

CAPITAL UNIVERSITY OF SCIENCE AND
TECHNOLOGY, ISLAMABAD



Evaluating the Role of Vildagliptin Against Piroxicam - Induced Nephrotoxicity in Experimental Animal Model

by

Laiba Abdul Qudooes

A thesis submitted in partial fulfillment for the
degree of Master of Philosophy

in the

Faculty of Pharmacy

Department of Pharmacology

2026

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CERTIFICATE OF APPROVAL

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Acknowledgement

In the name of Allah, the Most Gracious, the Most Merciful. All praise is due to Allah, the Lord of the worlds. With a heart full of humility and gratitude, I begin by offering my eternal thanks to Almighty Allah (SWT), the ultimate source of knowledge, patience, and strength. I send peace and blessings upon our beloved Prophet Muhammad (PBUH), whose life remains a radiant beacon of faith, whose teachings of compassion, knowledge, and perseverance continue to illuminate the path for seekers of truth and knowledge around the world.

I am profoundly grateful to my distinguished supervisor, Dr. Fazlullah Khan, Associate Professor, Faculty of Pharmacy, CUST, for his insightful mentorship, inspiring leadership, and constant encouragement, which elevated the quality of my work and nurtured my growth as a researcher. I extend heartfelt thanks to Prof Dr. Muzaffar Abbas, Dean of the Faculty of Pharmacy, whose support, both academic and personal, helped shape this endeavor and made the environment at CUST rich with knowledge and encouragement. I would like to express my sincere appreciation to my friends Ms. Iqra Farooq, and Mr. Muhammad Rehan Zahoor for their sincere support throughout the journey, your kind words and motivating presence have been a source of strength and confidence in my academic pursuits. I am also deeply grateful to Ms. Samia Siddique for her constant encouragement and support during this time period.

To my colleague Mr. Anees Murtaza, thank you for your companionship, your valuable insights and assistance during the lab work. Special thanks to my friend Ms. Aqeela Khurshid for turning this rigorous path into a collaborative, your guidance and support have been a true blessing in my academic path. I extend my deepest gratitude to my family, the foundation of all my achievements. To my father, whose strength, wisdom, and unwavering belief shaped my journey, and to my mother, whose boundless love, prayers, and sacrifices sustained me through every challenge. I owe everything to you both. Their silent support and selfless devotion are behind every success. I am also thankful to all who offered kind words, prayers, and support along the way; your presence made a lasting

impact. This work is dedicated to those who believed in me, prayed for me, and stood beside me when the path was unclear. May Allah (SWT) bless each of you abundantly

With sincere gratitude and love thank you

(Laiba Abdul Qudooes)

Abstract

The potential nephrotoxic effects that piroxicam has on the kidneys are a major clinical problem due to the production of reactive species leading to oxidative stress, inflammatory response, and damage to the renal parenchyma, which subsequently causes decreased renal function. Piroxicam inhibits COX-1 and COX-2, reducing renal prostaglandin synthesis and potentially contributing to renal hemodynamic disturbances, oxidative stress, and inflammatory responses. Vildagliptin, a DPP-4 inhibitor, has demonstrated antiinflammatory and antioxidant properties and may have potential as a nephroprotective agent against drug-induced renal injury. In this experiment, we studied the ability of vildagliptin to provide nephroprotection against renal toxicity in mice induced by administration of piroxicam at a dose of 50 mg/kg orally once daily for nine days. Animals receiving vildagliptin at low (10 mg/kg) and high (20 mg/kg) doses, as well as animals receiving the standard nephroprotective agent silymarin (100 mg/kg), were treated for nine consecutive days. After the treatment period was completed, blood samples were collected for biochemical testing and renal injury was assessed using serum creatinine, serum urea, TNF- α , and histopathological examination of kidney tissue. ELISA was used to quantify the secretion of the pro-inflammatory cytokine tumor necrosis factor-Alpha (TNF- α). Following administration of piroxicam alone, most animals showed significantly increased levels of serum creatinine 1.78 ± 3.5 mg/dL ($p < 0.05$ vs. control), serum urea 78 ± 3.5 mg/dL ($p < 0.05$ vs. control) and serum TNF- α , indicating acute renal injury. Additionally, the administration of 20 mg/kg vildagliptin caused a significant reduction in the levels of renal injury markers as it decrease serum urea level to 35 ± 1.7 mg/dL ($p < 0.001$ vs. piroxicam) and normalized serum creatinine levels to 0.60 ± 0.04 mg/dL ($p < 0.001$ vs. piroxicam) compared with both the 10 mg/kg group of vildagliptin and the untreated group receiving nephrotoxic drugs ($p < 0.05$). Furthermore, histopathological evaluations indicate the maintenance of normal renal architecture with decreased tubular degeneration and decreased glomerular damage. All of these findings demonstrate that vildagliptin, at an effective dose of 20 mg/kg,

significantly protects against piroxicam-induced renal injury via inhibition of oxidative stress and inflammatory pathways; improvement in the renal function and the reduction of inflammatory mediators demonstrates its therapeutic potential as a safer and more effective treatment option for the prevention or management of NSAID-induced nephrotoxicity. Thus, vildagliptin may have promise as a nephro-protective compound against piroxicam-induced nephrotoxicity.

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Abbreviations

ADH	Antidiuretic Hormone
AKI	Acute Kidney Injury
AVMA	American Veterinary Medical Association
BCA	Bicinchoninic Acid (Protein Assay)
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
CKD	Chronic Kidney Disease
CMC	Carboxymethyl Cellulose
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic Acid
DPP-4	Dipeptidyl Peptidase-4
ELISA	Enzyme-Linked Immunosorbent Assay
GFR	Glomerular Filtration Rate
GLDH	Glutamate Dehydrogenase
H&E	Hematoxylin and Eosin (Staining)
IACUC	Institutional Animal Care and Use Committee
IL-6	Interleukin-6
NF-κB	Nuclear Factor-kappa B
NSAIDs	Non-Steroidal Anti-Inflammatory Drug(s)
PBS	Phosphate-Buffered Saline
PPE	Personal Protective Equipment
TNF-α	Tumor Necrosis Factor-alpha
WR	Working Reagent

Symbols

α	Alpha (Greek letter used in TNF- α)
κ	Kappa (Greek letter used in NF- κ B)
<i>p.o.</i>	*Per os* (Latin term meaning administered orally)
<i>i.p.</i>	Intraperitoneal (administered via injection into the peritoneum)
<i>mg/kg</i>	Milligrams per kilogram (dosage unit)
<i>mg/dL</i>	Milligrams per deciliter (concentration unit)
<i>Ml</i>	Milliliter (volume unit)
μ L/ <i>ul</i>	Microliter (volume unit)
<i>G</i>	Gram (weight unit)
<i>Rpm</i>	Revolutions per minute (centrifugation speed unit)
<i>Nm</i>	Nanometer (wavelength measurement unit)
<i>C</i>	Degrees Celsius (temperature unit)
1X	One times (denoting normal working strength concentration)

Chapter 1

Introduction

1.1 Background

Kidneys are subservient organs of human physiology serving the purpose of filtering out toxins and drugs. Kidneys help remove waste products and toxins such as urea, creatinine and uric acid from the human body [1]. The kidneys help maintain the body's extracellular fluid volume, regulate serum osmolality and electrolyte balance, and produce essential hormones such as erythropoietin, renin, and the active form of vitamin D [2]. Tens of medications used in medical treatment have potential for causing nephrotoxicity resulting in the loss of their effectiveness. Although there have been considerable advancements in the fields of medicine in treating this illness, acute kidney injury (AKI) is still associated with devastating consequences [3]. When we talk about nephrotoxicity, we refer to kidney damage that occurs when drugs cause problems in kidney function. There are several mechanisms causing nephrotoxicity such as oxidative stress, DNA adducts, inflammation, mitochondria dysfunction, and nephrotoxicity because of the effect of drugs on tubular epithelial cells [4]. Piroxicam is one of the most popular non-steroidal anti-inflammatory medications with anti-inflammatory, analgesic, and antipyretic properties, but its adverse effects include heart failure, retention of fluids, and kidney problems [5]. The kidneys produce

prostaglandins and the prostaglandins produced from kidney tissues are involved in the regulation of kidney blood circulation, renal water retention, and the regulation of the renin-angiotensin system [6]. The use of non-steroidal anti-inflammatory drugs such as piroxicam inhibits the functioning of cyclooxygenase, the first step in the production of the prostaglandins. These medications may cause the retention of sodium, increased potassium levels in the blood, and various types of acute and chronic kidney failure in some cases [7]. NSAID-induced inhibition of COX enzymes decreases prostaglandin-mediated vasodilation in the renal vasculature, thereby leading to ischemic damage and upregulation of inflammatory factors like TNF- α and Piroxicam-induced nephrotoxicity is mediated through oxidative stress and inflammation IL-6. These changes are reflected by increased levels of blood urea nitrogen (BUN), serum creatinine, and histopathological alterations like glomerular shrinkage, tubular damage, and interstitial inflammation [8].

Vildagliptin is a selective dipeptidyl peptidase-4 (DPP-4) inhibitor, clinically used in type 2 diabetes mellitus for its role in enhancing incretin activity. However, beyond its glycemic control, vildagliptin has demonstrated anti-inflammatory, antioxidant, and organ-protective effects in various preclinical models. Vildagliptin modulates oxidative stress markers, reduces cytokine production, and inhibits NF- κ B pathway activation, which may offer renoprotective effects against drug-induced nephrotoxicity [9].

1.2 Kidney and Renal Physiology

The kidneys are essential paired organs situated in a retroperitoneal position in the upper part of the belly. They only compose about 0.5% of the human body, yet receive almost 20% of the blood supply from the heart. The reason is due to their numerous functions, that allow them to keep the body's internal environment stable. Each kidney contains two distinct parts: the outer cortex; and the inner medulla. Within each kidney there are approximately one million nephrons that function in blood filtration, reabsorption of essential components, waste secretion, and urine production [10].

The nephron consists of 2 main parts: A renal corpuscle where blood is filtered through the tuft of capillaries called the glomerulus, and an inductive effector setting of nephron (proximal tubular), which passes the almost 100% of the blood that is filtered through to the Proximal Tubule, Loop of Henle, Distal Tubule, and Collecting Duct. Each section has a specific part in the fine tuning of the urine constituents, through selective reabsorption of water and sodium, and/or secretion of waste [11]. The Proximal Tubule reabsorbs approximately 70% of the filtered sodium and water, virtually all of the glucose and the majority of amino acids. The Loop of Henle allows the kidneys to retain water by creating a hypertonic gradient (increased concentration of sodium and other solutes), which helps retain water in the kidneys, ultimately enabling them to conserve body fluid. Hormonal regulation is achieved mainly through the proximal tubular sodium and potassium reabsorption, Distal tubular reabsorption of sodium, potassium and H^+ , all of which are influenced by hormones including Aldosterone and Antidiuretic hormone (ADH) [12].

Due to various siren processes, the kidney keeps control of extracellular fluid volume, osmolarity, acid-base balance, and electrolyte concentrations within certain limits, regardless of the amount of food and drink consumed and what's happening in the body metabolic-wise. Kidney physiology and function can be impaired significantly through illness, getting older, or when certain substances (toxins) affect the kidney, which causes an imbalance in fluids and electrolytes and causes your blood pressure to be high or low, anemia, or to have problems with your bones [13].

1.3 Effects of Drugs on the Kidney

Because the kidneys play such a big role in eliminating drugs from the body, there is an increased risk of having an adverse reaction to drugs through the kidneys. Most of the commonly used drugs will affect how blood goes through your kidneys or how the kidneys work by filtering out waste; they will also affect the levels of electrolytes and acid/base levels in the body. Drug induced kidney damage can

occur all at once or over many months or years and can cause mild damage or cause you to lose 100% of kidney function [14]. A very common cause of drug-induced damage to the kidneys is through the use of NSAID medications. When used, they block the COX enzymes from making renal prostaglandins, which makes the afferent arterioles dilate or to open up, causing a decrease in blood flow and glomerular filtration rates; this can be very harmful when you are dehydrated, or have congestive heart failure, or chronic kidney disease; thus causing prerenal azotemia and/or acute kidney injury [15]. Kidney tubular cells can be damaged due to nephrotoxicity caused by cisplatin and aminoglycoside drugs through their effects on the nephron proximal tubule, which causes increased levels of serum creatinine and injury to tubule cells [16].

1.4 Nephrotoxicity and Its Causes

As the kidneys filter a very large amount of blood, with relative concentrations of very high solute and liquid volume, they are susceptible to injury by nephrotoxic agents. Different agents can injure kidneys, such as medications, environmental toxins, and the end products of metabolism. Nephrotoxic agents can produce different types of kidney injury, including acute kidney injury or chronic kidney disease; electrolyte imbalances; decreased amounts of urine; and/or ultimately, death [14].

1.5 Mechanisms of Nephrotoxicity

Several mechanisms underlie nephrotoxic injury.

1.5.1 Direct Tubular Toxicity

Direct tubular toxicity due to nephrotoxic agents can result from the accumulation of drug residues or metabolites in the tubular epithelial cells of the nephron proximal tubule. The most likely site of accumulation for drug residues or metabolites

is in the proximal tubular epithelial cell. The drug and its metabolites can be concentrated in the proximal tubular epithelial cell because of high rates of cellular metabolism of the drug and its active transport system within the kidney.

As a result of this accumulation, the proximal tubular epithelial cell may develop mitochondrial dysfunction, oxidative stress, and/or apoptotic and/or necrotic effects [17].

1.5.2 Hemodynamic Alterations

Hemodynamic changes are still another important factor in causing AKI. The alteration of either the afferent or efferent arteriolar tone associated with some medications can reduce renal blood flow creating ischemia and thereby decreasing glomerular filtration. For example, some medications interfere with vasodilatory prostaglandin production or produce excessive vasoconstriction due to modulating the renin-angiotensin pathway thus reducing renal perfusion [18].

