Effect of cilnidipine as an antihypertensive agent on two forms (L-NAME and L-NAME+4%NaCl) of hypertension in rats.

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Doctor of Philosophy
In
Medical Physiology

By

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Dedication

To my Parents

Mr & Mrs Mohamed Umar and

Najamunnisa Umar

To my Father and Mother in Law

Mr & Mrs Babu Sab Shaikh and

Rasool Bi Shaikh

And

To my Husband, Children, my brother, and colleagues

Whose constant support, encouragement, affection and love make me able to succeed.

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LIST OF ABBREVIATIONS

%	per cent
µl	microlitre
µM/L	Micro Moles per Litre
ACE	Angiotensin Converting Enzyme
Akt	Protein kinase B
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ANS	Autonomic nervous system
ATP	Adenosine 5'-Triphosphate
AT1	Angiotensin II type 1 receptors
AT2	Angiotensin II type 2 receptors
b. wt	Bodyweight
BCA	Bovine Serum Albumin
BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
BP	Blood Pressure
BSA	bovine serum albumin
BUN	Blood Urea Nitrogen
Ca^{2+}	Calcium
CCB	Calcium channel blocker
cells/cumm	cells per cubic millimetre
Cil	Cilnidipine
Cm	Centimetre
cGMP	Guanosine 3',5'-cyclic monophosphate
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on animals
CKD	chronic kidney disease
$CuSO_4$	Copper sulphate
DBP	Diastolic blood pressure
DF	Degree of freedom

DNA	Deoxyribonucleic acid
DSS	Dahl salt-sensitive rats
DMSO	Dimethyl sulfoxide
ECG	Electrocardiogram
ECP	Extracellular matrix
ECs	Endothelial cells
EDRF	Endothelial derived relaxing factor
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial Nitric Oxide Synthase
ERKs	Extracellular signal regulated kinases
ET-1	Endothelin-1
ETA	Endothelin receptor type A
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
Fe^{2+}	Ferrous ion
FFA	Free Fatty Acids
FGF-2	Fibroblast growth factor -2
FMN	Flavin mononucleotide
g/dl	grams/decilitre
GND	Ground
GFR	Glomerular filtration factor
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
Hb	Haemoglobin
HCl	Hydrochloric acid
Hct	Haematocrit
HDL	High-density lipoprotein
HF	High frequency
HIFs	Hypoxia-Inducible Factors
HPE	Histopathological examination
HR	Heart rate

HRP	Horseradish peroxidase
HRV	Heart rate variability
i.p	Intraperitoneal
IF-kβ	Inhibitory factor-kappa beta
IGF	Insulin-like growth factor
iNOS	Inducible Nitric oxide synthase
K^+	Potassium channels
Kg	Kilogram
K_f	ultrafiltration coefficient
LF	Low frequency
L-NA	N^w-nitro-L-arginine
L-NAME	N^G- Nitro L-Arginine methyl Ester
L-NMMA	N^w- iminoethyl-L-ornithine
LVA	Low voltage-activated
LVH	Left ventricular hypertrophy
m^2	meter square
MAP	Mean Arterial Pressure
MAPKs	Mitogen- activated protein kinases
MDA	Malondialdehyde
mg	Milligram
mg/dl	milligram per decilitre
mg/kg	milligram per kilogram
mI U/L	Milli International Units per Litre
mm Hg	Millimetre of mercury
mm^3	cubic millimetre
MMPs	Matrix Metalloproteinases
N_2	Nitrogen
Na^+	Sodium
NaCMC	Sodium Carboxymethyl cellulose
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide

NE	Norepinephrine
NF-kβ	Nuclear factor-kappa beta
NIBP	Non-invasive blood pressure
nm	Nanometre
nNOS	neuronal Nitric oxide synthase
NO	Nitric Oxide
NOS	Nitric oxide synthase
NOS2	Inducible NOS
O_2	Oxygen
O_2^-	superoxide anion
OD	Optical density
$ONOO^-$	Peroxynitrite
p38 MAPK	p38 mitogen-activated protein kinases
PCR	Polymerase chain reaction
PDGF	Platelet- derived growth factor
PEGME	Polyethylene- glycol- methyl ether
pg/ml	picogram per millimetre
PGI2	Prostaglandin I2
PHDs	Prolyl hydroxylases
PMSF	Phenyl methylene sulfonyl fluoride
PI3-K	Phosphoinositide-3 kinase
PLGF	Placental growth factor
PUFAs	Polyunsaturated fatty acids
RAS	Renin Angiotensin System
RAAS	Renin Angiotensin Aldosterone System
RBC	Red Blood Cell
RIPA	Radioimmunoprecipitation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Respiratory rate
RVLM	Rostro ventrolateral medulla
SA	Sinoatrial
SBP	Systolic blood pressure

SD	Standard deviation
SDS	Sodium dodecyl sulfate
Sec	Seconds
SHRs	Spontaneously hypertensive rats
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline with Tween 20
TBS	Tris buffered saline
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptors
VSMCs	Vascular smooth muscle cells
V_T	Tidal volume
$ZnSO_4$	Zinc Sulphate
α-cells	Alpha-cells
β-cells	Beta-cells

ABSTRACT

Background: A calcium channel blocker (CCB) of the fourth generation, cilnidipine, is dihydropyridine. It is inhibiting calcium channels of both the L- and N-type. Vascular smooth muscle contains L-type calcium channels, while presynaptic nerve terminals contain N-type calcium channels. Vasodilating effects from cilnidipine are slow-acting and persistent. Very few research studies have been done to clarify how N-type calcium channel blockers affect hypertensive rats whose nitric oxide production has been suppressed.

Aim and objectives: The purpose of our study was to show protective effect of cilnidipine on L-NAME or L-NAME plus salt induced experimental hypertension rats. Objectives of our study are to assess cardiovascular hemodynamic parameters like heart rate, mean arterial blood pressure, symapathovagal balance by heart rate variability analysis (HRV), systemic and renal oxidative stress (serum and kidney tissue MDA), renin angiotensin system activity (Kidney and serum VEGF, NOS3 and ACE protein expression and serum and urinary Ang II level) in N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) induced hypertensive rat model. Further we investigated (24hour protein excretion, Creatinine clearance, Renal fibrosis/glomerulosclerosis) as markers of renal injury in response to L-NAME and L-NAME plus salt induced hypertensive rats with or without cilnidipine treatment.

Material and methods: 36 male Albino Wister rats were collected from institutional animal house (six rats in each group). Group1 vehicle treated (control), group2 cilnidipine (2mg/kg body weight/day) treated, group3 treated with L-NAME (40 mg/kg body weight/day), group4 treated with L-NAME and cilnidipine, group 5 treated with L-NAME and 4% sodium chloride (4% NaCl), group 6 L-NAME, cilnidipine and salt treated. All experimental animals underwent gravimetry and after

28-day percentage of body weight gain calculation was made. Estimates were made for haematological variables such as Haemoglobin (g/dl), RBC count (million cells/mm3), and haematocrit (percentage). HRV analysis was performed to evaluate changes in the cardiovascular autonomic system, Heart rate (HR) and blood pressure (BP) were monitored every week for 28 days as examples of cardiovascular hemodynamic events. Blood pressure was recorded by non-invasive tail cuff method. Oxidative stress was assessed by estimating serum and kidney tissue MDA levels. Serum and kidney tissue nitric oxide levels were measured as nitrosative stress markers. Proteinuria and creatinine clearance were measured. kidney function parameters (serum urea and creatinine) were also measured. Serum eNOS, Ang II and urinary Ang II levels were quantitatively measured by ELISA technique. Relative expression of serum and kidney tissue NOS3, ACE and VEGF protein levels were done by Western Blotting. Histopathological examination of the Aorta and kidney tissue was done.

Results: Lower percentage body weight gain was seen after L-NAME administration, and changes in the sympathovagal balance and sympathetic overactivity were discovered by HRV analysis. Heart rate was lower and mean arterial pressure was higher in Hypertensive rats. There is increase in serum and kidney tissue oxidative stress. Marked decreased in serum and kidney tissue NO and NOS3 levels. There is increased serum and kidney tissue VEGF and ACE protein expression. There is increase in serum and urinary Ang II levels. We also observed proteinuria and decreased creatinine clearance in NO deficient and salt supplemented hypertensive rats.

Cilnidipine treatment was able to 1) reduce MAP and Heart rate 2) reduce sympathetic activity 3) decrease serum and kidney tissue oxidative stress 4) increase

the bioavailability of NO 5) decreased proteinuria and improvement in creatinine clearance value 6) increase in eNOS/NOS3 protein expression in serum and kidney tissue 7) decreased ACE and VEGF protein expression 8) decrease in serum and urinary angiotensin II levels 9) reduced renal glomerulosclerosis and tubular degeneration 10) ameliorate vascular and kidney remodelling resulting from L-NAME induced hypertension.

Conclusion: The present study demonstrates that L-NAME induced hypertension in rats, cause systemic and kidney tissue oxidative stress and increased sympathetic activity causing alteration in the sympathovagal balance. These alterations further proceed to activation of renin angiotensin system (RAS) and further enhances oxidative stress in kidney tissue leading to renal injury. As a dual L/N type calcium channel blocker with added antioxidant capacity, cilnidipine presumably has a helpful function in reducing the pathophysiology of the vascular and renal systems in rats that have been subjected to L-NAME and L-NAME + 4% NaCl-induced hypertension. These findings suggest that cilnidipine may act as renal protective agent and reduces glomerular injury in NO deficient hypertensive rats.

CHAPTER 1
INTRODUCTION

1.0 INTRODUCTION

Due to its high prevalence, hypertension which is brought on by both genetic and environmental factors is a significant public health issue. The risk of cardiovascular diseases is increased by hypertension. It is still unknown exactly how essential hypertension develops. The most prevalent and significant environmental risk factor for hypertension is excessive dietary salt consumption (Hoon Young Choi. et al., 2015). A necessary, last common pathway in the pathogenesis of hypertension, according to Guyton and his co workers, involves the kidney's improper management of salt and the subsequent dysregulation of body fluid levels. (Crowley SD et al., 2006).

Evidence from the previous research indicates that renal oxidative stress and subsequent NO deficit are crucially linked to the emergence of hypertension and cardiovascular disease. Nitric oxide (NO) is a fundamental regulator of renal and cardiovascular function. (Araujo M, Wilcox CS., 2014).

Reactive oxygen species (ROS) generation is excessive during oxidative stress, and the oxidation-reduction (redox) state is altered. Inflammation, proliferation, apoptosis, migration, and fibrosis are important processes that contribute to impaired vascular function, cardiovascular remodelling, renal dysfunction, immune cell activation, and sympathetic nervous system excitation in hypertension. These molecular events also cause protein oxidation and dysregulated cell signalling. (Oparil S et al., 2018; Stanley CP et al., 2019).

By substituting one or both of the terminal guanidino (G or w) nitrogen, analogues of L-arginine act as inhibitors of nitric oxide synthase isoform 3 (NOS3). L-isomers alone are typically thought to be the only active inhibitors, although there is evidence that N^{G}-nitro-L-arginine may prevent endothelial relaxation of the aortal rings, and L-NAME reduced NOS activity in both the rat heart and aorta. (Jana Kopincova et al., 2012).

Elevated levels of reactive oxygen species such as superoxide, hydrogen peroxide, peroxynitrite, and hydroxyl radicals are also produced by kidney tissue due to hypertension. (Qiangwei Fu et al., 2016).

These species are created by elevated intrarenal type 1 angiotensin II receptors that bind to increased intrarenal angiotensin II and transmit signals to activate the pro-oxidant nicotinamide adenine dinucleotide phosphate oxidase. Reduced expression of the antioxidant enzymes superoxide dismutase 1 and 3 and isoforms of nitric oxide synthase further increases the production of reactive oxygen species. (Simao S et al., 2011).

Chronic hypertension gradually progresses to chronic renal failure by damaging the glomeruli and causing several morphological and quantitative changes in the kidney. Because renin-angiotensin system dysregulation is the most common cause of hypertension. The two main medications used to treat chronic nephropathy brought on by hypertension are angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (Bae EH et al., 2010). The development and management of renal lesions similar to those reported in hypertension in humans can be studied using the nitric oxide deficient rat hypertension model (Girardi JM et al., 2011).

The goal of treatment should be to reduce hypertensive renal injury because the kidneys are the main target organs where complication of chronic hypertension mostly manifest. An important factor in the progression of renal damage is sympathetic overactivity. The management of chronic renal disease may benefit from medication that modulates sympathetic nerve activity (Toba H et al., 2011).

With its dual L/N-type Ca^{2+} channel-blocking action, cilnidipine is a promising 4^{th} generation Ca^{2+} channel blocker. By blocking N-type Ca^{2+} channels, cilnidipine effectively suppresses the cardiovascular neurohumoral regulation system including that of the

sympathetic nervous system and the renin-angiotensin-aldosterone system. As a result, cilnidipine is anticipated to be beneficial for a variety of hypertension related complications. A dual L & N type calcium channel blocker (CCB) cilnidipine, may be more effective than a solely L-type CCB in hypertensive renal damage rats. (Konno Y, Kimura K.,2008; Fan YY et al.,2010).

Hence the NOS3 inhibitor (L-NAME) act as stressors and alter the pathophysiology of vascular and renal microvasculature. Perhaps, L/N-type dual calcium channel blocker cilnidipine may act as vascular and renal protective agent.

Very few research have been done to clarify how N-type calcium channel blockers affect hypertensive rats whose nitric oxide production has been suppressed. In order to learn more about how cilnidipine protects the kidneys in rats with hypertension caused by L-NAME and L-NAME plus salt, the current investigation was conducted.

CHAPTER II
REVIEW OF LITERATURE

2.1 Hypertension

In India, hypertension is the most significant noncommunicable disease, with an estimated 200 million people suffering from it. Men (24.5%) had higher age-adjusted hypertension than women (20%). Greater socioeconomic level, metropolitan populations, and developed states of the nation all have higher rates of hypertension. Unique findings include the confluence of urban and rural areas and higher hypertension in younger men and women (Gupta R, Ram CVS., 2019).

Prevalence of hypertension continuously increasing across the globe. In spite of being high prevalence disease, the awareness, management, and control of hypertension in the community are very less (Chakraborty RN et al., 2021). Risk factors for primary hypertension include advancing age, obesity, high dietary sodium chloride (NaCl) consumption. (Lerman LO et., 2019).

2.2 Role of Nitric Oxide (NO) and Nitric Oxide Synthases (NOS) in Hypertension

In vertebrates, nitric oxide (NO) is an essential signalling chemical. The enzyme nitric oxide synthase (NOS) synthesis nitric oxide. Endothelial NOS (eNOS, also known as NOS3), neuronal NOS (nNOS, also known as NOS1), and inducible NOS (iNOS, also referred as NOS2) are the three isoforms of NOS that have been discovered in vertebrates. The endothelium lining of blood arteries expresses the endothelial isoform constitutively. Additionally, neuronal NOS is constitutively expressed and primarily located in the central nervous system, heart and skeletal muscles. When activated by a variety of proinflammatory stimuli, macrophages and some other cell types express inducible NOS. Through the calmodulin-calcium interaction, the calcium level regulates the activity of eNOS and nNOS. On the other hand, calmodulin is permanently linked to iNOS (S. Daff., 2010).

It has been demonstrated that momentary increases in blood pressure cause an enhanced release of NO into the bloodstream, which may be identified by tracking minute changes in plasma nitrate. On the other side, a drop in systemic pressure results in less nitric oxide production. It has been demonstrated that a genetic model of hypertension produces more NO and has more active constitutive nitric oxide synthase (NOS) than normotensive controls. These results imply that generation of NO is enhanced by high blood pressure. It is not yet clear what mechanisms are involved. Blood flow, shear stress, and other mechanical stimuli may have an impact on NO generation and endothelial NOS expression, according to certain theories.

The discovery that eNOS plays a important role in hypertension was supported by the fact that mice with the eNOS gene disrupted developed the hypertension. Additionally, it has been noted that critical hypertensive patients produced less NO than normotensive people did. Additionally, acute administration of L-arginine antagonists in normal animals results in a sharp and sudden increase in blood pressure as well as a reduction in renal plasma flow and glomerular filtration rate (GFR). For several weeks, rats were given NG-nitro L-arginine methyl ester (L-NAME), an antagonist of L-arginine, in the water. This caused the systemic blood pressure, glomerular capillary pressure, and ultrafiltration coefficient (Kf) to rise in experimental rats. These alterations were associated with proteinuria and the emergence of glomerulosclerosis.

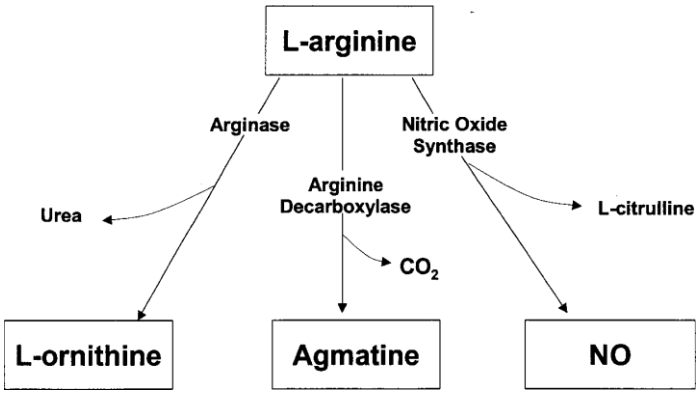


Fig. 2.2. Pathways of L-arginine metabolism. L-Arginine may be metabolized by the urea cycle enzyme arginase to L-ornithine and urea, by arginine decarboxylase to agmatine and CO2, or by nitric oxide synthase to nitric oxide (NO) and L-citrulline.

Source: Klahr S. The role of nitric oxide in hypertension and renal disease progression. Nephrol Dial Transplant. 2001;16 (1):60-2.

Numerous investigations have revealed the presence of NOS in a specific areas of the brain involved in a neurogenic regulation of blood pressure. The transduction pathways that tonically inhibit the sympathetic outflow from the brainstem involve the neuronal isoform of NOS (nNOS). In normal rats local NO generation controls the activity of the central sympathetic nervous system. When compared to control rats, the brains of rats with chronic renal failure exhibit more nNOS this may reduce the enhanced sympathetic nerve firing. L-NAME was administered to Munich-Wister rats in drinking water for a month to inhibit nitric oxide production, they discovered that the rats developed hypertension, glomerulosclerosis, and renal interstitial fibrosis (Klahr S., 2001). Kashiwagi et al (2000) also reported similar results.

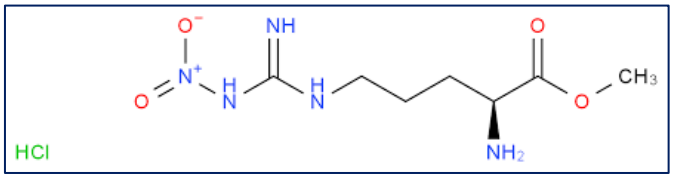
2.3 Hypertensive Rat Model

The pathophysiology of the early animal models of hypertension closely resembled that of its human analogues and entailed constriction of renal arteries (Goldblatt kidney) or parenchyma (Page kidney). However, Page kidney and renovascular hypertension only make for a small portion of overall human hypertension. Therefore, understanding the mechanisms of primary hypertension has been the main focus of most animal experiments studying hypertension (Bae EH et al., 2010). Additionally, models for the risk of hypertension-related conditions like left ventricular hypertrophy (LVH), metabolic abnormalities, heart failure, renal damage, and stroke are available (e.g., spontaneously hypertensive rats (SHRs) and Dahl salt-sensitive rats (DSS) (Girardi JM., 2011).

2.3.1 N^G-Nitro- L-Arginine Methyl Ester (L-NAME)

We developed the nitric oxide deficient hypertensive rat model by giving L-NAME in our laboratory of Vascular Physiology and Medicine.

Structure of L-NAME



Molecular Formula-C7H15N5O4

Figure:2.3.1 Chemical structure of L-NAME

L-arginine analogues are frequently utilised as in vivo and in vitro inhibitors of nitric oxide synthase (NOS) function. On the one hand, nitric oxide (NO) bioavailability is lowered by acute and chronic L-NAME therapy, which affects blood pressure and vascular reactivity. But if given over a longer period of time, smaller dosages of L-NAME may also stimulate NO production through feedback regulatory mechanisms (Jana Kopincova et al., 2012).

The competitive bonding between substrate analogues and the enzyme is the mechanism by which NOS is inhibited, however the chemical basis of the inhibitory activity varies for different analogues. After L-arginine replenishment, analogue-mediated suppression of NO production is typically in vivo reversible. However, some compounds, including L-NMMA, N^w- iminoethyl-L-ornithine, or N^w-allyl-L-arginine, can be used by NOS to produce intermediates tightly linked to enzyme molecules and so block the production irreversibly, that is why they deserved the name "suicide inhibitors" (Overend J, Martin W., 2007).

The carboxyl group of N^{w}-nitro-L-arginine(L-NA) is esterified increase water solubility, which makes it easier to employ this variant in experiments. On the other hand, specific esterase is required for the inhibitory activity to fully manifest in the tissue, which may limit the impact of L-NAME for specific tissues. L-NAME is frequently utilised in both acute and long-term in vitro and in vivo research when the consequences of NO production restriction are being studied, despite having an inhibitory efficiency that is 30-100 times lower than that of L-NA (Toba H., 2011).

2.3.2 L-NAME Induced Hypertensive Rat Model

The creation of an animal model of human hypertension caused by NO deficiency was made possible by giving long-term L-NAME treatment to experimental animals. This was done in response to the theory of "endothelial dysfunction" and the consequent insufficient NO production in human essential hypertension. Inducing so-called "NO-deficient hypertension" in normotensive rats required the long-term administration of NOS inhibitor in relatively high doses (10, 20, 25, 40, 50, 65, 80, and 100 mg/kg of body mass/day). Nitric oxide deficient hypertensive rat model is now a popular tool for research in cardiovascular disorders (Hobbs AJ et al.,1999). Higher dosages of L-NAME taken for 3-6 weeks lowered the in vitro relaxant response to carbachol or acetylcholine in aorta, femoral artery, and small mesenteric arteries (Desai KM et al., 2006). Moreover, the doses of 10 to 100 mg of L-NAME per kg/day to normotensive rats led to dramatic decrease of cGMP content in the aorta (Jana Kopincova et al., 2012).

2.3.3 L-NAME and NOS activity

By giving Sprague-Dawley rats 300 mg/kg/day of L-NAME by continuous infusion into the portal vein by an osmotic pump over the course of seven days, the first in vivo observation of L-arginine analogue-induced enhancement of NO generation was made. As a

result, there was a 38-percent increase in iNOS activity and an increase in iNOS expression in the liver tissue (Paulis L et al., 2008). Reduced NOS activity was consistently discovered in the aorta, numerous brain regions, left ventricle, or kidney following 4-week L-NAME treatment of 40 mg/kg/day, which was linked to a worsened relaxant response of the aorta, renal, and pulmonary artery to acetylcholine (Tucker EJ et al., 2000).

2.3.4 L-NAME and NOS expression

They hypothesise that higher NOS activity observed in specific tissues in vivo studies originates from higher level of NOS gene expression since L-NAME treatment limits NO production. In fact, both iNOS and eNOS expression rates are accelerated in vitro by the presence of L-NAME. Possibly via altering the interaction between NO and the transcription factor nuclear factor kB (NF-kB), a crucial regulator of NOS expression (Grumbach IM et al., 2005).

NO is thought to affect the rate of NOS transcription by either inhibiting NF-kB activation, its binding to DNA, or by inducing and stabilising NF-kB inhibitor. In this way, the L-NAME-induced decrease in NO level causes NF-kB activation, which in turn causes an increase in NOS expression (Kopincova J et al 2011; Hu W et al., 2011).

2.4. Oxidative and Nitrosative Stress in Hypertension

Reactive oxygen species (ROS) generation is excessive during oxidative stress, and the oxidation-reduction (redox) state is altered. Inflammation, proliferation, apoptosis, migration, and fibrosis are important processes that contribute to impaired vascular function, cardiovascular remodelling, renal dysfunction, and sympathetic nervous system activation in hypertension. These molecular events also cause protein oxidation and dysregulated cell signalling (Oparil S et al., 2018; Knock GA., 2019). A family of nonphagocytic NADPH

oxidases (Nox1, Nox2, and Nox4 in rodents and Nox1, Nox2, Nox4, and Nox5 in humans) is a significant source of cardiovascular ROS (Lushchak VI., 2014).

Oxidative stress encourages post-translational protein modification (oxidation and phosphorylation) in hypertension, as well as faulty signalling that leads to cell and tissue damage. A disturbance of redox signalling and control as well as molecular damage results from unchecked ROS production in pathological situations, which is defined as "an imbalance between oxidants and antioxidants in favour of the oxidants." The most significant ROS in the cardiovascular system are superoxide anion (O_2^-), peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Sies H et al.,2017; Lushchak VI., 2014).

Super oxide anion (O_2^-) is highly unstable and impermeable to cell membranes, but H_2O_2 is permeable to cell membranes, stable, and has a longer half-life than O_2^-, making it a useful signalling molecule (Sies H., 2018). The original endothelial-derived vasodilator is NO, which is created enzymatically by NOS (Touyz RM et al., 2020). NO and O_2^- combine to generate $ONOO^-$, a potent oxidant that is very unstable. Peoxynitrite is permeable to the cell membrane when protonated (HOONO). A change in cellular redox status and oxidative damage to cells and tissues are brought on by the interaction between O_2^- and NO as well as the dysregulated synthesis of O_2^- and H_2O_2 (Incalza MA et al., 2018).

ROS alter proteins by post-translational processes such oxidation (sulfenylation, nitrosylation, glutathionylation, and carbomylation) and phosphorylation to affect cell function (Hawkins CL, Davies MJ., 2019). ROS are essential signalling molecules that allow vasoactive substances to exert their effects on cells, including angiotensin II (Ang II), endothelin-1 (ET-1), aldosterone, and prostanoids. They also control intracellular calcium homeostasis, which is crucial for initiating and maintaining vasoconstriction and cardiac contraction (Ray PD et al., 2012).

In patients with hypertension, increased vascular ROS generation, oxidative stress, and vascular inflammation are linked to endothelial dysfunction, a hallmark of vascular injury (Gonzalez J et al., 2014). When compared to cells from normotensive counterparts, vascular smooth muscle cells (VSMCs) from the arteries of hypertension patients showed increased NOx activity, higher levels of O_2^- and H_2O_2, improved Ang II triggered redox signalling, and inflammatory responses (Touyz RM, et al., 2005).

It is well known that NOXes are the main ROS producer in the cardiovascular system and that they play a significant role in the development of oxidative stress in hypertension. However, growing evidence indicates that secondary sources are also important that is NOS uncoupling NO. (Tejero J et al., 2019). Through S-nitrosylation, NO also communicates through cyclic guanosine monophosphate-independent routes by changing the structure and function of proteins. Under physiological circumstances, NOS catalyses the conversion of L-arginine to L-citrulline plus NO in the presence of a number of cofactors, including NADPH and BH4 (tetrahydrobiopterin).

However, under situations of oxidative stress, NOS donates an electron from NADPH to O_2 in order to produce O_2^- instead of NO (Daiber A et al., 2019). This procedure, known as "NOS uncoupling," involves phosphorylating and S-glutathionylating eNOS as well as oxidising BH4 (Zweier JL et al., 2011). Depletion of BH4 due to ROS-mediated mechanisms is a significant catalyst for NOS uncoupling. Superoxide directly converts BH4 to BH2, which destabilises NOS and causes it to uncouple. Since BH4 controls all NOS isoforms, all of them are capable of uncoupling when under stress (Tejero J et al., 2019).

2.4.1 Oxidative stress and Vascular remodelling

Hypertension is both a cause and a result of vasculopathy, which includes reduced endothelium-dependent relaxation, increased arterial stiffness, heightened contractility,

inflammation, vascular calcification, and remodelling. These dysfunctions are a result of oxidative stress and redox-sensitive signalling, as has been shown both in vitro and in vivo. Vascular damage is caused when a variety of prohypertensive stimuli increase the generation of ROS in endothelial cells, vascular smooth muscles, adventitia, and perivascular adipose tissue (Touyz RM et al., 2017; Virdis A et al., 2016).

The formation of ROS in vascular cells in vitro and in the aortas of hypertensive mice was originally demonstrated to be induced by Ang II, a vasoactive drug. Resistance arteries from hypertension individuals and vessels from Ang II-infused animals exhibit elevated NOX1 and NOX2 vascular expression and ROS generation, which in turn activate complicated signalling pathways such MAP kinases and Ca^{2+} channels. These mechanisms result in fibrosis, increased wall stiffness, endothelial dysfunction, vascular remodelling, influx of inflammatory cells into the artery wall, and vascular hyperreactivity (Martinez Revelles S et al., 2017).

Aldosterone, ET-1, growth hormones, immunological factors, salt, and others are ROS-dependent causes of hypertension (Ren J, Crowley SD., 2019). Aldosterone signals via genomic and nongenomic routes to enhance ROS in VSMC and endothelial cells by activating NOX1 and NOX4, respectively, through mineralocorticoid receptors. It is possible that these two prohypertensive systems interact since AT1 receptor signalling is necessary for aldosterone-induced oxidative stress while mineralocorticoid receptor inhibition reduces Ang II-induced ROS generation in the vasculature (Lemarie CA et al., 2009). The growth factor receptors PDGF, EGF, and IGF-(insulin-like growth factor) 1 are transactivated by Ang II and endothelin 1 (ET-1), which signal through G-protein-coupled receptors. This amplifies redox-dependent vascular processes, such as medial hypertrophy, vascular remodelling, inflammation, and fibrosis (Ushio Fukai M et al., 2009).

2.4.2 Oxidative Stress and Renal System remodelling

Through a variety of ROS-involved processes, the kidneys play a significant part in the pathophysiology of hypertension. In experimental models, increased ROS production in the kidney can cause blood pressure to rise and hasten the onset of hypertension. Despite the fact that other NOXes also contribute, NOX4 is the main generator of renal ROS. While H_2O_2 appears to be significant in regulating pressure natriuresis by reducing preglomerular vascular reactivity and boosting medullary blood flow, increased O_2^- appears to produce vascular and tubular dysfunction. O_2^- and NO interact extensively in the macula densa to control afferent arteriolar tone via the tubuloglomerular feedback response, according to in vivo studies of single nephron function and in vitro studies using the double-perfused juxtaglomerular apparatus preparation.

