganti
Dean, School of Medical Sciences,
University of Hyderabad

Dr. Swathi Kaliki
Head, Oculoplasty & Oncology Services,
LVPEI, Hyderabad

CERTIFICATE

OF PARTICIPATION

This is to certify that

J. Ram Mukka Raju

has participated in the workshop on "Statistical Machine Learning for Biologists" held at the Centre for Molecular Simulation and Design (CMSD) from 19 – 22 December 2022, organized by DBT-Centre for Microbial Informatics (DBT-CMI), University of Hyderabad

Prof. H A Nagarajaram
PI
DBT-CMI

Dr. D Pankaj Singh
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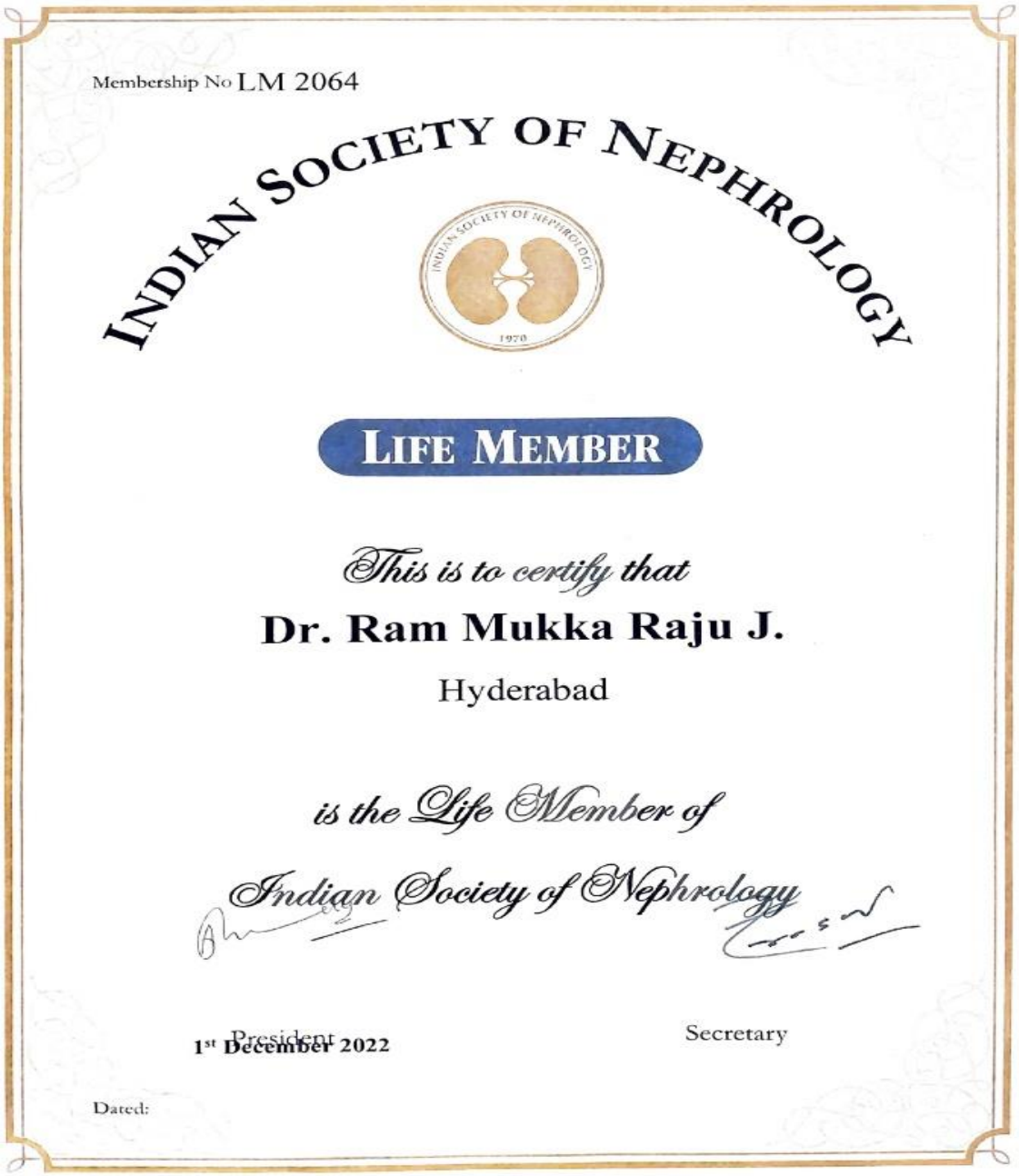
Coordinator