1.5.3 Inflammatory and Immune-mediated Mechanisms

Nephrotoxicity is also influenced by inflammatory and immune mediated processes. For example, certain drugs act as haptens triggering immune response resulting in interstitial nephritis and/or injury to glomerular capillaries. Immune activation produces inflammatory cell infiltration, the release of cytokines, and tissue injury [19].

1.5.4 Crystal-induced Nephropathy

Crystal-induced nephropathy occurs when poorly soluble medications precipitate in renal tubules creating obstruction and localized injury. Antiviral agents and sulfonamides are examples of such agents that produce crystal-induced nephropathy [20].

1.6 Role of Drugs in Causing Nephrotoxicity

Drugs are responsible for a large number of nephrotoxic incidents in practice. Nephrotoxicity can be caused by drugs with predictable dose-dependent toxicity or idiosyncratic reactions. Drug-induced nephrotoxicity risk factors include drug pharmacokinetics and route of elimination, duration of exposure to the drug and patient-related factors [21].

1.6.1 Nonsteroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used drugs to cause kidney injury. NSAIDs work by the inhibition of cyclooxygenase enzymes, which inhibits the production of prostaglandins from the arachidonic acid pathway. This prevents the dilation of blood vessels in the kidney, resulting in decreased renal perfusion and glomerular filtration rate (especially in patients who are hypovolemic, in heart failure or have chronic kidney disease) [22].

1.6.2 Aminoglycoside Antibiotics

An example of a direct tubular injury drug is gentamicin (an aminoglycoside antibiotic). The mechanism of gentamicin toxicity results from receptor-mediated endocytosis, leading to accumulation in proximal tubular cells, lysosomal rupture, oxidative stress and ultimately apoptosis. Clinically, this injury manifests as non-oliguric acute kidney injury with gradual elevations in serum creatinine [23].

1.6.3 Chemotherapeutic Agents

The nephrotoxic effects of chemotherapeutic agents (e.g., cisplatin) result from tubular uptake resulting in DNA damage, mitochondrial injury and inflammation. Nephrotoxic radiocontrast agents used in diagnostic imaging also lead to

oxidative injury and vasoconstriction causing acute kidney injury associated with radiocontrast agents, particularly in elderly patients and diabetic patients [24].

1.7 Piroxicam and its Pharmacological Properties

Piroxicam (an oxicam) is a long-acting nonsteroidal anti-inflammatory drug used primarily for treating rheumatoid arthritis and osteoarthritis. The prolonged half-life of piroxicam permits daily administration (one dose can provide up to 24 hours of treatment) [25].

1.7.1 Pharmacokinetics of Piroxicam

When taken orally, Piroxicam is almost all absorbed, which gives it almost 100 percent bioavailability and over 99 percent of the drug is also bound to plasma proteins; therefore, Piroxicam undergoes very extensive hepatic metabolism by means of hydroxylation and conjugating with drugs/metabolites, and is then excreted in urine and feces. Due to its long half-life of about 45 to 50 hours, the dosing for this medication may be performed once daily [26].

1.7.2 Pharmacodynamics of Piroxicam

As a non-selective Cox-1 and Cox-2 inhibitor, Piroxicam inhibits the metabolism of arachidonic acid and consequently inhibits the formation of both prostaglandins and thromboxanes. Thus, through these precursory inflammatory substrates, such as normal inflammation and symptoms associated with pain; such as pain, fever or swelling, Piroxicam is also capable of providing analgesia, anti-inflammatory effects, and antipyretic effects [27].

1.8 Uses and Effects of Piroxicam

The above characteristics make Piroxicam an ideal treatment for chronic systemic inflammatory disease and musculoskeletal pain, such as those related to long duration of action; non-steroidal anti-inflammatory (NSAIDs); proven effective for inflammatory pain and can be used with confidence for long duration therapies, such as injured and surgical acute pain and dysmenorrhoea [28].

1.9 Role of Piroxicam in Causing Nephrotoxicity

Piroxicam is a non-selective NSAID that can cause kidney problems (nephrotoxicity) when used by people who are more likely to have them. Its primary mechanism of action is by inhibiting renal synthesis of prostaglandins. Two prostaglandins that maintain renal blood flow and glomerular filtration rates (GFR) primarily are PGE₂ and PGI₂, which cause dilation of the renal afferent arterioles when circulating volume is low [22].

Piroxicam may induce renal injury primarily through inhibition of cyclooxygenase (COX-1 and COX-2), resulting in reduced synthesis of renal prostaglandins, particularly prostaglandin E₂ (PGE₂).

Under conditions of renal stress, PGE₂ helps maintain renal hemodynamics and tubular function through four G-protein-coupled EP receptors (EP1-EP4), with EP2 and EP4 receptors contributing to vasodilatory and renoprotective responses.

Inhibition of COX by piroxicam reduces PGE₂ availability and consequently diminishes EP2/EP4-mediated renal protection, favoring renal vasoconstriction, reduced renal blood flow and glomerular filtration, and subsequent ischemic and tubular injury.

Prolonged prostaglandin depletion may further promote oxidative stress and inflammatory responses, contributing to the structural and functional renal damage

observed with NSAID exposure. Thus, piroxicam-induced nephrotoxicity is primarily a consequence of COX inhibition and subsequent disruption of the renal PGE₂–EP receptor pathway rather than direct antagonism of EP receptors [26].

The use of piroxicam leads to the inhibition of COX activity, which in turn inhibits the synthesis of prostaglandins and causes vasoconstriction of the afferent arterioles.

The use of piroxicam leads to the inhibition of COX activity, which in turn inhibits the synthesis of prostaglandins and causes vasoconstriction of the afferent arterioles. This will reduce renal perfusion and GFR, leading to acute kidney injury (AKI). Elderly, dehydrated patients, and patients with heart failure, liver disease, or chronic kidney disease (CKD) are more susceptible to the development of kidney injury due to renal perfusion being dependent on prostaglandins [29].

1.10 Vildagliptin and its Pharmacological Properties

Vildagliptin is an oral DPP-4 inhibitor that is used in the treatment of type 2 diabetes mellitus. The agent has good oral bioavailability, has minimal binding to proteins, and acts in a glucose-dependent manner. The safety profile for vildagliptin is favorable when used by patients with different levels of renal function; therefore, it should be considered for long-term management of glycemia [30].

1.11 Pharmacokinetic and Pharmacodynamic Profile of Vildagliptin

Vildagliptin is rapidly absorbed into the system following oral administration; its bioavailability rate is approximately 85%. It exhibits low levels of binding to plasma proteins (~9%), and the majority of it is not metabolized by using

cytochrome P450 enzymes but rather metabolized through hydrolytic metabolism; therefore, there are a lower number of drug-drug interactions that occur with it.

The majority (about two-thirds) of the drug and its metabolites are eliminated from the body through the kidneys. The remainder is eliminated from the body through feces [31].

Vildagliptin is considered to be a pharmacologic agent that helps to better control blood sugar levels (glycemic control) through the inhibition of DDP-4; thereby increasing the activity of GLP-1 and GIP (the incretin hormones). By doing so, it stimulates the production of insulin in response to glucose and decreases the amount of glucagon released, resulting in lower blood sugar levels and decreased risks of having hypoglycemia [32].

1.12 Uses and Effects of Vildagliptin

There is currently a large patient population who is targeted by vildagliptin for treatment of type 2 diabetes mellitus. Patients who are not able to take metformin alone, or patients who require combination therapy with other glucose-lowering medications, can benefit from this medication. Due to its glucose-dependent nature, vildagliptin is beneficial for treating elderly patients and for patients who are at risk of hypoglycemia [33].

1.13 Mode of Action of Vildagliptin

The mechanism of action of vildagliptin involves reversible inhibition of the enzyme DPP-4 which is responsible for breaking down the incretin hormones (GLP-1 and GIP) very quickly. The incretin hormones are produced in the intestine right after the ingestion of food and are important for maintaining the balance of glucose levels [34].

In healthy individuals, GLP-1 and GIP are disabled by DPP-4 within a few minutes of their release. Vildagliptin inhibits DPP-4, maintaining the levels of active incretin, prolonging their beneficial effects. Increased levels of GLP-1 result in the stimulation of insulin production by the pancreas's β -cells but only when blood glucose levels are above normal. The suppression of glucagon from the pancreas's α -cells causes a decrease in hepatic (liver) glucose output. This explains the low incidence of hypoglycemia that occurs with use of the drug [34].

GLP-1 also slows gastric emptying and may have a role in improving satiety. These special effects can give better control of postprandial (after meal) glucose levels in patients using vildagliptin. Vildagliptin preserves the ability of the pancreas to produce insulin over time, unlike other classes of diabetes medications called insulin secretagogues, which cause forced (independent of blood sugar) release of insulin [33].

Studies have indicated that DPP-4 inhibitors (vildagliptin included) may provide protection to the kidneys (renoprotection) by dropping the effects of inflammation, oxidative stress, and fibrosis within the renal (kidney) tissues via the incretin mediated activity. The presence of GLP-1 receptors (GLP-1R) in the kidneys may also promote improvement in the function of the endothelial cells lining the inside walls of blood vessels and may decrease the amount of protein (albumin) lost into the urine (albuminuria) [35].

1.13.1 Inhibition of DPP-4 Enzyme

Vildagliptin blocks Dipeptidyl peptidase 4 (DPP-4) which contributes to renal injury as the DPP-4 enzyme is especially abundant on the proximal tubular cells, podocytes and glomerular endothelium. When the kidney is injured the DPP-4 activity is increased and enhances inflammation, oxidative stress and renal fibrosis. By inhibiting DPP-4, vildagliptin is able to reduce these pathways and provide protection to renal tissue [36].

In renal injury there will be increased levels of reactive oxygen species (ROS) which further damage the tubular cells and mitochondrial structures through oxidative damage. Vildagliptin helps to enhance the antioxidant properties of the tissue by increasing the levels of superoxide dismutase, catalase and glutathione (decreasing malondialdehyde, a marker of lipid peroxidation). This will help preserve mitochondrial function and may provide protection to the nephron structure [37].

Nephrotoxic agents activate pro-inflammatory pathways (e.g. $\text{NF-}\kappa\text{B}$) which increase the levels of cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and IL-6 which lead to renal inflammation. Vildagliptin inhibits these conduits and lessens the infiltration of inflammatory cells (and therefore provides protection) from both immune- and toxin-mediated injurious processes [38].

This study aimed to assess whether vildagliptin has renoprotective properties using a mouse model developed using piroxicam to cause nephrotoxicity. The outcome of the experiment was measured using the following biochemical (serum creatinine, serum urea, $\text{TNF-}\alpha$) and histopathological parameters. The results of the current study suggest that vildagliptin's renoprotective effects are mediated by its ability to modulate oxidative stress and inflammation due to piroxicam exposure in kidney tissue. Therefore, this study may help increased understanding of the mechanism leading to the therapeutic use of DPP-4 inhibitors via upregulation of GLP-1 through DPP-4 inhibition resulting in improved kidney hemodynamics and possibly leading to safer and more accurate treatments for kidney inflammation.

Chapter 2

Literature Review

2.1 Background

The problem of drug-induced nephrotoxicity is a serious issue for healthcare providers; drug-induced nephrotoxicity can lead to AKI and can be one of the leading causes of AKI worldwide. There are many drugs that can cause renal function problems including nonsteroidal anti-inflammatory drugs (NSAIDs) such as Piroxicam. NSAIDs inhibit cyclooxygenase enzyme activity and thus inhibit formation of prostaglandins which results in less renal blood flow, increased oxidative damage, increased inflammation, and damage to the renal tubules due to their nephrotoxic effects. Studies have shown that piroxicam causes significant nephrotoxicity in experimental animals after prolonged renal exposure as evidenced by an increase in serum creatinine, serum urea, degeneration of renal tubules and increased oxidative stress [39]. Identifying potential agents to prevent NKIDs continues to be an area of great interest; one drug that has demonstrated promise in preventing NSAID-induced renal injury is Vildagliptin, a member of a group of glucose lowering drugs known as Dipeptidyl Peptidase-4 (DPP-4) Inhibitors. These classes of drug have been found to have antioxidant, anti-inflammatory, and anti-apoptotic properties in addition to their ability of lowering blood glucose levels. There is also recent evidence that suggests Vildagliptin protects other organs as

well as the kidney. There is experimental evidence to support that Vildagliptin may protect the kidney from injury due to NSAIDs through reduction of oxidative stress markers, cytokine inhibition (anti-inflammatory) and modulation of apoptotic pathways.

2.2 Vildagliptin Against Cisplatin - induced Nephrotoxicity

The research assessed whether vildagliptin could prevent cisplatin toxicity on kidneys of rats. Cisplatin is a chemotherapeutic medication known to cause acute renal failure via oxidative stress and apoptosis by damaging the kidney. A total of 4 groups of rats received vildagliptin orally for 14 days. Control group, cisplatin group, vildagliptin + cisplatin group, and vildagliptin only.

Vildagliptin-treated rats showed significant reductions in serum creatinine, urea, TNF-alpha and caspase-3 levels (indicating acute renal failure), whereas control rats had an increase in these same indices of renal failure and damage. In addition, vildagliptin administration also increased total antioxidant capacity (TAC). Histopathological examinations showed vildagliptin administration improved renal tissue architecture by decreasing tubular degeneration and necrosis [35].

2.3 Vildagliptin Against Doxorubicin - induced Nephrotoxicity

This research assessed the protective advantages through the administration of either vildagliptin concerning the development of renal damage in rats when they were administered doxorubicin. Doxorubicin exerts nephrotoxic effects by inducing an rise in oxidative stress through the upregulation of inflammatory signals caused by the NLRP3 inflammasome; levels of inflammatory cytokines such as

TNF- α and IL-1 β , as well as an increased amount of inducible nitric oxide synthase (iNOS) and tubulointerstitial damage, had been identified in the plasma and renal pathological studies performed on rats treated with doxorubicin. The administration of vildagliptin resulted in significantly diminished levels of inflammatory mediators and will result in decreased activation of the NLRP3 Inflammasome. Consideration of histological samples have shown a return to normal renal morphology.

