Role of Nesfatin-1 in Acute Kidney Injury and Diabetic Nephropathy

THESIS

Submitted in partial fulfilment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

Srashti Gopal Goyal

ID No. 2018PHXF0456H

Under the supervision of

Prof. Arti Dhar

BITS Pilani

Pilani | Dubai | Goa | Hyderabad

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

2024

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis titled **"Role of Nesfatin-1 in Acute Kidney Injury and Diabetic Nephropathy",** submitted by **Srashti Gopal Goyal** ID No. **2018PHXF0456H** for award of Ph.D. of the Institute, embodies the original work done by him under my supervision.

Signature of the Supervisor:

Name in capital letters: **ARTI DHAR**

Designation: Professor

Date: 02/05/2024

ACKNOWLEDGMENTS

This thesis represents not only my research work done at the lab but also involves valuable time spent at BITS-Pilani, Hyderabad campus, since my first day, 8th December 2018, which made me feel at home. I have been given abundant facilities to learn and work all these years. This thesis is also the product of my experiences at the BITS campus with numerous outstanding people, all of whom I would like to thank.

First and foremost, I would like to thank my supervisor**,** Prof. Arti Dhar, Professor, Department of Pharmacy, BITS Pilani, Hyderabad campus. She has been supportive since I met her for an opportunity to work under her guidance. Her patience, motivation, and immense knowledge helped me personally and professionally to complete this work. After my supervisor, I must thank our research team members Suresh Babu Mangali, Jaspreet Kalra, Dasari Deepika, Trupti Ghatage, Ganesh Lahane, Jegadheeswari V for being great companions in and out of the lab for sharing research ideas and joy. Your company drove me to never give up in rough times.

I sincerely thank the Doctoral Advisory Committee members Prof. D Sriram and Prof. Venkata Vamsi Krishna Venuganti for evaluating my thesis and for valuable suggestions. I would also like to thank Prof. V. Ramgopal Rao, Vice-Chancellor;Prof. Soumyo Mukherji, Director, BITS Pilani, Hyderabad Campus; Col. Soumyabrata Chakraborty (Retd), Registrar; Prof. Venkata Vamsi Krishna Venuganti, Dean, Academic Graduate Studies Research, Prof. P Yogeeswari, Dean, General Administration, Hyderabad Campus and Prof. Sanket Goel, Dean, Sponsored Research and Consultancy Division for providing excellent research facility.

It’s my honor to thank HOD Prof. Sajeli Begum, Department of Pharmacy, BITS Pilani, Hyderabad campus, for providing all the necessary facilities for completing the research

work. I also thank Dr. Nirmal J, Asst. Professor, Convener, Departmental Research Committee (DRC), for his exceptional support.

I am indebted to all the faculty members of the Department of Pharmacy, Prof. Swati Biswas, Prof. Onkar Prakash Kulkarni, Prof. Punna Rao Ravi, Prof Balaram Ghosh, Prof. P. Yogeeswari, Prof. Ahmed Kamal, Dr. Srinivas Prasad, Dr. Abhijeet Joshi.

I would also like to extend my thanks to colleagues of the Pharmacy Department: A special thanks to my seniors Dr. Shubham Dwivedi, Dr. Girdhary Roy, Dr. Pravesh Sharma, and my colleagues Ashutosh Mahale, Milan Paul, Tarun Patel, Vandana K for immense help in experimentation.

I thank the technical staff of the central analytical lab facility, Mr. Uppalaiah and Mr. Mallesh, for their coordination and support during the research work. Also, thanks to the team of BITS Pilani Hyderabad, Mr. Praveen, Mrs. Saritha, Mrs. Rekha, Mrs. Chandrakala, and Mrs. Sunitha for their kind support during this work.

I sincerely thank the **Government of India,** the Council of Scientific & Industrial Research (CSIR), and the BITS Pilani-Hyderabad Campus for providing me with the fellowship.

I owe a debt of gratitude to my family. Words fall short of expressing gratitude to my wife, Dr. Priya Sharma, for her sacrifices on my behalf. Finally, I thank the almighty “Sri Krishna” and my parents for the blessings that have sustained me thus far.

Srashti Gopal Goyal

ABSTRACT

Kidney injury, both Acute and Chronic, is a major disease load on human health the world over. It is a major mortality factor in the non-communicable disease category after metabolic diseases. It has been reported that one in five adults and 1 in 3 children are hospitalized due to AKI. While 700 million people are affected by CKD in the world, 20% of them are due to diabetes. In India, AKI is associated with significant morbidity and mortality. Reports suggest 0.8% to 4.1% of hospital admissions and up to 20% of Intensive Care Unit (ICU) admissions are accounted for AKI with a high mortality rate. While more than 1.2 million people live with diabetic CKD.

Nesfatin-1 is a newly discovered anorexic peptide that was found to be expressed initially in the hypothalamus, while new research suggests it is expressed in multiple organs. It affects feeding behavior in animals irrespective of the presence or absence of leptin(the primary entity regulator). Its links as a therapeutic peptide in multiple diseases and its antioxidant, anti-inflammatory, and anti-apoptotic potentials have been studied. Making it a target molecule to study. It is endogenously produced as NUCB2 protein, which is 482 amino acids long and is post-translationally cleaved into three peptides, one of which is nesfatin-1(nes-1).

Our primary target was to assess the expression of NUCB2 in different disease conditions related to kidneys and observe the effect of exogenous nesfatin-1 treatment in these diseases. For this, we used in vitro models of chemo-toxicity and myoglobin-induced rhabdomyolysis. For *in vivo* analysis, we used chemo-toxicity-induced AKI models using Doxorubicin and Cisplatin, rhabdomyolysis-induced AKI by glycerol toxicity, and Diabetic nephropathy.

In all these models, we checked the effect of exogenous nesfatin-1 to evaluate the pathogenic

processes and molecular mechanisms of NUCB2 downregulation in disease conditions and its recovery after peptide treatment. To demonstrate the positive impact of nes-1 treatment, we used expression studies of oxidative stress genes, apoptotic genes, and inflammation genes using RT-PCR or western blot. *In vitro* morphology study was done by crystal violet assay, while for in vivo tissue sections, H&E staining was done of kidney sections. For assessing fibrosis in diabetic nephropathy conditions, Sirius red staining was done of tissue sections. NUCB2 expression was evaluated by both RT-PCR and western blot. The translocation study and expression study of NUCB2 in NRK52E cells was done by confocal microscopy.

In *in vitro* and *in vivo* models of AKI by chemo-toxicity or rhabdomyolysis, we found that NUCB2 is downregulated during disease conditions, suggesting exogenous nesfatin-1 can be used as replacement therapy. We observed that in our models of AKI, nesfatin-1 replacement could upregulate the expression of NUCB2 or, due to its protective effect, it is stopping disease aggravation. In turn, NUCB2 expression was higher compared to disease conditions. The protective effect of nesfatin-1 and its role as an anti-oxidative stress, anti-inflammatory, and anti-apoptotic molecule was observed.

For molecular mechanisms, we used the diabetic nephropathy model of CKD. Type I diabetes was induced in male Wistar rats using multiple low doses of streptozotocin, which gradually developed into nephropathy. Nesfatin-1 peptide treatment was given to diseased animals for four weeks. At regular intervals, various parameters were recorded, like serum parameters of kidney health, feeding, and water intake behavior due to the satiety activity of nesfatin-1 and fasting blood glucose to assess the diabetic condition and weight was recorded. Animals were sacrificed at the end of the protocol, and tissues were isolated. Western blotting, RT-PCR, and tissue histology were performed to demonstrate the disease induction and observe the effect of nesfatin-1 on different parameters and gene markers.

Experiments were also done to elucidate the molecular mechanisms of nestin-1-mediated protection of kidneys during diabetic nephropathy.

Table of Contents

List of Tables:

List of Figures:

Abbreviations:

αGST	α-glutathione S-transferase
πGST	π-glutathione S-transferase
ACEI-	Angiotensin-converting enzyme inhibitor.
AGE	Advanced glycation end products
AKI-	Acute kidney injury
AKIN	Acute Kidney Injury network
ARB	Angiotensin receptor blocker
Bcl-2	B-cell lymphoma
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cDNA	complementary DNA
cGMP	Cyclic guanosine monophosphate
CALU	Calumenin
CAMK1D	CAMK1D calcium/calmodulin-dependent protein kinase 1 D
CKD, Chronic kidney disease	
CKI	Chronic Kidney Injury
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DAPI	4',6-diamidino-2-phenylindole
DM	Diabetes mellitus

eGFR	estimated Glomerular filtration rate.
ECM	Extracellular matrix
ECG	Electrocardiogram
ERK1/2-	Extracellular signal-regulated protein kinase
ESRD	End Stage Renal Disease
FBG	Fasting Blood Glucose
GAPDH	Glyceraldehyde phosphate dehydrogenase
GAD	Glutamic acid decarboxylase
GFR	Glomerular Filtration Rate
GLP-1	Glucagon-like peptide 1
GPx	Glutathione Peroxidase
Gm	Gram
ICU	Intensive Care Unit
IDDM	Insulin Dependent Diabetes mellitus
IL-1β	Interleukin-1 beta
I/R	Ischemia reperfusion
IRS-1	Insulin receptor substrate 1
IGFBP7	Insulin-Like Growth Factor-Binding Protein 7
JNK	Jun N-terminal kinase
MC4R	Melanocortin receptor 4

KIM-1	Kidney Injury Marker- 1
mg	Milligram
mg/dL	milli gram per decilitre
mL	Millilitre
MMP2	Matrix metalloproteinase2
mRNA	messenger RNA
Nes-1	Nesfatin-1
NIDDM	Non-Insulin Dependent Diabetes Mellitus (
NF-kB	nuclear factor-κ kappa beta
NGAL	Neutrophil-Gelatinase-Associated Lipocalin
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2–related factor 2
NSAIDs, non-steroidal anti-inflammatory drugs	
NUCB2	Nucleobindin 2
NHE-3	Sodium-hydrogen exchanger 3
NLRP3	Nucleotide-Binding Domain, Leucine-Rich–Containing Family, Pyrin Domain–Containing-3
NRK52E	Normal rat kidney epithelial 52 cells
RIFLE	Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline.

PKC	Protein Kinase C
PVDF	Polyvinylidene fluoride
RBC	Red blood cells
RIPA	Radio immune precipitation assay
ROS	reactive oxygen species
RT-PCR	Real-Time Polymerase Chain reaction
SOD1	Superoxide dismutase 1
SDS	Sodium Dodecyl Sulphate
STZ	Streptozotocin
T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus
TIMP2	Tissue inhibitor of Metalloproteinase inhibitor 2
TFG-β1	Transforming growth factor beta 1.
TNF-α	Tumour necrosis factor α
UUO	Unilateral Ureteral Obstruction
WBC	White blood cells
α-SMA	α-smooth muscle actin

Chapter 1 Introduction

International Classification of Diseases (ICD)-10 defines 40 diseases related to the renal system. In sequence N00-N39 define diseases related to renal system like Glomerular diseases, Renal tubulo-interstitial diseases, Acute kidney failure and chronic kidney disease, Urolithiasis and Other disorders of kidney and ureter. (1)

End-stage kidney injury disease is a term used mainly for chronic kidney disease but can be extended to acute kidney injury characterized by—sudden loss of kidney function. (2) As in both acute kidney injury (AKI) and chronic kidney injury (CKI), the progression of disease to end-stage renal disease (ESRD) leads to disability, high long-term monetary cost of treatment, and impaired quality of life for patients. (3,4) As in both cases, uremia, i.e., recirculation of unfiltered blood containing excess nitrogenous waste and its accumulation in the body, happens. If left untreated or dialyzed out of the body, it may lead to seizures or coma and may lead to death. (5)

AKI can be defined as a sudden loss of kidney function that could be caused by a sudden blockage of blood vessels in or out of the kidney, blockage of the ureter or chemical toxicity, or internal blockage in the kidney due to stone formation, which in turn causes rapid deterioration of internal kidney morphology and hinder kidney function. (6) Conversely, CKD is caused by the gradual building of chemical toxins or metabolic waste due to metabolic disease and long-term pharmaceutical drug usage, which damage the kidneys and their function over the long term. (7)

1.1 Epidemiology of Kidney Injury

In India, AKI is associated with significant morbidity and mortality. AKI accounts for 0.8% to 4.1% of hospital admissions (8,9) and up to 20% of ICU admissions. (10) Mortality rate for AKI is approximately 12.5%-19.6% (8,9), and in ICU patients, it reaches 49% (10), and it increases with the severity of AKI and the duration of hospitalization. Sepsis (48-79%) is

the most common cause of AKI, followed by trauma from auto accidents and heart illnesses. (8,10) AKI outcome is influenced by factors such as the severity of AKI, underlying comorbidities, access to healthcare, and availability of renal replacement therapy (RRT).

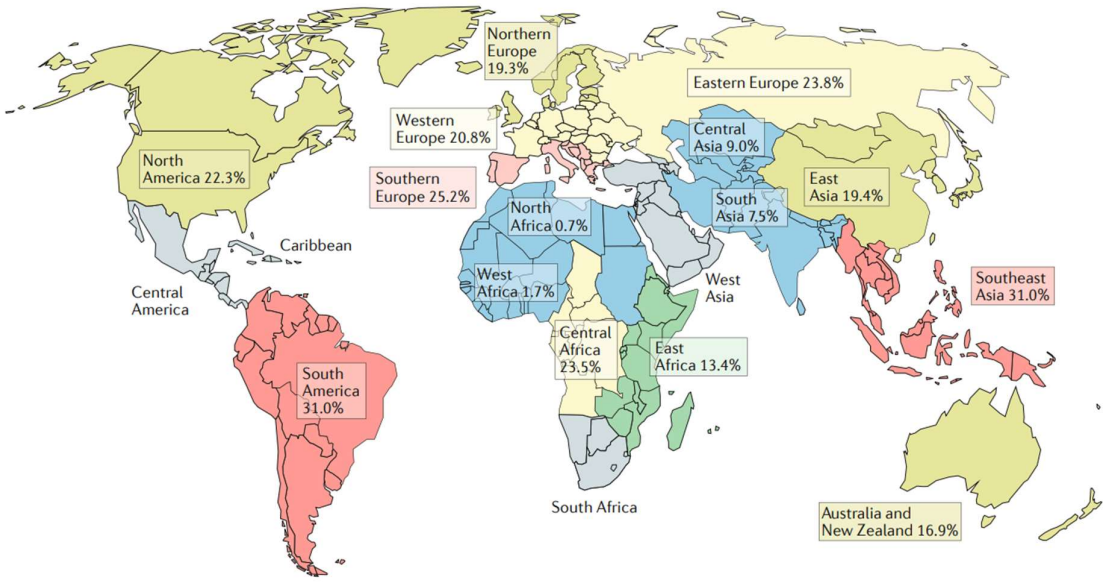


Figure 0-1 AKI prevalence in population of different countries

While in the general population, AKI incidence varies from state to state, from 0.8% in Himachal Pradesh(8), 0.83% in Karnataka(11), and 4.1% in Tamil Nadu (9). This increases drastically in critically ill patients, 33% of whom are in Delhi (12). A study from southern states on pediatric AKI states that the incidence of AKI was 5.2 % in the pediatric wards and 25.1 % in the pediatric ICU. (13) A high mortality of 17.5 % was observed while dialysis was required for 14.5 % of admitted patients. (8–10) AKI may be community-acquired (CA-AKI) or hospital-acquired (HA-AKI). CA-AKI has an incidence rate of 0.41% to 2.5% and is majorly caused due to diarrhea, malaria, and sepsis. (14,15) HA-AKI patients who developed AKI after a hospital stay due to any other disease were studied, with an AKI rate of 0.88% (North India (16)) and 2.1% (Central India (17)). The primary cause of HA-AKI

was found to be nephrotoxic drugs, decreased renal perfusion, major surgery, and sepsis. (16,17)

A global comprehensive analysis of 312 cohort studies comprising 49 million patients (mainly from high-income nations) concluded that one in five adults and one in three children hospitalized with acute illness suffer from AKI, accounting for an incidence rate of 20-33%. (18) This underscores the significant health burden AKI causes on the health care system. Studies have reported mortality rates of AKI ranging from 2% to 5% in different settings and grades of kidney damage. While up to 67% of patients admitted to the intensive care unit experience AKI development. (19) In another study from 2014 of 72 countries concluded from 320 participating centers that overall, nearly 65 percent of AKI cases were community-acquired rather than developing in the hospital setting. (20) When considering only developing, the figures reverse, i.e., the hospital acquired-AKI figure is higher than community-acquired–AKI. (21)

Acute kidney injury (AKI) is a commonly encountered syndrome associated with various aetiologies and pathophysiological processes leading to decreased kidney function. In addition to retention of waste products, impaired electrolyte homeostasis, and altered drug concentrations, AKI induces a generalized inflammatory response that affects distant organs. Complete recovery of kidney function is uncommon, which leaves these patients at risk of long-term morbidity and death. Estimates of AKI prevalence range from <1% to 66%. Population differences and inconsistent use of standardized AKI classification criteria can explain these variations. (19) The etiology and incidence of AKI also differ between high-income and low-to-middle-income countries. High-income countries show a lower incidence of AKI than do low-to-middle-income countries, where contaminated water and endemic diseases such as malaria contribute to a high burden of AKI. Outcomes of AKI are similar to or more severe than those of patients in high-income countries. (21) In all resource

settings, suboptimal early recognition and care of patients with AKI impede their recovery and lead to high mortality, which highlights unmet needs for improved detection and diagnosis of AKI and for efforts to improve care for these patients (19)

In India, the incidence of end-stage renal disease (ESRD) is 150–200 per million population, which with India's population comes to more than 0.3 million patients of ESRD, which require repeated dialysis and may require a kidney transplant in the future, while the approximate prevalence of CKD is 800 per million populations, i.e., more than 1.2 million living with CKD. (22) Chronic illness fatalities in India are predicted to increase from 3.78 million in 1990 (40.4% of all deaths) to 7.63 million in 2020 (66.7% of all deaths). (23) According to the Indian CKD Registry data, the overall prevalence of CKD in India is approximately 17%. The prevalence increases with age, and CKD is more common in older individuals. (23)

The disease burden of diabetes in the Indian population is increasing drastically with upward mobilization in economic terms and rapid urbanization. India had around 7.1% of its population diabetic in 2009, which increased to 8.9% in 2019. (24) Predisposition of diabetes enhances the chances of getting CKD drastically. Diabetic nephropathy is the most common cause of chronic kidney disease (CKD) in India, almost at par with hypertension in multiple population-based research. Diabetic nephropathy accounted for 26.1% - 41% of the cases of chronic kidney disease (CKD) in the Indian population. (22,25)

The global prevalence of CKD varies across different regions and countries. Estimates suggest that more than 10% of the worldwide population, greater than 800 million, is affected by CKD. (26) The prevalence is influenced by population demographics, healthcare access, and the prevalence of risk factors like diabetes, hypertension, and obesity. While comparing based on income levels, the prevalence of CKD in high-income countries is

9.1%, while in low- and middle-income countries, it is 11.5%; within the countries, significant regional variations in household incomes play a major role in the prevalence of CKD.(27)

Diabetes affected 537 million people globally in 2021 (eleven percent of the world's population); by 2045, that figure will rise to 783 million people (12%). (28) Compared to developed countries (5-7% now), the epidemiological load of diabetic patients will be very high for developing countries in the upcoming decades. 36% of the additional 154 million diabetics reside in China and India. (29) Reports suggest thirty to fifty percent of persons with type 2 diabetes will have high urine albumin excretion, and one in five will have an eGFR of less than 60 ml/min/1.73 m2. (30) The cumulative risk of end-stage renal disease (ESRD) due to diabetes varies significantly among populations within and between nations, ranging from less than 1% to up to 13%. (31)

1.2 Renal system

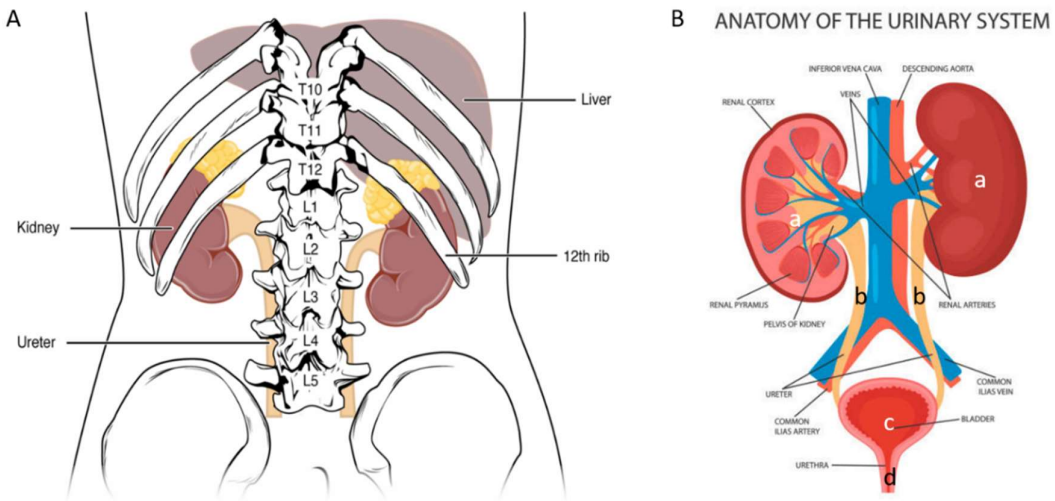


Figure 0-2 Urinary System overview A) Position of kidneys in the abdomen of human B) Anatomy of Urinary system showing a) Kidneys b) Ureter c) Bladder d) Urethra

The renal system removes metabolic waste from the human body. In humans, it mainly consists of the kidneys, ureter, bladder, and urethra. As an overview, blood pumped through the heart is filtered by ultrafiltration in the kidneys. The filtered urinary waste goes to the urinary bladder for collection via the ureter, a tubing that connects the kidneys and the bladder. Before being released outside the body, it is collected in the urinary bladder. And is passed outside the body through the urethra. (32)

1.3 Anatomy of Kidneys

Kidneys are vital and complex organs of the human body. They are bean-shaped organs located as a pair in the thoracolumbar region of the abdominal cavity, just below the ribcage on each side of the spine in higher eukaryotes. They have an average size of 9-11 cm and a volume of 150-190 cm3 (for human males).

1.4 Physiology of Kidney

Each kidney comprises several layers, including the outer cortex and inner medulla. The functional units of the kidneys are called nephrons, tiny structures within the kidney that filter the blood. Each nephron consists of a glomerulus, a small network of blood vessels, and a tubule. The glomerulus acts as a filter, allowing water and small molecules like salt, glucose, and urea to pass through into the tubule, preventing larger molecules like proteins and blood cells from entering. The tubule then reabsorbs specific molecules back into the bloodstream while excreting others in the urine. The kidneys' primary function is to filter out blood with all the accumulated metabolic waste produced in the body. Its other primary functions are maintaining blood pressure by controlling fluid balance, producing hormones, and controlling RBC production, essential in regulating homeostas

When blood is pumped via the suprarenal abdominal aorta in the heart to the lower abdomen, a fourth of it gets disturbed to the kidneys via renal arteries. The blood is redistributed into multiple segmental arteries and, in turn, into interlobar arteries in the cortex of the kidneys. Blood from here is redistributed into 1000s of nephron via an afferent arteriole. The nephron is the basic unit of blood filtration and reabsorption in kidneys, consisting of the glomerulus, proximal and distal convoluting tubule, and collecting duct. The blood enters the glomerulus, a cluster of tiny blood vessels that filter blood. Through differences in the internal pressures, the small molecules in the blood are extracted from it, and the filtrate is pushed to the proximal convoluting tubule.

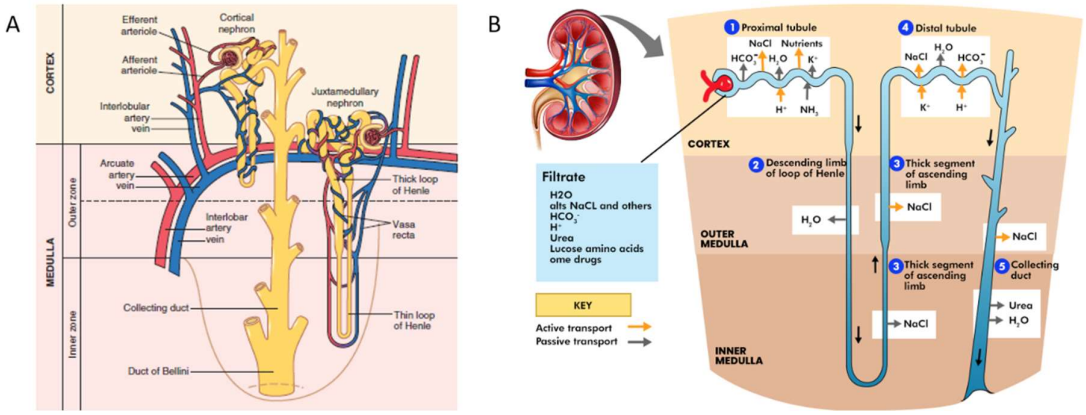


Figure 0-3 Inside the kidney A) Structure of nephron B) Figure explaining reabsorption in kidney after filtration at glomerulus

The heavier molecules, i.e., plasma proteins antibodies, etc., and cells, i.e., RBC and WBC, come out of the glomerulus and move to the efferent arteriole. This tubing consists of multiple segments with highly specialized functions and shapes (cellular morphology and diameter of lumen) and moves in the cortex and medulla of the kidney. (34) In these different segments, filtrate is extracted from water, small molecules, sugars, and electrolytes via active or passive transportation back to the blood via a mesh of tiny capillaries surrounding these tubules. The filtered blood, devoid of waste and consisting of reabsorbed nutrients and

electrolytes, goes back to the body via the renal vein. The final concentrated waste empties into the collecting duct, which finally empties into the ureter. The amount of micromolecule absorption is controlled according to blood and body homeostasis. (35)

The kidneys also produce several hormones that help regulate blood pressure and red blood cell production. One of these hormones, renin, is released when blood pressure is low and triggers a cascade of reactions that produce angiotensin II, a potent vasoconstrictor that raises blood pressure. Another hormone the kidneys produce, erythropoietin, stimulates the bone marrow to produce red blood cells. (36)

1.5 Acute Kidney Injury

Acute kidney damage is characterized by a sharp reduction in excretory capacities and a decline in renal shape, worsening the electrolyte imbalance and build-up of nitrogenous waste in the body. (37) Renal malfunctioning and dysregulation of its homeostatic state may develop due to ischemia, crystal nephropathy, sepsis, hypoxia, or nephrotoxicity, which subsequently triggers the inflammatory response, tubular obstruction, necroptosis, and apoptosis. Kidney Injury decreases renal efficiency, consequently increasing the risk of development of cardiovascular diseases and uremia and may lead to mortality. (38) Early detection and treatment of AKI are essential for the body's normal functioning. Based on etiologist, AKI can be classified as pre-renal and characterized by azotemia, intrinsic kidney disease (involving acute tubular necrosis, glomerular nephritis, interstitial as well as vascular nephritis), and post-renal obstruction of the urinary tract, e.g., Ureter obstruction. (37)

1.5.1 Stages of Assessment of Acute Kidney Injury

AKI involves diverse functional kidney conditions ranging from mild and self-limiting conditions to severe end-stage renal disease and can be classified by two methods: RIFLE and AKIN (Acute Kidney Injury Network) (39,40)

Table 1 Different stages of acute kidney injury according to RIFLE criteria.

Stage	Creatinine / GFR	Urine output
R (isk)	**1.5-fold increase** in serum creatinine / GFR reduction of 25% or more	**less than 0.5** ml/kg/h for at least **6 hours**
I (njury)	**2-fold increase** in serum creatinine / GFR reduction of 50% or more	**less than 0.5** ml/kg/h for at least **12 hours**
F(ailure)	**3-fold increase** in serum creatinine / GFR reduction of 75% or more	**less than 0.3** ml/kg/h for at least **24 hours**
L (oss)	**persistent renal failure** (after week 4)	
E (SRD)	**chronic kidney disease** (after month 3)	

Table 2 Different stages of acute kidney injury according to AKIN criteria

Stages	Serum Creatinine levels	Urine output
1	1.5- 2-fold increase by 0.3 mg/dL from baseline	Less than 0.5 mL/kg/h for six h
2	2-3- 3-fold increase from baseline	Less than 0.5 mL/kg/h for 12 h
3	More than 3-fold increase from baseline or ≥4.0 mg/dL with an acute rise of ≥0.5 mg/dL	Less than 0.3 mL/kg/h for 24 h or anuria for 12 h

Both the above assessment criterion is based on serum creatinine and urinary output. The degree to which urine production decreases or the serum creatinine level rises indicates the severity of AKI, as creatinine is considered a marker for kidney filtration efficiency, i.e., glomerular efficiency and removal of metabolic waste. The urinary output indicates the filtration rate of the kidneys, and its decrease suggests an obstruction in the kidneys.

1.5.2 Pathophysiology of AKI

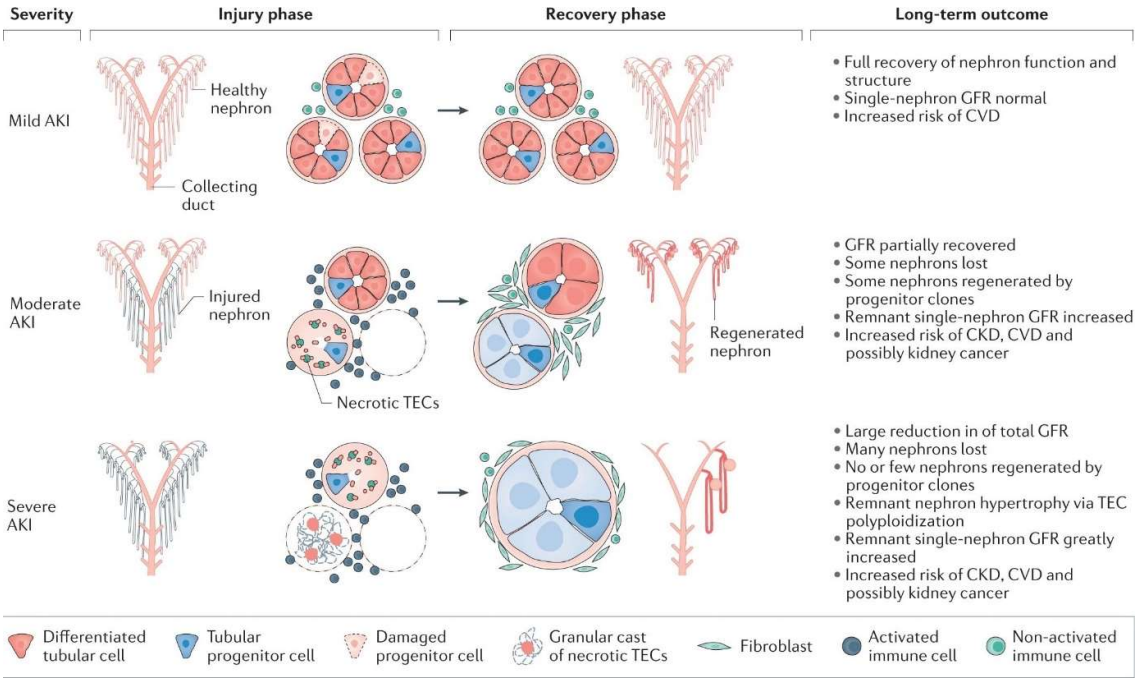


Figure 0-4 Pathophysiology of Acute Kidney Injury

During the initial stages of Acute kidney injury (AKI) development, temporary reduction in excretory function or urine production and minimal loss or necrosis of kidney cells are observed. After which, immune cells are activated in the interstitial compartment, and afflicted cells are irreversibly lost due to acute inflammation. Some wounded nephrons may recover due to the clonal growth of renal progenitor cells. When damaged nephron segments do not heal, they die irreversibly and are replaced by fibrous tissue that preserves the remaining nephrons' structural integrity. Loss of filtration capacity stimulates remaining nephrons to grow in size. When nephrons are lost permanently, kidney lifespan shortens, predisposing the kidney to CKD. With severe AKI, a significant number of nephrons die and are accompanied by widespread tubular necrosis. The diameters of the few remaining nephrons (megalonephrons that have increased in size) significantly expand due to adaptation to filtration and metabolic demands. With this significant damage, the remaining

nephrons work at their extremes and often cause secondary localized segmental glomerulosclerosis and eventual loss of the remaining nephrons, or progressive chronic kidney disease development. Interstitial fibrosis and progressive kidney atrophy are caused by polyploidization and senescence related to cellular adaptability, as well as scarring associated with nephron loss. (38,41,37)

During renal diseases, there may be systematic problems in the whole kidney, like in the case of diabetic kidney disease, where, due to glucotoxicity, the working of the entire kidney deteriorates. In some disease conditions, only a segment of kidney formation of internal kidney stone or a single kidney is disrupted, like in the case of stone formation or case of unitary urinary obstruction (UUO), or case of the renal ischemic condition. (39)

1.5.3 Chemically induced acute kidney Injury.

Chemically induced acute kidney injury (AKI), also known as drug-induced acute kidney injury, is a significant clinical concern that can occur as a result of exposure to certain drugs or chemicals. It is characterized by a sudden decrease in renal function over a variable period, ranging from hours to days. The chemical toxicity causes changes in glomerular hemodynamic, tubular cell toxicity, inflammation, and crystal formation in the kidneys.(42)

Drug-induced nephrotoxicity is a major cause of kidney dysfunction and can potentially lead to fatal consequences. The incidence of drug-related acute kidney injury (AKI) may be as high as 60% in hospitalized patients. (43) A wide range of drugs and chemicals have been associated with AKI, including aminoglycosides (such as tobramycin, gentamicin, amikacin, and others), antimicrobial agents, Chemotherapy drugs in cancer patients, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), and certain Chinese herbal medicines. Drug-induced AKI accounts for 19–26% of all hospitalized cases of AKI, depending upon the drug used and its dose.(44)

The kidney is a primary target for drug-induced toxicity due to its role in the excretion of waste products, including drugs and their metabolites. It also has a high blood flow and contains transporters that can actively uptake drugs, thereby concentrating them and increasing the risk of toxicity.