Activation of renal afferent neurons, malfunction of glomerular cells, disruption of Na^+ and water balance, and promotion of renin release are additional effects of oxidative stress in the kidney that contribute to the development of hypertension (Araujo M, Wilcox CS., 2014; Ratliff B et al., 2016). Additionally, generation of O_2^- in particular nephron segments raises Ang II reactivity. According to recent research, renal dopamine receptors (D1–D5 R) control the redox state by reducing ROS production, and dopamine receptor malfunction increases oxidative stress and blood pressure (Qaddumi WN, Jose PA.,2021). Due to the harmful role that oxidative stress plays in the embryonic programming of kidney disease, the effects of renal oxidative stress on hypertension manifest early in life (Hsu CN, Tain YL.,2020). Using ROS scavengers, antioxidants, and other methods to reduce oxidative stress in the kidney lead to improved renal function and ameliorates blood pressure elevation in experimental hypertension (Hong YA, Park CW.,2021).

2.5 L-NAME induced hypertension and Autonomic Nervous System

N^G-nitro-L arginine methyl ester (L-NAME) induces hypertension in rats by inhibiting nitric oxide synthase (NOS) in an acute or chronic manner. The basic mechanisms that mediate hypertension are not well known, yet. It appears that the sympathetic drive is more significant in the chronic stage of L-NAME hypertension than in the early stage. (Victoria Biancardi et al., 2007)

Acute infusion of L-NAME activated the sympathetic nervous system in the kidneys of conscious rats, as demonstrated by Augustyniak et al. (2006), when the confounding effect of the sinoaortic baroreceptors was removed by barodenervation. These findings support the hypothesis that sympathetic activation was brought about in conscious animals by acute L-NAME infusion (Augustyniak et al., 2006).

Since the vasodilator effects of NO were first thought to be the only cause of the rise in blood pressure, research of its effects on the vascular system were prioritised over its effects on other tissues. The sympathetic vasomotor activation has been identified as a key mechanism underpinning the hypertension caused by NO synthesis inhibition. (Peotta et al., 2007; Esler et al.,2010). Strong evidence points to brain-derived NO production as a key mechanism for effectively reducing sympathetic vasomotor activity (Zucker et al.,2004).

An important brain area involved in cardiovascular control, the rostral ventrolateral medulla (RVLM), has been shown to significantly increase glutamanergic neurotransmission when NO synthesis is inhibited, indicating that NO actions in the brain may be necessary to control the level of sympathetic vasomotor activity and, consequently, the systemic arterial blood pressure (Machado et al., 2016). Additionally, it has been postulated that the sympathetic hyperactivity that results from NO synthesis restriction may arise at a later stage rather than at the beginning of the development of hypertension. According to one theory that

could explain this phenomena, the powerful baroreceptor stimulation brought on by the sudden rise in blood pressure acts as a significant inhibitor of the rise in sympathetic vasomotor activity during the initial phase of NO production suppression (Peotta et al. 2007).

Furthermore, systemic NOS inhibition greatly enhanced cutaneous sympathetic vasomotor activity in humans, which is not controlled by baroreceptors, in a time-dependent way, supporting the notion that NO inhibition causes sympathoexcitation that results in hypertension (Young et al., 2009). The renin-angiotensin system, a powerful source of reactive oxygen species, is activated after NO inhibition, which may be one mechanism causing sympathoexcitation (Crowley, 2014). In reality, angiotensin II can activate the NADPH oxidase enzyme, which increases the production of intracellular superoxide anion. This anion serves as a potent cellular signalling mechanism in the brain that causes sympathoexcitation (Collister et al., 2016).

The issue of whether sympathetic vasomotor activity increases after NO synthesis suppression and causes hypertension is still up for debate despite the abundance of evidence. After two and fourteen days of L-NAME therapy in conscious rats, Dos Santos and colleagues reported that there is no increase in renal sympathetic nerve activity (dos Santos et al., 2010). The varying outcomes of these investigations may be due to variances in NO bioavailability or a differential reaction brought on by blocking certain NO synthase isoforms (endothelial, neuronal, inducible, and mitochondrial). Therefore, variable NO blocking effects on particular sympathetic targets could not be ruled out. Consequently, in this research, we look into the effects of the calcium channel blocker cilnidipine on NO deficient hypertensive rats.

2.6 Vascular endothelial growth factor (VEGF) and Hypertension

An essential transcriptional target of HIF-1 is vascular endothelial growth factor (VEGF) (Shibuya, 2011). VEGF controls lymphangiogenesis, angiogenesis, and vasculogenesis (Beeghly-Fadiel et al., 2011). The word "vasculogenesis" refers to the process by which new blood vessels form from precursor cells seen in the early stages of embryogenesis. Angiogenesis is the later development of new blood vessels from already-existing vessels (Shibuya, 2011). Adults develop vessels through a process called angiogenesis. The placental growth factor (PLGF), VEGF-A, B, C, and D are all members of the human VEGF family. The three types of VEGF receptors-VEGFR-1, VEGFR-2, and VEGFR-are tyrosine kinases that are expressed in numerous vascular and extravascular tissues (Ramakrishnan et al., 2014). Out of the three receptors, VEGFR-2 primarily mediates the permeability and cell proliferation effects of VEGF. (Kimura and Esumi, 2003).

A family of secreted proteins called VEGFs is required for healthy blood vessel development. Three layers make up blood vessels. The single layer of endothelial cells (ECs) that lines the lumen and forms the innermost endothelium is in direct touch with the blood and all medications that are circulated throughout the body. Vascular smooth muscle cells (SMCs) that constrict or relax to govern peripheral vascular resistance, lumen diameter, and blood flow make up the medial layer and help to lower blood pressure. The outer adventitia gives the interior layers stability and structure. Tyrosine kinase receptors called VEGFRs, which are mostly expressed on the surface of ECs (although they can also be expressed on ijured SMC), are the means by which VEGFs signals (Pruthi D et al., 2014).

The normal endothelium has anti-inflammatory and anti-thrombotic properties, and it also secretes substances that relax SMC and control vessel diameter. Nitric oxide (NO) and prostacyclin, also known as prostaglandin I2 (PGI2), as well as vasoconstrictors like

endothelin-1 (ET-1), which promotes vasoconstriction by interacting with SMC endothelin A receptors (ETAs) to phosphorylate myosin light chain, which results in vasoconstriction, are factors released by ECs to control vessel tone. The activation of VEGFR signalling encourages EC proliferation and survival, increases vascular permeability, and promotes angiogenesis in addition to causing ECs to secrete vasodilatory factors (Simons M et al, 2016).

The ECs and podocytes that make up the kidney's glomeruli are responsible for filtration and barrier functions of the kidney. Renal pressure-natriuresis, a process that normalises blood pressure, occurs when the healthy kidney is exposed to elevated vascular tone brought on by renal vasoconstriction. Podocytes release VEGF, and both ECs and podocytes express VEGF receptors. Regulating renal perfusion and salt reabsorption depends heavily on VEGF-induced glomerular NO generation. Therefore, VEGF signalling suppression negatively affects renal function and renovascular balance, which adds to hypertension (Camarda N et al. 2022).

The most notable effect of VEGFR-2 activation is the recruitment of PI3-kinase, which then activates AKT, which directly phosphorylates eNOS to enhance NO generation. To activate guanylate cyclase and enhance cGMP synthesis, NO diffuses in a paracrine manner to nearby vascular smooth muscle cells. This causes vasodilation. The pressure-natriuresis relationship, which describes the kidney's capacity to drain sodium in response to long-term increases in arterial pressure, has been widely recognised as a crucial regulatory mechanism for managing high blood pressure. All hypertension related studies have a deficiency in renal sodium excretion, which is necessary for chronic hypertension to develop. NO directly influences how pressure natriuresis and tubuloglomerular feedback are regulated. Therefore, altering the set point for sodium excretion due to improper eNOS

functioning by VEGF suppression may result in sodium retention and an increase in extracellular fluid volume (Robinson ES et al, 2010).

2.7 Angiotensin converting enzyme (ACE) in Hypertension

ACE was first identified as a "hypertensin converting enzyme" in 1956. The somatic form of ACE, which is present in many tissues, and the smaller germinal form of the enzyme, which is only present in the testes and plays a key role in fertility, are the two unique forms of ACE that are now recognised in humans. Both types of ACE are found as ectoenzymes at the cell surface where they hydrolyze circulating peptides. Although a soluble version of ACE, which is produced from the membrane form by the action of a secretase, is also available in serum and other body fluids, gene-targeting studies in mice have demonstrated that the tissue-bound form of ACE controls both blood pressure and kidney anatomy and function. ACE is particularly prevalent in its somatic form on endothelial surface of the lungs and on brush-border membranes of kidney, intestine, placenta and choroid plexus (Turner AJ et al, 2002).

The captopril production, the first ACE inhibitor utilised in clinical settings, is a prime example of rational drug development. Since then, a large number of effective dipeptide and tripeptide ACE inhibitors, or their prodrugs, have been synthesized that contain thiol, carboxylate (such as cilazapril and ramipril) or phosphate (such as ceranapril) groups as zinc chelators. Endothelial dysfunction, hypertension, congestive heart failure, myocardial infarction, renal disease, especially diabetic nephropathy, and myocardial infarction are now all treated therapeutically with ACE inhibitors (Turner AJ et al, 2002).

2.8 Role of Angiotensin II (Ang II) in hypertension

The only precursor protein and the source of all angiotensin peptides is angiotensinogen, which is produced in the liver. Renin (a protease produced in the kidney) largely breaks down angiotensinogen in the bloodstream to make angiotensin I (Ang I),

which is then processed by angiotensin converting enzyme (ACE) (secreted in the lung) to create the active octapeptide, angiotensin II (Ang II). The major mechanism of action of Ang II is the interaction of its two G protein-coupled receptor superfamily members, type 1 (AT1R) and type 2 (AT2R) receptors. In target tissues such as blood vessels, kidney, brain, and heart, AT1R predominately mediates the key cardiovascular activities of Ang II, such as regulation of arterial blood pressure with short-term vasoconstriction, aldosterone secretion, and water and salt balance. (De Gasparo M et al., 2000).

2.9 Kidney function tests in hypertension

The production of hormones like erythropoietin, 1,25-dihydroxyvitamin D, and renin, as well as the excretion of waste products and toxins like urea, creatinine, and uric acid, as well as the regulation of extracellular fluid volume, serum osmolality, and electrolyte concentrations, all depend on the kidneys. In the treatment of patients with kidney disease or other illnesses that impact renal function, assessment of renal function is crucial. Renal function tests are helpful in detecting the presence of renal disease, tracking the kidneys healing progress, and monitorig how the disease is progressing. The National Institutes of Health estimate that 14% of people worldwide have chronic kidney disease (CKD). The most frequent causes of CKD globally are diabetes and hypertension (Okoro RN, Farate VT., 2019; Nwose EU et al, 2019).

A variety of clinical laboratory tests can be performed to look into and assess kidney function. Estimating the glomerular filtration rate (GFR) and looking for proteinuria (albuminuria) are the clinical tests that are most useful for determining how well the kidneys are functioning.

2.9.1 Glomerular Filtration Rate

Glomerular filtration rate (GFR) is the most accurate overall measure of glomerular function. GFR, or the clearance of a material from the blood, is the rate in millilitres per minute at which compounds in plasma are filtered through the glomerulus. An adult male's GFR should be between 90 and 120 mL per minute. The following traits make for a perfect GFR marker: (Gounden V, Bhatt H, Jialal I., 2022)

- It should be continuously present endogenously in the plasma,
- It should not undergo extrarenal elimination.
- It should be freely filtered at the glomerulus,
- It should not be reabsorbed or secreted by the renal tubule,

2.9.2 Creatinine clearance

The most commonly used endogenous marker for the assessment of glomerular function is creatinine. The calculated clearance of creatinine is used to provide an indicator of GFR. This involves the collection of urine over a 24-hour period. Creatinine clearance is then calculated using the equation:

Creatinine is the endogenous marker that is most frequently used to evaluate glomerular function. An indicator of GFR is provided by the calculated creatinine clearance. 24hour urine was gathered for this purpose. The following equation is then used to determine creatinine clearance:

$$C = (U \times V) / P$$

Where C = clearance, U = urinary concentration, V = urinary flow rate (volume/time in ml/min), and P = plasma concentration

Correcting creatinine clearance for body surface area is necessary. One of the main problems affecting the accuracy of this test is improper or partial urine collection, hence

timed collection is helpful. Furthermore, creatinine overestimates GFR by 10% to 20% as a result of tubular secretion.

The body continuously produces creatinine, a byproduct of the breakdown of creatine phosphate in muscle. Most of the time, the kidney is the only organ that removes creatinine from the blood. Increased blood creatinine is the result of decreased renal clearance. The daily production of creatinine is influenced by muscle mass. As a result, males and females have different creatinine ranges with lower creatinine readings in children and those with less muscle mass. Creatinine levels are also influenced by diet. Red meat consumption can cause a 30% change in creatinine. Lower creatinine readings are observed in pregnancy when GFR rises. Furthermore, serum creatinine is a later sign of renal impairment since renal function declines by 50% before a rise in serum creatinine is observed. (Gounden V, Bhatt H, Jialal I., 2022).

2.9.3 Blood Urea Nitrogen (BUN)

Urea is a a nitrogen-containing substance sometimes known as BUN, is produced in the liver as a byproduct of protein metabolism in the urea cycle. The kidneys remove around 85% of the urea; the remainder is expelled through the GI tract. When renal clearance declines (as in acute and chronic renal failure/impairment), serum urea levels rise. Other disorders include upper GI hemorrhage, dehydration, catabolic states, and high protein diets that are unrelated to renal illnesses can also cause a rise in urea. In starvation, a low-protein diet, and severe liver illness, urea production may be reduced. Although urea is elevated early in renal disease, serum creatinine is a more precise measure of renal function. (Gounden V, Bhatt H, Jialal I. 2022).

2.9.4 Proteinuria

In diabetics, proteinuria is a sign for the early stages of nephropathy. It is a marker for chronic renal impairment as well as an independent indicator of cardiovascular disease because it denotes increased endothelial permeability. Urine albumin or total protein can be determined as an albumin/creatinine ratio in 24-hour urine collections or early morning/random sample. When a urinary tract infection is ruled out and albuminuria or proteinuria is present on two separate occasions, glomerular dysfunction is the likely cause. Albuminuria for three months or longer is a sign of chronic renal disease. Greater than 300 mg of protein per day is referred to as frank proteinuria.

Up to 150 mg of normal urine protein can be found daily (30% albumin, 30% globulins, and 40% Tamm Horsfall protein). Increased protein levels in the urine could be caused by:

- Glomerular proteinuria: Resulting from problems with the glomerular filtration barrier's permeability to plasma proteins (for example, glomerulonephritis or nephrotic syndrome)
- Tubular proteinuria: A condition brought on by insufficient tubular protein reabsorption (for example, interstitial nephritis)
- Overflow proteinuria: Caused by increased plasma concentration of proteins (for example, multiple myeloma-Bence Jones protein, myoglobinuria)
- Overflow proteinuria: Elevated protein levels in the blood (for example, multiple myeloma-Bence Jones protein, myoglobinuria)
- Tumour or Urinary tract inflammation (Gounden V, Bhatt H, Jialal I., 2022)

2.10 Voltage- Gated Calcium Ca_{2+} Channels

Voltage-gated calcium channels open as a result of membrane depolarization, allowing calcium ions to enter cells. They are a member of the multimeric transmembrane protein family. L, N, P, Q, R, and T types are six different subtypes of calcium channels. These calcium channels are hetero-oligomeric structures made up of structurally different subunits α1, α2, δ, β and γ. The calcium conduction channel is formed by the larger α1 subunit. While the remaining Ca^{2+} channels are high voltage-activated Ca^{2+} channels that are triggered by bigger depolarizations, T-type Ca^{2+} channels are low voltage-activated and are activated by tiny depolarizations. (Soderlund., 2010; Takahara., 2009). Heart and blood vessels of the cardiovascular system mostly possess L-type calcium channels, where they control cardiac contractility, sinus nodal activity, and vascular tone. In the sympathetic and central nervous systems, N-type Ca2+channels are located at the nerve terminals and control the release of neurotransmitters (Takahara., 2009). Ca^{2+} channel blockers are frequently used to treat various cardiovascular conditions.

2.11 Calcium Channel Blockers (CCBs)

Although certain CCBs also block other $^{Ca2+}$ channels, most CCBs primarily restrict Ca^{2+} ion transport across membranes by blocking L-type Ca^{2+}channels. As a result, cells in the sinoatrial (SA) and atrioventricular (AV) nodes, as well as smooth and cardiac muscle, contract less forcefully. CCBs dilates coronary and peripheral arteries, and also have negative inotropic, chronotropic, and dromotropic effects. (McDonagh et al., 2005).

There are three kinds of calcium channel blockers based on chemical structure.

1. Benzothiazepines: Diltiazem
2. Phenylalkylamines: Verapamil

3. Dihydropyridines: Amlodipine, nifedipine, nicardipine, bepridil, felodipine, isradipine

Since dihydropyridines block smooth muscle calcium channels at lower doses than are necessary to have substantial cardiac effects, they show a higher selectivity for vascular smooth muscle than cardiac muscle. As a result, they are less inotropically adverse than verapamil or diltiazem. Compared to dihydropyridines, benzothiazepines and phenylalkylamines have a higher effect on the heart, depresses the conduction of SA and AV node, their vasodilator efficacy also is less selective (McDonagh et al., 2005). Hence in the treatment of cardiovascular disorders dihydropyridines, have been utilised extensively. They have undergone a number of modifications since their debut in the 1960s in order to increase their effectiveness and safety.

2. 12 Cilnidipine: L/N type calcium channel blocker:

Dual L/N-type Ca2+ channel blockage is a property of cilnidipine. Norepinephrine (NE) release from sympathetic nerve terminals is controlled by the N-type voltage dependent Ca^{2+} channels, which play a significant part in sympathetic neurotransmission. N-type Ca^{2+} channels are found in the brain and dispersed along nerves. According to theories, cilnidipine has a specific effect on nerve activity, such as inhibiting the sympathetic nervous system (Chandra KS, G Ramesh., 2013). It has also been demonstrated to possess cardio-protective, renal protective and neuro-protective actions (Fan L et al., 2011).

Studies have also shown that cilnidipine has beneficial effects on renal function, glucose and lipid metabolism, and hypertension in people with type II diabetes (Masuda T et al., 2011). Recent studies have shown that increasing endothelial nitric oxide synthase activity results in increased NO generation, which relaxes human arteries (Fan L et al., 2011). Therefore, cilnidipine is a promising fourth generation calcium channel blocker with a dual L/N type Ca^{2+} channel blocking effect.

Dihydropyridine calcium antagonist cilnidipine has been found to have a sustained antihypertensive effect. After receiving first approval in Japan in 1995, cilnidipine went got approval from other nations, becoming one of the most popular anti-hypertensive medications available today. The L/N-type calcium channel blocker cilnidipine decreases blood pressure by inhibiting sympathetic nerves at peripheral sympathetic nerve endings in vivo (Kishi T et al., 2009). It has been demonstrated to lower both systolic and diastolic blood pressure (SBP and DBP), but not pulse rate (PR) or plasma catecholamine levels. Additionally, it has been demonstrated to reduce spontaneously hypertensive rats (SHR) pressor reaction to acute cold stress (Chakraborty RN et al., 2022). In hypertensive individuals with morning hypertension when sympathetic nerve overactivity was perhaps present, cilnidipine was reported to be beneficial. Cilnidipine has also been demonstrated to considerably decrease blood pressure in hypertensive individuals with aberrant nocturnal blood pressure, particularly while they are sleeping when the sympathetic nervous system is overactive (Kario K et al., 2013). Additionally, cilnidipine reduces vascular endothelial dysfunction, making it helpful for the long-term treatment of cardiovascular diseases (Fan L etal., 2011).

Cilnidipine has considerable prolong anti-hypertensive effects and have good oral absorption. Drug concentrations after oral administration reach their maximum at 1.8 to 2.2 hours and have a half-life of 7.5 hours. Cilnidipine, however, demonstrates a prolonged duration of antihypertensive activity while having a lower half-life. The duration of cilnidipine action is thought to be prolonged by its strong protein binding of 98%. Comparing cilnidipine activity to that of nifedipine and nicardipine, in-vitro and animal investigations have revealed that cilnidipine effect is slower in development and longer in duration (Chakraborty RN et al., 2022).

Effect of cilnidipine on renal function and micro vessels.

Clinical investigations have shown that cilnidipine, which is comparable to the angiotensin converting enzyme inhibitor benazepril, dramatically reduced urine albumin excretion without changing blood creatinine concentration in hypertensive patients. Evidence from the multicenter, open-label, randomised trial of cilnidipine versus amlodipine (CARTER) has revealed that when used in conjunction with a renin-angiotensin system inhibitor, cilnidipine is more effective than amlodipine at slowing the progression of proteinuria in patients with hypertension and chronic renal disease (Fujita T et al., 2007).

According to a study, cilnidipine relaxes human arteries by inhibiting Ca^{2+} channels and increases endothelial nitric oxide synthase in the internal thoracic artery, which enhances nitric oxide production (Fan L et al., 2011). With its dual L/N-type Ca^{2+} channel-blocking action, cilnidipine is a promising Ca^{2+} channel blocker of the fourth generation. The neurohumoral control of renin-angiotensin-aldosterone system, sympathetic nervous system, and cardiovascular system are effectively suppressed by blocking N-type Ca^{2+} channels. As a result, cilnidipine is anticipated to be beneficial for a variety of hypertension-related problems (Chandra KS, G Ramesh., 2013).

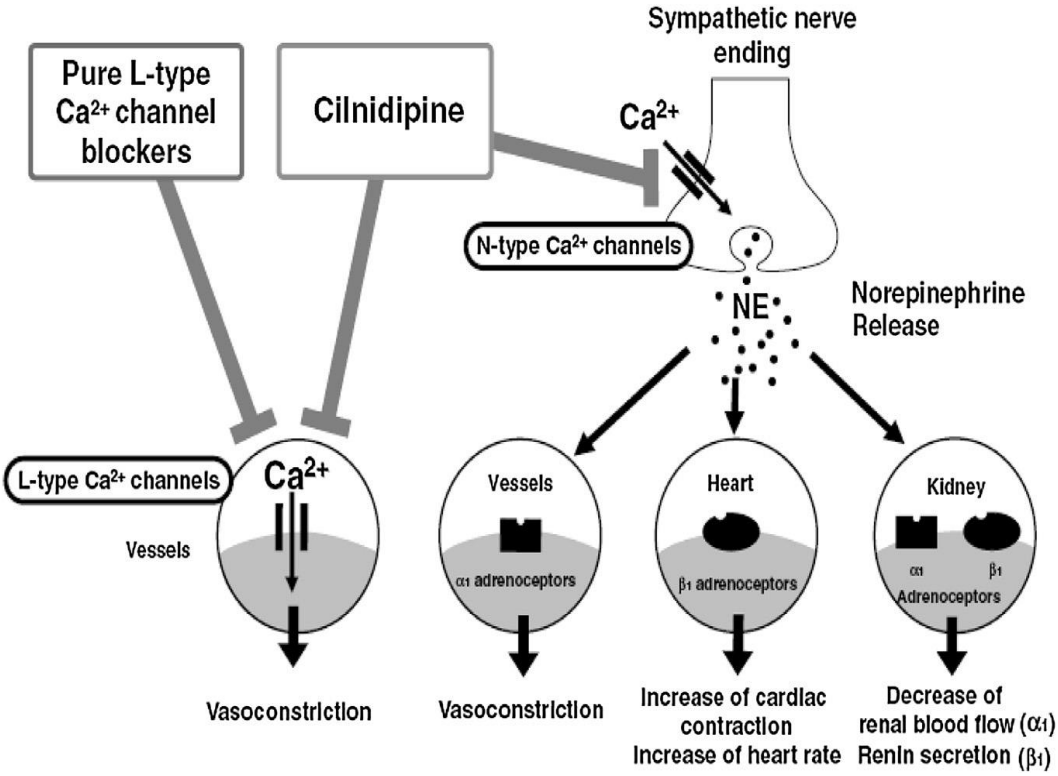


Figure 2.12: Diagrammatic showing cilnidipine L/N dual calcium channel action

Ref: Takahara A. Cilnidipine; a new generation calcium channel blocker with inhibitory action on sympathetic neurotransmitter release. Cardiovasc Ther 2009; 27 (2): 124-139.

CHAPTER III
AIM AND OBJECTIVES OF THE STUDY

3.1 AIM OF THE STUDY

To assess the impact of both L-type and N-type dual calcium channel blocker, cilnidipine, on experimental rats with NOS inhibition and salt supplementation with reference to their vascular and renal pathologies.

3.2 OBJECTIVES OF THE STUDY

1. To develop two forms of hypertensive rat models, nitric oxide deficient hypertension and salt -sensitive hypertension. (L-NAME & L-NAME + 4% NaCl induced hypertension).
2. To study the effect of L-NAME along with 4%NaCl on oxidative and nitrosative stress markers in experimental animals.
3. To evaluate the effect of NOS blocker(L-NAME) along with 4%NaCl treatment on vascular pathophysiology through cell signaling mechanism (vascular endothelial growth factor (VEGF), Nitric oxide synthase 3(NOS-3), Angiotensin converting enzyme (ACE) protein expression) in male albino rats.
4. To evaluate the effect of NOS blocker (L-NAME) along with 4%NaCl treatment on renal pathophysiology through cell signaling mechanism (VEGF, NOS-3, ACE expression) in male albino rats.
5. To find out whether there is any rule to modulate signal transduction through calcium channel blocker (Cilnidipine) on vascular and renal pathophysiology in experimental animals.

3.3 HYPOTHESIS

3.3.1 Null Hypothesis

Cilnidipine - dual L/N type calcium channel blocker has no significant effect on renal and vascular pathophysiology in L-NAME and L-NAME plus salt induced hypertensive rats.

3.3.2 Alternate Hypothesis

Cilnidipine treatment reduces renal and vascular damage caused by L-NAME and L-NAME plus salt induced hypertensive rats.

CHAPTER IV
MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Experimental animals

Rats for this study procured from the animal house of, Shri B. M. Patil Medical College, Hospital & Research Centre, BLDE (Deemed to be University), Vijayapura, Karnataka, India. Healthy adult male Wistar strain Albino rats (Rattus Norvegicus) laboratory, aged 60-70 days, weighing about 180g-250g were included in this study. For seven days, all the animals were allowed to adjust to the laboratory environment. The animals were kept at a temperature of 22–24 °C, exposed to a 12-hour cycle of light and darkness, and given unlimited access to food and water.

All the animals were acclimated to the lab environment for a week prior to the start of the experiment.

4.2 Sample size: Using the resource equation approach sample size has been calculated (Affrin et al., 2017).

Sample size=DF/k +1 Where DF=Degree of Freedom

k= No. of groups= 6 (present study)

- Minimum n/group= 10/k +1= 10/6+1=2.6=3
- Maximum n/group=20/k +1=20/6+1= 4.33=5

A minimum of 3 and a maximum of 5 animals per group are needed for the proposed study. In other words, study requires 18 to 30 rats overall to maintain the DF between 10 and 20.

Present study n=6 rats/group- Total 36 rats for 6 groups was taken.

4.3 Experimental groups

The six groups shown in table 4.3 were chosen at randomly among the acclimatised animals.

Table 4.3: Experimental groups of rats

GROUPS (n=6 in each group)	**Intervention**
Group 1 (Control)	Vehicle (0.5% sodium carboxy methyl cellulose) by oral gavage for 28 days
Group 2 (Cilnidipine)	Cilnidipine, 2mg/kg/day (Bagali S, et al. 2020) in 0.5% Na CMC by oral gavage for 28 days
Group 3 (L-NAME)	L-NAME, 40mg/kg/day (Ji Hoon S et al. 2013) orally in distilled water for 28 days
Group 4 L-NAME+ Cilnidipine	L-NAME, 40mg/kg/day orally in distilled water for 28 days Cilnidipine 2mg/kg/day in 0.5% Na CMC orally for 28 days
Group 5 L-NAME + 4% NaCl	L-NAME, 40mg/kg/day orally in distilled water for 28 days 4% Salt diet (Kopkan L et al. 2010) for 28 days
Group 6 L-NAME+ 4% NaCl + Cilnidipine	L-NAME, 40mg/kg/day orally in distilled water for 28 days Cilnidipine 2mg/kg/day in 0.5% Na CMC orally for 28 days 4% Salt diet for 28 days

4.4. Experimental Protocol: The experimental protocol followed has been summarized in figure 4.4

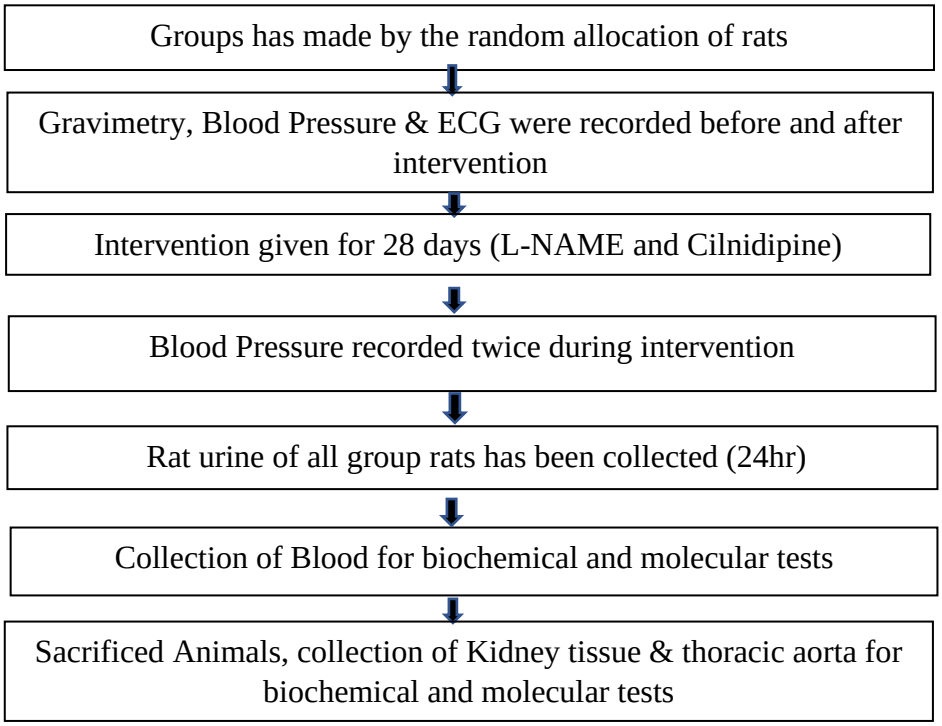


Fig 4.4: Experimental Protocol followed

4.5 L-NAME induced Rat Hypertensive model

1. ***L-NAME induced hypertensive rat model***

L-NAME was administered orally (40mg/kg/day) in distilled water for four consecutive weeks to induce NO deficient hypertension. (Hoon Young Choi et al. 2015).