The significance of this study is to demonstrate that vildagliptin prevents the activation of inflammatory signalling pathways and consequently, prevents the induction of cell death (apoptosis) in the renal tissues. Considering that piroxicam-induced nephrotoxicities are also caused by oxidative stress and inflammation, there is potential for using vildagliptin to prevent renal injury attributable to non-steroidal anti-inflammatory drug (NSAID) use [40].

2.4 Vildagliptin in Diabetic Renal Injury

The effects of vildagliptin on diabetic-C-shaped rats were the primary focus of this study. There is strong evidence demonstrating that oxidative stress is a major contributor to the development of renal injury due to diabetes mellitus. This study showed that diabetic-C-shaped rats exhibited increased markers of oxidative stress, had an increase in renal dysfunction and had changes in renal structure after vildagliptin treatment. Treatment of diabetic-C-shaped rats with vildagliptin significantly reduced radiological indicators of renal impairment and increased renal biochemical measures of renal dysfunction when compared with untreated diabetic rats.

Importantly, treatment of diabetic-C-shaped rats with vildagliptin also lowered MDA levels while significantly increasing activity levels of SOD, which supports the findings of other studies that provided similar data. Additionally, renal histological evaluations indicated that vildagliptin therapy significantly improved renal histologic findings compared with untreated diabetic-C-shaped rats. The findings

from this study provide evidence that vildagliptin may act as a reno-protective substance mainly through its antioxidant properties [41].

2.5 Studies on DPP-4 Inhibitors and Renal Protection

Research investigating injury to the kidneys using various models has demonstrated that vildagliptin (as well as saxagliptin and linagliptin, two other DPP-4 inhibitors), all exhibit anti-inflammatory effects through their ability to lessen the levels of proinflammatory cytokines, injury due to oxidative stress, apoptosis, and fibrosis in the kidney's tissues. While the renoprotection provided by vildagliptin, saxagliptin, and linagliptin involves a number of mechanisms (such as increasing GLP-1 activity; inhibition of NF- κ B signaling pathways within the kidney; decrease the assembly of reactive oxygen species (ROS) within the kidney; and alteration in the expression of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins), current evidence suggests that DPP-4 inhibitors may confer additional protective effects to the kidney beyond their capacity as antihyperglycemic agents. Additional studies are required to establish whether or not vildagliptin has protective qualities with respect to piroxicam-induced nephrotoxicity [42].

2.6 Anti-senescence Effect of Vildagliptin

Doxorubicin can lead to vascular toxicity and lead to the occurrence of senescence, and it is thought that vildagliptin has the ability to reduce the development of cellular senescence due to doxorubicin toxicity. Doxorubicin caused an increase in markers of cellular senescence (e.g., p16, p27), as well as cytokines that promote inflammation (i.e., IL-6 and IL-8). Treatment with vildagliptin decreased the levels of these markers and enhanced relaxation of vascular smooth muscle. Therefore, the authors of this study concluded that the use of GLP-1 via the action of vildagliptin might help reduce the oxidative damage and inflammatory

processes that lead to cellular senescence. This particular study focused on tissues of the vascular system; however, vildagliptin has been shown to have cytoprotective effects related to the reduction of inflammation and cellular aging, which could potentially also help to protect the kidneys from the damaging effects of doxorubicin [43].

2.7 Objectives

- i. To evaluate biochemical markers ,serum urea and Creatinine after Piroxicam induced nephrotoxicity using commercially available kit.
- ii. To determine morphological changes in renal tissues by histopathological examination.
- iii. To investigate the role of TNF-alpha in piroxicam induced nephrotoxicity by ELISA
- iv. To explore the potential anti-inflammatory effect of vildagliptin against piroxicam induced nephrotoxicity in experimental animal model.

Chapter 3

Material and Methods

3.1 Overview

This chapter provides a comprehensive description of the study design, experimental procedures, selection of the animal model, dosing regimen, analytical techniques, and ethical protocols employed to evaluate the potential protective effects of vildagliptin against piroxicam-induced kidney toxicity in laboratory animals. In designing the study, an effort was taken to ensure the experimental design reflected the pathophysiological mechanisms associated with renal injury due to drug exposure while maintaining scientific merit, reproducibility, objectivity, and clinical translatability. Nephrotoxic effects of piroxicam were produced by repeatedly giving piroxicam to the animals with the intention of mimicking extended periods of exposure to non-steroidal anti-inflammatories (NSAIDs) while reproducing the critical pathogenic processes typically found in cases of drug-induced renal injury (i.e. inflammation, oxidative stress, Renal biomarker Elevation, tubular degeneration, and necrotic changes in kidney tissue). Adult Balb/C mice were selected as the laboratory animals for this study due to their known stable genetic background, predictable physiological responses to toxic agents, and being a widely used model for nephrotoxicity and other pharmacological studies [44].

The nephroprotective and anti-inflammatory properties of vildagliptin (DPP-4 inhibitor) were evaluated using a dosing protocol developed for use with the piroxicam nephrotoxicity model. Renal damage was assessed biochemically (i.e. serum creatinine, serum urea), and inflammatory response (TNF- α) was evaluated using an ELISA. In addition, total protein was measured in serum from animals in order to detect systemic biochemical alterations using the Bicinchoninic Acid (BCA) protein assay.

Morphological changes in kidney tissues were identified using histopathological examination via hematoxylin and eosin (H&E) staining, assessing parameters such as inflammatory cell infiltration, glomerular damage, tubular degeneration, and necrotic lesions.

The combination of in vivo pharmacological studies with biochemical and histological analyses provided valuable insight into the mechanism by which piroxicam-induced renal injury may occur, as well as the relative potencies of nephroprotection provided by vildagliptin.

The procedures defined in this section create a consistent format for measuring vildagliptin's biochemical, histopathological and anti-inflammatory properties against piroxicam-induced nephropathy. All animals were treated following established ethical procedures at international standards and institutionally adopted standards for laboratory animals.

3.2 Ethical Statement

All experiments involving animals were conducted in accordance with national regulations governing the ethical use and care of laboratory animals, as well as the institutional guidelines for animal welfare. The experimental protocol (REC/FOP/CUST/2025 /15) received approval from the Research and Ethical Committee of the CUST, Islamabad, prior to the commencement of the study.

3.3 Experimental Animals

The experimental animals consisted of 4-week-old mice with body weights ranging from 25 to 30 g, obtained from CUST. The animals were maintained under controlled laboratory conditions at a temperature of $22 \pm 2^\circ\text{C}$ and relative humidity of $50 \pm 10\%$, with a 12-hour light/12-hour dark cycle. Throughout the study period, the animals had free access to standard laboratory chow and water. Before the commencement of experimental procedures, the mice were allowed to acclimatize to the laboratory conditions for a period of 7 days[35].

3.3.1 Composition of Animal Feed

The experimental mice were provided with a standard diet formulated to fulfill their dietary requirements. This feed generally contained approximately 18-24% protein, 4-6% fat, and 40-60% carbohydrates, essential vitamins and minerals necessary for normal growth and maintenance. The specific composition of the diet may vary depending on the source and formulation of the feed ingredients.

3.3.2 Bedding of Experimental Animals

Mice were provided with bedding materials that allow them to be comfortable, absorb moisture, and help to control odor. Examples of bedding include wood shavings (e.g., aspen).

3.3.3 Handling of Mice

To minimize stress during handling, mice were restrained using a combination of the tail and scruff restraint techniques. The thumb and index finger of one hand was used to gently held the base of tail, while the other hand was used to apply gentle forward pressure toward the neck. The restraint technique provided

adequate immobilization of the animals while reducing handling-induced stress during experimental procedures [45].



FIGURE 3.1: Illustration of the tail and scruff handling techniques employed for the safe restraint of mice during experimental procedures.

3.4 Experimental Design and Grouping



FIGURE 3.2: Accleimatization Of mice prior to experimental study

This figure depicts the gentle handling techniques used to minimize stress and promote the welfare of animals during routine experimental procedures.

As a mean of reducing selection bias, mice were allocated randomly into treatment groups using a basic randomization technique. Individual mice were identified using a tail-marking method in which unique combinations of circles and horizontal

lines were drawn on their tails. This identification system enabled each animal to be distinguished throughout the experimental period. Tail markings were applied before the mice were randomly assigned to their respective treatment groups. Throughout the study, the animals were observed daily to monitor their general health status and any changes in behavior.



FIGURE 3.3: Identification and Marking Protocol of Mice.

Images depict the brief tail-marking system for the identification of individual laboratory mice during experimental studies of short duration.

3.5 Randomized Controlled Method

The nephroprotective effect of vildagliptin was evaluated using a randomized controlled experimental design with a placebo-treated control group. This approach was employed to determine the ability of vildagliptin to protect against piroxicam-induced acute renal injury.

Random assignment of animals to the different treatment groups minimized selection bias and improved the reliability and validity of the experimental findings [46].

3.5.1 Allocation of Mice

Thirty mice, whose weight were between 26 and 30 g, were randomly allocated into five experimental groups, with six animals assigned to each group.

All animals were maintained under similar controlled laboratory conditions and received their designated treatments once daily for a period of nine consecutive days. Nephrotoxicity was induced using piroxicam, whereas vildagliptin was administered as the investigational treatment to assess its potential protective effects against kidney injury.



FIGURE 3.4: Experimental grouping of mice By Randomization.

TABLE 3.1: Treatment Description Of Experimental Groups

Group	Treatment Received
Group I	Received Distilled water, served as the control group.

Table 3.1 continued from previous page

Group	Treatment Description
Group II (Piroxicam Control)	Received Piroxicam (50 mg/kg, p.o) once daily for 09 consecutive days to induce nephrotoxicity.
GroupIII (Piroxicam+ Vildagliptin low dose)	Received Piroxicam (50 mg/kg, p.o) followed by Vildagliptin (10 mg/kg, p.o) 30 minutes later.
GroupIV (Piroxicam Vildagliptin High Dose)	Received Piroxicam (50 mg/kg, p.o.) followed by Vildagliptin (20 mg/kg, orally) 30 minutes later.
Group V (Piroxicam + Standard Drug)	Received Piroxicam (50 mg/kg, i.p.) followed by Silymarin (100 mg/kg, p.o.) as a standard nephroprotective drug.

3.6 Routes of Administration of Drugs

In terms of how therapeutic agents are administered, the route of administration can greatly influence their pharmacokinetic/pharmacodynamic characteristics. For the purposes of this study, both piroxicam and vildagliptin were chosen to be administered via the oral route in the same way that they are typically used clinically because piroxicam and vildagliptin are generally prescribed to humans and are conventionally administered orally.

Thus, the use of the oral route for the administration of these medications ensures that the experimental results have increased clinical relevance/translational value. Furthermore, the oral administration of both medications is non-invasive, quick/easy to perform and gives a cost-effective way to eliminate the potential for tissue damage or stress associated with multiple injections into experimental animals. Lastly, since both medications can be given via the oral route, it is possible to achieve the same dosing of both agents throughout this study.

3.7 Chemicals Used

The chemicals used for these activities were all analytical grade materials. Piroxicam was the drug that caused nephrotoxicity. Piroxicam was obtained from Biorex Pharmaceuticals, Islamabad. Vildagliptin was generously gifted by Karsons Pharmaceuticals Rawat to examine the potential of Vildagliptin to have a nephroprotective effect against renal injury induced by Piroxicam.

3.8 Solubility of Drugs

Due to the little solubility of the test drugs in water, a co-solvent was required to administer the tested drugs. For the creation of Piroxicam, this preparation involved dissolving the drug in carboxymethylcellulose (CMC) sodium and then diluting to its final volume with sterile distilled water and then mixing to create a clear, homogenous, uniform preparation for oral administration. Vildagliptin was found to dissolve readily in sterile distilled water and therefore was prepared by simply dissolving the test drug in sterile distilled water and then mixing gently. Silymarin served as the standard compound and was prepared by dissolving the compound in 0.5% of CMC sodium and then triturating to formulate a suspension.

3.9 Drug Preparation

3.9.1 Calculation Of Dose

$$\text{Dose (mg)} = \text{Body Weight in grams} \times \text{Dose (mg/kg)} / 1000$$

Example Calculations for a 30 g Mouse

$$\text{Piroxicam (50 mg/kg): Dose} = (30 \times 50)/1000 = 1.5 \text{ mg}$$

$$\text{Vildagliptin (10 mg/kg): Dose} = (30 \times 10)/1000 = 0.3 \text{ mg}$$

$$\text{Vildagliptin (20 mg/kg): Dose} = (30 \times 20)/1000 = 0.6 \text{ mg}$$

$$\text{Silymarin (100 mg/kg): Dose} = (30 \times 100)/1000 = 3 \text{ mg}$$

All formulations were freshly prepared daily before administration.

3.10 Dosing Protocol

All drugs were given to the animals once a day for 09 straight days with oral fittings using 30-gauge gavage needles. Animals' individual weight readings were taken daily to accurately calculate dose amounts given. Piroxicam was the first drug to induce nephrotoxicity. To evaluate nephroprotective effect, Vildagliptin was administered 30 minutes after Piroxicam treatment.



FIGURE 3.5: Measurement of weight of mice.

Body weight was measured throughout the entire study period to evaluate general health, identify any adverse drug reactions, and to modify dose amounts accordingly (Photograph taken at CUST).