In addition to drugs, exposure to certain environmental chemicals can also lead to nephropathy. For instance, exposure to heavy metals such as mercury can cause severe injury to the kidney.(45) It's important to note that the severity and progression of chemically induced nephropathy can vary greatly among individuals. This can be influenced by several factors, including the dose and duration of exposure, the individual's health status, and their genetic predisposition to kidney disease.(46) In some cases, the damage may be reversible if the chemical exposure is stopped and appropriate treatment is initiated. However, in other cases, the damage may be permanent and can lead to end-stage renal disease (ESRD). (47)

Chemically produced xenobiotic drugs have been known to have toxic effects that depend upon the duration and dosage of the drug. In the case of nephrotoxicity, drugs can affect different parts of the kidney. For instance, it can cause damage to the tubules, interstitium, or both. (48) Calcineurin inhibitors and vasopressors contributes to the nephrotoxicity of afferent and efferent arteriolar vasoconstriction.(49) Altered intra-glomerular hemodynamics has been observed in patients prescribed with nonsteroidal anti-inflammatory drugs (NSAID), angiotensin converting enzyme inhibitors (ACEI), and angiotensin receptor blockers (ARB) and are responsible for the decline in renal function in them.(50) The proximal tubule plays a major role in concentrating and reabsorbing the glomerular filtrate. Due to high concentration of excreted drugs and their dose dependent toxicity causes kidney injury associated with multiple drugs like aminoglycosides, Calcineurin inhibitors, cisplatin, antiretroviral, etc.(51) pH-dependent crystal formation in

the distal tubular lumens accounts for the nephrotoxicity of acyclovir, sulphonamide, methotrexate, indinavir, and triamterene.(52) Tumor lysis syndrome is a condition that is most frequently seen after chemotherapy for high-grade lymphoproliferative cancers. The release of cellular metabolite after chemotherapy, like potassium, phosphorus, and nucleic acids converting to uric acid. These can also precipitate as calcium phosphate crystals and uric acid crystals in kidneys causing AKI. (53,54)

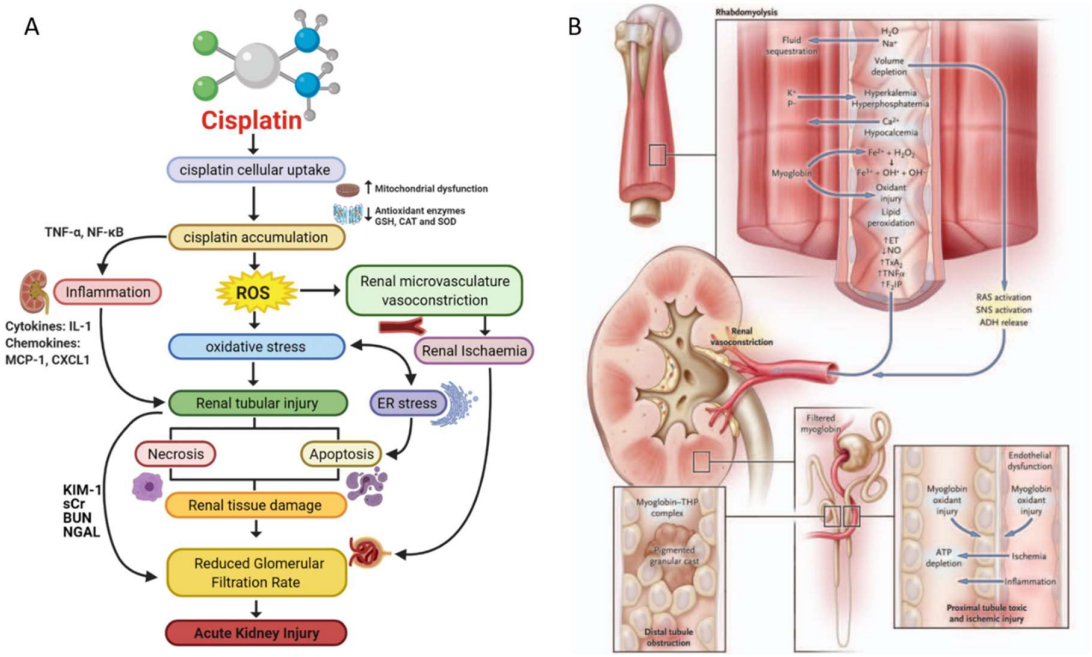


Figure 0-5 Physiological and Molecular manifestation of Acute Kidney Injury due to A) Chemo-toxicity B) Rhabdomyolysis

1.5.4 Rhabdomyolysis

Rhabdomyolysis is a condition characterized by the breakdown of skeletal muscle fibres, causing the release of intracellular myoglobin, into the bloodstream and then excreted in the urine. This can result in a range of complications, including acute kidney injury, electrolyte imbalances, and metabolic acidosis. In severe cases, rhabdomyolysis can be life-threatening.

One of the most serious complications of rhabdomyolysis is acute kidney injury (AKI). (55) The pathophysiology of AKI in rhabdomyolysis is complex and involves a combination of factors, including direct toxicity of myoglobin to renal tubular cells, intrarenal vasoconstriction, and tubular obstruction. The severity of AKI in rhabdomyolysis is directly proportional to the degree of muscle damage and the amount of myoglobin released in the bloodstream. Myoglobin can damage the kidneys by blocking the small tubes that filter waste products from the blood, causing inflammation in the kidneys, and directly damaging the kidney cells.(56)

Rhabdomyolysis can be caused by a number of things, including crush injuries, overexertion, certain medications, illegal drugs, and medical conditions such as diabetes and thyroid disease.(57) Clinical manifestation of myoglobinuria (i.e. presence of myoglobin in urine) induced AKI, may include dark red or brown urine, decreased urination, muscle pain and weakness, abdominal pain, nausea and vomiting, fever, rapid heart rate, confusion, and dehydration.(58)

In normal conditions the glomerulus filters myoglobin and is reabsorbed by tubular epithelial cells by endocytosis but when its violently released during muscle injury it overwhelm the kidney reabsorption. This excess myoglobin causes intrarenal vasoconstriction, direct and ischemic tubule injury, and tubular obstruction. Myoglobin becomes concentrated along the renal tubules, a process that is exacerbated by volume depletion and renal vasoconstriction. Myoglobin precipitates when it interacts with the Tamm–Horsfall protein, a process that is promoted by acidic urine forming miniature crystals and hindering urine flow.(59) Direct tubule cytotoxicity mostly affects the proximal tubules, while tubule blockage mostly affects the distal tubules. Heme containing proteins like myoglobin include iron in the form of ferrous oxide (Fe^{2+}), which is required for the binding of molecular oxygen. Molecular oxygen, on the other hand, might encourage the

oxidation of Fe2+ to ferric oxide (Fe3+), producing a hydroxyl radical in the process. This oxidative potential is counter acted by effective intracellular antioxidant molecules. However, cellular release of myoglobin leads to uncontrolled leakage of reactive oxygen species, and free radicals further enhancing cellular injury. (56)

1.6. Chronic Kidney Injury

1.6.1 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder that causes persistent high blood glucose levels in a patient's body. It may be caused due to disease condition, surgery, lifestyle changes and other environmental factors. This is one of the leading causes for mortality in humans in metabolic diseases category and excluding cancers and infectious diseases. India a large country which was marred with infectious diseases mortality from 50‘s to 90’s has become the epicentre of diabetes in these 20 years for this century. With rapid urbanization and lifestyles/food preference changes India will be the capital of diabetes in the next 30 years. (60) Now 11.4 % of India’s population in diabetic i.e. 101 million people while 15.3% i.e. 136 million are prediabetes. The load of this disease will increase with time. (61)

Discussing diabetes, it is important to know how the blood sugar is controlled in the body. As a small overview, we have checks and balances in metabolism of the body. Whenever we eat the body starts digesting and breaking down the food into small constituent molecules that can be easy absorbed by the gut. These absorbed micro molecules/ nutrients are transported by the blood all over the body.(62) When in reaches pancreas it senses the increased level of glucose in blood and release insulin molecules in the blood stream. (63) It is important to note that glucose is the energy molecule of the body and it has to be stored and consistent levels maintained in the body and if the same molecule is high for long in the

body it becomes and toxin. (64) As a result of release of this insulin it starts acting on hepatic cells in the liver and excess glucose is absorbed from the blood to be stored as polymer of glucose in form of glycogen molecules. (65) This acts as a feedback loop and lowering of blood glucose stops release of insulin from pancreas. If this feedback does not stop the release of insulin the person can go in hypoglycaemic shock. While if this regulation is somehow disturbed and constant high blood glucose levels are maintained this known as diabetes. (63)

There are many types of diabetes mellitus which were categorised but we will be discussing on only two for relation of our work i.e. Type I and Type II. (66)

Type I diabetes mellitus is known as Insulin Dependent Diabetes mellitus (IDDM) as it is caused due to non-formation of insulin. This is also known as adolescent diabetes as it mainly starts at the age of 12-16. This is caused by genetic factors during adolescence and later life due to surgical removal of pancreas. In this condition the body loses the ability to produce Insulin molecules, and this causes diabetes. Type-1 diabetes can be further classified as Type-1A and Type-1B. Type-1 A is an autoimmune disorder, characterized by the presence of antibodies against islet cells, anti-glutamic acid decarboxylase (anti-GAD), IA-2, IA-2f3, and anti-insulin antibodies. Replacement of insulin from the outside in form of drug is prescribed to type I diabetes patients, as a long term treatment. (67)

The Type II of diabetes is Non-Insulin Dependent Diabetes Mellitus (NIDDM). This type of diabetes as the name suggests is independent of amount of insulin in the blood. Type-2 diabetes is the most common and accounts for 80-95% of diabetic cases worldwide. Type-2 diabetes is more complex in pathogenesis and characterized by an abnormality in insulin action and secretion. It is typically diagnosed in the middle-aged population, likely in the fourth decade of life but can have an early onset. In initial stages of insulin resistance in

T2DM results in decreased glucose absorption in muscle, hepatic and adipose tissue and increased glucose synthesis in the liver in a feedback loop. As the disease progresses, there is an abundant production of insulin from the pancreas, while its effect is low due to the development of insulin resistance. This causes the failure of β-cell in the pancreas as the disease aggravates and insulin production drops, causing permanent malfunction in keeping blood sugar levels within normal range. Taken together persistent hyperglycaemia causes deleterious effects at micro and microvasculature in whole body. Important risk factors associated with the development of type-2 diabetes include a sedentary lifestyle, smoking, excessive alcohol consumption, and physical inactivity. T2DM is also attributed to several metabolic changes, including obesity, hypertension, dyslipidaemia, and an increased risk of atherosclerosis. (68)

1.6.2 Diabetic Nephropathy

As discussed above ESRD can also be caused due to long term gradual deterioration of kidney health that may be caused due to drug toxicity, repetitive stone formation, metabolic disorders and can amalgamate into a form of chronic kidney injury (CKI). This gradual decrease in normal kidney function due to consistent high glucose levels in blood causes damage to small blood vessels in the kidney manifesting the disease into diabetic nephropathy. (69)

It is important to understand that disease condition i.e. in kidney may not show within a month or year of been diabetic but may take several years to reach the level of been termed as diabetic nephropathy. This condition is very closely connected to level of control of diabetes and may or may not develop into diabetic nephropathy. People with both type 1 and type 2 diabetes are at risk for developing diabetic nephropathy. (69)

It begins with increase in kidney and glomerular volume. Significant structural changes particularly thickening of glomerular basement membrane and mesangial expansion occur after years of being diabetic.(70) With turns into microalbuminuria, a condition in which small amounts of albumin leak into the urine i.e. plasma protein have started to seep into urine because of decrease in filtration capacity of kidneys. As the disease progresses, more albumin leaks into the urine. This stage is called microalbuminuria or overt nephropathy. (71) During the development of diabetic nephropathy new types of collagens appear gradually substituting the physiologically occurring collagen types. This is due to the death of natural occurring cell linings dying due to hyperglycaemia and the space been filled with new collagen to repair the damage. (72) Similar morphologic and structural changes can also be observed in renal interstitium and the glomerular arterioles. (73)

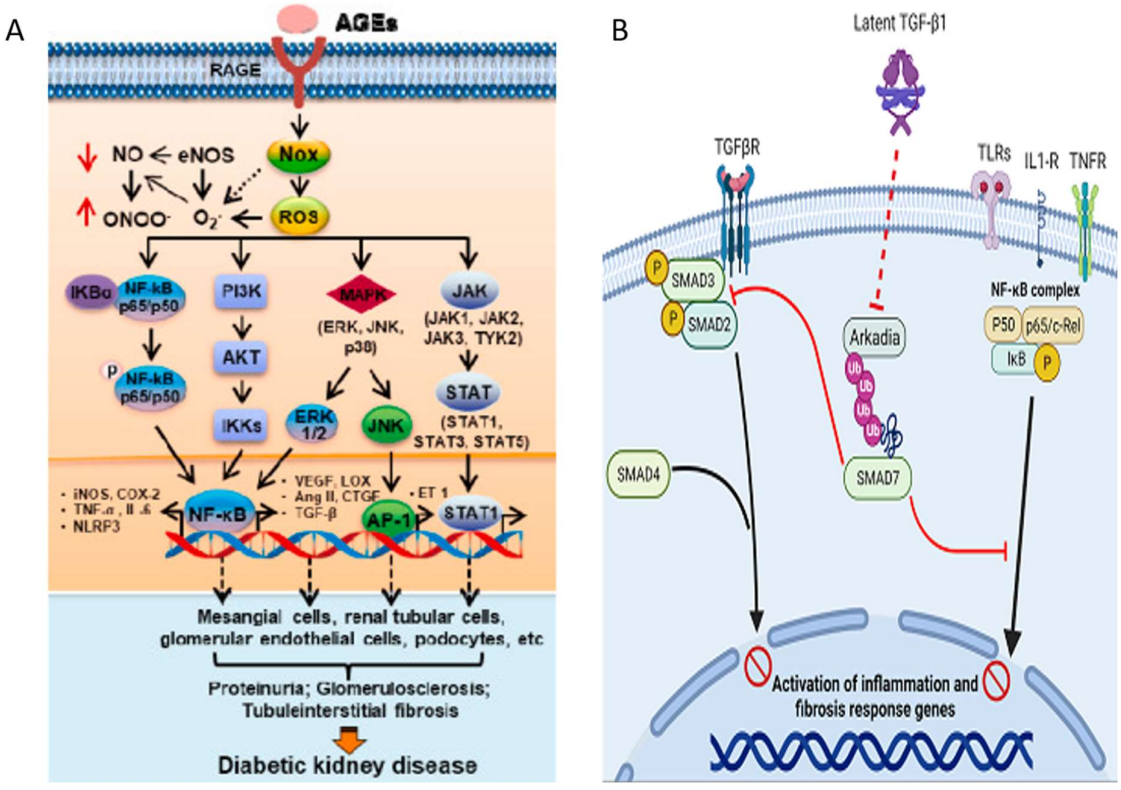


Figure 0-6 Diabetic Nephropathy Glucotoxicity due to formation of A) Advance Glycated End Products B) Activation of TGF-β1

Two different mechanisms of glucotoxicity are discussed for development of DN in kidneys.

A) Formation of Advance Glycosylated End products

Due to presence of high amount of glucose molecules, they non-enzymatically react with proteins by the chemical reactivity of its carbonyl group, glycosylation of proteins happens. A well studies glycosylation of long life-time proteins like collagens is well documented. The glycations of proteins proceed further resulting in the formation of advanced glycation end products (AGE products). These AGE products exert pathophysiological relevant activity in renal cells by getting accumulated in cortex of kidneys and sclerosis of glomeruli is observed in diabetic patients. (74) Reports indicate that accumulation of AGE in tissues is associated with their possibly toxic effects. These include either cross-linking of long-lived matrix proteins or quench nitric oxide, both by chemical reactions. (75) It has been shown that AGEs increase vascular permeability, promote the influx of mononuclear cells, and induce the production of growth factors and cytokines.(76) This AGE-mediated kidney damage happens through a receptor for AGE (RAGE) . The cells include macrophages, vascular endothelial and smooth muscle cells and mesangial cells express these receptors. Stimulation of this receptor by AGE-modified proteins results in increased oxidative stress and activation of the transcription factor NF-kB.(77) Activation of this factor is involved in the expression of genes of the inflammatory response, such as cytokines, growth factor inducible nitric oxide synthesis and RAGE. (78)

B) Glucose mediated toxicity

Elevated glucose concentrations may also exert toxic effects by glucose transporter-mediated entrance into the cell and subsequent enzymatic conversion. Several pathways have been identified which are activated upon high ambient glucose concentration like pertaining to activation of TGF-B1. (79) The activation of sorbitol pathway is one of these

causing increase in oxidative stress, activation of protein kinase C (PKC), and activation of the hexosamine pathway. (80) These pathways lead to increased cytokine and growth factor production. This increased oxidative stress has also been shown to induce cytokine expression in diabetes. The experiments show that hyperglycaemia-induced formation of oxygen radicals is mediated by glucose metabolism giving it a feedback loop. Transforming growth factor b1 (TGF-b1) is the first and most prominently induced growth factor during glucotoxicity in renal cells. (79) One of the key features of diabetic nephropathy is the accumulation of extracellular matrix (ECM) proteins, leading to the development of renal fibrosis. TGF-β1 is a potent inducer of ECM production and deposition, primarily through the activation of Smad signalling pathways. Increased TGF-β1 expression in the diabetic kidney promotes the synthesis of collagens, fibronectin, and other ECM components, leading to the progressive expansion of the renal interstitium and impaired kidney function. TGF-β1 signalling pathway involves the phosphorylation and activation of Smad proteins, which then translocate to the nucleus to regulate gene expression. (81) In addition to the canonical pathway, TGF-β1 can also activate non-Smad signalling pathways, such as the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway, which further contribute to its biological effects. (82)

1.7 Biomarkers of AKI and CKI

AKI biomarkers can be divided into functional or damage biomarkers. Function can be assessed as markers that can be assessed without any biopsy and those can be assessed with biochemical analysis of body fluids like blood and urine. These can be increased serum creatinine levels and blood urea nitrogen levels, presence of plasma proteins in urine (a marker of kidney excretory function) and reduced urinary output (oliguria). Loss of kidney excretory function implies disturbances in the main function of the kidneys (maintaining

homeostasis), for example, through excretion of metabolic waste products. Manifestation of AKI is not necessary show up in above biochemical markers as kidney being one of the most important organ for maintaining homeostasis.(83) Markers like decline of glomerular filtration rate (GFR) can activate renin-angiotensin system promoting fluid retention, which can present as peripheral oedema, third-space effusions, and pulmonary congestion not directly presenting as AKI. (84) Potassium excretion is determined by urinary output and when the kidney loses its ability to concentrate or dilute urine as needed, both hyponatraemia and hypernatremia causing hyperkalaemia showing ECG abnormities, AKI is considered a medical emergency during emergency hospitalization. (85)

Damage markers that can predict the extent of damage are mostly molecular based and some require biopsy of kidney tissue. Biomarkers like TIMP-2, IGFBP-7,(86) insulin-like growth factor-binding protein 7; IL-18, interleukin 18; KIM-1, kidney injury molecule 1; (87) NGAL, neutrophil-gelatinase-associated lipocalin; α-GST, π-GST,(88) Sodium Hydrogen exchanger isoform (NHE-3) (89) are available and most of them have a very good predictive value, but limitations exist if timing of the kidney insult is unknown.(90)

One which stands out is neutrophil-gelatinase-associated lipocalin (NGAL) as its isoforms are released by the kidney and by immune cells which can be detected in urine and blood sample. It has been a high predictive, sensitive, versatile, non-invasive biomarker for AKI. In the urine of healthy individuals, the concentration of NGAL is very low. After an insult, NGAL plasma and urine levels increase considerably, suggesting a role of NGAL for the kidneys that is analogous to that of troponin for the heart. (91)

These biomarkers play critical role in development of intended remedies and mapping clinical trials for AKI patients. Tissue inhibitor of Metalloproteinase inhibitor 2 (TIMP2) and insulin-like growth factor-binding protein 7 (IGFBP7) are detectable in urine very early

phases of AKI development. KIM-1 is a type-1 membrane protein which helps in adhesion, growth and differentiation of epithelial cells. Significant increase in KIM-1 expression levels is observed in proximal tubule following ischaemic and nephrotoxic drugs induced damage. Cytoplasmic enzymes, α-GST and π-GST are expressed in proximal and distal tubular epithelial cells and presence of these enzymes in urine indicates tubular injury. Sodium transporter NHE-3 is abundantly present in apical membrane of proximal tubule, thin and thick ascending loop of Henle and any injury to the kidney results its increased excretion.(86–89)

1.8 Nesfatin-1

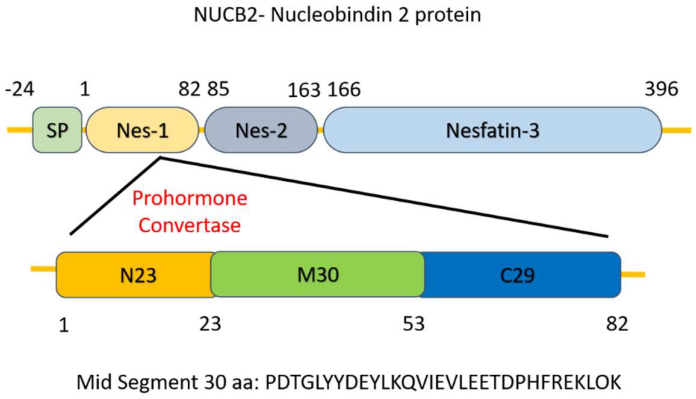


Figure 0-7 NUCB2 and its post translationally cleaved subunits

It is a recently identified peptide hormone, that has garnered much interest due to its possible functions in controlling several physiological processes. Nesfatin-1 is an 82–amino acid polypeptide derived from the precursor protein, nucleobindin 2 (gene: NUCB2), of 420 amino acid lengths. The parent protein is post-translationally cleaved into three smaller peptides, namely nesfatin-1, -2, and -3, for the last two peptides' function is unknown. (92)

It has been reportedly having diverse effects and functions, including regulation of appetite, energy homeostasis, stress response, cardiovascular function, and reproductive processes.

One of the major areas of research surrounding nesfatin-1 is its role in regulating appetite and energy homeostasis. (93) Studies have shown that nesfatin-1 acts as an anorexic hormone, suppressing appetite and promoting satiety. This anorexic effect of nesfatin-1 is independent of leptin, a very well-studied anorexic peptide. Administration of nesfatin-1 has been found to reduce food intake and body weight in animal models. (94) Additionally, nesfatin-1 has been implicated in regulating glucose homeostasis and insulin secretion, suggesting its involvement in metabolic processes beyond appetite control. (95) The exact mechanisms by which nesfatin-1 influences appetite and energy balance are still being elucidated, but it is believed to act through the modulation of neuropeptides and neurotransmitters involved in feeding behaviour and energy regulation. (96)

1.8.1 Nesfatin-1 and its effect on different organs

Initially, it was thought that nesfatin-1 is released from brain-only neurons (hypothalamic paraventricular nucleus, supraoptic nucleus, arcuate nucleus, lateral hypothalamic area, and spinal cord) regarding its diet control effect. Further research described its expression in peripheral tissues i.e. pancreas, liver, subcutaneous and visceral fat tissues, brown adipose tissue, and skeletal muscles. (92)

In Brain and Central Nervous System, nesfatin-1 is predominantly produced in the hypothalamus, a region of the brain involved in the regulation of appetite, energy balance, and other physiological processes. Nesfatin-1 was shown to cross the blood-brain barrier in both directions, supporting a humoral route of signalling.(97) Nesfatin-1 acts within the central nervous system to regulate food intake, body weight, and energy homeostasis. It influences the activity of brain regions involved in appetite control, such as the arcuate and paraventricular nuclei. (98) Knockdown of NUCB2 specifically in the paraventricular

nucleus of the hypothalamus resulted in a stimulation of food intake and a subsequent increase in body weight in rats.(99)

In gastrointestinal Tract nesfatin-1 has been detected in various regions of the gastrointestinal tract, including the stomach, intestine, and pancreas. (100) Nesfatin-1 has been shown to influence gastrointestinal motility, which refers to the movement of food through the digestive tract. It may regulate the contraction and relaxation of smooth muscles in the gastrointestinal tract, thereby affecting the rate of food transit. (101) In experimental animals it has been implicated in the regulation of gut hormone secretion and gut-brain signalling. Gut hormones such as ghrelin, cholecystokinin (CCK), peptide YY (PYY), and glucagon-like peptide-1 (GLP-1) play important roles in appetite regulation, satiety, and glucose homeostasis.(102,103) It plays and important role in release of insulin from B-cells of pancreas.(104)

In Adipose Tissue, nesfatin-1 has been shown to regulate adipocyte (fat cell) differentiation, lipid metabolism, and adipokine secretion. (105) By effecting adipo-genesis, nesfatin-1 regulate and stimulate adipose differentiation from pre-adipocytes into mature adipocytes. It has a role to play in regulation of lipid metabolism in adipose tissue. It influences the breakdown (lipolysis) and storage (lipogenesis) of fats in adipocytes by modulating the expression of genes involved in lipid metabolism. (106) Adipose tissue secretes various bioactive molecules called adipokines, which play a role in regulating metabolism, inflammation, and other physiological processes. Nesfatin-1 has been shown to act as an adipokine synthesized from adipose tissue. It should the potential to become biomarker for early detection of gastric diabetes mellitus. (107) It has been involved in the regulation of energy balance and body weight. It has been shown to affect food intake and energy expenditure, which can have implications for adipose tissue metabolism. (93)

Nesfatin-1 has a profound effect on pancreas and has been implicated in the regulation of insulin secretion from pancreatic beta-cell. It appears to have both stimulatory and inhibitory effects on insulin release, depending on the experimental context. This in turn shows its role in modulating glucose homeostasis and play a role in the regulation of blood sugar levels.

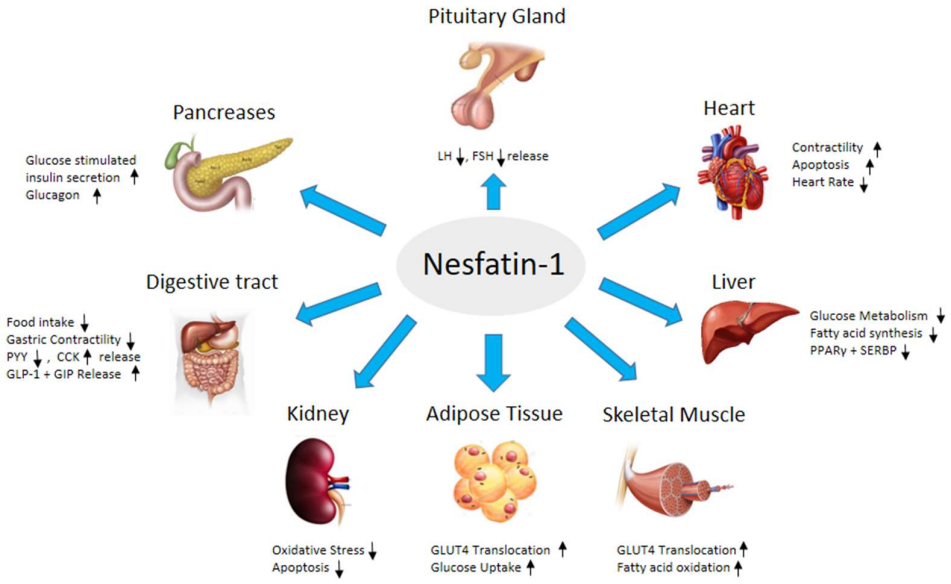


Figure 0-8 Effect of nesfatin-1 peptide on different organs

The mechanism of glucose-induced insulin secretion from the pancreas by nesfatin-1 is due to its effect by Ca2+ influx through L-type channels in mouse islet β-cells and release of insulin containing vacuoles in the blood stream.(104)

Nesfatin-1 has been found to exert cardiovascular effects, including the regulation of blood pressure and heart rate. It may act on blood vessels and the heart to influence vascular tone, cardiac contractility, and cardiac output. (108) Nesfatin-1 has been reported to elicits influence on the heart by depressing contractility and relaxation via a cGMP-linked pathway, and by inducing cardio protection against Ischemia-Reperfusion injury. (109) In a study nesfatin -1 has produced cardio protective effect against isoproterenol-induced

myocardial infarction caused heart damage by exerting its inhibiting oxidative stress, cell apoptosis, and myocardial fibrosis.(110)

1.8.2 Nesfatin – 1 and its role in Renal system and disease conditions

Nesfatin-1, a peptide hormone initially discovered for its role in appetite regulation and energy balance, has also been studied in relation to kidney disease. While the understanding of the exact mechanisms involved is still evolving, research suggests that nesfatin-1 may have implications for kidney function, inflammation, and the development and progression of kidney diseases.(111,112)

Nesfatin-1 expression have been found in various parts of the kidney, including the renal tubules, glomeruli, and collecting ducts. This indicates that nesfatin-1 may have a local regulatory role within the kidney itself. Studies have shown that nesfatin-1 administration can increase urine output and decrease sodium reabsorption in the kidney, suggesting a potential role in the regulation of water and electrolyte balance. (113)

Its effect in the regulation of blood pressure. Animal studies have demonstrated that nesfatin-1 can affect renal blood flow and vascular tone, potentially influencing blood pressure control. Dysregulation of nesfatin-1 levels or signalling may contribute to hypertension, a common risk factor for kidney disease. (113)

Expression of nesfatin-1 in different organs of body during neonatal to adult development shows its importance during organ development. In contrast expression of nesfatin-1 in adult kidneys is higher comparison to fetal kidneys in mice comparison to other organs like heart, lungs, and intestine. (114)

Inflammatory processes and fibrosis play a significant role in the development and progression of kidney diseases. Nesfatin-1 has been shown to have anti-inflammatory

properties and can modulate the production of pro-inflammatory cytokines. Additionally, studies have suggested that nesfatin-1 may inhibit renal fibrosis, which is a hallmark of chronic kidney disease. (115)

In population study of diabetic kidney disease, positive correlation between the quantity of nesfatin-1 in plasma and stage of disease has been observed. (116) Nesfatin-1 expression has been investigated in *in vitro* model of high glucose induced renal injury and it has shown to protect against by reducing oxidative stress, inflammation, and fibrosis. (111)

In kidneys, anti-oxidative and anti – apoptotic effect of nesfatin-1 has been studied in acute kidney injury model of renal ischemia-reperfusion injury. (112) In an another study of AKI unilateral ureteral obstruction models in animals its anti-oxidative properties and it effect on inhibition of renal fibrosis has been observed. (115) Studies have reported loss of nesfatin-1 expression in *in vitro* model of H_2O_2 induced oxidative stress and effect of exogenous nesfatin-1 in its reversal. (111)

Research related to nesfatin-1 and kidney disease is relatively limited, and many aspects of its involvement in renal physiology and pathology are under and have to be investigated. The potential therapeutic applications of nesfatin-1 in kidney disease and its precise mechanisms of action have yet to be fully understood. Existing studies reported above highlight potential of nesfatin - 1 as a target for future research and therapeutic interventions in the field of kidney disease.

