NEAR EAST UNIVERSITY

HEALTH SCIENCES INSTITUTE

# EFFECT OF PROTOCATECHUIC ACID ON KIDNEY TISSUE IN EXPERIMENTAL DIABETIC RATS

TAHANI ELSAHLY
MASTER THESIS

HISTOLOGY AND EMBRYOLOGY DEPARTMENT

THESIS SUPERVISOR

Prof. Dr. AYSEL KÜKNER

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This work has been adopted as a master thesis in the program of Histology and Embryology by the jury.

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This thesis has been approved by the above jury members in accordance with the relevant articles of the NEU postgraduate education, training and examination regulations and has been accepted by the decision of the board of the Institute.

Prof. Dr. Hüsnü Can BAŞER

Director of Health Sciences Institute

## DECLARATION

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**Student Name: Tahani Elsahly**

**Signature:** _____

**Date:** _____

COMPLIANCE AND APPROVAL

Her master thesis "**Effect of protocatechuic acid on kidney tissue in experimental diabetic rats"** was written in accordance with the NEU Postgraduate thesis proposal and thesis writing directive.

| Prepared by Thesis | Supervisor |
| --- | --- |
| Tahani ELSAHLY | Prof. Dr. Aysel KÜKNER |

Prof. Dr. Aysel KÜKNER
Head of Histology and Embryology

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Thanks for believing in me.

**Dedication**

**To my parents…**

**Effect of protocatechuic acid on kidney tissue in experimental diabetic rats"**

Tahani Elsahly

Supervisor: Aysel KÜKNER

Department of Histology and Embryology

## ABSTRACT

**Aim:** The potential beneficial effects of Protocheuic acid on human health have aroused considerable interest and initially attributed to their antioxidant activities. Recent studies have shown that they have antioxidant and anti-hyperglycemic roles that have seen to ameliorate diabetes. In this study, protecheuic acid (PCA) extract was investigated to determine its effects on diabetes model of rats.

**Materials and Methods:** The rats were induced to be diabetic by administering streptozotocin. The rats were divided into four groups (Control, Diabetus Mellitus, Diabetus Mellitus + PCA and PCA). Rats were sacrificed after three weeks and tissues and blood samples were collected. Blood samples were tested for urea and creatine levels of the kidney. The kidney tissue was washed in tap water and dehydrated in ascending grades of ethyl alcohols, cleaned in xylene and finally embedded in paraffin wax at 60 °C. The paraffin was sectioned at 5-6 μm thickness and then stained with Periodic Acid Schiff (PAS) Stain, Masson Trichrome stain and Hematoxylin Eosin Staining methods and viewed under the microscope at X10 and X40 magnifications. The data extracted was analysed using Statistical Package for Social Sciences.

**Results:** A significant elevation of blood urea nitrogen (BUN) was found in the DM group. The significant increase was attributed to the STZ treatment however with PCA treatment there was a significant reduction of BUN levels (P=0.041). There was a significant increase in the serum creatine levels of the diabetic mice compared to the control group and the PCA group. The rats showed a significant improvement creatine levels in the DM+PCA treatment group when compared with the DM group as p=0.026 whilst the control and PCA group the calculated p value was 0.02. Histological examination revealed that there is no thickening of the proximal and distal tubule basement membranes and bowman membrane in the kidney tissue of the control group. In the diabetic group however, there was enlargement of the kidney cortex in a large number of tubules, irregularities in some proximal tubular epithelium, disfigured glomeruli and congestion in the tubular epithelial cells. In diabetic and PCA-treated group kidney tissue, it was determined that the enlargements in the tubules and inter-tubular congestion decreased. There was no increase in intraglomerular and extraglomerular connective tissue.

**Conclusion:** We demonstrated that PCA has the ability to ameliorate kidney damage caused by diabetes and therefore has a potential to be an adjuvant for diabetic therapy.