2. ***L-NAME + 4% NaCl induced hypertensive rat model***

L-NAME (40 mg/kg/day) was administered orally combined with a 4% NaCl diet for four consecutive weeks to induce NO deficient and salt supplemented hypertension. (Hoon Young Choi et al. 2015).

4.5.1 Administration of L-NAME

L-NAME was purchased from Pro Lab Marketing PVT, Limited, in New Delhi, India. L-NAME was kept in a -20 ^{O}C freezer for further usage. The computed daily dose of L-NAME is 40 mg/kg/day. To rats in Group 3 (L-NAME), Group 4 (L-NAME + Cil), and Group 6 (L-NAME+ Salt + Cil) L-NAME was administrated by oral gavage at once in distilled water every morning for 28 days.

4.5.2 Administration of 4% sodium chloride (4%NaCl)

Procured 4% salt diet from VRK Nutritional Solutions, Miraj, Karnataka. High salt diet has given to the group 5 (L-NAME + Salt) and group6 (L-NAME + Salt + Cilnidipine).

4.6 Administration of Cilnidipine

Cilnidipine was purchased from Gujarat, India's Laksh Finechem Pvt. Ltd. As cilnidipine is sparingly soluble in water, every day cilnidipine suspension in 0.5% sodium carboxymethyl cellulose (Na CMC) was prepared and it was given oral gavage once every morning.

4.6.1. Calculation of dose of cilnidipine

Using the given formula, the dose of cilnidipine for rats was calculated (Nair and Jacob, 2016).

Rat equivalent dose (mg/kg) = Human dose (mg/kg) x K_m ratio

= 20/60 x 6.2

~ 2.0 mg/kg/day

K_m ratio is obtained by dividing human K_m factor by animal K_m factor or vice versa. The K_m ratio for the rat is 6.2 and 0.162 obtained by dividing 37 (human K_m factor) by 6 (rat K_m factor) and vice versa respectively.

K_m - correction factor. It is estimated by dividing the average body weight (Kg) of species to its body surface area (m^2) (Nair and Jacob, 2016)

The dose of cilnidipine for each rat was calculated to be 2.0mg/kg/day.

4.7 Method of data collection

4.7.1 Gravimetry

On day 1 of the study (starting body weight) and day 29(final body weight) of the intervention, the body weight of each rat was recorded using an electronic balance (Practum 1102-10IN, Sartorius Lab Instruments, Germany). The weights of the rats in each group were identical before the study began.

The following formula was used to calculate the percentage change in body weight.

$$\text{Change in body weight (\%)} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}} \times 100$$

4.7.2. Recording of Pneumogram

Using the respiratory pad transducer, the respiratory rate was measured by observing the abdomen movements during breathing (BioPac Student lab system). Pre-intervention (day 0) and post-intervention (day 29th) respiration rates were measured. (Zehendner CM et al., 2013).

4.7.3. Evaluation of cardiovascular electrophysiology

4.7.3.1 Recording of Blood Pressure

The conscious rats blood pressure (systolic and diastolic) was measured every week for four weeks. Prior to monitoring blood pressure, animals were kept in the restainer for 10–20 minutes every day for 5 days. For a better detection of the pulsations in the tail artery, the tail of the animals were warmed for 30 minutes. Blood pressure was measured non-invasively (NIBP) using a Bio Pac non-invasive tail cuff sensor (Bio Pac MP 100: PC Windows based animal electrophysiology system). Bio Pac Student Lab 4.1 Software was used to analyse each parameter. Systolic and diastolic blood pressure was recorded for rats of all groups weekly. All measures were taken three times, and for each rat, the mean of the three measurements was taken into consideration (Ji Hoon S et al. 2013). Using a formula, the mean arterial pressure (MAP) was determined.

MAP =Diastolic blood pressure+1/3Pulse pressure.

4.7.3.2 Recording of ECG

Utilizing a PC-based BSL 4.1 (Biopac Student Lab 4.1) software and an MP45 Biopac device, ECG was obtained using needle subcutaneous electrodes. Following an overnight fast, all of the recordings were made in the morning. The needle electrodes were inserted in the right (negative) and left (positive) front legs, as well as the right rear leg

(Ground), of anaesthetized rats in dorsal recumbency to record an ECG for 10 minutes. The recorded ECG was used to calculate heart rate.

4.7.3.3 Heart rate variability analysis

The heart works as a powerful pump. The intervals between each subsequent heartbeat differ. Heart rate variability analysis is the study of such variations in the inter beat intervals. A popular non-invasive method to evaluate cardiovascular autonomic functions is HRV analysis. Both time and frequency domain indices are used in HRV analysis. Two basic frequency bands are used in the frequency domain investigation of RR interval oscillations. High frequency (HF) (0.15–0.4 Hz) band reflects parasympathetic activity, while low frequency (LF) band (0.04-0.15Hz) mostly reflects sympathetic activity. A measure of cardiac sympathovagal balance is the LF/HF ratio. (Shaffer F et al. 2017).

Offline examination of the recorded ECG for artefacts and manual removal of ectopic beats from the recording was done. To do an HRV analysis, the RR intervals acquired from the recorded ECG were exported to the Kubios software version 2.0 (created by the Department of Physics at the University of Kuopio in Finland). A short-term heart rate variability (HRV) analysis using 5 min ECG RR interval data was carried out to assess cardiac autonomic balance. To measure the degree of sympathetic activity, parasympathetic activity, and sympathovagal balance, HRV analysis using the frequency-domain approach was performed.

Table 4.7.3.3: Components of HRV analysis using the frequency domain

Sl. No	Component (nu)	Indicator of
1.	LF-Low frequency component	Sympathetic activity
2.	HF-High frequency component	Parasympathetic activity
3.	LF/HF ratio	Sympathovagal balance

All of the aforementioned variables were recorded twice: before (day 0) and after) (day 29th) of intervention.

4.7.4 24hour Rat Urine Collection:

Keeping the rats individually in metabolic cage, 24-hour urine samples were collected from all six groups of rats. Urine was collected continuously from 10:00 a.m. to 10:00 a.m. the following day. All urine samples were centrifuged to eliminate any sediments. With an auto analyser (VITROS 5.1/FS chemistry system), the 24-hour protein concentration and 24-hour urine creatinine were determined. Creatinine Clearance of all the rats was measured by using formula **(urine creatinine in mg/ml ×urine volume / day in mL ÷ 1440 min ÷ serum creatinine in mg/ml ÷ both kidney weight in gm)** (Lu J, et al. 2003).

4.7.5: Collection of Blood

All of the animals were starved overnight before blood was drawn from anaesthetized rats on day 29 of after 28-days of intervention for further haematological, biochemical, and molecular marker analyses, respectively, in EDTA and plain tubes.

4.7.6: Haematological Analysis

In commercial tubes with around 40µl of potassium EDTA as an anticoagulant, about 1 ml of blood was drawn. Sysmex K4500 Automated Haematology Analyzer was used to analyse the collected blood samples to determine the RBC count (million/mm3), haematocrit (%), and haemoglobin (g/dl).

4.7.7: Biochemical Parameters

4.7.7.1: Oxidative stress parameters

1. Estimation of serum malondialdehyde (MDA)

2. Estimation of malondialdehyde (MDA) in kidney tissue homogenate.

1. Serum and kidney tissue MDA was estimated by using the method of Buege and Aust (Buege and Aust, 1978)

Introduction

Malondialdehyde (MDA) is a marker of oxidative stress in biological systems. It is a by product of the lipid peroxidation process. It is produced when a free radical chain reaction breaks down polyunsaturated fatty acids (PUFA). It is one of the numerous reactive electrophile species that cause oxidative stress and is a reactive aldehyde.

Principle

MDA, which is produced when PUFA break down, functions as an easy way to gauge how much lipid peroxidation has occurred. It reacts with thiobarbituric acid (TBA) to produce a pink colour that may be detected at 535 nm.

Sample: Serum and kidney tissue homogenate

Chemicals required:

1. Trichloroacetic acid (TCA)
2. 2-Thiobarbituric acid (TBA)
3. Hydrochloric acid (HCl)
4. Malonaldehyde (dimethyl acetal)

Preparation:

1. TCA-TBA-HCl reagent

2. 21 ml of concentrated HCl was diluted to 100 ml with 0.25 N HCl (DW). 100ml of 0.25N HCl were used to dissolve 15% TCA and 0.375% TBA. The reaction mixture was heated and maintained at 40°C in order to dissolve the components.

2. MDA standard (stock-164µg/ml)

16. To make 100 ml, 4µl of the standard MDA solution were diluted with distilled water.

3. MDA working standard (working- 1.64µg/ml)

The stock was made 10ml from 100µl l using distilled water.

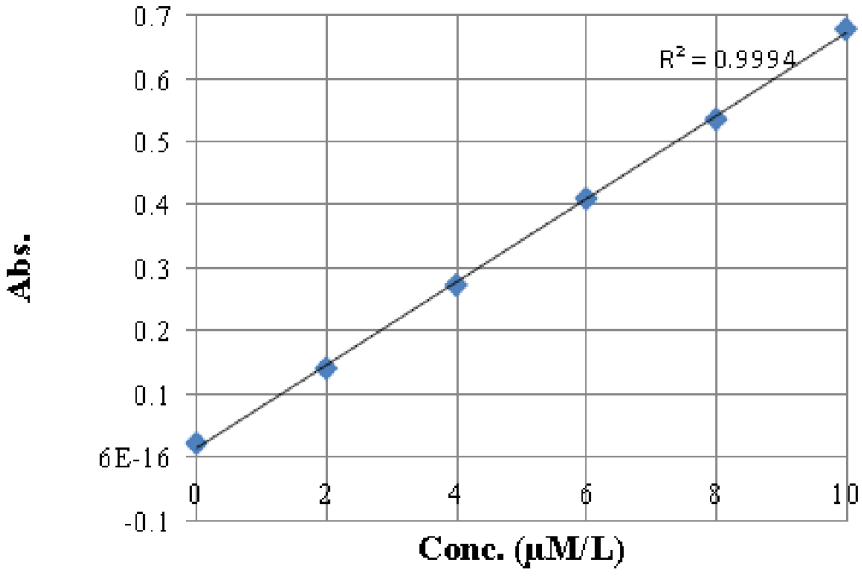
The procedure for standardization:

Standardisation (Range 2-10µM/L)

All of the reagents were added in accordance with the values listed in the table and standardisation was performed using the table.

S. No.	Vol of MDA (ml)	Vol of DW (ml)	Conc. Of MDA(µM/L)	TBA-TCA-HCl (ml)	
B	0.0	I	0.0	1	Keep in boiling water bath for 15 min
1	0.2	0.8	2.0	1	
2	0.4	0.6	4.0	1	
3	0.6	0.4	6.0	1	
4	0.8	0.2	8.0	1	
5	1	-	10.0	1	

Standard Curve



Estimation of MDA in the sample:

Preparation of sample:

Serum: 500µl of serum was obtained by diluting 100µl of serum with distilled water

Kidney Tissue Homogenate: 10% kidney tissue homogenate was used about 500µl was taken (By combining 500 mg of kidney tissue with 5 ml of 0.1 M phosphate buffer, homogenising the combination for a few minutes, centrifuging the mixture, and using the supernatant for estimation, 10% tissue homogenate was made).

Procedure

1. One millilitre of TCA-TBA-HCl reagent was added to the diluted sample.
2. For 15 minutes, the samples were maintained in a boiling water bath.
3. The reaction mixture was centrifuged after cooling.
4. The optical densities of the pink colour generated in the supernatant were measured at 535 nm by a UV visible spectrophotometer (Shimadzu, Model: UV 1800).
5. Comparing the acquired absorbance to the reference graph allows one to determine the MDA concentration in the sample. The optical density of the resulting pink colour was closely directly associated with the amount of MDA present in the sample.

Calculations

Plotting against the reference graph and multiplying by the respective dilution factors yielded the optical densities of the test samples. They were directly correlated with the concentration of MDA in the samples. MDA final concentration was measured as µM/L.

4.7.7.2: Nitrosative stress parameters

1. Estimation of serum Nitric Oxide (NO)
2. Estimation of kidney tissue Nitric Oxide (NO)

Griess Reaction method (Moshage Han et al., 1995; Cortas and Wakid., 1990)

Principle

The stable by product of nitric oxide, nitrate, was coupled to N-naphthyl ethylene diamine and then reduced to nitrite using the cadmium reduction process. A spectrophotometer was used to measure the coloured complex produced at 540 nm.

Reagents

1. Cadmium granules: About 2.5-3gm of cadmium granules are stored in 0.1M/L H_2SO_4
3. Glycine-NaOH buffer (pH-9.7): 7.5g of glycine were dissolved in 200ml of distilled water. After adjusting the pH to 9.7 using 2M NaOH, distilled water was used to dilute the solution to 500ml.
4. Sulfanilamide: 250ml of heated 3M/L HCl was used to dissolve 2.5g of sulfanilamide, which was then allowed to cool.
5. N-Naphthyl ethylene diamine: Distilled water was used to dissolve 50 mg of N-Naphthyl ethylene diamine, and the amount was then increased to 250ml.
6. Standard sodium nitrite solution:

a. Stock standard (0.1mol/L)

In 100 ml of a sodium borate solution containing 10 mmol/L, 690 mg of sodium nitrite were dissolved.

b. Working standard (10µmol/L)

10mmol/L sodium borate solution was used to dilute 10µl of stock sodium nitrite ($NaNO_2$) to 100ml.

6. $ZnSO_4$ solution (75mmol/L)

7. NaOH solution (55mmol/L)

8. H_2SO_4 solution (0.1mol/L)

9. $CuSO_4$ solution (5mmol/L)

The glycine-NaOH buffer 100ml was used to dissolve 125 mg of CuSO4.

Procedure

a. Deproteinization:

Serum (0.5 ml) was collected and placed in a fresh, dry centrifuge tube. This was combined with 2.0 ml of a zinc sulphate solution that contained 75 mmol/L. After the sample was thoroughly mixed with 2.5 ml of the 55 mmol/L NaOH reagent, it was centrifuged for 10 minutes. Protein-free filtration was carried out using the supernatant.

b. Activation of cadmium granules:

The cadmium granules were kept in 0.1mol/L H_2SO_4 solution and then rinsed three times in distilled water before the analysis. The granules were then agitated for one to two minutes in a 5 mmol/L CuSO4 solution. The glycine-NaOH buffer was used to drain and clean the copper-coated granules. Ten minutes after activation, these granules were utilised. The granules were rinsed with distilled water after use and then placed in a solution containing 0.1 mmol/L H2SO4.. The same granule activation method was applied each time.

c. Nitrite Assay:

1. We took three Erlenmeyer flasks and assigned them the labels Blank (B), Test (T), and Standard (S).
2. Each Erlenmeyer flask, 1 ml of glycine-NaOH buffer was taken. 1 ml of deionized water, 1 ml of the deproteinized sample, and 1 ml of the working standard were added to the flasks marked B (Blank), T (Test), and S (Standard), respectively.
3. Each flask was filled with 2.5–3 gramme of freshly activated cadmium granules using a spatula.

4. The granules were swirled throughly.
5. The mixture in each of the three flasks was diluted to 4ml with distilled water after 90 minutes.
7. 3 clean, dry test tubes with the letters B, S, and T were each pipetted with 2 ml of solution from the corresponding flasks.
8. To each tube, 1 ml of sulfanilamide and 1 ml of N-naphthyl ethylene diamine solution were added.
9. All three tubes were well shaken before the OD of S and T was measured at 540 nm against a blank after 20 minutes. Following formula was used to determine serum nitric oxide.

Serum Nitric oxide(µmol/L) = OD of Sample/ OD of Standard x conc. Of Standard x DF

4.7.7.3 Kidney function tests.

1. Estimation of Serum Urea

1. Method used

VITROS Chemistry Products BUN/UREA Slides quantitatively measure urea concentration, in serum, plasma, and urine using VITROS 250/350/5,1 FS/4600/XT 3400 Chemistry Systems and the VITROS 5600/XT 7600 Integrated Systems.

2. Principles of the Procedure

A multi-layered analytical component coated on a polyester support is the VITROS BUN/UREA Slide. A drop of sample is placed on the slide, and the spreading layer evenly distributes it to the underlying layers. The urease process creates ammonia as water and non-proteinaceous components move to the lower reagent layer. Only ammonia can flow past the semipermeable membrane and into the color-forming layer, where it interacts with the

indicator to form a dye. It is possible to measure the dye's reflection density, which correlates with the sample's urea concentration.

Test Type and Conditions

Test Type	VITROS System	Approximate incubation time	Temperature	Wavelength	Reaction Sample Volume
Colorimetric	5600, 4600,5,1 FS,250/350, XT 7600, XT 3400	5 minutes	37^{O}C (98.6^{o}F)	670 nm	5.5 µL

Reaction Scheme

$$H_2NCONH_2 + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{ammonium indicator} \longrightarrow \text{dye}$$

Slide layers

1. Upper slide mount
2. Spreading layer (TiO_2)
3. Reagent layer

• urease, buffer, pH 7.8

4. Semipermeable membrane
5. Indicator layer: ammonia indicator
6. Support Layer
7. Lower slide mount

Regents used

Slide ingredient

Reactive Ingredients per cm^2

Urease (jack bean) 1.2 U and N-propyl-4-(2,6-dinitro-4-chlorobenzyl)-quinolinium ethane sulfonate (ammonia indicator) 0.26 mg.

Other Ingredients

Pigment, binders, buffer, surfactants, stabilizers, chelator and cross-linking agent

2. Estimation of creatinine

Principles of the Procedure

The VITROS CREA Slide is an analytical component with multiple layers that is coated on a polyester support. A drop of sample is placed on the slide, and the spreading layer evenly distributes it to the underlying layers. In the rate-determining step, creatinine diffuses to the reagent layer and is hydrolysed to creatine. Creatine amidino hydrolase breaks down the creatine into sarcosine and urea. Sarcosine is converted into glycine, formaldehyde, and hydrogen peroxide when sarcosine oxidase is present. In the final step, a leuco dye is oxidised under the influence of peroxidase to create a coloured product.

Following addition of the sample, the slide is incubated. During the initial reaction phase, endogenous creatine in the sample is oxidized. The resulting change in reflection density is measured at 2 time points.

The difference in reflection density is proportional to the concentration of creatinine present in the sample.

Test Type and Conditions

Test Type	VITROS System	Approximate incubation time	Temperature	Wavelength	Reaction Sample Volume
Two point rate	5600, 4600,5,1 FS,250/350, XT 7600, XT 3400	5 minutes	$37^{O}C$ ($98.6^{o}F$)	670 nm	6 µL

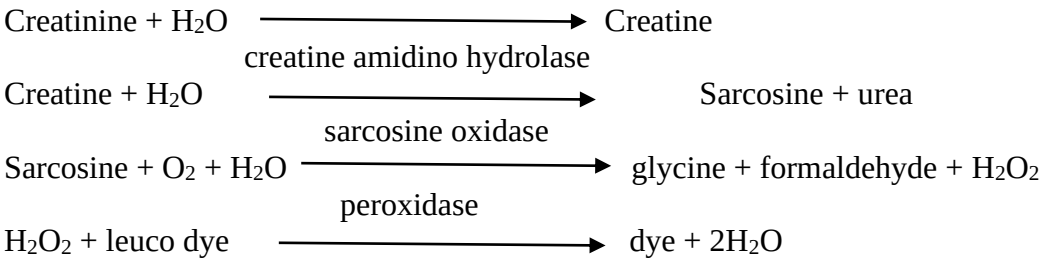
Calculation:

Total protein concentration (gm/dl) =

OD Test-OD Blank x Concentration of Std X 100

OD Std-OD Blank effective vol of sampl

Reaction Scheme



4.7.7.4: Estimation of 24hr urinary total protein

Principle: Total protein calorimetric measurements based on the principles of Biuret reaction (copper salt in alkaline medium). When treated with cupric ions in an alkaline solution, the protein in the serum sample forms a complex that is blue in colour. The concentration of proteins is proportional to the intensity of blue colour.

Normal Range: 6.2- 8.0gm/dL

Reagent composition:

Potassium iodide- 6 mmol/l, Potassium sodium tartrate- 21 mmol/l, Copper sulphate- 6 mmol/l, Sodium hydroxide- 58 mmol/l, total Protein Standard- 6 g/dl.

Procedure: Taken three clean dry test tube label them as T(Test), S(Standard), and B(Blank). Add the solution as shown in the table below.

Reagents	Test	standard	Blank
Serum	100 µl		
Standard protein solution(5gm/dl)		100 µl	
Distilled water	-		100 µl
Biuret reagent	30ml	30ml	30ml

The mixture was incubated at 37^{O}C for 10 minutes and the absorbance was measured at 530 nm using a biochemical analyser.

4.7.8: Quantitative Analysis of Molecular Markers (ELISA)

4.7.8.1: Serum NOS3 (eNOS), Estimated by using Rat NOS3 ELISA kit (Rat eNOS Kit No: K11-0466)

Serum NOS3 was estimated by ELISA using Rat NOS3 ELISA Kit (Cat No: K11-0466 KINESIS Dx, Los Angeles, CA 90007, USA). The product manual's protocol was followed.

Principle of the Assay

This kit utilises a sandwich enzyme-linked immune-sorbent assay method. We used 96-well plates that have been precoated with the pure anti-NOS3 antibody. As detecting antibodies, biotin-labelled anti-NOS3 antibodies were utilised. The wells were filled with the standards, test samples, and HRP conjugate, which was then mixed and incubated. Unbound conjugates were then removed with wash buffer. The HRP enzymatic process is visualised using the substrates (A & B). TMB is catalysed by HRP to yield a blue product that, when combined with acidic stop solution, turns yellow. The amount of NOS3(eNOS) in the sample that was captured on the plate is proportional to the yellow density. Reading O.D absorbance at 450 nm using a microplate reader concentration of NOS3(eNOS) was calculated.

Range: 6ng/ml-96ng/ml

Kit components:

a. One pre-coated 96-well plate with anti-rat NOS3 antibody
b. eNOS Ab Biotin Conjugate
c. Standard (192ng/ml)
d. Standard diluent buffer
e. Wash buffer (30X): Dilution (1:30)
f. Sample diluent buffer
g. HRP conjugated anti-rat NOS3 antibody

h. Stop solution

i. TMB substrate A

j. TMB substrate B

k. Plate sealer

l. Hermetic bag

Protocol

Preparation of sample and reagents

1. **Sample:** The collected blood is allowed to coagulate at room temperature for 10-20 min, and then centrifuged at the speed of 2000-3000rpm for 20 min to collect the supernatant. The supernatant is aliquoted and stored at -20^{0}C. Multiple freeze-thaw cycles were avoided.

2. **Wash buffer:** Concentrated wash buffer was diluted 30fold (1:30) with distilled water.

3. **Standard: Prepared the standards as per the table given below**

96ng/ml	Standard No 5	120µl Original Standard + 120 µl Standard diluent
48ng/ml	Standard No 4	120µl Standard 5 + 120 µl Standard diluent
24ng/ml	Standard No 3	120µl Standard 4 + 120 µl Standard diluent
12ng/ml	Standard No 2	120µl Standard 3 + 120 µl Standard diluent
6ng/ml	Standard No 1	120µl Standard 2 + 120 µl Standard diluent

Assay Procedure

1. Equilibrate kit components at room temperature for 15–30 minutes.

2. The wells on the pre-coated plate were set with the standard, test sample, and control (zero), respectively. Their positions were noted. Standard wells received 50 µl of diluted

standards (96 ng/ml, 48 ng/ml, 24 ng/ml, 12 ng/ml, and 6 ng/ml). The control (zero) well received 50 µl of the usual diluent buffer.

3. 40 µl of sample and 10 µl of the biotin conjugate were added to the test sample wells. Without touching the side well, the solution was poured into each well at the bottom. Plates were gently l shaken properly combine properly.

4. Pipette out 50µl of HRP conjugate into each sample and standard well but not into blank well.

5. For 60 minutes, the plate was incubated at 37^0C with a plate sealer covering it.

6. The plate was manually washed after the sealer was taken off. Without touching the sidewalls, the solution in the plate was thrown out for this. The plate was clapped upon some filter paper that was absorbent. The contents of the plate were aspirated after each well had been completely filled with wash buffer (1x) and lightly vortexed on an ELISA shaker for 2 min. The plate was clapped upon some filter paper that was absorbent. For a total of five washes, the identical process was repeated four more times.

7. Each well received 50 µl of TMB substrate A, then 50 µl of TMB substrate B. The plate was incubated at 37^0C in the dark for 10 minutes after being gently shaken by hand for 30 seconds. The wells displayed the blue shade.

8. Each well received 50µl of Stop solution, which was carefully mixed in. The colour immediately turned yellow.

9. The O.D. Within 15 minutes of adding the stop solution, absorbance was measured at 450 nm in a microplate reader (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.).

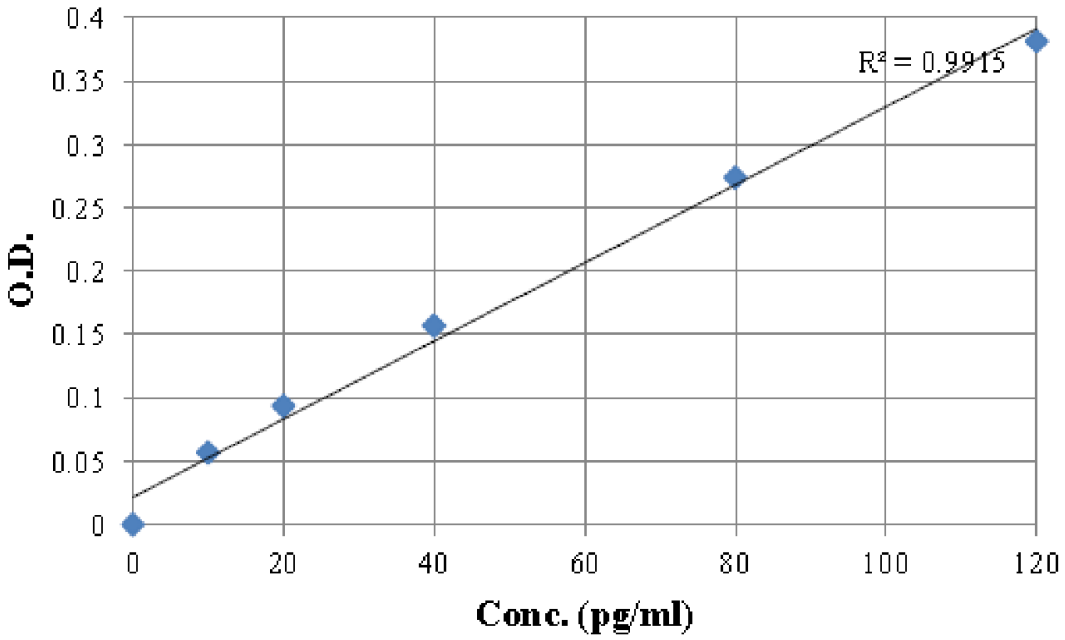
Calculations

Relative O.D.450= (O.D. 450 of each well) – (O.D. 450 of Zero well). The normal distribution was as the relative O.D. 450, on a graph. of each standard solution (Y) versus.

the corresponding standard solution concentration (X). The standard curve was interpolated to determine the NOS3 content in the samples.

Standard Curve

X	ng/ml	0	6	12	24	48	96
Y	OD_{450}	0	0.0567	0.0935	0.1568	0.2737	0.3812



4.7.8.2: Serum and urinary Angiotensin II estimation by ELISA Kit method

Estimation of serum and urinary Angiotensin II

Rat Ang II ELISA Kit (Cat No. K11-0656 KINESIS Dx, Los Angeles, CA 90007, USA) was used to estimate the levels of Ang II in the serum and urine in accordance with the recommended procedure given in instruction manual.

Principle of the Assay

This kit utilises a sandwich enzyme-linked immune-sorbent assay method. We use 96 well plates that have been precoated with the pure anti-Ang II antibody. As detecting antibodies, anti-Ang II biotin-labelled antibodies were utilised. The wells were filled with

the standards, test samples, and HRP conjugate, which was then mixed and incubated. Unbound conjugates were then removed with wash buffer. The HRP enzymatic process is visualised using the substrates (A & B). TMB is catalysed by HRP to produce a blue product that, when an acidic stop solution is added, turns yellow. The amount of Ang II in the sample that was collected in the plate correlates with the density of yellow colour. By reading the O.D. at 450 nm in a microplate reader, concentration of Ang II in the serum and 24-hour urine was computed.

Range: 40pg/ml-640pg/ml

Kit components:

a. One 96-well plate pre-coated with anti-rat ANGII antibody
b. ANG II Ab Biotin Conjugate
c. Standard (1280pg/ml)
d. Standard diluent buffer
e. Wash buffer (30X): Dilution (1:30)
f. Sample diluent buffer
g. HRP conjugated anti-rat Ang II antibody
h. Stop solution
i. TMB substrate A
j. TMB substrate B
k. Plate sealer
l. Hermetic bag

Protocol

Preparation of sample and reagents

1. **Sample:** The collected blood is allowed to coagulate at room temperature for 10-20 min, and then centrifuged at the speed of 2000-3000rpm for 20 min to collect the

supernatant. The supernatant is aliquoted and stored at -20^{0}C. Multiple freeze-thaw cycles were avoided.

2. **Wash buffer:** Concentrated wash buffer was diluted 30fold (1:30) with distilled water.

3. **Standard: Prepared the standards as per the table given below**

640pg/ml	Standard No 5	120µl Original Standard + 120 µl Standard diluent
320pg/ml	Standard No 4	120µl Standard 5 + 120 µl Standard diluent
160pg/ml	Standard No 3	120µl Standard 4 + 120 µl Standard diluent
80pg/ml	Standard No 2	120µl Standard 3 + 120 µl Standard diluent
40pg/ml	Standard No 1	120µl Standard 2 + 120 µl Standard diluent

Assay Procedure

1. Equilibrate kit components for 15-30 min at room temperature
2. Standard, test sample and control (zero) were set in the wells on pre-coated plate respectively. Their positions were recorded. 50µl of diluted standards (640pg/ml, 320pg/ml, 160pg/ml, 80pg/ml, 40pg/ml) were added into standard wells. 50µl of the standard diluent buffer was added into control (zero) well.
    1. 3. For test sample wells, 40µl of sample and then 10µl of the biotin conjugate was added. The solution was added at the bottom of each well without touching the side well. The plate was mildly shaken to mix thoroughly.
3. Pipette out 50µl of HRP conjugate into each sample and standard well but not into blank well.
4. The plate was covered with a plate sealer and incubated at 37^{0}C for 60 min.

5. The sealer was removed and the plate was washed manually. For this, the solution in the plate was discarded without touching the sidewalls. The plate was clapped on absorbent filter paper. Each well was filled completely with wash buffer (1x) and vortexed mildly on ELISA shaker for 2 min, the contents of the plate were then aspirated. The plate was clapped on absorbent filter paper. The same procedure was repeated four more times for a total of five washes.