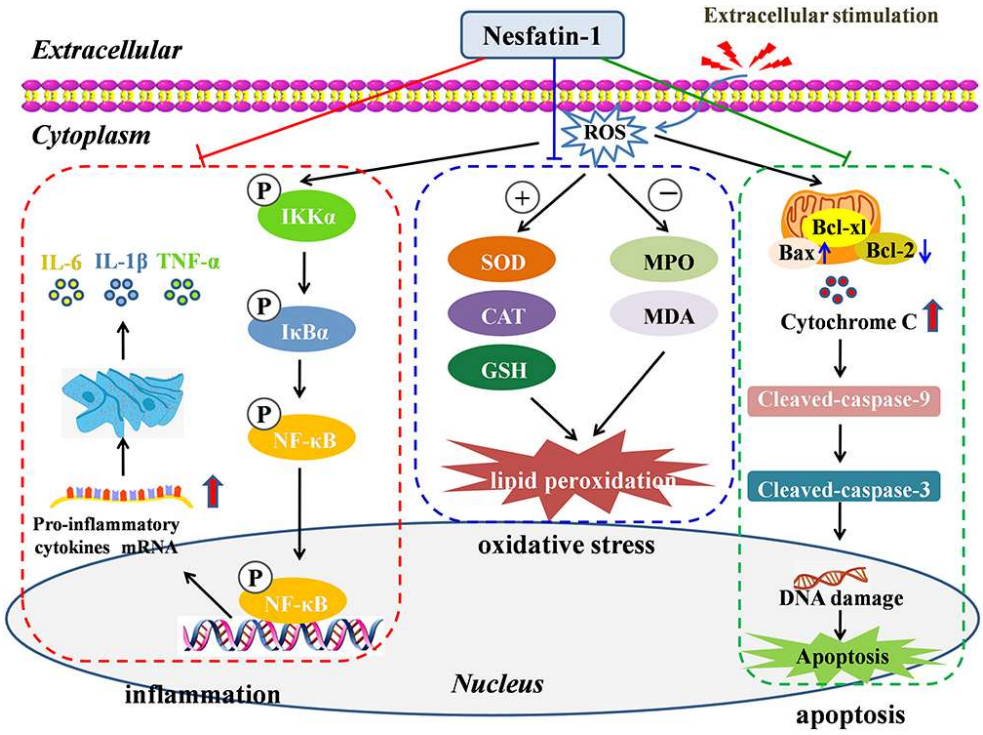


Figure 0-9 Nesfatin-1 treatment and its action on ROS, inflammation, and apoptosis

1.8.3 Oxidative stress and nesfatin -1

Oxidative stress is a condition characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defence mechanisms. ROS are highly reactive molecules that can cause damage to cells, including lipids, proteins, and DNA. It is constitutively produced in a cell and is successfully chelated under normal homeostatic conditions. Excessive oxidative stress has been implicated in the development and progression of various diseases, including cardiovascular disease, neurodegenerative disorders, and kidney disease.

Multiple publications give insight on nesfatin-1 effect on reducing oxidative stress by enhancing the body's antioxidant defence mechanisms. Nesfatin-1 has been found to increase the production of endogenous antioxidants, such as superoxide dismutase (SOD),

Catalase and glutathione peroxidase (GPx). These enzymes help neutralize ROS and protect cells from oxidative damage.

Experimental studies have demonstrated that nesfatin-1 can protect against oxidative damage in various tissues. For example, in the context of cardiovascular disease, nesfatin-1 has been shown to attenuate oxidative stress-induced injury to cardiac cells. Similarly, in neurodegenerative disorders, nesfatin-1 has shown neuroprotective effects by reducing oxidative stress and preserving neuronal function.

Effect on mitochondria, as it is energy-producing organelles within cells, but is also a major source of ROS. Dysfunctional mitochondria can lead to increased ROS production and oxidative stress. Nesfatin-1 has been found to regulate mitochondrial function and maintain mitochondrial integrity, which in turn can help mitigate oxidative stress.

1.8.4 Inflammation and Nesfatin-1

Inflammation is part of defence mechanism of an organism from internal or external agents, acting as a site recognition step. It may cause due to toxin bacterial infections injury. At the site of inflammation, the immune cells are rushed making it act as a location of problem. Excessive inflammation also has its down side causing edema at the side, chronic inflammation can also cause diseases like rheumatoid arthritis, cardiovascular disease etc. (117) Research suggests that nesfatin-1 has anti-inflammatory properties and can modulate inflammatory processes in various tissues and disease conditions. (118)

Oxidative stress often goes hand in hand with inflammation.(119) Nesfatin-1 has been shown to possess anti-inflammatory properties, and by reducing inflammation, it can indirectly decrease oxidative stress. (120)

Nesfatin-1 has been found to regulate the production and release of pro-inflammatory cytokines, which are key mediators of inflammation. In experimental studies, nesfatin-1 administration has been shown to inhibit the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), causing reduction in inflammation.(112)

It has been observed that nesfatin -1 can modulate the activity of immune cells by suppressing the activation and inflammatory response of macrophages and inhibit the proliferation of lymphocytes, suggesting an immunomodulatory role. (121) Chronic inflammation in gut can cause inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis. Nesfatin-1 has been shown to reduce the production of pro-inflammatory cytokines in the gut, inhibit the migration of immune cells to the inflamed tissues, and promote the healing of intestinal epithelial cells. (122)

Inflammation in the central nervous system, neuro-inflammation is involved in various neurological disorders. Nesfatin-1 inhibits the activation of microglial cells, the immune cells of the central nervous system, and reduce the production of pro-inflammatory cytokines in neuro-inflammatory conditions. This suggests a potential role for nesfatin-1 in modulating neuro-inflammation and protecting against neurological disorders. (123)

Chronic low-grade inflammation is associated with obesity and contributes to the development of obesity-related complications such as insulin resistance and cardiovascular disease. (124)Nesfatin-1 has been investigated in the context of obesity-related inflammation and show reduction in release of pro-inflammatory cytokines from adipose tissue and inhibit the activation of inflammatory pathways associated with obesity. (125)

The mechanisms underlying the anti-inflammatory effects of nesfatin-1 are still being investigated. It is believed to act through various signalling pathways, including the

modulation of nuclear factor-kappa B (NF-κB) signalling, which is a key regulator of inflammation. (126) However, the exact mechanisms and receptors involved in mediating nesfatin-1's anti-inflammatory effects are not fully understood.

1.8.5 Apoptosis and nesfatin -1

Apoptosis is a programmed cell death process that plays a crucial role in maintaining tissue homeostasis, eliminating damaged or unwanted cells, and regulating development and immune responses. Nesfatin-1, a peptide hormone, has been investigated for its potential role in modulating apoptosis. (118)

Studies have suggested that nesfatin-1 has anti-apoptotic properties in various tissues and cell types and same has been observed in animal models. In neuronal cells, nesfatin-1 has been found to protect against apoptosis induced by various insults, including oxidative stress and neurotoxic agents. (127)

Nesfatin-1 can modulate the activity of key signalling pathways involved in apoptosis. It has been shown to regulate the expression and activity of pro- and anti-apoptotic proteins. For instance, nesfatin-1 has been found to upregulate the expression of anti-apoptotic proteins, such as B-cell lymphoma 2 (Bcl-2), while downregulating the expression of pro-apoptotic proteins, such as Bcl-2-associated X protein (Bax) and caspases. (126)

Apoptotic effect of nesfatin -1 was also studied in Ischemia/reperfusion injury, occurs when blood supply to an organ or tissue is temporarily interrupted and then restored. This process can induce apoptosis in the affected cells. Nesfatin-1 has shown protective effects against ischemia/reperfusion injury in various organs, including the heart, liver, and kidney. (112,128,129)

Nesfatin-1 has been investigated its effects on apoptosis in tumor cells. With some ambiguous reports, it shows both pro-apoptotic and anti-apoptotic effects, depending on the specific cancer type and cellular context. In some cases, nesfatin-1 has been found to induce apoptosis and inhibit tumor growth, (130) while in other cases, it has been shown to promote cell survival and enhance tumor progression. (131,132)

It's important to note that the precise mechanisms by which nesfatin-1 modulates apoptosis are still being elucidated. The hormone likely acts through multiple signalling pathways, including those involving protein kinases, transcription factors, and cell survival and death regulators.

1.8.6 Fibrosis and Nesfatin -1

Fibrosis is a pathological process characterized by excessive accumulation of extracellular matrix components, such as collagen, leading to tissue scarring and organ dysfunction. Nesfatin-1, a peptide hormone, has been studied in relation to fibrosis.

Several studies have indicated that nesfatin-1 exhibits anti-fibrotic properties in various organs and tissues. It has been shown to attenuate fibrosis and reduce the deposition of extracellular matrix components. For instance, in experimental models of lung fibrosis, Nesfatin-1 treatment prevented BLM-induced collagen synthesis in lungs and suppressed the activation of lung fibroblasts by inhibiting TGF-β1/Smad signalling pathway in an AMPKα-dependent manner.(133) Similarly, in models of cardiac and renal fibrosis, nesfatin-1 has been shown to reduce fibrotic tissue remodelling and inhibit the expression of fibrotic markers.(115,129)

Nesfatin-1 can modulate signalling pathways involved in fibrosis. It has been shown to inhibit the transforming growth factor-beta (TGF-β) pathway, a key pathway implicated in fibrotic processes.(133) TGF-β is a potent inducer of fibrosis, promoting the activation of

fibroblasts and the production of extracellular matrix components. In a model of doxorubicin induced cardiotoxicity nesfatin-1 has been found to suppress TGF-β signalling, thereby inhibiting fibrotic responses.(134)

Chronic inflammation and fibrosis are closely linked. Nesfatin-1's anti-inflammatory effects may contribute to its anti-fibrotic properties. By modulating inflammatory responses, nesfatin-1 can indirectly influence fibrotic processes. It can inhibit the production of pro-inflammatory cytokines and reduce the infiltration of immune cells, which contribute to the initiation and progression of fibrosis.

Chapter 2 Research Objectives

The principal objectives of our studies are to investigate the role of Nesfatin-1 peptide in emolliating acute kidney injury and diabetic nephropathy and study its molecular mechanism.

1. To correlate expression of NUCB2 in *in vitro* cultured cells and acute kidney injury models.
2. To check possible effects of nesfatin-1 treatment in rhabdomyolysis model of AKI in *in vitro* and *in vivo*.
3. To investigate the direct effect of nesfatin-1 in *in vivo* Diabetic Nephropathy disease model and investigate it's the molecular mechanism.

Chapter 3 Role of nesfatin-1/NUCB2 in acute kidney disease models in wistar rats and *in vitro* cultured cells.

3.1. Introduction:

Acute renal injury (AKI) formerly known as acute renal failure is a heterogeneous group of disorders characterized by sudden loss of kidneys excretory function caused by hypovolemia, renal ischemia, kidney stones, ureter blockage and chemotherapeutic agents that result in significant morbidity and mortality. This sudden onset of renal failure causes a sudden spike in serum creatinine and urea levels while decreasing glomerular filtration rate. The pathogenesis of AKI is essentially unknown and is linked to worse outcomes (6,135,136). Currently there is not specific treatment available for AKI that can either prevent the progression or slow down the progression of the disease. AKI patients are also at increased risk of subsequent chronic kidney disease. Several hormones and peptides are reported to be altered in AKI. Thus, new therapeutic avenues like renal biomarkers might help with early diagnosis of AKI (137,138).

Recently, a new peptide, nesfatin-1 expression was identified in pancreatic β cells, gastric mucosa and adipose tissue of humans and rodents. Nesfatin-1 is reported to possess anorexic and anxiogenic properties (92,139). Nesfatin-1 is an 82 amino acid peptide discovered in 2006 by Oh-I et al in the rat hypothalamus and is post-translationally processed from nucleobindin2 (NUCB2).(92) Nesfatin-1 exhibits pleiotropic actions in the cardiovascular system as well as plays a role in stress response.(139) Nesfatin-1 was reported to be expressed in the brain stem, cerebrospinal fluid and hypothalamic nuclei initially and involved in control of appetite in rats. (97) Since nesfatin-1 immuno-reactive cells were reported to be expressed in pancreatic β-cells of mouse and rats (95), possibly suggesting a potential role for nesfatin-1 in pancreatic islet and glucose regulation (140). Nesfatin-1 expression was also detected in the subcutaneous fat tissue of humans and rodents (141). Also, nesfatin-1 expression has been reported in the male reproductive system of humans and rodents (142). Korani and Sonbol reported significantly higher plasma nesfatin-1 levels

in diabetic kidney patients with positive correlation between plasma nesfatin-1 levels and the grade of diabetic kidney disease (143).

Although the pathogenesis of acute kidney injury (AKI) consists of many mechanisms, there is increasing evidence for an important role of inflammation, oxidative stress and apoptotic responses in the complex process of AKI (135). Even though, several studies have reported nesfatin-1 is expression in various organs in humans and rodents (144,145), however, more is needed to know about nesfatin-1/NUCB2 expression in kidneys and under AKI condition. Therefore, we examined the expression of nesfatin-1 in kidneys of AKI models and compared them with the expression in healthy rats. We first investigated whether nesfatin-1 is expressed in kidneys by western blotting analysis. Thereafter, the nesfatin-1 protein expression in the kidneys of AKI models were measured and compared to expression levels in healthy rats. Moreover, we also used *in vitro* model of AKI by using NRK-52E renal epithelial cells and whether nesfatin-1 treatment can attenuate the changes induced by doxorubicin. Thus, the aim of this study was therefore to determine nesfatin-1 expression in AKI models in rats and to evaluate plausible relationships of nesfatin-1 with oxidative stress, inflammation and morphological changes *in vitro* in renal epithelial cells.

3.2. Methodology and Chemicals:

3.2.1 Animals

Male Wistar rats at eight weeks of age (180–210 g) were purchased from Sainath Agencies, Hyderabad, India and housed under standard conditions, with food and water available ad libitum, in a room with a 12/12-h light/dark cycle and controlled temperature of 24-26 ºC and humidity of 60-65%. Animals were acclimatized for one week before start of the experiments. Animals were randomly divided into four different groups (Control n=6, Cisplatin n=6, Doxorubicin n=6, and Glycerol n=6). All the animals used in the study were approved by Institutional Animal Ethical Committee (IAEC) of Birla Institute of Technology and Science Pilani, Hyderabad Campus bearing a protocol approval number: BITS/HYD/IAEC/2019/21. We also confirm that all the experiments were carried out in accordance with the guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Environment, Government of India, for care and use of experimental animals. Moreover, animals used were purchased from registered CPCSEA breeder to ensure proper maintenance and health of the animals.

3.2.2 Chemicals

Cisplatin and doxorubicin were purchased from Sigma Aldrich, India and glycerol from Himedia, India. Nefatin-1 peptide from Novus Biologicals (cat no: NBP2-35072). Eosin form Sigma chemicals (cat no: E4009), and haematoxylin from Himedia (cat no: 51275), Blood urea nitrogen kit cat no. 120241 and creatinine cat no. 120246 were procured form Trans Asia Bio-medicals Ltd, India. Paraformaldehyde, RIPA buffer (Sigma Aldrich, India) Immuno-Blot PVDF Membrane was purchased from Bio-Rad (Hercules, CA, USA), Bovine serum albumin from Himedia, India. Protease inhibitor cocktail and PMSF from Sigma

Aldrich, India. cDNA synthesis kit PrimeScript™ RT reagent (RR037A), Sybr Green containing PCR master mix, Trizol reagent RNAiso plus (9109) were purchased from Gene Bioscience, India.

3.2.3 Antibodies

A primary antibody for nesfatin-1 (PA5-77384) was purchased from Thermofisher Scientific, Invitrogen (MA, USA). β-actin (sc-47778) and goat anti-mouse IgG-HRP (sc-2005) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA)

Experimental design and Tissue collection

For inducing AKI, male wistar rats were administered cisplatin (7.5mg/kg) and doxorubicin (15mg/kg) intraperitoneally (i.p.). For inducing rhabdomyolysis for the glycerol group, animals were fasted and dehydrated 24 hours prior to injecting glycerol (50% prepared in normal saline) intramuscularly at 10ml/kg divided between both thigh muscles/hind limb muscles. For cisplatin and doxorubicin group, blood samples were collected 24 hours after injections while for glycerol group blood was collected 24-48 hours after injections. Serum creatinine and blood urea nitrogen were accessed using assay kits. Kidney tissues were collected kidneys were collected for histopathology, RNA isolation and protein estimation. (146,147)

Collection of serum samples and estimation of biochemical parameters:

Blood was withdrawn from the retro orbital venous plexus of rats, after 30 minutes' serum was separated from the blood by centrifugation at 2500 rpm for 15 min and serum was analysed for creatinine, blood urea nitrogen (BUN) using assay kits (Erba Liquixx Pvt. Ltd. India).

3.2.4 Western blotting

Total protein from rat kidney tissues was extracted in snap freezing in RIPA buffer after which it was subjected to homoginization using bead beater. The sample was centriguged to remove tissue particulates while dissolved protein was extracted as supernatant. Further protein was quantified using BCA assay. Normalized equal quantity of protein was loaded on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for saperation on basis of Molecular weight before subjecting to western blot. Protein was transferred to Polyvinylidene difluoride (PVDF) membranes in chilled transfer buffer under the conditions of 70 V for 3 hrs at 4°C. Protein bands were stained with ponceau S solution in order to check the transfer of proteins to PVDF. Subsequently, the membrane was washed three times with TBST buffer with 5 min interval. The membrane was blocked with 5% skim milk for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibody. Then the membrane was incubated with secondary antibody for 2 h at room temperature. Thereafter, the membranes were incubated with a secondary antibody for two h at room temperature, and after washing thrice with PBS-T protein bands were detected with a chemiluminescence kit using an Eppendorf fusion solo chemidoc machine (148). .

3.2.5 Cell Culture

Normal rat kidney epithelial cell line (NRK-52E) was obtained from National Centre for Cell Sciences, Pune, India. After receiving cells were maintained in T25 flasks, using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin antibiotic solution. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C. $5x10^6$ cells per ml were seeded in T25 flasks, and grown up to 80% confluency before treatment was given. Cells were co-treated with nesfatin-1 (1nM) in the presence of nephrotoxic agent doxorubicin for 24h.

3.2.6 *In vitro* Cell culture treatment

Rat NRK-52E epithelial cells were treated with different concentrations of doxorubicin (0.5 µM, 1 µM, 10µM) and nesfatin-1 (0.5nM, 1nM and 10nM) for 24h. Based on the MTT cell viability assay, doxorubicin-1 µM and nesfatin-1 1nM was used for further studies.

3.2.7 Real time Polymerase Chain Reaction (RT-PCR)

RT-PCR was done for the determination of mRNA expression. Protocol for RNA isolation is taken from Invitrogen RNA isolation protocol with minor changes were done wherever required. For tissues, small section of tissue was cut while isolating it from animal and directly added to 500ul trizol reagent and kept in -80 °C till further usage. Trizol containing tissue was subjected to bead beater for its disruption and centrifuged to settle cellular debris. Supernatant was transferred to new tube. While for cells after finishing treatment protocol, media from cells was removed and cells washed with PBS. Trizol 500ul was added to each well and plates left for 5 minutes are RT cellular disruption by trizol. After this protocol for tissue and cells are same. 100ul of chloroform was added to the samples and mixed and left for 5 min at RT to settle. Samples were subjected to centrifugation at 13000 rpm for 30 mins at 4 °C. Aqueous top layer was transferred gently to a new tube and 500ul of 100% isopropanol was added to the tubes. After mixing well sample were subjected to centrifugation as above. RNA pellet will settle down and supernatant was discarded. The pellet was subjected two washes of 70% ethanol with centrifugation after each step. At the end pellet was and air dried and quantified. 1000ng of total RNA was converted to cDNA using Bio-Rad iScript cDNA synthesis kit as per manufacturers protocol. mRNA expression of KIM-1, NGAL, nesfatin-1, SOD, catalase, caspase-3 and IL-1β were detected from synthesized cDNA. Reaction mixture of cDNA, Primers and SYBR Green was prepared according to the manufacturing instructions of iTaq Universal SYBR® Green Supermix

(Bio-rad). (149) Primers were designed by using integrated DNA technology. Primer sequences for nesfatin-1, SOD1, catalase, KIM1 and NGAL is mentioned in the given Table-1.

Table 3. Primer Sequence Chapter 3

Gene	Primer Sequence Forward	Primer Sequence Reverse
SOD1	GGTCCACGAGAAACAAGATGA	CAATCCCAATCACACCACAAG
Catalase	CATGGATCTGCTTAGGACTTCTG	CCAGGCTGTGAGGTAACATAA
KIM1	GCCATTTCCACTCCACTTCT	CCTGCTCTCTCTCCTTTCTTTC
NGAL	CCCTCAGATACAGAGCTACGA	CTTCCGTACAGGGTGACTTTG
IL1b	GGAACCCGTGTCTTCCTAAAG	CTGACTTGGCAGAGGACAAA
NUCB2	GGAAAGAGGAAGAAGCCAAGT	GGTCCAATCCATCAGTCTCTTC
Caspase3	TGGAAAGCATCCAGCAATAGG	GACTCAGCACCTCCATGATTAAG
18s	CATTCGAACGTCTGCCCTAT	GTTTCTCAGGCTCCCTCTCC
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA

3.2.8 Immunofluorescence Staining

NRK-52E cells were seeded on glass cover slips which were placed in six well plate and followed by incubation with doxorubicin and nesfatin-1 for 24 hr and subjected to nesfatin-1 staining. The treated cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed twice with 0.01 N phosphate buffered saline (PBS). Cells were permeabilize with 0.1% Triton X-100 for 5 min and three washes with PBS, the cells were incubated with 3%bovine serum albumin (BSA) (in PBST) for 1 h to block non-specific binding sites. After BSA was removed from the wells, cells were incubated overnight at a dilution of 1:200 with the nesfatin-1 antibody and washed three times with PBST. Then cells

were incubated with a Texas red/ Alexa Fluor-conjugated secondary antibody for 1hr, counter stained with DAPI, placed cover slips on glass slide and analysed under laser confocal microscope (LAS-X). Slides from four different experiments were analysed with 5 fields per slide observed and averaged.

3.2.9 Crystal Violet staining

NRK-52E renal epithelial cells were seeded on coverslips till they reached confluency of 70-75%. Afterwards, cells were treated with doxorubicin (1μM) or nefatin-1 (1nM) for 24h. After 24hrs of treatment media was removed, washed with PBS and fixed with ice cold methanol. Crystal violet stain (0.5% w/v in methanol) was added to cells for 4-5 minutes. Excess of stain was removed, and cells were washed with PBS and coverslips were mounted on slides and observed under light microscope for cell morphology.

3.2.10 Statistical Analysis

The data obtained in the present study is expressed as mean± SEM, n corresponds to number of animals for each independent experiment. Results were statistically analysed by using one-way ANOVA followed with post hoc Bonferroni's test using Graph Pad Prism Software Version 5 (San Diego, CA, USA). A p value of less than 0.05 was considered as statistically significant for all the experiments.

3.3 Results:

3.3.1. Induction and establishment of AKI in wistar rats:

We first induced the AKI in wistar rats by three different drug molecules: cisplatin, doxorubicin and glycerol. Thereafter, creatinine and BUN levels which are markers of AKI were measured in serum of these rats. Significant increase in creatinine and BUN was observed in cisplatin, doxorubicin and glycerol administered rats (Fig. 3-1), indicative of establishment of AKI. Also, creatinine and BUN levels were much higher in cisplatin group compared to doxorubicin and glycerol (Fig. 3-1).

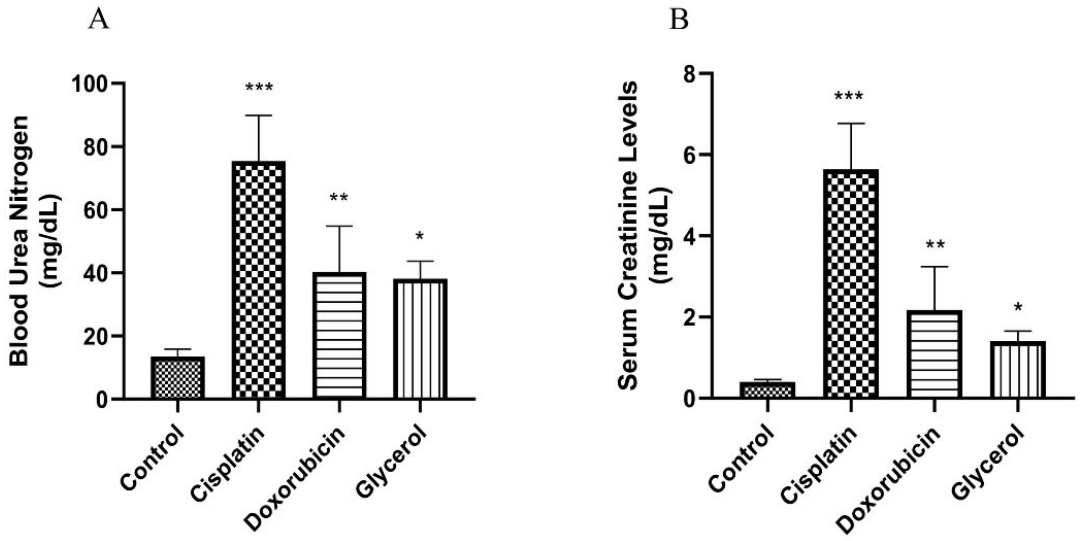


Figure 3-1 : Chemo-toxic agents, cisplatin, doxorubicin and glycerol increase serum creatinine and BUN levels.

Male wistar rats were treated with cisplatin, doxorubicin and glycerol for 24-48h. Serum creatinine and BUN levels were measured using assay kits **(A, B)**. *p<0.05, **p<0.01, ***p<0.001 *vs* control. Data is expressed as mean ± SD with n=6 in each group.

3.3.2. Upregulation of early biomarkers of AKI:

Number of protein molecules such as kidney injury marker (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) can serve as early biomarkers for the detection of AKI. KIM-1 is a type of transmembrane glycoprotein, and its expression is increased significantly in the apical membrane of proximal tubule cells of nephron after nephrotoxic injury and ischemic injury. Urinary KIM-1 levels in the urine may facilitate sensitive, specific and accurate prediction of human nephrotoxicity in preclinical drug screening apart from serum creatinine, BUN and urinary NAG. (150) NGAL, also known as lipocalin-2, was found in activated neutrophils, and other tissue while in case of kidneys it is produced in distal nephrons. NGAL has been found to have a role in kidney development and tubular regeneration after injury. NGAL may eventually have prognostic value in predicting not only acute, but also chronic, worsening in renal function in patients already affected by chronic nephropathies. (151) We examined the expression of KIM-1 and NGAL in different models of AKI. Significant increase in mRNA expression of KIM-1 and NGAL was observed in kidneys of cisplatin, doxorubicin and glycerol induced AKI (Fig. 3-2).

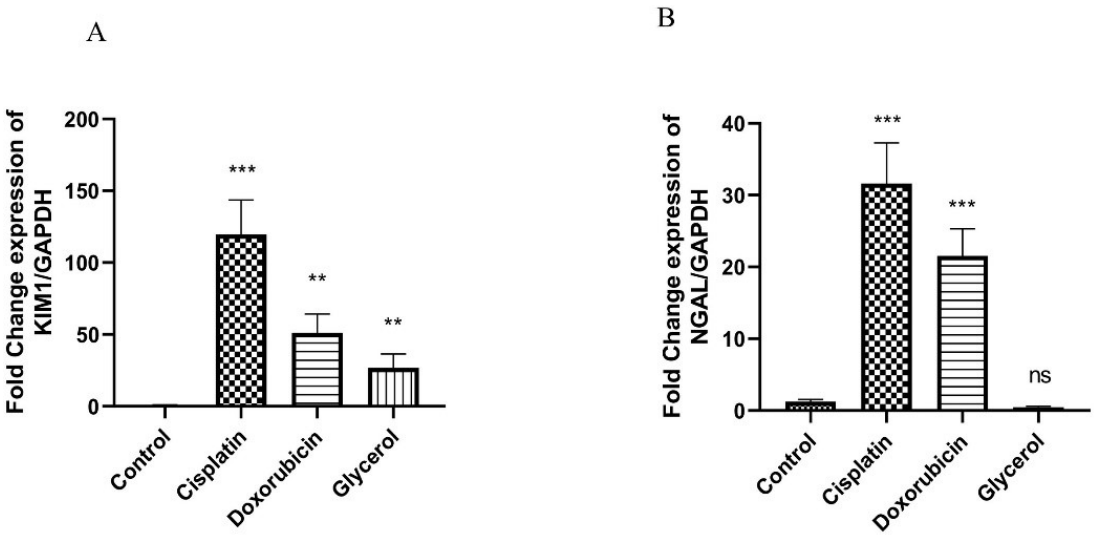


Figure 3-2 : Cisplatin, doxorubicin and glycerol increase the expression of acute kidney injury markers.

Male wistar rats were treated with cisplatin, doxorubicin and glycerol for 24-48h. mRNA expression of kidney injury markers KIM-1 and NGAL was measured by RT-PCR (A, B). **p<0.01, ***p<0.001 *vs* control. Data is expressed as mean ± SD with n=6 in each group.

3.3.3 Downregulation nesfatin-1 expression *in vivo* AKI models and *in vitro* in NRK-52E cells:

We checked whether there was change in nesfatin-1 expression in AKI models in rats. In this study male wistar rats were induced AKI with cisplatin, doxorubicin and glycerol. In all three models of AKI i.e., cisplatin, doxorubicin and glycerol, there was a significant decrease in nesfatin-1 protein expression compared to control (Fig. 3-3).

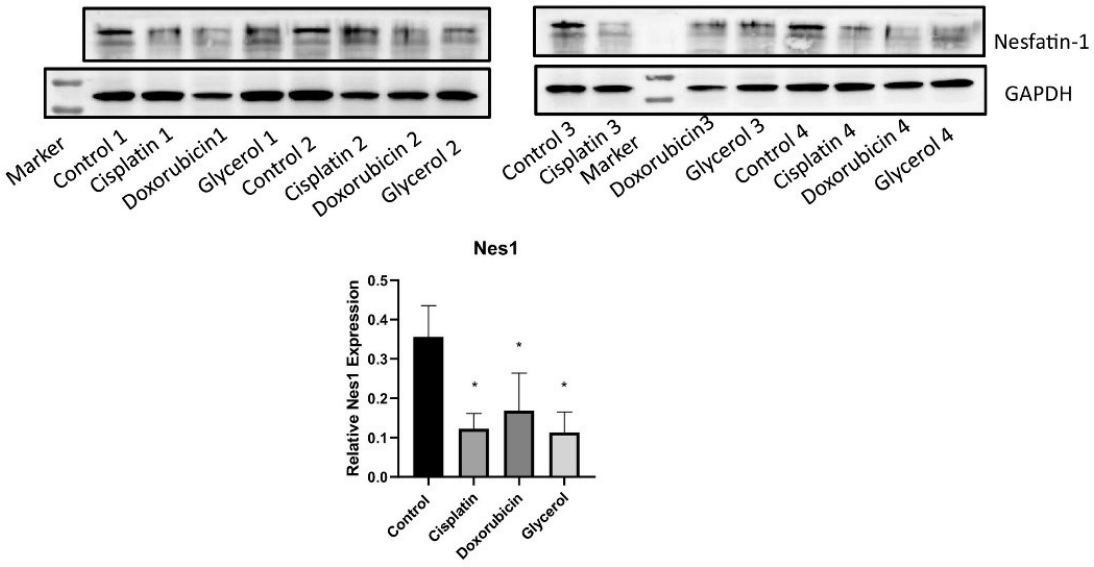


Figure 3-3 Acute kidney injury decreased the expression of nesfatin-1.

Male wistar rats were treated with cisplatin, doxorubicin and glycerol for 24-48h. Nesfatin-1 protein expression was measured by western blotting (A, B). *p<0.05 *vs* control. Data is expressed as mean ± SD with n=4 in each group

3.3.4 Altered expression of antioxidant enzymes in AKI induced models

Oxidative stress is one of the hallmark player in the pathogenesis of AKI as well as CKD. ROS are involved in a number of signalling pathways regulating cell growth and differentiation, mutagenic responses,inflammation and apoptosis. Antioxidant enzymes, such as, glutathione peroxidase, glutathione S-transferase decompose lipid hydro peroxides to alcohols. Catalase and superoxide dismutase (SOD) and glutathione peroxidase also reduce hydrogen peroxide to nontoxic substances. We checked whether nephrotoxic agent's cisplatin, doxorubicin and glycerol had influence on antioxidant enzymes, catalase and superoxide dismutase (SOD) in the kidneys of AKI induced rats. Significant decrease in the mRNA expression of catalase and SOD was observed in cisplatin and doxorubicin induced rats while significant increase was observed in glycerol induced rats compared to control (Fig. 3-4 A, B).

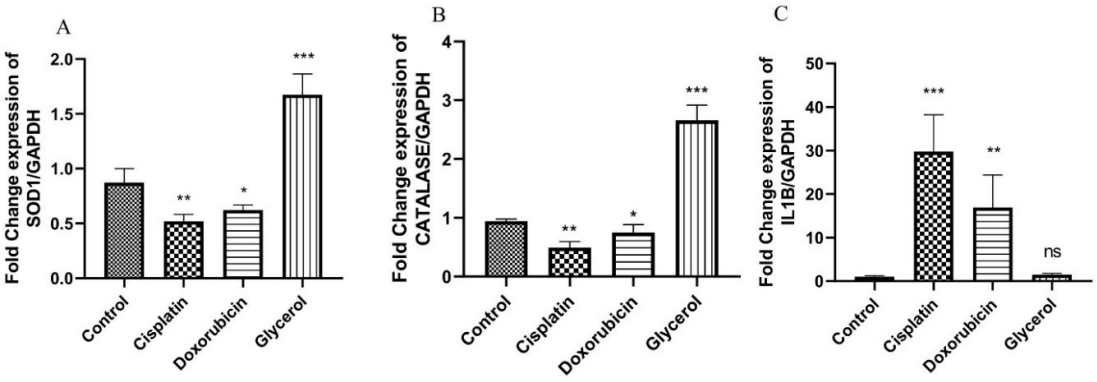


Figure 3-4 Acute kidney injury decreased the expression of antioxidant enzymes and increased expression of inflammatory markers.

