SUBACUTE TOXICITY STUDIES OF MAX GLP-1 ON KIDNEY FUNCTION

HEMATOLOGICAL AND GLUCOSE LEVELS

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BENIN CITY

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MEDICAL BIOCHEMISTRY, SCHOOL OF BASIC MEDICAL SCIENCES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE, B.S.c. (HONS) MEDICAL BIOCHEMISTRY OF THE UNIVERSITY OF BENIN, BENIN CITY

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CERTIFICATION

This is to certify that this undergraduate project was carried out by DANIEL OMOKAGBHO RUFUS with Matriculation Number **BMS2101461** in the Department of **Medical Biochemistry, School of Basic Medical Sciences, University of Benin, Benin city,** in partial fulfillment of the requirements for the award of Bachelor of Science (BSc) degree in Medical Biochemistry.

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(Project supervisor)

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(Head of Department)

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EXTERNAL EXAMINER

DEDICATION

This work is humbly and lovingly dedicated to **God Almighty,** for the gift of life, wisdom, and strength to complete this academic journey, to my beloved **Mother Mrs. Rufus Gladys** whose unwavering love, prayers, and immense financial sacrifice laid the foundation for my success and to my siblings for their support and encouragement.

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) is an incretin hormone widely recognized for its role in enhancing insulin secretion and improving glycemic control. Beyond its antidiabetic effects, emerging evidence suggests that GLP-1 may influence renal physiology, hematological and glucose levels. This study investigates the subacute toxicity effects of MaxGLP-1 a novel analogue of GLP-1 administration on the kidney, hematological and blood glucose levels in experimental models over a 28 day period. A total of 20 rats were divided into four groups and were fed and given water daily. Group 1 was the control and was exposed to standard feeding and water only, Group 2 was administered 10mg/kg of MaxGLP-1, Group 3 was administered 60mg/kg MaxGLP-1 drug while Group 3 was administered 600mg/kg doses of MaxGLP-1. At the end of the study, animals were sacrificed, the kidneys were harvested and taken to the laboratory to be examined, blood samples were also collected and centrifuged to obtain the serum and were subjected to biochemical assays.

Findings showed dose-related changes in serum creatinine and urea, indicating possible renal stress. Haematological analysis revealed mild but notable shifts in erythrocyte and leukocyte indices, while glucose levels decreased significantly across treatment groups. Overall, Max GLP-1 exhibited hypoglycaemic effects with minimal haematological disturbances, though higher doses suggested early signs of renal compromise. These results highlight the need for cautious dose optimization and further investigation into long-term safety.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by intestinal L-cells following nutrient intake. It plays a critical role in glucose homeostasis by stimulating glucose-dependent insulin secretion, inhibiting glucagon release, slowing gastric emptying, and enhancing satiety (Drucker, 2018). Due to these effects, GLP-1 receptor agonists (GLP-1RAs) such as liraglutide, semaglutide, and exenatide have become cornerstone therapies for type 2 diabetes mellitus and obesity (Davies *et al., 2021*).

The global prevalence of Type 2 Diabetes Mellitus and obesity necessitates continuous development of safe and effective pharmacological agents (Williams *et al., 2024*). A key therapeutic area is the incretin system, specifically targeting the Glucagon-like Peptide-1 (GLP-1) pathway.

The therapeutic success of native GLP-1 is limited by its rapid degradation by the enzyme Dipeptidyl Peptidase-4 (DPP-4). This led to the development of GLP-1 Receptor Agonists (GLP-1 RAs), which are modified analogues designed for enhanced stability and extended duration of action (Giugliano *et al., 2019*). MAX GLP-1 represents a novel formulation or analogue within this class, requiring rigorous preclinical evaluation.

According to international regulatory standards, such as the OECD guidelines, any novel

pharmaceutical must undergo comprehensive subacute toxicity studies (typically 28 days) to characterize its safety profile, identify target organs of toxicity, and establish the No-Observed-Adverse-Effect Level (NOAEL) (OECD, 2018)

Since GLP-1 directly affects glucose metabolism, monitoring fasting blood glucose levels serves as a marker of therapeutic efficacy and glucose regulatory stability.Subacute toxicity studies, typically spanning 28 days, provide essential insight into biochemical and physiological responses to repeated exposure (OECD, 2018). Such studies are crucial for establishing safety margins for therapeutic agents administered chronically. Therefore, this study investigates the subacute toxicity of GLP-1 on kidney function, hematological indices, and blood glucose levels in an animal model to provide insight into systemic safety and potential adverse effects associated with prolonged MAX GLP-1 use.

1.2 Statement of problem

Although licensed GLP-1 RAs are considered generally safe, their safety profiles vary by compound, dose and duration; some analogues have been associated with organ-specific findings in long-term toxicology. The absence of published toxicological information for MaxGLP-1 means that its safety in terms of renal and haematological effects and glucose homeostasis remains unknown. Without this data, moving toward longer-term chronic toxicity studies or human trials would lack an important safety foundation.

1.3 Justification of study

Conducting a 28-day subacute toxicity study of MaxGLP-1 is important because:

It will supply foundational safety data on kidney function markers (e.g., serum creatinine, BUN, electrolytes) and haematological parameters (e.g., RBC, WBC, Hb) under repeated exposure. It will also evaluate any unintended effects on glucose levels and metabolic regulation in non-disease male Wistar rats, which is relevant given GLP-1's glucose-modulating actions.

Another importance is that it will be useful in identifying early signs of target-organ toxicity (especially kidney and haematopoietic system) and guide dose-selection, risk assessment, and subsequent chronic or reproductive toxicology studies.

Lastly, it will also add to the body of knowledge on GLP-1 receptor agonists and their preclinical safety profiles, particularly for newer analogues such as MaxGLP-1

1.4 Research Questions

This study is guided by the following research questions:

i Does repeated administration of MaxGLP-1 affect renal function in male Wistar rats?

ii Does MaxGLP-1 alter haematological indices after 28 days of exposure?

III What is the effect of MaxGLP-1 on glucose levels in rats

1.5 Aim of study

The main aim of this study is to evaluate the subacute (28-day) toxicity profile of MaxGLP-1, a

novel glucagon-like peptide-1 (GLP-1) analogue, in male Wistar rats, with particular emphasis on its effects on kidney function, haematological parameters, and blood glucose levels

1.6 Objectives

To assess the subacute (28-day) toxicity effects of MaxGLP-1 on renal function, haematologicals, and glucose levels in male Wistar rats.

1.6.1 Specific Objectives

1. To assess the effect of 28-day MaxGLP-1 administration on renal function by measuring serum creatinine, urea, and electrolytes in male Wistar rats.
2. To evaluate the impact of MaxGLP-1 on haematological parameters, including RBC count, WBC count, haemoglobin, haematocrit, and platelet count.
3. To determine the effect of MaxGLP-1 on blood glucose levels during the 28-day subacute exposure.
4. To investigate whether changes in renal function, haematological indices, and blood glucose levels are dose-dependent.
5. To monitor general health, body weight, and behavior of rats throughout the 28-day period to identify any signs of toxicity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Incretin Hormones

Incretin hormones are a class of gastrointestinal peptides released from enteroendocrine cells in the gut in response to nutrient ingestion. Their primary function is to enhance or "potentiate" the body's glucose-dependent insulin secretion, a phenomenon critical for maintaining blood glucose homeostasis known as the incretin effect (Drucker, 2018).

The incretin effect explains why oral ingestion of glucose results in a significantly greater insulin response compared to an equivalent amount of glucose administered intravenously (IV) (Giugliano *et al.,* 2019). This difference is due to the rapid release of incretins from the gut as food passes through the digestive tract, signaling the pancreas to prepare for the glucose ingested.

Key Incretin Hormones

The two most physiologically relevant incretin hormones in humans are:

1. **Glucagon-like Peptide-1 (GLP-1)**

Source: It is usually released from L-cells found mainly in the distal ileum and colon (Drucker, 2018).

Action: Potently stimulates insulin secretion, inhibits glucagon release from alpha-cells, slows gastric emptying, and promotes satiety. GLP-1 also exhibits pleiotropic effects, including

potential cardiovascular and renoprotection (Kristensen *et al*., 2019).

2. Glucose-dependent Insulinotropic Polypeptide (GIP):

Source: Primarily released from K-cells located in the proximal small intestine (duodenum and jejunum) (Drucker, 2018).

Action: Strongly stimulates insulin secretion, but its effects on glucagon and gastric emptying are less pronounced than those of GLP-1.

Inactivation Mechanism

Both GLP-1 and GIP are rapidly degraded by the enzyme Dipeptidyl Peptidase-4 (DPP-4}), giving them a very short half-life (usually about 1–2 minutes). This rapid inactivation necessitates the development of specialized drug therapies to sustain their benefits (Drucker, 2018).

2.1.1 Incretins in Type 2 Diabetes Mellitus(T2DM)

In patients with T2DM, the incretin effect is significantly diminished. While GIP levels are often preserved, its ability to stimulate insulin release is severely impaired (GIP resistance). Conversely, the secretion of GLP-1 itself is often reduced (Giugliano *et al.,* 2019). This deficiency and resistance make the incretin system a prime pharmacological target for treating T2DM.

The mechanism of action for Glucagon-like Peptide-1 Receptor Agonists (GLP-1 RAs) involves

mimicking the effects of the natural incretin hormone, GLP-1, but with enhanced pharmacological properties to ensure prolonged stability and duration of action (NCBI, 2024).

1. **Core Mechanism:** Prolonged Receptor Activation GLP-1 RAs like semaglutide and liraglutide are synthetic peptide analogues that are chemically modified to resist rapid degradation by the Dipeptidyl Peptidase-4 (DPP-4) enzyme (Williams *et al.,* 2024).

- **Binding and Activation:** The drugs bind directly to and activate the ubiquitous GLP-1 receptor. This receptor is widely expressed, most significantly on pancreatic cells, neurons in the brain, and cells lining the gastrointestinal tract (Kristensen *et al.,* 2023).
- Signaling: Receptor activation triggers intracellular signaling pathways, primarily increasing cyclic adenosine monophosphate (cAMP) within the target cells, which mediates the downstream biological effects (Frontiers, 2022).

2. Pancreatic Actions: Glucose Homeostasis Control

The most critical and classic actions occur in the pancreas, leading to improved glycemic control:

- **Glucose-Dependent Insulin Secretion:** GLP-1RAs potently stimulate the release of insulin from pancreatic beta cells. This effect is crucially glucose-dependent: the drug's influence significantly diminishes as blood glucose levels approach the normal range (euglycemia), providing a built-in safeguard against severe hypoglycemia (NCBI, 2024).
- **Glucagon Suppression:** GLP-1 RAs suppress the secretion of glucagon from pancreatic

alpha cells. Since glucagon raises blood sugar by promoting hepatic glucose production, its inhibition is vital for achieving lower plasma glucose levels (Williams *et al.,* 2024).

3. Extra-Pancreatic and Pleiotropic Effects:

GLP-1 RAs exert powerful effects on non-pancreatic systems, which underpin their success in treating obesity and preventing cardiovascular complications:

Satiety and Appetite Regulation: The drug acts on GLP-1 receptors in the brain, particularly within the hypothalamus. This action increases feelings of satiety (fullness) and reduces hunger, leading to decreased calorie intake and sustained weight loss (Kristensen et al., 2023).