TABLE 3.2: Daily Treatment Schedule and Observations

Group	Treatment		Route	Monitoring	Expected Observation
Control	Distilled		Oral	Body weight, Behavior	Normal Behavior And stable Body weight
Piroxicam	50mg/kg		Oral	Body Weight, Behavior	Weight loss, nephrotoxicity signs
Piroxicam	+	Both	Oral	Body weight, Behavior	Mild Nephroprotection
Vildagliptin (10mg)		drugs			
Piroxicam	+	Both	Oral	Body Weight, Behavior	Improved renal protection and body weight retention
Vildagliptin (20mg)		Drugs			
Piroxicam + Silymarin (100mg)		Both	Oral	Body weight, Behaviour	Sufficient Nephroprotection
		drugs			

3.11 Personal Protective Equipment and Biosafety Measures

Appropriate personal protective equipment (PPE) was used during all experimental procedures, including animal handling, drug administration, blood sampling, and tissue collection, in accordance with institutional biosafety policies and ethical guidelines for laboratory animal research.

These measures were adopted to minimize the risk of exposure to biological specimens, chemicals, and pharmaceutical agents used throughout the study. Furthermore, disposable gloves were changed between treatment groups to prevent

cross-contamination and maintain experimental integrity. All contaminated materials, including needles, syringes, and biological waste, were disposed of immediately in designated biohazard and puncture-resistant sharps containers according to institutional biosafety protocols [47].

3.12 Euthanasia and Methods of Euthanasia

Euthanasia processes were done according to the recommendations of the American Veterinary Medical Association Guidelines.

The euthanasia procedure was selected to achieve a rapid onset of unconsciousness while minimizing pain and distress, thereby ensuring a humane death. All procedures were carried out in accordance with established ethical guidelines to reduce animal suffering and preserve tissue quality for subsequent experimental analyses [48].

3.13 Methods of Euthanasia

Euthanasia techniques are broadly classified into two categories [49].

3.13.1 Physical Methods

Physical methods include cervical dislocation, decapitation, and other techniques that induce immediate loss of consciousness through mechanical disruption of vital neurological functions.

3.13.2 Chemical Methods

Chemical methods involve administration of anesthetic or inhalational agents that induce unconsciousness followed by respiratory and cardiac arrest. The current

research uses chloroform to put mice to sleep via inhalation before they are killed, and to take blood and tissue samples from the animals.

For euthanasia, approximately 2-3 mL of chloroform was applied to an absorbent material placed inside a sealed desiccator. Mice were individually exposed to the chloroform vapors until complete loss of consciousness was achieved, followed by cessation of respiration. Death was confirmed by the absence of breathing, heartbeat, and corneal or ocular reflexes. Following confirmation of death, the thoracic cavity was carefully opened to expose the heart, and blood samples were collected via cardiac puncture for subsequent biochemical analyses [50].

3.13.3 Techniques For Blood Withdrawal

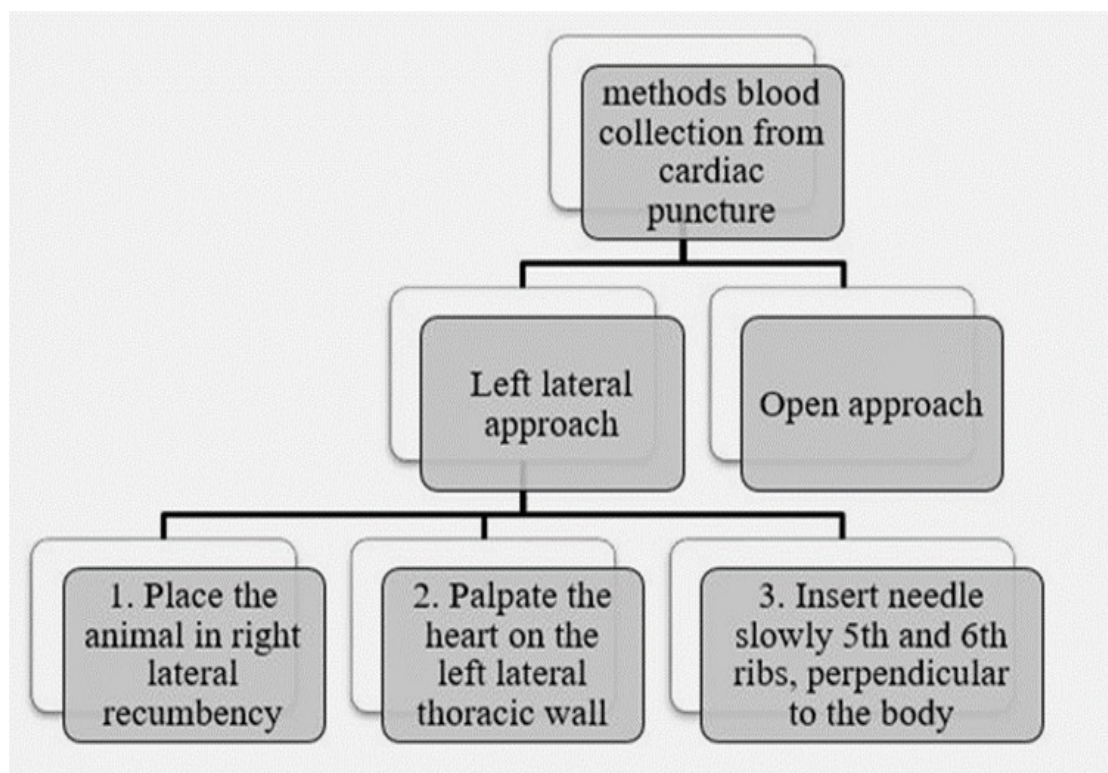


FIGURE 3.6: Techniques employed for blood sample collection from mice during the experimental study.

The figure demonstrates commonly used blood collection methods in mice, including tail vein, saphenous vein, retro-orbital sinus, and cardiac puncture.



FIGURE 3.7: Collection of blood by cardiac puncture technique.

3.13.4 Cardiac Puncture Procedure for Blood Withdrawal

Chloroform was used to deeply anesthetize the mouse by way of inhalation. Once anesthetized and laid in a dorsal recumbent position on a clean sterile working surface, a sterile 1mL syringe with a 23G needle attached was inserted bevel side up at a 30-40 degree angle on the left of the sternum toward the heart [51, 52].

After the needle was inserted into the body cavity, gentle negative pressure was applied by slowly retracting the syringe plunger while carefully advancing the needle until blood entered the syringe. Once blood flow was established, aspiration was continued until the required volume of blood had been collected. The collected samples were then immediately transferred into appropriately labeled collection tubes for further analysis.



FIGURE 3.8: Microcentrifuge tubes utilized for the collection of blood specimens from mice during the experimental study.

3.13.5 Extraction of Kidney

The abdominal cavity was opened through a midline incision after cardiac puncture and euthanasia and was surgically entered through the incision. The subcutaneous fat and skin were cut with scissors and the fascia and connective tissue over the peritoneum were dissected bluntly to prevent major damage to underlying tissues. The peritoneum was then cut longway to view the abdominal cavity. A suture was used to elevate the abdominal cavity at the xiphoid process of the sternum to provide the surgeon with a greater view of the organs in the abdomen. The suture was held with moderate tension to prevent falling back down after it was elevated. A retractor was used to expose the retroperitoneal organs. The contents of the gastrointestinal tract were moved out of the way to view the kidneys, located in the retroperitoneal space, bilaterally adjacent to the spine. The kidneys were carefully separated from their surrounding adipose and connective tissues.

The removal of each kidney from the body using surgical technique required the removal of blood vessels and ureter (collectively termed "renal blood vessels and ureters") from the renal pedicle (the junction of blood supply and urine outflow) prior to ligating and transecting each renal vessel; all fibers associated with the

kidneys must be carefully dissected away so that they can be completely mobilized and removed together as an entire unit; after removing the kidneys; the kidneys themselves were washed again with sterile 0.9% sodium chloride solution immediately after removing them (to remove blood and contaminants) before being blotted dry with paper towels; tissue samples intended of analysis for histopathological purposes were fixed using 10% phosphate buffer formalin in order maintain cellular morphology and tissue architecture at the time of histochemical processing and the specimens are to be maintained at 4 degrees C until histochemical / immunologic processing is done; tissue samples remaining in each kidney that are to be used only for biochemical assays were appropriately preserved and maintained at -80 degrees C until analysis [53].

3.14 Biochemical Estimation of Urea and Creatinine

At the conclusion of the experimental period, renal function was appraised by measuring serum creatinine and serum urea concentration. Cardiac puncture was used to obtain blood for each sample of approximately 3-5mL. The procedure was performed as per the aseptic technique.

Following collection, blood was placed in a plain tube for centrifugation. To allow blood to clot, blood was left at room temperature for 30 minutes prior to centrifugation at 3000 for 10 minutes. During centrifugation, serum and red cells were separated in the tube. After centrifugation, the serum was poured off into another container, placed in a -20 freezer, until biochemical testing (serum creatinine) could be performed.

Serum urea concentration was determined using a commercial kit with the enzymatic colorimetric method (urease-GLDH method). The major steps involved with the testing method include: (1) Hydrolysis of urea to ammonia and CO₂ by urease; (2) reaction of ammonia with α -ketoglutarate and NADH in the presence of GLDH; and (3) measurement of the reduction in absorbance due to NADH

oxidation (340 nm) using a spectrophotometer. Serum creatinine concentration was estimated using the alkaline picrate Jaffe method of measurement.

Using a UV-Visible Spectrophotometer, we measured the absorbance (abs) of 520 nm (nanometers) of the orange colored creatinine/picric acid complex reacted in an alkaline (basic) solution (sodium bicarbonate) to quantify the amount of urea and creatinine in the serum (specimens or samples) of the patient(s) (blood).

The elevated levels of creatinine and urea in the serum of patients indicate the presence of damaged kidneys (renal failure) or nephrotoxicity; while complete normalization of these two (2) creatinine and urea markers has the potential to prove the agent (vildagliptin) to have renoprotective properties [54].

The kidney tissue was analyzed for pro-inflammatory cytokines Tumor Necrosis Factor-alpha (TNF- α) by ELISA.

3.14.1 Preparation of Kidney Homogenate

Kidney tissue samples were washed thoroughly with phosphate-buffered saline and placed into pre-weighed Eppendorf tubes. The required amount of Tween 80 was added according to the tissue weight (50-100 mg), with an example ratio of 166.67 μ L of buffer used for 50 mg of tissue. The samples were then homogenized using a D160 homogenizer (China) [55]. Following homogenization, the samples were centrifuged using an SCI-24 high-speed microcentrifuge at 8500 revolution per minute for 30 minutes. The resulting supernatant was carefully obtained and used for subsequent assay.

To prepare the Tween 80-PBS solution, 0.1 mL (100 μ L) of Tween 80 was added to the desired volume of phosphate-buffered saline. The mixture was stirred thoroughly to ensure complete dissolution and uniform distribution of Tween 80 throughout the buffer. Mixing was continued until the solution appeared clear and consistent. The prepared solution was then appropriately labeled with the solution name, concentration, and preparation date. For storage, the solution was

kept either at room temperature or refrigerated at 4°C in a tightly sealed container [56].

3.14.1.1 Kidney Tissue Collection and Preparation

All kidney tissue samples were obtained with care, weighed, and transferred directly into pre-labeled and pre-weighed Eppendorf tubes. Post weighing, each Eppendorf tube with kidney tissue had Tween 80 was added based on the weight of the kidney sample at 50 mg to 100 mg in order to establish a ratio of kidney tissue sample weight to the total volume of 0.1% Tween 80 present in the Eppendorf tube with kidney tissue to obtain information on homogenization.

An example would be if a kidney sample weighed 50 mg, then 166.67 μL of 0.1% Tween 80 would be used for that sample. The same 0.1% Tween 80 was used for all kidney tissue samples. The kidney tissue homogenate was homogenized using a homogenizer and centrifuged at 8500 rpm for 30 minutes at room temperature to remove large, visible debris. The supernatant was collected.

3.14.1.2 Standards Preparation

A series of standard dilutions was prepared from a BSA stock solution (2 mg/mL) in a 96-well plate to generate a standard curve. An aliquot of 20 μL from each dilution was added to the wells for standard preparation. The dilution series consisted of 20, 15, 10, 7, 5, 2.5, and 0.00 μL of BSA solution, with distilled water added as required to adjust the final volume of each sample to 20 μL .

3.14.1.3 Sample Preparation

For sample preparation, we took the super-natant of the sample (1:10 dilution) with 1-part sample to 9 parts distilled water.

3.14.1.4 Preparation of Working Reagent

Working reagent was prepared by mixing Reagent A and Reagent B in a 50:1 ratio. The required amount of each reagent was freshly combined before use to ensure optimal performance during the experiment. Subsequently, 200 μL of the prepared working reagent was added to each well containing the samples and standards. The plate was gently agitated by tapping for approximately 30 seconds to ensure proper mixing of the contents.

3.14.1.5 Incubate

The prepared microplate, containing the samples, standards, and working reagents (WRs) as described in the preceding procedure, was incubated at 37°C for 30 minutes. After completion of the incubation period, the plate was allowed to equilibrate and cool to room temperature before proceeding with the subsequent steps of the assay.

3.15 ELISA Test

The concentration of TNF- α in kidney homogenate samples was determined using a commercially available ELISA kit (Ref. No. PRS-2050Mo; Lot No. 202313). The assay was performed according to the instructions and procedures provided by the manufacturer.

3.15.1 Procedure of ELISA Assay

A serial dilution of the sample was prepared by adding 50 μL of the sample to the designated standard wells (10 wells in total). Initially, 100 μL of standard solution was added to the first and second wells, followed by the addition of 50 μL of dilution buffer to achieve the required dilution volume. The contents were mixed, and 100 μL of the resulting mixture was transferred from the first two wells to the third

and fourth wells. This serial transfer process was continued sequentially from wells 3-4 to 5-6, 5-6 to 7-8, and finally from 7-8 to 9-10, maintaining the same dilution procedure throughout.