Male wistar rats were treated with cisplatin, doxorubicin and glycerol for 24-48h. mRNA expression of catalase, SOD and IL-1β was measured by RT-PCR (A, B, C). *p<0.05, **p<0.01, ***p<0.001 *vs* control Data is expressed as mean ± SD with n=5 in each group.

3.3.5 Upregulation of inflammatory marker in AKI induced models.

Inflammatory cytokines such as IL-1β, IL-6 and TNF-α play a critical role in inflammation and lead to activation of various inflammatory pathways. We checked whether nephrotoxic agent's cisplatin, doxorubicin and glycerol have an effect on inflammatory marker, IL-1β in the kidneys of AKI induced models. Significant increase in the mRNA expression of IL-1β was observed in AKI induced rats compared to control (Fig. 3-4 C).

3.3.6 Cell viability and decreased expression of nesfatin-1 in renal NRK52E epithelial cells treated with doxorubicin, attenuation with nesfatin-1 treatment

We first checked cell viability by MTT assay using different concentrations of doxorubicin and nesfatin-1 for 24 h. We observed 66 % cell viability with 1 μM of doxorubicin and 80 % cell viability with 1 nM of nesfatin-1 (Fig. 3-5 A). We also checked the expression of nesfatin-1 in renal NRK-52E epithelial cells in the presence of cytotoxic agent doxorubicin. Significant decrease in protein (Fig. 3-5 B), immunofluorescence and mRNA expression of nesfatin-1 was observed in doxorubicin induced cells (Fig. 3-6 A, B) compared to control group. We also checked the nesfatin-1 mRNA expression in renal NRK-52E epithelial cells in the presence of cisplatin and myoglobin (Fig. 3-6 C, D). Significant decrease in mRNA expression of nesfatin-1 was observed in cisplatin as well as myoglobin induced cells. The mRNA expression was attenuated in doxorubicin, cisplatin and myoglobin induced cells co-treated with nesfatin-1 (Fig. 3-6). We also checked the expression of nesfatin-1 in renal NRK-52E epithelial cells in the presence of cytotoxic agent doxorubicin. Significant decrease in immunofluorescence and mRNA expression of nesfatin-1 was observed in doxorubicin induced cells (Fig. 3-5) compared to control group.

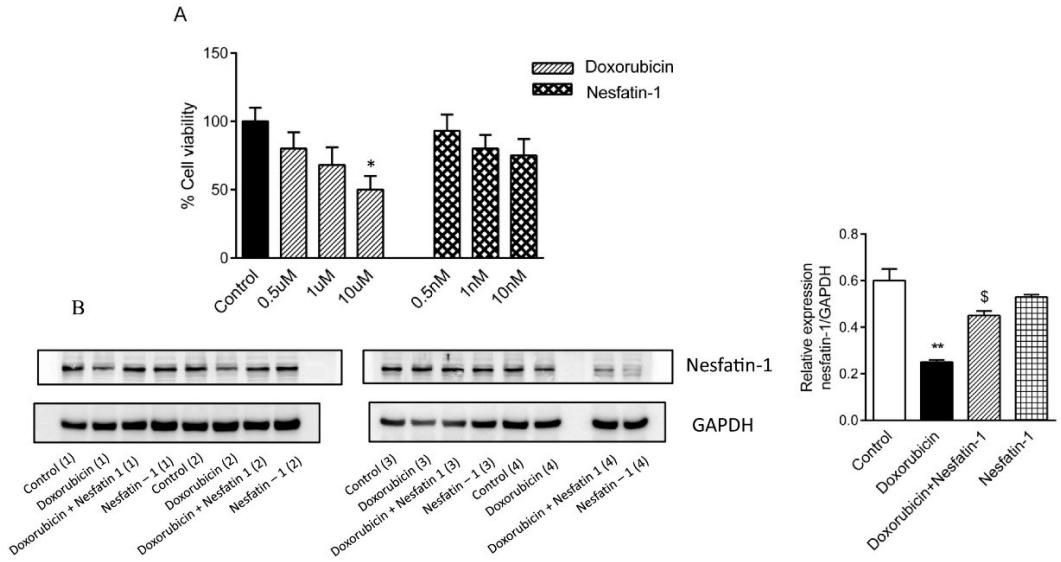


Figure 3-5 Cell viability and decreased expression of nesfatin-1 in renal NRK52E epithelial cells treated with doxorubicin, attenuation with nesfatin-1

Cell viability and protein expression of nesfatin-1 in renal NRK-52E epithelial cells: Renal NRK-52E epithelial cells were treated with different concentrations of doxorubicin (0.5 μM, 1 μM, 10 μM) and nesfatin-1 (0.5 nM, 1 nM, 10 nM) for 24 h. Cell viability was measured by MTT assay (A) (n = 8). Nesfatin-1 protein expression cultured cells treated with doxorubicin (1 μM) and nesfatin-1 (1 nM) for 24 h was measured by western blotting (B). Data is expressed as mean ± SD with n = 5 in each group. *$p < 0.05$, **$p < 0.01$ vs control; $$p < 0.05$ vs. doxorubicin group. Data is expressed as mean ± SD with n = 5 in each group

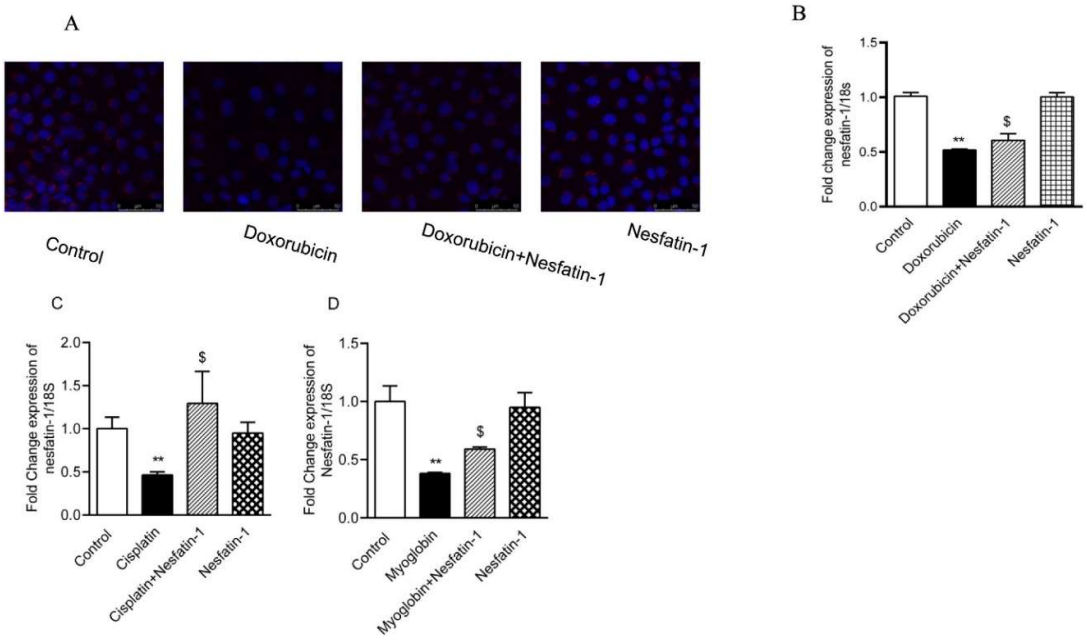


Figure 3-6 Doxorubicin decreased the expression of nesfatin-1 in renal NRK-52E epithelial cells.

Renal NRK-52E epithelial cells were treated with doxorubicin for 24 h. Nesfatin-1 immunofluorescence was measured by confocal microscopy. Nesafatin-1 mRNA expression was measured by RT-PCR. $^{**}p<0.01$ *vs* control. Data is expressed as mean ± SD with n=5 in each group

3.3.7 Nesfatin-1 protects against doxorubicin induced downregulation of antioxidant enzymes in renal NRK-52E epithelial cells

To confirm whether nesfatin-1 has any effect on anti-oxidant enzymes, NRK-52E rat renal epithelial cells were incubated with nesfatin-1 in the presence of nephrotoxic agent doxorubicin. Interestingly here also doxorubicin induced group showed significant decrease in catalase and SOD expression compared to control and significant increase in catalase and SOD expression was observed in doxorubicin induced cells cotreated with nesfatin-1 compared to doxorubicin alone group (Fig. 3-7).

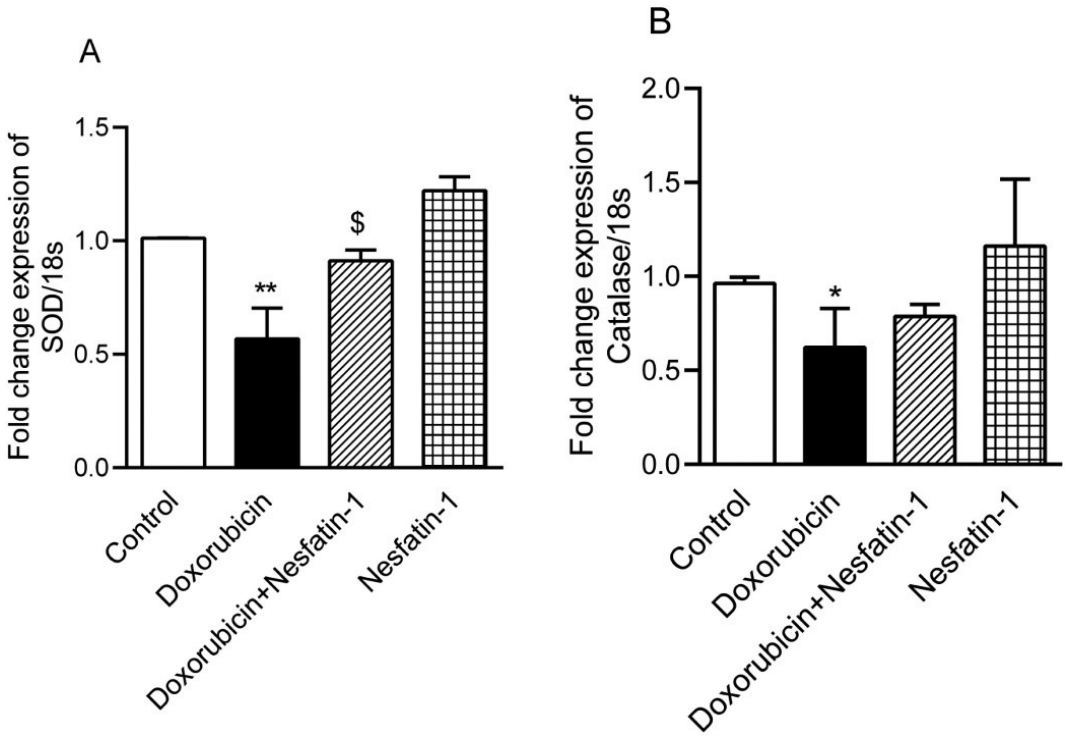


Figure 3-7 Nesfatin-1 treatment attenuated the decrease in antioxidant enzymes.

Renal NRK-52E epithelial cells were treated with doxorubicin for 24 h. Catalase and SOD mRNA expression was measured by RT-PCR. **$p<0.01$ *vs* control; $^{\$}p<0.05$ *vs*. doxorubicin group. Data is expressed as mean ± SD with n=5 in each group.

3.3.8 Nesfatin-1 protects against doxorubicin induced upregulation of inflammatory markers in renal NRK-52E epithelial cells

To confirm whether nesfatin-1 has any protective role in the regulation of inflammatory pathway, NRK-52E rat renal epithelial cells were incubated with nesfatin-1 in the presence of nephrotoxic agent doxorubicin. The doxorubicin induced group showed significant increase in IL-1β and TNF-α expression compared to control and significant decrease in IL-1β and TNF-α expression was observed in doxorubicin induced cells co-treated with nesfatin-1 compared to doxorubicin alone group (Fig. 3-8).

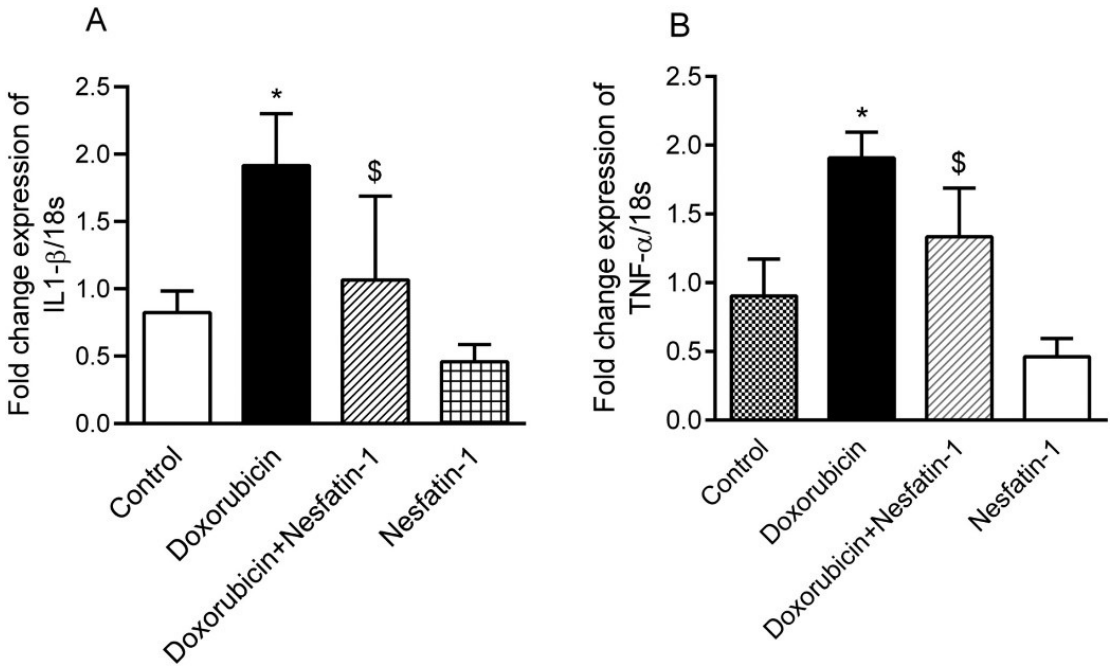


Figure 3-8 Nesfatin-1 treatment attenuated the increase in inflammatory cytokines.

Renal NRK-52E epithelial cells were treated with doxorubicin for 24 h. TNF-α and IL-1β mRNA expression was measured by RT-PCR. *p<0.05, **p<0.01 vs control; $p<0.05, $$$p<0.001 vs. doxorubicin group. Data is expressed as mean ± SD with n=5 in each group.

3.3.9 Nesfatin-1 protects against doxorubicin induced morphological changes in renal NRK-52E epithelial cells.

We also determined structural changes in NRK-52E renal epithelial cells in doxorubicin and nesfatin-1 co-treated group. Morphology of NRK-52E renal epithelial cells was observed by crystal violet staining (Fig. 3-9). In control group we observed well established morphology and well-defined nucleus whereas in doxorubicin group, we observed the cells with morphological change and nuclear swelling. In doxorubicin induced cells co-treated with nesfatin-1, morphology was preserved.

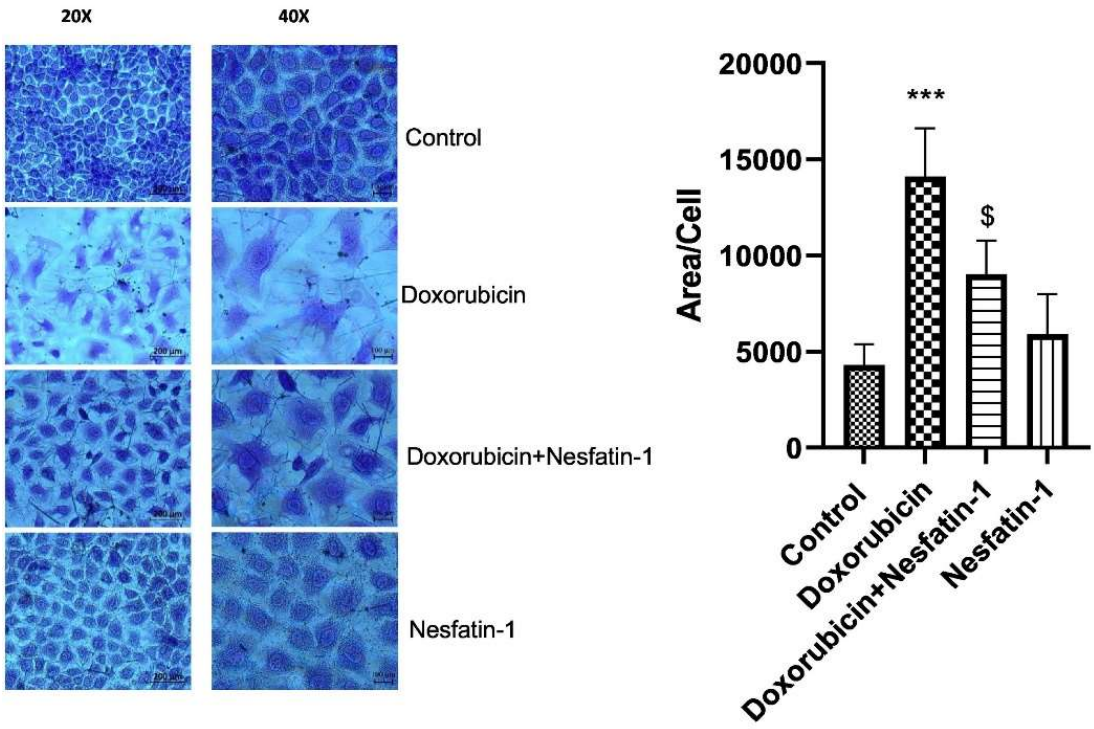


Figure 3-9 Nesfatin-1 protects doxorubicin induced structural changes renal NRK-52E epithelial cells:

NRK-52E epithelial cells were treated with doxorubicin (1 μM) (A, B) for 24 h. Nesfatin-1 (1 nM) was incubated alone or with doxorubicin for 24 h. Structural changes were observed by Crystal violet staining. Data is of at least three separate experiments ***$p < 0.001$ vs control; $\$p < 0.05$ vs. doxorubicin group. Data is expressed as mean ± SD with $n = 5$ in each group.

3.4 Discussion and Conclusions:

In the present study we report for the first time decreased nesfatin-1 expression in AKI models of rats (Fig. 3-3). Further, to investigate whether nesfatin-1 treatment can reverse, inflammation oxidative stress and morphological changes, renal epithelial NRK-52E cells were incubated with nephrotoxic agent doxorubicin for 24 h in the presence of nesfatin-1. Interestingly, we found significant decrease in gene markers of inflammation and oxidative stress in NRK-52E renal epithelial cells in doxorubicin treated cells co-incubated with nesfatin-1 (Fig. 3-6,7), which possibly suggests nesfatin-1 plays a protective role in AKI.

Nesfatin-1 is a satiety peptide that is expressed not only in neurons about also in peripheral tissues such as GIT tract, pancreatic beta cells, heart and subcutaneous fat tissue. Nesfatin-1 is reported to have various functions in different systems and metabolic processes, including blood glucose regulation, satiety, cardiovascular system and lipid metabolism. (152,153) However, the expression and role of nesfatin-1 in AKI has yet to be investigated to date. AKI was induced in rats with nephrotoxic agents, cisplatin, doxorubicin and glycerol and significant increase in biochemical markers of AKI: BUN and creatinine was observed in these models (Fig. 3-1). Moreover, significant increase in gene marker of AKI: KIM1 was observed in all three models of AKI (Fig. 3-2) indicative of the development and establishment of AKI. Kidney injury molecule -1 (KIM-1) is a robust marker for assessing kidney damage in acute conditions as it is positively correlated with extent of kidney damage. Release of KIM-1 is suggested to be sensitive to damage in proximal tubular injury which is observed in all of the three models studied. (154) Significant increase in gene expression of NGAL was observed in cisplatin and doxorubicin induced kidney injury rats (Fig. 3-2), however no significant change was observed in glycerol induced kidney injury rats (Fig. 3-2). It is an interesting observation and usually in AKI, NGAL is upregulated in glycerol induced rhabdomyolysis. In case of rhabdomyolysis there are two mechanisms of

pronged damage; first is formation of ischemic conditions in proximal tubules and myoglobin-THP complex causing distal tube obstruction. NGAL has an iron chelating property from the environment, and it is a known bacteriostatic agent due to chelation of iron causing growing bacteria deficient of iron. Possibly, when NGAL expression is increased, there is decrease in ferric iron present in the environment which will hinder formation of myoglobin-THP complex which in turn is important for formation of AKI.(56,155) This hypothesis can suggest, maybe there is a feedback present as in this study: BUN, Creatinine levels in serum and KIM-1 expression in rhabdomyolysis kidney tissue are suggestive of kidney damage while in NGAL there is not so significant change, this however, needs further investigation. Our results are also in agreement with previous studies which reported renal protein and tissue NGAL did not increase until 48 h after glycerol induced AKI. NGAL is thought currently to maintain the balance in iron levels in cells. Thus, it seems possible that when proximal tubular cells are exposed to iron-mediated oxidative stress (resulting from glycerol-mediated rhabdomyolysis and release of heme from circulating myoglobin in the tubular lumen) increased NGAL may be produced. It also is documented that NGAL is up-regulated during the time of kidney damage and participates in nephrogenic repair and regeneration.(155) Our study reports for the first time that nephrotoxic agents, cisplatin, doxorubicin and glycerol significantly decreased the protein expression of nesfatin-1 in kidneys of rats (Fig. 3-3). One study had reported nesfatin-1 was expressed in the kidney, pituitary gland, lungs, ovary, testis and stomach of fetal and adult mice. Also, the levels of nesfatin-1 increased significantly in the lung, stomach, and kidney of the mouse foetus at embryonic day 17.5. These studies suggest that nesfatin-1 may play an important role in development and other physiological processes in mouse foetus. However, further studies are required on the function of nesfatin-1 in physiological and pathological conditions.(156) One previous study reported positive correlation between

circulating nesfatin-1 and microalbuminuria in type 2 diabetic patients independent of other established risk factors of diabetic kidney disease. However, the possible molecular mechanisms and the prognostic significance of this association still needed to be investigated. (157)

Next our study analysed the correlation between decreased nesfatin-1 and catalase, SOD and IL-1β expression in kidneys of AKI. Significant decrease in catalase and SOD was observed in cisplatin and doxorubicin induced AKI (Fig. 3-4) while significant increase in catalase and SOD was observed in glycerol induced AKI (Fig. 4). Moreover, significant increase in IL-1β gene expression was observed in cisplatin and doxorubicin induced AKI while partial increase was observed in glycerol induced AKI (Fig. 3-4). To investigate the role of nesfatin-1 on antioxidant defence, inflammation and morphology, renal epithelial cells NRK-52E were co-treated with nesfatin-1 in the presence of nephrotoxic agent, doxorubicin. First nesfatin-1 immunofluorescence and mRNA expression were checked after incubation with doxorubicin and also after co-incubation with nesfatin-1. Doxorubicin significantly decreased the nesfatin-1 immunofluorescence and mRNA expression of nesfatin-1 in NRK-52E cells. Co-incubation with nesfatin-1 attenuated the decreased nesfatin-1 expression (Fig. 3-5). Interestingly, co-treatment of nesfatin-1 with doxorubicin significantly increased the expression of antioxidant enzymes SOD and catalase (Fig. 3-6). Tang et al reported that nesfatin-1 has anti-oxidative and anti-apoptotic effects through its neuro-protective effects against subarachnoid haemorrhage that induced brain injury and inhibited apoptosis in rats.(158) Our observations are in in agreement with previous studies done by other researchers. Previous studies have reported that nesfatin-1 can improve gastric injury through its antioxidant effects. (159,160) Furthermore, Jiang et al showed that nesfatin-1 could protect the kidney from ischaemia reperfusion injury by decreasing the

oxidative stress and inhibiting apoptosis, inflammatory cytokines and increased the reactive oxygen species and malondialdehyde content following high glucose treatment.(112)

In our study, we found that doxorubicin significantly increased the expression of IL-1β and TNF-α in cultured NRK-52E cells (Fig. 3-7). Cytokines such as IL-1β and TNF-α are pro-inflammatory markers and they play a critical role in various inflammatory pathways such as JNK, and NF-kB pathway activation further downstream. We observed that treatment with nesfatin-1 could prevent activation of TNF-α and IL-1β (Fig. 3-7) in renal NRK-52E cells. Pro-inflammatory cytokines, especially TNF-α, IL-6, and IL-1 exert directs harmful effects on the kidney. (161) Moreover, change in morphology with decreased cell viability and nuclear swelling was observed in renal epithelial cells treated with doxorubicin. The cell structure and number were preserved in doxorubicin group co-treated with nesfatin-1 (Fig. 3-8). AKI induced by chemotherapeutic agents is often associated with morphological changes and apoptotic and necrotic death of renal tubular cells. (162) Doxorubicin is a potent anticancer agent and is reported to cause oxidative stress in numerous cells. Doxorubicin leads to activation of mitochondrial dependent as well as mitochondrial independent pathway of apoptosis. Additionally, doxorubicin causes unfolding of the chromatin structure of the DNA, which possibly explains increased nuclear swelling observed in doxorubicin treated NRK-52E cells. (163) These findings suggest that nesfatin-1 might be a novel therapeutic agent for attenuating AKI induced renal injury.

In conclusion, our data shows that cisplatin, doxorubicin and glycerol induce AKI in rats along with decreased expression of nesfatin-1 and activate the oxidative stress and inflammatory pathways via down-regulation of SOD, catalase and upregulation of IL-1β and TNF-α. Treatment with nesfatin-1 attenuated the doxorubicin induced oxidative stress inflammation and morphological changes in NRK-52E renal epithelial cells. Thus, treatment

with nesfatin-1 can be one of the possible approaches to inhibit various inflammatory and oxidative stress pathways in AKI.

Chapter 4 Protective effect of nesfatin-1 treatment in rhabdomyolysis model of AKI in *in vitro* and *in vivo*.

4.1. Introduction

Rhabdomyolysis is a condition in which skeletal muscle tissue breaks down, releasing large amounts of cellular content including electrolytes, myoglobin, and other sarcoplasmic proteins into the bloodstream. It can be caused by a variety of factors, including injury, infection, drug toxicity, and muscle exertion. When untreated causes acute kidney injury and in known as myoglobin-induced acute kidney injury.(56)

In rhabdomyolysis, its onset is early and kidney health deteriorates within hours of muscle damage. As myoglobin reaches the kidney it gets concentrated in renal tubules due to water reabsorption and renal vasoconstriction, and precipitates after interacting with Tamm–Horsfall protein causing obstruction in distal tubules. (164) Myoglobin being a heme protein contains iron ion required for binding to oxygen. This molecular oxygen can promote the oxidation of Fe2+ to ferric oxide (Fe3+), thus generating a hydroxyl radical causing damage to proximal tubules. Due to this damage, the renal capacity decreases causing a sudden spike in serum creatinine and urea levels and decreases glomerular filtration rate. (165)

Nesfatin-1 a peptide hormone, initially found to have expressed in hypothalamus and has anorexic properties, independent of the leptin pathway, was found to be expressed in multiple organs of the body including pancreatic β cells, gastric mucosa and adipose tissue of humans and rodents. (92,99) Nesfatin-1 exhibits pleiotropic actions in multiple diseases like in the cardiovascular system stress response (139), blood glucose regulation by acting on the pancreas (95), fat metabolism (105) and also in male reproductive system(166).

Endogenous expression of NUCB2 has also been discovered in kidneys. (167) Therapeutic potential of exogenous nesfatin-1 with respect to kidney diseases have been observed in case of renal ischemia (112), unilateral ureter obstruction (115) and also in in vitro models of oxidative stress and high glucose toxicity(111). Like other AKI models, Rhabdomyolysis

induced AKI have similar pathophysiology and inflammation, oxidative stress and renal cell death plays an important role in it.

We checked the effect of nesfatin-1 in rhabdomyolysis models of AKI in both *in vitro* and *in vivo* model of rhabdomyolysis-induced acute kidney injury. We mimicked rhabdomyolysis *in vitro* by exposing renal epithelial NRK52E cells to myoglobin (168) and for *in vivo* model we injected glycerol in hind limbs of male wistar rats imitating muscle damage which releasing myoglobin in bloodstream.(169) We examined the expression of NUCB2 in epithelial cells and kidneys under disease conditions and effect of exogenous nesfatin-1 treatment on its expression. We further assessed the expression of oxidative stress markers, inflammatory markers and apoptosis markers and with cellular morphology analysis for cells and histology for kidney sections.

4.2. Materials and methods

4.2.1. Cell culture

Rat renal epithelial cells (NRK-52E) were used for cell culture experiments. Cells were seeded into 6 well plates for expression studies and on coverslips in 12 well plates for morphology and confocal studies. After cells attain 80% confluency and good morphology is observed cells are subjected to starvation for 10-12 hr in 1% FBS media. Myoglobin stock (500uM) was prepared in 1% media and used as a disease-causing agent in cell culture experiments. After 2 hrs of treatment of cells with 100μMof myoglobin, nesfatin-1 peptide 1nM was added to the media and left for 24 hrs.

4.2.2. Animals

Male eight weeks old wistar rats (180–200 g) were purchased from Sainath Agencies, Hyderabad, India and housed under standard conditions, with food and water available ad libitum, in a room with a 12/12-h light/dark cycle and controlled temperature of 24-26 ºC and humidity of 60-65%. All the animals used in the study were approved by Institutional Animal Ethical Committee (IAEC) of Birla Institute of Technology and Science Pilani, Hyderabad Campus bearing a protocol approval number: BITS/HYD/IAEC/2022/51.

4.2.3. Chemicals and antibodies

Glycerol from Sigma (G5516) was procured—nesfatin-1 peptide from Novus Biologicals (cat no: NBP2-35072). Blood urea nitrogen kit (Cat no: 120241) and creatinine (Cat no: 120246) were procured form Trans Asia Bio-medicals Ltd., India. Takara first strand cDNA synthesis kit (Cat no: RR037A), Sybr Green containing PCR master mix from takara (TB Green Premix Ex Taq II Cat no. RR820A), Trizol reagent RNAiso plus (cat no: 9109) were purchased from Takara Gene Bioscience, India. Myoglobin from equine

skeletal muscle (Cat no. M0630) was bought from Sigma-Aldrich. Primary antibody for nesfatin-1 (Cat no: PA5-77384) and Secondary Goat Anti-Rabbit, Texas Red (Cat no. # T-2767) was purchased from Thermofisher Scientific, Invitrogen (MA, USA).

4.2.4. Experimental design and tissue collection

After procurement animals were quarantined for one week of acclimatization according to guidelines of the Indian Council on Animal Care. Rats will be randomly divided into IV groups (n=6 in each group) Control, Acute Kidney Injury (AKI), AKI + Treatment, Treatment (Nesfatin -1) alone. For inducing rhabdomyolysis for the glycerol group, animals were fasted and dehydrated 24 hours prior to injecting glycerol (50% prepared in normal saline) intramuscularly at 10ml/kg divided between both thigh muscles/hind limb muscles. In treatment groups, Nesfatin-1 peptide (1µg/kg) after 2 hrs of glycerol injection was given via intraperitoneal route.

Blood was withdrawn from retro orbital plexus route in anesthetized rat and serum was separated after centrifugation. Serum was collected to evaluate BUN and creatinine. Animals will be sacrificed after 24 hrs of nesfatin-1 treatment and kidney tissue will be isolated for further isolation of RNA and proteins and histopathological studies like H&E staining (morphology). Protein and RNA expression studies will be done for possible mechanisms.

4.2.5. Histopathology

After keeping the collected tissues in buffered saline paraformaldehyde solution for 10-12 hrs, they were processed in different concentrations of ethanol and infiltration of xylene and paraffin infiltration and embedding. After which 5-micron sections of tissues were made using microtome and transferred to slides. These sections were subjected to H&E

staining. Protocol of rehydration of sections staining and then rehydration was done, and sections were observed under the microscope.

4.2.6. Real time polymerase chain reaction (RT-PCR)

RT-PCR was done for the quantification of mRNA expression. Briefly total RNA was isolated from kidney tissue by trizol method and 1ug of total RNA was converted into cDNA using Takara first strand cDNA synthesis kit. mRNA expression of KIM-1, NGAL, nesfatin-1, SOD, Catalase are detected from the synthesized cDNA. Reaction mixture of cDNA, Primers and SYBR Green was prepared according to the manufacturing instructions.