- **Slowing Gastric Emptying:** GLP-1 RAs slow the rate at which food leaves the stomach. This mechanism not only enhances satiety but also ensures a slower, more gradual absorption of nutrients and glucose into the bloodstream, minimizing postprandial glucose spikes (Frontiers, 2022).
- **Cardio-Renal Protection:** Independent of glucose and weight loss, GLP-1 RAs directly modulate cardiovascular risk. They improve vascular endothelial function, reduce inflammation, and possess renoprotective effects, evidenced by decreased albuminuria and reduced risk of major adverse cardiovascular events in high-risk patients (Williams *et al.,* 2024).

2.1.2 Therapeutic Exploitation and Drug Classes

The short half-life of natural incretins led to the development of two major drug classes that revolutionized T2DM treatment:

1. GLP-1Receptor Agonists (GLP-1RAs)

These drugs are analogues of GLP-1 that are chemically modified to resist degradation by DPP-4. This modification allows for longer-lasting effects, enabling once-daily or even once-weekly dosing (Williams *et al.,* 2024).

Mechanism: Directly bind to and activate the GLP-1 receptor.

Benefits: Excellent glycemic control, significant weight loss (due to central satiety effects), and confirmed reduction in major adverse cardiovascular events and kidney disease progression (Kristensen *et al.,* 2019).

2. **DPP-4 Inhibitors (Gliptins)**

These agents block the activity of the DPP-4 enzyme.

Mechanism: By inhibiting DPP-4, they prevent the breakdown of endogenous GLP-1 and GIP, thereby increasing the circulating levels and half-life of the naturally secreted incretins.

Benefits: Effective for glycemic control with a low risk of hypoglycemia, but they are generally weight-neutral and have a less pronounced effect on cardiovascular outcomes compared to GLP-1RAs.

2.1.3 Pharmacology of GLP-1

The therapeutic interest in the incretin axis stems from the potent actions of GLP-1, secreted by

intestinal L-cells. GLP-1 stimulates glucose-dependent insulin secretion, inhibits glucagon release, and reduces appetite and gastric motility (Drucker, 2018). The development of MAX GLP-1 as a novel GLP-1 RA aims to capitalize on these benefits while overcoming the rapid degradation of native GLP-1 by the DPP-4 enzyme.

Development of GLP-1 Receptor Agonists (GLP-1 RAs)

Native GLP-1 has a biological half-life of only 1–2 minutes, primarily due to rapid degradation by the Dipeptidyl Peptidase-4 (DPP-4) enzyme. The development of GLP-1 RAs, such as liraglutide and semaglutide, involved structural modifications (e.g., fatty acid acylation or substitution of amino acids) to enhance resistance to DPP-4 and prolong plasma half-life, allowing for once-daily or once-weekly dosing. The success of this class is evidenced by its expanded use from T2DM treatment to include cardiovascular risk reduction and chronic weight management (Williams *et al.,* 2024). MAX GLP-1, as a novel analogue, must demonstrate comparable safety and efficacy profiles.

2.1.4 The Regulatory Mandate for Novel Agents

Before a novel agent can proceed to human trials, its safety profile should be rigorously established according to international guidelines (OECD, 2018). The subacute toxicity study (28-day repeated dosing) is the standard method for identifying target organ toxicity and determining the No-Observed-Adverse-Effect Level (NOAEL). This process is essential to ensure that any

structural modifications in MAX GLP-1 have not introduced unanticipated toxicological risks compared to established GLP-1RAs (Gad et al., 2021). The male Wistar rat model is used due to its established biological and toxicological database, allowing for reliable and comparative safety assessment of MAX GLP-1.

2.2 SUPPLEMENT OF GLP-1 BOOSTER

2.2.1 Max GLP-1 as a supplement GLP-1 booster

Max GLP-1 is a nutritional supplement consisting of both oral capsules and a transdermal patch, marketed as a "natural GLP-1 booster." The product asserts that it supports appetite regulation, metabolic balance, and glucose homeostasis by enhancing the body's endogenous production or responsiveness to the incretin hormone GLP-1 (glucagon-like peptide-1). Unlike prescription GLP-1 receptor agonist drugs (such as semaglutide or liraglutide), Max GLP-1 does not contain synthetic GLP-1 agonist molecules. Rather, it relies on selected botanical, mineral and nutrient-based ingredients purported to modulate GLP-1 secretion, insulin sensitivity, and metabolic markers.

The supplement is thus positioned as a support for weight management, metabolic health and glycaemic control rather than a pharmaceutical treatment for diabetes. However, a key limitation is that there is currently no publicly available, peer-reviewed toxicology or long-term clinical safety study specifically on Max GLP-1 itself, which raises important scientific and regulatory questions about its efficacy and safety.

Figure 1.0 MaxGLP-1 marketed container sample

Source: *MaxGLP-1. com*

Constituents of Max GLP-1 (Active Ingredients)

1. Capsule Formula

According to the manufacturer

- Eriomin® – a standardized lemon bioflavonoid complex (eriocitrin, hesperidin, diosmin) suggested in some studies to increase GLP-1 secretion, reduce inflammation, and support healthy glucose metabolism.

- Chromium Picolinate – an essential trace mineral complex that may enhance insulin sensitivity and help regulate carbohydrate cravings.

- PoZibio® – a patented postbiotic compound designed to promote gut microbiome balance and intestinal GLP-1 production.

- ReDaxin™ – a red sorghum extract rich in anthocyanins and polyphenols with antioxidant and anti-inflammatory properties.

2. **Transdermal Patch Formula**

The patch is marketed as a companion delivery system and includes:

- L-Carnitine – involved in fatty-acid metabolism and energy production.

- Alpha-Lipoic Acid (ALA) – an antioxidant that supports glucose utilization and mitochondrial function.

- Ashwagandha Extract (Withania somnifera) – an adaptogenic herb proposed to modulate stress responses and metabolic rate.

- Decaffeinated Green Tea Extract – a source of catechins that may support thermogenesis and fat oxidation.

- Quercetin – a polyphenolic flavonoid with anti-oxidant and anti-inflammatory potential.

- Vitamin B12 and Vitamin B6 – water-soluble vitamins critical for energy metabolism and nervous system health.

2.2.2 Proposed Mechanism of Action

Max GLP-1 claims to act through nutritional support of the gut–pancreas axis by enhancing endogenous GLP-1 secretion and promoting satiety signals.

- Eriomin and PoZibio are marketed as stimulating L-cells in the intestine to secrete GLP-1, which may improve insulin secretion and slow gastric emptying.
- Chromium picolinate may reduce appetite by stabilizing post-meal glucose and insulin responses.

- Polyphenols (from ReDaxin and quercetin) and ALA may protect pancreatic β-cells and improve oxidative balance.

Patch ingredients may further support metabolism and mood during caloric restriction

Purported Benefits

According to product literature and marketing materials:

- Appetite suppression and reduced sugar cravings
- Enhanced satiety (feeling of fullness)
- Improved postprandial glucose stability
- Weight management support (fat reduction while maintaining lean mass)
- Increased daily energy and mental focus
- Support for healthy insulin sensitivity

Common Side Effects

The most common side effects are gastrointestinal (GI)-related, often mild-to-moderate and tend to lessen over time as the body adjusts:

- Nausea
- Vomiting
- Diarrhea or constipation

2.2.3 Safety and Scientific Evidence

- As of 2025, there are no peer-reviewed toxicological or clinical studies published specifically on Max GLP-1 capsules or patches.
- The individual ingredients (e.g., Eriomin®, chromium picolinate) have been studied in isolation, with some evidence for modest improvements in glucose control and oxidative balance (Silva et al., 2020; Pimenta et al., 2019).
- The supplement is not approved by the U.S. FDA or the European EMA for the treatment or prevention of any disease.
- Potential side effects are unknown; users with kidney disease, diabetes, or haematological conditions should exercise caution and consult healthcare professionals before use.

2.3 MAXGLP-1 AND NEPHROTOXICITY

2.3.1 The Kidney

The kidney is a vital organ in the human body responsible for maintaining homeostasis by regulating fluid balance, electrolyte levels, and the removal of metabolic wastes. Humans typically have two kidneys located retroperitoneally on either side of the vertebral column, at the level of the twelfth thoracic to the third lumbar vertebrae. Each kidney plays a crucial role in the body's excretory and endocrine systems (Guyton & Hall, 2020).

Functions of the Kidney

1. **Excretion of Metabolic Waste**

The kidney removes waste substances formed during metabolism. These include:

- Urea, produced from protein metabolism.
- Creatinine, from muscle metabolism.
- Uric acid, from nucleic acid breakdown.
- Ammonia and other toxins.

These waste products are filtered from the blood in the glomeruli and excreted through urine

2. **Regulation of Fluid and Electrolyte Balance**

The kidney controls the body's water and ion balance by adjusting reabsorption and secretion processes within the nephrons. It conserves water during dehydration and excretes excess during overhydration.

It regulates electrolytes such as sodium (Na^+), potassium (K^+), chloride (Cl^-), calcium (Ca^{2+}), and phosphate (PO_4^{3-}).

This balance is vital for normal cell function, nerve conduction, and muscle contraction (Jha *et al.,* 2020).

3. Regulation of Acid–Base Balance

The kidney helps maintain a normal blood pH (7.35–7.45) by:

Reabsorbing bicarbonate (HCO_3^-) and

Excreting hydrogen ions (H^+).

This function complements the respiratory system's regulation of carbon dioxide and ensures stable pH for enzyme and metabolic activity (Levey et al., 2020).

4. Detoxification and Drug Excretion

The kidney plays a role in detoxifying harmful substances, including drugs, environmental toxins, and metabolic byproducts. It helps eliminate these through filtration and secretion, preventing toxic accumulation in the blood (Jha *et al.,* 2020).

5. **Endocrine Functions**

The kidney functions as an endocrine organ by producing and secreting several hormones:

- Erythropoietin: Stimulates red blood cell production in the bone marrow in response to low oxygen levels (hypoxia).
- Renin: Regulates blood pressure and electrolyte balance.
- Calcitriol (active vitamin D): Promotes calcium and phosphate absorption in the intestines, maintaining bone health (Levey *et al.,* 2020).

6. **Regulation of Blood Pressure**

The kidneys regulate blood pressure through the renin–angiotensin–aldosterone system (RAAS). When blood pressure drops, juxtaglomerular cells in the kidney release renin. Renin converts angiotensinogen to angiotensin I, which is then converted to angiotensin II, a potent vasoconstrictor. Angiotensin II also stimulates aldosterone secretion from the adrenal cortex, leading to sodium and water retention, which increases blood volume and pressure (Kovesdy & Kalantar-Zadeh, 2017).

7. **Glucose Homeostasis**

Although the liver is the primary site of gluconeogenesis, the kidney also contributes by synthesizing glucose from non-carbohydrate sources during fasting or starvation. It also reabsorbs filtered glucose in the proximal tubule to prevent glucose loss in urine (Guyton & Hall,

2020).

Clinical Importance

Kidney dysfunction can result in conditions such as acute kidney injury (AKI) or chronic kidney disease (CKD), leading to impaired waste elimination, electrolyte imbalance, and systemic complications. Early detection and management are essential to prevent progression to end-stage renal disease (ESRD), which often requires dialysis or transplantation (Levey *et al*., 2020).