10. 50µl of TMB substrate A was added into each well, followed by 50µl of TMB substrate B. The plate was gently shaken by hand for 30 sec and incubated in dark at 37^0C for 10 min. The shades of blue were seen in the wells.

11. 50µl of Stop solution was added into each well and mixed thoroughly. The colour changed into yellow immediately.

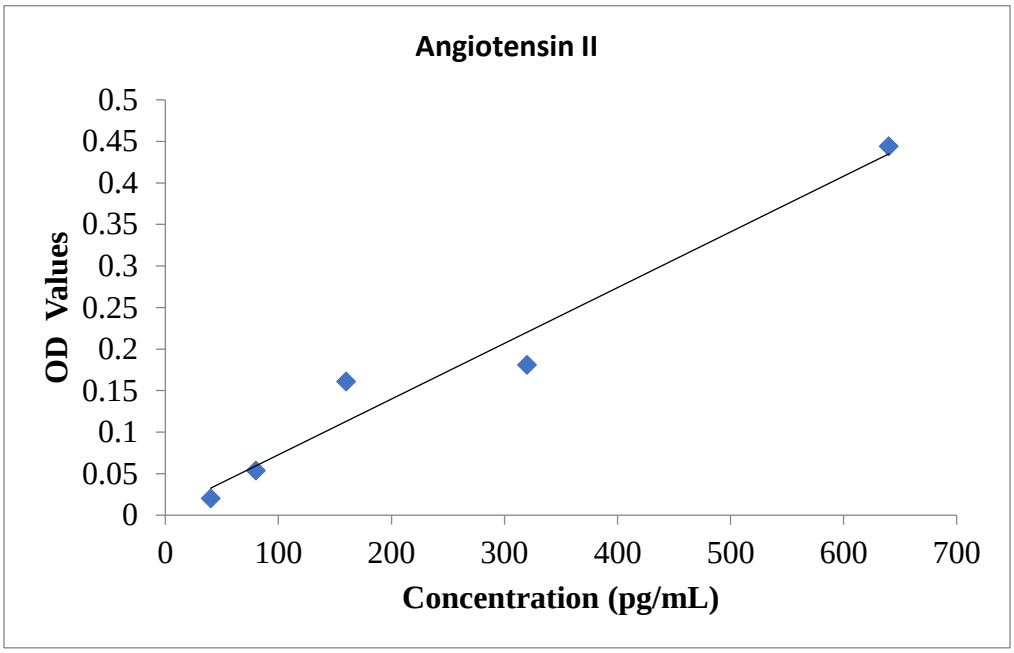
12. The O.D. within 15 minutes of adding the stop solution, absorbance was measured at 450 nm in a microplate reader (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.).

Calculations

Relative O.D.450= (O.D. 450 of each well) – (O.D. 450 of Zero well). The standard curve was

plotted as the relative O.D. 450 of each standard solution (Y) vs. the respective concentration of the standard solutions (X). The Ang II concentration of the samples was interpolated from the standard curve.

Standard curve



4.7.9: Relative Expression of Molecular Markers by Western blotting

A: Serum eNOS and kidney tissue eNOS protein expression

Primary antibody used- Cat No- **AHP2305**

B. Serum ACE and Kidney tissue ACE and protein expression

Primary antibodies- Cat No-**ACE (2E2): sc-23908**

C: Serum VEGF and Kidney tissue VEGF protein expression

Primary antibodies-Rabbit anti rat **VEGF-Cat No-AAR42**

Secondary Antibodies- Goat anti mouse IgG1: HRP -**Cat No-STAR132P**

Common Bio-Rad Western Blotting Protocol followed

Key Solutions and Reagents

i. Loading buffer: 2x Laemmli buffer

4% SDS (Sodium dodecyl sulfate)

10% 2-mercaptoethanol

20% glycerol

0.004% bromophenol blue

0.125 M Tris-HCl

Check the pH and adjust to pH 6.8 if necessary.

ii. Running buffer: Tris/Glycine/SDS

25 mM Tris

190 mM glycine

0.1% SDS

iii. Transfer buffer

25 mM Tris

190 mM glycine

20% methanol

For proteins larger than 80kD, SDS be included at a final concentration of 0.1%.

iv. **Ponceau S staining buffer** 0.2%(w/v) Ponceau S, 5% glacial acetic acid

v. Tris-buffered saline with Tween 20 (TBST) buffer

20 mM Tris, pH 7.5

150 mM NaCl

0.1% Tween 20

vi. **Blocking buffer**

3% bovine serum albumin (BSA) in TBST

Sample Preparation

To prepare sample for running on a gel, cells and tissue need to be lysed to release the protein of interest. This solubilizes the protein so they can migrate individually through a separating gel.

Tissue sample preparation

Animals are dissected and right and Left kidney was isolated and washed in ice cold Tris buffered saline (TBS). Tissue is homogenized by using RIPA(Radioimmunoprecipitation)

Lysis buffer (Sc-24948). Per gram tissue 3ml RIPA buffered is used. Homogenate is centrifuged for 20min at 12000 rpm at 4^{O} C in a microcentrifuge. Gently removed the tubes from the centrifuge and placed on ice, aspirated the supernatant and place in a fresh tube kept on ice, and stored at -20^{O}C for further use.

RIPA buffer has strong denaturing capabilities. This high quality product includes protease inhibitors, making it ready for use in mammalian cell and tissue lysis.

Composition: Four (4) vials

VIAL 1: 1x Lysis Buffer: 1x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide.

VIAL 2: PMSF (Phenyl methylene sulfonyl fluoride) in DMSO (Dimethyl sulfoxide).

VIAL 3: Protease inhibitor cocktail in DMSO.

VIAL 4: Sodium orthovanadate in water.

Usage: Combine 10 µl PMSF solution, 10 µl sodium orthovanadate solution and 10–20 µl protease inhibitor cocktail solution per ml of 1x RIPA Lysis buffer to prepare complete RIPA.

Use 3 ml complete RIPA per gram of tissue.

Determination of protein in serum and tissue homogenate

Protein concentration was estimated by Bovine Serum Albumin (BCA) assay in serum and kidney tissue homogenate. Once the protein concentration of each sample is determined. It can be stored at -20º C or -80º C for further use. 20ug of protein in each sample should be loaded on a gel.

Procedure

1Taken 20µg of protein from each serum and tissue homogenate sample and added an equal volume of 2x Laemmli sample buffer.

2. Boiled each sample in sample buffer at 95°C for 5min.

Protein separation by gel electrophoresis

1. Loaded equal amounts of protein (20µg) into the wells of a midi (13.3x8.7cm) format SDS- PAGE gel, along with molecular weight markers.
2. Run the gel for 5 min at 50V.
3. Increased the voltage to 100–150V to finish the run in about 1hr.

   We used in our laboratory voltage of 120V for one hour
4. Gel percentage selection depends on the size of the protein of interest. A 4–20% gradient gel separates proteins of all sizes very well.

Transferring the protein from the gel to the membrane

1. Placed the gel in1x transfer buffer for10–15min.
2. Assembled the transfer sandwich and make sure no air bubbles are trapped in the sandwich. The blot should be on the cathode and the gel on the anode.
3. Place the cassette in the transfer tank and place an ice block in the tank.
5. Transfer overnight in a cold room at a constant current of 10mA.

Note: Transfer can also be done at 100 V for 30 min–2 hr, but the method needs to be optimized for proteins of different sizes.

We transferred by using 100 V for 90 minutes in our laboratory.

Antibody incubation

1. Briefly rinsed the blot in water and stain it with Ponceau S solution to check the

1. transfer quality.

2. Rinsed off the Ponceau S stain with three washes with TBST.

3. Blocked in 3% BSA in TBST at room temperature for1 hr.

4. Incubate overnight in the respective primary antibody solution against the target protein at 4°C.

5. Note: The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. Primary antibody may be applied to the blot for 1–3 hr at room temperature depending on antibody quality and performance.
6. We incubated primary antibodies overnight, and kept the membrane at 4^{O} C on a rotator.
6. Rinsed the blot 5 times for 5 min with TBST.
7. Incubated in the HRP-conjugated secondary antibody solution for 1 hr at room temperature.
8. Rinsed the blot 5 times for 5 min with TBST.

Imaging and data analysis

1. Applied the chemiluminescent substrate to the blot according to the manufacturer's recommendation.
2. Captured the chemiluminescent signals using a CCD camera-based imager.
3. Used image analysis software to read the band intensity of the target proteins.

4.8 The sacrifice of animals and collection of tissues:

Following the collection of blood, After 28 days of treatment, the animals were killed by an overdose of ketamine (150 mg/kg, ip). Rats were dissected with care. Kidney tissue and thoracic aorta were separated. The kidney was immediately weighed individually. For each kidney, the organosomatic index was determined.

Organosomatic index= Kidney weight/Body weight x 100

For additional histopathological examination (HPE), some of the tissues were kept in 10% neutral buffered formalin while the remainder was kept at -20^{o} C for tissue homogenate preparation.

4.9: Histopathological examination:

The thoracic aorta and kidney tissue were preserved in 10% neutral buffered formalin, embedded in paraffin blocks, cut into sections with a microtome (0.7 thickness), and then stained with haematoxylin and eosin for histological analysis.

4.10: Statistical Analysis :

SPSS 16.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. The presentation of all the parameters is as Mean ±SD. A post hoc test was used to identify the significant difference between groups after one-way analysis of variance (ANOVA) to assess the statistical significance of data across several groups. For the examination of data within groups, paired t-tests were used. Statistical significance was defined as a p-value of 0.05 or lower.

4.11: Ethical Clearance:

The study was approved by the Institutional animal ethical committee

Ref No: (BLDE/BPC/641/2016-2017) dated 21.10.2016. After changing title: Ref No (BLDE(DU)/REG/R&D/RGC/2019-20/937) dated: 15/July/2019.

CHAPTER V
RESULTS

5.1 GRAVIMETRY

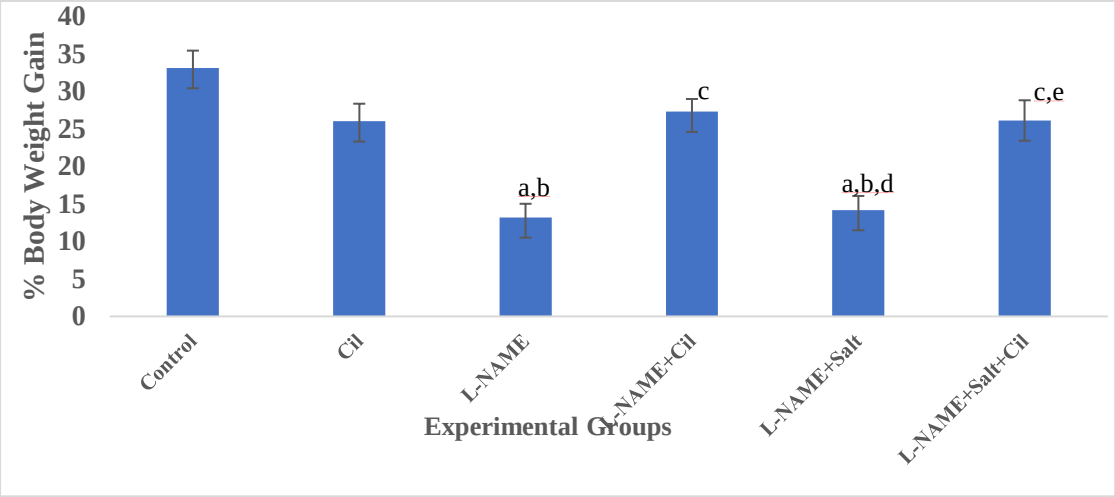
5.1 GRAVIMETRY

5.1.1: Comparison of Body Weight Gain among groups

Body weight (g) (n= 6)	Group 1 Control	Group 2 Cilnidipine	Group 3 L-NAME	Group 4 L-NAME + Cil	Group 5 L-NAME + Salt	Group 6 L-NAME + Salt +Cil	F value	p-Value
1st day	206.66 ± 3.55	205.16 ± 3.92	209.83± 6.46	207.5 ± 1.87	205.83 ± 4.91	209.5± 3,72	1.184	0.34
29th day	276.83 ± 4.95	258.5 ±2.66[a]	237.5 ± 6.28[a,b]	264.16 ± 2.85[c]	235 ± 3.74[a,b,d]	264.16±2.31[a, c,e]	98.98	0.001 *
% body weight gain	33.12 ± 2.32	26.02±2.34[a]	13.20 ± 1.82[a,b]	27.81±1.84[c]	14.19 ±1.87[a,b,d]	26.12±2.70[a,c,e]	78.95	0.001 *

Table 5.1.1: Comparison of body weight gain among groups (n=6 in each group)

The values are shown as mean ± SD. Following a one-way ANOVA, a Post Hoc Tukey's test is used. Significant differences between groups are indicated by the superscripts a, b, c, d, and e. Comparison with group 1 is depicted as a, group 2 depicted as b, group 3 is depicted as c, group 4 is depicted as d, group 5 is depicted as e. * Statistically significant t test, p≤0.05. Cil-Cilnidipine.



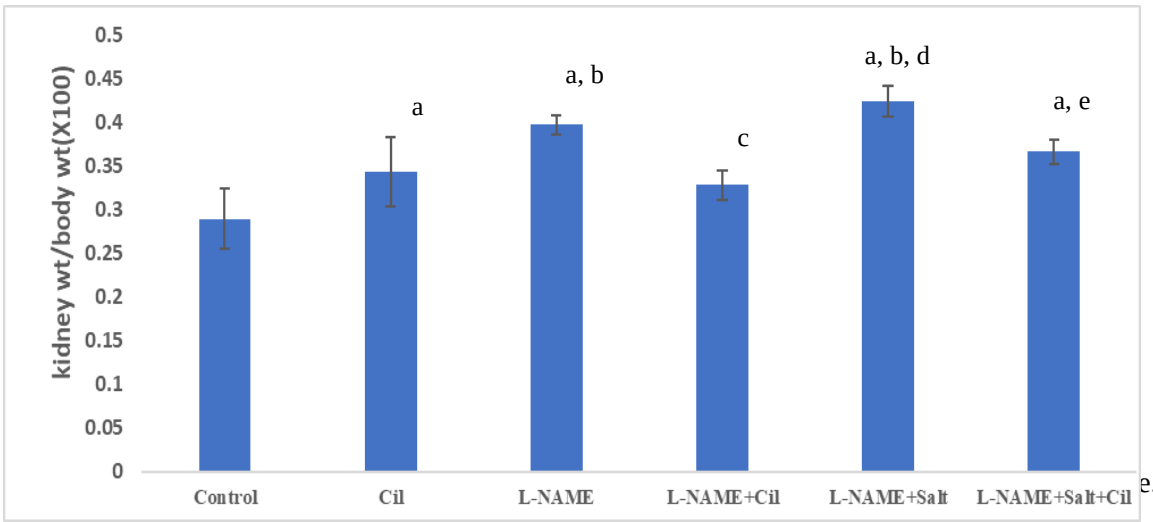
Superscript a, b, c, d, e indicates significant difference between groups. a depicts comparison with group 1, b depicts comparison with group 2, c depicts comparison with group 3, d depicts comparison with group 4. e depicts comparison with group5. *p≤0.05 was considered statistically significant. Cil-cilnidipine

Figure 5.1.1 Comparison of Percentage of Body Weight Gain among groups

Table 5.1.1 depicts the body weights of rats, measured before (day1) and after intervention (day 28). At the start of the experiment, the body weights of the rats in each group were matched. Between groups, there were no appreciable differences in initial body weight. The mean body weight of the rats in all groups significantly increased after 28 days, as shown by the paired t-test. Significant disparities in final body weight between groups were found using one-way ANOVA, showing that the increase in body weight was not uniform. Further when percentage of body weight gain is calculated there was less increase in percentage body weight (**Figure 5.1.1**) in L-NAME and L-NAME+ Salt treated rats compared to control group. A 33.12% increase in body weight was observed in the control group after 4 weeks. The rate of weight increase was less (13.2%) in the L-NAME group compared to that in the control group, which was significantly prevented by treatment with cilnidipine (27.81%).

5.1.2 Organosomatic Index

Figure 5.1.2.A. depicts the renal somatic index of left kidney of rats of all the experimental groups. We observed there is increased in weight of kidney in L-NAME and L-NAME+ Salt treated rats.

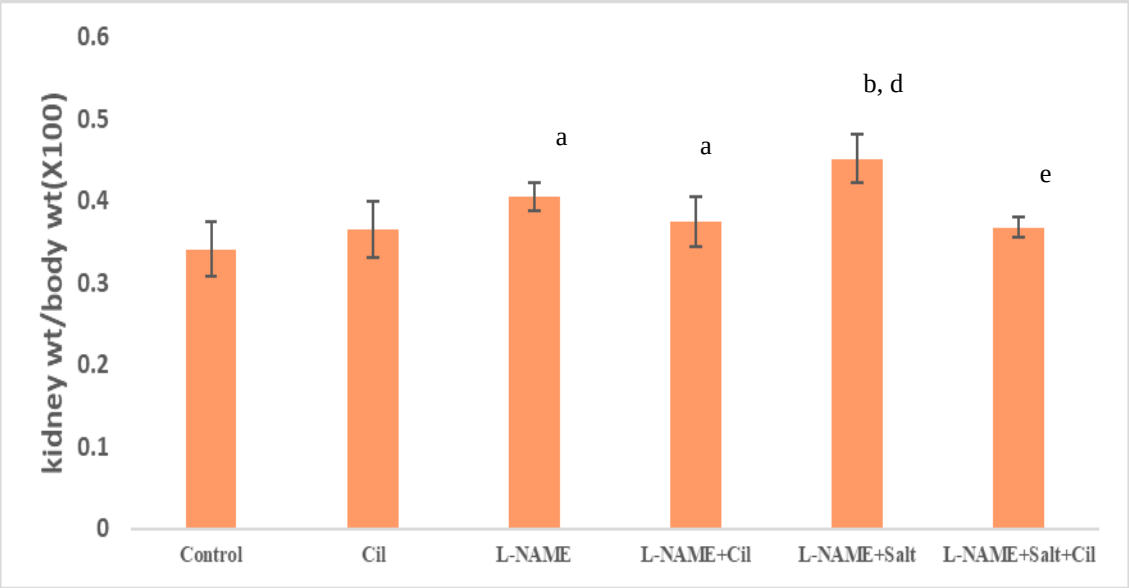


e.

Comparison with group 1 is shown as a, group 2 is shown as b, group 3 is shown as c, and group 4 is shown as d. Comparison with group 5 is shown as e. * $p<0.05$ was considered statistically significant. Cil-cilnidipine

Fig. 5.1.2.A: Renal somatic Index Left kidney

Figure 5.1.2.B depicts renal somatic index of right kidney of rats of all groups**.** We found there is increased in weight of right kidney in L-NAME and L-NAME + Salt treated rats.



Significant differences across groups are denoted by superscript letters a to e. a shows a comparison to group 1, b shows a comparison to group 2, c shows a comparison to group 3, and d shows a comparison to group 4. e shows a comparison to group 5. *p<0.05 was considered statistically significant. Cil-cilnidipine

Fig. 5.1.2 B: Renal somatic Index Right kidney

Fig 5.1.2.A and B, depicts renal somatic index for both left and right Kidney respectively. We observed there is a significant increase in weight of right and left kidney in L-NAME and L-NAME+ Salt treated rats compare to control group. Treatment with cilnidipine there is less increased in kidney weight compare to L-NAME treated rats.

5.2 HEMOGRAM

5.2 HEMOGRAM

Table 5.2: Comparison of haematological parameters among groups

Groups		Hb-gm/dl	RBC (million/cumm)	WBC (thousands/ cumm)	HCT (%)	Platelet count (lakhs/cumm)
Control		18.01±0.39	8.66±0.33	9883±495	52.81±1.87	6.34±0.318
Cilnidipine		18.05±0.53	8.61±0.40	9850±372	52.80±1.47	6.06±0.32
L-NAME		17.9±0.35	8.41±0.23	9793 ±579	52.24±1.64	5.88±0.426
L-NAME + Cil		17.70±0.4	8.21±0.44	9765 ±375	51.28±2.93	6.06±0.332
L-NAME + Salt		17.60±0.35	8.11±0.27	9428 ±329	50.66±0.4	6.09±0.26
L-NAME +Salt +Cil		17.44±0.43	8.21±0.23	9765 ±556	50.51±0.54	6.15±0.34
ANOVA	F value	1.977	2.886	0.752	2.238	1.166
	p value	0.111	0.03	0.592	0.076	0.349

Values are expressed as Mean ±SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests. RBC, red blood cells; WBC, wight blood cells; Hb, haemoglobin.

Table 5.2 depicts haematological parameters of rats of all experimental groups. We didn't observe any significant variation in haematological parameters among group except RBC count in L-NAME + Salt group is decreased compare to control group.

5.3 RESPIRATORY RATE

5.3 RESPIRATORY RATE (Pneumogram)

Figure 5.3 shows a comparison or respiratory rate among groups. Respirate rate of rats of all the group is within normal range (15.33±1.01-15.66±1.03). There is no significant variation among groups.

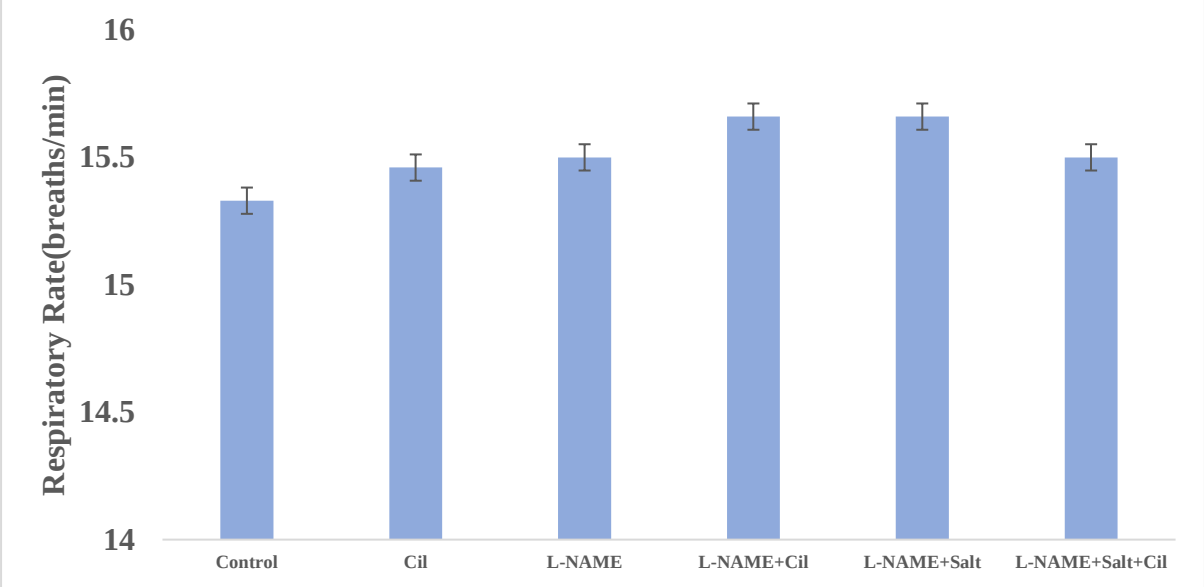


Fig. 5.3 Comparison of respiratory rate among groups (n=6 in each group)

5.4 CARDIAC ELECTROPHYSIOLOGY

5.4.1 HEART RATE

Figure 5.4.1 shows a comparison of small animal heart rate among all six groups of rats post intervention recording. We observed a decrease in HR in L-NAME and L-NAME+ Salt treated rats even though it is statistically non significant.

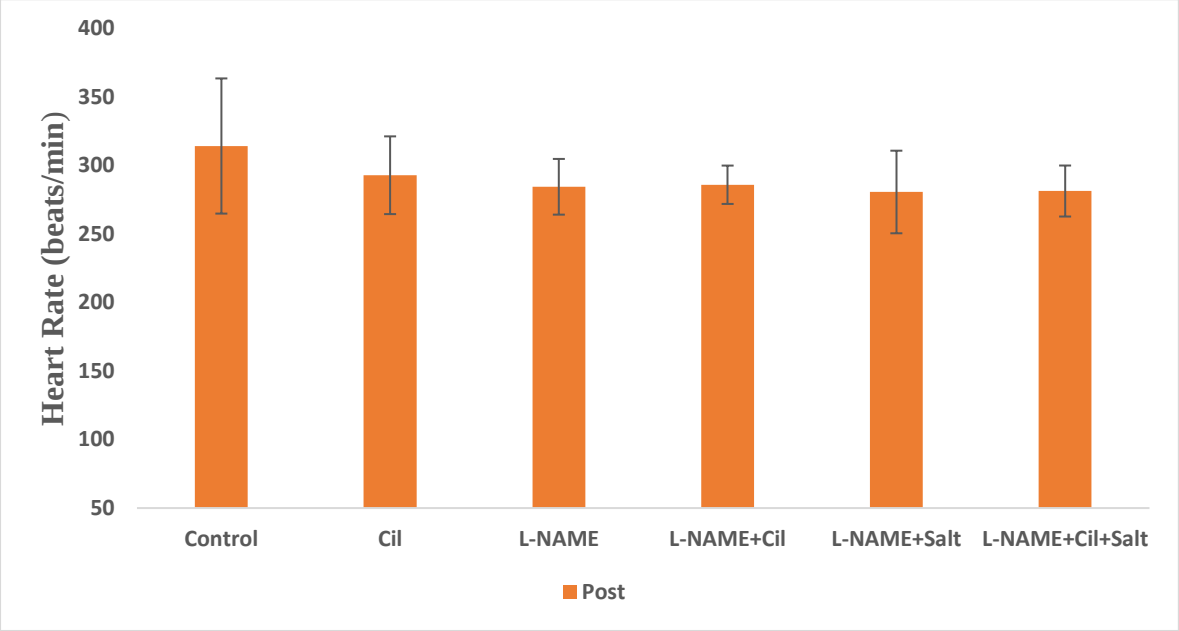
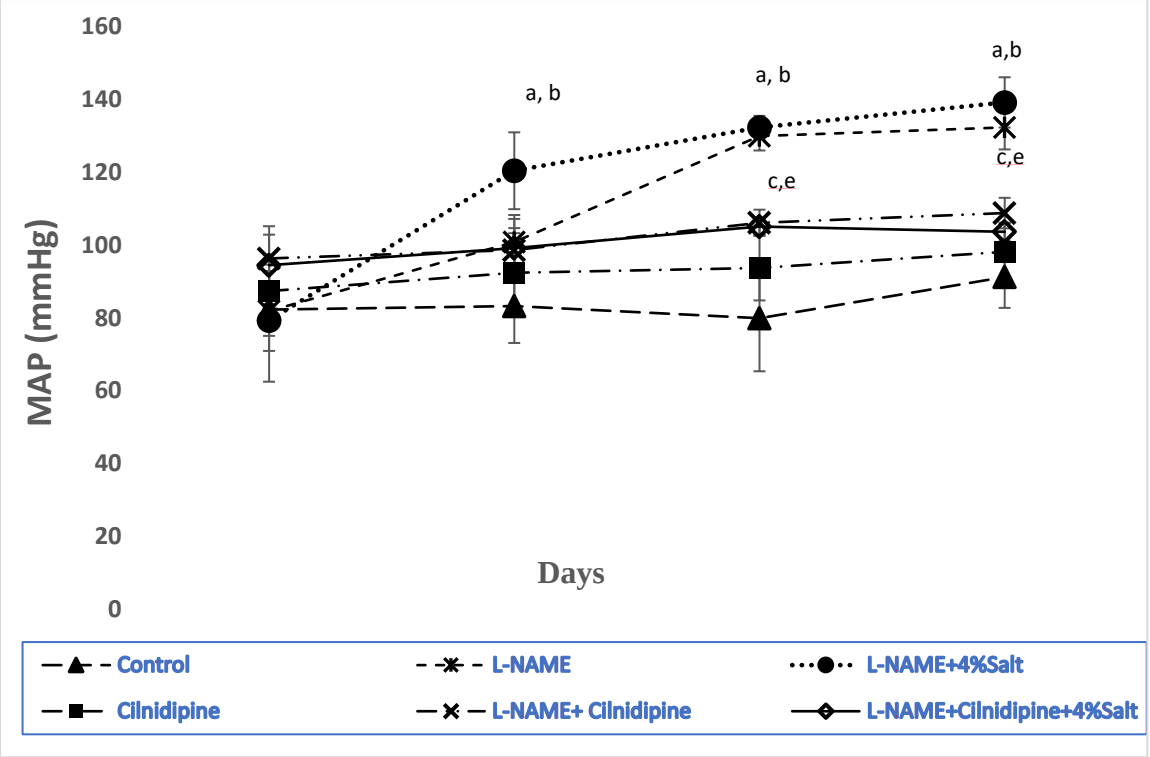


Fig. 5.4.1: Comparison of Heart Rate among groups

5.4.2 MEAN ARTERIAL BLOOD PRESSURE (MAP)

Figure 5.4.2 shows how the mean arterial pressure in each of the experimental groups was compared statistically. L-NAME (40 mg/kg) was given orally once per day, and this gradually increased MAP. From the ninth day on, we discovered a significant increase in MAP in the rats treated with L-NAME and L-NAME+ Salt (groups 3 and 5) as compared to the control group. We found that rats treated with cilnidipine (groups 4 and 6) had lower mean arterial pressure (MAP) values compare to rats treated with L-NAME and L-NAME+ Salt.



Significant differences between groups are indicated by the superscripts a, b, c, d, and e. Comparisons with groups 1 to 5 are shown as letters a, b, c, d, and e, respectively. *p≤0.05 was considered statistically significant. MAP-mean arterial pressure.