Table 4 Primer Sequence Chapter 4

Gene	Primer Sequence Forward	Primer Sequence Reverse
SOD1	GGTCCACGAGAAACAAGATGA	CAATCCCAATCACACCACAAG
Catalase	CATGGATCTGCTTAGGACTTCTG	CCAGGCTGTGAGGTAACATAA
KIM1	GCCATTTCCACTCCACTTCT	CCTGCTCTCTCTCCTTTCTTTC
NGAL	CCCTCAGATACAGAGCTACGA	CTTCCGTACAGGGTGACTTTG
IL1b	GGAACCCGTGTCTTCCTAAAG	CTGACTTGGCAGAGGACAAA
NUCB2	GGAAAGAGGAAGAAGCCAAGT	GGTCCAATCCATCAGTCTCTTC
Caspase3	TGGAAAGCATCCAGCAATAGG	GACTCAGCACCTCCATGATTAAG
18s	CATTCGAACGTCTGCCCTAT	GTTTCTCAGGCTCCCTCTCC
β-actin	GAGGCCCCTCTGAACCCTAA	ACCAGAGGCATACAGGGAACAA
Bcl2	CTGGTGGACAACATCGCTCTG	GGTCTGCTGACCTCACTTGTG
NLRP3	ACCTGTACCTACGAAGCAATG	GTGAGGCTGCAGTTGTCTAA

4.2.7. Crystal Violet staining

NRK-52E renal epithelial cells were seeded on coverslips in 12 well plate till they reached confluency of 80%. After starvation of 10-12 hrs cells were treated with Myoglobin 100uM, after 2 hrs in treatment groups nesfatin-1 peptide final conc. 1nM was added. 24hrs after treatment media was removed, and cells were slowly washed with PBS and fixed with ice cold methanol for 15 mins in deep freezer. Crystal violet stain (0.5% w/v in methanol) was added to cells for 4-5 minutes. Stain was removed and cells were washed with PBS till cells release excess stain. Coverslips were mounted on slides and observed under light microscope for cell morphology.

4.2.8. Immunofluorescence Staining

NRK-52E cells were seeded on glass cover slips in 12 well plate till they reached confluency of 80%. After starvation of 10-12 hrs cells were treated with Myoglobin 100uM, after 2 hrs in treatment groups nesfatin-1 peptide final conc. 1nM was added. 24hrs after treatment media was removed, and cells were slowly washed with PBS and subjected to NUCB2 immuno-staining. The treated cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed twice with phosphate buffered saline (PBS). Cells were permeabilize with 0.1% Triton X-100 for 10 min and three washes with PBS, the cells were incubated with 3% filtered bovine serum albumin (BSA) (in PBST) for 1 h to block non-specific binding sites. After BSA was removed from the wells, cells were incubated overnight at a dilution of 1:200 with the NUCB2 antibody. Next day cover slips are washed three times with PBS. Then cells were incubated with a Texas red-conjugated secondary antibody (1:100 dilution) for 1hr, after which they are washed 3 times with PBS. Counter staining of nucleus was done with DAPI for 10 min, cover slips are mounted on

glass slide and analysed under laser confocal microscope (LAS-X). Slides from three different experiments were analysed with 5 fields per slide observed and averaged.

4.2.9 Statistical analysis

The data obtained in the present study is expressed as mean ± SD, n corresponds to number of animals for each independent experiment. Results were statistically analysed using one-way ANOVA followed by post hoc Bonferroni's test using Graph Pad Prism Software Version 5 (San Diego, CA, USA). A p value of <0.05 was considered as statistically significant for all the experiments.

4.3 Results

4.3.1. Cell viability and NUCB2 expression

For *in vitro* rhabdomyolysis model, myoglobin is used as a disease-causing agent in NRK52E cells. We assessed toxicity of myoglobin for 100μMdose and treatment of nesfatin-1 of 1nM for 24 hrs using MTT. We observed 74 % cell viability with 100 μM of myoglobin. In nesfatin-1 treatment case after myoglobin the viability increased to 87%.

After this we checked the NUCB2 mRNA expression in renal NRK-52E epithelial cells in the presence of myoglobin and nesfatin-1 treatment. Significant decrease in mRNA expression of nesfatin-1 was observed in myoglobin induced cells.

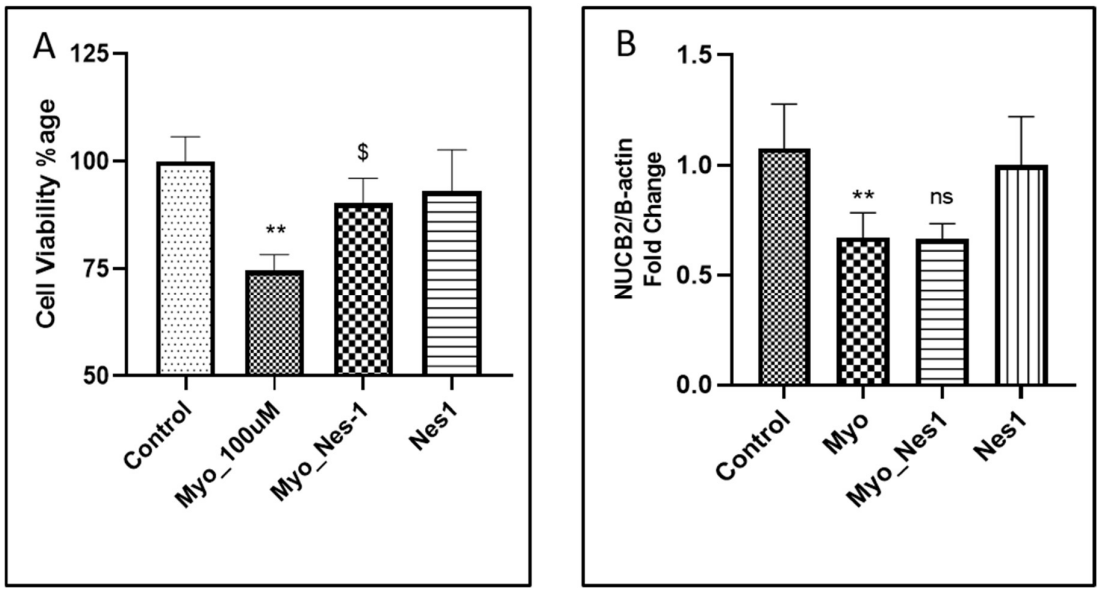


Figure 4-1 Dose selection using cytotoxicity and effect on NUCB2 expression.

Cell viability and NUCB2 expression in myoglobin-treated cells renal NRK-52E epithelial cells: Renal NRK-52E epithelial cells were treated with 100μM of myoglobin and after 2 hr nesfatin-1 (1μM) was added for 24 Hr. Cell viability was measured by MTT assay (A) (n = 6). (B) NUCB2 mRNA expression was measured by RT-PCR. Data is expressed as mean ± SD with n = 4 in each group. **$p < 0.01$ vs control; $\$p < 0.05$ vs. myoglobin group. Data is expressed as mean ± SD with n = 4 in each group.

4.3.2. Improvement in expression of oxidative stress marker Catalase in *in vitro* model of rhabdomyolysis.

Expression analysis of oxidative stress markers was done in NRK52E cells after myoglobin and nesfatin-1 treatment. Due to disease condition in myoglobin groups both the genes Catalase and SOD1 were downregulated. While we observed significant improvement in only catalase gene expression after nesfatin-1 treatment in disease+nesfatin-1 group

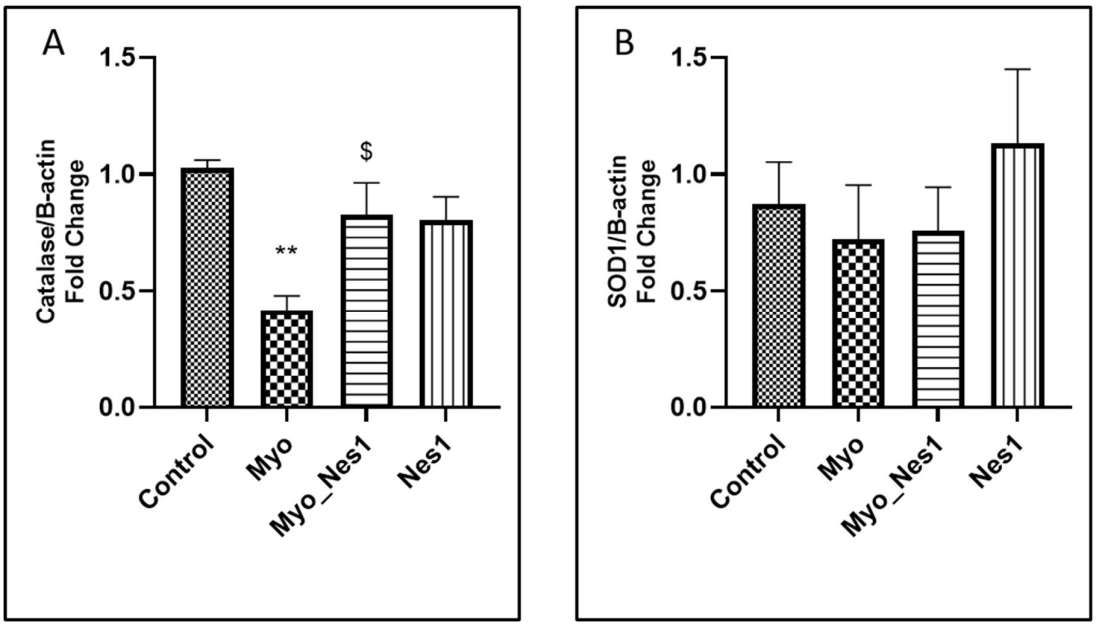


Figure 4-2 Effect of myoglobin and nesfatin-1 on Oxidative stress markers

Nesfatin-1 treatment attenuated the decrease in antioxidant enzymes: Renal NRK-52E epithelial cells were treated with myoglobin and nesfatin-1 for 24 h. Catalase and SOD mRNA expression was measured by RT-PCR. **$p < 0.01$ vs control; \$$p < 0.05$ vs. myoglobin group. Data is expressed as mean ± SD with $n = 4$ in each group.

4.3.3. Positive effect of nesfatin-1 treatment on pro- and anti–apoptotic genes expression in *in vitro* model of rhabdomyolysis

Expression of anti-apoptotic protein Bcl-2 and pro-apoptotic Caspase-3 was observed in NRK52E cells treated with myoglobin and with co-treatment of nesfatin-1 peptide for 24 h. As in myoglobin group we observed significant downregulation of anti-apoptotic Bcl-2 while significant increase in pro-apoptotic Caspase-3. These conditions were ameliorated in myoglobin+nesfanti-1 treatment group suggesting protective effect of nesfatin-1.

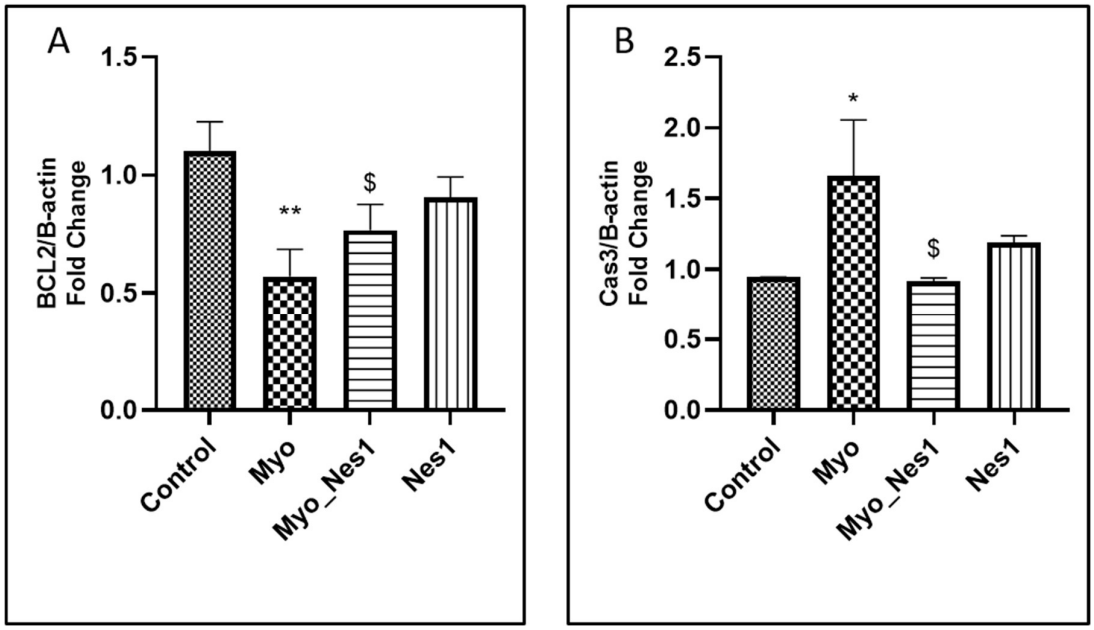


Figure 4-3 Effect of myoglobin and nesfatin-1 on apoptotic markers.

Myoglobin treatment decreased the expression of anti-apoptotic protein Bcl-2 while increasing pro-apoptotic Caspase-3: Renal NRK52E cells were treated with myoglobin and nesfatin-1 peptide for 24 h. mRNA expression of Bcl2 and Caspase-3 was measured by RT-PCR (A, B). *$p < 0.05$, **$p < 0.01$ vs control group, \$$p < 0.05$ vs. myoglobin group. Data is expressed as mean ± SD with $n = 4$ in each group.

4.3.4. Downregulation of inflammatory markers after nesfatin-1 treatment in *in vitro* model of rhabdomyolysis

Due to myoglobin treatment, we observed overexpression of pro inflammatory cytokines and pro inflammatory complex in NRK52E cells. These cytokines IL-1b and NLRP3 complex play major role in disease progression due to sustained inflammation and cellular injury. While in group with nesfatin-1 treatment after myoglobin treatment, these inflammatory markers decreased this correlates with protective effect of nesfatin-1.

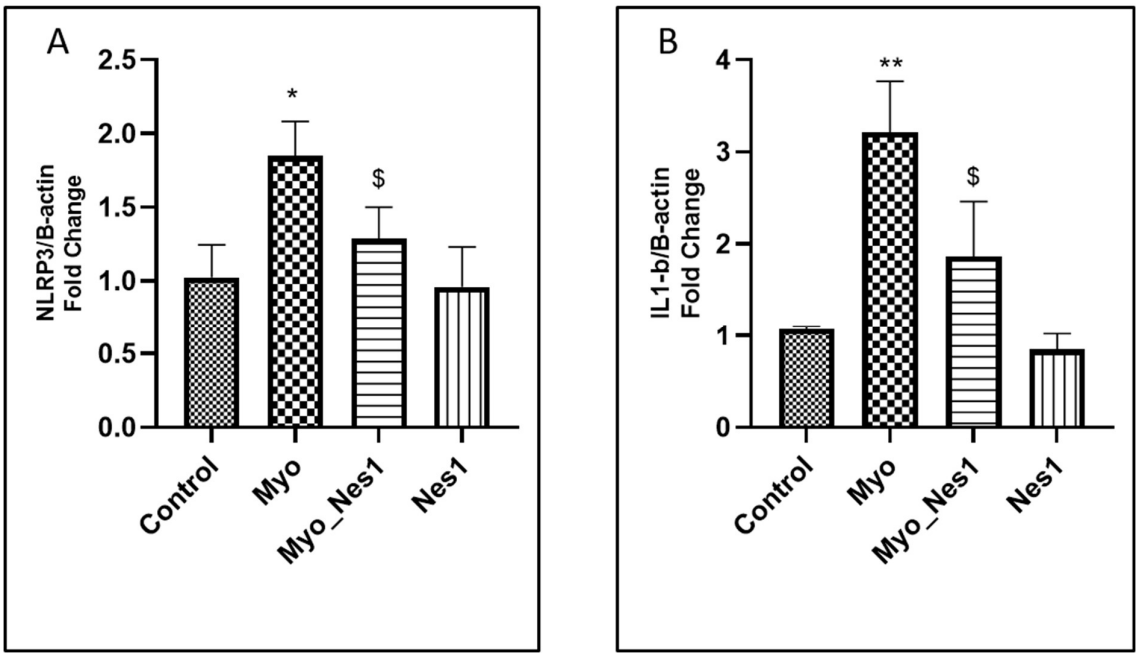


Figure 4-4 Effect of myoglobin and nesfatin-1 on inflammatory markers

Nesfatin-1 treatment attenuated the increase in inflammatory proteins and cytokines: Renal NRK-52E epithelial cells were treated with myoglobin and nesfatin-1 for 24 h. NLRP3 (A) and IL-1b (B) mRNA expression was measured by RT-PCR. *$p<0.05$, **$p < 0.01$ vs control; \$$p < 0.05$ vs. myoglobin group. Data is expressed as mean ± SD with $n = 4$ in each group.

4.3.5. Nesfatin-1 protects against myoglobin induced morphological changes in renal NRK-52E epithelial cells.

We determined structural changes in NRK-52E renal epithelial cells in myoglobin induced rhabdomyolysis and cellular toxicity and nesfatin-1 co-treated group. Morphology of NRK-52E renal epithelial cells was observed by crystal violet staining. In control group we observed well established morphology and well-defined nucleus whereas in myoglobin group, we observed that the cells have shrunk and decrease in cell size. While in treatment group the cells have been protected against complete shrinkage of cells.

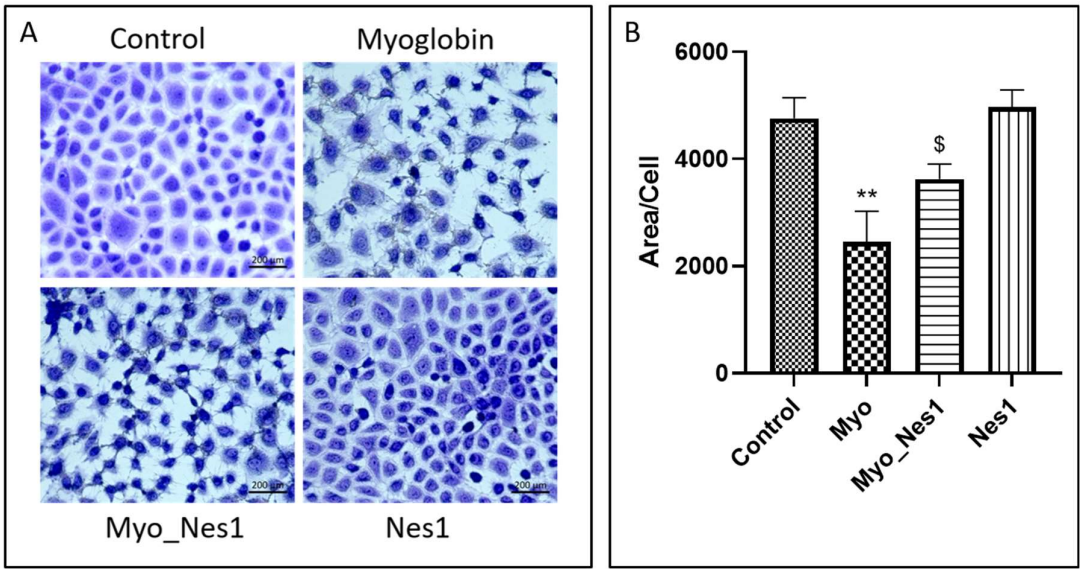


Figure 4-5 Effect of myoglobin and nesfatin-1 on inflammatory markers

Nesfatin-1 protects from myoglobin induced structural changes in renal NRK-52E epithelial cells: NRK-52E epithelial cells were treated with rhabdomyolysis 100 µM (A, B) for 24 h. Structural changes were observed by Crystal violet staining. **$p < 0.01$ vs control; $\$p < 0.05$ vs. myoglobin group. Data is expressed as mean ± SD with n = 3 in each group.

4.3.6. Expression quantification of NUCB2 and its cellular localization by confocal microscopy

To observe expression of NUCB2 and its localization in the cell we performed immunofluorescence experiment with myoglobin and myoglobin-nesfatin-1 co treatment group. In our results downregulation in expression of NUCB2 in myoglobin group and nuclear localization was observed. While in co treatment group we assessed decrease in NUCB2 expression was less than disease group and its localization also out of the nucleus.

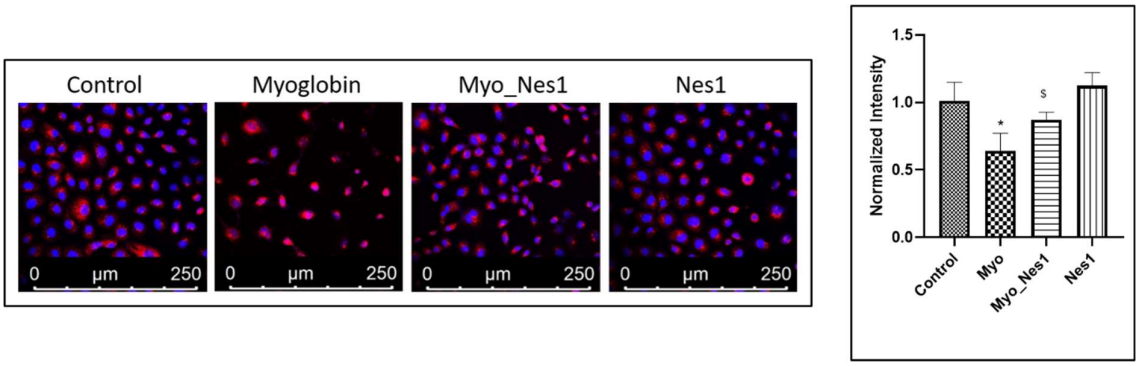


Figure 4-6 Expression of NUCB2 using microscopy in renal epithelial cells.

Myoglobin decreased the expression of nesfatin-1 in renal NRK-52E epithelial cells: Renal NRK-52E epithelial cells were treated with myoglobin and nesfatin-1 peptide for 24 h. NUCB2 immunofluorescence was captured by confocal microscopy. *p<0.05 *vs* control; $^{\$}p$< 0.05 *vs* myoglobin group. Data is expressed as mean ± SD with n = 4 in each group.

4.3.7. Development of rhabdomyolysis model *in vivo* by glycerol

Male wistar rats were subjected to dehydration of 24 hr before injecting 50% glycerol 10ml/kg in its hind limb's thigh muscles. After 2 hrs intra peritoneum doses of nesfatin-1 1 µg/kg peptide were given to disease + treatment group animals. After 24 hrs blood was withdrawn and animals were sacrificed. Serum parameters Creatinine and Blood Urea Nitrogen (BUN)

was assessed for kidney damage by biochemical method. Significant increase in both the parameters was observed while in co-treatment group it was less compared to disease group.

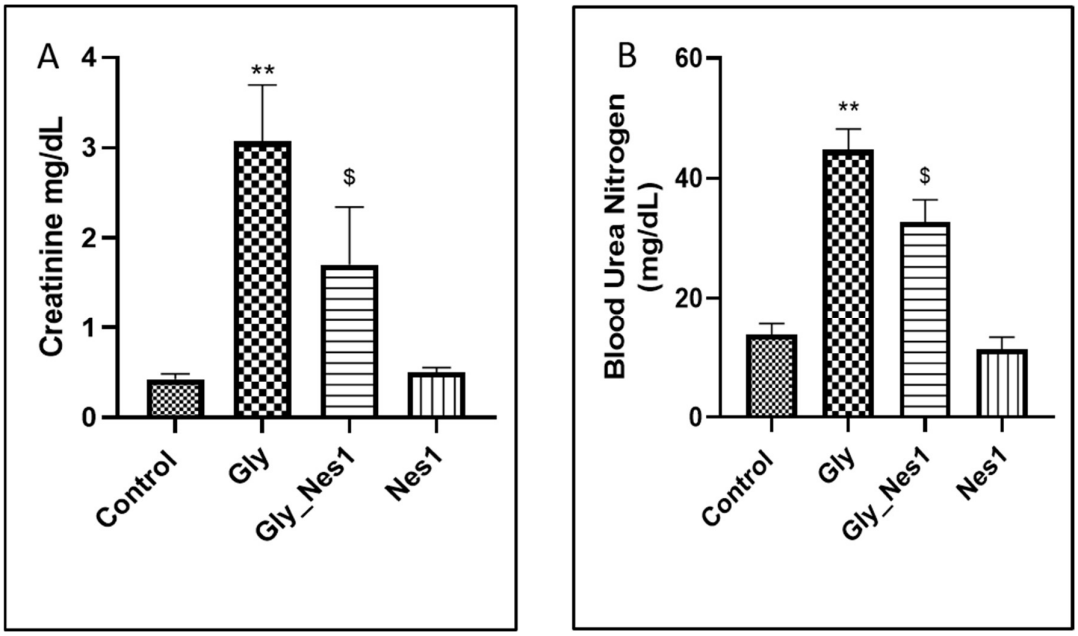


Figure 4-7 Rhabdomyolysis model of AKI causes kidney damage and increases serum creatinine and BUN levels.

Male wistar rats were injected with glycerol in lower thigh muscles rupturing them for 24 Hr. Treatment of Nesfatin-1 peptide was given 2 hrs after glycerol injection I.P. Serum creatinine and BUN levels were measured using assay kits (A, B). **p < 0.01 vs control; $p < 0.05 vs. glycerol group. Data is expressed as mean ± SD with *n* = 5-6 in each group

4.3.8. Effect of nesfatin-1 on molecular markers of AKI induced by rhabdomyolysis.

KIM-1 and NGAL are versatile markers of kidney injury. Expression analysis of KIM-1 and NGAL gene was done to assess the extent of damage due to rhabdomyolysis and effect of nesfatin-1 on disease condition. We observed significant high expression of both the markers

in disease condition suggesting successful induction of disease model. While in case of nesfatin-1 co-treatment group the severity of disease as less than diseased group.

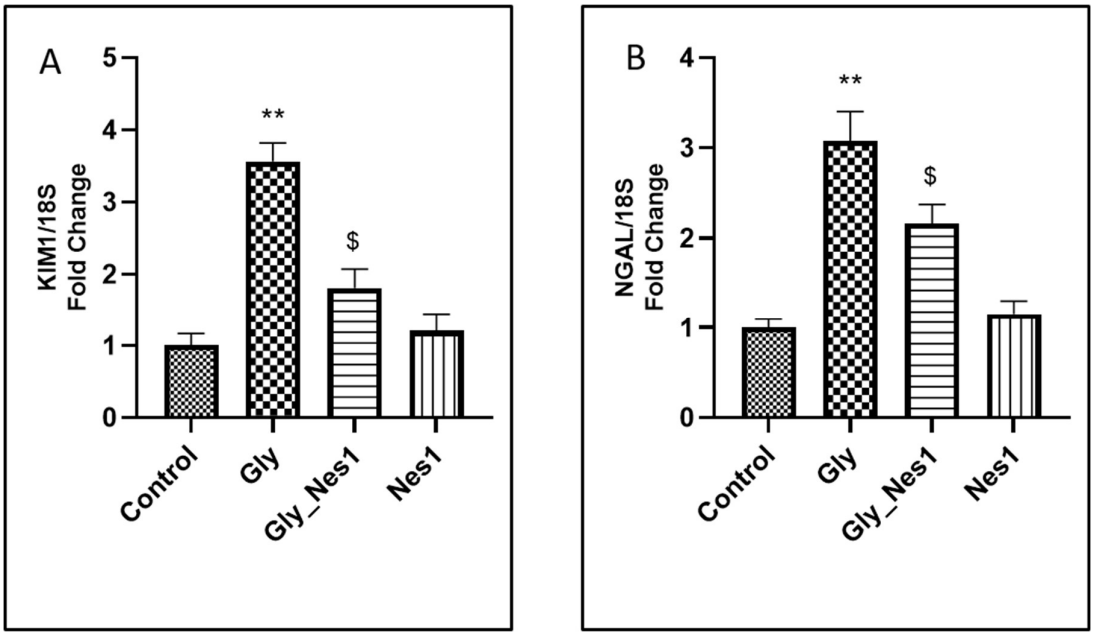


Figure 4-8 Confirmation of Rhabdomyolysis induced AKI using molecular markers.

Rhabdomyolysis model of AKI causes kidney damage and increases in expression of kidney injury markers: Male wistar rats were injected with glycerol in lower thigh muscles rupturing them for 24 Hr. Treatment of Nesfatin-1 peptide was given 2 hrs after glycerol injection. mRNA expression of kidney injury markers KIM-1 and NGAL was measured by RT-PCR (A, B). **$p < 0.01$ vs control; \$$p < 0.05$ vs. glycerol group. Data is expressed as mean ± SD with $n = 6$ in each group.

4.3.9 Effect of nesfatin-1 treatment in rhabdomyolysis induced AKI on NUCB2, inflammatory and pro apoptotic markers expression

After sacrifice of animals the expression of NUCB2 (Fig 4-9A) and pro-inflammatory cytokine IL-1b (Fig 4-9B) and pro-apoptotic gene Caspase 3 (Fig 4-9C) was assessed in all the groups by RT-PCR. We found that the NUCB2 expression was down regulated in disease group while in treatment group it is higher than compared to diseased group. This has been observed with

IL-1b and cas3 expression was less than diseased group in nesfatin-1 treatment suggesting reduced inflammation and apoptosis.

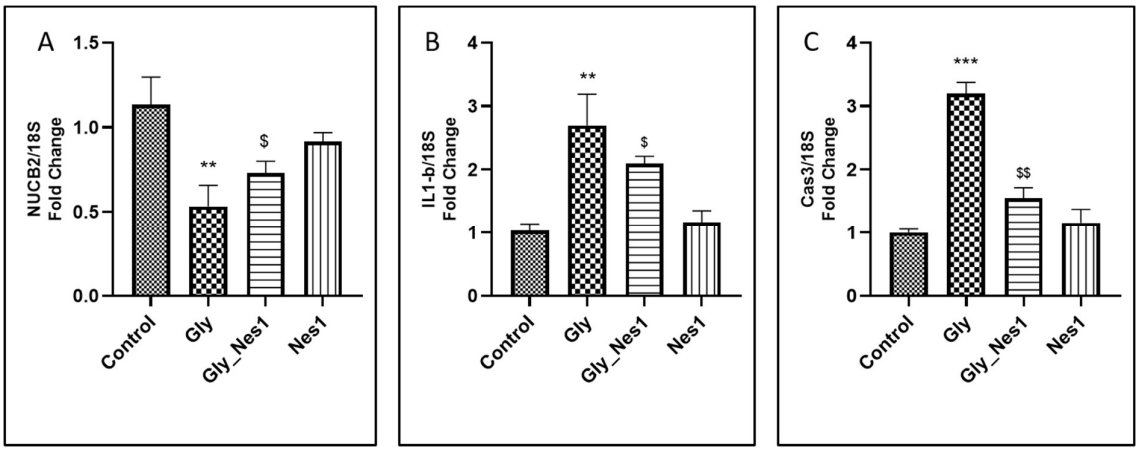


Figure 4-9 Effect of Nesfatin-1 peptide treatment on Rhabdomyolysis induced AKI on NUCB2, inflammatory and pro-apoptotic markers.

Rhabdomyolysis model of AKI causes decrease in expression of NUCB2 and increases in expression of inflammatory and pro-apoptotic markers: Male wistar rats were injected with glycerol in lower thigh muscles rupturing them for 24 Hr. Treatment of Nesfatin-1 peptide was given 2 hrs after glycerol injection. mRNA expression of NUCB2, inflammatory cytokine IL1b and pro-apoptotic markers Caspase-3 was measured by RT-PCR (A, B, C). $^{**}p<0.01$, $^{***}p<0.001$ vs control; \$p < 0.05 vs. glycerol group, Data is expressed as mean ± SD with n = 6 in each group.

4.3.10. Histopathological improvement in rhabdomyolysis induced AKI after nesfatin-1 treatment

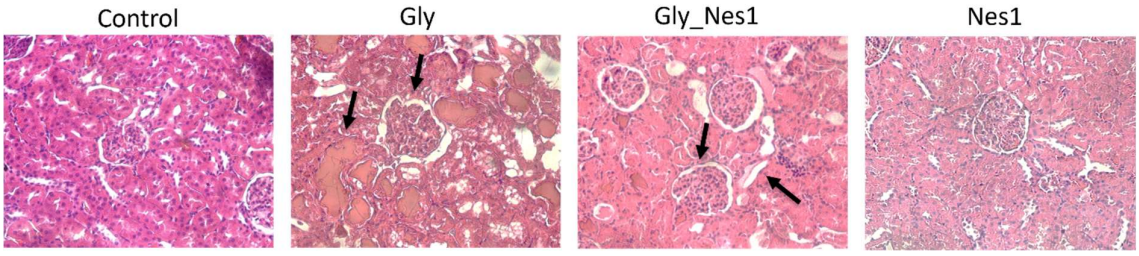


Figure 4-10 Effect of Nesfatin-1 peptide treatment in DN kidneys on histology of kidneys

In Rhabdomyolysis, kidneys histological changes in morphology and internal structure of kidneys are observed by H&E staining. The loss of interstitial lining and deformation of the glomerulus can be observed in the disease group. While in nesfatin-1 treated rhabdomyolysis group it is less even though some damage can be seen.

4.4. Discussion

This present study is extension of our previous work in which we have assessed expression of NUCB2 in effect to myoglobin on rat epithelial NRK52E cells and in glycerol induced rhabdomyolysis kidneys.(167) Protective effect of nesfatin-1 in Unilateral Ureter Obstruction models (UUO) regarding oxidative stress and inflammation has been reported. While in another report nesfain-1 effect on reduction in apoptosis inflammation and oxidative stress in renal ischemia reperfusion injury has been observed. In this work we assessed effect of nesfatin-1 treatment in rhabdomyolysis model of AKI both *in vitro* and *in vivo*

Cellular toxicity due to myoglobin (100uM) and its protection when co treated with Nesfatin-1 (1nM) (Fig 4-1) provided us with effective dose of myoglobin and nesfatin-1 for our experimentation. We observed decrease in expression of NUCB2 in NRK52E cells subjected to myoglobin induced toxicity and its increase in co-treatment group. (Fig 4-1 B) These results were correlated with immunofluorescence experiments in cells with same conditions (Fig 4-6).