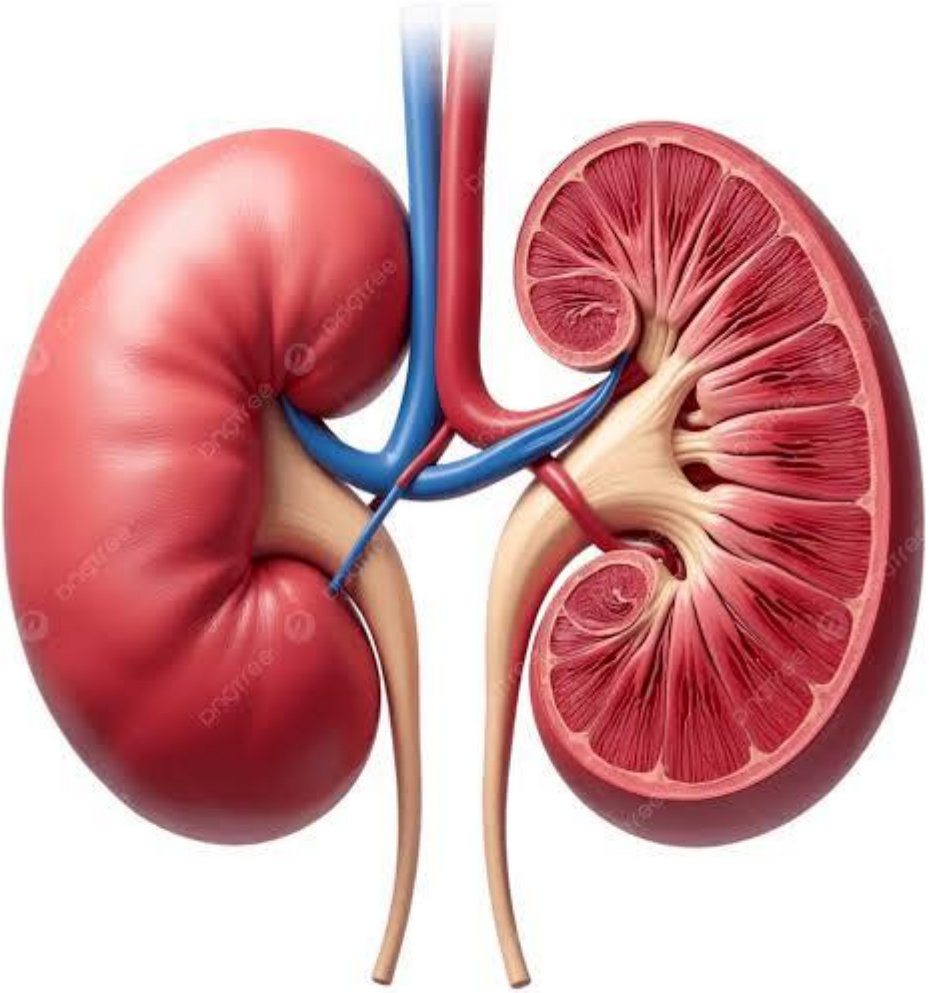

Figure 2 Structure of the kidneys

Source: Google images

2.3.1 Nephrotoxicity

Nephrotoxicity refers to structural or functional kidney damage resulting from exposure to harmful substances such as drugs, contrast media, heavy metals, or herbal compounds. It commonly manifests as acute kidney injury (AKI), chronic renal impairment, or tubular dysfunction depending on the toxic agent and duration of exposure (Perazella, 2022).

Major Causes

Common nephrotoxic agents include aminoglycosides, vancomycin, amphotericin B, cisplatin, NSAIDs, calcineurin inhibitors, radiographic contrast media, and certain chemotherapeutic agents (Campbell, 2023). These agents induce various patterns of injury such as acute tubular necrosis, acute interstitial nephritis, hemodynamic alterations, and obstructive crystal nephropathy (Connor, 2024).

Pathophysiology

The mechanisms of nephrotoxicity vary but often involve direct tubular epithelial cell injury, mitochondrial dysfunction, oxidative stress, inflammatory responses, and intrarenal vasoconstriction (Connor, 2024). Chemotherapeutic agents like cisplatin particularly cause oxidative stress–mediated apoptosis and mitochondrial impairment in proximal tubular cells (Perazella, 2022).

Prevention and Management

Prevention strategies include dose adjustment in renal impairment, avoidance of multiple concurrent nephrotoxins, adequate hydration before contrast exposure, and therapeutic drug monitoring for high-risk medications (Campbell, 2023). If nephrotoxicity occurs, management focuses on immediate discontinuation of the offending agent, hemodynamic stabilization, and supportive AKI care following KDIGO recommendations (KDIGO, 2021).

2.3.2 MAX GLP-1 and Renal Function

The GLP-1 receptor is expressed in the kidney, specifically in the renal vasculature (afferent and efferent arterioles) and tubular cells. Activation of these receptors is linked to promoting natriuresis (sodium excretion) and diuresis, which contributes to its blood pressure-lowering effects (Hviid & Sørensen, 2017). Given this direct interaction, the potential for high doses of MAX GLP-1 to exert off-target effects on renal tissue must be investigated and analysed.

The Issue of Renoprotection and Acute Kidney Injury Risk

Established GLP-1 RAs have demonstrated profound renoprotective benefits in large-scale clinical trials, showing a reduction in albuminuria and a stabilization of the glomerular filtration rate (GFR) in patients with T2DM (Kristensen *et al.,* 2019; Williams *et al.,* 2024). This protective mechanism is hypothesized to involve improved glucose control, reduced blood

pressure, and anti-inflammatory action. However, clinical concerns have been raised regarding the risk of Acute Kidney Injury (AKI), often related to the severe gastrointestinal side effects (vomiting/diarrhea) of some GLP-1 RAs, leading to dehydration and pre-renal azotemia (Hess *et al.,* 2019). The subacute study on MAX GLP-1 must differentiate these indirect clinical risks from any intrinsic nephrotoxicity caused by the compound itself at high doses. This is achieved by monitoring:

- Serum Creatinine: A reliable measure inversely correlated with GFR.
- Blood Urea Nitrogen: Elevated levels, especially relative to creatinine, can indicate dehydration or impaired filtration.

Any significant, dose-dependent changes in these parameters following repeated administration of MAX GLP-1 would signal potential renal toxicity.

2.4 HAEMATOLOGICAL PARAMETERS

2.4.1 Haematological Parameters and it Functions

Haematological parameters refers to the measurable components of blood that provide valuable information about the health, physiological status, and function of the circulatory and immune systems. These parameters are assessed through haematological tests, commonly performed using automated hematology analyzers or manual methods. They are essential in diagnosing diseases, monitoring treatment, and evaluating the effects of drugs or toxic substances on blood and bone marrow (Jain *et al.,* 2020).

Composition of Blood

Blood is a specialized body fluid that consists of:

- Plasma (about 55%) the liquid component containing proteins, electrolytes, hormones, and nutrients.

- Red blood cells (RBCs or erythrocytes)

- White blood cells (WBCs or leukocytes)

- Platelets (thrombocytes)

Each of these components has specific physiological roles that are reflected in haematological parameters (Guyton & Hall, 2020).

Importance of Haematological Parameters

- **Diagnosis of Disease**s:

Abnormal values help in diagnosing anaemia, leukemia, infection, and blood disorders.

- **Monitoring Treatment**:

Used to assess patient response to therapy, such as in chemotherapy, malaria, or anaemia management.

- **Toxicological Studies:**

In experimental animals, haematological parameters are used to evaluate the safety or toxicity of drugs and chemicals (Eze *et al*., 2019).

- **Assessment of Nutritional Status:**

Changes in Hb, RBC, or MCV can indicate iron, vitamin B_{12}, or folate deficiency.

- **Evaluation of Immune Function:**

WBC count and differential give information on immune system activity and infection status.

2.4.2 MAX GLP-1 and Hematological Parameters (Systemic Toxicity)

The hematopoietic system, which encompasses the blood and blood-forming tissues (primarily the bone marrow), is an exceptionally sensitive indicator of systemic toxicity (Gad *et al.,* 2021). Because of the high proliferation rate of blood cells, this system is one of the first to display adverse effects from drug exposure, making a Complete Blood Count (CBC) analysis mandatory

in subacute toxicity screening (OECD, 2018). Changes in blood composition are rapid, quantifiable, and can often precede morphological changes observed in organ pathology. The assessment serves as a critical biological signal, revealing potential myelotoxicity (bone marrow damage), inflammatory responses, or fluid dynamics changes induced by MAX GLP-1.

2.4.3 Analysis of the Red Blood Cell (RBC) Series

The RBC series evaluates the efficacy of oxygen transport throughout the body. Analysis includes:

- **Red Blood Cell Count (RBC**): A decrease signals anemia, resulting from potential drug-induced hemolysis (RBC destruction) or myelosuppression (inhibition of RBC production in the bone marrow). Conversely, an increase in RBC count, along with corresponding increases in Hb and Hct, often suggests hemoconcentration, frequently a secondary effect of severe fluid loss (e.g., dehydration due to the gastrointestinal side effects sometimes associated with GLP-1 RAs) (Hess *et al*., 2019).

- **Hemoglobin (Hb) and Hematocrit (Hct):** These measures confirm the severity and type of anemia or hemoconcentration. Hct represents the volume percentage of RBC in blood.

- **RBC Indices (MCV, MCH, MCHC):** These metrics, such as Mean Corpuscular

Volume (MCV) (average cell size), help classify the type of anemia (e.g., microcytic, normocytic, or macrocytic). Abnormal MCV or Mean Corpuscular Hemoglobin (MCH}) could point toward specific toxic effects on cell maturation or nutrient utilization related to MAX GLP-1 exposure.

2.4.4 **Analysis of the White Blood Cell (WBC) Series**

The WBC or leukocyte series provides direct insight into the integrity of the immune system and the body's inflammatory status.

- **Total White Blood Cell Count (TWBC):** An increase (leukocytosis) indicates an immune reaction, often due to drug-induced irritation or inflammation. A decrease (leukopenia) is a hallmark sign of immunosuppression or generalized bone marrow suppression—a serious toxicological outcome that must be ruled out for MAX GLP-1

- **WBC Differential Count:** This breakdown into specific leukocyte types (Neutrophils, Lymphocytes, Monocytes, Eosinophils) is crucial for pinpointing the mechanism of toxicity.

An increase in Neutrophils (neutrophilia) is typically seen in acute inflammation or stress.
A decrease in Lymphocytes (lymphopenia) often accompanies systemic stress or direct toxic damage to lymphatic tissues. Since GLP-1 receptors are expressed on immune cells (Drucker, 2018), high doses of MAX GLP-1 could cause subtle, dose-dependent shifts in these differential

counts, warranting close examination.

2.4.5 Platelet Series and Coagulation Risk

Platelets are cell fragments essential for blood clotting. The platelet count is monitored for two key reasons:

Thrombocytopenia (low count) is a severe adverse effect indicating either direct bone marrow suppression or increased peripheral platelet consumption, dramatically raising the risk of spontaneous hemorrhage.

Thrombocytosis (high count) can reflect inflammation or increased risk of thrombosis.