**KEYWORDS: Diabetes Mellitus, Kidney, Protocheuic Acid**

## ÖZET

**Amaç:** Protekheik asit insan sağlığı üzerindeki potansiyel yararlı etkileri büyük ilgi uyandırmış ve başlangıçta antioksidan aktivitelerine atfedilmiştir. Son çalışmalar, diyabetin iyileştirdiği görülen antioksidan ve anti-hiperglisemik roller ortaya koyduklarını göstermiştir. Bu çalışmada sıçanların diyabet modeli üzerindeki etkilerini belirlemek için protekheik asit (PCA) ekstresi araştırılmıştır.

**Gereç ve Yöntem:** Sıçanlar, streptozotosin verilerek diyabetik olarak uyarıldı. Sıçanlar 4 gruba (Kontrol, Diyabetik Mellitus (DM), DM + PCA ve PCA) ayrıldı. Sıçanlar 3 hafta sonra kurban edilmiş ve dokular ve kan örnekleri toplanmıştır. Kan örnekleri böbreğin üre ve kreatin düzeyleri açısından test edildi. Böbrek dokusu musluk suyunda yıkandı ve artan derecelerde etil alkollerde dehidre edildi, ksilende temizlendi ve son olarak 60 ° C'de parafin mumu içine gömüldü. Parafin 5-6 um kalınlıkta kesildi ve daha sonra Periyodik Asit Schiff (PAS) Lekesi, Masson Trikrom boyası ve Hematoksilin Eosin Boyama yöntemleri ile boyandı ve X10 ve X40 büyütmelerinde mikroskop altında incelendi. Elde edilen veriler Sosyal Bilimler için İstatistiksel Paket kullanılarak analiz edilmiştir.

**Sonuçlar:** DM grubunda kan üre nitrojeninde (BUN) anlamlı bir artış bulundu. Önemli artış STZ tedavisine bağlandı, ancak PCA tedavisi ile BUN düzeylerinde anlamlı bir azalma vardı (P = 0.041). Diyabetik farelerin serum kreatin düzeylerinde kontrol grubu ve PCA grubuna göre anlamlı bir artış vardı. Sıçanlar DM + PCA tedavi grubunda DM grubu ile karşılaştırıldığında p = 0.026 olarak anlamlı bir iyileşme kreatin seviyesi gösterirken, kontrol ve PCA grubu hesaplanan. Histolojik inceleme, kontrol grubunun böbrek dokusunda proksimal ve distal tübül taban membranlarında ve bowman membranında kalınlaşma olmadığını ortaya koydu. Bununla birlikte, diyabetik grupta çok sayıda tübülde böbrek korteksinin genişlemesi, bazı proksimal tübüler epitelde düzensizlikler, şekilsiz glomerüller ve tübüler epitel hücrelerinde tıkanıklık vardı. Diyabetik ve PCA ile tedavi edilen grup böbrek dokusunda tübüllerde genişlemelerin ve tübülerler arası tıkanıklığın azaldığı belirlenmiştir. İntraglomerüler ve ekstraglomerüler bağ dokusunda artış yoktu.

**Sonuç:** olarak, PCA'nın diyabetin neden olduğu böbrek hasarını iyileştirme yeteneğine sahip olduğunu ve bu nedenle diyabetik tedaviye yardımcı olma potansiyeli olduğu tespit edilmistir.



**ANAHTAR KELİMELER: Protocheuic Acid, Diabetes Mellitus, Böbrek**

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

**LIST OF ABBREVIATIONS**

DM (Diabetes mellitus)

WHO (World Health Organisation)

ROS Reactive Oxygen Species

AGE (Advance-Glycation End Product)

DN (Diabetic Nephropathy)

PCA (Protocatechuic Acid)

PTs (Proximal Tubules)

FS (Filtration slits)

BM (Basement membrane)

F (Fenestrations)

C (Capillary)

$P_1$ (Primary process)

$P_2$ (Secondary process)

E (Endothelium)

CS (Fused cytoplasm)

MC (Messengial cell)

MM (Molecular matrix)

BM (Basement Membrane)

PD (Podocytes)