Fig. 5.4.2: Comparison of MAP among groups

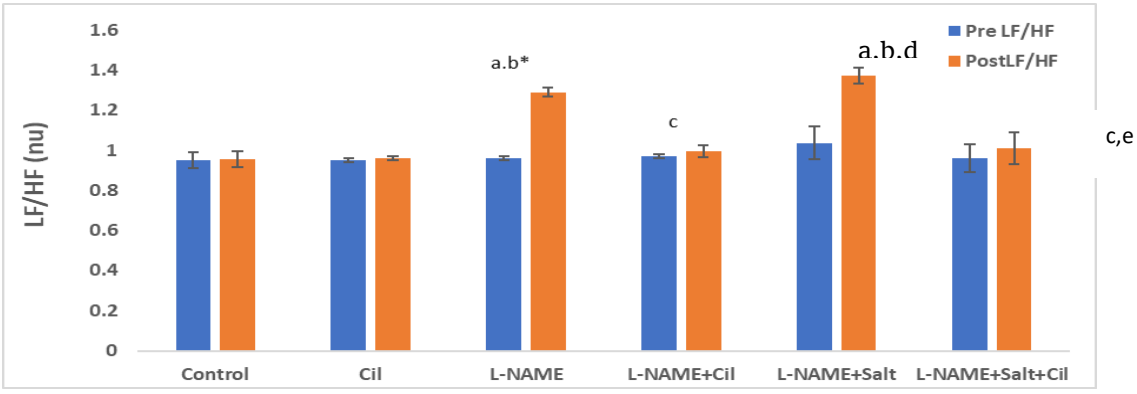
5.4.3 HEART RATE VARIABILITY MEASURES (HRV)

Table 5.4.3 shows heart rate variability (HRV) data in frequency domain for all experimental groups. When compared to control and cilnidipine treatment, rats treated to L-NAME and L-NAME+ Salt showed increased sympathetic activity (LF), decreased parasympathetic activity (HF), and a shift in the sympathovagal balance (LF/HF ratio) towards greater sympathetic activity.

Table 5.4.3 Comparison of Heart Rate Variability (HRV) parameters among groups

Parameters (nu)	Control	Cil	L-NAME	L-NAME + Cil	L-NAME +Salt	L-NAME +Salt+ Cil	ANOVA F	ANOVA P
Pre LF	48.55±1.6	47.73 ±3.03	49.17±2.09	50.13±1.6	52.39±2.25	50.11±1.81	2.31	0.087
Post LF	49.37±4.01	49.45 ±1.86	61.40±1.73[a,b]	54.14±3.54	68.62±2.63 [a,b,d]	54.13±2.48[a,b,e]	28.04	0.000 *
Pre HF	51.47±2.27	50.67 ±2.9	50.67±1.36	51.27±2.37	51.64±3.33	52.16±3.37	0.184	0.965
Post HF	50.2±3.4	51.4± 2.38	47.26±2.28[a.b]	54.35±4.58	38.29±3.02 [a.b.c.d]	53.38±3.64[c,e]	12.66	0.000 *
Pre LF/HF	0.95±0.07	0.94± 0.12	0.96±0.025	0.97±0.02	1.018±0.09	0.96±0.09	0.715	0.620
Post LF/HF	0.98±0.08	0.96± 0.016	1.29±0.03[a ,b]	0.99±0.04[c]	1.79±0.12[a,b,c,d]	1.01±0.10 [c,e]	70.29	0.000 *

The values are shown as Mean SD. Following a one-way ANOVA, Post Hoc Tukey's multiple comparison tests was used. Significant differences between groups are indicated by the superscripts a, b, c, d, and e. The comparisons with groups 1, 2, 3,4, and 5 was shown as letters a, b, c, d and e respectively. * Statistically significant *p≤0.05. Cil- cilnidipine. LF-low frequency, HF-high frequency.



Superscript a, b, c, d, e indicates significant difference between groups. a depicts comparison with group 1, b depicts comparison with group 2, c depicts comparison with group 3, d depicts comparison with group4, e depicts comparison with group 5. * Statistically significant paired t test. *p≤0.05. Cil-cilnidipine. LF-low frequency, HF-high frequency.

Fig. 5.4.3: Comparison of LF/HF among experimental groups

5.5 BIOCHEMICAL PARAMETERS

5.5.1 OXIDATIVE STRESS MARKERS (SERUM AND KIDNEY TISSUE MALONDIALDEHYDE LEVELS) (MDA)

Table 5.5.1 Shows comparison of serum MDA and Kidney Tissue MDA levels**.** We found there is significant increase in MDA levels in serum and kidney tissue in L-NAME and L-NAME +Salt treated rats compare to control. Treatment with cilnidipine there is significant decrease in MDA levels.

Table 5.5.1: Comparison of Serum MDA and Kidney tissue MDA levels among groups

Parameters	**Control**	**Cil**	**L-NAME**	**L-NAME + Cil**	**L-NAME +Salt**	**L-NAME + Salt + Cil**	**ANOVA**	
							F value	**P value**
Serum MDA µmoles/L	0.86± 0.05	0.90 ± 0.34	1.58±0.05[a,b]	1.21±0.22[a,b,c]	1.78±0.05[a,b,c,d]	1.23±0.1[a,b,c,e]	64.01	0.001*
Tissue MDA µmoles/gm	24.67 ± 0.52	24.57± 0.76	31.25±0.45[a,b]	28.96±0.59[a,b,c]	39.01±0.79[a,b,c,d]	30.95±0.53[a,b,e]	439.22	0.001*

The values are shown as Mean ± SD. Following a one-way ANOVA, Post Hoc Tukey's multiple comparison tests are used. Significant differences between groups are indicated by the superscripts a, b, c, d, and e. The comparisons shown with groups 1, 2, 3, 4 and 5 are with letters a, b, c, d, e respectively. * Statistically significant, $p \leq 0.05$. Cil-cilnidipine. MDA-Malondialdehyde.

5.5.2: SERUM UREA AND CREATININE

Table 5.5.2. depicts serum urea and creatinine levels among groups. We observed there is significant increase in serum urea and creatinine in L-NAME and L-NAME +Salt treated rats. With cilnidipine treatment there is significant decrease in serum urea and creatinine levels.

Table 5.5.2: Comparison of Serum urea and creatinine levels among groups

Parameters	Control	Cil	L-NAME	L-NAME +Cil	L-NAME +Salt	L-NAME +Salt +Cil	ANOVA F value	ANOVA P Value
Serum urea mg/dl	41.37± 2.72	43.13± 3.26	44.01± 2.92	41.00± 3.34	52.35± 3.08 a,b,c,d	47.64±4.6 d	9.927	0.0001*
Serum Creatinine in mg/ml	0.40±0 .03	0.52±0. 04 a	0.611± 0.03 a,b	0.40±0. 03 b,c	0.50±0. 03 a,c,d	0.50±0.05 c,d	23.69	0.0001*

The values are shown as Mean± SD. One-way ANOVA is used to compare the groups, and then a Post Hoc multiple test is used. Significant comparisons with groups 1, 2, 3, 4, and 5 are denoted by the superscripts a, b, c, d, and e, respectively. *p≤0.05 consider as statistically significant. Cil-cilnidipine.

5.5.3 PROTEINURIA AND CREATININE CLEARANCE

Table 5.5.3 Show the 24hr urinary protein concentration and creatinine clearance in experimental group. We found that rats treated with L-NAME & L-NAME + Salt had significantly higher 24-hour urine protein excretion than control rats. Protein excretion is significantly reduced after using cilnidipine.

We also found there is significant decrease in creatinine clearance in hypertensive rats (group 3 and 5) compare to control rats. Treatment with cilnidipine (group 4 & 6) there is significant increase in creatine clearance.

Table 5.5.3: Comparison of 24hr urinary protein & creatinine clearance levels among groups

Parameters	Control	Cil	L-NAME	L-NAME + Cil	L-NAME + Salt	L-NAME + Salt + Cil	ANOVA	
							F value	P value
24 hr Urinary protein in mg/ml	1.56±0.043	1.66±0.098	4.58±0.28[a,b]	3.38±0.23[a.b,c]	4.65±0.29[a,b,d,]	3.08±0.17[a,b,c,e]	251.79	0.0001*
Creatinine Clearance in ml /min /gm of kidney tissue	0.078±0.0043	0.079±0.003	0.06±0.0018[a,b]	0.077±0.0026[c]	0.063±0.0025[a,b,d]	0.076±0.03[c,e]	44.421	0.0001*

The values are shown as Mean± SD. One-way ANOVA is used to compare the groups, and then a Post Hoc multiple test is used. Significant comparisons with groups 1, 2, 3, 4, and 5 are indicated by the superscripts a, b, c, d, and e, respectively. *p≤0.05 consider statistically significant. Cil-cilnidipine

5.5.4 NITROSATIVE STRESS MARKERS (SERUM AND KIDNEY TISSUE NITRIC OXIDE (NO) LEVELS)

Table 5.5.4 Shows comparison of serum and kidney tissue NO levels**.** We observed there is significant decrease in NO levels in serum and kidney tissue in L-NAME & L-NAME + Salt treated rats compare to control. With cilnidipine there is significant increase in NO levels.

Table: 5.5.4: Comparison of serum and kidney tissue nitric oxide (NO) levels among groups

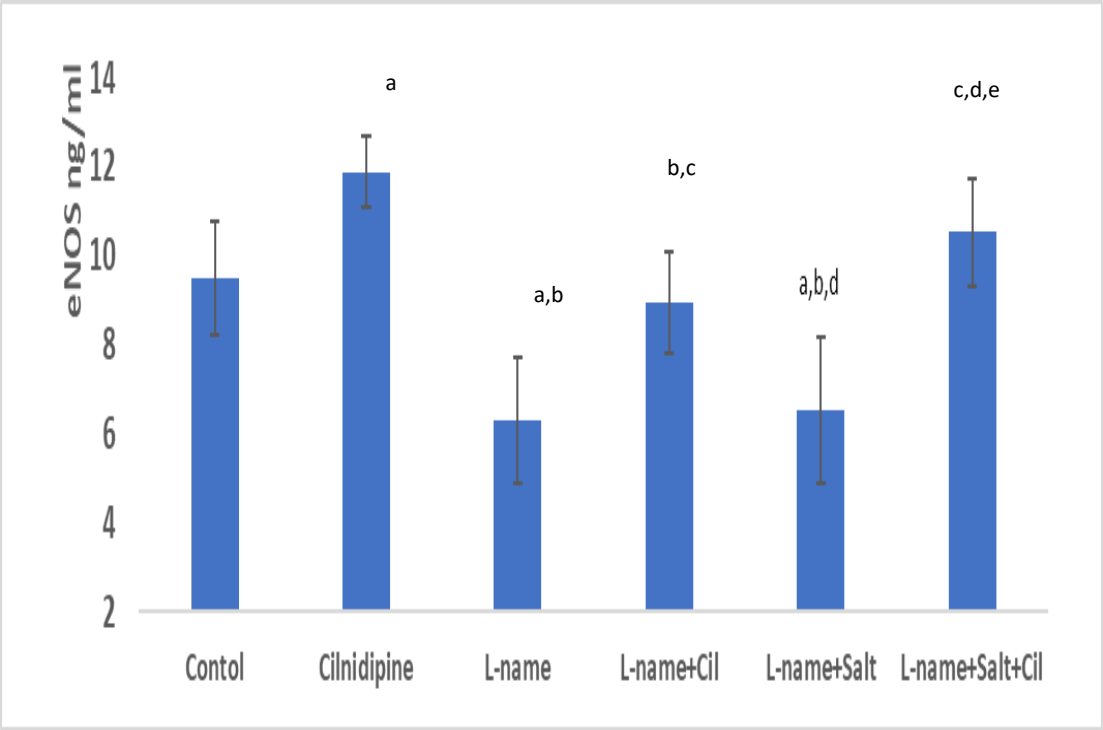
Parameters	Control	Cilnidipine	L-NAME	L-NAME + Cil	L-NAME + Salt	L-NAME + Salt +Cil	ANOVA	
							F value	P value
Serum NO µmol/L	7.46±1.0	7.09±0.43	4.21±0.66 a,b	7.01±0.61 c	4.26±0.70 a,b,d	7.35±0.62 c,e	29.85	0.001*
Tissue NO µmol/gm	0.55±0.06	0.52±0.07	0.42±0.06 a,b	0.51±0.06 c	0.41±0.07 a,b,d	0.48±0.07 c,e	4.253	0.005*

The values are shown as Mean±SD. Following a one-way ANOVA, Post Hoc Tukey's multiple comparison tests are used. Significant differences between groups are indicated by the superscripts a, b, c, d, and e. The comparisons shown in letters a, b, c, d and e are with groups 1, 2, 3, 4, and 5 respectively. Statistically significant *p≤0.05. Cil- cilnidipine, NO- Nitric oxide,

5.6 QUANTITATIVE ESTIMATION OF MOLECULAR MARKERS BY ELISA

5.6.1 SERUM NOS3 (eNOS)

Fig 5.6.1 depicts serum eNOS levels among group. We found there is significant decrease in eNOS protein expression in L-NAME and L-NAME +Salt treated rats compare to control. Treatment with cilnidipine there is significant increase in eNOS protein expression compare to L-NAME and Salt treated rats.



Superscript a, b, c, d, e indicates significant difference between groups. a depicts comparison with group 1, b depicts comparison with group 2, c depicts comparison with group 3, d depicts comparison with group 4, e depicts comparison with group 5. Statistically significant *p≤0.05, Cil-cilnidipine. eNOS- endothelial nitric oxide synthase.

Fig. 5.6.1: Comparison of eNOS protein among groups

5.6.2 SERUM AND URINARY ANGIOTENSIN II (Ang II)

Table 5.6.2 depicts quantitative estimation of serum and urinary Angiotensin II levels. We observed significant increase in serum and Kidney tissue Ang II level in L-NAME and L-NAME + Salt treated rats compare to control. With cilnidipine supplementation there is significant decrease in serum and kidney tissue Ang II levels.

Table 5.6.2: Comparison of Serum and urinary Ang II levels among groups

Parameters	Control	Cil	L-NAME	L-NAME + Cil	L-NAME +Salt	L-NAME+ Salt + Cil	ANOVA	
							F value	P Value
Serum Ang II in pg/ml	152.53±25.2	161.12 ±42.59	321.61±25.86[a,b]	245.45±14.62[a,b,c,e]	385.47 ±33.40[a,b,c,d]	257.88±21.35[a,b,c,e]	49.70	0.0001*
Urinary Ang II in pg/ml	329.74±7.13	321.99 ±4.58[a,c]	450.84±10.30[a, b]	410.49±6.8[a.b,c]	457.42 ±9.28[a,b,d]	433.77±11.2[a,b,c,d,e]	166.12	0.0001*

The values are shown as Mean ± SD. One-way ANOVA is used to compare the groups, and then a Post Hoc multiple test is used. Significant comparisons with groups 1, 2, 3, 4, and 5 are indicated by the superscript letters a, b, c, d, and e, respectively.*$p<0.05$ is considered as statistically significant. Ang II-Angiotensin II, Cil-cilnidipine.

5.7 RELATIVE EXPRESSION OF MOLECULAR MARKERS BY WESTERN BLOTTING

5.7.1: Serum and Kidney tissue vascular nitric oxide synthase (eNOS/NOS3) protein expression

Figure 5.7.1.A. shows relative expression of Serum eNOS protein by Western blotting. We observed less expression in L-NAME and L-NAME +Salt treated rats. There is relatively more expression with cilnidipine treatment.

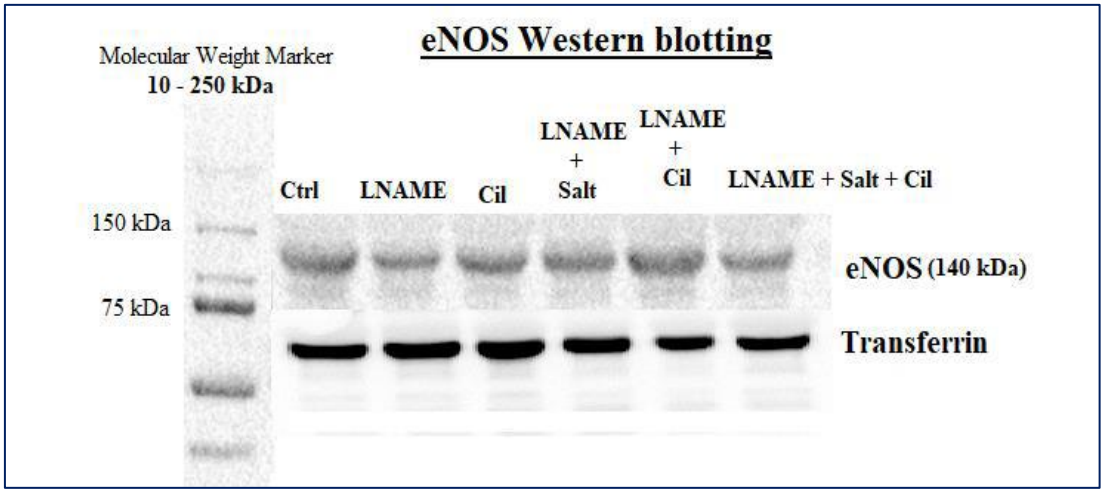


Fig. 5.7.1. A: Serum NOS3 (eNOS) protein expression among groups

Figure 5.7.1 B. depicts NOS3 protein expression in Kidney tissue. We observed there is relatively less expression of NOS3 protein in L-NAME and L-NAME +Salt treated rats compare to control. With cilnidipine there is relative increase in expression of NOS3 protein.

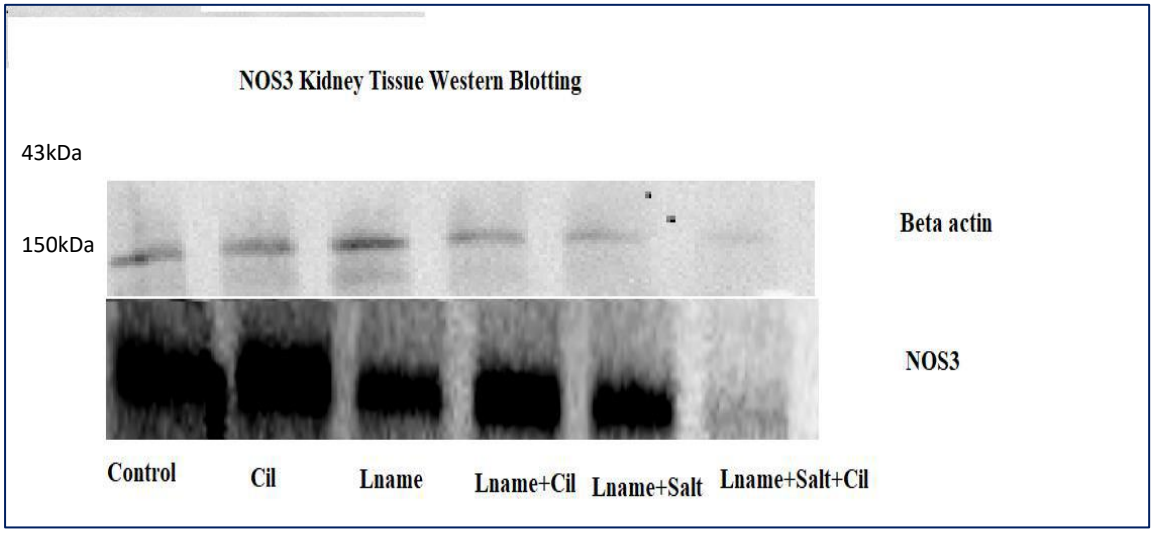


Fig 5.7.1. B: Relative expression of NOS3 protein in kidney tissue among groups

5.7.2: Serum and Kidney Tissue Angiotensin converting enzyme (ACE) protein expression

Figure 5.7.2. A. depicts ACE protein expression in serum by Western Blotting. We observed there is relatively more expression of ACE protein in L-NAME and L-NAME +Salt treated rats compare to control. There is less expression of ACE protein in cilnidipine supplemented rats.

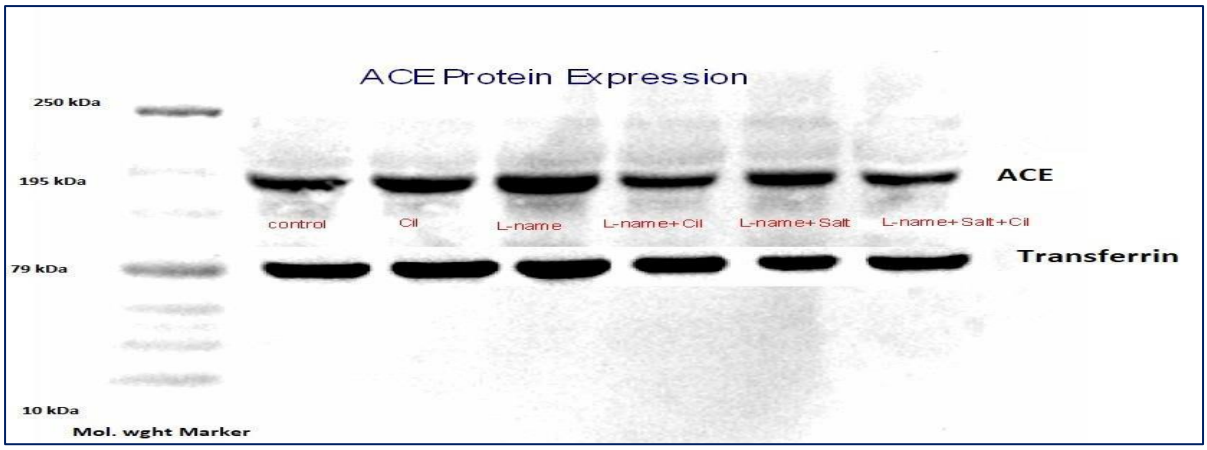


Fig. 5.7.2 A: Serum ACE protein expression among groups

Figure 5.7.2 B. Shows ACE protein expression in Kidney tissue among experimental groups. We observed there is more expression of ACE protein in L-NAME and L-NAME +Salt treated rats compare to control. With cilnidipine supplementation there is relatively less expression of ACE protein.

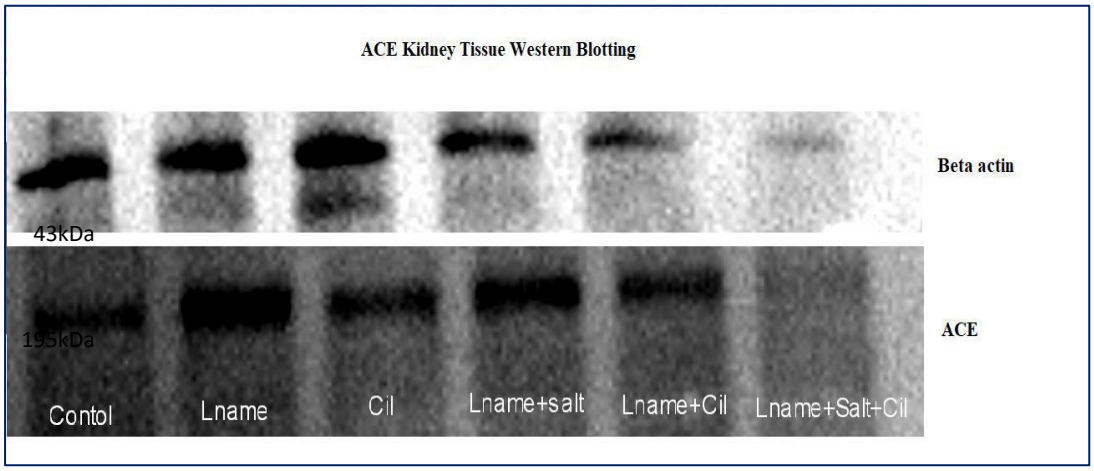


Fig 5.7.2 B: Relative expression of ACE protein in kidney tissue among groups

5.7.3: Serum and Kidney Tissue vascular endothelial growth factor (VEGF) protein expression

Figure 5.7.3.A. depicts VEGF protein expression in serum by Western Blotting**.** We observed relatively increase expression of VEGF in L-NAME and L-NAME +Salt treated rats compare to control. Treatment with Cilnidipine there is decrease in expression of VEGF.

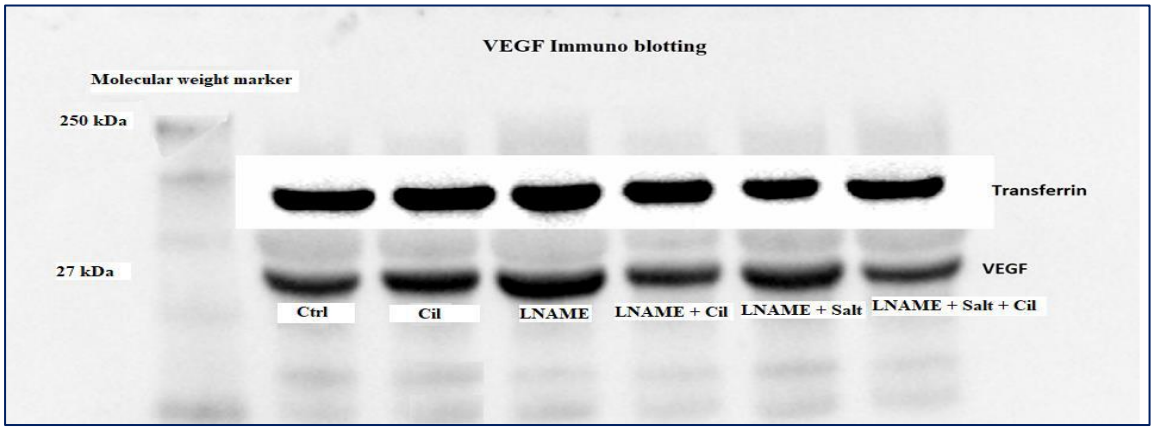


Fig. 5.7.3 A: Serum VEGF protein expression among groups

Figure 5.7.3.B. Shows VEGF protein expression in kidney tissue in all experimental groups. We found there is increase in VEGF levels in kidney tissue in L-NAME and L-NAME + Salt treated rats compare to control rats. Simultaneous treatment with cilnidipine there is relative decrease in VEGF level.

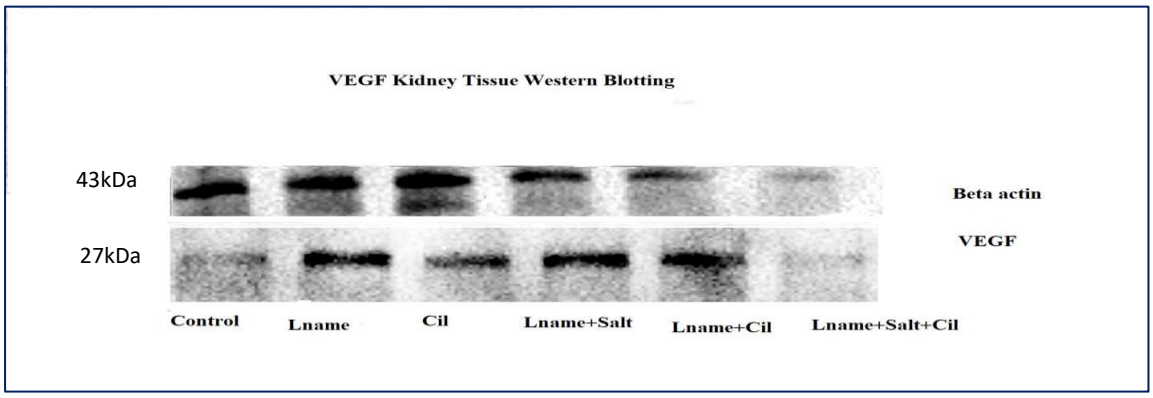


Fig 5.7.3 B: Relative expression of VEGF protein in kidney tissue among groups

5.8 HISTOPATHOLOGICAL EXAMINATION

Figure 5.8.1. Shows histopathological examination of elastic artery

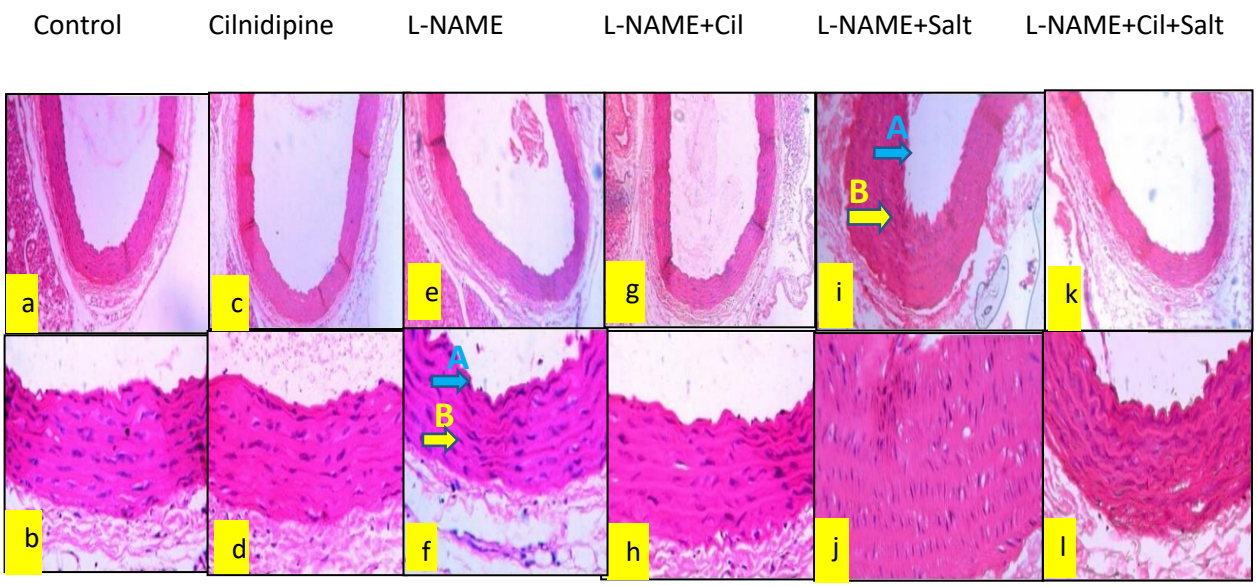


Fig. 5.8.1: Histopathological examination of aorta

(a) Group1(control) (x10); (b) group1 (control) (x40); (c) group2 (Cil) (x10);(d)

(b) group2 (Cil) (x40); (e) group3 (L-NAME) (x10); (f) group3(L-NAME) (x40); (g) group4 (L-NAME+Cil) (x10); (h) group4 (L-NAME+Cil)(x40);(i)group5(L-NAME+Salt)(x10); (j)group5(L-NAME+Salt) (x40); (k)group6 (L-NAME + Salt +Cil) (x10); (l) group6 (L-NAME + Salt +Cil) (x40); Arrow A- **Mild thickening of tunica intima.** Arrow B- The tunica media is consisting of **hyperplastic smooth muscle cells** with elastic fibres. Cil-cilnidipine

Figure 5.8.2. depicts histological examination of kidney tissue among experimental groups

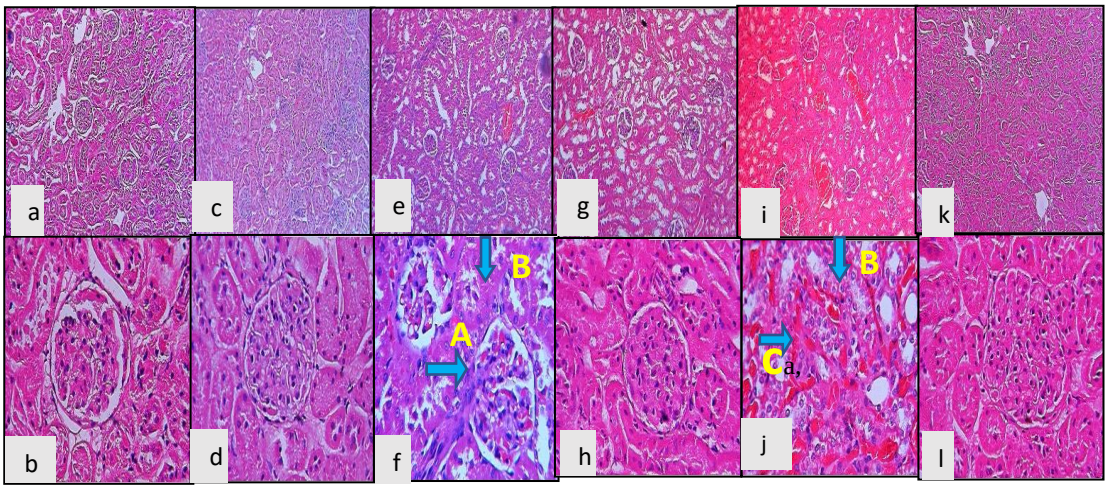


Fig 14: Histopathological examination of kidney tissue among groups

Fig 5.8.2: Histopathological examination of kidney tissue among groups

(a)Group1(control) (x10); (b) group1 (control) (x40); (c) group2 (Cil) (x10); (d) group2 (Cil) (x40); (e) group3 (L-NAME) (x10); (f) group3(L-NAME) (x40); (g) group4 (L-NAME+Cil) (x10); (h) group4 (L-NAME+Cil)(x40);(i)group5(L-NAME+Salt)(x10); (j)group5(L-NAME+Salt) (x40); (k)group6 (L-NAME + Salt +Cil) (x10); (l)group6 (L-NAME + Salt +Cil) (x40); **Arrow A- Glomeruli are hyper cellular with increased mesangial proliferation. Arrow B- Focal tubular epithelial hydropic degeneration. Arrow C –Interstitial congestion.** Cil-cilnidipine

CHAPTER VI
DISCUSION

6.1 GRAVIMETRY
6.2 HAEMOGRAM
6.3 RESPIRATORY RATE

6.1 GRAVIMETRY

6.1.1: Comparison of Body Weight Gain among groups

In our study weight gain was lower in L-NAME treated rats that is group 3 (L-NAME) and group 5 (L-NAME + Salt)) rats indicating disturbance of autonomic nervous balance and increased in sympathetic activity following L-NAME treatment. Interestingly simultaneous treatment with cilnidipine (group 4 and group 6) rats demonstrated a better % body weight gain when compared only L-NAME treated rats. This can be a result of the cilnidipine treatment. By inhibiting N-type calcium channels on sympathetic nerve terminals, cilnidipine decreases sympathetic neurotransmission and, as a result, sympathetic drive. (Takahara., 2009). Because of this function, cilnidipine may likely have an impact on sympathetic activity and, in turn, body weight. In our study also sympathetic inhibition with cilnidipine treatment might have caused the better percentage of body weight again compare to L-NAME treated hypertensive rats.