Any significant deviation in the MAX GLP-1 treated groups' platelet counts or Mean Platelet Volume (which reflects platelet production kinetics) must be thoroughly examined as a potential toxic effect.

2.5 GLUCOSE AN IMPORTANT SOURCE ENERGY

Glucose is a monosaccharide that serves as the principal metabolic substrate and primary energy source for cellular activities. It is predominantly derived from dietary carbohydrates and plays a critical role in sustaining normal physiological and biochemical functions. The concentration of glucose within the bloodstream, termed the blood glucose level, is tightly regulated through complex hormonal and metabolic mechanisms to maintain homeostasis (Guyton & Hall, 2020;

World Health Organization, 2021).

The brain, erythrocytes, and renal medulla are particularly dependent on glucose as their main or energy source because they either lack mitochondria or have limited capacity to oxidize alternative fuels (DeFronzo et al., 2021). During fasting or prolonged exercise, the liver maintains blood glucose through glycogenolysis (breakdown of glycogen) and gluconeogenesis (synthesis of glucose from non-carbohydrate sources such as lactate, glycerol, and amino acids) to ensure a continuous energy supply (Trefts & Wasserman, 2019).

The brain, erythrocytes, and renal medulla are particularly dependent on glucose as their main or exclusive energy source because they either lack mitochondria or have limited capacity to oxidize alternative fuels (DeFronzo et al., 2021). During fasting or prolonged exercise, the liver maintains blood glucose through glycogenolysis (breakdown of glycogen) and gluconeogenesis (synthesis of glucose from non-carbohydrate sources such as lactate, glycerol, and amino acids) to ensure a continuous energy supply (Trefts & Wasserman, 2019).

2.5.1 Normal Blood Glucose Levels

Blood glucose concentration is commonly measured in milligrams per deciliter (mg/dL) or millimoles per liter (mmol/L) and serves as an essential indicator of metabolic health. Under normal physiological conditions, fasting blood glucose levels are maintained within a narrow range through precise hormonal regulation.

According to the World Health Organization (2021) and recent clinical guidelines, fasting blood

glucose levels between 70 and 99 mg/dL (3.9–5.5 mmol/L) are considered normal. Values between 100 and 125 mg/dL (5.6–6.9 mmol/L) are classified as prediabetic, indicating impaired glucose tolerance or early dysregulation of carbohydrate metabolism. Levels equal to or exceeding 126 mg/dL (≥7.0 mmol/L) in a fasting state, or 200 mg/dL (≥11.1 mmol/L) two hours after glucose intake, are diagnostic for diabetes mellitus (American Diabetes Association, 2022; WHO, 2021).

Maintaining glucose within these physiological limits is crucial, as deviations may reflect impaired insulin function, metabolic disorders, or endocrine abnormalities (Adewale *et al.,* 2022; Hall *et al.,* 2021).

2.5.2. The Mechanism: Glucose-Dependence and Hypoglycemia Risk.

The fundamental therapeutic action of MAX GLP-1 centers on its ability to positively regulate glucose homeostasis, a complex process achieved through two key pancreatic effects: the stimulation of insulin secretion from beta cells and the concurrent suppression of glucagon release from alpha cells (NCBI, 2024). However, the superior safety profile of the GLP-1 Receptor Agonist (GLP-1 RA) class, which includes MAX GLP-1, is intrinsically tied to the principle of glucose-dependence. This mechanism dictates that the drug's powerful insulinotropic effect is only fully operational when blood glucose concentrations are elevated (hyperglycemic). As the circulating glucose levels are corrected and begin to fall into the normal, euglycemic range, the drug's influence on insulin release diminishes significantly (Williams *et al.,* 2024).

This inherent, self-regulating biological safeguard prevents the over-secretion of insulin when it is not needed, thereby acting as a powerful brake against excessive glucose lowering. Consequently, this class of drugs, and by extension MAX GLP-1, carries a substantially lower risk of inducing severe, uncontrolled hypoglycemia compared to older antidiabetic medications like sulfonylureas, which stimulate insulin release regardless of the current blood glucose concentration. This mechanism not only confirms the efficacy of MAX GLP-1 but also provides the critical basis for its robust clinical safety (Kristensen *et a*l., 2023).

CHAPTER THREE

MATERIALS AND METHODS

3.1. MATERIALS:

3.1.1 Test materials

MaxGLP-1, a dietary supplement formulated from a combination of medicinal plants, minerals and microbial ingredients procured from a licensed pharmacy. The product is registered with the National Agency for Food, Drug Administration and Control (NAFDAC), with verified manufacturing and expiry dates and intact manufacturer seals.

3.1.2. Equipment

Apparatus and equipments	Producer/maker
Beakers (50, 150 and 250ml)	Pyrex (England)
Retort Stand	
Tripod Stand, Bunsen Burner and Gas Supply	
Pippettes (1,10 and 25ml)	Pyrex (England)
Automated micropippette (0-100 µl, 0-1000µl).	Micropet and Accumax PRO.
Conical flasks.	Pyrex (England)
Filter paper (0.45µm and 125mm)	Whatman (England)
Cuvettes	Pyrex (England)

Needles and syringes (1ml, 2ml, 5ml, 10ml)	
Paper tapes, cardboard papers and pins	
Cotton wool and Methylated spirit	
Animal cages	UNIBEN MEDBCH Dept. (Nigeria)
Oro-gastric Gavage	UNIBEN MEDBCH Dept. (Nigeria)
Stop watch	
Test tube racks and test tubes	UNIBEN MEDBCH Dept. (Nigeria)
Volumetric flasks (100, 250 and 500ml)	Technics (England)
HH-W Constant Temperature Water Bath	B. Bran Sc. Inst. Company, England.
Analytical weighing balance	Metter H-80 (Germany)
Water distiller	B. Bran Sc. Inst. Company, England.
Simple Weighing Balance	Adventurer OHAUS AR1530
T70UV/VISSpectrophotometer	PG Instruments Ltd., UK.
microplate reader	PG Instruments Ltd., UK.
Refrigerator	Citizens PRC4246
80-2 model Electric Centrifuge.	B.Bran Scientific and Instrument Company, England

3.1.3 Chemicals/Reagents:

Reagent/Enzyme kits and other reagentsused were of standard quality and were purchased from qualified/accredited dealers/suppliers or their manufacturers' representative in Nigeria. The Chemicals used were of analytical grade and an accredited dealer - Pyrex Laboratories, Benin,Nigeria.

3.1.3.1 Reagents/chemicals used for assessment of the status of glucose metabolism

Fasting blood glucose determination:

This is determined using the Glucose kit (Randox Lab.UK; purchased from manufacturer's representative in Nigeria). The kit contains the following reagents:

R1a. Buffer {Phosphate buffer (0.1mol/l, pH 7.0)}; Phenol (11mmol). R1b. GOD-PAP reagent {4-aminophenazone (0.77mmol/l), Glucose oxidase (≥ 1.5kU/l), Peroxidase (≥ 1.5kU/l)}. CAL. Standard (5.49mmol/l), Glucose (99mg/dl).

3.1.3.2. Reagents/chemicals used for the assessment of kidney function status

a. Urea kit. (RandoxLab.UK;Purchased from manufacturer's representative in Nigeria). The kit contains the following reagents:

- **R1-EDTA** (116mmol/l); Sodium Nitroprusside (6mmol/l); Urease (1g/l)
- **R2-Phenol (diluted)** (120mmol/l)
- **R3- Sodium hypochloride (diluted)** (27mmol/l); Sodium hydroxide (0.14N)

- **Urea standard (CAL)** (13.4mmol/l or 80.5mg/dl)

b. Creatinine kit. (RandoxLab.UK); Purchased from manufacturers representative in Nigeria). The kit contains the following Reagents:

- **R1a: Picric Acid** (35mmol/l)
- **R1b: Sodium Hydroxide** (0.32mol/l)
- **Creatinine Standard** (174µmol/l or 2mg/dl).

3.2. METHODS

3.2.1. Test Substance

MaxGLP-1 was obtained in its commercial formulation. The preparation of the dosing solution followed manufacturer guidelines to ensure accuracy and stability. Oral administration (per os) was given via gavage using a calibrated stainless-steel cannula.

3.2.2 Dose Justification and Calculation

The manufacturer's recommended human oral dose is 9.33 mg/kg (MaxGLP-1, 2024). To convert this to an appropriate rat equivalent dose (RED), body surface area normalization was employed using the FDA-recommended Km values for dose translation (Reagan-Shaw et al., 2008):

RED (mg/kg) = Human dose × (Km human / Km rat)

RED = 9.33 mg/kg × (37 / 6) ≈ 57.54 mg/kg

The rat-equivalent dose (RED) of 57.54 mg/kg, approximated to 60 mg/kg, served as the basis for the sub-acute dosing regimen. This dose was designated as the mid-dose (recommended) level for the study. The low and high dose levels were set at 10 mg/kg/day and 600 mg/kg/day, respectively, in accordance with OECD guidelines recommending the use of multiple dose levels to evaluate dose–response relationships and to establish a No-Observed-Adverse-Effect Level (NOAEL) (OECD, 2008)

Dosage Preparation

Separate stock solutions were prepared for each dose level (10, 60, and 600 mg/kg). The powdered supplement was reconstituted in distilled water to achieve the required concentrations for each group. Each rat received its calculated dose once daily by oral gavage, with the administered volume standardized to approximately 1 mL per rat to ensure dosing uniformity and to remain within the recommended safe oral volume limit (≤ 2 mL/100 g body weight). The doses were administered using a sterile syringe fitted with a blunt-ended gavage needle, once daily for 28 consecutive days.

3.2.3 Sub-Acute Toxicity Study (28-Day Repeated Dose)

This study was designed to assess the potential sub-acute toxicological effects of MaxGLP-1, a proprietary dietary supplement, following 28 consecutive days of oral administration in Wistar rats. The study followed methods modified from the internationally recognized guidelines

outlined by the Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (2008) for repeated-dose 28-day oral toxicity studies in rodents**.**

3.3 Experimental Animals and Housing Conditions

3.3.1 Experimental Animal and Grouping

Adult male *Wistar* rats (Rattus norvegicus), weighing approximately 150–180 g, was obtained from the Anatomy department, University of Benin, Benin City, a registered animal facility. Rats was housed in clean polypropylene cages under standard laboratory conditions: 12-hour light/dark cycles, ambient temperature of 25–29°C, and relative humidity of 50–70%. Animals were fed standard laboratory chow and given water *ad libitum*. Acclimatization was carried out for 7 days prior to the commencement of dosing to ensure physiological stabilization.

Animal care and handling complied with the guidelines outlined by the National Research Council (NRC, 2011) and OECD principles of Good Laboratory Practice (GLP).

Grouping of Animals

Male W*istar* rats was randomly assigned into groups (n = 5 per group) to reduce selection bias.

Sub-AcuteToxicity Groups (28 Days):

- Group 1: Control (vehicle only)

- Group 2: 10 mg/kg MaxGLP-1

- **Group 3:** 60 mg/kg MaxGLP-1

- Group 4: 600 mg/kg MaxGLP-1

Justification for use of single sex rats (Male):

Only male *Wistar* rats were used in this study to avoid the physiological variability associated with the estrous cycle in females, which could introduce confounding effects into hematological, biochemical, and histological outcomes. Given the absence of prior toxicological data on MaxGLP-1 this approach allows for an initial evaluation of its safety while reducing the number of animals used, in accordance with the 3Rs principle (Replacement, Reduction, and Refinement). This design aligns with OECD Test Guideline 407 (2008), which permits single-sex studies when scientifically justified.