Following completion of the serial dilution, 50 μL of the prepared samples was added to each respective well before the addition of ELISA reagents. Blank wells were maintained as negative controls and received neither sample nor HRP-conjugated reagent, while all other assay steps were performed identically. For sample preparation, 40 μL of sample diluent was added to each designated sample well, followed by 10 μL of the test sample. Care was taken to avoid contact with the sides of the wells during sample addition, and gentle mixing was performed to ensure uniform distribution.

After sample loading, the microplate was sealed with an adhesive membrane and incubated at 37°C for 30 minutes. The washing buffer was prepared by diluting the concentrated wash solution 20- or 30-fold with distilled water according to the required protocol. After incubation, the plate seal was removed, and the contents of each well were discarded. The wells were washed by adding wash buffer, allowing it to remain for approximately 30 seconds, and then removing the liquid by gentle aspiration or decanting. The washing procedure was repeated five times, or until the wells were free of residual liquid. After washing, the wells were dried using absorbent paper.

Subsequently, 50 μL of HRP-conjugated enzyme reagent was added to each well, except the blank wells. The plate was then incubated and washed following the same procedure described previously. Chromogenic substrates, including 50 μL of Chromogen Solution A and 100 μL of Chromogen Solution B, were added to each well. The plate was protected from light and incubated at 37°C for 15 minutes to allow color development. The reaction was terminated by adding 50 μL of stop solution to each well, resulting in a color change from blue to yellow. Absorbance was measured at 450 nm using the blank well as the reference within 15 minutes of adding the stop solution. The recorded optical density values were used to generate a standard curve, which was subsequently applied for determining the protein concentrations of the samples [57].



FIGURE 3.9: Schematic representation of ELISA assay using primary antibody-coated 96-well plates.

The figure illustrates the application of 96-well microplates pre-coated with antibodies specific to TNF- α . During the process, the prepared samples are added to the wells, allowing TNF- α present in the samples to bind with the immobilized antibodies. The bound TNF- α is then quantified using an enzyme-linked detection system, enabling its measurement through the resulting enzymatic reaction.

3.16 Evaluation of Kidney Histopathological Changes

To evaluate the kidneys and liver tissues morphometrically, they were first preserved with 10% neutral-buffered formalin for approximately 24-48 hours at room temperature to preserve the gross architecture of the respective tissues. Subsequently, the tissues were processed using standard histological protocols. Tissues were dehydrated through a series of graded alcohols, cleared in xylene, and embedded in paraffin wax. The paraffin blocks were cut into sections of approximately 4-5 μm using a rotary microtome. The sections were placed onto clean glass slides, and then dried at 37 °C to enhance the adhesion of the tissue to the slides.

For routine histological examination, divisions were, rehydrated through an ethanol series (100%, 95%, 70%), and washed in distilled water before staining with hematoxylin/eosin (H&E). The cells' nuclei were stained with Harris Hematoxylin for 5-7 minutes, and differentiated with 1% acid alcohol (if needed), and then blued with alkaline water. After staining was completed, the slides were dehydrated through ascending alcohol grades, cleared in xylene, and mounted with a permanent mounting medium [58].

For assessment of fibrosis and collagen deposition, Masson's trichrome staining was performed using standard protocols. Stained sections were examined under a bright-field light microscope at 10× and 40× magnifications, and representative photomicrographs were captured for histopathological analysis [59].

3.17 Statistical Analysis

Data obtained from biochemical analyses, oxidative stress assessments, inflammatory markers, and histopathological examinations were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was carried out using GraphPad Prism software (version 8.0.1). Variations among the experimental groups were assessed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test for post hoc analysis. A p-value of <0.05 was considered statistically significant. Statistical assessments were performed to determine the nephroprotective potential of vildagliptin against piroxicam-induced renal damage by analyzing biochemical, histological, and inflammatory parameters. The obtained findings were summarized and illustrated in the form of tables and graphical representations..

Chapter 4

Results

4.1 Background

The chapter includes portion of results obtained from the experiment which determined the nephroprotective and anti-inflammatory properties of vildagliptin and silymarin during nephrotoxicity induced by piroxicam in mouse. The levels of inflammatory markers were studied via to obtain the data corresponding to the standard curve. Results were interpreted in terms of substance dose-response effects and mechanisms of action of the substances studied connected with nephrotoxicity caused by piroxicam. In our research we employed the model of piroxicam nephrotoxicity in mice in order to assess the therapeutic potential of vildagliptin, which is a DPP4 inhibitor.

4.2 Biochemical Assessment of Kidney Damage

In the present work, blood samples were procured in a sterile fashion using cardiac puncture from the mice model of piroxicam nephrotoxicity and processed for serum biochemistry. Serum creatinine and urea were estimated using standard enzymatic colorimetric detection techniques in a certified laboratory. Since high

serum creatinine and urea levels are noticed following piroxicam injection, it suggests that there is some renal stress, poor glomerular filtration, and kidney injury. This change in biochemistry indicates piroxicam nephrotoxicity.

In the post-treatment phase of the study, the administration of a selective Vildagliptin (20 mg/kg) has shown better nephroprotective effects in a dose-dependent manner. The increase in renal function parameters was reduced. This corresponds with the anti-inflammatory activity. Treatment with Vildagliptin at a lower dose (10 mg/kg) and its high dose (20 mg/kg) along with the standard nephroprotective agent Silymarin(100 mg/kg) led to a significant decrease in serum creatinine and urea levels, which indicates improved kidney activity.

4.2.1 Serum Urea and Creatinine Level

Serum urea is a sensitive biochemical marker of renal dysfunction and reflects the kidney's ability to excrete nitrogenous waste products. The serum urea levels in the Piroxicam-only group were considerably elevated compared to the control group, indicating severe renal impairment and nephrotoxicity. Vildagliptin administration at both low dose (10 mg/kg) and high dose (20 mg/kg), as well as the standard reference drug Silymarin (100 mg/kg), significantly reduced Piroxicam induced elevations in serum urea levels, suggesting a nephroprotective effect against renal injury.

The control group exhibited a serum urea level of 28 ± 1.8 mg/dL. Administration of piroxicam (50mg/kg) resulted in a substantial rise in serum urea to 78 ± 3.5 mg/dL ($\#p < 0.05$ vs. control). Silymarin administration (100 mg/kg) significantly reduced serum urea levels to 42 ± 2.4 mg/dL ($p < 0.001$ vs. piroxicam group). Vildagliptin at a dose of 10 mg/kg lowered serum urea levels to 58 ± 2.1 mg/dL ($p 0.01$ vs. piroxicam), while the higher dose of 20 mg/kg further reduced serum urea to 35 ± 1.7 mg/dL ($p 0.001$ vs. piroxicam), approaching normal values.

The marked elevation of serum urea following piroxicam administration confirms significant renal dysfunction and impaired excretory capacity. Treatment with

Vildagliptin significantly attenuated serum urea levels, with 10 mg/kg and 20 mg/kg doses reducing serum urea by 25.6% and 55.1%, respectively, compared to the piroxicam-treated group. Silymarin reduced serum urea by 46.2%. These findings indicate that Vildagliptin, particularly at the higher dose, effectively protects against piroxicam-induced nephrotoxicity.

Serum creatinine is another important marker of renal injury and glomerular filtration efficiency. Raised serum creatinine levels are suggestive of impaired kidney function and nephron damage.

Piroxicam administration caused a significant increase in serum creatinine levels, whereas treatment with Vildagliptin and Silymarin effectively reduced these elevations. The nephroprotective effect of Vildagliptin was more pronounced at the higher dose.

The normal control group showed a serum creatinine level of 0.45 ± 0.03 mg/dL. Piroxicam administration (50 g/kg) significantly increased serum creatinine to 1.78 ± 3.5 mg/dL ($p < 0.05$ vs. control). Treatment with Silymarin (100 mg/kg) significantly decreased serum creatinine to 0.78 ± 0.05 mg/dL ($p < 0.001$ vs. piroxicam).

Vildagliptin at 10 mg/kg reduced serum creatinine to 1.15 ± 0.06 mg/dL ($p < 0.01$ vs. piroxicam), while the 20 mg/kg dose further normalized serum creatinine levels to 0.60 ± 0.04 mg/dL ($p < 0.001$ vs. piroxicam), approaching the values observed in the control group.

The significant elevation of serum creatinine in the piroxicam-treated group confirms piroxicam-induced nephrotoxicity and compromised renal filtration. Treatment with Vildagliptin at doses of 10 mg/kg and 20 mg/kg reduced serum creatinine levels by 30.3% and 63.6%, respectively, whereas Silymarin produced a reduction of 52.7% compared to the piroxicam group.

These results suggest that high-dose Vildagliptin provides superior protection against piroxicam-induced renal damage and effectively restores kidney function.

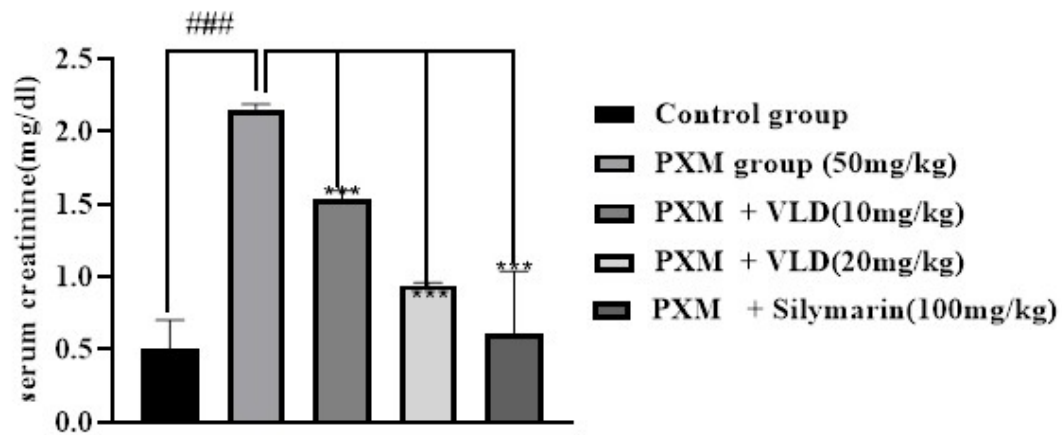


FIGURE 4.1: Effect of Piroxicam induced nephrotoxicity and vildagliptin treatment on Serum Creatinine levels (mg/dl) in experimental mice. Data is presented as mean with SEM

Figure 4.1 shows that the serum Creatinine levels were increased in the Piroxicam group (50 g/kg) than control group, which indicates renal impairment ($p < 0.001$ versus controls). Vildagliptin at (10 mg/kg) and (20 mg/kg) plus Silymarin (100 mg/kg) significantly decreased serum Creatinine levels compared to the Piroxicam group ($*** p < 0.001$). Values are presented as mean $\pm$ standard deviation (SD) with six samples per group ($n = 6$). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post hoc test.

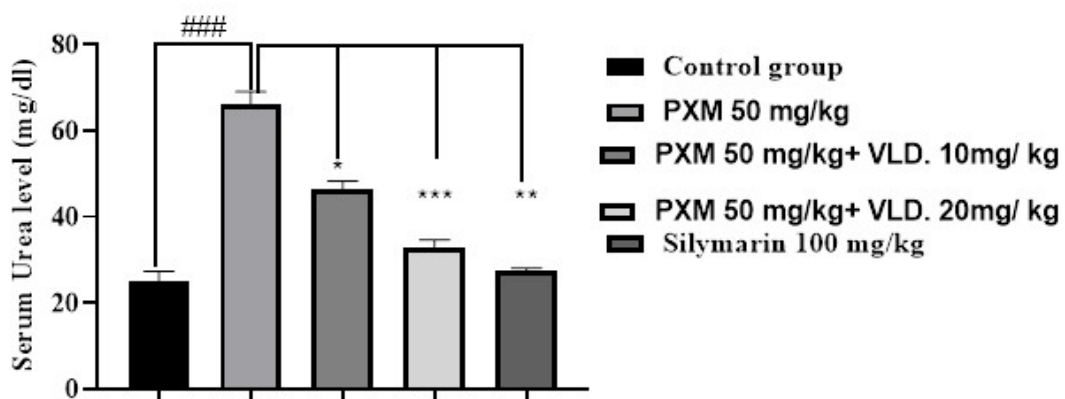


FIGURE 4.2: Effect of Piroxicam induced nephrotoxicity and vildagliptin treatment on Serum Urea levels (mg/dl) in experimental mice.

As illustrated in the figure, the Piroxicam group exhibited elevated serum urea concentrations when compared to the control ($p < 0.001$). Administration of

both doses of Vildagliptin (10 mg/kg and 20 mg/kg) and Silymarin at 100 mg/kg resulted in statistically significant reductions in serum urea concentration when evaluated against the Piroxicam group ($p < 0.001$).

4.3 Analysis of Inflammatory Marker

Following the collection of blood samples via cardiac puncture, the mice were euthanized, and the kidneys were carefully excised. The isolated kidney tissues were stored in PBS buffer (pH 7.4) for further biochemical investigations. Subsequently, ELISA analysis was performed to determine the levels of TNF- α in the tissue samples.

4.3.1 TNF- α Levels

The standard calibration curve demonstrated strong linearity with an R^2 value of 0.99, confirming the accuracy, reliability, and reproducibility of the assay used for TNF- α quantification. The TNF- α concentrations in the experimental samples were determined by comparing their absorbance values with those obtained from the standard curve.

The findings indicated that the assay was effective for assessing piroxicam-induced renal inflammation and for evaluating the potential anti-inflammatory effects of therapeutic agents, including silymarin and vildagliptin..