Increases in pro-inflammatory mediators leading to advancement of renal illness by L-NAME and high salt diet treatment, and angiotensin II are linked to decrease in percentage of weight gain in rats. Instead, by increasing the expression and activity of vital elements of the ubiquitin-proteasome proteolytic interaction of the 20S proteasome with 19S regulation, angiotensin II directly drives protein degradation in myocyte cultures. (Aritomi S et al., 2010). Similarly we also observed increased in serum and urinary angiotensin II levels might have contributed to less body weight gain in L-NAME treated hypertensive rats.

6.1.2: Renal somatic index of right and left kidney

We also observed there is significant increase in the weight of kidney in L-NAME and L-NAME+ Salt treated rats compare to control group.

In accordance with our study, Ji Hoon et al and Sahar Mohamed also observed renal hypertrophy in L-NAME treated rats (Ji Hoon et al., 2013; Sahar Mohamed Kamal.,2014).

Nitric oxide inhibition by L-NAME in our study might have caused the renal vasoconstriction, stimulation of renin angiotensin system and increased production of Ang II leading to hypertrophy of nephron and increased kidney weight.

A lack of NO in the kidney may have caused the renal artery to constrict, stimulating the synthesis of renin and angiotensin II, which in turn caused systemic vasoconstriction and hypertension (Ji Hoon et al., 2013). Nephron hypertrophy is likely to be caused by L-NAME-induced oxidative stress through an increase in angiotensin II synthesis, which is mediated by reactive oxygen species (ROS). Additionally, it may cause fibroblast growth, interstitial matrix deposition, and inflammatory cell infiltration, all of which contribute to progressive tubulointerstitial fibrosis (Sahar Mohamed Kamal.,2014).

6.2: HAEMOGRAM

We didn't observe any significant variation in hematological parameters among groups except RBC count in L-NAME + Salt group which is shown as decreased compare to control group.

6.3: RESPIRATORY RATE (Pneumogram)

Respirate rate of rats of all the group is within normal range (15.33±1.01-15.66±1.03). There is no significant variation among groups.

6.4 CARDIAC ELECTROPHYSIOLOGY

6.4: CARDIAC ELECTROPHYSIOLOGY

To determine the impact of the L/N dual calcium channel blocker cilnidipine on the cardiac hemodynamics of nitric oxide deficient hypertensive rats, electrophysiological parameters were recorded.

6.4.1 HEART RATE (HR)

In our study we observed a decrease in HR in L-NAME and L-NAME+ Cilnidipine treated rats compare to control rats. (vide chapter: 5.4; Figure: 5.4.)

In accordance with our study, Kobayashi et al (2000), Sadek SA et al (2015), Ji Hoon S et al(2013) also found decreased in HR in L-NAME treated hypertensive rats. (Kobayashi et al., 2000; Ji Hoon S et al., 2013; Sadek SA et al., 2015). One possibility of decrease HR may be because of baroreceptor sensitivity is altered as a consequence of the chronic increase in blood pressure. A second possibility is that nitric oxide produced by eNOS could play a direct role in the modulation of heart rate (Ji Hoon S et al, 2013).

We also found decrease in HR in cilnidipine treated rats compare to control. Similarly, Das A et al (2016) also found the decrease in HR with cilnidipine treatment. The likely cause of the cilnidipine reducing heart rate is the control of cardiac sympathetic overactivity by blocking N-type calcium channels (Das A et al., 2016). In spontaneously hypertensive rats (SHR) and individuals with essential hypertension, cilnidipine had a sustained antihypertensive impact without raising heart rate or circulating plasma norepinephrine concentrations. (Toba H et al., 2011). Lida T et al (2017) also observed decrease in HR with cilnidipine in dialysis patients. They propose that cilnidipine inhibit reflex tachycardia due to antihypertensive treatment by depressing sympathetic activity (Lida T et al, 2017).

6.4.2: MEAN ARTERIAL BLOOD PRESSURE (MAP)

Globally, hypertension is a major problem that ranks third in terms of reducing the number of years of life with a disability. More than 600 million individuals worldwide are affected by hypertension, which accounts for 13% of all global fatalities. By 2025, it is predicted that 29% of adults worldwide will have hypertension.

Chronic NO suppression using N^G-nitro-L-arginine methyl ester (L-NAME) may result in elevated blood pressure, increased regional vascular resistance, and kidney impairment from oxidative stress. The chronic inhibition of basal nitric oxide with an orally active nitric oxide synthase (NOS) inhibitor (L-NAME) is a particularly interesting model of hypertension because the main functions of nitric oxide are the regulation of vascular tone, inhibition of vascular smooth muscle cells proliferation, and platelet aggregation (Veerappan R, Malarvili T, 2019)

We developed L-NAME and L-NAME plus salt induced hypertensive rat model, first time in our Laboratory of Vascular Physiology and Medicine.

L-NAME (40 mg/kg) was given orally once per day, and this gradually increased MAP. From the ninth day onwards, we observed a significant increase in MAP in the L-NAME and L-NAME plus Salt treated groups compared to the control group (vide chapter: 5.4; Figure: 5.4.2).

In accordance with our study, Zhou X et al(2005), Sadek SA et al(2015), Ji Hoon S et al(2013), Veerappan R et at(2019), also developed L-NAME induced hypertension in rats, they also found the progressive increase in blood pressure in rats due to chronic administration of L-NAME. (Zhou X, Frohlich ED, 2005; Sadek SA et al., 2015; Ji Hoon S et al., 2013; Veerappan R et al, 2019).

L-NAME and Salt administration lead to an increase in MAP. A higher MAP results from a multiple factors. Three important elements were identified in our study as causes of greater MAP., crucial being decreased the bioavailability of NO (vide chapter: 5; table: 5.5.2) by inhibiting eNOS, we observed both quantitative and qualitative decrease in eNOS/NOS3 protein in serum as well as kidney tissue. (vide chapter: 5; Fig: 5.6.1and Fig 5.7.1 A,5.7.1 B).

Decrease over all nitric oxide causes dysfunction of endothelium (Calbet., 2003). NO is essential for maintaining arterial blood pressure because of its strong vasodilator effects. As a counter-regulatory agent, NO also inhibits the vasoconstrictor effects of endothelin, angiotensin II, and renal sympathetic nerve activity. Additionally, NO plays a part in pressure natriuresis. (Barton et al., 2003). Other factors might be increased sympathetic drive as depicted by Heart Rate Variability analysis (vide chapter:5.4; Table:5.4.3) and increased oxidative stress (vide chapter:5.5; Table:5.5.1)

In accordance with our study, other studies also observed that increase in MAP may be because of decreased bioavailability of nitric oxide NO (Zhou X, Frohlich ED, 2005), increased sympathetic drive (Bagali S, Das KK, 2021) and increased oxidative stress (Kopkan L et al, 2010).

When compared to L-NAME & L-NAME+ Salt treated rats (groups 4 and 6), we saw a decrease in the MAP in cilnidipine treated rats.

The calcium channel blocker cilnidipine blocks both N- and L-type calcium channels concurrently. The predominately dispersed N-type calcium channels of sympathetic nervous system regulate the release of neurotransmitters from the nerve ends of sympathetic neurons. (Toba H., 2011).

Many other study (Takahara A., 2009; Aritomi S et al., 2010; Takai S et al., 2013; Sahar Mohamed Kamal., 2014) also observed that cilnidipine decreases blood pressure in

hypertensive rats. Possibly because of increasing bioavailability of NO (Takai S, 2013; Leung HS, 2006), suppressing sympathetic stimulation (Konda T et al, 2006; Takahara A et al, 2002) and reducing oxidative stress (Bagali S et al 2019). Vasodilation through the inhibition of L-type calcium channels on vascular smooth muscle and decreased sympathetic drive through the inhibition of N-type calcium channels on sympathetic nerve terminals and neurotransmitter release (Takahara A et al, 2002). Hence in present study we also proposed that cilnidipine by increasing bioavailability of NO, reducing oxidative stress and suppressing sympathetic stimulation might have caused decreased in MAP.

6.4.3: HEART RATE VARIABILITY ANALYSIS (HRV)

There are oscillations in the interval of time between subsequent heartbeats. Heart rate variability (HRV) analysis is the term used to describe these variations in the inter beat intervals. A popular non-invasive method to evaluate cardiovascular autonomic functions is HRV analysis. HRV time and frequency domain indices are used in the analysis. Two main frequency bands are used in frequency domain analysis of RR interval oscillations: the low frequency (LF) band (0.04-0.15 Hz), which mostly reflects sympathetic activity, and the high frequency (HF) band (0.15-0.4 Hz) parasympathetic activity is reflected. A measure of the heart sympathovagal balance is the LF/HF ratio. (Shaffer et al., 2017).

In our study we observed there is significant increased in LF value, decreased in HF value and significant increase in LF/HF ratio in L-NAME & L-NAME +Salt treated rats. Treatment with cilnidipine there is significant decrease in LF/HF ratio (vide chapter: 5.4; Table: 5.4.3, Figure:5.4.3).

Increase in sympathetic vasomotor activity, has been noted as an important mechanism underlying the hypertension induced by inhibition of NO (Biancardi VC et al., 2007; Esler et al.,2010; Zambrano LI et l., 2019). In contrast to our study Dos Santos et al (2010) described that there is no increase in renal sympathetic nerve activity after two and fourteen days of L-NAME treatment in conscious rats (dos Santos et al.,2010).

According to a previous studies changes in glutaminergic neurotransmission in the nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM) are promoted by low concentrations of NO from endothelial nitric oxide synthase (eNOS), which results in sympathoexcitation in response to NO inhibition. (Chan and Chan., 2014). On the other side, sympathoinhibition is caused by a high concentration of NO produced by iNOS, which enhances GABAergic neurotransmission in the NTS and RVLM (Chan and Chan., 2014). Accordingly, one theory is that in L-NAME-treated rats, increases in NO concentration in the brain are likely a key mechanism causing sympathoexcitation and hypertension. Considering that inhibiting the RVLM causes a decrease in blood pressure to a level comparable to that seen following ganglionic blocking by hexamethonium in normotensive rats, it is reasonable to conclude that sympathetic drive is the primary cause of all hypertension in L-NAME-treated rats.

The stimulation of the renin-angiotensin system (RAS) in response to NO inhibition is one more factor that might be responsible for sympathoexcitation in rats after L-NAME treatment (Crowley, 2014). The oxidative stress-activating enzyme NADPH oxidase, which results in the production of intracellular superoxide anion and hypertension, can be increased by angiotensin II.

In current study we also observed increase in oxidative stress in kidney and increase in serum and urinary ANG II levels. (vide chapter: 5.5; Table: 5.5.1; Chapter 5.6: Table: 5.6.2)

We found simultaneous treatment with cilnidipine there is decreased in sympathetic derive.

Interestingly, in both vivo and vitro examinations and also in clinical practice cilnidipine shows antisympathetic profiles (Takahara A., 2009; Takahara A., 2002; Konda T., 2006). Numerous in vitro investigations have shown that cilnidipine reduces the release of norepinephrine from sympathetic nerve terminals. (Nap A., 2004). Such effects have been also observed in anesthetized rats in vivo experiments (Takahara A., 2002) and dogs (Konda T., 2006). Therefore, during antihypertensive therapy, cilnidipine may rarely activate sympathetic function (Takahara A., 2009).

Ability of cilnidipine to inhibit N-type calcium channels offers some potential avenues for preventing increasing kidney damage.

6.5 BIOCHEMICAL PARAMETERS

6.5: BIOCHEMICAL PARAMETERS

6.5.1: OXIDATIVE STRESS MARKERS (Serum and kidney tissue MDA levels)

Reactive oxygen species (ROS) generation is excessive during oxidative stress, and the oxidation-reduction (redox) state is changed. Inflammation, proliferation, apoptosis, migration, and fibrosis are important processes that contribute to impaired vascular function, cardiovascular remodelling, renal dysfunction, immune cell activation, and sympathetic nervous system excitation in hypertension. These molecular events also cause protein oxidation and dysregulated cell signalling (Oparil S et al.,2018; Stanley CP et al., 2019).

The oxidative degradation of lipid, protein, carbohydrate, and nucleic acid macromolecules, among other effects of oxidative stress, can cause cell death in extreme cases (Hanna Ali H., 2012). When free radicals or non-radical species attack lipids with carbon-carbon double bonds, notably polyunsaturated fatty acids (PUFA), lipid peroxidation occurs, producing lipid peroxyl radicals and hydroperoxides. A vast range of oxidation products are produced by lipid peroxidation. Lipid hydroperoxides are the principal by products of lipid peroxidation, and various aldehydes, such as malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE), are the secondary by products (Ayala et al., 2014). According to Ayala et al. (2014), As an indirect biomarker of oxidative stress, MDA, a by product of the peroxidation of omega-3 and omega-6 fatty acids, is commonly used. (Buege and Aust, 1978).

In this work, tissue oxidative stress was determined by calculating MDA in kidney tissue, while systemic oxidative stress was determined by estimating MDA in serum. We observed that animals treated with L-NAME and L-NAME plus Salt had significantly higher blood and kidney MDA levels than control rats. MDA levels significantly decreased after using cilnidipine. (vide chapter: 5.5; Table 5.5.1)

In accordance with our study many other studies (Ji Hoon S., 2013; Veerappan R, Malarvili T.,2019) also proved that L-NAME induces oxidative stress by inhibiting NO. Many different forms of hypertension pathophysiology have been linked to the oxidative stress (L. Kopkan, 2006). Endothelial dysfunction may result from the increased effect of reactive oxygen species (ROS) produced by vascular NADPH oxidase caused by suppression of nitric oxide synthesis by L-NAME (Zalba G.,2001). Because it breaks lipophilic chains, cilnidipine is a lipophilic antioxidant. The greatest antioxidant property among dihydropyridine calcium channel blocker have been found in cilnidipine, which has been shown to be highly lipophilic. (Hishikawa et al., 2009). Additionally, in our investigation, cilnidipine-treated hypertensive rats demonstrated decreased oxidative stress, suggesting the its antioxidant properties. cilnidipine demonstrates strongest lipophilicity and has highest antioxidant actions among all the dihydropyridine derivatives (Bagali S., 2019).

6.5.2: SERUM UREA AND CREATININE

In L-NAME plus Salt treated rats, we saw a considerable rise in serum urea, and in L-NAME and L-NAME plus Salt treated rats, we saw a significant rise in serum creatinine. Serum urea and creatinine levels significantly decrease during cilnidipine therapy. (vide: chapter; 5.8, Table: 5.8.1)

Usually rat blood urea nitrogen (BUN) is resistant to age and gender related variation and it is within the range of 15–22 mg/dL and rat serum creatinine levels are in the range of 0.4–0.8 mg/dL. (Thammitiyagodage MG., 2020)

The balance between body salts and water is crucially regulated by the kidney, and pathophysiological disorders like hypertension are characterised by an imbalance of salt and water due to dysregulation of renal activities. Endogenous synthesis of creatinine results in its release into bodily fluids, and its clearance serves as a indicator of

glomerular filtration rate. Primary nitrogen-containing metabolic by product protein metabolism is urea, while uric acid is a main product of purine nucleotide. (Kumar S et al., 2012). Elevated serum creatinine and urea in present study indicates hypertension induced renal damage. In accordance with our study other studies also found increased in serum urea and creatinine levels in L-NAME induced hypertensive rats (Tirani SA, et al., 2015; Kumar S et al., 2012). We observed with cilnidipine treatment there is decrease in serum urea and creatinine levels indicating improvement in renal function. Konda et al (2006) showed that increase in blood urea nitrogen and creatinine levels were inhibited by cilnidipine. Toba et al (2011) also observed that "administration of Cilnidipine to the DOCA-salt hypertensive rat normalized the levels of creatinine.

6.5.3: PROTEINURIA AND CREATININE CLEARANCE

We observed there is significant increase in 24hr urinary protein excretion and decrease in creatinine clearance in L-NAME and L-NAME + Salt treated rats compared to control rats. Treatment with cilnidipine there is significant decrease in protein excretion and increase in creatinine clearance value. (vide chapter:5.8; Table 5.8.2). Other kidney cells also express calcium channels in addition to vascular smooth muscle cells. such as T-type calcium channels found in collecting ducts and L-type calcium channels found primarily in arteries but also in tubular cells. It is known that N-type calcium channels are expressed in nerve terminals and that by preserving the intracellular calcium level, they play a role in the regulation of nerve activity. (Fan YY et al., 2010). These observations corroborate and support the findings of our results.

The first evidence of the expression of N-type calcium channels in podocytes, which are key cells in the glomerular filtration barrier, is shown by Fujisawa T et al (Fujisawa T et al., 2007). It is possible that suppression of N-type calcium channels by

cilnidipine in podocytes will prevent podocyte damage and result in antiproteinuric effect in SHR/ND. In numerous organs, including the kidney, Ang II activates NADPH oxidase, causing superoxide to be produced. This mechanism is thought to induce proteinuria and renal damage in experimental hypertension (Konoshita T et al.,2010).

In our study we observed decrease in nitric oxide in kidney tissue (vide chapter:5.5: Table; 5.5.2) might have led to oxidative stress (vide chapter: 5.5; Table 5.5.1) and increase in serum and urinary Ang II levels (vide chapter:5.6; 5.6.2) may contribute to the renal injury and proteinuria and decrease in creatinine clearance.

In nitric oxide synthase-inhibited spontaneously hypertensive rats (SHR), As demonstrated by Zhou et al. (2002), cilnidipine alleviated glomerular hypertension and prevented proteinuria by lowering single nephron filtration fraction, glomerular capillary pressure, afferent and efferent arteriole resistance, and proteinuria. Both the afferent and efferent arteries were dilated by cilnidipine, indicating that it may cause efferent vasodilation by inhibiting N-type calcium channels (Zhou X et al., 2002). In our study we also found with cilnidipine treatment there is a decrease in 24hour protein excretion and improvement in creatinine clearance which supports the above explanation and mechanism.

The L/N-type calcium channel blocker cilnidipine reduced proteinuria and restored normal levels of creatinine clearance when administered to DOCA-salt hypertensive rats. which is found to be similar with our findings. (Toba H et al., 2011). According to another study, mibefradil protected against renal structural damage as measured by scoring glomerular, tubulointerstitial, and vascular lesions in the L-NAME/SHR model. It also prevented the development of proteinuria and decreased creatinine clearance (Zhou X, Frohlich ED., 2005).

Comparing CCBs cilnidipine and L-type CCB amlodipine in a clinical trial revealed that cilnidipine is significantly more effective than other CCBs at stopping the progression of proteinuria in hypertension patients. Additionally, they demonstrated that cilnidipine was superior to L-type CCB amlodipine at maintaining the glomerular slit membrane and preventing kidney damage (Mori Y et al., 2014). Taken together, it seems likely that cilnidipine elicits renoprotective effect by regulating glomerular hemodynamics.

In the current study antiproteinuric impact of cilnidipine is partially explained by its greater antioxidant activity. According to Lei et al. (2012), cilnidipine reduced podocyte damage, which in turn reduced proteinuria and albuminuria. According to Soeki et al. (2012), cilnidipine likely has a stronger antioxidative effect due to its renoprotective effects.

6.6 ESTIMATION OF MOLECULAR MARKERS

6.6: ESTIMATION OF MOLECULAR MARKERS

6.6.1: SERUM AND KIDNEY TISSUE (NO) AND eNOS/NOS3 LEVELS

Nitric oxide (NO), a biological mediator involved in a variety of physiological and pathological processes and a key regulator of vascular tone, is a vasodilatory factor generated from the endothelium. An established NO synthase inhibitor called L-NAME is known to cause hypertension that is characterised by endothelial dysfunction and a marked nitric oxide deficit (Zhu et al., 2016).

L-arginine NO pathway plays a crucial role in the regulation of blood pressure (Seth et al., 2016). It is an established regulator in the circulation and cardiovascular system (Kunes J., 2004). L-arginine is changed into L-citrulline by the enzyme NO synthase in vascular endothelial cells to produce endogenous NO. The endothelium continuously releases NO, which is involved in the control of vascular tone. (Seth et al., 2016). NO modifies sympathetic neurotransmission in addition to having a direct impact on the vascular system. In fact, the mechanism by which the acetylcholine (ACh) receptor induces vasorelaxation depends on NO (Macarthur H. et al., 2008). In addition, a number of other substances (such as histamine, bradykinin, 5-hydroxytryptamine, thrombin, etc.) can relax the smooth muscles of the arteries directly or indirectly through activating endothelium receptors, with the latter having a stronger effect than the former (Torok J., 2008). An established model of experimental NO-deficient hypertension is formed in rats by the chronic injection of the L-arginine analogue N^G-nitro-L-arginine methyl ester (l-NAME), which blocks all three NO synthase isoforms of the pathway (Dabire H. et al., 2012).

In Our study we also observed there is significant decrease in nitric oxide levels in serum L-NAME and L-NAME +Salt treated rats compare to control. Simultaneous

treatment with cilnidipine there is significant increase in nitric oxide levels (vide chapter: 5; table: 5.5.2).

Further we evaluated eNOS/NOS3 protein expression in serum and kidney tissue by both quantitative (ELISA) and qualitative (Western Blotting) techniques. (vide chapter: 5; Fig: 5.6.1and Fig 5.7.1 A,5.7.1 B). We observed decrease in eNOS/NOS3 protein expression in L-NAME and L-NAME + Salt treated rats. And simultaneous treatment with cilnidipine (group 4, and 6) there is decrease in eNOS/NOS3 protein expression compare to L-NAME treated hypertensive rats.

The mechanism of NOS inhibition by L-NAME consists in competitive bonding to enzyme (Jana Kopincova et al., 2012). Cilnidipine demonstrated higher serum NO which can be attributed to either higher bioavailability of NO or due to increased production or release of NO. By enhancing endothelial nitric oxide synthase (eNOS) in elastic vascular tissues, cilnidipine may increase NO production. Additionally, it was discovered that the cilnidipine therapy increased the release of NO from vascular endothelium. Fan et al demonstrated that eNOS gene expression was significantly augmented after treatment with cilnidipine in the human internal thoracic artery (Fan L et al., 2011). Our results also corroborate with these observations.

6.6.2: SERUM AND KIDNEY TISSUE ANGIOTENSIN CONVERTING ENZYME (ACE) PROTEIN EXPRESSION

The levels of the zinc metallopeptidase angiotensin converting enzyme, which is membrane-anchored, are raised in hypertension and cardiovascular diseases such as myocardial infarction and atherosclerosis. There is evidence that rats with spontaneous hypertension have significantly increased ACE levels. The prevention of L-NAME-

induced hypertension by ACE inhibitors like captopril shows the importance of ACE inhibitors in the NO pathway (Seth et al., 2016).

To observe the effect of CCB cilnidipine on renin angiotensin system we estimated the relative expression of ACE in L-NAME induced hypertensive rats. We found there is relative increase in serum and kidney tissue ACE protein expression in L-NAME treated rats compare to control. With cilnidipine treatment there is decrease in ACE protein expression. (vide chapter: 5 .7; Figure: 5.7.2 A and 5.7.2 B). The decrease in ACE protein expression may be because of sympathetic inhibition by cilnidipine as proved by HRV analysis (Vide chapter: 5.4: Table:5.4.3 and) and may also be because of decrease in oxidative stress as depicted in (vide, chapter5.5: Table; 5.5.1).

There are studies which reveal the reciprocal relation between ACE inhibition and NO level. (Zicha J et al., 2006; Pechanova O., 2007). Even without a decrease in afterload and coronary artery enlargement, Angiotensin Converting Enzyme (ACE) whose levels are elevated in hypertension, effectively prevents left ventricular hypertrophy. According to some reports, the proliferative effects of Ang II or the anti-proliferative effects of ACE inhibition may include both the local and systemic renin-angiotensin system (RAS). (Seth et al., 2016).

By chronically administering L-NAME (Kobayashi N et al., 2000) shown that mRNA expression of ACE is stimulated in long-term NOS blockage. Activation of ACE mRNA expression by L-NAME is not clear. The activation of ACE mRNA would, however, increase the synthesis of angiotensin II, which in turn directly causes the proliferation of vascular smooth muscle and myocyte hypertrophy as well as the release of platelet-derived growth factor. There is evidence that when NOS activity is reduced, the angiotensin II type 1 receptor may be increased, contributing to the proliferation of

vascular smooth muscle cells and the matrix synthesis triggered by angiotensin II. They also showed that NO prevents angiotensin II induced vascular smooth muscle cells migration. Chronic L-NAME injection enhances cardiac tissue and plasma ACE activity, and it has been shown that increased local ACE expression plays a significant role in the pathophysiology of the vascular and myocardial remodelling brought on by long-term NOS blockage in the rat model. (Kobayashi N et al., 2000). Cilnidipine, but not amlodipine reduce the expression of the ACE gene in the kidney of hypertensive rats. They suggest that N-type calcium channel blocking with cilnidipine, would protect the kidney by inhibiting sympathetic nerve activity. (Toba et al., 2011).

Hence we proposed that sympathoinhibitory effects of cilnidipine would reduce renal activity and ACE expression. Additionally, we hypothesise that the renoprotective action of cilnidipine would be accompanied by direct antioxidant characteristics in addition to sympathoinhibition.

6.6.3: SERUM AND URINARY ANG II LEVEL

Angiotensin II is a potent vasoconstrictor and biological agent that plays a key role in controlling blood pressure and renal function. Long-term administration of low dose of angiotensin II, which does not immediately raise blood pressure, causes hypertension to gradually develop along with an increase in oxidative stress. Increased intrarenal angiotensin II levels impair renal function, which results in salt retention and helps to develop and maintain hypertension (Kopkan L., 2006).

Further to confirm the inhibition of renin and angiotensin system by L/N type CCB cilnidipine we quantitatively estimated the serum and urinary angiotensin II levels. When compared to control rats, rats treated with L-NAME and L-NAME plus Salt had significantly higher levels of Ang II in both urine and serum. Angiotensin II levels in the

blood and urine significantly decline with cilnidipine supplementation. (vide chapter: 5.6; Table: 5.6.2).

Renin angiotensin aldosterone system imbalance was caused by NO deficient hypertension (Veerappan R, Malarvili T., 2019). By preventing the release of renin and the activity of the angiotensin converting enzyme (ACE), cilnidipine may have reduced the production of angiotensin II. In spontaneously hypertensive rats, cilnidipine inhibits the rise in plasma renin activity (PRA), angiotensin II levels, and plasma aldosterone levels that signify the activation of the renin-angiotensin-aldosterone system (Aritomi S et al., 2015). These findings are consistent with the current research. The vicious cycle of renin-angiotensin system and kidney oxidative stress are both inhibited by cilnidipine. In rats with spontaneous hypertension, it might also decrease angiotensinogen expression in the kidneys (Onozato ML et al., 2002).

When we estimated urine Ang II levels, we observed that rats treated with L-NAME and L-NAME plus Salt had higher urinary Ang II levels than control rats. The level of Ang II significantly decreases after taking cilnidipine. (vide chapter: 5.6; Table: 5.6.2).

Oxidative stress, which may be brought on by a deficiency of NO, seems to enhance the expression of the AGT gene, the synthesis of Ang II, and additional oxidative stress in the kidney. Cilnidipine suppresses the vicious cycle of RAS and oxidative stress in the kidney and may lower AGT expression in the kidneys of spontaneously hypertensive rats. (Fan YY, et al., 2010). These observation suggests that cilnidipine suppress renin-angiotensin system (RAS) through its N-type calcium channel blocking action.

6.6.4: SERUM AND KIDNEY TISSUE VEGF PROTEIN EXPRESSION

We observed relatively increase expression of VEGF in L-NAME & L-NAME +Salt treated rats compare to control. Treatment with cilnidipine there is decrease in expression of VEGF (vide chapter: 5.7; 5.7.3 A & 5.7.3 B).