3.3.2 Housing and Husbandry

Animals were housed in standard polycarbonate cages with stainless steel tops under controlled environmental conditions, including a 12-hour light/dark cycle, temperature maintained at 22 ± 3 °C, and relative humidity between 50% and 60%. Rats were acclimatized for 2weeks prior to the commencement of the study and were provided with standard rodent chow and filtered tap water *ad libitum* throughout the study period.

Clinical Observations and Monitoring

All animals were observed twice daily for signs of morbidity and mortality and once daily for clinical signs of toxicity. Body weight and food consumption were recorded weekly

3.4 Hematological and Biochemical Analyses

On the 29th day, blood samples were collected under anesthesia (using chloroform) via cardiac puncture in to heparinized and non-heparinizee sample bottles for Hematological and biochemical investigations. Hematological parameters assessed included hemoglobin concentration, red and white blood cell counts, hematocrit, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Clinical biochemistry parameters measured included:

- Renal function: Urea, creatinine and electrolytes
- Metabolic markers: Fasting blood glucose

Commercially available diagnostic kits (e.g., Randox, Sigma-Aldrich) was used, following manufacturers' protocols.

3.4.1. Relative Organ Weight:

At the end of the 28-day experimental period, just after the rats have been weighed, sacrificed (using chloroform anaesthesia) and blood samples collected from them, different organs namely the heart, liver, and kidneys of the respective rats from all groups was carefully dissected out and weighed (this weight was designated as the absolute organ weight). The relative organ weight was then calculated using the formula:

$$\text{Relative Organ Weight} = \frac{\text{Absolute organ weight (g)}}{} \times 100$$

Body weight of rat on sacrifice day (g)

(Aniagu *et al*., 2005).

3.4.2. Observations (symptoms, signs and mortality):

The animals were observed for toxic symptoms such as weakness or aggressiveness, food refusal, loss of weight, diarrhoea, discharge from the eyes and ears, noisy breathing and other physiological changes including mortality, (Fielding and Metheron, 1991; Vijoyalakshmi et al, 2000).

Clinical signs that were assessed before dosing, immediately and 4hrs after dosing, include level of sedation, restlessness, changes in nature of stool, urine and eye colour, excretion of worms, diarrhoea, haematuria, uncoordinated muscle movements, etc. (Fielding and Metheron, 1991; Vijoyalakshmi et al, 2000).

3.4.3. Blood Sample Collection and Preparation:

Three specimen bottles was used for collection of blood from each animal. Anticoagulant bottles containing **fluoride oxalate** for blood glucose test and **lithium heparin** for assay of parameters for liver function and kidney function statuses, while the plain bottle was used for other biochemical parameters.

The last dose of the respective diets, drugs and bitters was administered on morning of the 28th day. All meals where stopped by 7pm on the 28th day. After an overnight fast, blood samples

were collected from the animals (following chloroform anaesthesia and sacrifice/opening up of the animals), using syringes and needles via the inferior vena cava and cardiac puncture, and put into already labelled fluoride oxalate,lithium heparin and plain bottleswithout undue pressure to either the arm or the plunger of the syringe. The samples where be mixed by gentle inversion.

The sample in the fluoride oxalate bottles was immediately analysed for the fasting blood glucose. The samples in the lithium heparin and plain bottles where centrifuged at 4000r/min for 10mins to obtain plasma and serum respectively. The plasma and serum supernatants where separated into **sterile plain bottles** and used for assay of the required biochemical parameters.

3.4.5. Biochemical Analyses

Clinical biochemistry parameters measured included:

- Renal function: Urea, creatinine and electrolytes
- Metabolic markers: Fasting blood glucose

3.4.6 . Tissue Sample Collection and Preparation:

After blood was collected from the anaesthesized rats, the kidney was harvested and fixed in a 10% formal-saline solution until they are needed for processing into slides for histological evaluation.

3.5 HEMATOLOGICAL ANALYSES

Hematological parameters were determined within 2 hours of blood collection to ensure accuracy and prevent artifactual changes that can occur with prolonged storage. The analyses were performed at the Department of Biochemistry, University of Benin. Both manual methods using established protocols and standardized commercial kits were employed for comprehensive hematological evaluation.

3.5.1 Hemoglobin Concentration

Hemoglobin concentration was determined using the cyanmethemoglobin method, which is the gold standard recommended by the International Committee for Standardization in Haematology.

Principle: Hemoglobin is oxidized to methemoglobin by potassium ferricyanide present in Drabkin's reagent. Methemoglobin then combines with potassium cyanide to form cyanmethemoglobin (also called hemiglobincyanide), a stable colored compound that absorbs light maximally at 540 nm. The intensity of the color produced is directly proportional to the hemoglobin concentration in the sample.

Reagent: Drabkin's reagent containing:

- Potassium ferricyanide: 200 mg/L
- Potassium cyanide: 50 mg/L
- Potassium dihydrogen phosphate: 140 mg/L

- Distilled water to 1000 ml (pH 7.0-7.4)

Procedure:

1. Drabkin's reagent (5.0 ml) was dispensed into a clean, dry test tube.
2. Exactly 20 µl (0.02 ml) of well-mixed EDTA blood was added to the reagent using a calibrated micropipette to give a 1:251 dilution.
3. The mixture was mixed thoroughly by gentle inversion 8-10 times and allowed to stand for 5 minutes at room temperature (20-25°C) to allow complete conversion to cyanmethemoglobin.
4. The absorbance was measured at 540 nm using a UV-Visible spectrophotometer against a reagent blank (Drabkin's reagent without blood).
5. A standard solution of cyanmethemoglobin with known hemoglobin concentration (provided in the commercial kit) was similarly processed, and its absorbance was measured.

Calculation: **Hemoglobin concentration (g/dL) = (Absorbance of sample / Absorbance of standard) × Concentration of standard (g/dL)**

Normal reference range for male Wistar rats: 12-18 g/dL

3.5.2 Red Blood Cell (RBC) Count

RBC count was performed using the improved Neubauerhemocytometer method, a manual counting technique that provides accurate cell enumeration.

Principle: Red blood cells are diluted in an isotonic solution (Hayem's solution) that prevents hemolysis while maintaining cell integrity. The diluted cells are counted microscopically in a counting chamber of known volume, and the concentration is calculated based on the dilution factor and chamber dimensions.

Reagent - Hayem's Solution:

- Mercuric chloride: 0.25g
- Sodium chloride: 0.50g
- Sodium sulfate: 2.50g
- Distilled water: 100ml

Procedure:

1. Well-mixed EDTA blood (20 µl) was drawn into a clean, dry red blood cell (RBC) pipette up to the 0.5 mark.
2. The external surface of the pipette was carefully wiped clean with tissue paper to remove excess blood.

3. The tip of the pipette was immediately immersed in Hayem's solution, and the solution was drawn up to the 101 mark, giving a 1:200 dilution.

4. The ends of the pipette were sealed with the index finger and thumb, and the pipette was gently rotated and shaken for 2-3 minutes to ensure thorough mixing of blood and diluent.

5. The first 3-4 drops were discarded to clear the capillary tube of unmixed fluid.

6. A small drop of the diluted blood was placed on the counting chamber of an improved Neubauerhemocytometer.

7. A clean coverslip was carefully placed over the counting area, and the chamber was allowed to stand for 2-3 minutes to allow cells to settle.

8. The hemocytometer was examined under a light microscope using the 40× objective (high power).

9. Red blood cells were counted in the five small corner squares of the central ruled area (each small square = 1/25 mm^2).

10. Cells touching the top and left boundaries of the squares were included in the count, while those touching the bottom and right boundaries were excluded to avoid double counting.

11. The count was performed in duplicate (both chambers of the hemocytometer), and the average was calculated. Counts differing by more than 10% were repeated.

Calculation: The number of RBCs per microliter (µl) was calculated using the formula:

RBC count ($\times 10^6$/µl) = (Number of cells counted × Dilution factor) / (Area counted × Depth of chamber)

Where:

- Number of cells counted = Total cells in 5 small squares
- Dilution factor = 200
- Area counted = 5 small squares = 5 × 1/25 = 1/5 mm²
- Depth of chamber = 0.1 mm

Simplified formula: **RBC count ($\times 10^6$/µl) = N × 10,000**

Where N = average number of cells counted in 5 small squares

Normal reference range for male Wistar rats: 7-10 × 10^6/µl

3.5.3 Packed Cell Volume (Hematocrit)

Hematocrit (PCV) was determined using the microhematocrit method, which provides a measure of the proportion of blood volume occupied by red blood cells.

Principle: When whole blood is centrifuged at high speed, the cellular components separate from the plasma based on density. Red blood cells, being the densest and most numerous, pack at

the bottom of the tube. The height of the red cell column relative to the total blood column represents the hematocrit.

Procedure:

1. Plain capillary tubes (75 mm length, 1 mm internal diameter) were filled with well-mixed EDTA blood by capillary action to approximately three-quarters full (about 50-60 mm).
2. One end of the capillary tube was sealed with plasticine (modeling clay) by pressing it firmly into the sealant.
3. The sealed tubes were placed in a microhematocrit centrifuge with the sealed end facing outward toward the periphery and the open end toward the center.
4. Tubes were balanced in the centrifuge to prevent vibration.
5. The centrifuge lid was secured, and samples were centrifuged at 12,000 rpm for 5 minutes.
6. After centrifugation, three distinct layers were visible:
    - Bottom layer (red): Packed red blood cells
    - Middle layer (thin, whitish): Buffy coat containing white blood cells and platelets
    - Top layer (clear/straw-colored): Plasma

7. The tubes were read using a microhematocrit reader or a standard ruler with the scale positioned so that the bottom of the red cell column aligned with zero and the top of the plasma column aligned with 100.

8. The hematocrit was read at the junction between the red cell column and the buffy coat.

9. The measurement was performed in duplicate, and the average was calculated. Values differing by more than 2% were repeated.

Calculation: **Packed Cell Volume (%) = (Height of red cell column / Total height of blood column) × 100**

Normal reference range for male Wistar rats: 40-54%

3.5.4 Mean Corpuscular Volume (MCV)

MCV represents the average volume of individual red blood cells and was calculated from the hematocrit and RBC count values.

Principle: MCV provides information about the size of red blood cells and helps classify anemia (microcytic, normocytic, or macrocytic).

Formula: **MCV (fL) = [Hematocrit (%) × 10] / RBC count ($\times 10^6$/µl)**

The factor of 10 converts the units appropriately (1 fL = 10^{-15} L).

Example Calculation: If Hematocrit = 45% and RBC count = 8.0 × 10^6/µl MCV = (45 × 10) / 8.0 = 56.25 fL

Normal reference range for male Wistar rats: 50-60 fL

Interpretation:

- MCV < 50 fL: Microcytic (small red cells)
- MCV 50-60 fL: Normocytic (normal-sized red cells)
- MCV > 60 fL: Macrocytic (large red cells)

3.5.4. Mean Corpuscular Hemoglobin (MCH)

MCH represents the average amount of hemoglobin per red blood cell and was calculated from the hemoglobin concentration and RBC count.