TABLE 4.1: TNF- α Standard Concentration Curve

Sr.no	optical density	Concentration ($\mu\text{g}/\mu\text{l}$)
1.	0.055	0
2.	0.407	7.5
3.	0.701	15
4.	0.981	30
5.	1.721	60
6.	2.532	90

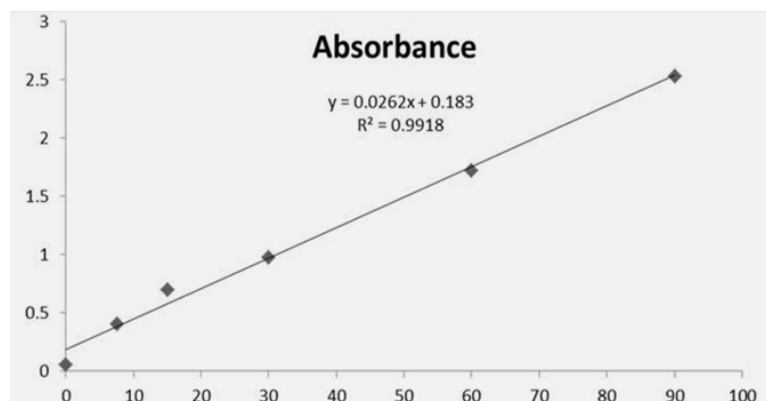


FIGURE 4.3: Standard Calibration curve of tumor necrosis factor-alpha generated using serial dilutions of TNF-alpha standards in ELISA assay.

A calibration curve was generated to determine protein concentrations in the ELISA assay. The obtained protein concentrations were plotted against the corresponding optical density (OD) readings measured at 450 nm. The resulting curve exhibited a high degree of linearity ($R^2 = 0.99$), indicating the reliability of the assay and enabling accurate estimation of sample concentrations within the working range of the method.

4.3.1.1 Level of TNF- α in Experimental Groups

The ELISA assay was employed to evaluate the levels of inflammatory biomarkers in mice subjected to piroxicam-induced nephrotoxicity. The findings were interpreted in relation to the dose-dependent effects of the administered treatments and their potential mechanisms in mitigating renal injury. Administration of piroxicam (50 mg/kg) resulted in a significant elevation of TNF- α levels, indicating the induction of an inflammatory response in renal tissue.

Treatment with a combination of low-dose vildagliptin (10 mg/kg) and piroxicam (50 mg/kg) produced a reduction in TNF- α concentration compared with the piroxicam-treated group. A more pronounced decrease in TNF- α levels was observed following the administration of high-dose vildagliptin (20 mg/kg) in combination with piroxicam (50 mg/kg). Similarly, silymarin (50 mg/kg) co-administered with piroxicam demonstrated a moderate protective effect by reducing inflammatory marker levels.

Furthermore, administration of vildagliptin at doses of 10 mg/kg and 20 mg/kg, along with silymarin at 100 mg/kg, resulted in a significant decline in TNF- α levels compared with animals receiving piroxicam alone. These findings demonstrate the potential anti-inflammatory activity of the tested compounds in attenuating piroxicam-induced TNF- α elevation. Piroxicam treatment significantly increased TNF- α production in renal tissue, confirming the development of an inflammatory state associated with nephrotoxicity.

Relative to the piroxicam-only group, vildagliptin at doses of 10 mg/kg and 20 mg/kg reduced TNF- α levels by 31.6% and 44.5%, respectively, whereas silymarin treatment produced a reduction of approximately 33.8%. Following measurement of absorbance at 450 nm, regression analysis was performed using the standard curve equation to determine TNF- α concentrations in renal tissue samples. TNF- α levels were quantified based on the absorbance readings obtained from each experimental sample.

TABLE 4.2: TNF- α concentration in experimental group by ELISA

Groups	Dose	Concentration ($\mu\text{g}/\mu\text{l}$) mean $\pm$ SD
Normal control group	-	2.48 ± 0.10
Piroxicam group	50mg/kg	6.17 ± 0.69 #
Vildagliptin (low dose)	10 mg/kg	4.30 ± 0.71 *
Vildagliptin (high dose)	20 mg/kg	3.48 ± 0.71 ***
Silymarin	100mg/kg	4.14 ± 0.17 **

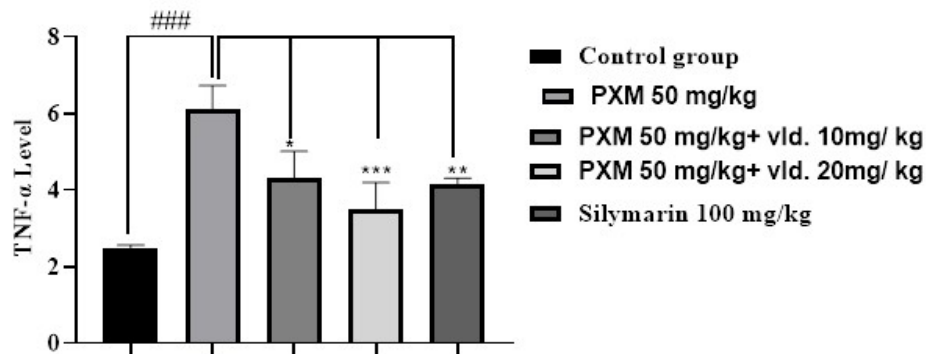


FIGURE 4.4: Effect of Piroxicam induced nephrotoxicity and vildagliptin treatment on Serum Urea levels (mg/dl) in experimental mice .Data is presented as mean with SEM

The assessment of TNF- α levels revealed that administration of piroxicam (50 mg/kg) significantly elevated TNF- α expression compared with the control group, indicating the activation of an inflammatory response ($p < 0.001$). In contrast, treatment with vildagliptin at doses of 10 mg/kg and 20 mg/kg, as well as silymarin (100 mg/kg), resulted in a significant reduction in TNF- α levels compared with the piroxicam-treated group.

Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test, with significance levels represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. All values were expressed as mean $\pm$ SD ($n = 6$ animals per group).

Piroxicam administration caused a marked increase in TNF- α expression in renal tissue compared with the control group, demonstrating the development of an inflammatory condition associated with nephrotoxicity. The mean TNF- α concentration in the piroxicam-treated group (6.17 ± 0.69) was considerably higher than that observed in the control animals (2.48 ± 0.10), confirming the upregulation of this pro-inflammatory cytokine.

The elevation of TNF- α following piroxicam exposure suggests activation of inflammatory signaling pathways involved in renal injury. Increased TNF- α production promotes pro-inflammatory responses, which may contribute to oxidative stress, apoptosis, and progression toward fibrotic changes in kidney tissue. Co-administration of vildagliptin (10 mg/kg) with piroxicam reduced TNF- α levels to 4.30 ± 0.71 , suggesting a moderate anti-inflammatory effect.

Furthermore, treatment with a higher dose of vildagliptin (20 mg/kg) resulted in a greater reduction in TNF- α concentration (3.48 ± 0.71), indicating improved suppression of piroxicam-induced inflammatory activity. These findings suggest that vildagliptin exhibits dose-dependent anti-inflammatory potential and may contribute to renal protection by regulating inflammatory cytokine production.

Silymarin administration (100 mg/kg) also decreased TNF- α levels (4.14 ± 0.17)

and demonstrated anti-inflammatory activity; however, its protective effect appeared less pronounced compared with vildagliptin treatment. The beneficial effects of silymarin may be associated with its antioxidant properties, including free radical scavenging and inhibition of inflammatory mediators.

In contrast, vildagliptin may provide nephroprotection through modulation of dipeptidyl peptidase-4 (DPP-4) activity and incretin-related pathways, resulting in reduced oxidative stress and inflammatory signaling.

These findings indicate that vildagliptin, particularly at higher doses, may represent a promising therapeutic approach for attenuating inflammatory responses in piroxicam-induced nephrotoxicity.

4.4 Histopathological Changes in Piroxicam - induced Nephrotoxicity and the Nephroprotective Effect of Vildagliptin

Kidney tissues from histopathological examination for each animal showed that kidneys from normal controls had an unaltered renal architecture with glomeruli and renal tubules that were normal. Administration of piroxicam resulted in significant renal damage characterized by tubular epithelial degeneration, glomerular shrinkage; vascular congestion; and infiltration of interstitial inflammatory cells.

Co-administration of vildagliptin at both the low and high dose levels progressively reduced histopathological changes in a dose-dependent manner, with the high dose vildagliptin group showing almost complete restoration of normal renal histology and apparent nephroprotective effects.

The standard therapy group also maintained renal morphology similar to the normal control group, i.e., the standard therapy group had similarly verified protective properties to piroxicam-induced nephrotoxicities. After staining of Kidney sections they were stained, then evaluated (H&E) at 40× magnification.

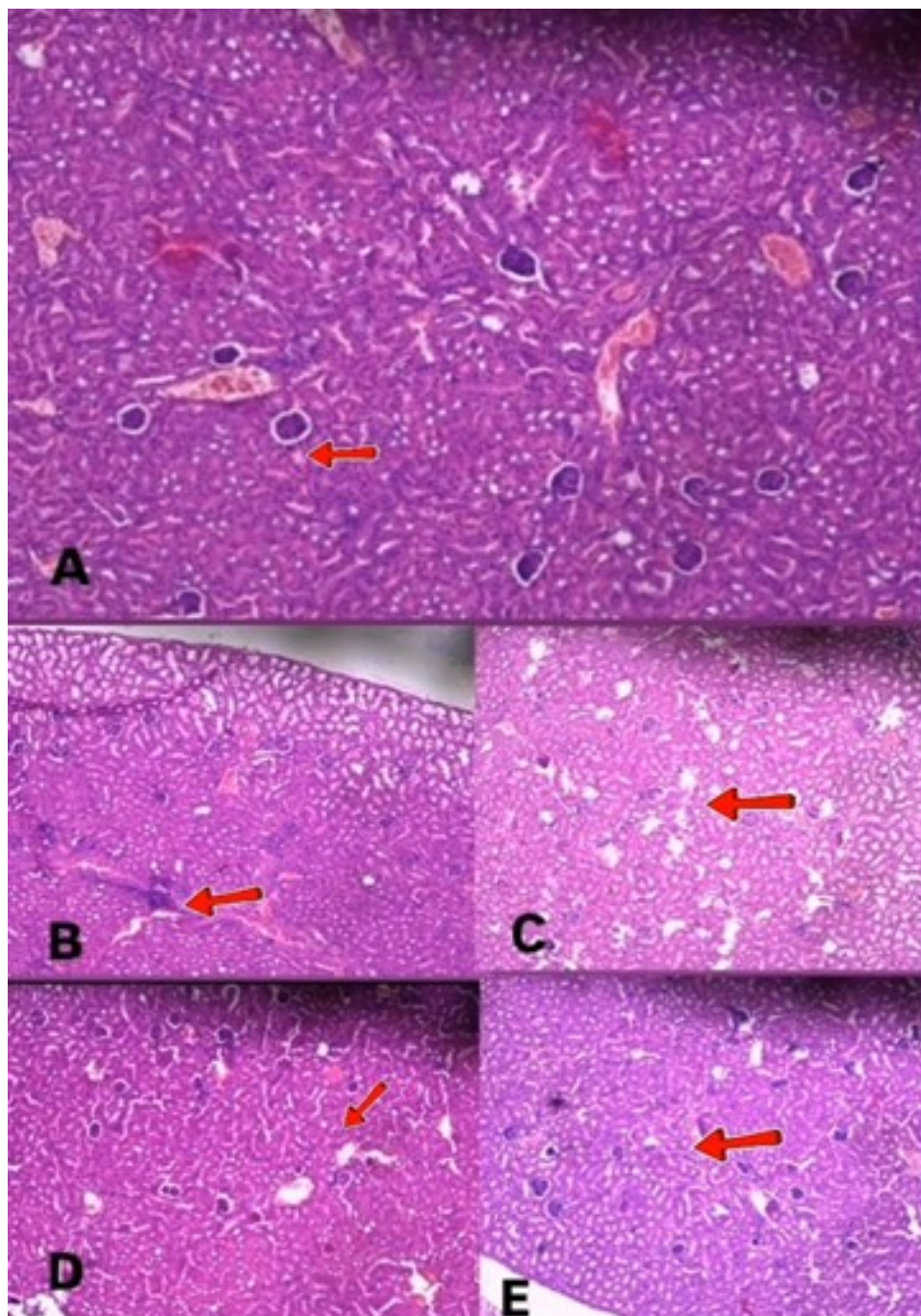


FIGURE 4.5: Hematoxylin and eosin (H&E)-stained kidney sections showing the histopathological changes induced by piroxicam and the protective effects of vildagliptin in experimental mice (40× magnification).

4.4.1 Normal Control Group

The histology of the renal cortex is normal, and the renal corpuscles and glomeruli are well defined (as shown in Fig 4.5a). The proximal and distal convoluted tubules are normal and lined with normal cuboidal epithelium with normal lumina. There

is no indication of tubular degeneration, infiltration of inflammatory cells, vascular congestion, or interstitial edema; therefore, the renal morphology is normal.

4.4.2 Piroxicam-Treated Group

The kidney sections show severe histopathological changes, including tubular epithelial degeneration, vacuolation of the cytoplasm, dilatation of some tubules, and shrinkage of the glomeruli (as shown in Fig 4.5b).

There is mild to moderate vascular congestion and interstitial inflammatory cell infiltration, confirming that piroxicam has nephrotoxic effects.

4.4.3 Piroxicam + Vildagliptin Low Dose Group

Some evidence of normal renal architecture has returned to the renal tissue compared with the piroxicam group post-treatment (as shown in Fig 4.5c). The degree of degeneration in the tubules has decreased, but there are some scattered areas of vacuolation in the epithelium, and some mild dilatation of the tubules.

Glomeruli have shown a relative preservation of structure and minimal interstitial inflammatory cells; indicating vildagliptin low dose is moderately renoprotective.

4.4.4 Piroxicam + Vildagliptin High-dose Group

Histological renal sections from the vildagliptin plus piroxicam group show an almost entirely restored histological appearance with nearly normal glomeruli and renal tubule architecture (as shown in Fig 4.5d). The sections show only minor tubule degeneration (1-2 tubules degenerating per section) and little to no vascular congestion. The amount of interstitial inflammatory disease noted in these sections is negligible at best.