During our study we analysed the correlation between decreased NUCB2 expression and oxidative stress (SOD1 and Catalase) markers, apoptotic markers (BCL2 and caspase 3) and inflammatory markers (IL1b and NLRP3) as all three factors are correlated to each other, in myoglobin induced cytotoxicity model of AKI in NRK52E cells. The role of nesfatin-1 in antioxidant defence, inflammation, apoptosis and morphology changes, in renal epithelial cells NRK-52E when co-treated with nesfatin-1 in the presence of myoglobin was observed.

Oxidative stress markers like SOD1 and Catalase were found to be downregulated in our myoglobin toxicity model in NRK52E cells. While it was reversed in cells co-treated with myoglobin and nesfatin-1 (Fig 4-2). This correlates with our previously reported effect of renal toxicity using doxorubicin(167). Anti- apoptotic marker BCL2 was also found to be downregulated in myoglobin treated group while its counterpart a pro-apoptotic gene was

upregulated suggesting that overall myoglobin promotes apoptosis in NRL52E cells treated with myoglobin. While expression of both the anti and pro-apoptotic genes were reversed in myoglobin_nesfatin-1 treatment group (Fig 4-3). After which we checked for inflammation in myoglobin treated cells, we found that both pro-inflammation genes IL1b and NLRP3 were upregulated in the cells and in nesfatin-1 treated myoglobin group cells it was less as compared to alone myoglobin group (Fig 4-4). Tang et al reported effect of nesfatin-1 as an anti-oxidative and anti-apoptotic molecule and its neuro-protective effects against subarachnoid haemorrhage that induced brain injury and inhibited apoptosis in rats.(158)

Cellular morphology after myoglobin treatment was observed using crystal violet assay. We observed that cell got shrink in myoglobin treatment group while in co-treatment group the cell size was bigger as compared to disease group (Fig 4-5). This shrinkage of cells was observed in immune fluorescence imaging of treated cells to check expression and localization of NUCB2. From imaging we concluded that NUCB2 expression was downregulated as shown by RT-PCR results and protein got localized to inside the nucleus which was a very interesting observation (Fig 4-6).

We induced rhabdomyolysis in male wistar rats by dosing glycerol 50% in their hind limb muscles which rupture them, and cause inflow of myoglobin released from them to kidneys causing AKI. We also gave animals 1ug/kg dose of nesfatin-1 to glycerol dosed animals as a separate treatment group. Using BUN and creatinine as serum parameters we assessed kidney health of these animals and high levels of both were regarded as induction of disease. BUN and creatinine levels of co-treatment animals was less the disease group but still higher than control group suggesting protective effect of nesfatin-1 (Fig 4-7).

After sacrifice of animal's molecular markers of kidney injury KIM1 and NGAL were assessed in kidneys using RT-PCR. Upregulation of both the genes was observed in disease case while

in treatment group it was reduced as compared to disease group (Fig 4-8). Correlating the biochemical results of BUN and creatinine in serum with kidney injury molecular markers.

We checked NUCB2 expression in rhabdomyolysis kidneys and as reported in our earlier publication it was reduced in disease conditions. As an addition to previous experiment, we added a Nesfatin-1 treatment group to see its effect on disease. We observed that with reduction in kidney injury there was increase in NUCB2 expression as compared to diseased group (Fig 4-9 A). This may be due to protective effect of nesfatin-1 treatment or exogenous nesfatin-1 provides positive feedback to NUCB2 expression enhancement.

We further checked expression of pro inflammatory marker IL1b and pro-apoptotic marker caspase 3. As expected in disease conditions they are upregulated as has been reported earlier. While in treatment group they were decreased suggesting positive effect of nesfatin-1 treatment in ameliorating kidney injury due to rhabdomyolysis (Fig 4-9 B, C). We also observed histopathological changes in kidney tissue at cellular level. Protective effect of nes1 can be clearly observed in disease + treatment group showing reduction in PCT and tubular damages (Fig 4-10). Gharanei et al suggest that nesfatin-1 treatment reduces inflammation in white adipose tissues of mice caused due to diet induced factors. (125)

Protective effect of exogenous nesfatin-1 in rhabdomyolysis induced AKI model *in vitro* and *in vivo* is a start in positive direction regarding opening great potential of this peptide. It is an addition to anti-oxidative stress, anti-inflammatory, and anti-apoptotic properties of this peptide in addition to effect on endogenous NUCB2 expression. Further work on its mechanism of action can be explored

Chapter 5 Investigation into nesfatin-1 treatment in *in vivo* Diabetic Nephropathy and investigate it's the molecular mechanism.

5.1. Introduction

Diabetic nephropathy, also known as diabetic kidney disease, is a long-term complication of diabetes that affects the kidneys. It is a leading cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) worldwide. (26)It starts with diabetes, having high levels of consistent blood sugar levels; over time this hyperglycemia damages the small blood vessels in the kidneys and permanently damaging the tissue and hampering its normal functioning. As kidneys play a crucial role in filtering waste products from the blood, and help regulate blood pressure, hyperglycemia caused damage leads to kidney failure.(69)

Nesfatin-1 is a polypeptide of 82 amino acids which is first discovered to be expressed in the hypothalamus and has been reported to possess anorexic properties. NUCB2 is the parent protein which in post translationally cleaved in three peptides, one of them is nesfatin-1.(92) The satiety effect of nesfatin-1 is as effective as leptin and works independently of leptin pathway.(99) Nesfatin-1 expression was identified in pancreatic β cells, gastric mucosa and adipose tissue of humans and rodents. It has been reported that it has positive effect on secretion of insulin and glucagon in pancreases in T2D.(104)

Studies have investigated the relationship between nesfatin-1 and acute kidney disease in animal models and suggest that there is a negative correlation between nesfatin-1 levels and disease aggravation.(167) A study from our group suggest that there is a correlation between nesfatin-1 treatment and diabetic nephropathy model of *in vitro* in NRK52E cells opening up avenues for animal studies.(111) Clinical studies have suggested conflicting results regarding correlation of nesfatin-1 levels in serum and diabetes.(143,170) While protective effect of nesfatin-1 treatment in the Unilateral Ureter Obstruction model and Renal Ischemia-Reperfusion model has been observed.

In this study we induced Type I diabetes in male wistar rats using multiple low doses of streptozotocin a toxic drug which specifically damage b-cells of pancreas. We regularly checked animals for their fasting blood glucose and kidney condition by assessing BUN and creatinine. We also recorded feeding and water intake quantities for animals.

We assessed the effect of nesfatin-1 peptide treatment which was for last 4 weeks of 10-week protocol on expression of NUCB2 protein, inflammation markers, oxidative stress markers and apoptosis markers. Histopathological assessment of internal kidney morphology and fibrosis was also assessed in this study. We also assessed the possible effect of nesatin-1 treatment on expression of known receptor of nesfatin-1 peptide and its downstream effector proteins.

As the ever-expanding knowledge increase regarding this peptide, we in this work assessed its effect on animals with type I diabetes and in turn nephropathy caused due to it. We will be looking into the pathways which are helping it to reduce inflammation, oxidative stress, and apoptosis and inhibiting the progression of disease in the animals. This study adds to the existing knowledge of nesfatin-1 with regard to diabetic nephropathy.

5.2. Materials and methods

5.2.1. Animals

Male eight weeks old wistar rats (180–200 g) were purchased from Sainath Agencies, Hyderabad, India and housed under standard conditions, with food and water available ad libitum, in a room with a 12/12-h light/dark cycle and controlled temperature 24-26 ºC and humidity of 60-65%. All the animals used in the study were approved by Institutional Animal Ethical Committee (IAEC) of Birla Institute of Technology and Science Pilani, Hyderabad Campus bearing a protocol approval number: BITS/HYD/IAEC/2021/31.

5.2.2. Chemicals and antibodies

Streptozotocin (Cat no: TC418) procured from Himedia labs, India. Nesfatin-1 peptide from Novus Biologicals (cat no: NBP2-35072). Blood urea nitrogen kit (Cat no: 120241) and creatinine (Cat no: 120246) were procured from Trans Asia Bio-medicals Ltd., India. RIPA buffer, Direct Red 80 Cat no 365548 Haematoxylin solution Cat no 03971 Eosin Y Solution Cat no 318906 from Sigma Aldrich, India. Immuno-Blot PVDF Membrane was purchased from Bio-Rad (Hercules, CA, USA). Takara first strand cDNA synthesis kit (Cat no: RR037A), PCR master mix containing SYBR Green from takara (TB Green Premix Ex Taq II Cat no. RR82WR), Trizol reagent RNAiso plus (cat no: 9109) were purchased from Takara Gene Bioscience, India.

5.2.3. Antibodies

The primary antibody for nesfatin-1 (cat no: PA5-77384) was purchased from Thermofisher Scientific, Invitrogen (MA, USA). GAPDH (cat no: sc-365062), goat anti-rabbit IgG-HRP (cat no: sc-2004), rabbit anti-mouse HRP (Cat no: sc-2357), caspase 3 (Cat no: sc-136219) and

TGF-β (Cat no: sc-130348) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

5.2.4. Experimental design and tissue collection

After procurement, animals were quarantined for one week of acclimatization according to the guidelines of the Indian Council on Animal Care. For inducing diabetes mellitus Type I in male Wistar rats were randomly divided into four groups: I) control, II) disease (diabetes), III) disease + treatment (disease + nesfatin – 1), and IV) drug control (nesfatin-1). In groups II and III, Type I diabetes, nephropathy was induced by injecting Streptozotocin (STZ) freshly prepared in citrate buffer (20 mg/kg) for five days intraperitoneally in a volume of 10 mL/kg after fasting animals for 6-8 Hrs. All the dosing, weighing, blood withdrawal, and blood glucose check are done at the same time of day/week every time. After nine days after the start of the first dose of STZ, fasting blood glucose levels of animals are checked via tail snip method, and if it is above 300mg/dl, the animals were used in the preceding experiments. After establishing diabetes via fasting blood glucose levels, every two-week blood was withdrawn from all the animals via retro-orbital plexus, and a Renal function test was performed by accessing Blood urea nitrogen (BUN) and creatinine to observe the level of renal damage due to diabetes. After six weeks from day 0 of protocol, Nesfatin-1 (1μg/kg) was given intraperitoneally for four weeks every alternate day to groups III and IV.

Animals were sacrificed at the end of the study i.e. end of week 10, and kidney tissues were isolated for isolation of RNA and protein, and for further histopathological studies like Haematoxylin & Eosin staining(morphology) and Sirius red staining (fibrosis). Western blot, and RT-PCR was performed for confirmation of molecular pathways.

5.2.5. Histopathology

After keeping the collected tissues in buffered saline paraformaldehyde solution for 10-12 hrs, they were processed in different concentrations of ethanol and infiltration of xylene and paraffin infiltration and embedding. After which, 5-micron sections of tissues were made using a microtome and transferred to slides. These sections were subjected to H&E staining and Sirius red staining. Protocol of rehydration of sections staining and then rehydration was done, and sections were observed under the microscope.

5.2.6. Western blotting

Total protein from rat kidney tissues was isolated after homogenising small tissue section in a bead beater with RIPA buffer added to it. After quantifying equal amount of protein was loaded on a 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for saperation on basis of molecular weight. Protein was transferred from the gel to Polyvinylidene difluoride (PVDF) membranes in a chilled transfer buffer under the conditions of 60 V for two hours at four °C. The membranes were blocked with 3 % BSA prepared in PBS-T for two hrs at room temperature and then incubated with primary antibody overnight at four °C. Next day membrane was washed three times with PBS-T for 20 mins each. Thereafter, the membranes were incubated with a secondary antibody for two h at room temperature, and after washing thrice with PBS-T protein bands were detected with a chemiluminescence kit using an Eppendorf fusion solo chemidoc machine.

5.2.7. Real-time polymerase chain reaction (RT-PCR)

RT-PCR was done for the quantification of mRNA expression. Total RNA was isolated from kidney tissue by the trizol method, previously explained # and 1ug of total RNA was converted into cDNA using First Strand cDNA synthesis kit from Takara. mRNA expression of genes given in below table were detected from the synthesized cDNA. A reaction mixture of cDNA,

Primers, and SYBR Green was prepared according to the manufacturing instructions of Sybr Green containing PCR master mix from Takara.

Table 5 Primer Sequence Chapter 5

Gene	Primer sequence Forward	Primer sequence Reverse
SOD1	GGTCCACGAGAAACAAGATGA	CAATCCCAATCACACCACAAG
Catalase	CATGGATCTGCTTAGGACTTCTG	CCAGGCTGTGAGGTAACATAA
KIM1	GCCATTTCCACTCCACTTCT	CCTGCTCTCTCTCCTTTCTTTC
NGAL	CCCTCAGATACAGAGCTACGA	CTTCCGTACAGGGTGACTTTG
NUCB2	GGAAAGAGGAAGAAGCCAAGT	GGTCCAATCCATCAGTCTCTTC
18s	CATTCGAACGTCTGCCCTAT	GTTTCTCAGGCTCCCTCTCC
MC4R	GGCGAGGCTTCACATTAAGA	GCAGACAACAAACACTCCAATC
CALU	CCCAGTCGTGGTGTAACTTT	GGCAGTGAGGGATGTTCATTA
CAMK1D	GAGGCAGAAAGAGCAGCTAATA	TGCCTCTGTTGCTTCCTAAC
β-actin	GAGGCCCCTCTGAACCCTAA	ACCAGAGGCATACAGGGAACAA

5.2.8. Statistical analysis

The data obtained in the present study is expressed as mean ± SD, n corresponds to number of animals for each independent experiment. Results were statistically analysed using one-way ANOVA followed by post hoc Bonferroni's test using Graph Pad Prism Software Version 5 (San Diego, CA, USA). A p value of more than <0.05 was considered as statistically significant for all the experiments.

5.3. Results:

5.3.1. Nesfatin-1 treatment cause reducing in blood glucose level in diabetic rats:

Persistent high glucose level in a hallmark of diabetes. From week 0 to week 10 every two weeks fasting blood glucose of all the animals were checked. After 10 days of last dose of streptozotocin (STZ), animals with Fasting Blood Glucose (FBG) of more than 250mg/dL i.e. STZ and STZ+Nes1 groups were used further in the experiments. We observed that after treatment of nesfatin-1 treatment from week 6 there is gradual decrease in FBG of animals in treatment group. At 10 weeks fasting blood glucose of diabetic animals who have been treated with nesfatin-1 for 4 weeks have significant less FBG than untreated animals.

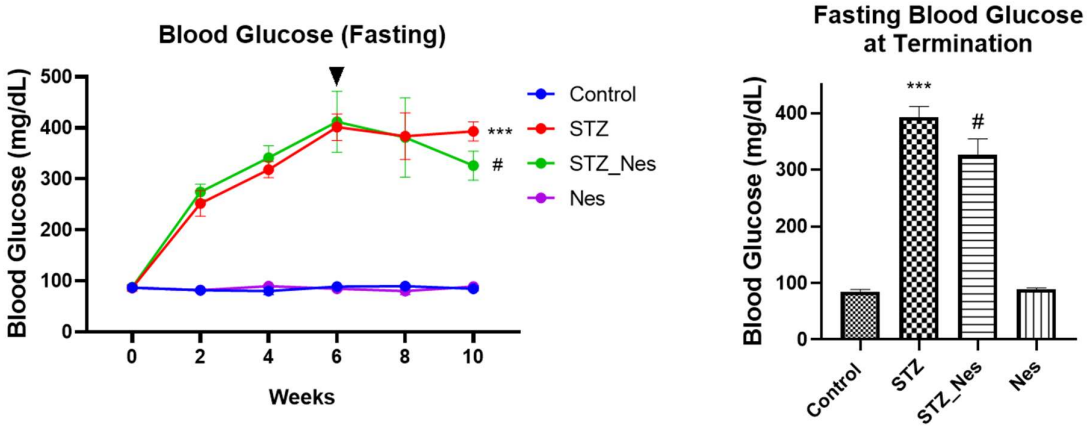


Figure 5-1 Effect of Nesfatin-1 peptide on Blood Glucose of Diabetic Animals

In the same Wistar rats Fasting Blood Glucose was checked every two weeks. After the start of treatment of Nesfatin-1 peptide, there were changes in FBG of diabetic animals receiving the nesfatin-1 peptide. After the end of the protocol FBG in nesfatin-1 treated diabetic animals was less than non-treatment diabetic group. ***$p < 0.001$ vs control #$p < 0.05$ *vs* STZ. Data is expressed as mean ± SD with n = 4-6 in each group.

5.3.2. Upregulation of biochemical biomarkers of Diabetic nephropathy:

After manifestation of diabetes, hyperglycaemia starts effecting kidneys. We started observing Serum BUN and creatinine of all the animals from week 0. Till week 4 small changes in biochemical parameters were observed in STZ dosed animals but were not significant. We waited for 2 more weeks for the establishment of diabetic nephropathy. After week 6 significant changes were observed, only then nesfatin-1 treatment was started. Establishment of Diabetic nephropathy using BUN (Fig 5-2) and creatinine (Fig 5-3) was necessary as it tells us overall health of kidneys without invasive procedures. We observed that after treatment started the rate of change in these parameters starts stagnating for nesfatin-1 dosed diseased animals suggesting protection in hyperglycaemia induced damage to them.

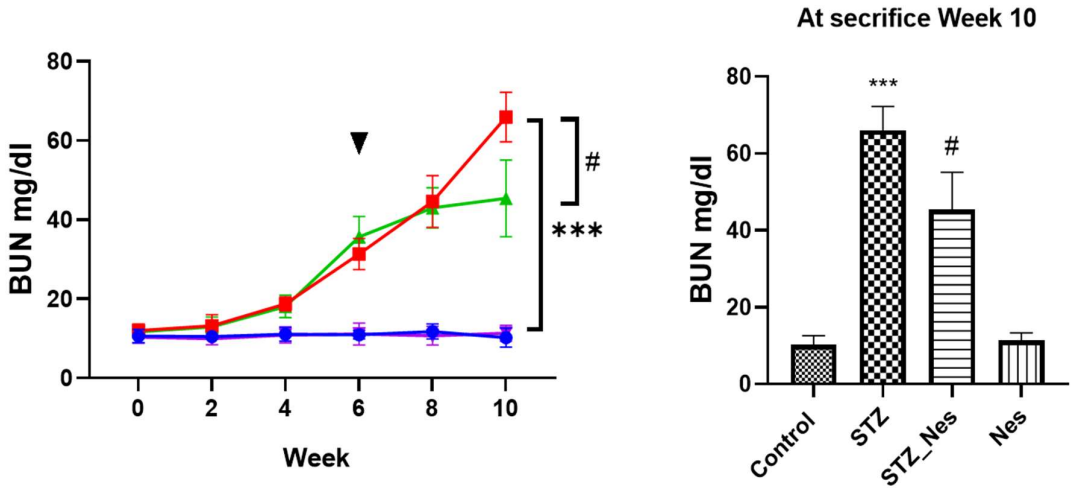


Figure 5-2 Effect of Nesfatin-1 peptide on Blood Urea Nitrogen of Diabetic Animals

Diabetes in male Wistar rats was induced with multiple low doses of Streptozotocin (20mg/kg) for 5 days. Nesfatin-1 peptide treatment was given for last 4 weeks (week 7-10) every two days. Blood of rats was isolated, and BUN recorded every two weeks for the whole duration of the protocol. After the start of treatment of Nesfatin-1 peptide, the rate of increase

of BUN decreased in those diabetic animals. After end of protocol BUN in nesfatin-1 treated diabetic animals was less than diabetic animals. ***$p < 0.001$ vs control #$p < 0.05$ *vs* STZ. Data is expressed as mean ± SD with n = 4-6 in each group.

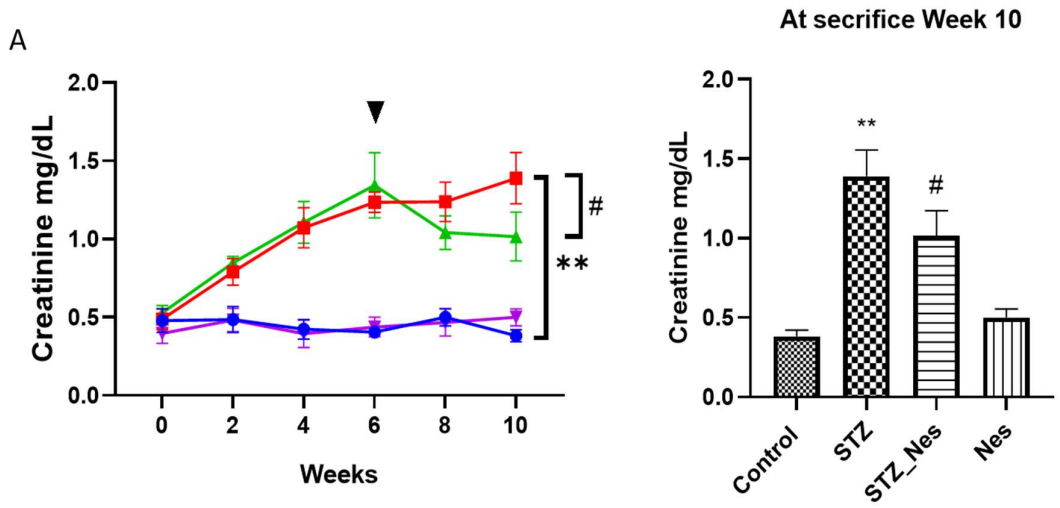


Figure 5-3 Effect of Nesfatin-1 peptide on Creatinine of Diabetic Animals

Diabetes in male Wistar rats was induced with multiple low doses of Streptozotocin (20mg/kg) for 5 days. Nesfatin-1 peptide treatment was given for last 4 weeks (week 7-10) every two days. Blood of rats was isolated, and creatinine recorded every two weeks for the whole duration of the protocol. After the start of treatment of Nesfatin-1 peptide, the rate of increase of creatinine decreased in those diabetic animals. After end of protocol creatinine in nesfatin-1 treated diabetic animals was less than diabetic animals. **$p < 0.01$ vs control #$p < 0.05$ *vs* STZ. Data is expressed as mean ± SD with n = 4-6 in each group.

At sacrifice of animals (Week 10) BUN and creatinine of the animals was also checked. Diabetic animals in which Nesfatin-1 treatment of 4 weeks was given they had lower BUN and creatinine in their blood stream as compared to untreated group suggesting its protective effect.

5.3.3. Nesfatin-1 treatment to diabetic animals and its effect on food and water intake and body weight of animals

As anorexic effect of nesfatin-1 is widely established it is important that it should not affect its therapeutic effect in diabetic nephropathy. For this we recorded the food and water intake and weight of animals of all the groups. In two groups in which STZ was given we can see there is dip in food intake and increase in water intake than normal animals this may be due to initial toxicity of STZ. While we can see that in these animals the diet does not change much during the whole protocol, comparing to normal animals as their diet increased with time. Due to polydipsia diseased groups water intake was very high. Interesting effects were seen after week 6 when nesfatin-1 treatment started in both diabetes + nesfatin-1 and nesfatin-1 alone treated group. In both the groups the food and water intake decreased and stagnated till the dose was given. This was an interesting observation suggesting that anorexic effect of nesfatin-1 plays part in food intake and cause decrease in FBG in these animals.

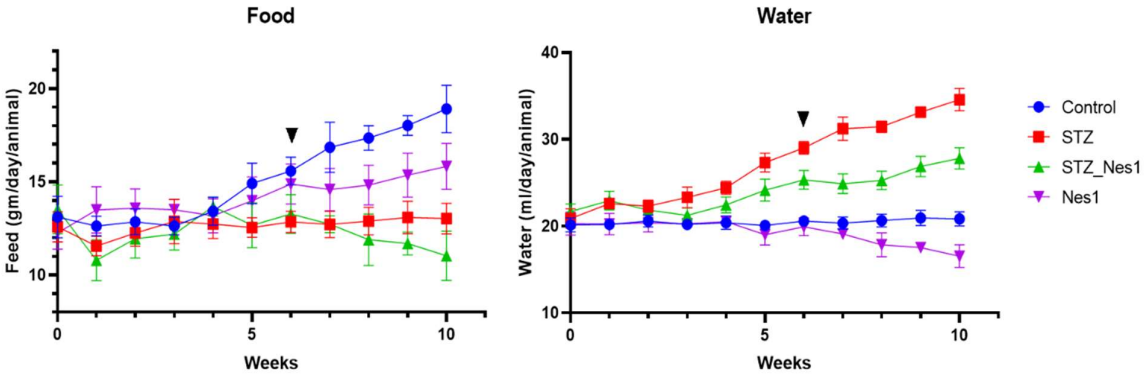


Figure 5-4 Effect of Nesfatin-1 peptide on food and water intake of Diabetic animals

Diabetes in male Wistar rats was induced with multiple low doses of Streptozotocin (20mg/kg) for 5 days. Nesfatin-1 peptide treatment was given for last 4 weeks (week 7-10) every two days. Food and water intake of rats was recorded every two days for the whole

protocol of 10 weeks. Due to the disease condition the food intake of diabetic animals was not changed while in diabetic and non-diabetic nesfatin-1 treatment (A, green, purple) we can observe a loss in the diet in subsequent weeks suggesting the anorexic effect of nesfatin-1. Data is expressed as mean ± SD with n = 4-6 in each group.

In our observation STZ dosed animals lost weight while control animals gained weight, due to type I diabetes. Weight change after nesfatin-1 dose started affected STZ+Nes1 and nes1 group due to decrease in food intake.

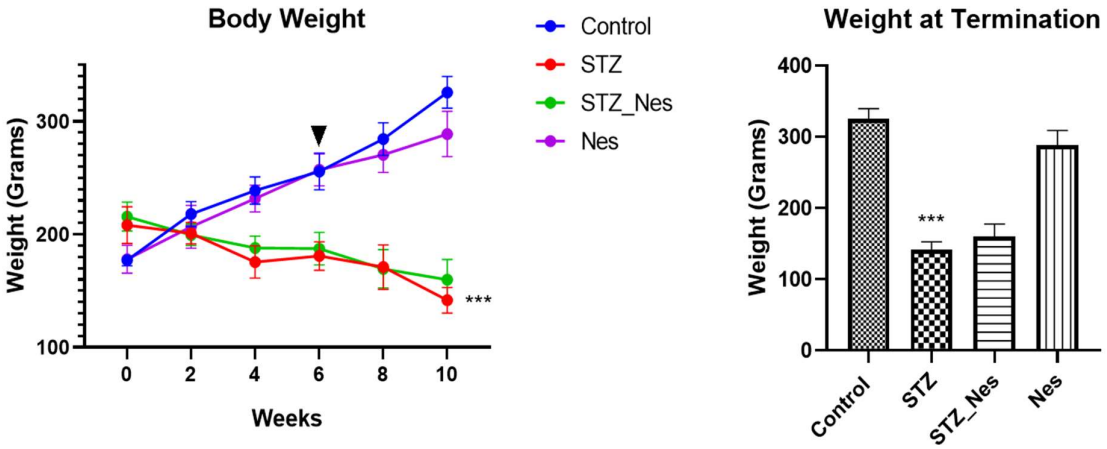


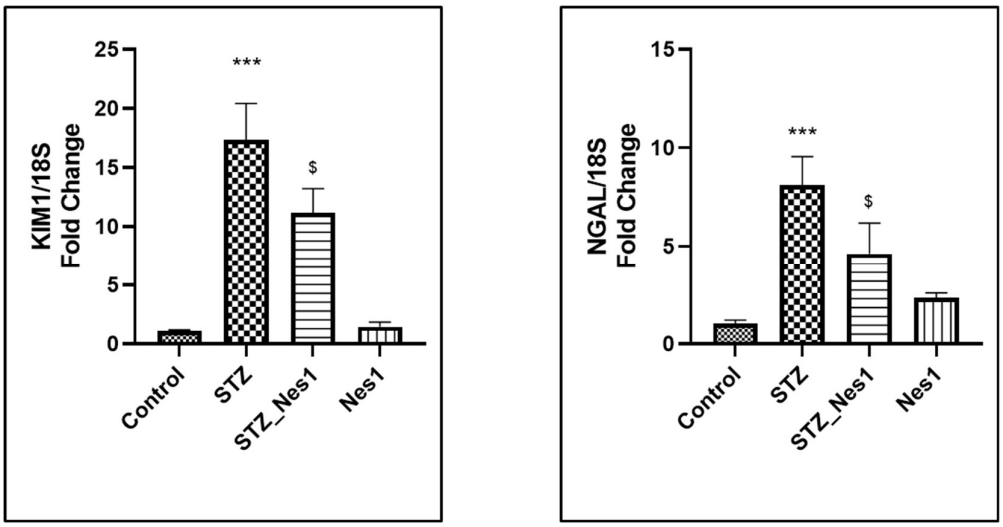
Figure 5-5 Effect of Nesfatin-1 peptide on Body Weight of Diabetic Animals

Same Wistar rats were weighed every two weeks during the protocol. After the start of treatment of Nesfatin-1 peptide, there was change in weight of both nesfatin-1 treatment groups but was not significant. ***$p < 0.001$ vs control. Data is expressed as mean ± SD with n = 4-6 in each group.

5.3.4. Confirmation of kidney injury using biomarkers

Kidney injury marker (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) are known to be potent markers of kidney injury. After 10 weeks' animals were sacrificed and expression analysis of KIM-1 and NGAL was done using RT-PCR. Significant change as

observed in expression of both the markers in STZ group compared to control group. While in treatment group it was less than disease group.



Fi

Figure 5-6 Confirmation of Kidney injury in Diabetic animals using molecular markers.

After sacrifice of animals at 10th week the kidney damage was accessed using expression of kidney injury markers: mRNA expression of kidney injury markers KIM-1 and NGAL was measured by RT-PCR (A, B). ***$p < 0.001$, \$$p < 0.05$ *vs* STZ. Data is expressed as mean ± SD with n = 6 in each group.

5.3.5. Downregulation NUCB2 expression in diabetic nephropathy and its effect on oxidative stress markers

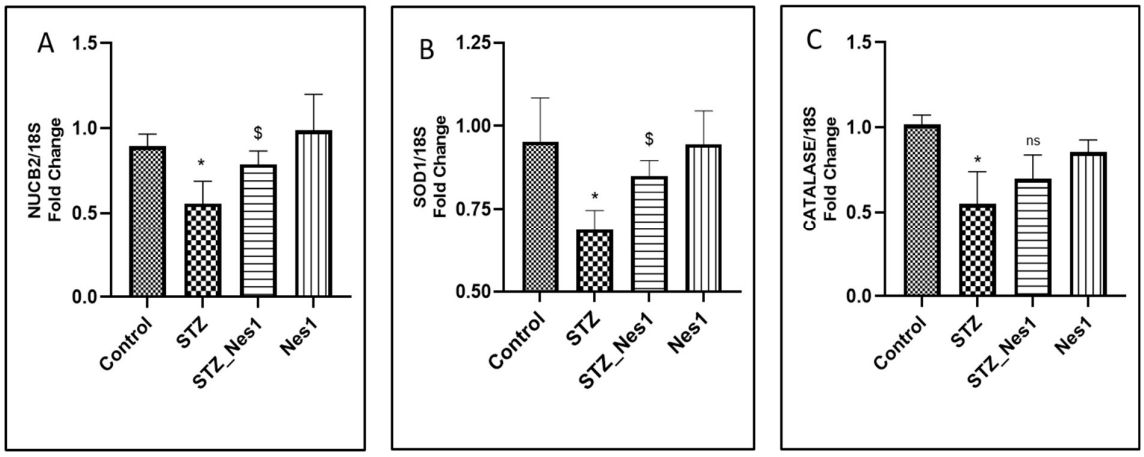


Figure 5-7 Effect of Nesfatin-1 peptide treatment in kidneys of Diabetic nephropathy animals on NUCB2 and oxidative stress markers

In Diabetic nephropathy, a decrease in expression of NUCB2 and oxidative stress markers was observed: In DN animals there is a decrease in expression of NUCB2, anti-oxidative stress proteins SOD1 and Catalase was observed. Which was ameliorate in nesfatin-1 treated diabetic animals. mRNA expression of NUCB2, anti-oxidative stress genes SOD1 and Catalase was measured by RT-PCR (A, B, C). *$p < 0.05$ vs control, $p<0.05 *vs* STZ. Data is expressed as mean ± SD with n = 6 in each group.

At the end of the protocol we checked, for changes in NUCB2 expression in disease condition i.e. in diabetic kidneys. From our results we observed that in DN there is decrease in NUCB2 expression while after treatment of 4 weeks in diabetic animals the NUCB2 expression gets attenuated.

We checked expression of oxidative stress markers SOD and Catalase kidneys of our DN induced animals. Significant decrease in the mRNA expression of catalase and SOD was observed in kidneys of diabetic animals. While improvement in expression of these genes was observed in treatment group.