L (Lumen)

EC (Endothelial cell)

US (Urinary Space)

STZ (Streptozotocin)

FBG (Fasting Blood Glucose)

MCE *(Myrciaria cauliflora* extract)

TAS (Total antioxidant status)

MDA (Malondialdehyde)

SOD (Superoxide Dismutase)

TNF-α (Tumor Necrosis Factor Alpha)

CHYS (Chaihuang-Yishen granule)

HPLC (High Performance Liquid Chromatography)

IP (Intraperitoneal injection)

PAS (Periodic Acid Schiff)

MT (Masson Trichrome stain)

HE (Hematoxylin Eosin Staining)

## 1. INTRODUCTION AND AIM

Diabetes mellitus (DM) is characterized hyperglycemia which is a disease of the endocrine system. The pathogenesis of this disease is characterized by defective secretion of insulin, insulin action, and in some cases both are known as systemic disease. The WHO notes that the number of diabetes-populated people is projected to rise from 171 to 300 million in 2030. The most prevalent demographic affected by this disease are the older age group above 45 years, but recent trends indicate that sedentary lifestyles and eating habits have increased in the younger age group as (20-34 years), which is why it is urgently needed to counter this disease. Patients with DM are vulnerable to long-term complications, such as nephropathy, retinopathy and neuropathy. Such long-term complications have led to a drop in the life expectancy of diabetic patients as two thirds of the general diabetes population die of these complications.

Hyperglycemia, characteristic of diabetes, is thought to generate reactive oxygen species (ROS), which leads in time to oxidative stress and micro-variety complications in several organs through activation of the pathway of polyol, hexosamine, advance-glycation end product (AGE) and activation of the protein kinase C (Brownlee, 2003). Glucose penetration into the kidneys can induce diabetic nephropathy (DN) because insulin is not regulating glucose. DN affects different types of cells, including glomeruli and mesangial and endothelial cells, interstitial cells, interstitial cell, tubular epithelial cells, and interstitial fibroblasting. DN is a step-by-step process that begins with hyperfiltration and microalbuminuria following deterioration in the renal function and changes in the structure of glomeruli (Reddy, 2004).

The kidney is a key organ. The primary function is the bloodstream elimination of waste and the control of water, electrolytes and oxygen. In contrast to many other body organ systems such as the skin, intestine, tumor and liver, which are subject to permanent cellular turnover (Pellettieri and Sánchez Alvarado, 2007), adult stem cell populations in the kidney have not yet been definitively identified following kidney development. To understand the etiology of diseases and identifying potential therapeutic strategies for their care it is important to continue to recognize and understand fundamental mechanisms that underlie the production of a functional renal system.

Protocatechuic acid derived from dried Hibiscus sabdariffa Linens flora (PCA, 3,4-dihydroxybenzoic acid),is a phenolic material. Studies on this compound showed that PCA

possessed some anti-carcinogenic properties (Yin et al., 2009; Yip et al., 2006; Lin et al., 2007), anti-hyperglycemia (Lin et al., 2009, Harini and Puge 2010), anti-oxidant and anti-inflammatory properties (Zhang et al., 2011; Sroka and Cisowski 2003; Liu et al., 2002). No scientific study on the impact of PCA on diabetes-induced nephropathy in the kidneys, which is also triggered by oxidative stress, has yet been done. This research will focus on the study of antihyperglycemic activities and the impact of Streptozocin with PCA on diabetic rats on diabetes-induced renal dies and other associated metabolic parameters.

## 1.1 Hypothesis

**$H_0$:** Protocatechuic acid does not have a protective effect on kidney tissue in streptozotocin -induced diabetic rats.

**$H_1$:** Protocatechuic acid does have a protective effect on kidney tissue in streptozotocin -induced diabetic rats.

## 1.2 Problem Statement

Kidney diabetic nephropathy causes substantially more deaths every year worldwide than kidney cancer (Luyckx et al 2017). The incidence of diabetic nephropathy is constantly increasing, despite efforts being made to help affected patients. Treatment options are becoming sparse and thus inversely increasing the healthcare costs. There is therefore need to search for new natural products that can work together with the available medicine in combination so as to regenerate the damaged tissues caused by diabetes. If kidney function is not restored this will cause major problems for the affected individuals as waste products cannot be excreted which would often lead to death.