Collectively, these observations demonstrate that high-dose vildagliptin had a marked reduction in piroxicam-induced renal damage.

4.4.5 Standard Treatment Group

Renal sections from the standard treatment group show nearly normal renal histology with intact glomeruli and tubule architecture (as shown in Fig 4.5e). The tubular epithelial cells are intact without significant evidence of inflammatory infiltration, tubular necrosis or vascular congestion, demonstrating that the standard treatment has nephroprotective properties.

Chapter 5

Discussion

Vildagliptin has been shown to protect against renal damage by the use of piroxicam through several protective mechanisms. Its protective effect on the kidneys with respect to piroxicam is evident from the fact that, despite the significant amount of damage caused to the kidneys by piroxicam, Vildagliptin was able to prevent this damage. Piroxicam produced significant changes to kidney structure as observed histologically, while the protective effect of Vildagliptin resulted in little to no changes in kidney structure and preserved normal renal architecture. This provides evidence that Vildagliptin has a protective effect on the kidneys from nephrotoxic injury due to the use of piroxicam, and is likely due to the ability of Vildagliptin to produce anti-oxidative, anti-inflammatory, and anti-apoptotic effects. Piroxicam is an NSAID that is widely used due to its analgesic and anti-inflammatory effects through COX inhibition, which results in the inhibition of the synthesis of prostaglandins. Chronic use of NSAIDs can lead to the development of renal toxicity, because prostaglandins are necessary for maintaining renal blood flow/renal perfusion, regulating glomerular filtration, and tubular processing. The inhibition of prostaglandin synthesis leads to reductions in renal blood flow, reduced renal perfusion, ischemic injury/ischemia, and increased oxidative stress/inflammatory responses, culminating in tubular apoptosis leading to renal failure. Some of the mechanisms described in recently published reviews regarding drug-induced nephrotoxicity support the above mechanisms as either contributing

factors to the development of renal toxicity or as consequences of chronic use of piroxicam [60].

The findings from the present study indicate that exposure to piroxicam elicits persistent degeneration of tubular epithelium, shrinkage of glomeruli, congestion of blood vessels, dilation of tubules, and infiltration of inflammatory cells into the tubules in these animals. Other experimental studies have also shown similar nephrotoxic effects for NSAIDs. Bonet-Monné *et al.* (2024) indicated that long-term use of NSAIDs caused worsening of renal function and damage to the structure of the kidneys [61]. According to Dada *et al.* (2023), although piroxicam and diclofenac are chemically different from each other, both drugs will cause nephrotoxic effects from the inhibition of prostaglandin production and oxidative injury [62].

The histological examination of renal tissue from both treatment groups indicated marked changes in renal histology. For instance, there were significant improvements with respect to the preservation of glomeruli structure, decreased tubule degeneration and improved vascular congestion relative to piroxicam-treated groups as well as a marked reduction in inflammatory infiltrates when compared with the piroxicam-treated animals. Furthermore, the high-dose group exhibited nearly complete restoration of normal renal structure, which illustrates a dose-dependent renoprotective effect of vildagliptin.

Our results are consistent with the findings of Watife *et al.* (2024), who studied the nephroprotective effects of vildagliptin in a model of cisplatin-induced nephrotoxicity. In the current study, vildagliptin significantly decreased blood urea nitrogen, creatinine and TNF- α levels and caspase-3 expression while improving histopathological changes in the kidney. In addition, administration of vildagliptin significantly reduced the number of tubular necrosis and inflammatory cells after cisplatin administration. The mechanisms by which cisplatin and piroxicam induce nephrotoxicity are different; however, both processes share a common end pathway of oxidative stress and inflammation, which likely accounts for the protection produced by vildagliptin in both sets of data [35].

Also, Honma *et al.* (2024) showed that vildagliptin reduced renal interstitial fibrosis and inflammation in mice with unilateral ureteral obstruction. Their data indicate that vildagliptin inhibits activation of fibroblasts, production of extracellular matrix and activation of inflammatory signaling pathways, leading to the preservation of renal structure. Collectively, these studies provide additional evidence that renal protection through DPP-4 inhibition occurs independently from improvements in glycemic control [63].

Oxidative stress is a major contributor to drug-related renal damage as evidenced from the results of experimental studies. Following administration of the non-steroidal anti-inflammatory drug, oxidative stress through the action of reactive oxygen species, leads to the occurrence of lipid peroxidation, mitochondrial dysfunction, DNA damage, and activation of inflammatory pathways. Furthermore, one of the consequences of oxidative stress is the activation of nuclear factor-kappa B (NF- κ B) resulting in the release of pro-inflammatory cytokines such as TNF- α , interleukin-1 β , and interleukin-6, all of which exacerbate tissue damage as well as promote apoptosis. Piko *et al.* (2023) have shown that oxidative stress is one of the primary mechanisms whereby acute and chronic kidney injury occurs. Based upon our results, it is reasonable to conclude that the reduction in histologic injury, observed following vildagliptin treatment, is a result of the inhibition of oxidative stress and restoration of endogenous antioxidant defenses [64].

Aside from diminishing oxidative stress, vildagliptin has the capacity to stop cell death from occurring in tubular epithelial cells found within the kidneys. Studies have shown that by inhibiting DPP-4, vildagliptin reduces activation of the apoptotic protein (caspase-3), and subsequently increases levels of Bcl-2 (the anti-apoptotic protein), resulting in a decrease in programmed cell death and an overall protection of renal tissue. The protection observed in tubular epithelial cells in this study supports this proposed mechanism.

Another possible reason for the nephroprotective aspect observed is via improving functionality of the endothelial cells lining blood vessels. As GLP-1 stimulates nitric oxide production, it also enhances microcirculation through renal blood vessels and reduces poor functioning of endothelial cells. Improving blood flow

in the kidneys may reduce ischemic injury due to the depletion of prostaglandins secondary to NSAIDs, thus mitigating structural damage. In support of the role of the kidneys in the mechanism of action of vildagliptin, Guo *et al.* (2022) demonstrated that renal function can also alter the pharmacokinetics (how much of a drug is in the bloodstream after taking it) of vildagliptin [65].

It is anticipated that histopathological evidence obtained from this study will correlate positively with improvement in biochemical indicators of kidney function; as multiple prior experiments using vildagliptin consistently show decrease in serum creatinine, blood urea nitrogen, and markers of oxidative stress. While histopathological analysis was the primary focus of this study, also supporting the therapeutic efficacy of vildagliptin against NSAID-related nephrotoxicity is the preservation of kidney architecture, which provides strong evidence for improved renal function.

Other than the histopathological findings the creatinine in serum is frequently used as a clinical laboratory measure of renal (kidney) function and glomerular filtration rate (GFR). In the current study, the serum creatinine level from the piroxicam-treated group was significantly higher than that of the normal control group and is an indication of diminished renal (kidney) filtration. The increase in serum creatinine could relate to both diminished renal perfusions resulting from cyclooxygenase inhibition and a decrease in the production of vasodilatory prostaglandins, resulting in the renal tissue becoming ischemic injury. Additionally, when vildagliptin was offered, there was a significant decrease in the serum creatinine level compared to that of the toxic control suggesting renal (kidney) function was improved. Measurably, these data are in agreement with previous studies that reported that vildagliptin provides a protective effect on the kidneys through anti-inflammatory, antioxidant and cytoprotective processes. This decrease in serum creatinine supports the idea that vildagliptin has maintained the structural integrity of the glomerulus and decreased the functional renal impairment associated with renal injury [66]. Watife *et al.* (2024) found that treating cisplatin-induced nephrotoxic rats with vildagliptin resulted in a significantly decreased serum creatinine level. Reduced serum creatinine indicates

improvement in the renal architecture/function as indicated by reduced oxidative stress/inflammation. Therefore, the reduction in serum creatinine seen in this study supports the protective effect/vildagliptin on functional renal impairment from piroxicam [35].

Likewise, serum levels of urea were substantially elevated with increased administration of piroxicam due to a decrease in renal clearance caused by impairment to kidney function. Serum urea is often raised in cases of acute kidney injury due to an overall reduction in glomerular filtration and additional increased levels of protein catabolism. The relative decrease in serum urea in the vildagliptin group suggests that the renal excretory function had been restored. The mechanism by which protection occurred could be related to improved renal hemodynamics, reduction of inflammation, and preservation of tubular architecture. Similar findings have been documented in studies using DPP-4 inhibitors whereby renal biochemical measurements improved following nephrotoxic agents delivered below recommended doses [67].

Inflammation is a major contributor to both the onset and progression of nephrotoxicity caused by certain drugs. One of the key cytokines associated with inflammation in the kidney is tumor necrosis factor alpha (TNF- α). Increased levels of TNF- α lead to an increase in the entry of white blood cells to the area, as well as an increase in the number of cells undergoing apoptosis, increased oxidative stress, and destruction of tissue within the kidney.

In this study, a statistically significant increase in TNF- α levels was seen following administration of the non-steroidal anti-inflammatory drug (NSAID), piroxicam, indicating that the inflammatory pathways in the kidneys were activated. TNF- α levels were also reduced following treatment with the Dipeptidyl Peptidase 4 (DPP-4) inhibitor, vildagliptin, indicating that vildagliptin has anti-inflammatory properties. This is consistent with previous studies that have shown that DPP-4 inhibitors decrease TNF- α and other inflammatory mediators by inhibiting the activation of nuclear factor kappa B (NF- κ B). Further, it was shown that endogenously released glucagon-like peptide-1 (GLP-1), due to inhibition of DPP-4,

exhibits anti-inflammatory properties and may protect renal tissue from injury due to inflammation.

Interleukin-1 beta (IL-1 β) is another important inflammatory cytokine involved in renal inflammation and tissue injury. Elevated IL-1 β contributes to activation of inflammatory cascades, infiltration of immune cells, and progression of renal fibrosis. In the present study, piroxicam significantly increased IL-1 β levels compared with the control group, confirming the involvement of inflammatory mechanisms in nephrotoxicity. Administration of vildagliptin significantly lowered IL-1 β concentrations, suggesting suppression of inflammatory responses. These findings agree with previous investigations demonstrating that vildagliptin inhibits activation of the NLRP3 inflammasome and reduces IL-1 β production, thereby limiting inflammatory damage within renal tissues. The observed reduction in IL-1 β further supports the anti-inflammatory properties of vildagliptin in experimental nephrotoxicity [68].

According to Samson *et al.*, the activated NLRP3 inflammasome promotes IL-1 β formation and increases renal inflammation; however, inhibiting it resulted in decreased inflammatory renal injury. As demonstrated by other recent reviews, targeting NLRP3-mediated IL-1 β signaling is an effective means of preventing renal injury and potentially presents a new therapeutic option. The actions of DPP-4 inhibitors are known to be anti-inflammatory, therefore, the decreased level of IL-1 β found in the present study can, at least in part, be attributed to the inhibition of NLRP3 activation by vildagliptin [69].

The renoprotective effect of vildagliptin observed in this study may be explained through multiple complementary mechanisms. Inhibition of DPP-4 increases circulating GLP-1 levels, which not only improve glucose metabolism but also exert direct protective effects on renal cells. GLP-1 signaling reduces oxidative stress, suppresses inflammatory cytokine production, improves endothelial function, and decreases apoptosis. Moreover, vildagliptin has been reported to reduce reactive oxygen species generation, inhibit NF- κ B activation, and preserve mitochondrial function. Collectively, these mechanisms contribute to the attenuation of renal inflammation and improvement of kidney function observed in the present study.

To summarize, the results of our experiment show that vildagliptin provides considerable protection to the kidneys from injury caused by piroxicam due to a number of interdependent actions. Vildagliptin can prevent loss of kidney structure as observed by histology, decrease serum creatinine and urea concentrations, and inhibit multiple inflammatory mediators such as TNF- α and IL-1 β . The actions of vildagliptin are primarily attributable to its antioxidant, anti-inflammatory, anti-apoptotic, and endothelial protective effects due to DPP-4 inhibition and GLP-1 enhancement. The kidney-protective effects of vildagliptin were observed through inhibition of oxidative stress, by inhibiting NF- κ B and by activating NLRP3 inflammasome; maintenance of mitochondrial function; and improved microcirculation to the kidneys.

The results of the current study are in agreement with findings reported in recent international studies, which suggest that vildagliptin may have beneficial effects against experimentally induced renal injury. In our study, treatment with vildagliptin was associated with better preservation of renal architecture, along with reductions in serum creatinine, urea, TNF- α , and IL-1 β following piroxicam administration. These observations are comparable with the findings of Watife *et al.* (2024), who observed that vildagliptin treatment improved renal histopathological changes and reduced serum creatinine, urea, TNF- α , and caspase-3 expression in rats with cisplatin-induced nephrotoxicity. Although the initial mechanisms of renal injury produced by cisplatin and piroxicam differ, oxidative stress, inflammatory responses, and apoptosis are common processes involved in the progression of renal damage. The comparable response to vildagliptin in these different experimental models may therefore indicate that its protective effects involve pathways that are shared across different forms of renal injury [35].

The histological findings of the present study are also comparable with those reported by Honma *et al.* (2024), who found that vildagliptin reduced renal inflammation and interstitial fibrosis in mice with unilateral ureteral obstruction. In the present study, vildagliptin similarly helped maintain the normal appearance of the glomeruli and reduced tubular degeneration, vascular congestion, and inflammatory-cell infiltration. Collectively, these findings support the possibility

that the renoprotective effects of vildagliptin extend beyond a single type of renal injury and may involve several complementary protective mechanisms [63]. Overall, these data support further investigation of vildagliptin as a potential nephroprotective agent against NSAID-associated renal injury.