5.3.6. Upregulation of pro apoptotic and inflammatory markers in Diabetic kidneys models:

Cell death by apoptosis plays part in glomerular and tubular damage during hyperglycaemic toxicity in kidneys. Caspase 3 is a well kwon pro-apoptotic marker that is upregulated diabetic kidney injury. In nesfatin-1 treated diabetic group we observed decrease in cas-3 expression compared to disease group suggesting positive effect of nesfatin-1 treatment. (Fig 5-8A, B)

Activation of inflammatory pathways by inflammatory cytokines such as IL-1β, IL-6 and TNF-α has been widely studied. In a disease like diabetic nephropathy, inflammation in kidneys is well known. We checked effect of nesfatin-1 on inflammation maker TNF-α in disease + nesfatin-1 treatment group. Significant decrease in mRNA expression of TNF-α in kidneys of diabetic nephropathy animals treated with nesfatin-1 peptide compared to disease group was observed. (Fig 5-8A, C)

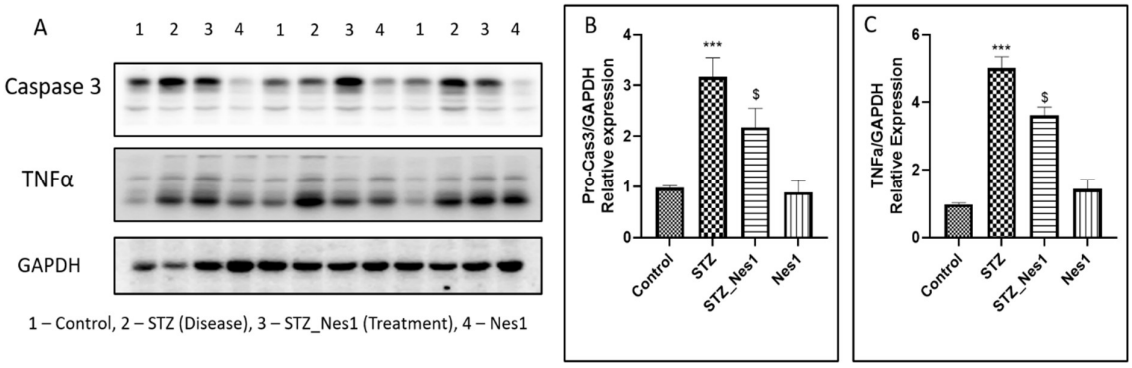


Figure 5-8 In DN kidneys there is increased expression of pro-apoptotic and inflammatory markers.

Expression of pro-apoptotic markers pro-caspase-3 and inflammatory cytokine TNF-α was measured by western blot (A – WB, B, C - Quantification). ***$p < 0.001$ vs control,

$p < 0.05$ *vs* STZ. Data is expressed as mean ± SD with n = 6 in each group.

5.3.7. Downregulation of putative receptor and effector genes in diabetic kidneys.

Nesfatin-1 peptide is known to acts through Melanocortin Receptor – 4 (MC4R) in hypothalamus. Its effect through calcium modulation suggesting putative effector genes Calumenin (CALU) and Calcium/calmodulin-dependent protein kinase I D (CAMK1D). We observed effect of disease and nesfatin-1 treatment on these 3 genes. While we observed that they are downregulated in disease condition. It is also downregulated in nesfatin-1 also group also. While being slightly but non significantly upregulated in treatment group compared to disease group. These results are interesting as they add new information on possible pathway of mode of action of nesfatin-1.

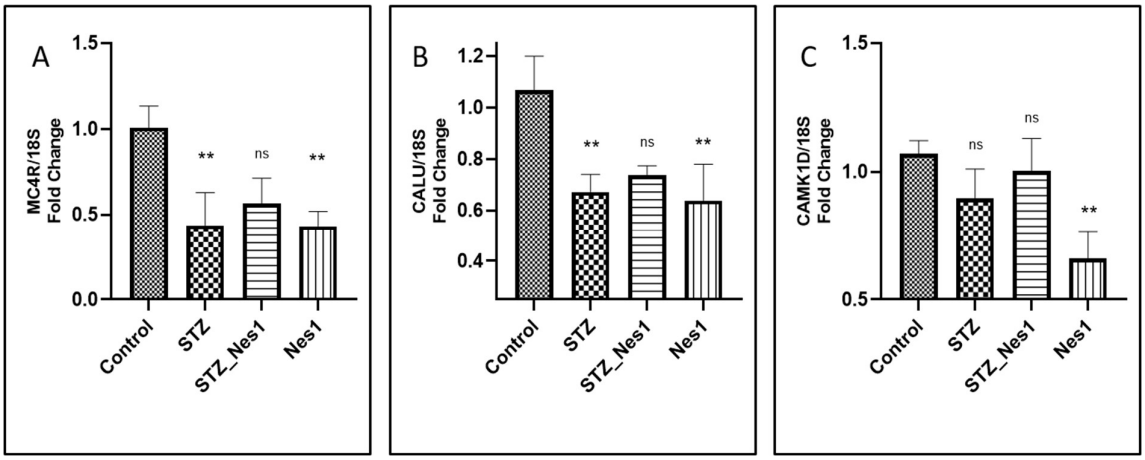


Figure 5-9 In DN there is attenuation in a known receptor of Nesfatin-1 peptide and its downstream effector genes.

In kidneys expression of MC4R, CALU and CAMK1D gene is assessed. While in DN models the genes were downregulated; after nesfatin-1 treatment in diabetic animals there is no significant increase in the expression of these genes. While in the control nesfatin-1 group

there is a significant decrease in their expression suggesting nesfatin-1 effect on them. MC4R, CALU and CAMK1D expression was measured by RT-PCR. **p < 0.01 vs control. Data is expressed as mean ± SD with n = 4-6 in each group

5.3.8. Histopathological analysis of kidney sections

Histopathological examination of kidney tissue in diabetic nephropathy model by Haematoxylin and eosin staining shows significant damage in disease group while in treatment group damage is less. This co-relates with all the previous kidney damage/gene expression data showing protective effect of nesfatin-1 in kidneys of diabetic nephropathy animals. In DN, kidneys histological changes in morphology and internal structure of kidneys are observed by H&E staining. The loss of interstitial lining, damage and deformation of the glomerulus can be observed in the disease group. In nesfatin-1 treated diabetic group it is less than STZ group.

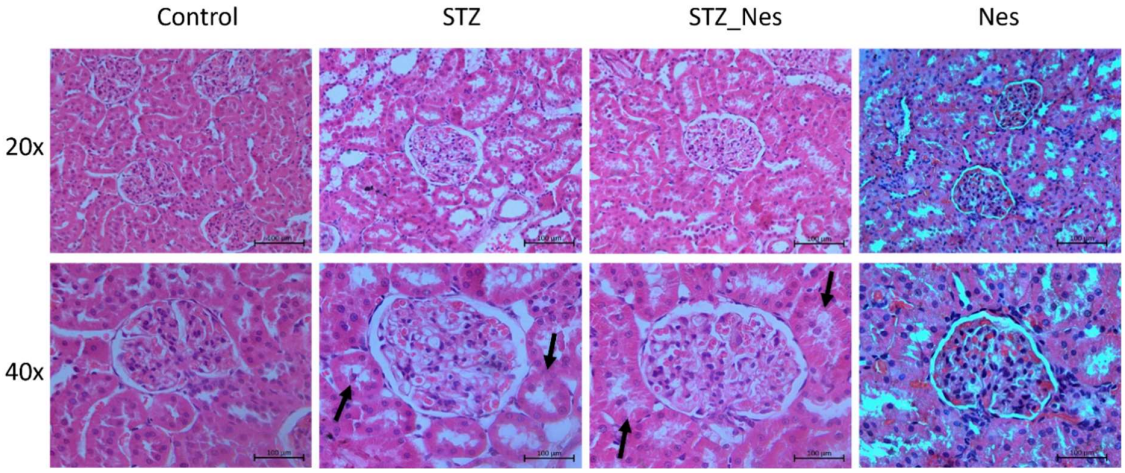


Figure 5-10 Effect of Nes-1 peptide treatment in DN kidneys on histology of kidneys

5.3.9. Assessment of fibrosis by Sirius red staining.

In long term diseases fibrosis of interstitium happened in organs. The same is observed in kidneys of diabetic patients. Collagen deposition is one of the major factors suggesting

fibrosis of tissues. Sirius red stain binds to this collagen and stains is bright red. Histological damage and fibrosis was observed in disease kidneys while in treatment fibrosis was less.

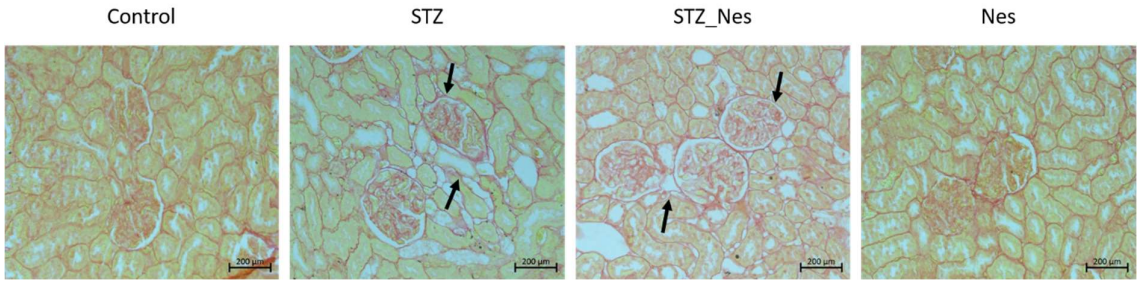


Figure 5-11 Effect of Nesfatin-1 peptide treatment in DN on fibrosis in kidneys

Nesfatin-1 treatment ameliorated renal fibrosis in DN kidney model. Histological analysis by Picrosirius staining for collagen deposition was done. More structural changes and high collagen deposition can be observed in Diabetic nephropathy group as compared to nesfatin-1 treatment group.

5.4 Discussion

The objective of this study was to investigate effect of long term nesfatin-1 treatment on kidneys of animals with diabetic nephropathy and its possible mechanism of action. It is known that nesfatin-1 treatment positively impacts release of insulin from isolate Langerhans cells in presence of glucose(171) while in another study the same has been observed in *in vivo* pancreases in mice. (172) Nesfatin-1 when inject in hypothalamus of Type II diabetic mice increases peripheral tissues' absorption of glucose and inhibits gluconeogenesis through various mechanisms, and increases hepatic and peripheral insulin sensitivity. (173)

While positive effect of nesfatin-1 in disease models effecting kidneys like Unilateral Ureter Obstruction (UUO) (115) and Renal Ischemia-Reperfusion injury(112) have been studied *in vivo*. Reports from our group suggesting nesfatin-1 positive effect in *in vitro* models of

glucotoxicity (111) and chemo-toxicity (167) have been observed. Aligning both the effects we devised a protocol to observe long term effect of nesfatin-1 treatment in diabetic nephropathy model in laboratory animals and find out possible mechanism of action.

For this work we induced diabetes in male wistar rates using multiple low doses of streptozotocin. Animals were checked for induction of disease at week 2 and animals with FBG 250mg/dL were selected for further experimentation. Our results suggest that after start of treatment of nesfatin-1 at week 6 disease + treatment group saw slight lowering of blood glucose levels and at week 10 FBG of STZ+Nes1 treatment animals was significantly less than STZ group (Fig 5-1). This may be due to reduction in food intake (Fig 5-4) as due to STZ loss of b-cells of pancreas was certain and known positive effect of nesfatin-1 treatment on insulin release can't be correlated suggesting direct role of nes1 in decrease in FBG in treatment group.

We observed renal health by checking regularly serum BUN (Fig 5-2) and creatinine (Fig 5-3) levels for 10 weeks every fortnight. As diabetic nephropathy is a chronic kidney disease, we observed that it took 6 weeks for significant kidney damage to be seen, after which treatment of nesfatin-1 was started. It is evident from our results that even though treatment does not reverse the increased BUN and creatinine levels in blood it definitely restricts further deterioration of kidneys due to diabetes possibly by protecting against deleterious effect of hyperglycaemia or a possible different mechanism. At week 10 significant less BUN and creatinine levels were seen in animals with diabetic nephropathy whom nesfatin-1 was given as treatment.

Gene expression study was carried out in kidney tissue samples using RT-PCR for biomarkers of kidney injury i.e. KIM1 and NGAL which get elevated during trauma related to kidneys. Our results were collaborate with previous studies suggesting increased KIM-1

and NGAL expression compared to control animals suggesting kidney injury while in animals with diabetes and nesfatin-1 treatment it was less than nephropathy animals (Fig 5-6). (174)

As we know that nesfatin-1 has anorexic effect, food and water intake of the animals under study was recorded. Food and water consumption of animals was recorded every two days for 10 weeks (Fig 5.4). (138) Food intake of STZ dosed groups remained stagnant till 6 weeks while it increased gradually for both control groups. Water intake for STZ dosed groups increased due to development of diabetes and increased polydipsia while for non-diseased groups it was consistent. A fascinating observation was observed after week 6 (nesfatin-1 dose started) in both the groups (disease + nesfatin-1) and nesfatin-1 alone their food and water intake decreased as compared to their co-groups (STZ vs STZ-Nes1) and (Control vs. Nes1 alone) group. These results showed us that nesfatin-1 dose was showing its anorexic effect and positive effects we are observing regarding kidneys can be correlated with it positive effect.

We also recorded weight of animals (Fig 5-5) and loss of weight in diabetic animal was consistent with weight loss in animals with Type I diabetes. A slight loss in weight in animals with nes1 dose was observed adding to low feed intake results in our study. But change was not significant at week 10. (175)

Our study first time report decreases in expression of NUCB2 due to DN in kidneys and it correlated with rate of kidney damage protection in STZ-Nes1 group (Fig 5-7 A). During diabetes induced CKD oxidative stress plays an important role in aggravating disease and is an important factor which causes kidney damage. Cellular protection mechanism against these ROS molecules involves antioxidant enzymes, such as catalase and superoxide dismutase (SOD) and glutathione peroxidase. The effect of kidney damage was also

observed in downregulation of oxidative stress genes SOD1 and Catalase while in treatment group they were less downregulated than diseased group (Fig 5-7 B, C). These results suggest that nesfatin-1 treatment was beneficial in restricting disease growth.

During disease progression multiple factors play part like inflammation and apoptosis. Using western blot analysis, we observed upregulation in expression of pro-inflammatory marker TNF-α (Fig 5-8 A) and pro-apoptotic marker Caspase-3 (Fig 5-8 B) in diseased kidneys and their reduction in treatment group. As it is well known that in diabetic nephropathy, podocyte apoptosis results in glomerular injury and apoptosis of the epithelial cells of the proximal convoluted tubules is common. While hyperglycaemia and correlated oxidative stress have been known to activate inflammation in diabetic kidneys. Positive effect of nesfatin-1 treatment in UUO and Renal Ischemia treatment in kidneys with respect to inflammation and apoptosis adds into our observation of protective effect of peptide in diabetic kidneys.

It is imperative that nesfatin-1 treatment has protective effects on diabetic kidneys. These effects should reciprocate in histopathological imaging and fibrosis staining of kidney tissues. To observe this, we stained kidneys tissues with Haematoxylin and Eosin (Fig 5-10) to observe changes in morphology of tissues while to observe fibrosis i.e. collagen deposition tissue sections were stained with Sirius red (Fig 5-11). Anti-fibrotic effect if nesfatin-1 treatment in lungs and kidneys are previously observed. (115,133) The results of biochemical, expression studies correlated our histopathological data and tissue in treatment group have significant less damage and collagen deposition in them.

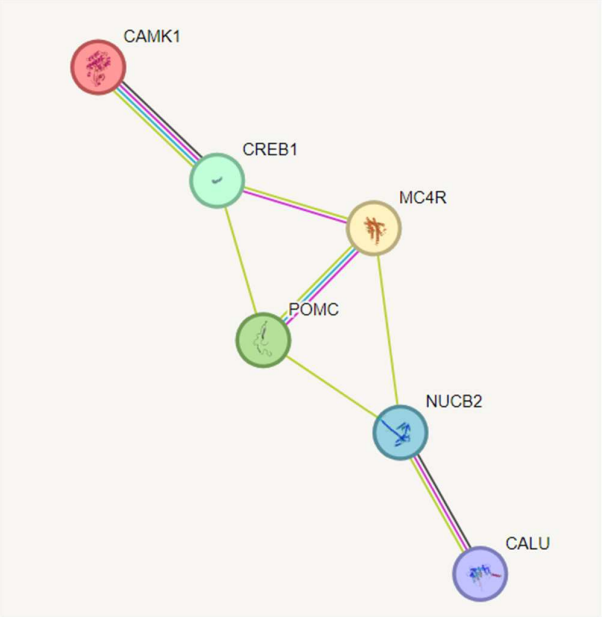


Figure 5-12 Interacting partners of NUCB2 (StringDB)

Figure explaining interaction between genes/proteins. Green line represents interaction is via text mining i.e. search in large datasets of research papers while purple represents experimental validation of interactions.

The mechanism of action of positive effect of long-term dosing of nesfatin-1 peptide in animals with diabetic nephropathy must assessed. For this known hypothalamic target of nesfatin-1 Melanocortin Receptor 4 (MC4R) (Fig 5-9A) (108,176)which plays a central role in affecting its anorexic effect is selected# Nesfatin-1 effect through calcium modulation and release of insulin in pancreas through it is also studied. (177)Suggesting putative effector genes Calumenin (CALU) (Fig 5-9B) and Calcium/calmodulin-dependent protein kinase I D (CAMK1D) (Fig 5-9C) have a role in its downstream actions. Downregulation in disease condition in all the three genes was observed. Interesting observation was that they are also downregulated in nesfatin-1 group also. Which is suggesting effect of nes1 treatment on them. While being slightly but non significantly upregulated in treatment group

compared to disease group. These results are interesting as they add new information on possible pathway of mode of action of nesfatin-1.

From our findings, it is evident that nesafin-1 treatment in diabetic kidney disease helps in protection in further aggravation of kidney disease. In kidneys it is evident that NUCB2 is downregulated diabetic kidneys and its expression correlated with damage to the kidneys. Long term treatment of Nesfatin-1 in diabetic animal's effect their food and water intake and weight and blood glucose levels. While it also hinders progression of disease by increasing anti-oxidative stress genes while decreasing pro-apoptotic and pro-inflammatory genes. This protective effect can have been seen in histopathology and fibrosis imaging of diseased kidneys. Putative receptors of nesfatin-1 and its downstream effector molecule can be further explored for elucidating its mechanism of action. We suggest Nesfatin-1 as a novel therapeutic peptide which can be further explored in diabetic kidney injury disease.

Chapter 6 Conclusion and Future Prospective

Acute Kidney Injury and Chronic Kidney Injury are leading causes of kidney related hospitalization and mortality in the world. With them at rise it's essential to develop and screen therapeutic interventions which can be beneficial to ameliorating their severity and controlling further tissue damage during these conditions. The primary goal of this thesis is to evaluate the expression of NUCB2 an endogenous protein which post translationally cleaves into three peptides, one of which Nesfatin-1, and use it as an exogenous peptide in treating and protecting agent in different disease conditions and what will be its possible molecular mechanism of action.

We found that in chemo toxic models of AKI (Doxorubicin and Cisplatin) and rhabdomyolysis NUCB2 expression was found to be downregulated and correlated with elevated kidney injury genes and biochemical parameters and inflammatory and oxidative stress markers also. We also checked effect of nesfatin-1 on NRK52E cells based *in vitro* models of these conditions and in those we found that NUCB2 is downregulated in disease conditions. We observed protective effect of nesfatin-1 cotreatment by oxidative stress markers and inflammation markers. The study findings revealed nesfatin-1 protected renal NRK52E cells against chemo toxicity -induced ROS and inflammation.

We used rhabdomyolysis model to check effect of Nesfatin-1 treatment in both *in vitro* and *in vivo* conditions. We induced *in vitro* rhabdomyolysis in NRK52E cells using myoglobin. We found Nesfatin-1 cotreatment has protective effect against myoglobin induced rhabdomyolysis and NUCB2 expression was found to be increased as compared to disease group. Protective effect of nesfatin-1 was collaborated with oxidative stress markers, anti-inflammatory markers and apoptotic markers, with positive indications in expression of these markers as compared to disease group. For *in vivo* experiments rhabdomyolysis model

was induced and nesfatin-1 treatment was given to one group of diseased animals. Effect of nesfatin-1 on these animals was checked using biochemical and injury markers of kidneys and protective effect of nesfatin-1 was elucidated using inflammatory markers and histology. We concluded that nesfatin-1 has a protective effect in *in vitro* and *in vivo* models of rhabdomyolysis.

We from our group have reported that nesfatin-1 has protective effect in *in vitro* models of glucotoxicity. Here we assess nesfatin-1 effect in diabetic CKI in rats. For this we induced diabetes in rats with low doses of STZ and nesfatin-1 dose for 4 weeks was given and its long-term effect was observed. We observed protection against aggravation of kidney disease and stopping of further deterioration in kidney health. It was an interest observation regarding anorexic effect of nesfatin-1 dosing on food and water intake of these animals. We also observed reduced pro-inflammatory marker and increase in antioxidant genes while increased in anti-apoptotic genes in co-treatment group as compared to disease group. Positive outcome of nesfatin-1 treatment in co-treatment group was observed in histopathology and reduced collagen deposition suggesting reduction in fibrosis via Sirius red staining. We saw that all the three disease, co-treatment and nesfatin-1 alone group expression of putative receptor and its downstream effectors was also reduced. Suggesting that these genes play some part in as receptors and effector molecules and in positive outcome of its treatment. Further studies have to be performed for the same.

Therefore, our overall study concludes that nesfatin-1 treatment has a protective effect in AKI and CKI models of kidney injury in both *in vitro* and *in vivo* conditions.

Future perspectives:

Treatment of nesfatin-1 has proven to be beneficial to ameliorate AKI and Diabetic nephropathy. However, the possible side effects associated due to its anorexic effect has to be studied. This is also imperative to do further research to elucidate clear picture of mechanism of action of nesfatin-1. It has been firmly established that nesfatin-1 treatment imparts protection to kidneys in different models but complete picture of metabolic effect of this anorexic peptide has to be elucidated.

Further experiments with using inhibitors of MC4R like SHU9119 can be used to observe how nesfatin-1 treatment is effective and is it able to protect after inhibiting the receptor. To minimize these side effects and to increase the benefits, further research needs to be done to design and evaluate nesfatin-1 like peptides with longer half-life and effectiveness in renal injury. Effect of nesfatin-1 treatment on ER and Ca2+ channels have to be done for its better understanding of working.

References

1. ICD. ICD-10 Version:2019 [Internet]. [cited 2023 Nov 7]. Available from: https://icd.who.int/browse10/2019/en#/XIV

2. Hsu RK, Hsu C yuan. The Role of Acute Kidney Injury in Chronic Kidney Disease. Seminars in Nephrology. 2016 Jul 1;36(4):283–92.

3. Zelmer JL. The economic burden of end-stage renal disease in Canada. Kidney International. 2007 Nov 1;72(9):1122–9.

4. Kimmel PL, Patel SS. Quality of Life in Patients with Chronic Kidney Disease: Focus on End-Stage Renal Disease Treated with Hemodialysis. Seminars in Nephrology. 2006 Jan 1;26(1):68–79.

5. Kleeman CR. Metabolic coma. Kidney International. 1989 Dec 1;36(6):1142–58.

6. Bellomo R, Kellum J, Ronco C. Acute kidney injury. Lancet. 2012;380(9843):756–66.

7. Romagnani P, Remuzzi G, Glassock R, Levin A, Jager KJ, Tonelli M, et al. chronic kidney disease. Nat Rev Dis Primers. 2017 Nov 23;3(1):1–24.

8. Vikrant S, Gupta D, Singh M. Epidemiology and outcome of acute kidney injury from a tertiary care hospital in India. Saudi J Kidney Dis Transpl. 2018;29(4):956–66.

9. Jayakumar M, Ram Prabahar M, Fernando EM, Manorajan R, Venkatraman R, Balaraman V. Epidemiologic Trend Changes in Acute Renal Failure—A Tertiary Center Experience from South India. Renal Failure. 2006 Jan 1;28(5):405–10.

10. Korula S, Balakrishnan S, Sundar S, Paul V, Balagopal A. Acute kidney injury-incidence, prognostic factors, and outcome of patients in an Intensive Care Unit in a tertiary center: A prospective observational study. Indian Journal of Critical Care Medicine: Peer-reviewed, Official Publication of Indian Society of Critical Care Medicine. 2016 Jun;20(6):332.

11. Mohammed ZA, Suresh AA, Kumar P, Attur RP. Acute Kidney Injury: Prevalence and Outcomes in Southern Indian Population. Journal of clinical and diagnostic research. 2018;12(5):5–10.

12. Paudel MS, Wig N, Mahajan S, Pandey RM, Guleria R, Sharma SK. A Study of Incidence of AKI in Critically Ill Patients. Renal Failure. 2012 Nov 1;34(10):1217–22.

13. Krishnamurthy S, Mondal N, Narayanan P, Biswal N, Srinivasan S, Soundravally R. Incidence and Etiology of Acute Kidney Injury in Southern India. Indian J Pediatr. 2013 Mar 1;80(3):183–9.

14. Prakash J, Singh TB, Ghosh B, Malhotra V, Rathore SS, Vohra R, et al. Changing epidemiology of community-acquired acute kidney injury in developing countries: analysis of 2405 cases in 26 years from eastern India. Clinical Kidney Journal. 2013 Apr 1;6(2):150–5.

15. Kaul A, Sharma RK, Tripathi R, Suresh KJ, Bhatt S, Prasad N. Spectrum of Community-Acquired Acute Kidney Injury in India: A Retrospective Study. Saudi Journal of Kidney Diseases and Transplantation. 2012 Jun;23(3):619.

16. Jha V, Malhotra HS, Sakhuja V, Chugh KS. Spectrum of hospital-acquired acute renal failure in the developing countries--Chandigarh study. Q J Med. 1992 Jul;83(303):497–505.

17. Goswami S, Pahwa N, Vohra R, Raju BM. Clinical Spectrum of Hospital Acquired Acute Kidney Injury: A Prospective Study from Central India. Saudi Journal of Kidney Diseases and Transplantation. 2018 Aug;29(4):946.

18. Susantitaphong P, Cruz DN, Cerda J, Abulfaraj M, Alqahtani F, Koulouridis I, et al. World incidence of AKI: a meta-analysis. Clin J Am Soc Nephrol. 2013 Sep;8(9):1482–93.

19. Hoste EAJ, Kellum JA, Selby NM, Zarbock A, Palevsky PM, Bagshaw SM, et al. Global epidemiology and outcomes of acute kidney injury. Nat Rev Nephrol. 2018 Oct;14(10):607–25.

20. Wonnacott A, Meran S, Amphlett B, Talabani B, Phillips A. Epidemiology and Outcomes in Community-Acquired Versus Hospital-Acquired AKI. Clin J Am Soc Nephrol. 2014 Jun 6;9(6):1007–14.

21. Cerdá J, Bagga A, Kher V, Chakravarthi RM. The contrasting characteristics of acute kidney injury in developed and developing countries. Nat Rev Nephrol. 2008 Mar;4(3):138–53.

22. Jha V. End-Stage Renal Care in Developing Countries: The India Experience. Renal Failure. 2004 Jan 1;26(3):201–8.

23. Agarwal SK, Srivastava RK. Chronic Kidney Disease in India: Challenges and Solutions. Nephron Clinical Practice. 2009 Feb 5;111(3):c197–203.

24. Pradeepa R, Mohan V. Epidemiology of type 2 diabetes in India. Indian J Ophthalmol. 2021 Nov;69(11):2932–8.

25. Unnikrishnan R, Rema M, Pradeepa R, Deepa M, Shanthirani CS, Deepa R, et al. Prevalence and Risk Factors of Diabetic Nephropathy in an Urban South Indian Population: The Chennai Urban Rural Epidemiology Study (CURES 45). Diabetes Care. 2007 Aug 1;30(8):2019–24.

26. Kovesdy CP. Epidemiology of chronic kidney disease: an update 2022. Kidney Int Suppl (2011). 2022 Apr;12(1):7–11.

27. Suriyong P, Ruengorn C, Shayakul C, Anantachoti P, Kanjanarat P. Prevalence of chronic kidney disease stages 3-5 in low- and middle-income countries in Asia: A systematic review and meta-analysis. PLoS One. 2022;17(2): e0264393.

28. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Res Clin Pract. 2022 Jan; 183:109119.

29. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Research and Clinical Practice. 2010 Jan 1;87(1):4–14.

30. Retnakaran R, Cull CA, Thorne KI, Adler AI, Holman RR, UKPDS Study Group. Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74. Diabetes. 2006 Jun;55(6):1832–9.

31. Sheen YJ, Sheu WH. Risks of rapid decline renal function in patients with type 2 diabetes. World Journal of Diabetes. 2014 Dec 15;5(6):835–46.

32. Randall Thomas S. Kidney modeling and systems physiology. WIREs Mechanisms of Disease. 2009 Sep;1(2):172–90.

33. Soriano RM, Penfold D, Leslie SW. Anatomy, Abdomen and Pelvis: Kidneys. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2023.

34. Pavenstädt H, Kriz W, Kretzler M. Cell Biology of the Glomerular Podocyte. Physiological Reviews. 2003 Jan 1;83(1):253–307.

35. Sands JM, Layton HE. The Physiology of Urinary Concentration: an Update. Semin Nephrol. 2009 May;29(3):178–95.

36. Sahay M, Kalra S, Bandgar T. Renal endocrinology: The new frontier. Indian J Endocrinol Metab. 2012;16(2):154–5.

37. Makris K, Spanou L. Acute Kidney Injury: Definition, Pathophysiology and Clinical Phenotypes. Clin Biochem Rev. 2016 May;37(2):85–98.

38. Basile DP, Anderson MD, Sutton TA. Pathophysiology of Acute Kidney Injury. Compr Physiol. 2012 Apr;2(2):1303–53.

39. Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, Warnock DG, et al. Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. Crit Care. 2007 Mar 1;11(2): R31.

40. Bellomo R, Ronco C, Kellum J, Mehta R, Palevsky P. Acute renal failure – definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. Crit Care. 2004;8(4): R204–12.

41. Kellum JA, Romagnani P, Ashuntantang G, Ronco C, Zarbock A, Anders HJ. Acute kidney injury. Nat Rev Dis Primers. 2021 Jul 15;7(1):1–17.

42. Perazella MA, Rosner MH. Drug-Induced Acute Kidney Injury. Clinical Journal of the American Society of Nephrology. 2022 Aug;17(8):1220.

43. Ghane Shahrbaf F, Assadi F. Drug-induced renal disorders. J Renal Inj Prev. 2015 Sep 1;4(3):57–60.

44. Hinson JS, Ehmann MR, Al Jalbout N, Ortmann MJ, Zschoche J, Klein EY. Risk of Acute Kidney Injury Associated with Medication Administration in the Emergency Department. J Emerg Med. 2020 Mar;58(3):487–96.

45. Lentini P, Zanoli L, Granata A, Signorelli SS, Castellino P, Dell'Aquila R. Kidney and heavy metals - The role of environmental exposure (Review). Molecular Medicine Reports. 2017 May 1;15(5):3413–9.

46. Bicalho MD, Soares DB, Botoni FA, Reis AMM, Martins MAP. Drug-Induced Nephrotoxicity and Dose Adjustment Recommendations: Agreement Among Four Drug Information Sources. Int J Environ Res Public Health. 2015 Sep;12(9):11227–40.

47. Izzedine H, Perazella MA. Anticancer Drug-Induced Acute Kidney Injury. Kidney International Reports. 2017 Jul 1;2(4):504–14.

48. Dobrek L. A Synopsis of Current Theories on Drug-Induced Nephrotoxicity. Life (Basel). 2023 Jan 24;13(2):325.

49. Naesens M, Kuypers DRJ, Sarwal M. Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol. 2009 Feb;4(2):481–508.

50. Markowitz GS, Perazella MA. Drug-induced renal failure: a focus on tubulointerstitial disease. Clinica Chimica Acta. 2005 Jan 1;351(1):31–47.

51. Hall AM, Trepiccione F, Unwin RJ. Drug toxicity in the proximal tubule: new models, methods and mechanisms. Pediatr Nephrol. 2022;37(5):973–82.

52. Perazella MA. Crystal-induced acute renal failure. Am J Med. 1999 Apr;106(4):459–65.

53. Howard SC, Jones DP, Pui CH. The Tumor Lysis Syndrome. N Engl J Med. 2011 May 12;364(19):1844–54.

54. Pui CH. Childhood Leukemias. New England Journal of Medicine. 1995 Jun 15;332(24):1618–30.

55. Torres PA, Helmstetter JA, Kaye AM, Kaye AD. Rhabdomyolysis: Pathogenesis, Diagnosis, and Treatment. The Ochsner Journal. 2015 Spring;15(1):58.

56. Bosch Esteban; Grau Josep M. XP. Rhabdomyolysis and Acute Kidney Injury. The New England journal of medicine. 2009;361(1):62–72.

57. Nance JR, Mammen AL. Diagnostic Evaluation of Rhabdomyolysis. Muscle Nerve. 2015 Jun;51(6):793–810.

58. Trujillo MH, Fragachán G. C. Rhabdomyolysis and Acute Kidney Injury due to Severe Heat Stroke. Case Rep Crit Care. 2011; 2011:951719.