AWARDS / ACHIEVEMENTS



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Hyderabad

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1st December 2022

Dated:

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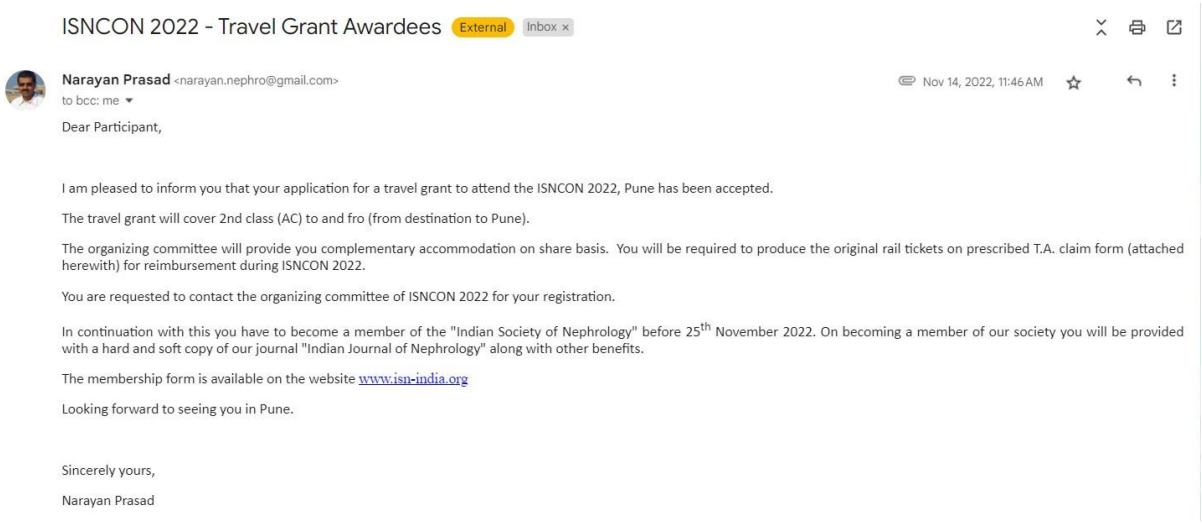

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Narayan Prasad <narayan.nephro@gmail.com> Nov 14, 2022, 11:46 AM

to bcc: me

Dear Participant,

I am pleased to inform you that your application for a travel grant to attend the ISNCON 2022, Pune has been accepted.

The travel grant will cover 2nd class (AC) to and fro (from destination to Pune).

The organizing committee will provide you complementary accommodation on share basis. You will be required to produce the original rail tickets on prescribed T.A. claim form (attached herewith) for reimbursement during ISNCON 2022.

You are requested to contact the organizing committee of ISNCON 2022 for your registration.

In continuation with this you have to become a member of the "Indian Society of Nephrology" before 25th November 2022. On becoming a member of our society you will be provided with a hard and soft copy of our journal "Indian Journal of Nephrology" along with other benefits.

The membership form is available on the website www.isn-india.org

Looking forward to seeing you in Pune.

Sincerely yours,

Narayan Prasad

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Dated: 9-10-2023

PLAGAIRISM FREE CERTIFICATE

This is to certify that the thesis entitled **"Exploring the Altered Functions of Renin-Angiotensin System (RAS) Under Diabetic Conditions"** submitted by Mr. J. Ram Mukka Raju, bearing Reg No: 17BMPH02, in partial fulfillment for the requirements of the award of Doctor of Philosophy in the School of Medical Sciences, is free from Plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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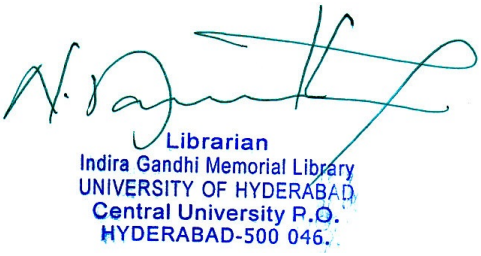
by Ram Mukka Raju Jogula



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