Principle: MCH indicates the hemoglobin content of individual red blood cells and complements MCV in characterizing red cell morphology.

Formula: **MCH (pg) = [Hemoglobin (g/dL) × 10] / RBC count ($\times 10^6$/µl)**

The factor of 10 converts grams to picograms appropriately (1 pg = 10^{-12} g).

Example Calculation: If Hemoglobin = 14.5 g/dL and RBC count = 8.0 × 10^6/µl MCH = (14.5 × 10) / 8.0 = 18.13 pg

Normal reference range for male Wistar rats: 17-24 pg

3.5.5 Mean Corpuscular Hemoglobin Concentration (MCHC)

MCHC represents the average concentration of hemoglobin in a given volume of packed red blood cells and was calculated from the hemoglobin concentration and hematocrit values.

Principle: MCHC reflects the hemoglobin concentration within red blood cells and is useful in distinguishing between different types of anemia.

Formula: **MCHC (g/dL) = [Hemoglobin (g/dL) / Hematocrit (%)] × 100**

Example Calculation: If Hemoglobin = 14.5 g/dL and Hematocrit = 45% MCHC = (14.5 / 45) × 100 = 32.2 g/dL

Normal reference range for male Wistar rats: 30-36 g/dL

Interpretation:

- MCHC < 30 g/dL: Hypochromic (decreased hemoglobin concentration)
- MCHC 30-36 g/dL: Normochromic (normal hemoglobin concentration)
- MCHC > 36 g/dL: Hyperchromic (increased hemoglobin concentration, rare)

3.5.6 White Blood Cell (WBC) Count

Total WBC count was performed using the improved Neubauerhemocytometer method with Turk's solution, which lyses red blood cells while preserving white blood cells.

Principle: White blood cells are diluted in a solution (Turk's solution) that lyses red blood cells through the action of acetic acid, making white cells easier to visualize and count. The diluted white cells are counted microscopically in a counting chamber of known volume.

Reagent - Turk's Solution:

- Glacial acetic acid: 2 ml
- Gentian violet (1% aqueous solution): 1 ml
- Distilled water: 100 ml

The acetic acid lyses red blood cells, while gentian violet stains the nuclei of white blood cells, making them easier to identify.

Procedure:

1. Well-mixed EDTA blood (20 µl) was drawn into a clean, dry white blood cell (WBC) pipette up to the 0.5 mark.
2. The external surface was carefully wiped clean.
3. The tip of the pipette was immediately immersed in Turk's solution, and the solution was drawn up to the 11 mark, giving a 1:20 dilution.
4. The pipette was sealed and gently rotated for 2-3 minutes to ensure thorough mixing and complete lysis of red blood cells.
5. The first 3-4 drops were discarded.
6. The counting chamber of an improved Neubauerhemocytometer was loaded with the diluted blood.
7. A clean coverslip was placed over the chamber, and the preparation was allowed to stand for 2-3 minutes for cells to settle.
8. The hemocytometer was examined under a light microscope using the 10× objective (low power).

9. White blood cells were counted in the four large corner squares of the ruled area. Each large corner square has an area of 1 mm^2 and is subdivided into 16 smaller squares.

10. All white blood cells within the four large corner squares were counted, including those touching the top and left boundaries but excluding those touching the bottom and right boundaries.

11. The count was performed in duplicate (both chambers), and the average was calculated. Counts differing by more than 10% were repeated.

Calculation: **WBC count ($\times 10^3/\mu l$) = (Number of cells counted × Dilution factor) / (Area counted × Depth of chamber)**

Where:

- Number of cells counted = Total cells in 4 large corner squares
- Dilution factor = 20
- Area counted = 4 mm^2
- Depth of chamber = 0.1 mm

Simplified formula: **WBC count ($\times 10^3/\mu l$) = N × 50**

Where N = average number of cells counted in 4 large corner squares

Normal reference range for male Wistar rats: 6-12 × $10^3/\mu l$

3.5.7. Differential White Blood Cell Count

Differential leukocyte count was performed to determine the relative percentages of different types of white blood cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils).

Principle: A thin blood film is prepared, stained with Leishman's stain (a Romanowsky-type stain), and examined microscopically. Different types of white blood cells are identified based on their morphological characteristics and staining properties, and their relative proportions are determined.

Reagent - Leishman's Stain: Commercial Leishman's stain powder (a mixture of eosin and methylene blue) dissolved in absolute methanol.

Procedure:

***Blood Film Preparation*:**

1. A clean, grease-free glass slide was used.
2. A small drop of well-mixed EDTA blood (about 2 mm diameter) was placed near one end of the slide.
3. A spreader slide (another clean slide) was held at a 30-45° angle and backed into the blood drop until the blood spread along the edge.
4. The spreader slide was then pushed forward smoothly and rapidly to create a thin, even film.
5. The film was air-dried by waving it gently or allowing it to dry at room temperature.

***Staining*:**

1. The dried blood film was placed on a staining rack.
2. Leishman's stain (undiluted) was poured onto the film to cover it completely and left for 2 minutes to fix the cells.
3. An equal volume of buffered distilled water (pH 7.2) was added to the stain on the slide and mixed by gentle rocking. The diluted stain was left for 10 minutes to stain the cells.
4. The stain was washed off gently with tap water or buffered distilled water.
5. The slide was allowed to air dry completely.

***Microscopic Examination*:**

1. The dried, stained film was examined under a light microscope.
2. The film was first scanned using the 10× objective to assess overall quality and distribution of cells.
3. The examination area was located using the 40× objective—the area where red blood cells are close together but not overlapping (the feather edge or tail of the film).
4. The examination was then continued using the 100× oil immersion objective.
5. A drop of immersion oil was placed on the film, and the oil immersion lens was carefully lowered into the oil.

6. White blood cells were systematically identified and counted across the film in a serpentine pattern (moving back and forth across the slide).

7. A total of 100 white blood cells were counted and classified based on morphological characteristics:

Neutrophils:

- Size: 12-15 μm
- Nucleus: Multi-lobed (2-5 lobes), purple
- Cytoplasm: Pink with fine granules

Lymphocytes:

- Size: 7-12 μm (small) or 12-16 μm (large)
- Nucleus: Round or slightly indented, dark purple
- Cytoplasm: Scant, pale blue

Monocytes:

- Size: 15-22 μm (largest white cell)
- Nucleus: Kidney-shaped or horseshoe-shaped, purple
- Cytoplasm: Blue-gray with fine granules

Eosinophils:

- Size: 12-17 µm
- Nucleus: Usually bi-lobed, purple
- Cytoplasm: Filled with large, red-orange granules

Basophils:

- Size: 10-14 µm
- Nucleus: Obscured by granules
- Cytoplasm: Filled with large, deep purple-black granules

8. Each cell type was recorded using a differential counter (manual or electronic tally counter).
9. The percentage of each cell type was calculated.

Calculation: **Percentage of each cell type = (Number of that cell type / Total WBC counted) × 100**

Absolute count for each cell type = (Percentage × Total WBC count) / 100

Normal reference ranges for male Wistar rats:

- Neutrophils: 9-34% (absolute: 0.54-4.08 × 10^3/µl)
- Lymphocytes: 65-85% (absolute: 3.90-10.20 × 10^3/µl)

- Monocytes: 0-5% (absolute: 0-0.60 × 10^3/µl)
- Eosinophils: 0-6% (absolute: 0-0.72 × 10^3/µl)
- Basophils: 0-2% (absolute: 0-0.24 × 10^3/µl)

3.5.8 Platelet Count

Platelet count was estimated using the direct microscopic method on stained blood films.

Principle: Platelets are counted in relation to red blood cells on a stained blood film, and the platelet count is estimated based on the red blood cell count.

Procedure:

1. A well-prepared and stained blood film (prepared as described in section 3.5.8) was examined under oil immersion (100× objective).
2. Ten fields with an even distribution of red blood cells were selected.
3. In each field, the number of platelets and red blood cells were counted.
4. The average number of platelets per field was calculated.

Calculation: **Platelet count (×10^3/µl) = (Average number of platelets per field / Average number of RBCs per field) × RBC count (×10^6/µl) × 1000**

Alternative method (Rule of Thumb): If there are an average of 8-20 platelets per oil immersion field in an area where red cells are close but not touching, the platelet count is considered adequate (150-400 × 10^3/µl).

Normal reference range for male Wistar rats: 500-1,300 × 10^3/µl

Note: Rat platelet counts are normally higher than human values.

3.6. BIOCHEMICAL ANALYSIS

3.6.1 Assessment of status of glucose metabolism

Determination of the fasting blood glucose (FBG).

Principle:

The Randox glucose kit was used. The principle applied is the glucose oxidase method as described by Barham and Trinder (1972). Glucose is determined after enzymatic oxidation in the presence of glucose oxidase (GOD). The hydrogen peroxide formed reacts, under catalysis of peroxidase (POD), with phenol and 4-aminophenazone to form a red-violet quinoneimine dye indicator. Using a visible spectrophotometer set at 500nm, the absorbance of the dye which is equivalent to the plasma glucose concentration was read. Using the known absorbance and concentration of the standard, the concentration of glucose in the sample was then determined.

glucose + O_2 + H_2O $\xrightarrow{\text{GOD}}$ gluconic acid + H_2O_2"

H_2O_2 + 4-aminophenazone + phenol $\xrightarrow{\text{POD}}$ quinoneimine + $4H_2O$

Procedure:

Three tubes were labelled standard, sample and reagent blank respectively. 20µl of the standard

solution was pipetted into the tube labelled standard, 20µl of the sample solution was also pipetted into the tube labelled sample, while nothing was put in the tube labelled reagent blank. 2000 µl of Reagent 1 was then added to each tube. Mixing, incubation for 10min at 37^{o}C was done, afterwhich the absorbance of the standard ($A_{standard}$) and the sample(A_{sample}) against the reagent blank was measured within 60 minutes using a spectrophotometer set at 500nm.

Calculation

$$\text{Glucose concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{Standard conc. (mg/dl)}$$

3.6.2. Assessment of kidney function status

a. Determination of plasma electrolytes: sodium (Na^+), potassium (K^+), chloride (Cl^-) and bicarbonate (HCO_3^-)

The plasma sodium, potassium, chloride and bicarbonate ion levels were determined using Ion Selective Electrode Device (Tietz *et. al.,* 1996).

Principle

An ion-selective electrode (ISE), also known as a specific ion electrode (SIE), is a transducer (or sensor) that converts the activity of a specific ion dissolved in a solution into an electrical potential, which can be measured by a voltmeter or pH meter. The ion selective membrane is a membrane across which only the intended ion can be transported. The transport of

ions from a high concentration to a low one through a selective binding with some sites within the ion selective membrane creates a potential difference/reduction potential or voltage which is theoretically dependent on the logarithm of the ionic activity (Nernst equation). This explains the relationship of the ionic activity to the free energy change that is captured by the voltmeter (Tietz *et. al.,* 1996).