59. Hebert JF, Burfeind KG, Malinoski D, Hutchens MP. Molecular Mechanisms of Rhabdomyolysis-Induced Kidney Injury: From Bench to Bedside. Kidney Int Rep. 2022 Sep 30;8(1):17–29.

60. Jha RP, Shri N, Patel P, Dhamnetiya D, Bhattacharyya K, Singh M. Trends in the diabetes incidence and mortality in India from 1990 to 2019: a joinpoint and age-period-cohort analysis. J Diabetes Metab Disord. 2021 Jul 5;20(2):1725–40.

61. Anjana RM, Unnikrishnan R, Deepa M, Pradeepa R, Tandon N, Das AK, et al. Metabolic non-communicable disease health report of India: the ICMR-INDIAB national cross-sectional study (ICMR-INDIAB-17). The Lancet Diabetes & Endocrinology. 2023 Jul 1;11(7):474–89.

62. Gromova LV, Fetissov SO, Gruzdkov AA. Mechanisms of Glucose Absorption in the Small Intestine in Health and Metabolic Diseases and Their Role in Appetite Regulation. Nutrients. 2021 Jul 20;13(7):2474.

63. Röder PV, Wu B, Liu Y, Han W. Pancreatic regulation of glucose homeostasis. Exp Mol Med. 2016 Mar;48(3): e219.

64. Rossetti L, Giaccari A, DeFronzo RA. Glucose toxicity. Diabetes Care. 1990 Jun;13(6):610–30.

65. Han HS, Kang G, Kim JS, Choi BH, Koo SH. Regulation of glucose metabolism from a liver-centric perspective. Exp Mol Med. 2016 Mar;48(3): e218.

66. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2010 Jan;33(Suppl 1): S62–9.

67. Katsarou A, Gudbjörnsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. Nat Rev Dis Primers. 2017 Mar 30;3(1):1–17.

68. DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ, et al. Type 2 diabetes mellitus. Nature Reviews Disease Primers. 2015 Jul 23;1(1):15019.

69. Thomas MC, Brownlee M, Susztak K, Sharma K, Jandeleit-Dahm KAM, Zoungas S, et al. Diabetic kidney disease. Nat Rev Dis Primers. 2015 Jul 30;1(1):1–20.

70. Tyagi I, Agrawal U, Amitabh V, Jain AK, Saxena S. Thickness of glomerular and tubular basement membranes in preclinical and clinical stages of diabetic nephropathy. Indian J Nephrol. 2008 Apr;18(2):64–9.

71. Tobe SW, McFarlane PA, Naimark DM. Microalbuminuria in diabetes mellitus. Canadian Medical Association Journal. 2002 Sep 3;167(5):499–503.

72. Kim Y, Kleppel MM, Butkowski R, Mauer SM, Wieslander J, Michael AF. Differential expression of basement membrane collagen chains in diabetic nephropathy. Am J Pathol. 1991 Feb;138(2):413–20.

73. Pourghasem M, Shafi H, Babazadeh Z. Histological changes of kidney in diabetic nephropathy. Caspian J Intern Med. 2015;6(3):120–7.

74. Forbes JM, Cooper ME, Oldfield MD, Thomas MC. Role of Advanced Glycation End Products in Diabetic Nephropathy. Journal of the American Society of Nephrology. 2003 Aug;14(suppl_3): S254.

75. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. J Clin Invest. 1991 Feb 1;87(2):432–8.

76. Sun L, Kanwar YS. Relevance of TNF-α in the context of other inflammatory cytokines in the progression of diabetic nephropathy. Kidney International. 2015 Oct 1;88(4):662–5.

77. C. Tobon-Velasco J, Cuevas E, A. Torres-Ramos M. Receptor for AGEs (RAGE) as Mediator of NF-kB Pathway Activation in Neuroinflammation and Oxidative Stress. CNS Neurol Disord Drug Targets. 2014 Nov 1;13(9):1615–26.

78. Tan ALY, Forbes JM, Cooper ME. AGE, RAGE, and ROS in Diabetic Nephropathy. Seminars in Nephrology. 2007 Mar 1;27(2):130–43.

79. Wu L, Derynck R. Essential Role of TGF-β Signaling in Glucose-Induced Cell Hypertrophy. Developmental Cell. 2009 Jul;17(1):35–48.

80. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative Stress and Stress-Activated Signaling Pathways: A Unifying Hypothesis of Type 2 Diabetes. Endocrine Reviews. 2002 Oct 1;23(5):599–622.

81. Hills CE, Squires PE. TGF-beta1-induced epithelial-to-mesenchymal transition and therapeutic intervention in diabetic nephropathy. Am J Nephrol. 2010;31(1):68–74.

82. Lu Q, Wang WW, Zhang MZ, Ma ZX, Qiu XR, Shen M, et al. ROS induces epithelial-mesenchymal transition via the TGF-β1/PI3K/Akt/mTOR pathway in diabetic nephropathy. Experimental and Therapeutic Medicine. 2019 Jan 1;17(1):835–46.

83. Vaidya VS, Ferguson MA, Bonventre JV. Biomarkers of Acute Kidney Injury. Annu Rev Pharmacol Toxicol. 2008; 48:463–93.

84. Salih M, Bovée DM, Roksnoer LCW, Casteleijn NF, Bakker SJL, Gansevoort RT, et al. Urinary renin-angiotensin markers in polycystic kidney disease. American Journal of Physiology-Renal Physiology. 2017 Oct;313(4): F874–81.

85. Gaibi T, Ghatak-Roy A. Approach to Acute Kidney Injuries in the Emergency Department. Emergency Medicine Clinics. 2019 Nov 1;37(4):661–77.

86. Schanz M, Kimmel M, Alscher MD, Amann K, Daniel C. TIMP-2 and IGFBP7 in human kidney biopsies in renal disease. Clinical Kidney Journal. 2023 Sep 1;16(9):1434–46.

87. Ganda IJ, Kasri Y, Susanti M, Hamzah F, Rauf S, Albar H, et al. Kidney injury molecule type-1, interleukin-18, and insulin-like growth factor binding protein 7 levels in urine to predict acute kidney injury in pediatric sepsis. Front Pediatr. 2022; 10:1024713.

88. Seabra VF, Perianayagam MC, Tighiouart H, Liangos O, dos Santos OFP, Jaber BL. Urinary α-GST and π-GST for prediction of dialysis requirement or in-hospital death in established acute kidney injury. Biomarkers. 2011 Dec;16(8):709–17.

89. du Cheyron D, Daubin C, Poggioli J, Ramakers M, Houillier P, Charbonneau P, et al. Urinary measurement of Na+/H+ exchanger isoform 3 (NHE3) protein as new marker of tubule injury in critically ill patients with ARF. Am J Kidney Dis. 2003 Sep;42(3):497–506.

90. Ferguson MA, Vaidya V, Bonventre JV. Biomarkers of Nephrotoxic Acute Kidney Injury. Toxicology. 2008 Mar 20;245(3):182–93.

91. Devarajan P. Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of kidney disease. Scand J Clin Lab Invest Suppl. 2008; 241:89–94.

92. Oh-I S, Shimizu H, Satoh T, Okada S, al et. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature. 2006 Oct 12;443(7112):709–12.

93. Weibert E, Hofmann T, Stengel A. Role of nesfatin-1 in anxiety, depression and the response to stress. Psychoneuroendocrinology. 2019 Feb; 100:58–66.

94. Shimizu H, Oh-I S, Hashimoto K, Nakata M, Yamamoto S, Yoshida N, et al. Peripheral administration of nesfatin-1 reduces food intake in mice: the leptin-independent mechanism. Endocrinology. 2009 Feb;150(2):662–71.

95. Gonzalez R, Tiwari A, Unniappan S. Pancreatic beta cells colocalize insulin and pronesfatin immunoreactivity in rodents. Biochem Biophys Res Commun. 2009 Apr 17;381(4):643–8.

96. Dore R, Krotenko R, Reising JP, Murru L, Sundaram SM, Di Spiezio A, et al. Nesfatin-1 decreases the motivational and rewarding value of food. Neuropsychopharmacology. 2020 Sep;45(10):1645–55.

97. Pan W, Hsuchou H, Kastin AJ. Nesfatin-1 crosses the blood-brain barrier without saturation. Peptides. 2007 Nov;28(11):2223–8.

98. Stengel A, Taché Y. Role of brain NUCB2/nesfatin-1 in the regulation of food intake. Curr Pharm Des. 2013;19(39):6955–9.

99. Darambazar G, Nakata M, Okada T, Wang L, Li E, Shinozaki A, et al. Paraventricular NUCB2/nesfatin-1 is directly targeted by leptin and mediates its anorexigenic effect. Biochem Biophys Res Commun. 2015 Jan 24;456(4):913–8.

100. Zhang AQ, Li XL, Jiang CY, Lin L, Shi RH, Chen JD, et al. Expression of nesfatin-1/NUCB2 in rodent digestive system. World J Gastroenterol. 2010 Apr 14;16(14):1735–41.

101. Bonnet MS, Ouelaa W, Tillement V, Trouslard J, Jean A, Gonzalez BJ, et al. Gastric distension activates NUCB2/nesfatin-1-expressing neurons in the nucleus of the solitary tract. Regul Pept. 2013 Nov 10; 187:17–23.

102. Prinz P, Stengel A. Control of Food Intake by Gastrointestinal Peptides: Mechanisms of Action and Possible Modulation in the Treatment of Obesity. J Neurogastroenterol Motil. 2017 Apr 30;23(2):180–96.

103. Ramesh N, Mortazavi S, Unniappan S. Nesfatin-1 stimulates cholecystokinin and suppresses peptide YY expression and secretion in mice. Biochem Biophys Res Commun. 2016 Mar 25;472(1):201–8.

104. Nakata M, Manaka K, Yamamoto S, Mori M, Yada T. Nesfatin-1 enhances glucose-induced insulin secretion by promoting Ca (2+) influx through L-type channels in mouse islet β-cells. Endocr J. 2011;58(4):305–13.

105. Luo JJ, Wen FJ, Qiu D, Wang SZ. Nesfatin-1 in lipid metabolism and lipid-related diseases. Clin Chim Acta. 2021 Nov; 522:23–30.

106. Wang Y, Li Z, Zhang X, Xiang X, Li Y, Mulholland MW, et al. Nesfatin-1 promotes brown adipocyte phenotype. Sci Rep. 2016 Nov 8; 6:34747.

107. Mierzyński R, Poniedziałek-Czajkowska E, Dłuski D, Patro-Małysza J, Kimber-Trojnar Ż, Majsterek M, et al. Nesfatin-1 and Vaspin as Potential Novel Biomarkers for the Prediction and Early Diagnosis of Gestational Diabetes Mellitus. Int J Mol Sci. 2019 Jan 4;20(1).

108. Yosten GLC, Samson WK. Nesfatin-1 exerts cardiovascular actions in brain: possible interaction with the central melanocortin system. Am J Physiol Regul Integr Comp Physiol. 2009 Aug;297(2): R330-336.

109. Su RY, Geng XY, Yang Y, Yin HS. Nesfatin-1 inhibits myocardial ischaemia/reperfusion injury through activating Akt/ERK pathway-dependent attenuation of endoplasmic reticulum stress. J Cell Mol Med. 2021 Jun;25(11):5050–9.

110. Tasatargil A, Kuscu N, Dalaklioglu S, Adiguzel D, Celik-Ozenci C, Ozdem S, et al. Cardioprotective effect of nesfatin-1 against isoproterenol-induced myocardial infarction in rats: Role of the Akt/GSK-3β pathway. Peptides. 2017 Sep; 95:1–9.

111. Lahane GP, Dhar A. Nesfatin-1 peptide protects rat renal epithelial cells against high glucose and H (2)O(2) induced injury via inhibition of oxidative stress, apoptosis, and fibrosis. Peptides. 2023 Jul; 165:171013.

112. Jiang G, Wang M, Wang L, Chen H, Chen Z, Guo J, et al. The protective effect of nesfatin-1 against renal ischemia-reperfusion injury in rats. Ren Fail. 2015 Jun;37(5):882–9.

113. Mori Y, Shimizu H, Kushima H, Hiromura M, Terasaki M, Tanaka M, et al. Increased blood pressure in nesfatin/nuclebindin-2-transgenic mice. Hypertens Res. 2017 Oct 5;40(10):861–7.

114. Chung Y, Jung E, Kim H, Kim J, Yang H. Expression of Nesfatin-1/NUCB2 in Fetal, Neonatal and Adult Mice. Dev Reprod. 2013 Dec;17(4):461–7.

115. Tezcan N, Özdemir-Kumral ZN, Özkan Yenal N, Çilingir-Kaya ÖT, Virlan AT, Özbeyli D, et al. Nesfatin-1 treatment preserves antioxidant status and attenuates renal fibrosis in rats with unilateral ureteral obstruction. Nephrol Dial Transplant. 2022 Jun 23;37(7):1238–48.

116. Xu D, Yu Y, Xu Y, Ge J. Plasma Nesfatin-1: Potential Predictor and Diagnostic Biomarker for Cognitive Dysfunction in T2DM Patient. Diabetes Metab Syndr Obes. 2021; 14:3555–66.

117. Netea MG, Balkwill F, Chonchol M, Cominelli F, Donath MY, Giamarellos-Bourboulis EJ, et al. A guiding map for inflammation. Nat Immunol. 2017 Aug;18(8):826–31.

118. Xu Y, Chen F. Antioxidant, Anti-Inflammatory and Anti-Apoptotic Activities of Nesfatin-1: A Review. J Inflamm Res. 2020; 13:607–17.

119. Biswas SK. Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox? Oxid Med Cell Longev. 2016; 2016:5698931.

120. Wang ZZ, Chen SC, Zou XB, Tian LL, Sui SH, Liu NZ. Nesfatin-1 alleviates acute lung injury through reducing inflammation and oxidative stress via the regulation of HMGB1. Eur Rev Med Pharmacol Sci. 2020 May;24(9):5071–81.

121. Cheng H, Zhu Y, Chen L, Wang Y. Nesfatin-1 alleviated lipopolysaccharide-induced acute lung injury through regulating inflammatory response associated with macrophages modulation. J Cardiothorac Surg. 2022 Aug 26;17(1):206.

122. Zheng J, Han J, Wang Y, Tian Z. Role of brain NUCB2/nesfatin-1 in stress and stress-related gastrointestinal disorders. Peptides. 2023 Sep 1; 167:171043.

123. Jin X, Guan K, Chen Z, Sun Y, Huo H, Wang J, et al. The protective effects of nesfatin-1 in neurological dysfunction after spinal cord injury by inhibiting neuroinflammation. Brain Behav. 2022 Nov;12(11): e2778.

124. Castro AM, Macedo-de la Concha LE, Pantoja-Meléndez CA. Low-grade inflammation and its relation to obesity and chronic degenerative diseases. Revista Médica del Hospital General de México. 2017 Apr 1;80(2):101–5.

125. Gharanei S, Ramanjaneya M, Patel AH, Patel V, Shabir K, Auld C, et al. NUCB2/Nesfatin-1 Reduces Obesogenic Diet Induced Inflammation in Mice Subcutaneous White Adipose Tissue. Nutrients. 2022 Mar 28;14(7).

126. Jiang L, Xu K, Li J, Zhou X, Xu L, Wu Z, et al. Nesfatin-1 suppresses interleukin-1β-induced inflammation, apoptosis, and cartilage matrix destruction in chondrocytes and ameliorates osteoarthritis in rats. Aging (Albany NY). 2020 Jan 30;12(2):1760–77.

127. Shen XL, Song N, Du XX, Li Y, Xie JX, Jiang H. Nesfatin-1 protects dopaminergic neurons against MPP (+)/MPTP-induced neurotoxicity through the C-Raf-ERK1/2-dependent anti-apoptotic pathway. Sci Rep. 2017 Jan 20; 7:40961.

128. Yavuz M, Ozsoy M, Celep B, Gülsari Y, Aslan E, Bozbiyik O, et al. Anti-inflammatory and anti-apoptotic effect of nesfatin-1 on liver ischemia-reperfusion injury. Ann Ital Chir. 2020; 91:207–14.

129. Sharifi M, Nazarinia D, Ramezani F, Azizi Y, Naderi N, Aboutaleb N. Necroptosis and RhoA/ROCK pathways: molecular targets of Nesfatin-1 in cardioprotection against myocardial ischemia/reperfusion injury in a rat model. Mol Biol Rep. 2021 Mar;48(3):2507–18.

130. Ramanjaneya M, Tan BK, Rucinski M, Kawan M, Hu J, Kaur J, et al. Nesfatin-1 inhibits proliferation and enhances apoptosis of human adrenocortical H295R cells. J Endocrinol. 2015 Jul;226(1):1–11.

131. Suzuki S, Takagi K, Miki Y, Onodera Y, Akahira JI, Ebata A, et al. Nucleobindin 2 in human breast carcinoma as a potent prognostic factor. Cancer Sci. 2012 Jan;103(1):136–43.

132. Kan JY, Yen MC, Wang JY, Wu DC, Chiu YJ, Ho YW, et al. Nesfatin-1/Nucleobindin-2 enhances cell migration, invasion, and epithelial-mesenchymal transition via LKB1/AMPK/TORC1/ZEB1 pathways in colon cancer. Oncotarget. 2016 May 24;7(21):31336–49.

133. Zhang R, Liang H, Liu G, Jiang W, Tang Z, Fan Q, et al. Nesfatin-1, a novel energy-regulating peptide, alleviates pulmonary fibrosis by blocking TGF-β1/Smad pathway in an AMPKα-dependent manner. Int Immunopharmacol. 2023 May 22; 120:110369.

134. Patricelli C, Lehmann P, Oxford JT, Pu X. Doxorubicin-Induced Modulation of TGF-β Signaling Cascade in Mouse Fibroblasts: Insights into Cardiotoxicity Mechanisms. Res Sq. 2023 Jul 27; rs.3.rs-3186393.

135. Zuk Joseph V. AB. Acute Kidney Injury. Annual review of medicine. 2016;67(1):293–307.

136. Peerapornratana S, Manrique-Caballero C, Gómez H, Kellum J. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. Kidney international. 2019;96(5):1083–99.

137. Saldanha JF, Carrero JJ, Mafra D. The possible role of nesfatin-1 on appetite regulation in hemodialysis patients. Med Hypotheses. 2011 Oct;77(4):654–7.

138. Saldanha JF, Carrero JJ, Lobo JC, Stockler-Pinto MB, Leal VO, Calixto A, et al. The newly identified anorexigenic adipokine nesfatin-1 in hemodialysis patients: Are there associations with food intake, body composition and inflammation? Regul Pept. 2012 Jan 10;173(1–3):82–5.

139. Kuyumcu A, Kuyumcu MS, Ozbay MB, Ertem AG, Samur G. Nesfatin-1: A novel regulatory peptide associated with acute myocardial infarction and Mediterranean diet. Peptides. 2019 Apr; 114:10–6.

140. Foo KS, Brismar H, Broberger C. Distribution and neuropeptide coexistence of nucleobindin-2 mRNA/nesfatin-like immunoreactivity in the rat CNS. Neuroscience. 2008 Oct 15;156(3):563–79.

141. Ramanjaneya M, Chen J, Brown JE, Tripathi G, Hallschmid M, Patel S, et al. Identification of nesfatin-1 in human and murine adipose tissue: a novel depot-specific adipokine with increased levels in obesity. Endocrinology. 2010 Jul;151(7):3169–80.

142. Kim J, Sun S, Lee D, Youk H, Yang H. Gonadotropin regulates NUCB2/nesfatin-1 expression in the mouse ovary and uterus. Biochem Biophys Res Commun. 2019 Jun 4;513(3):602–7.

143. Korani Ahmed A MA; S. The relation between plasma levels of nesfatin-1 and different grades of diabetic kidney disease in patients with type 2 diabetes. The Egyptian Journal of Internal Medicine. 2018;30(2):68–71.

144. Prinz P, Stengel A. Expression and regulation of peripheral NUCB2/nesfatin-1. Curr Opin Pharmacol. 2016 Dec; 31:25–30.

145. Goebel-Stengel M, Wang L. Central and peripheral expression and distribution of NUCB2/nesfatin-1. Curr Pharm Des. 2013;19(39):6935–40.

146. Izuwa Jun Ichi; Horiuchi Mizuki; Aiba Tetsuya; Kawasaki Hiromu; Kurosaki Yuji YK. Comparative study of increased plasma quinidine concentration in rats with glycerol- and cisplatin-induced acute renal failure. Drug metabolism and pharmacokinetics. 2009;24(5):451–7.

147. Xiang Yi; Zhang Dingguo CY. Alleviation of the doxorubicin-induced nephrotoxicity by fasudil *in vivo* and *in vitro*. Journal of pharmacological sciences. 2020;145(1):6–15.

148. Mangali S, Bhat A, Jadhav K, Kalra J, Sriram D, Vamsi Krishna Venuganti V, et al. Upregulation of PKR pathway mediates glucolipotoxicity induced diabetic cardiomyopathy *in vivo* in wistar rats and *in vitro* in cultured cardiomyocytes. Biochem Pharmacol. 2020 Jul; 177:113948.

149. Udumula Sureshbabu; Kalra Jaspreet; Dasari Deepika; Goyal Srashti; Krishna Vandana; Bollareddy Srivarsha Reddy; Sriram Dharamrajan; Dhar Arti; Bhat Audesh MPM. High fructose and streptozotocin induced diabetic impairments are mitigated by Indirubin-3-hydrazone via downregulation of PKR pathway in Wistar rats. Scientific reports. 2021;11(1):12924.

150. Luo Meng-lu; Chen Zhengli; Huang Chao; Cheng Anchun; Fang Jing; Tang Li; Geng Yi QC. Evaluation of KIM-1 and NGAL as Early Indicators for Assessment of Gentamycin-Induced Nephrotoxicity *In vivo* and *In vitro*. Kidney & blood pressure research. 2016;41(6):911–8.

151. Zhao Panfilo; Fishpaugh Jeffrey R.; Rupprecht Kevin R.; Workman Ryan F.; Grenier Frank C.; Ramsay Carol CO. Structural characterization of glycoprotein NGAL, an early predictive biomarker for acute kidney injury. Carbohydrate research. 2010;345(15):2252–61.

152. Ayada C, Toru Ü, Korkut Y. Nesfatin-1 and its effects on different systems. Hippokratia. 2015 Mar;19(1):4–10.

153. Schalla MA, Stengel A. Current Understanding of the Role of Nesfatin-1. J Endocr Soc. 2018 Oct 1;2(10):1188–206.

154. Han Veronique; Abichandani Rekha; Thadhani Ravi; Bonventre Joseph V. WK; B. Kidney Injury Molecule-1 (KIM-1): A novel biomarker for human renal proximal tubule injury. Kidney international. 2002;62(1):237–44.

155. Zager C A RA; F. Effects of inorganic iron and myoglobin on *in vitro* proximal tubular lipid peroxidation and cytotoxicity. The Journal of clinical investigation. 1992;89(3):989–95.

156. Sun S, Yang H. Tissue-Specific Localization NUCB2/nesfatin-1 in the Liver and Heart of Mouse Fetus. Dev Reprod. 2018 Dec;22(4):331–9.

157. Irannejad A, Ghajar A, Afarideh M, Khajeh E, Noshad S, Esteghamati S, et al. Association of peripheral nesfatin-1 with early-stage diabetic nephropathy. Pathophysiology. 2017 Mar;24(1):17–22.

158. Tang CH, Fu XJ, Xu XL, Wei XJ, Pan HS. The anti-inflammatory and anti-apoptotic effects of nesfatin-1 in the traumatic rat brain. Peptides. 2012 Jul;36(1):39–45.

159. Özsavcí D, Erşahin M, Şener A, Özakpinar ÖB, Toklu HZ, Akakín D, et al. The novel function of nesfatin-1 as an anti-inflammatory and antiapoptotic peptide in subarachnoid hemorrhage-induced oxidative brain damage in rats. Neurosurgery. 2011 Jun;68(6):1699–708; discussion 1708.

160. Kolgazi M, Cantali-Ozturk C, Deniz R, Ozdemir-Kumral ZN, Yuksel M, Sirvanci S, et al. Nesfatin-1 alleviates gastric damage via direct antioxidant mechanisms. J Surg Res. 2015 Jan;193(1):111–8.

161. Masood Ruochen; Zhang Aihua HC. Inflammasomes in the Pathophysiology of Kidney Diseases. Kidney diseases (Basel, Switzerland). 2015;1(3):187–93.

162. Price Robert L.; Megyesi Judit PM; S. The cell cycle and acute kidney injury. Kidney international. 2009;76(6):604–13.

163. Sardão Paulo J.; Holy Jon M; Oliveira Catarina R.; Wallace Kendall B. VA; O. Morphological alterations induced by doxorubicin on H9c2 myoblasts: nuclear, mitochondrial, and cytoskeletal targets. Cell biology and toxicology. 2008;25(3):227–43.

164. Zager RA. Rhabdomyolysis and myohemoglobinuric acute renal failure. Kidney International. 1996 Feb;49(2):314–26.

165. Vanholder R, Sever MS, Erek E, Lameire N. Rhabdomyolysis. J Am Soc Nephrol. 2000 Aug;11(8):1553–61.

166. Kim J, Yang H. Nesfatin-1 as a new potent regulator in reproductive system. Dev Reprod. 2012 Dec;16(4):253–64.

167. Goyal SG, Dhar A. Downregulation of nesfatin-1 expression in acute kidney injury *in vivo* in wistar rats and *in vitro* in cultured cells. Life Sci. 2022 Sep 15; 305:120762.

168. Chen Z, Wang J, He J, Fan H, Hou S, Lv Q. Myoglobin Mediates Autophagy of NRK-52E in Rat Renal Tubular Epithelial Cells Via the Pink1/Parkin Signaling Pathway. Med Sci Monit. 2020 Jul 22;26: e923045-1-e923045-12.

169. Zurovsky Y. Models of glycerol-induced acute renal failure in rats. J Basic Clin Physiol Pharmacol. 1993;4(3):213–28.

170. Abaci A, Catli G, Anik A, Kume T, Bober E. The relation of serum nesfatin-1 level with metabolic and clinical parameters in obese and healthy children. Pediatr Diabetes. 2013 May;14(3):189–95.

171. Riva M, Nitert MD, Voss U, Sathanoori R, Lindqvist A, Ling C, et al. Nesfatin-1 stimulates glucagon and insulin secretion and beta cell NUCB2 is reduced in human type 2 diabetic subjects. Cell Tissue Res. 2011 Dec;346(3):393–405.

172. Gonzalez R, Reingold BK, Gao X, Gaidhu MP, Tsushima RG, Unniappan S. Nesfatin-1 exerts a direct, glucose-dependent insulinotropic action on mouse islet β- and MIN6 cells. J Endocrinol. 2011 Mar;208(3): R9–16.

173. Yang M, Zhang Z, Wang C, Li K, Li S, Boden G, et al. Nesfatin-1 action in the brain increases insulin sensitivity through Akt/AMPK/TORC2 pathway in diet-induced insulin resistance. Diabetes. 2012 Aug;61(8):1959–68.

174. Quang TH, Nguyet MP, Thao DP, Thi MH, Phuong Thi Dam L, Thi HH, et al. Evaluation of Urinary Neutrophil Gelatinase Associated Lipocalin and Kidney Injury Molecule-1 as Diagnostic Markers for Early Nephropathy in Patients with Type 2 Diabetes Mellitus. Diabetes Metab Syndr Obes. 2020 Jun 24; 13:2199–207.

175. King AJ. The use of animal models in diabetes research. Br J Pharmacol. 2012 Jun;166(3):877–94.

176. Beckers S, Zegers D, Van Gaal LF, Van Hul W. The role of the leptin-melanocortin signalling pathway in the control of food intake. Crit Rev Eukaryot Gene Expr. 2009;19(4):267–87.

177. Ozcan M, Gok ZB, Kacar E, Serhatlioglu I, Kelestimur H. Nesfatin-1 increases intracellular calcium concentration by protein kinase C activation in cultured rat dorsal root ganglion neurons. Neurosci Lett. 2016 Apr 21; 619:177–81.

Publications

List of publications (Thesis work)

1. Downregulation of nesfatin-1 expression in acute kidney injury *in vivo* in wistar rats and *in vitro* in cultured cells. **SG Goyal**, A Dhar. Life Sciences 305, 120762
2. Protective effect of nesfatin-1 treatment in rhabdomyolysis model of AKI in *in vitro* and *in vivo*. (Under Preparation)
3. Investigation into nesfatin-1 treatment in Diabetic Nephropathy and investigate it's the molecular mechanism (Under Preparation)

List of publications (Others)

1. Canagliflozin protects diabetic cardiomyopathy by mitigating fibrosis and preserving the myocardial integrity with improved mitochondrial function. D Dasari, **SG Goyal**, A Penmetsa, D Sriram, A Dhar. European Journal of Pharmacology 949, 175720
2. Nanomaterial for Kidney Disease Management. T Ghatage, **S Goyal**, D Dasari, J Jain, A Dhar, A Bhat. Functional Nanomaterials for Regenerative Tissue Medicines, 159-180
3. Novel therapeutics for the treatment of hypertension and its associated complications: peptide-and nonpeptide-based strategies. T Ghatage, **SG Goyal**, A Dhar, A Bhat. Hypertension Research 44 (7), 740-755
4. Miniaturized and IoT enabled continuous-flow-based microfluidic PCR device for DNA amplification. MB Kulkarni, **S Goyal**, A Dhar, D Sriram, S Goel. IEEE Transactions on NanoBioscience 21 (1), 97-104
5. High fructose and streptozotocin induced diabetic impairments are mitigated by Indirubin-3-hydrazone via downregulation of PKR pathway in Wistar rats. MP Udumula, S Mangali, J Kalra, D Dasari, **S Goyal,** V Krishna, A Dhar. Scientific Reports 11 (1), 12924
6. PKR inhibitor imoxin prevents hypertension, endothelial dysfunction and cardiac and vascular remodelling in L-NAME-treated rats J Kalra, D Dasari, A Bhat, S Mangali, **SG Goyal**, KB Jadhav, A Dhar. Life Sciences 262, 118436

7. SGLT1 inhibition boon or bane for diabetes-associated cardiomyopathy. J Kalra, SB Mangali, D Dasari, A Bhat, **S Goyal**, I Dhar, D Sriram, A Dhar. Fundamental & Clinical Pharmacology 34 (2), 173-188

Poster presentations

- **Srashti Gopal Goyal,** Arti Dhar**.** Endogenous Peptide Nesfatin-1, Role in Acute Kidney Injury Models: *In vivo* and Invitro Approach. Poster Presented at NIPER PHARMACON-2022 International Conference on Research, SAS Nagar, Punjab, INDIA. November 10-12, 2022.
- Deepika Dasari, **Srashti Gopal Goyal**, Arti Dhar, Canagliflozin Ameliorates *In Vitro* and *In Vivo* Diabetic Cardiomyopathy by Targeting Cardiac SGLT-1. Endogenous Peptide Nesfatin-1, Role in Acute Kidney Injury Models: *In vivo* and Invitro Approach. Poster Presented at NIPER PHARMACON-2022 International Conference on Research, SAS Nagar, Punjab, INDIA. November 10-12, 2022.

Biography of Candidate

Mr. Srashti Gopal Goyal completed his Bachelors and master's in technology in Biotechnology from Jaypee University of Information Technology, Solan H.P, India in the year 2014. with specialization in microbiology and molecular biology for bachelors and database management systems in bioinformatics for his master's thesis work. He worked as a Project fellow in biological sciences department in Indian Institute of Science Education and Research, Bhopal (IISER, Bhopal) from 2015 to 2017, working in microbiology and protein engineering. Later, Mr. Srashti joined Prof. Arti Dhar's lab at Birla Institute of Technology and Science, Pilani, Hyderabad Campus in 2018 for his doctoral work. His doctoral thesis work involved evaluation of a novel anorexic peptide Nesfatin-1 in invitro and *in vivo* models of Acute and Chronic kidney injuries. He has co-authored 8 scientific peer review publications in well-renowned international journals.

Biography of Supervisor Prof. Arti Dhar

Dr Arti Dhar is currently working as a Professor in the Department of Pharmacy at Birla Institute of Technology and Science (BITS), Pilani, Hyderabad campus. Dr. Dhar received her PhD from College of Medicine, University of Saskatchewan, Canada in the year 2010. During her PhD she received scholarships from Heart and Stroke Foundation of Canada (HSFC) and Arthur Smith Memorial Scholarship from University of Saskatchewan, Canada. Dr. Dhar also won numerous travel awards from Canadian Physiological Society and Canadian Hypertension Society. Her PhD thesis was nominated for Governor General's Gold medal and her thesis work was presented on CBC channel Canada in March 2011. She did her postdoctoral trainings from Lakehead University, Ontario, Canada and University of Saskatchewan, Canada from the year 2010 to 2013. After joining BITS Pilani, Hyderabad Campus in 2014, she has received research funding from DST-SERB, CSIR, ICMR and from BITS under additional competitive grant. She has published more than 48 research publications in peer-reviewed international journals. She has guided four doctoral students, six master's students and six undergraduate students in fulfilment of their dissertation work. Currently 4 students are pursuing PhD under her supervision, while 3 are working for master's dissertation. Her main research interests are centred on novel therapeutic targets for cardiovascular, metabolic disorders and cancer. She has won numerous awards at national and international level.