## 1.3 Significance of Study

Since diabetes leads to damage of the kidney causing diabetic nephropathy we aimed to test whether PCA has the ability to restore cell morphology of the kidney cells and also the endocrinology system. The intended outcome is to also evaluate if PCA has the potential to ameliorate cell proliferation in the damaged tissue. The results will be highly significant in that PCA can be given to diabetic patient's inorder to combat their disease.

## 1.4 Study Aim

To evaluate the effect Protocatechuic acid on kidney tissue in streptozocin induced diabetic rates.

## 2.1 Anatomy of Kidney

The kidney is the organ that acts as a waste collector and disposal device in the body. It also helps to cleanse the blood and remove body toxins. Using the heart to filter the kidneys, up to one third of all the blood passes before it flows into the remaining tissues. Most people have a couple of kidneys but only one kidney or one working kidney is able to live with a person (Arooj et al., 2019). The loss of both renal tissues will cause waste and death to occur in a couple of days.

The convex and concave sides of each kidney are shown in Figure 2.1. On the concave side, the small opening that leads into the renal artery, into the renal vein and into the ureter is known as the renal hilum. Renal capsule comprised by connective tissue, envelops the entire surface of the kidney (Rodrigues-Díez et al., 2017).The kidney is easily identifiable in two major areas by the sagittal part of the organ: (i) external cortex and, (ii) deeper medulla area (Taal et al., 2011). The kidneys on the left and right of the spinal cord as Figure 2.1 shows.

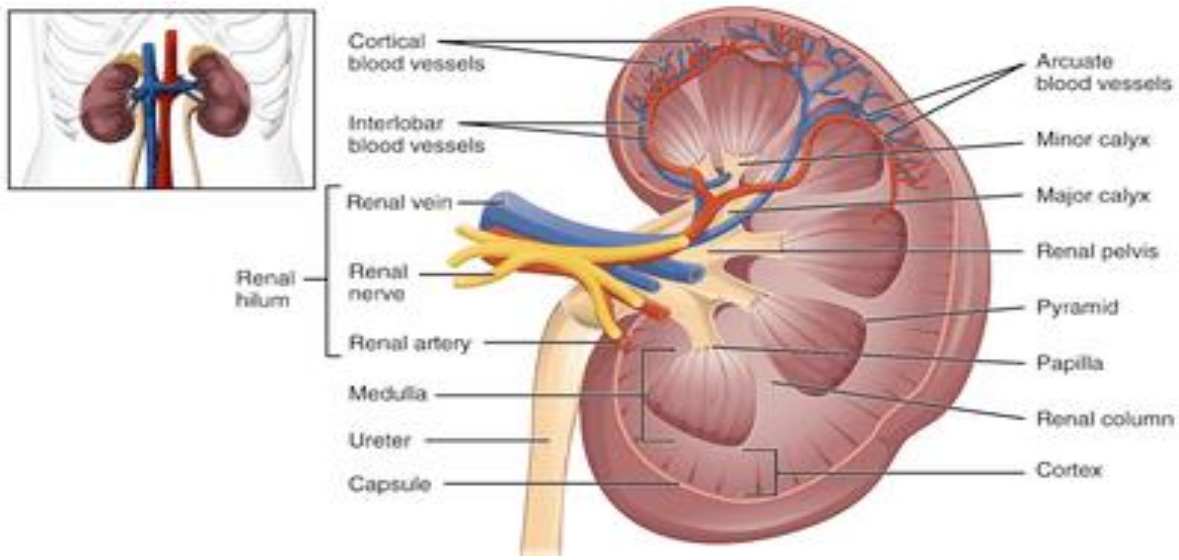


**Figure 2.1**: Illustration shows cross section of a human kidney - Image Credit: Anatomy & Physiology, Connexions Web site - http://cnx.org/content/col11496/1.6/