This potential difference/reduction potential (whose magnitude is in turn equivalent to the concentration of the ion generating it in the sample), is given by the Nernst equation:

$$E = E^{o} - \frac{N}{n} \log \frac{a_{red}}{a_{ox}} = E^{o} - \frac{0.0592V}{n} \log \frac{a_{red}}{a_{ox}}$$

where:

E = electrode potential of the half cell

E^{o}= standard electron potential when $a_{red}/a_{ox}= 1$

n = number of electrons involved in the reduction reaction

N = (R x T x ln 10)/F (the Nernst factor if n=1)

N = 0.0592V if T = 298.15 K (25°C)

N = 0.0615V if T = 310.15 K (37°C)

R = gas constant 8.31431 Joules x K^{-1} x mol^{-1}

T absolute temperature (unit: K, kelvin)

F = faraday constant (96,487 Coulombs x mol^{-1})

ln 10 = natural logarithm of 10 = 2.303

a = activity

"a_{red}/a_{ox} = product of mass action for the reduction reaction" (Tietz *et. al.,* 1996).

A difference in ionic composition of the two solutions involved in the ISE devise causes an electrical potential difference to develop across the ion selective membrane. A change in potential across the ion selective membrane are measured with respect to a reference electrode, the potential of which is constant.The change in potential difference between the reference electrode (E_{Ref})and the ion selective electrode (E_{ISE}) for the sample is proportional with the potential difference (E_{Cell}) already known for a calibrated solution of known composition and quantity. Hence the unknown solution is therefore said to be the same in terms of composition and quantity as the calibrated solution (Tietz *et. al.,* 1996).

The basic formula of the net potential generated by the unknown solution is given for the galvanic cell as:

$E_{Cell} = E_{ISE} - E_{Ref}$(Tietz *et. al.,* 1996).

The strength of this charge (E_{cell}) is directly proportional to the concentration of the selected ion.

Calculation of analyte concentration is doneautomatically by the ISE analyzer/devise. It calculates the analyte concentration from the potential developed and recorded by the voltmeter and presents it as a digital numberon the screen of the ISE analyser/devise (Tietz *et. al.,* 1996; Burtis*et al.,* 2008).

Procedure:

Depending on which electrolyte concentration is to be determined, with all the materials ready and the specific electrode with the ion selective membrane and electrode specific for the specific electrolyte installed in the ISE analyzer;and with the electrode wire connected to one terminal of a galvanometer or voltmeter and the other terminal connected to the reference electrode, both electrodes are then immersed in the solution to be tested. The passage of the ion through ion selective membrane creates an electrical current, which registers on the galvanometer; and because the galvanometer has already been calibrated against standard solutions of varying concentration, the ionic concentration in the test solution can be automatically derived from the galvanometer reading. Calculation of analyte concentration is doneautomatically by the analyzer which calculates the analyte concentration from the potential developed and recorded by the voltmeter/galvanometer. The concentration of the sodium, potassium, chloride, and bicarbonate depending on which is being measured then appears on the display screen of the ISE analyzer as a digital number (Tietz *et. al.,* 1996; Burtis*et al.,* 2008).

b. Determination of plasma urea: Randox lab. kit, UK, was used (Weatherburn, 1967)

Principle:

Urea in plasma is hydrolyzed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction (Weatherburn, 1967)

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

NH_3 + hypochloric acid + phenol → indophenol (blue compound).

The absorbance of the resulting solution (indophenol) is measured spectrophotometrically at a wavelength of 546nm. The absorbance (A) of the resulting solution being equivalent to the urea in the sample and its concentration was determined by the formula:

$$\text{Urea concentration} = \frac{A_{Sample}}{A_{standard}} \times \text{Standard conc. (mg/dl)}$$

Procedure:

Three cuvettes were labelled standard, sample and blank respectively. 10µl of the standard solution (CAL) was pipetted into the tube labelled standard, 10µl of the sample solution was also pipetted into the tube labelled sample, while 10µl of distilled water was put in the tube labelled reagent blank. 100 µl of the solution labelled Reagent 1 (a mix of 116mmol/l EDTA, 6mmol/l sodium nitroprusside and 1g/l urease), and 2.5ml of the solutions labelled Reagent 2 (diluted 120mmol/l phenol) and Reagent 3 (a mix of 27mmol/l sodium hypochloride (diluted) and 0.14N sodium hydroxide) in the kit, was then added to each tube sequentially.

Each tube was mixed immediately and incubated at 37^0C for 15mins.

The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) were read against the blank at 546nm. (The colour of the reaction is known to be stable for at least 8 hours)".

Calculation:

$$\text{Urea concentration} = \frac{A_{Sample}}{A_{standard}} \times \text{Standard conc. (mg/dl)}$$

c. Determination of plasma creatinine: Randox lab. kit, UK was used (Bartels and Bohmer, 1972).

Principle: Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The intensity of the complex formed is directly proportional to creatinine concentration (Bartels and Bohmer, 1972). The absorbance of the resulting solution is measured two times 2 minutes from each other spectrophotometrically at a wavelength of 492nm (Bartels and Bohmer, 1972). The change in absorbance of the standard and sample solutions is then determined and inputed with the known concentration of the standard into the prescribed formula "to determine the concentration of creatinine in the sample.

Procedure:

Three cuvettes were labelled standard, sample and blank respectively. 100µl of the standard solution was pipetted into the tube labelled standard, 100µl of the sample solution was also pipetted into the tube labelled sample, while 100µl of distilled water was put in the tube labelled reagent blank. 1000 µl of the solution labelled working reagent (Picric acid + Sodium Hydroxide) was then added to each tube sequentially and the resulting solutions mixed. **A**fter 30 seconds the absorbance A_1 of the standard and sample were read after zeroing the spectrophotometer set at 492nm, with the blank. Exactly 2 minutes later the absorbance A_2 of standard and sample were read again.

Calculation:

A_{sample} = $A_2 - A_1$ (for sample)

$\triangle A_{standard}$ = $A_2 - A_1$ (for standard)

Concentration of creatinine in plasma:

$$\frac{\triangle A_{sample}}{A_{standard}} \times \text{Concentration of standard (mg/dl).}$$

3.7 Data analysis

Results were expressed as mean ± SEM (Standard Error of Mean) from each experiment group of n=5 rats. Analysis of variance (ANOVA) was computed among the mean values of the experimental data for ascertain the level of significance at 95% confidence interval using Graph pad prism and SPSS statistical package and data with comparative mean of values of $p<0.05$ were significant.

CHAPTER FOUR

RESULTS

MAXGLP-1 SUBACUTE TOXICITY

The MaxGLP-1 supplementation at 10 mg/kg, 60 mg/kg, and 600 mg/kg caused a significant decrease ($p<0.05$) in urea levels in the rats when compared to the control group (Table 4.1). The decrease was more pronounced in the 60 mg/kg and 600 mg/kg treated groups, while the 10 mg/kg group showed a moderate reduction. Creatinine levels were not significantly altered ($p>0.05$) in all treated groups when compared with the control rats (Table 4.2). Potassium (K^+) and chloride (Cl^-) levels were not significantly different ($p>0.05$) in the treated groups compared with the control group (Tables 4.3 and 4.5). Sodium (Na^+) levels were significantly increased ($p<0.05$) in the 600 mg/kg treated group compared to the control, while the 10 mg/kg and 60 mg/kg groups showed no significant change (Table 4.1).

Table 4.1 Renal Function Parameters

Group	Urea (mg/dL)	Creatinine (mg/dL)	K^+ (mEq/L)	Na^+ (mEq/L)	Cl^- (mEq/L)
Control	142.12 ± 3.02 A	2.798 ± 0.132	0.884 ± 0.086	32.95 ± 0.93 B	23.84 ± 0.87
10 mg/kg	114.95 ± 6.01 B	2.639 ± 0.143	0.671 ± 0.025	36.14 ± 2.53 B	24.85 ± 1.25

60 mg/kg	78.30 ± 6.06 C	2.840 ± 0.099	0.677 ± 0.043	40.41 ± 3.56 AB	24.16 ± 0.43
600 mg/kg	78.00 ± 8.91 C	2.551 ± 0.087	0.653 ± 0.018	50.78 ± 6.03 A	25.39 ± 0.81

Table 4.1 showed the effects of the study drug MaxGLP-1 on the kidneys revealing mild changes in the parameters except Na^+ that showed significant increase in the 600mg/kg group

The administration of the MaxGLP-1 at 10 mg/kg, 60 mg/kg, and 600 mg/kg caused no significant change ($p>0.05$) in fasting blood glucose levels in the rats when compared with the control group (Table 4.2). Generally, all administered doses of the test substance maintained fasting blood glucose levels without any significant alteration ($p>0.05$) when compared to the control group, and their effects were not significantly different ($p>0.05$) from each other.

Table 4.2 Glucose levels

Group	Glucose
Control	72.60 ± 5.48 A
10 mg/kg	70.60 ± 4.48 A
60 mg/kg	75.50 ± 2.50 A
600 mg/kg	75.40 ± 4.23 A

Table 4.2 showed no significant change in the glucose level across the test subjects ($p> 0.05$)

The control group consumed significantly more feed than all the MaxGLP-1–treated groups ($p < 0.05$). Among the treated groups, rats administered 60 mg/kg consumed significantly less feed than both the 10 mg/kg and 600 mg/kg groups ($p < 0.05$), while feed consumption between the 10 mg/kg and 600 mg/kg groups did not differ significantly ($p > 0.05$).

Water intake increased progressively with dose. The 10 mg/kg, 60 mg/kg, and 600 mg/kg groups all consumed more water than the control rats, with the highest intake observed in the 600 mg/kg group. All differences were statistically significant ($p < 0.05$).

There was no significant difference in daily weight gain between the control group and any of the MaxGLP-1–treated groups ($p > 0.05$). Similarly, weight gain among the three treated groups did not differ significantly from each other ($p > 0.05$).

Table 4.3 Feed Consumed, Water Consumed, Weight Gain/Loss per day per rat

Group	Feed Consumed	Water Consumed	Weight Gain/Loss/day
Control	25.46 ± 0.00 A	35.37 ± 0.00 A	1.584 ± 0.11 A
10 mg/kg	22.22 ± 0.00 B	38.48 ± 0.00 B	1.332 ± 0.23 A
60 mg/kg	18.03 ± 0.00 C	39.54 ± 0.00 B	1.106 ± 0.22 A
600 mg/kg	22.33 ± 0.00 B	40.32 ± 0.00 B	0.966 ± 0.31 A

Table 4.3 revealed that there was no significant difference in daily weight gain between the

control group and any of the MaxGLP-1–treated groups ($p > 0.05$).

Relative kidney weight did not differ significantly between the control rats and any of the treatment groups ($p > 0.05$).There were also no significant differences among the 10 mg/kg, 60 mg/kg, and 600 mg/kg groups ($p > 0.05$).

Table 4.4 Relative Organ Weight

Group	Kidney Wt
Control	0.674 ± 0.05 A
10 mg/kg	0.642 ± 0.06 A
60 mg/kg	0.575 ± 0.04 A
600 mg/kg	0.612 ± 0.04 A

Table 4.4 shows the relative kidney weight did not differ significantly between the control rats and any of the treatment groups ($p > 0.05$).

Feed efficiency did not differ significantly between the control group and the 10 mg/kg, 60 mg/kg, or 600 mg/kg groups ($p > 0.05$). Likewise, no significant differences were observed among the treated groups themselves ($p > 0.05$).