Endothelial cells produce the strong vasodilator nitric oxide (NO) via the enzyme endothelial nitric oxide synthase (eNOS). The intracellular receptor tyrosine kinase is activated by VEGF binding to type 1 (VEGFR1) or type 2 (VEGFR2) VEGF receptors, which stimulates the PI3K/Akt signalling cascade and stimulates the production of NO. Evidence from cultured endothelium cells and preclinical animal models shows that VEGF receptor inhibitor reduces NO availability. Reduced NO synthesis by eNOS or increased oxidative stress, which inactivates NO, may be the cause of lower bioavailable NO. (Camarda N., et al 2022)

Complex inter relationship exist between oxygen sensitive HIF-1α, VEGF and nitric oxide synthase (NOS). Nitric oxide and HIF-1α have an inverse relationship. L-NAME decreases NO bioavailability resulting in increase in HIF-1α subsequently increase in VEGF by a negative feedback mechanism. (Chun YS., 2002).

In the present study cilnidipine treated hypertensive rats demonstrated decrease serum VEGF, increase serum NOS3 and improvement of serum NO. The higher bioavailability or increased generation of NO may have contributed to the improved serum NO levels in the cilnidipine-treated group. The reduced oxidative stress seen in cilnidipine-treated rats may have increased the bioavailability of NO. (vide chapter: 5.5; table: 5.5.1). These findings also support antioxidant properties cilnidipine. Additionally, it has been shown that cilnidipine increases NO production and NOS3 expression (Fan et al., 2011).

6.7 HISTOPATHOLOGICAL EXAMINATION

6.7: HISTOPATHOLOGICAL EXAMINATION

6.7.1: Histopathological examination of Aortic tissue

In current study histopathological examination of elastic aorta (vide chapter: 5.9, Figure 5.9.1) showed under Haematoxylin and Eosin stain large sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. Endothelial cells line the tunica intima. Elastic fibre covered smooth muscle cells make up the tunica media. Loose connective tissue makes up the tunica adventitia. In present study there is no evidence of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm or dysplasia in control rats.

In L-NAME treated rats, the tunica intima shows mild thickening. The tunica media which is consisting of normal smooth muscle cells with elastic fibres did not show any significant changes. Also there is no significant abnormality was observed in tunica adventitia.

In L-NAME plus Salt treated rats we observed the tunica intima is lined by endothelial cells and shows mild thickening. The tunica media is consisting of hyperplastic smooth muscle cells with elastic fibres. The tunica adventitia is composed of loose connective tissue where no significant changes were noticed. With cilnidipine treatment we observed near normal histology of aorta.

Nitric oxide bioavailability, increased oxidative stress, and mechanical stress with a rise in blood pressure may all have a direct impact on vascular endothelial function. In Wistar rats, long-term NOS inhibition alters the structure of thoracic aortic wall. These structural alterations most likely resulted from the decrease in NO production. Although Na-K-ATPase activity may also play a important role, NO inhibits the proliferation of vascular smooth muscle cells (VSMCs) through a cGMP-dependent mechanism. In

VSMCs, NO also prevents protein production. It is probable that the hyperplasia of the media in the thoracic aorta was caused by uncontrolled proliferation of vascular medial smooth muscle cells. (Vardi N et al., 2003). In present study also chronic NO inhibition and oxidative stress might have caused the mild intimal thickening and hyperplasia of smooth muscle cells. Similarly (Rossi MA et al.,2001) also observed prominent intima thickness in L-NAME-treated rat aorta.

Girardi et al. proposed that prolonged nitric oxide inhibition by the L-NAME administration in rats causes endothelial dysfunction, vascular hypertrophy, cardiac fibrosis, atherosclerosis, perivascular inflammation, renal failure, and increased vascular responses to adrenergic stimuli (Girardi JM et al., 2011).

In our study NO inhibition by L-NAME might have cause the vascular damage. Increased in blood pressure (vide chapter:5.4; Figure 5.4.2) may also lead to mechanical stress. Further the oxidative stress and increase in Ang II in serum might have also contributed to the vascular injury. Increase bioavailability of NO, reduction in oxidative stress and decrease in mean arterial pressure with cilnidipine treatment might have ameliorated the vascular pathophysiology in cilnidipine treated rats.

Takai S. et al. also discovered that NADPH oxidases of vascular smooth muscle and endothelial cells are acutly activated by mechanical stress, such as high blood pressure. On the other hand, the development of vascular dysfunction is significantly influenced by an increase in oxidative stress brought on by angiotensin II in the vascular tissue of hypertensive rats. They noticed that cilnidipine reduced the gene expression of a NADPH oxidase subunit in spontaneously hypertensive rats (SHR-SPs). Therefore, the potent attenuation of vascular NADPH oxidase by cilnidipine may be strongly related to the stronger attenuation of oxidative stress by this drug. (Takai S et al., 2013). The prime bioactive peptide of the RAAS, Angiotensin-II, has a important role in the regulation of

structure and functions of vascular tissue (Veerappan R, Malarvili T., 2019) which has having similarity with our study.

6.7.2: Histopathological examination of Kidney tissue

Section studied under Haematoxylin & Eosin stain shows normal renal parenchymal tissue (vide chapter: 5.9, Figure 5.9.2) which is composed of glomeruli and tubules separated by small amount of interstitial connective tissue containing peritubular capillaries. Each glomerulus is spherical collection of interconnected capillaries within a Bowman's space lined by flattened parietal cells.

In control rats the outer aspects of the glomerular capillaries are covered by a layer of visceral epithelial cells (podocytes). The capillary tufts are supported by the mesangium. Tubules appear normal. No evidence of tubular atrophy. Glomeruli appear normal in morphology and in cellularity. Interstitium and vessels appears normal.

In L-NAME treated rats we observed, there is moderate thickening of glomerular basement membrane. Tubules appear normal. No evidence of tubular atrophy. There is moderate thickening of tubular basement membrane. Interstitium appears normal. Vessels show moderate thickening of the wall.

In L-NAME + Salt treated rats we found glomeruli appear enlarged in size with hypercellularity. There is severe thickening of glomerular basement membrane. There is also a moderate thickening of tubular basement membrane. Interstitium shows congested blood vessels. Vessels show severe thickening of the wall with sclerotic changes.

Simultaneous treatment with cilnidipine we observed near normal histopathological study of kidney tissue.

L-NAME induced hypertension exhibits tubular cell necrosis, tubular lumen dilation, and congestion of the glomeruli (Kumar S et al., 2012). In accordance with our

study Girardi JM et al (2011) also found glomerulosclerosis, glomerular ischemia, and interstitial fibrosis, accumulation of macrophages in the glomeruli in the kidneys of nitric oxide deficient rats fed a high-salt diet. (Girardi JM et al., 2011).

To study the renal consequences of NO blockage L-NAME in drinking water was given to Munich-Wister rats for a month. They discovered that rats fed L-NAME developed kidney interstitial fibrosis, hypertension, and glomerulosclerosis. (Klahr S., 2000). Similar results were also reported by Kashiwagi M et al., 2001.

With cilnidipine we found improvement in renal histopathological study. In accordance with our study (Zhou X et al., 2002; Fan YY et al., 2010; Aritomi S et al., 2015; Toba H et al., 2011) also observed the renal protective effect of cilnidipine.

The cilnidipine treated rats showed significantly reduced glomerulosclerosis and interstitial fibrosis (Shizuka Aritomi et al., 2010; Toba H et l., 2011) which is similar to our findings. Hence calcium channel blocker cilnidipine in addition to its direct antihypertensive effects, have been reported to ameliorate organ damage through the antioxidant and other beneficial properties. Cilnidipine is a CCB that blocks N-type calcium channels in addition to L-type channels (Takahara A. 2009). Its effects on N-type channels give it a unique profile of beneficial actions that includes the suppression of sympathetic nervous system (SNS) hyperactivity and to inhibit renin transcription in juxtaglomerular cells. (Takahara A et al., 2002; Shiga T et al., 2007; Aritomi S et al., 2015). Furthermore, cilnidipine suppresses the elevation of plasma renin activity (PRA), angiotensin II (Ang II) levels, and aldosterone levels in plasma of spontaneously-hypertensive rats (Konda T et al., 2009). These findings suggest that cilnidipine by increasing the bioavailability of NO, inhibiting the sympathetic nervous system and oxidative stress and reducing the Ang II levels might have reduced the renal injury. Hence cilnidipine may act as a renal protective agent in hypertensive renal damage.

CHAPTER VI
SUMMARY AND CONCLUSION

7.1 SUMMARY

Hypertension is the most important noncommunicable disease in India with an estimated burden of 200 million persons. Hypertension is more common in developed countries, urban populations and better socioeconomic status individuals. Prevalence of hypertension continuously increasing across the globe. In spite of being high prevalence disease, the awareness, management, and control of hypertension in the community are very less. Risk factors for primary hypertension include advancing age, obesity, high dietary sodium chloride (NaCl) consumption.

Chronic hypertension progresses to chronic renal failure by causing the loss of glomeruli and several morphological and quantitative changes in the kidney. The main medications used to treat chronic nephropathy are angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. The development and management of renal lesions similar to those reported in hypertension in humans can be studied using the nitric oxide deficient rat hypertension model. The goal of treatment should be to decrease the hypertensive renal injury because the kidneys are where chronic hypertension complications mostly manifest. An important factor in the progression of renal damage is sympathetic overactivity. Chronic renal illness may benefit from treatments that control the activity of the sympathetic nervous system. In rats with hypertensive renal damage, cilnidipine, a double L&N type calcium channel blocker, may be more beneficial than a single L-type CCB. Very few studies have been done to clarify how N-type calcium channel blockers affect hypertensive rats whose nitric oxide production has been suppressed. Therefore, using L-NAME-induced hypertension rats, we investigated the renoprotective properties of cilnidipine.

We hypothesized that renal and vascular injury caused by L-NAME and L-NAME plus salt induced hypertension possibly be reduced by treatment with cilnidipine, both L-type and N-type calcium channel blocker.

We included 36 adult male Wistar strain albino rats in the study (Rattus Norvegicus), divided into six groups at random. Group 1 is the control group, Group 2 is treated with cilnidipine (2.0mg/kg/day) for 28 days, Group 3 is treated with L-NAME (40mg/kg/day orally in distilled water), Group 4 is treated with L-NAME and cilnidipine for 28 days, Group 5 is treated with L-NAME and Salt (4% NaCl), and Group 6 is treated with L-NAME and cilnidipine and salt. All experimental animals underwent gravimetry, and after 28 days, the percentage of body weight gain was measured. For haematological factors including RBC count (million cells/mm3 of blood), haemoglobin (g/dl), and haematocrit (percentage), estimates were calculated. The cardiovascular autonomic balance was assessed by analysing HRV data. By using the non-invasive tail cum approach (NIBP), hemodynamic parameters like heart rate and weekly blood pressure were measured. Systemic and kidney tissue oxidative stress were assessed by estimating MDA in serum and kidney tissue homogenate. Serum and kidney tissue nitric oxide (NO) levels were estimated as nitrosative stress markers. 24hour urine was collected by keeping the rats in metabolic cage individually. 24hour total protein excretion and creatine excretion were estimated. Creatinine clearance was calculated for rats of all group. Blood urea and creatinine levels were also estimate. Serum eNOS and serum and urinary angiotensin II levels were estimated by quantitative methods (ELISA).

Molecular markers like NOS3, ACE and VEGF were estimated in the serum and kidney tissue by Western Blotting as qualitative tool. Histopathological examination of Haematoxylin and Eosin stained sections was done for kidney tissue and Aorta.

L-NAME therapy led to a reduced percentage of body weight increase. The results of an HRV analysis showed altered sympathovagal balance and excessive sympathetic activity. Heart rate was lower and mean arterial pressure was higher when cardiovascular haemodynamics were assessed (MAP). There were increased in oxidative stress marker MDA indicating oxidative stress. We found there is increased in 24 hr urinary protein and creatinine excretion in L-NAME and salt induced hypertensive rats. Even we observed increased in serum and urinary angiotensin II levels in NO deficient hypertensive rats. Estimation of molecular markers revealed decreased in serum and kidney tissue NO and eNOS/NOS3 levels and more relative expression of VEGF, ACE protein by Western Blotting.

These results suggest a potential connection between the sympathetic overactivity, the renin-angiotensin system, and oxidative stress in the vascular and renal remodelling in NO deficient and salt supplemented hypertensive rats. Cilnidipine treatment was able to 1) decrease mean arterial pressure (MAP) 2) decrease sympathetic overactivity 3) decrease oxidative stress 4) increase the bioavailability of NO 5) increase the eNOS/NOS3 protein expression 6) decreased urinary protein excretion and improvement in creatinine clearance value, 7) decreased in ACE and VEGF protein expression 8) decreased in serum and urinary angiotensin II levels 9) ameliorate vascular and kidney remodelling resulting from L-NAME induced hypertension. Histopathological findings on kidney and aorta were also supportive of our findings from electrophysiological, biochemical and molecular analysis.

These effects of cilnidipine could be partly related to its antioxidant property and partly to its both L-Type and N-Type calcium channel blocking action. Thus, cilnidipine may be used as an additional therapy to treat L-NAME-induced NO deficient hypertensive renal damage, according to the findings of the present investigation.

7.2 CONCLUSION

In our Laboratory of Vascular Physiology and Medicine, we successfully developed a L-NAME induced nitric oxide deficient hypertensive rat model.

These data demonstrate that an enhanced oxidative stress activity induced by HS intake under deficient NO production by eNOS enzyme contributes substantially to the development of salt-sensitive hypertension.

We come to the conclusion that cilnidipine improves vascular pathophysiology by increasing nitric oxide bioavailability, reducing oxidative stress, reducing sympathetic activity, and suppressing the renin-angiotensin-aldosterone system. Additionally, we draw the conclusion that cilnidipine decreases kidney damage in hypertensive rats by lowering proteinuria, enhancing creatinine clearance, and minimising glomerular sclerosis.

According to the current research, people with hypertension and the metabolic syndrome who also have renal illness may benefit from treatment with cilnidipine.

7.3 GRAPHICAL ABSTRACT

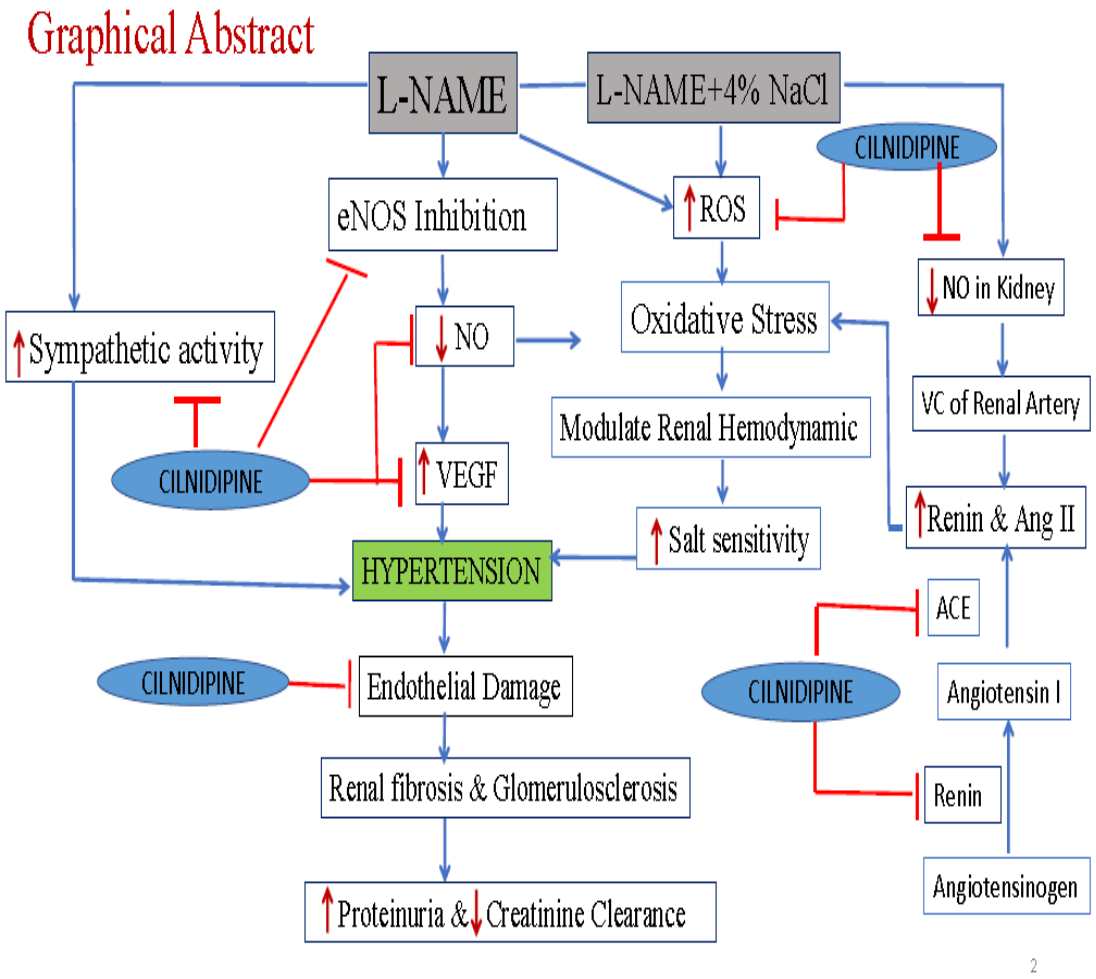


Figure: 7.2: Depicts the Effect of Cilnidipine in L-NANE and L-NAME plus salt induced hypertension

LIMITATION AND FUTURE PROSPECTIVE OF THE STUDY

Limitations of this study we have not estimated the inflammatory markers causing renal damage induced by NO suppression and salt supplementation.

Further molecular levels studies are needed to demonstrate that the functional eNOS activity provides a protective role in preventing oxidative stress. And to understand the mechanism of how cilnidipine dual L/N type calcium channel blocker protects inflammatory cytokine mediated renal injury and hypertension induced by activation of renin angiotensin aldosterone system.

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ANNEXURES

ANNEXURE I

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: Dr. GOUHER BANU SHAIKH Reg No: 16PHD003

2. Title of the Thesis: ***Effect of cilnidipine as an antihypertensive agent on two forms (L-NAME and L-NAME+4%NaCl) of hypertension in rats.***

3. Department: Physiology

4. Name of the Guide & Designation: Prof. Kusal K. Das, PhD, Distinguished Chair Professor

5. Name of the Co Guide & Designation: Dr Dewan S.A Majid, Professor of Physiology

The above thesis was verified for similarity detection. The report is as follows:

Software used: TURNITIN Date: 19. 10.2022.

Similarity Index (%): Ten percent (10%) Total word Count: 27521

The report is attached for the review by the Student and Guide.

The plagiarism report of the above thesis has been reviewed by the undersigned.

The similarity index is below accepted norms.

The similarity index is above accepted norms, because of following reasons:

Total 10% similarity was found out of which 1% is from her own publications, hence 10-1=9% similarity. The thesis may be considered for submission to the University. The software report is attached.

Signature of the Guide **Signature of Student**

Name & Designation

Verified by (Signature)

Name & Designation

ANNEXURE II

INSTITUTIONAL ANIMAL ETHICAL CLEARANCE CERTIFICATE

BLDE Association's

SHRI SANGANABASAVA MAHASWAMIJI COLLEGE OF PHARMACY & RESEARCH CENTRE

Post Box No. 40, BLDE University Campus, Solapur Road, VIJAYAPUR-586 103

Estd. 1982 Approved by Pharmacy Council of India(PCI), All India Council for Technical Education(AICTE), New Delhi.

Recognised by : Govt. of Karnataka & Affiliated to RGUHS, Bengaluru

Dr. Navanath V. Kalyane
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Ref. : BLDE/ BPC/644/2018-19 **Certificate** Date : 15/12/2018

This is to certify that the research project entitled **"Effect of clinidipine as an antihypertensive agent on two forms (L-NAME & L-NAME + 4% NaCl) of hypertension in rats"** of **Dr. Gouher Banu Shaikh** has been approved by the IAEC.

Dr. N V Kalyane

Name of Chairman/Memb. Secretary IAEC

Signature with date 15/12/18

IAEC CHAIRMAN

Chairman/Memb. Secretary IAEC

Dr. Chandrashekhar V M

Name of CPCSEA Nominee

CPCSEA Nominee

CPCSEA NOMINEE

(Kindly make sure that minutes of meeting duly signed by all the participants are maintained by office.)

ANNEXURE III

PRESENTATIONS

1. Effects of calcium channel blocker (cilnidipine) on cardiovascular electro physiology in experimentally induced hypertensive rats. ASSOPICON 2019, JSS Medical College, Mysuru, from 12-14 Sep 2019.

2. Effect of L/N-type Calcium Channel Blocker (Cilnidipine) on Oxidative Stress in Nitric Oxide-Deficient Hypertensive Rats. UNESCO/UNITWIN WEB SEMINAR-2020, on 6-7 August 2020, conducted by BLDE (Deemed to be university) Vijayapura. (Awarded Certificate of Merit)

3. Renoprotective effects of dual L/N- type Calcium Channel Blocker (Cilnidipine) on L-NAME induced Hypertensive Rats. Biennial SAAP VII & PSI-Conference. 25th march 2021. Conducted by department of Physiology, Jamia Hamdard Medical College, New Delhi.

ANNEXURE IV

PUBLICATIONS

1. Gouher Banu, Surekha hippargi, K.K Das, M. S Biradar, D.S.A Majid. L/N-type calcium Channel Blocker (cilnidipine) ameliorates Oxidative stress in NO Deficient hypertensive Rats. JKIMSU 2020; 9(2) :73-80. (SCOPUS)

2. Gouher Banu Shaikh, Surekha Hippargi, Dewan S. A Majid, K K Das. Protective Actions of Cilnidipine: Dual L/N-Type Calcium Channel Blocker Against Hypertensive Renal Injury in Rats. *Biomedical & Pharmacology Journal* 2021;14(4): 1887-1893. (SCOPUS)

ORIGINAL ARTICLE

Effect of L/N-type Calcium Channel Blocker (Cilnidipine) on Oxidative Stress in Nitric Oxide-deficient Hypertensive Rats

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Abstract:

Background: The sympathetic nervous system plays a major role on the renal function through the vasoactive system and the renin-angiotensin aldosterone system. Even though interest in the renal protective effects of sympathetic blocker has been increased, there are not much data to clarify this efficiency in nitric oxide deficient hypertensive rats. *Aim and Objectives:* To find out the effect of cilnidipine, L/N-type calcium channel blocker on oxidative stress of kidney in Nitric Oxide Synthase (NOS) inhibited experimental hypertensive rats. *Material and Methods:* Male Albino Wistar rats (n-24) were randomly allocated into four groups: Group 1 control received vehicle; Group 2 received Cilnidipine; Group 3 received N^G-nitro-L-Arginine Methyl Ester (L-NAME) hydrochloride; Group 4 received L-NAME and Cilnidipine; All drugs are given orally for 4 weeks. Blood pressure was measured before and after intervention and twice during intervention for all the rats. On 29th day, blood was collected and animals were sacrificed and kidneys were collected. Serum and kidney tissue Malondialdehyde (MDA) levels are estimated. *Results:* The results demonstrate that there is a significant increase in Mean Arterial Pressure (MAP) in L-NAME treated rats compared to control group. Treatment with cilnidipine significantly decreases the MAP in Group 4 rats. We also demonstrated the significant elevated serum and kidney tissue MDA levels in L-NAME treated rats. Treatment with Cilnidipine reduced serum and kidney tissue MDA levels in Group 4 rats as compared to Group 3 rats. *Conclusion:* The results demonstrate that cilnidipine has suppressive effects against progressive renal injury as evidenced by decrease oxidative stress indicator MDA levels in NO deficient hypertensive rats. This effect is explained by the L/N type calcium channel inhibition of Cilnidipine, the L-type calcium channel blocking action lowers blood pressure and N-type calcium channel blocking action leads to suppression of the sympathetic nerve activity and renin-angiotensin aldosterone system.

Keywords: Nitric Oxide Deficient Hypertension, Oxidative Stress, Mean Arterial Pressure, Malon-dialdehyde

Introduction:

Kidney is the major target organ for hypertensive complications, therefore major aims of anti-hypertensive therapy should be to reduce the progression of hypertensive renal damage[1]. In vivo, vasodilators and vasoconstrictors modulate the endothelial function. It is established that Nitric Oxide (NO) produced in vascular endothelial cells has a potent vasodilator effect and plays an important role in vascular resistance and growth. L-arginine analogues such as N^G-nitro-L-Arginine Methyl Ester (L-NAME) hydrochloride administration inhibits nitric oxide synthase activity and hence reduce nitric oxide biosynthesis, leading to hypertension [2]. Accumulation of superoxide anion in biological tissues can occur in the

condition of NO deficiency that can lead to alterations in organ function [3]. NO acts as an endogenous antioxidative agent by reacting with superoxide anion generated in the living tissues, thus it provides a protective function against the action of superoxide anion in many organs including kidney [4].

Cilnidipine, a dihydropyridine L/N type calcium channel blocker [5]. N-type calcium channels are predominantly distributed in the sympathetic nervous system, control neurotransmitter release from the nerve endings of sympathetic neurons [6]. N type calcium channel inhibitory actions of cilnidipine increase the possibility that cilnidipine may have a greater renoprotective effect than L-type calcium channel blockers, because glomerular efferent arterioles do not have L-type calcium channels [7]. Although cilnidipine is expected to suppress the renal injury by suppression of sympathetic nerve activity. Renal protective profile of cilnidipine is not much assessed in animal model of hypertension [8]. Since anti-hypertensive actions of cilnidipine has not much studied in animal model with renal injury, the present study was designed to clarify the renal protective effects of antisympathetic agent, cilnidipine in NO deficient hypertensive rats.

Material and Methods:

Experimental Animals:

Twenty four adult male Albino Wistar rats (*Rattus norvegicus*) weighing 180-250 g, brought from the animal house of BLDE (Deemed to be University). The animals were kept in a environmentally controlled room with a 12-h light/dark cycle and given standard rodent chow and tap water ad libitum. The rats were acclimated to handling. The animals were adapted to the laboratory conditions for a week before the onset of the experiment.

Ethical Considerations:

Institutional Animal Ethics Committee clearance certificate was obtained for the study (Ref: BLDE/BPC/644/2018-2019 dated 15.12.2018). All the experimental procedures were done in accordance with national guidelines (Committee for the Purpose and Control and Supervision of Experiments on Animals, Government of India).

Experimental Groups:

The experimental animals were randomly allocated to four groups as shown in Table 1.

Study Protocol: The experimental protocol followed

Table 1: Experimental Groups of Rats

Groups	No of rats	Intervention
Group 1 (Control)	6/set	Vehicle (0.5% Na CMC) orally for 28 days
Group 2 (Cilnidipine)	6/set	Cilnidipine, 2 mg/kg/day in 0.5% Na CMC orally for 28 days
Group 3 (L-NAME)	6/set	L-NAME, 40 mg/kg/day orally in distilled water for 28 days
Group 4 (L-NAME+ Cilnidipine)	6/set	L-NAME, 40 mg/kg/day orally in distilled water for 28 days, Cilnidipine 2 mg/kg/day in 0.5% Na CMC orally for 28 days

Na CMC- Sodium Carboxy Methyl Cellulose, L-NAME - N^G-nitro-L-Arginine Methyl Ester

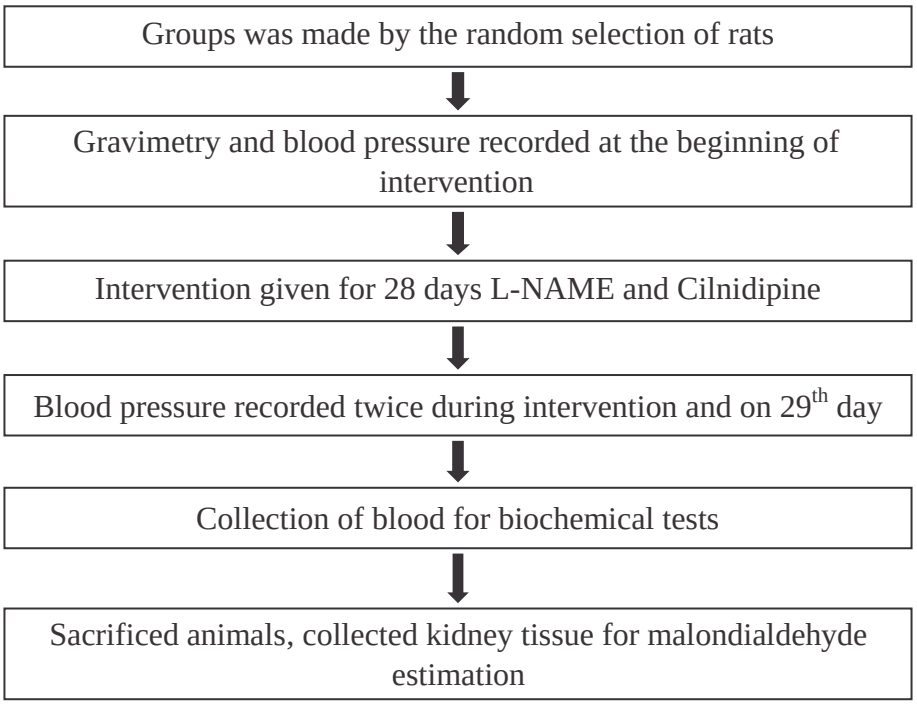

Fig. 1: Experimental Protocol

Body Weight

The body weight of all the rats was recorded on Day 1 and Day 29 using electronic balance (Practum 1102-10IN, Sartorius Lab Instruments, Germany). The weight of all the groups of rats were matched at the beginning of experiment (Table 2).

Table 2: Changes in Body Weight of the Rats

Body weight (g) (n= 6)	Group 1 Control	Group 2 Cilnidipine	Group 3 L-NAME	Group 4 L-NAME + Cilnidipine	*P*
1st day	206.5 ± 4.5	201.5 ± 5.8	209.5 ± 9.00	211.67 ± 4.08	0.191
29th day	275.75 ± 10.11	258.0 ± 0.95[a,c]	237.5 ± 7.5[a,b,d]	263.75 ± 2.62[c]	0.000*
% body weight gain	33.66 ± 2.73	32.97 ± 3.47[a,c]	13.42 ± 2.88[a,b,d]	25.02 ± 1.73[a,c]	0.000*

*L-NAME - N^G-nitro-L-Arginine Methyl Ester. Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test was done for comparison of multiple groups. Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with Group 1, 'b' denotes comparison with Group 2, 'c' denotes comparison with Group 3. *p<0.05.*

Administration of Drug:

1. Procured L-NAME from Pro Lab Marketing PVT, Limited, New Delhi, India. L-NAME was stored in -20^{o}C refrigerator for further use. L-NAME daily dose (40 mg/kg/day) was calculated and given in the morning by oral gavage at once in distilled water to Group 3 and Group 4 rats for 28 days. [2]
2. Procured cilnidipine from Laksh Finechem Pvt. Limited, Gujarat, India. Cilnidipine was stored in the refrigerator (-4^{o}C) until further use. Cilnidipine dose for rats was calculated using the formula: Rats (mg/kg) = Human dose×0.018×5 [9]. The daily dose (2 mg/kg body weight) of cilnidipine was calculated. A suspension of cilnidipine in 0.5% Sodium Carboxy Methyl Cellulose (0.5% Na CMC) was prepared freshly every day and was administered by oral gavage once in the morning to Group 2 and Group 4 rats for 28 days.