The kidney appears bean-like with a length of about 11-14 cm, a width of 6 cm and a thickness of 4 cm. The weight is 125-170 g per adult kidney in males and 115-155 g in females (Glodny et al., 2009). Via the renal artery, renal vein and the ureter, the kidney is connected to the blood system. Figure 1.1 also lets the ureter be easily recognized and the main function can easily be deduced on the kidneys. They filter the blood and produce the urine that will be put into the bladder using the

ureter. The most obvious part of this is that the kidneys are also responsible for other functions, such as electrolyte control, acid-base balance, and blood pressure.

There are various functions in the kidney structures shown in Figure 2.1 to ensure that the kidney works properly. The following is a short description of kidney structures:

i. Cortex: contains nephrons hence the appearance of reddish colour and is located at the the outer part.
ii. Medulla: comprises of renal pyramids and is the inner portion of the kidney. This area is striped red brown colour
iii. Renal pelvis: area at the centre which has a funnel-shaped basin (cavity) that collects urine into the ureter.
iv. Renal artery: Each kidney has renal artery and is the main vessel that supplies oxygenated to the kidney and nearby glands.
v. Renal vein: receives deoxygenated blood from the peritubular veins within the kidney and ureter to the inferior vena cava.
vi. Ureter: tube between kidney and bladder that allows urine to pass through.
vii. Calyce: these are parts of the kidney that collect urine before it passes further into the urinary tract. The calyces are part of the renal pelvis, a convex system of sinuses that connect the innermost part of the kidney to the ureters and, from there, to the bladder (Wan et al., 2012).

### 2.2 Kidney development

Three kidney systems, including pronephros, mesonephros and metanephros are used to develop the renal disease (Figure 2.2). The pronephros are the cranial set of tubes which will regress. Mesonephros is situated in the middle part of the embryo, and becomes mesonephric tubules, as well as a mesonephric duct. These tubules initially perform a certain kidney function, but then many tubules return. The mesonephric canal, however, continues and opens at the embryo's tail to the cloaca. The final and permanent kidneys are Metanephros. They start developing early as an outgrowth of the mesonephric caudal duct (uteric bud) as well as the blastemal metanephric condensation from the nearby renogenic intermediate mesoderm (Figure 2.2C).