5.1 Limitations

In the nephrotoxicity model generated by the administration of piroxicam, the data collected from these results showed that vildagliptin is able to provide renoprotection as well as anti-inflammatory properties; however, there are limitations in making these findings applicable to human clinical settings. The experimental model showed that by an acute administration of piroxicam, it produced renal injury and inflammatory responses; however, this model does not reflect the complexity of chronic kidney injury seen in patients over time, nor does it reflect the progressive nature of chronic kidney injury seen in patients. An example of this would be long-term NSAID-induced nephropathy. Long-term NSAID use produces chronic nephropathy through continued oxidative stress, glomerular changes, interstitial fibrosis, and the slow and progressive loss of renal function. The other area where there is a limitation in the present study is that the pharmacokinetics of vildagliptin were not examined in renal tissue. If the pharmacokinetic profile (absorption, distribution, metabolism, renal accumulation, clearance) had been completed on vildagliptin this would allow for the optimal dosing of vildagliptin in treating patients with CKD as well as assisting in the translation of the renoprotective effect of vildagliptin seen in the research study into clinical practice. Without the pharmacokinetic data on vildagliptin, it is not possible to determine the precise dose-response relationship or the therapeutic window.

There was use of histopathological assessment to establish a measure of renal architecture after vildagliptin treatment; however, the lack of advanced histopathological scoring systems limited their quantitative analyses of tubular injury, glomerular damage, interstitial inflammation and fibrosis to assess kidney protection. Evaluation of pertinent biomarkers related to oxidative stress and fibrogenesis

(e.g. malondialdehyde (MDA), reduced glutathione (GSH), super oxide dismutase (SOD), catalase (CAT), transforming growth factor- β (TGF- β) and kidney injury molecule-1 (KIM-1)), not performed, limited the understanding of potential antioxidant/antifibrotic mechanisms involved with vildagliptin's renoprotective effects. In addition, while the decreases in markers of inflammation indicate that vildagliptin modulates critical inflammatory pathways, there has been no mechanistic verification of the underlying mechanisms involved in this modulation. Without gene/protein analysis of key molecular targets (e.g. NF- κ B, TNF- α , IL-1 β , IL-6, Nrf2, HO-1 and DPP-4), there will not be further confirmation of the mechanistic role(s) of these targets in the modulation of renal protection by vildagliptin.

The precise molecular factors that mediate the renoprotective effects of Vildagliptin could not be determined with any degree of certainty. Another limitation of the study is that there were no comparisons between sexes regarding their response to treatment. The differences between male and female animals with respect to susceptibility to nephrotoxic drugs and their pharmacologic responses are likely due to the differences in hormones and physiology. Therefore, it will be important to utilize both sexes in order to increase the generality of the findings. Overall, these limitations emphasize the need for future studies using chronic models of piroxicam-induced renal injury, complete pharmacokinetic studies, and both complex histopathologic grading systems and more extensive oxidative stress, inflammatory and fibrotic biomarker panels. Also, additional molecular studies that evaluate gene and protein expression will be needed to provide a more complete understanding of how Vildagliptin confers renoprotection. These studies will help to further clarify the therapeutic potential of Vildagliptin and therefore aid in its potential use clinically for the prevention of and/or treatment of NSAID-induced nephrotoxicity.

Chapter 6

Conclusion And Future Perspectives

6.1 Conclusion

Vildagliptin, a Dipeptidyl Peptidase-4 (DPP-4) Inhibitor, was selected for evaluation as a potential nephroprotective and anti-inflammatory agent through the use of an established model of Piroxicam nephrotoxicity. Piroxicam is an NSAID that is commonly used to relieve pain and reduce inflammation, but its long-term use can cause damage to the kidneys due to oxidative stress, decreased blood flow to the kidneys, damage to the tubules, and ultimately inflammation in the kidneys. The present study will investigate whether the ability of vildagliptin to act as an anti-inflammatory, antioxidant, and cytoprotective compound will attenuate the renal injury that occurs after administration of Piroxicam and establish the potential use of using vildagliptin to treat drug-induced renal injury. The study provided compelling experimental data that support the use of vildagliptin as a nephroprotective agent against piroxicam-induced renal toxicity. Vildagliptin was shown to protect against renal toxicity, as determined by reductions in serum creatinine levels and urine protein. In contrast, serum creatinine levels and urine had high levels of protein when animals were treated only with piroxicam. When

animals were treated with both vildagliptin and piroxicam, serum creatinine levels returned to normal, but urinary protein concentrations increased slightly. Administration of vildagliptin did not prevent significant renal toxicity, as indicated by the presence of histopathological abnormalities in renal tissues, use of the nephrotoxin piroxicam.

This data supports the hypothesis that vildagliptin has a renoprotective effect via a dose-dependent manner where decreasing doses of vildagliptin result in progressively less protection against renal damage induced by piroxicam. Plasma creatinine concentrations were significantly lower in the vildagliptin treated compared with the piroxicam control.

Additionally, the urine protein concentrations were lower in the vildagliptin-treated than in the control groups. The renal protective effect of vildagliptin was less from the lower than the higher dose, but both doses provided some degree of protection against renal impairment induced by piroxicam.

The notable drop in TNF- α levels after administering vildagliptin is of particular interest since TNF- α plays a key role in the mechanism for drug-related damage to the kidneys. With vildagliptin treatment there was suppression of TNF- α production which may have disrupted downstream inflammatory signaling pathways, including NF- κ B activation, therefore limiting tubular cell death, inflammation of cells and the progressive damage that occurs to kidney tissues. These results are consistent with previous investigations indicating that the mechanism of action of DPP-4 inhibitors is anti-inflammatory and renoprotective, as both protect the kidneys through regulating cytokines and reducing oxidative stress. The reversal of renal protein homeostasis seen in the vildagliptin-treated group supports its protective function against cellular damage. The use of vildagliptin compared with the reference standard of care showed superior improvement in biochemical parameters and inflammatory parameters indicating that vildagliptin has a much broader mechanism of nephroprotection than conventional nephroprotective modalities. The ability of vildagliptin to modulate inflammatory mediators while at the same time maintain kidney structure may

have also contributed to its favorable efficacy in models of nephrotoxicity. Findings from this study further support that targeting inflammatory and oxidative pathways represents an excellent approach for management of piroxicam-induced renal injury.

Vildagliptin was shown to be effective in mitigating nephrotoxicity due to the results of the histopathology analysis of the kidney under a microscope with kidney tissue stains utilizing the hematoxylin-eosin (H&E) method. Kidney slides from the piroxicam-treated group exhibited prominent damage on histopathology consist of tubular degeneration, glomerular congestion, infiltration of inflammatory cells, interstitial edema, and necrosis. These findings were consistent with the findings seen in patients with NSAID-induced nephrotoxicity.

The administration of vildagliptin to these animals restored the structural architecture of the kidney, restored the structural integrity of the renal tubules, reduced the number of inflammatory cells present, and improved glomerular structure. Of all of the vildagliptin doses administered, the group that received the highest (20 mg/kg) of vildagliptin had the most amount of histological improvement, indicating greater nephroprotective activity.

In summary, data from this study demonstrated that vildagliptin protects experimental animals from nephrotoxicity induced by piroxicam. Vildagliptin treatment prevented the increases in serum creatinine, blood urea nitrogen, uric acid and tumor necrosis factor-alpha associated with piroxicam treatment and promoted the restoration of renal protein balance; in addition, vildagliptin preserved the architecture of the kidneys. The group that received the highest dose of vildagliptin had significantly better results than the lower dose treatment group, which indicated that vildagliptin has dose-dependent renoprotection.

The results provide solid evidence from experimental work to support that vildagliptin may be an important therapeutic agent for the treatment of nephrotoxicity due to NSAIDs. Additional studies with detailed molecular methods as well as clinical studies in humans are needed to determine the ability of vildagliptin to treat nephrotoxicity in humans.

6.1.1 Future Perspective

The results from this study suggest that vildagliptin, a selective DPP-4 inhibitor, greatly reduced renal damage due to piroxicam and significantly improved both biochemical and histopathological measures of nephrotoxicity. The large reduction in parameters including serum creatinine, serum urea, inflammatory markers, and histopathological changes suggests that vildagliptin provides a protective effect on the kidney. The data generated from this project will be used as an initial foundation for subsequent pharmacologic studies of vildagliptin. Future studies should examine dose-response strategies and pharmacological analyses of vildagliptin over time in larger animal models in order to optimize the dose and determine the efficacy of vildagliptin.

Detailed characterization of vildagliptin's renoprotective mechanism can be performed using advanced bioanalytical methods, such as LC-MS/MS, to define the concentration-time curves of vildagliptin and its metabolites in both systemic circulation and renal tissues. Because of the significant anti-inflammatory and antioxidant properties observed with vildagliptin, future studies should also examine molecular mechanisms beyond DPP-4 inhibition, including pathways involved in oxidative stress, apoptosis, and inflammation. Specific molecular targets for study should include NF- κ B, Nrf2/HO-1, TGF- β , and inflammasome pathways shown to be involved in NSAID-induced nephrotoxicity. Additionally, gene expression profiling, transcriptomics, proteomics, and metabolomics may reveal additional downstream molecular networks regulated by vildagliptin.

Future computational studies that use molecular dynamics simulations and binding free energy calculations could yield further evidence of the potency of constituents found within. These studies, including SBDD (structure-based drug design), can be utilized for the development of vildagliptin analogs that possess greater renal selectivity, better PK properties and less off-target activity. The application of high-throughput virtual screening and lead optimization may lead to the identification of new classes of renoprotective agents with enhanced efficacy and safety profiles.

This current study used an acute nephrotoxicity model utilizing piroxicam to induce nephrotoxicity; nevertheless, the vast majority of humans presenting for clinical care due to NSAID-related renal injury present with renal failure due to cumulative chronic kidney disease from chronic NSAID use. Future studies should, therefore, evaluate chronic nephrotoxicity models lasting from 4 - 12 weeks to provide a better approximation of the renal pathophysiology of humans. Future longitudinal studies should evaluate the sustained renoprotective effects of vildagliptin, and should further investigate the ability of vildagliptin to prevent the progression of AKI to CKD.

As a result of the large nephroprotective effects seen, recommendations will need to be developed for conducting combination therapy studies with vildagliptin and established nephroprotective agents that include N-acetylcysteine, Quercetin, Curcumin or Alpha-Lipoic Acid in order to assess their potential synergistic benefits through the simultaneous modulation of oxidative stress, inflammation, mitochondrial dysfunction and apoptotic pathway. This may be further defined by molecular studies examining caspase-3, Bax and Bcl-2, cytochrome c, and mitochondrial membrane potential, to provide further data on the anti-apoptotic mechanism involved in the vildagliptin-mediated renoprotection.

Extended duration studies would also provide a comprehensive histopathological assessment of renal architecture by evaluating glomerular injury; tubular degeneration; interstitial inflammatory changes; fibrosis; and alterations in vascular integrity. A quantitative assessment of renal fibrosis should be performed using a fibrosis scoring system to determine if vildagliptin has an antifibrotic effect using immunohistochemical analysis of markers of fibrosis as indicated by α -SMA, collagen I, collagen III, fibronectin, and TGF- β . These studies may assist in validating vildagliptin's future use in the treatment of chronic kidney disease or drug induced nephrotoxicities.

Piroxicam has been shown to induce nephrotoxicity by means of excessive ROS generation, lipid peroxidation, mitochondrial dysfunction, and ultimately, DNA damage to the kidney. Future studies should assess vildagliptin's effects on oxidative stress biomarkers, including MDA, GSH, SOD, CAT, GPx, and metabolites of

NO. In addition, combining vildagliptin with potent antioxidants may further enhance its efficacy in providing renoprotection by targeting anti-inflammatory and oxidative mechanisms of injury at the same time. Additionally, novel drug delivery systems could provide another opportunity for enhancing therapeutic effects (i.e., using targeted nanoparticles, such as liposomes, polymeric nanoparticles, solid lipid nanoparticles, and biodegradable nanocomposites) to improve renal targeting, increase bioavailability, limit systemic toxicity, and obtain sustained drug release. Nanoparticles that have been modified with ligands capable of selective renal accumulation may improve therapeutic efficacy and decrease effects outside the target organ as well. Patient studies that have been done to evaluate how vildagliptin protects patients from possible kidney (nephrotoxicity) damage caused by taking NSAIDs could be conducted in a more controlled setting (e.g., Clinical Trials) to assess whether or not the drug can provide protection against this type of injury.

The first phase of these studies would likely consist of patients that either have mild-to-moderate kidney function impairment or those that require prolonged treatment with an NSAID, and they would look at specific clinical end points such as: maintaining (or even improving) their predicted (or estimated) Glomerular Filtration Rate (eGFR), decreasing Serum Creatinine, decreasing the amount of protein in their urine (Albuminuria), decreasing levels of inflammatory markers, improving results of any renal imaging studies, and providing an improved quality of life for the patient. Future Phase I/II Clinical Trials will also include important safety data, tolerability data, pharmacokinetics data, and preliminary efficacy data. This present study indicates that vildagliptin protects the kidney from damage caused by NSAID use better than piroxicam (an NSAID) does. Future studies will need to compare vildagliptin's effectiveness to other DPP-4 inhibitors (sitagliptin, saxagliptin, linagliptin), as well as established nephroprotective agents such as N-acetylcysteine, losartan, empagliflozin, and curcumin. The results of these types of studies will allow researchers to determine which therapies are the most effective overall.

To conclude, long-term assessments of potential safety are still necessary and will

need to be part of future studies aimed at determining the long-term adverse effects associated with the use of vildagliptin such as those that may occur in the metabolic, hepatic, cardiovascular and renal systems from prolonged administration. Therefore, future research studies will need to assess and evaluate specific organ toxicity, as well as measure the metabolic burden of systemic oxidative injury, the electrical balance of electrolytes, and hemodynamic measures to achieve the most favorable balance between nephroprotection and systemic safety. Additionally, pharmacogenetic studies may aid in understanding the variability in metabolism, responses to therapy and susceptibility to adverse drug reactions, which could ultimately lead to the development of anti-vildagliptin therapy on a patient-specific basis for preventing and managing nephrotoxicity as a result of NSAID use.

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