L-NAME Induced Hypertensive Rat Model

Hypertension was induced by oral administration of L-NAME (40 mg/kg/day) in distilled water for 4 successive weeks [2].

Blood Pressure Recording

Blood Pressure (systolic and diastolic) of conscious rats was measured at the start and end of the experiment and twice during intervention. Animals were kept in the restainer for 10–20 min/day for 5 days prior to recording BP in the tail-cuff technique, and tail of the animals were warmed for 30 min for better detection of tail artery pulsations. BP was recorded using noninvasive tail cuff sensor (NIBP) Bio Pac Instrument (Bio Pac MP 100: PC windows based animal electro-physiology system) and all the parameters will be analysed by Bio Pac Student Lab 4.1 software. Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) was measured. All the measurements were made thrice and the mean of three measurements was considered for each rat. Mean Arterial Pressure (MAP) was calculated by using formula MAP = Diastolic Blood Pressure+ 1/3 Pulse Pressure [2].

Assessment of Oxidative Stress

Malondialdehyde (MDA) is a product of lipid peroxidation. Concentration of MDA are used frequently as a marker for oxidative stress. MDA concentration was estimated in the serum and kidney tissue homogenate by the method of Buege and Aust (1978) [10].10% of tissue homogenate was prepared in 0.1M phosphate buffer using tissue homogenizer (Remimotors, Bombay, India) and supernatant was used for the assay. MDA reacts with thiobarbituric acid to give a pink colour and absorbance was read at 535 nm using spectro-photometer (Schimadzu UV 800, Schimadzu Corporation, Japan).

Statistical Analysis:

Statistical analysis was done using SPSS16.0 (SPSS Inc., Chicago, USA). The values were presented as Mean ± SD. Statistical significance of multiple groups was analysed using One-way Analysis of Variance (ANOVA) followed by Post hoc Tukey's multiple comparison test. P-value < 0.05 was considered as statistically significant.

Results:

Effect of Cilnidipine on Systolic Blood Pressure and Diastolic Blood Pressure:

Hypertensive rat model by NOS3 inhibitor L-NAME was successfully developed in our laboratory (Table 3). Significant increase in SBP

in L-NAME treated rats from 9th day when compared to control while significant decrease in SBP in cilnidipine treated rats when compared to L-NAME treated was observed. Significant increase in DBP from 9th day in L-NAME treated group. There is a decrease in DBP in cilnidipine treated group on 9th and 18th day although not significant. We observed significant decrease in DBP on 29th day (Table 3).

Effect of Cilnidipine on Mean Arterial Pressure (MAP)

There is no significant difference in baseline mean arterial pressure among groups. No significant differences was observed in MAP in the control group over the 4 week experimental period. Administration of L-NAME (40 mg/kg/day) induced a progressive increase in mean arterial pressure. We found significant increase in MAP with L-NAME treated groups when compared to control group from 9th day onwards. We observed decrease in the MAP in cilnidipine treated rats on 9th day and 18th day when compared to L-NAME treated group although not significant but we found significant decrease in MAP on 29th day (Fig. 2).

Oxidative Stress

We observed significant increase in MDA levels in serum and kidney tissue of L-NAME treated rats when compared to control group. We also observed significant decrease in MDA levels in serum of cilnidipine group. MDA levels in kidney tissue of cilnidipine treated rats also decreased though not significant (Table 4).

Table 3: Effect of Cilnidipine on Systolic And Diastolic Blood Pressure

Groups	Measurement	1st Day	9th Day	18th Day	29th Day
Group 1 Control	SBP (mmHg)	107.32±3.19	112.685±8.19	99.78±8.05	111.01±9.5
	DBP (mmHg)	72.4±10.2	72.63±12.28	74.24±14.66	84.64±6.24
Group 2 Cilnidipine	SBP (mmHg)	104.20±6.43	111.21±4.09^{c}	111.71±6.43^{c}	114.37±4.87^{c}
	DBP (mmHg)	78.7±3.85	83.00±7.65	84.70±4.79^{c}	89.83±4.02^{c}
Group 3 L-NAME	SBP (mmHg)	103.33±3.32	124.11±3.96^{a,b,d}	142.73±13.42^{a,b}	152.24±8.38^{a,b}
	DBP (mmHg)	71.93±4.7	89.36±7.75^{a}	106.32±7.64^{a,b}	122.60±3.27^{a,b}
Group 4 L-NAME + Cilnidipine	SBP (mmHg)	110.29±4.14	113.46±6.71^{c}	128.25±4.23^{a,b,c}	130.24±4.03^{a,b,c}
	DBP (mmHg)	79.39±6.13	88.34±4.96^{a}	95.22±3.32^{a}	98.12±3.42^{a,b,c}
P	SBP (mmHg)	0.054	0.005*	0.000*	0.000*
	DBP (mmHg)	0.130	0.011*	0.000*	0.000*

*L-NAME - N^{G}-nitro-L-Arginine Methyl Ester. Values are expressed as Mean ± SD. One Way ANOVA followed by Post Hoc Tukey's multiple comparison test was done for multiple groups Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with Group 1, 'b' denotes comparison with Group 2, 'c' denotes comparison with Group 3. *p<0.05. SBP-Systolic blood pressure, DBP-Diastolic blood pressure*

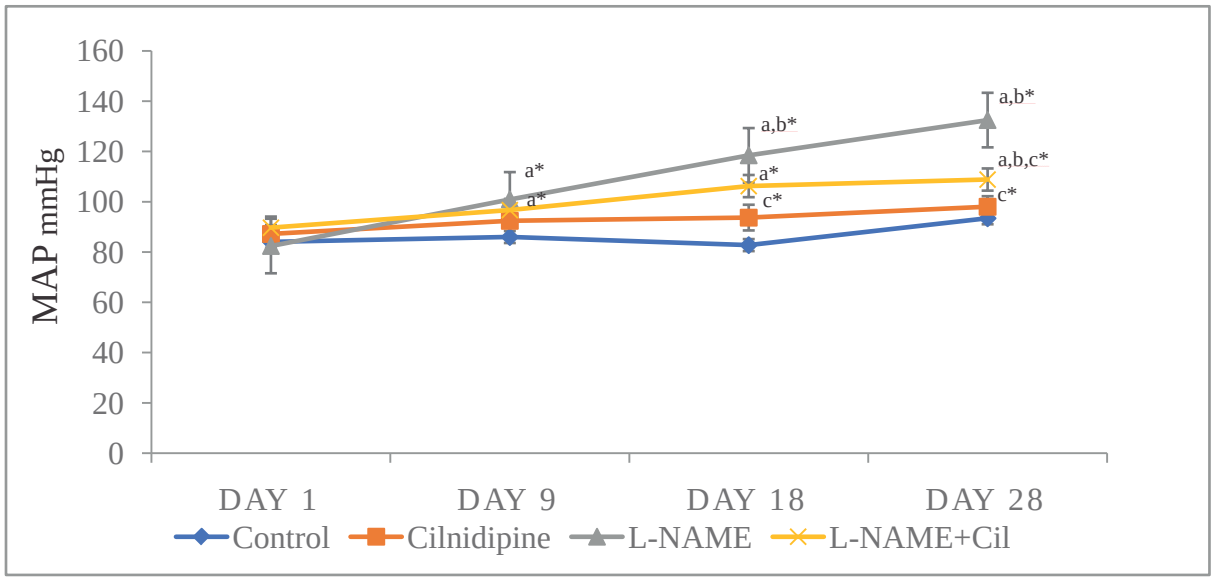

Fig. 2: Effects of Cilnidipine on Mean Arterial Blood Pressure

*Values are expressed as Mean ± SD. Oneway ANOVA followed by Post Hoc Tukey's multiple comparison test was done for multiple groups. Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with group 1, 'b' denotes comparison with group 2, 'c' denotes comparison with group 3. *p<0.05. L-NAME - N^G-nitro-L-Arginine Methyl Ester, Cil- Cilnidipine, MAP-mean arterial pressure*

Table 4: Malondialdehyde Levels in Serum and Kidney Tissue Homogenate among Groups

Parameters	Control	Cilnidipine	L-NAME	L-NAME + Cilnidipine	*P*
MDA in serum µmoles/L	1.21 ± 0.43	1.23 ± 0.14^{c}	$1.755 \pm 0.08^{a,b}$	0.819 ± 0.11^{c}	0.001*
MDA in kidney tissue µmoles/gm	24.67 ± 0.55	24.53 ± 0.9^{c}	31.25 ± 0.54^{a}	29.95 ± 1.05	0.000*

*Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test was done for comparison in multiple groups. Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with group 1, 'b' denotes comparison with Group 2, 'c' denotes comparison with Group 3. *p<0.05. MDA- Malondialdehyde, L-NAME - N^G-nitro-L-Arginine Methyl Ester.*

Discussion:

Cilnidipine has renoprotective effect in L-NAME-induced hypertensive rats. Chronic blockade of NO synthesis by L-NAME is a well-known model of hypertension. Although this model cannot be extrapolated to human hypertension, it provides the possibility of reducing the causes of increased blood pressure to a single factor, that is decrease in NO bio availability. Sufficient NO is needed for normal blood pressure. Thus, a failure to generate NO or an enhanced NO consumption can lead to hypertension. Deficiency of NO in the kidney might have caused vasoconstriction of the renal

artery and stimulated renin and angiotensin II production. This activation of renin angiotensin system may lead to vasoconstriction and hypertension [2]. Another mechanism of endothelial dysfunction might be NO synthase inhibition by L-NAME may have exaggerated the effect of Reactive Oxygen Species (ROS) generated by vascular NADPH oxidase [11]. Treatment with cilnidipine (Group 4) there was significant decrease in MAP observed compared to L-NAME treated rats.

In the kidney, renal sympathetic nerve activity contributes to the regulation of renal blood flow, glomerular filtration rate, electrolyte transport, and hormonal release. Sympathetic imbalance may lead to hypertension and progressive renal disease. Cilnidipine is a dihydropyridine calcium channel blocker, and it has been demonstrated to inhibit both N-type and L-type (long acting) calcium channels in various types of neurons. In one study on dogs, the increase in heart rate and plasma Norepinephrine (NE) level induced by bilateral carotid artery occlusion. This effect was blocked by cilnidipine through an inhibitory effect on sympathetic nerve overactivity. Cilnidipine has been shown to reduce NE secretion in response to renal nerve stimulation in dogs. This result was not observed by selective L-type calcium channel blocker nifedipine. Because of the N-type calcium channel blocking action of cilnidipine, there are some possibilities to suppress progressive renal injury [8].

MDA is a product of lipid peroxidation and has been used as a biomarker of oxidative stress [10]. Serum MDA levels in L-NAME treated hypertensive rats were increased when compared to control rats indicating high oxidative stress in L-NAME treated rats. It was found significant decrease in MDA level of serum of cilnidipine treated rats. We also found decrease in MDA level of kidney tissue of cilnidipine treated rats though not significant. Supporting this notion, previous studies also demonstrated that NOS inhibition enhances vascular super oxide release in rats [12] mice [13] and humans [14].

Along with L/N type calcium channel blocker, cilnidipine acts as a strong antioxidant. Cilnidipine demonstrates strongest lipophilicity and has highest antioxidant actions compare to other dihydropyridine derivatives [15]. The L/N type inhibitory actions of Cilnidipine would have a greater renoprotective effect than L-type calcium channel blockers, as there is absence of L-type calcium channels on glomerular efferent arterioles [16]. Oxidative stress can accompany hypertension in many animal studies, including Spontaneously Hypertensive Rats (SHR), angiotensin II-infused rats, renovascular hypertension and Deoxy-corticosterone Acetate (DOCA) salt hypertension.

Conclusion:

Result of our study demonstrate that the enhanced oxidative stress because of chronic NO synthase inhibition contributes to the impairment of renal function thus plays a role in the pathogenesis of NO-deficient form of hypertension. The L/N type calcium channel inhibitory actions of cilnidipine raise the possibility that cilnidipine would have a higher renoprotective effect by its strong anti-oxidant property compare to L-type calcium channel blockers.

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Protective Actions of Cilnidipine: Dual L/N-Type Calcium Channel Blocker Against Hypertensive Renal Injury in Rats

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Cilnidipine belongs to fourth generation dihydropyridine calcium channel blocker (CCB). It is a dual L & N-type CCB. L- type calcium channels are present on the vascular smooth muscle and N-type calcium channels are present on the presynaptic nerve terminals. Cilnidipine has a vasodilating effect, its action is slow and long lasting. Aim of present study was to demonstrate the beneficial effects of cilnidipine on the hypertensive renal injury rats. And our objectives is to assess renal injury parameters (Proteinuria, Creatinine clearance, Renal fibrosis/ glomerulosclerosis) in response to chronic N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) treatment in the presence or absence of cilnidipine treatment. Male albino Wister rats were procured from institutional animal house, divided into 4 groups (n=6 in each group). Group1 treated with vehicle (control), group2 treated with cilnidipine, group3 treated with L-NAME, group4 treated with L-NAME & cilnidipine. 24 hour urinary protein and creatinine clearance were measured. Serum urea and creatinine levels are also measured. Urinary and serum Angiotensin II levels were measured. Histopathological examination of kidneys was performed. Our results demonstrate that treatment with cilnidipine (group4) there is reduction in 24hr urinary protein, improvement in creatinine clearance. We observed there was renal glomerulosclerosis and tubular degeneration of kidney tubules in group3 rats and reduction of renal injury in group4 rats. We also found reduced urinary and serum Angiotensin II level in cilnidipine treated (group 4) rats. Conclusion: These findings indicated that cilnidipine act as renoprotective agent and reduces glomerular damage in L-NAME induced hypertensive rats.

Keywords: Creatinine Clearance; Hypertensive Arts; 24Hour Protein; L and N-type Calcium Channel Blocker; Renal Injury.

Persistent hypertension causes loss of glomeruli and several morphological and quantitative alterations in the kidney and progressively leads to chronic renal failure. The angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are the prime drugs used for the treatment of chronic nephropathy.[1] The nitric oxide deficient rat hypertension model has

been shown to be a useful tool for studying both the development and treatment of renal lesions resembling those found in human hypertension. [2]

As complications of chronic hypertension mainly occurs in the kidneys, hence purpose of the therapy should be to reduce hypertensive renal injury. Sympathetic over activity plays a major role in progressive renal injury. Treatment which modulate sympathetic nerve activity may be of benefit to chronic renal disease.

Cilnidipine as a double L&N type calcium channel blocker may have greater beneficial effect compare to only L-type CCB in hypertensive renal injury rats. [3, 4, 5]

There are very less studies to elucidate the effects of N-type calcium channel blocker against nitric oxide inhibited hypertensive rats.

Hence we investigated the renoprotective effects of cilnidipine on L-NAME induced hypertensive rats.

METHODS

Experimental Animals

Procured 24 male Albino Wister rats (Weighing 160-200gms) from institutional animal house. The rats were acclimated to handling for one week before intervention. Animals were housed in standard conditions, two rats in a cage, with 12-hour light and dark cycle and given rodent food and water. All experiments are conducted according to the guide lines of (CPCSEA) Committee for the Purpose and Control and Supervision of Experiments on Animals, Government of India.

Animal Intervention

After one week of acclimatisation rats were divided into 4 groups, group1 (Control) treated with vehicle (0.5% sodium carboxy methyl cellulose (Na CMC). Group2, treated with cilnidipine (2mg/kg/day) in 0.5% (Na CMC). Cilnidipine purchased from Laksh Fine chem. Pvt. Limited, Gujarat, India. Group 3, treated with L-NAME (40mg/kg/day in distilled water). Purchased L-NAME from Pro Lab Marketing Pvt. Limited, New Delhi, India. Group 4, treated with both L-NAME and cilnidipine. All drugs are given by oral gavage at morning hours for 28days.

Recording of Mean Arterial Pressure

Mean arterial pressure (MAP) of conscious rats was measured weekly during intervention. Animals were trained in the restainer everyday for one week before recording blood pressure. Blood pressure was recorded noninvasively by tail cuff (NIBP). Three readings were taken for each rat using Bio Pac Instrument (Bio Pac MP100:PC windows based animal electrophysiology). All the parameters will be analyzed by Bio Pac Student Lab 4.1 software.). Mean value of three recordings was considered.[6]

Assessment of Biochemical Parameters

For assessment of serum urea and creatinine, blood was collected from supra orbital plexus. Serum was separated and stored at -20 °C, serum urea and creatinine levels were assessed by fully automated dry chemistry analyser (VITROS 5.1/FS chemistry system).

Collection of Urine

Rats were kept individually in metabolic cages, 24 hour urine was collected from 10:00 a.m. to next day 10am to determine the 24hr urinary protein and creatinine excretion. Sediments were removed by centrifuging all urine samples.[7] The 24 hour protein concentration was measured with auto analyser (VITROS 5.1/FS chemistry system). Creatinine Clearance of all the rats was measured by using formula (urine creatinine in mg/ml ×urine volume / day in mL ÷ 1440 min ÷ serum creatinine in mg/ml ÷ both kidney weight in gm .[8]

Estimation of Angiotensin II Levels in Urine and serum

Urinary and serum Angiotensin II level were estimated in pg /ml by ELISA Kit method (Cat No-k11-0656).

Histological examination of Kidney

Kidneys were fixed by using 10% formalin at pH 7.4, paraffin embedded sections were made. Thin slices of 4µm were prepared. Histological examinations was done under 10x and 40x.

Ethical considerations

Taken permission from Institutional Animal Ethics Committee (IAEC) before commencement of experiment. Ethical clearance certificate (Ref: BLDE/BPC/644/2018-2019 dated 15.12.2018).

Statistical Analysis

Statistical analysis was done by using SPSS Soft ware (version 16 software). Values were expressed as the mean± SD. Statistical comparisons were made by one-way ANOVA.

p-value of less than or equal to 0.05 were considered to be statistically significant.

RESULTS

Effect of Cilnidipine on Mean Arterial Blood Pressure (MAP)

Basal mean arterial blood pressure among all the groups was not significant. There is no statistical significant difference was observed in mean arterial pressure of control group through out the intervention period. Mean arterial pressure was progressively increased in L-NAME administered rats. We found significant increase in MAP from first week onwards in group3 rats. There was significant decrease in MAP on third week and fourth week in group4 rats compared to group 3 rats. (Fig1)

Urinary protein and creatinine excretion

We found that there is significant increase in excretion of 24hour protein and decrease in creatinine clearance in group 3 rats, with simultaneous treatment of L-NAME & cilnidipine (group4 rats) there is significant reduction in excretion of 24 hour protein and increase in creatinine clearance compare to group3 rats. (Table 1)

Urinary Angiotensin II levels

We observed there is significant increase in Angiotensin II level in group 3 rats. There is significant decrease in Angiotensin II levels in urine with L-NAME & cilnidipine treated (Group 4) rats. (Table 1)

Serum urea, creatinine and Angiotensin II levels

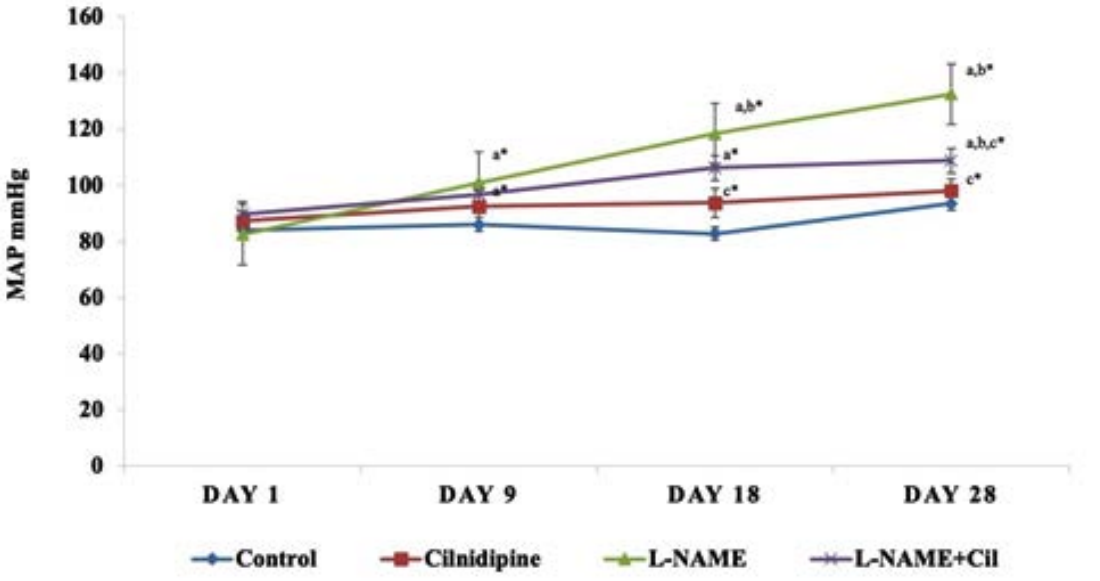
We found there is no significant change in serum urea levels in all four groups.

We observed there is significant increase in serum Angiotensin II levels in group 3 hypertensive rats, simultaneous treatment with L-NAME and cilnidipine there is significant decrease in Angiotensin II levels. (Table2)

Histological study of Kidney

Microscopic structure of kidney in each rat of all groups are stained with haematoxylin & eosin (10 x & 40x) depicted in two rows. Control group we observed normal renal histology. Cilnidipine group is also showing normal renal histological features. L-NAME group we observed (arrow A) glomeruli are hyper cellular with increased mesangial proliferation. Focal tubular epithelial hydropic degeneration (arrow B). L-NAME plus Cilnidipine treated group we found there is normal renal histology.

Histopathology of Kidney



Graph showing the effect of cilnidipine on mean arterial pressure in L-NAME induced hypertensive rats. Superscript a, b, c, indicate significant difference between group1, group2 and group3 respectively . *p<0.05 is statistically significant

Fig. 1. Effect of cilnidipine on mean arterial blood pressure

Table 1. Proteinuria, creatinine clearance & Urinary Angiotensin II level (n=6 in each group)

Parameters	Group1	Group2	Group3	Group4	ANOVA	
					F value	P value
24 hr Urinary protein in mg/ml	1.56±0.043	1.72±0.045^{c,d}	4.58±0.28^{a,b,d}	3.24±0.23^{a,b,c}	346	0.0001*
Creatinine Clearance in ml/min/gm of kidney tissue	0.073±0.0026	0.075±0.0032^{c}	0.063±0.0028^{a,b}	0.077±0.0026^{c}	28.499	0.0001*
Urinary Ang II in pg/ml	329.74±7.13	403.99±4.58	450.84±10.30^{a,b}	410.49±6.8^{a,c}	272.143	0.0001*

Values are expressed in (Mean ± SD). Groups are compared by One-way ANOVA followed by Post Hoc multiple test. Superscript a, b, c, indicate significant difference between groups. 'a, b, c denotes comparison with group1, group2 and group3 respectively. *p<0.05. Ang II-Angiotensin II

Table 2. Serum urea and creatinine and Angiotensin II levels among groups. (n=6 in each group)

Parameters	Group1	Group2	Group3	Group4	ANOVA	
					F value	P Value
Serum urea mg/ml	41.37±2.72	43.13±3.26	44.01±2.92	41±3.34	1.2093	0.304
Serum Creatinine in mg/ml	0.40±0.03	0.42±0.04	0.611±0.03^{a,b}	0.40±0.03^{b,c}	41.672	0.0001*
Serum Ang II in pg/ml	152.53±25.22	209.12±42.59^{a,c}	321.61±25.86^{a,b,}	245.45±14.62^{a,c}	36.12	0.0001*

Values are expressed in (Mean ± SD). One-way ANOVA done for comparison between groups. Superscript a, b, c, indicate significant difference between groups. *p<0.05. Ang II-Angiotensin II

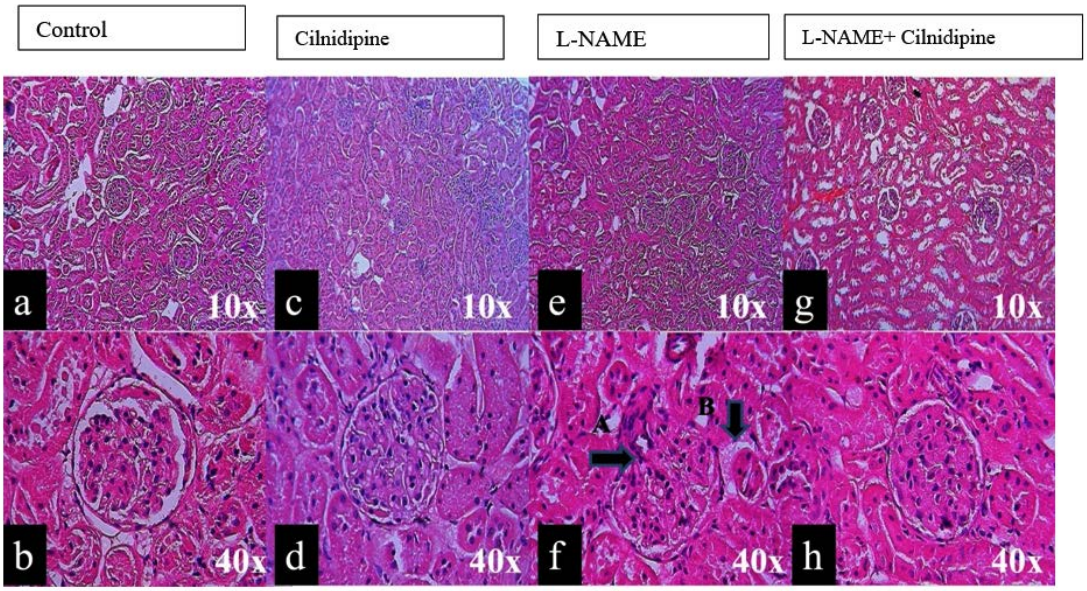


Fig. 2. Photomicrograph of the renal tissue stained with haematoxylin and eosin stain from (a) Group1(control) (x10); (b) group1 (control) (x40); (c) group2 (Cil) (x10); (d) group2 (Cil) (x40); (e) group3 (L-NAME) (x10); (f) group3(L-NAME) (x40); (g) group4 (L-NAME + Cil) (x10); (h) group4 (L-NAME + Cil) (x40). Arrow A- Glomeruli are hyper cellular with increased mesangial proliferation. Arrow B- Focal tubular epithelial hydropic degeneration. Cil-cilnidipine

DISCUSSION

We observed that administration of cilnidipine to the L-NAME induced renal injury hypertensive rat reduced proteinuria, improves creatinine clearance and reduces the glomerular damage.

Chronic nitric oxide synthesis inhibition induced by chronic L-NAME treatment, results in endothelial dysfunction, hypertrophy of vasculature, fibrosis of cardiac tissue, atherosclerosis and perivascular inflammation. Nitric oxide deficiency also contributes to renal failure and increased vascular responses to adrenergic stimuli. Several other factors, including renin angiotensin system (RAS), endothelial constrictor factors, sympathetic nervous system, arterial remodelling are also involved in these effects.[9]

Cilnidipine being both L and N-type calcium channel blocker, many of the actions are mediated by inhibiting specifically N-type calcium channels. Primary actions include the suppression of sympathetic nervous over activity,[10,11] cardiovascular and renal protective functions.[12,13] They also observed in spontaneously hypertensive rats, Plasma rennin activity and plasma angiotensin II levels doesn't increase with cilnidipine treatment. They also found that cilnidipine by inhibiting N-type calcium channels, directly suppresses secretion of aldosterone from adrenocortical cells.[14] These results suggest that N-type calcium channels plays a important role in regulation of renin–angiotensin–aldosterone (RAAS) activity. A clinical trial comparing CCBs cilnidipine and L-type CCB amlodipine, in combination with a RAAS inhibitor, showed that cilnidipine is much effective compared to other CCB in preventing the progression of proteinuria in hypertensive patients. They also proved that cilnidipine was more superior than L-type CCB amlodipine in preserving the glomerular slit membrane and preventing impaired kidney function.[15]

We found there is increase in Angiotensin II levels in plasma and urine in L-NAME treated rats and there is significant decrease with L-NAME and cilnidipine treatment.

Angiotensin-II, the prime bioactive peptide of the RAAS, has a important role in the regulation of structure and functions of vascular. RAAS activation leads to hypertension in rats treated with L-NAME. Angiotensin-II a potent vasoconstrictor can activates sympathetic nerve function. Sympathetic hyperactivity can cause vascular remodelling leading to heart failure in a hypertensive rat model. Nitric oxide deficient hypertension causes an imbalance of renin angiotensin aldosterone system.[16]

Treatment with cilnidipine decreases plasma Angiotensin-II level. It is evident from the previous study that cilnidipine is having antioxidant property.[17] and increases plasma NO bioavailability.[18] Cilnidipine attenuates angiotensin II formation by inhibiting renin release and angiotensin converting enzyme (ACE) activity.[19] Cilnidipine inhibits the vicious cycle of renin angiotensin system and oxidative stress in the kidney. It may also decrease the kidney expression of angiotensinogen in the spontaneously hypertensive rats.[20]

Konda *et al.* Observed that cilnidipine doesn't cause any change in Angiotensin II levels, plasma norepinephrine and plasma renin activity. Hence they concluded that cilnidipine, suppressed reflex sympathetic hyperactivity and rennin angiotensin system activation induced by hypotension, by specifically blocking N-type calcium channel. [21]

Similar findings was observed in our study that cilnidipine decreases the level of Angiotensin II in nitric oxide deficient hypertensive rats. Hence, it can be considered that cilnidipine as a regulator of rennin angiotensin system by its inhibitory action of N-type calcium channel in the kidney. These actions of cilnidipine proves the renal protective effects.

CONCLUSION

We conclude that cilnidipine reduce renal damage by reducing proteinuria, improvement in creatinine clearance, and preventing glomerular sclerosis in nitric oxide deficient hypertensive rats. Possibly, through the inhibition of N-type calcium channels and by inhibiting renal Renin Angiotensin System and reducing oxidative stress.

These observations suggest that both L and N- type calcium channel blocker (cilnidipine) could be a better drug for therapeutic purposes in hypertensive patients with chronic renal complications.

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Conflicts of interest

There are no conflicts of interest.

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