Table 4.5 Feed Efficiency

Group	Feed Efficiency
Control	**6.22 ± 0.45 A**
10 mg/kg	**6.00 ± 1.02 A**
60 mg/kg	**6.13 ± 1.23 A**
600 mg/kg	**4.33 ± 1.41 A**

Table 4.5 shows the feeding efficiency of the four groups and reveals no significant differences among the groups($p>0.05$)

Across all time points (Day 0, Week 1, Week 2, Week 3, Week 4), MaxGLP-1 at 10 mg/kg, 60 mg/kg, and 600 mg/kg did not cause any significant ($p > 0.05$) change in body weight when compared with the control rats. Body weight progression among treated groups also remained statistically similar ($p > 0.05$), indicating no dose-dependent effect on body weight throughout the 28-day exposure period.

Table 4.6 Body Weight Progression

Group	Day 0	Week 1	Week 2	Week 3	Week 4
Control	155.71 ± 8.47	165.67 ± 7.75	179.58 ± 9.64	185.29 ± 10.25	200.08 ± 10.97
10 mg/kg	154.98 ± 6.40	161.54 ± 5.59	168.08 ± 7.99	182.12 ± 8.76	192.37 ± 8.03
60 mg/kg	154.79 ± 6.26	156.57 ± 9.63	157.10 ± 13.11	164.75 ± 14.03	185.84 ± 8.90
600 mg/kg	154.78 ± 5.91	158.96 ± 5.82	168.15 ± 7.70	179.54 ± 5.67	181.89 ± 6.19

Table 4.6 shows the body weight progression across the 28-dayperiod (Day 0, Week 1, Week 2, Week 3, Week 4), MaxGLP-1 at 10 mg/kg, 60 mg/kg, and 600 mg/kg did not cause any significant ($p > 0.05$) change in body weight when compared with the control rats. Body weight progression among treated groups also remained statistically similar ($p > 0.05$), indicating no dose-dependent effect on body weight across all time points.

The administration of MaxGLP-1 at various doses did not cause any significant alterations in the measured hematological parameters (WBC, RBC, HGB, HCT, MCV, and MCH) when compared to the control group. Generally, all dose groups maintained levels that were comparable to the normal control rats, with no statistically significant differences ($p>0.05$) observed across these treatment groups. Minor variations were observed in some parameters, such as WBC and MCV, where the highest dose (600 mg/kg) showed a slight increase or

decrease compared to the control; however, these differences were not statistically significant and remained within the normal physiological range. Overall, these results indicate that MaxGLP-1 did not adversely affect the hematological profile of the rats during the study period, and any observed variations were not indicative of toxicity.

The administration of MaxGLP-1 at various doses did not cause any significant alterations in the hematological parameters measured, specifically MCHC and platelet count (PLT), when compared to the control group. For MCHC, all dose groups (10 mg/kg, 60 mg/kg, and 600 mg/kg) maintained levels that were comparable to the control rats, with no statistically significant differences observed across treatment groups ($p>0.05$).

Similarly, platelet counts (PLT) showed no significant differences between any of the dose groups and the control ($p>0.05$), despite slight numerical fluctuations across groups. Overall, these results show that MaxGLP-1 did not adversely affect the hematological profile of the rats during the study period.

Table 4.6 Haematological Parameters of Rats Administered MAXGLP-1

Parameter	Control	10 mg/kg	60 mg/kg	600 mg/kg
WBC (10^3/µL)	10.52 ± 0.88	12.68 ± 1.67	12.80 ± 1.49	14.14 ± 1.31
RBC (10^6/µL)	7.05 ± 0.31	7.19 ± 0.28	6.68 ± 0.52	7.62 ± 0.49
HGB (g/dL)	14.46 ± 0.45	14.24 ± 0.79	13.48 ± 1.02	15.00 ± 0.98
HCT (%)	42.22 ± 0.75	40.42 ± 1.92	38.62 ± 2.40	41.80 ± 2.61
MCV (fL)	60.24 ± 1.83	56.26 ± 1.58	55.84 ± 1.38	54.94 ± 1.06
MCH (pg)	20.50 ± 0.32	19.70 ± 0.51	19.34 ± 0.43	19.60 ± 0.09
MCHC (g/dL)	34.18 ± 0.57	35.14 ± 0.45	35.20 ± 0.74	35.84 ± 0.57
PLT (10^3/µL)	342.20 ± 19.18	361.00 ± 3.67	348.40 ± 13.57	350.80 ± 52.13

Table 4.6 shows the administration of MaxGLP-1 at various doses did not cause any significant alterations in the measured hematological parameters (WBC, RBC, HGB, HCT, MCV, and MCH) when compared to the control group. Although minor variations were observed in some parameters, such as WBC and MCV, where the highest dose (600 mg/kg) showed a slight increase or decrease compared to the control; however, these differences were not statistically significant and remained within the normal physiological range. Overall, these results indicate that MaxGLP-1 did not adversely affect the hematological profile of the rats during the study period, and any observed variations were not indicative of toxicity.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

SUMMARY OF FINDINGS

This 28-day subacute toxicity study investigated the safety and physiological effects of MaxGLP-1 supplementation in male Wistar rats at three dose levels (10 mg/kg, 60 mg/kg, and 600 mg/kg). The primary results from chapter four are summarized below:

Renal Function & Electrolytes: MaxGLP-1 induced a significant, dose-dependent decrease in serum urea ($p<0.05$). Critically, creatinine, potassium (K^+) and chloride (Cl^-) levels were unaffected ($p>0.05$). The highest dose, 600 mg/kg, caused a significant increase in serum sodium (Na^+) ($p<0.05$), indicating potential hypernatremia.

Somatic & Metabolic Indicators: There were no significant changes in daily weight gain, overall body weight progression, or fasting blood glucose levels ($p>0.05$). MaxGLP-1 significantly reduced feed consumption ($p<0.05$) but, conversely, resulted in a significant and progressive increase in water intake ($p<0.05$) across all treatment groups.

Safety Profile: Both relative kidney weight and all measured hematological parameters (WBC, RBC, HGB, etc.) were not significantly altered ($p>0.05$).

DISCUSSION

The findings for MaxGLP-1 present a mixed profile: demonstrating expected pharmacological

efficacy alongside a unique and concerning toxicological signal when compared to the existing class of Glucagon-Like Peptide-1 Receptor Agonists (GLP-1RAs), such as Liraglutide and Exenatide.

General Toxicity and Lack of Systemic Adverse Effects

The overall assessment of safety is favorable, mirroring the typical low-toxicity profile of GLP-1RAs. The stability of hematological parameters is a robust indicator that the test substance is not myelotoxic (toxic to the bone marrow) and does not induce systemic inflammation or blood dyscrasias over the 28-day period (Torella, 2021). Furthermore, the lack of change in relative kidney weight suggests the compound does not induce gross organ damage as seen in chapter four.

Metabolic and Satiety Modulation

The observation of reduced feed consumption by MaxGLP-1 is a signature effect of GLP-1RA therapy, confirming the compound's agonistic activity on the GLP-1 receptor (Drucker and Nauck, 2012). This effect is mediated by signals from the gastrointestinal tract and the central nervous system, promoting satiety (Holst, 2007).

The absence of significant body weight change is not necessarily a contradiction, as significant weight loss often requires a longer, chronic exposure period or is more pronounced in pre-diseased (e.g., obese or diabetic) animal models. Similarly, the stable fasting blood glucose levels confirm that MaxGLP-1's action is likely glucose-dependent, meaning it avoids causing hypoglycemia in healthy, normoglycemic subjects (Drucker and Nauck, 2006).

Divergence in Renal and Fluid Homeostasis

The most significant toxicological findings relate to renal function and fluid balance:

Urea Reduction and Creatinine Stability: The significant reduction in urea, while creatinine remains stable from the results in Chapter Four suggests the compound is not acutely nephrotoxic. This result is promising, as clinical GLP-1RAs are known to exert nephroprotective effects (e.g., in diabetic kidney disease models) and can improve parameters like BUN (Kim et al., 2022; Li et al., 2022). For MaxGLP-1, the urea drop is likely secondary to reduced protein intake or a modification of nitrogen turnover, but it is supported by the class's tendency toward renal benefit.

Unprecedented Fluid Imbalance: The finding of significantly increased water intake (polydipsia) and the ensuing hypernatremia at the high dose represents a major divergence from the expected pharmacology of GLP-1RAs:

Established GLP-1RAs are well-documented to induce a hypodipsic effect (suppressed water intake) in rats, independent of food intake, and often promote natriuresis (sodium excretion) (McKay et al., 2011; Tang-Christensen et al., 1996). The central GLP-1 receptor is a known regulator of fluid homeostasis, typically leading to decreased drinking behavior (McKay and Daniels, 2013).

MaxGLP-1's Effect: MaxGLP-1's actions—increased drinking and elevated serum Na+—suggest a mechanism that either overrides, antagonizes, or selectively modulates the central osmoregulatory effects of the GLP-1 receptor in a novel way. The hypernatremia could arise

from an exaggerated diuretic effect coupled with insufficient water excretion, or it could signal a disruption of osmoreceptor sensitivity, forcing the animals to drink excessively to compensate for a high tonicity state induced by the compound. This effect is a critical potential dose-limiting toxicity that requires immediate and detailed mechanistic elucidation, as sustained hypernatremia can lead to severe neurological and cardiovascular compromise.

CONCLUSION

In conclusion, the MaxGLP-1 subacute toxicity study demonstrates that the compound is generally well-tolerated across hematological endpoints, and its appetite-suppressant activity is consistent with its intended class. However, the unique and significant findings of dose-dependent polydipsia and hypernatremia at the highest dose level represent a substantial pharmacological deviation from established GLP-1RAs. These effects strongly suggest an unanticipated interaction with renal mechanisms controlling fluid and electrolyte balance, which must be resolved to establish the compound's long-term safety profile. The study's results indicate that the No-Observed-Adverse-Effect-Level (NOAEL) is likely constrained by this osmoregulatory disturbance.

RECOMMENDATIONS

Recommendations for Pre-Clinical Safety and Mechanistic Research

Targeted Renal Physiology Study: Conduct a specialized study to precisely measure urine

output, urinary osmolality, and fractional excretion of sodium and water. This will distinguish whether the hypernatremia is due to exaggerated water loss (diabetes insipidus-like effect) or a primary defect in sodium handling.

Neuroendocrine Investigation: Immediately investigate the levels of key osmoregulatory hormones, including Antidiuretic Hormone (ADH/Vasopressin) and Aldosterone, in the treated groups to pinpoint the hormonal axis disrupted by MaxGLP-1.

Chronic Toxicity Study: A 90-day chronic toxicity study must be performed, closely monitoring serum sodium and fluid intake, and incorporating histopathological evaluation of the kidneys, adrenal glands, and hypothalamus to assess for cumulative damage or morphological changes (Torella, 2021).

Recommendations for Future Compound Development

Receptor Selectivity Profiling: Comprehensive in vitro and in vivo studies should be executed to compare MaxGLP-1's binding and signaling efficacy at the GLP-1 receptor (GLP-1R) versus other relevant receptors (e.g., GIPR or other hypothalamic receptors) to determine if its unusual effect on fluid intake is due to off-target activity.

Dose Response Refinement: The maximum tested dose (600 mg/kg) should be characterized as the Lowest-Observed-Adverse-Effect-Level (LOAEL), and subsequent studies must focus on a dose range below this level to identify a true NOAEL based on the critical hypernatremia endpoint.

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