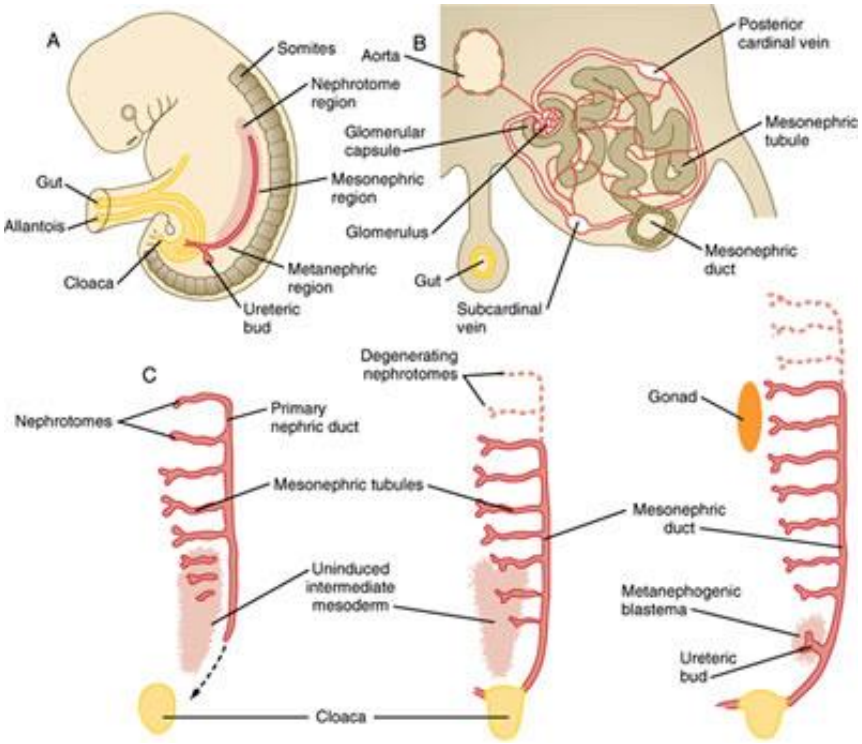


**Figure 2.2:** Development of the pronephros, the mesonephros, and the metanephros (Sadler, 2012).

The mesonephros slowly decreases early in the fifth week. At the end of the 9th week they start working when the metanephrons return entirely (Figure 2.2) and they start working. The cranial-caudal patterning establishes a "renogenic" region within the intermediate mesoderm in the tail of the embryo –this renogenic mesoderm is the metanephric blastemal. The metanephric blastema secretes growth factors that cause the ureteric budget to expand out of the caudal region of the mesonephric channel. The ureteric bud proliferate and respond by secreting factors of growth which stimulate proliferation and subsequent differentiation in glomeruli and renal tubules of the metanophric blasthema. Conversely, these structures can be doubled or are proliferated if the inductive factors increase in size.

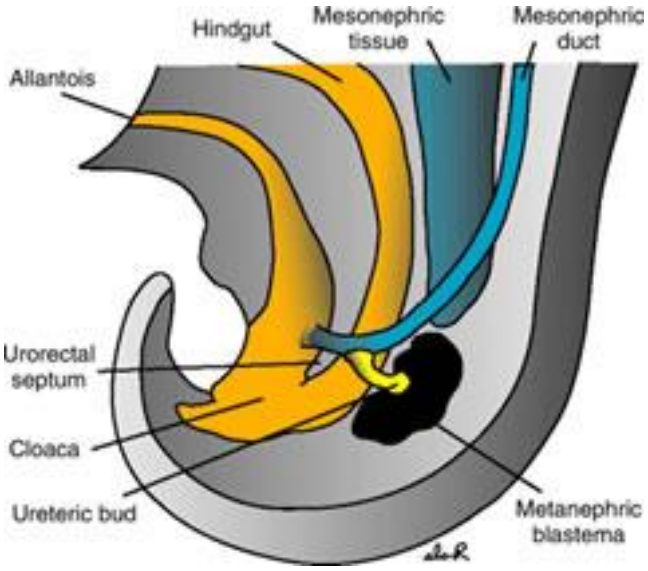


**Figure 2.3:** Schematic diagram of the formation of Uteric bud (Sadler, 2012).

The formation of metanephrons begins in the fifth week when the uteric bud from the distal portion of the mesonephric ducts. After four days the uteric bud penetrates the blastemal tissue. After penetration it bifurcates and each branch dilates at the end to form an ampulla. Each ampulla acquires a cap like aggregate of metanephric blastemal tissue which is bilobe in appearance. Each branch of the uteric bud divides further and further at the blastemal tissue (Ludwig and Landmann, 2005). At the end of each branch it will result in the mesonephros being lobulated. This lobulation usually fades during infancy because of: elongation of the proximal convoluted tubule and an increment of the interstitial tissue. When the uteric bud first contact with the metanephric blastemal its tip expands to form initial ampulla which later forms the renal pelvis.

During the 6th week the uteric bud bifurcates four times which will results in four generations of bifurcations that results in 16 branches. These branches will coalesce and forms the major calyx. In the 7th week further branching also occurs and bifurcation occurs four times having another four generations of bifurcations which will also coalesce and form a minor calyx. These branches continue to bifurcate until 32 weeks which results in approximately 11 additional generations of bifurcations which form between 1-3 million branches which are the future collecting tubules (figure 2.4). Therefore, the uteric bud gives rise to the: ureter, renal pelvis, major and minor calyces and 1 to 3 million collecting tubules.

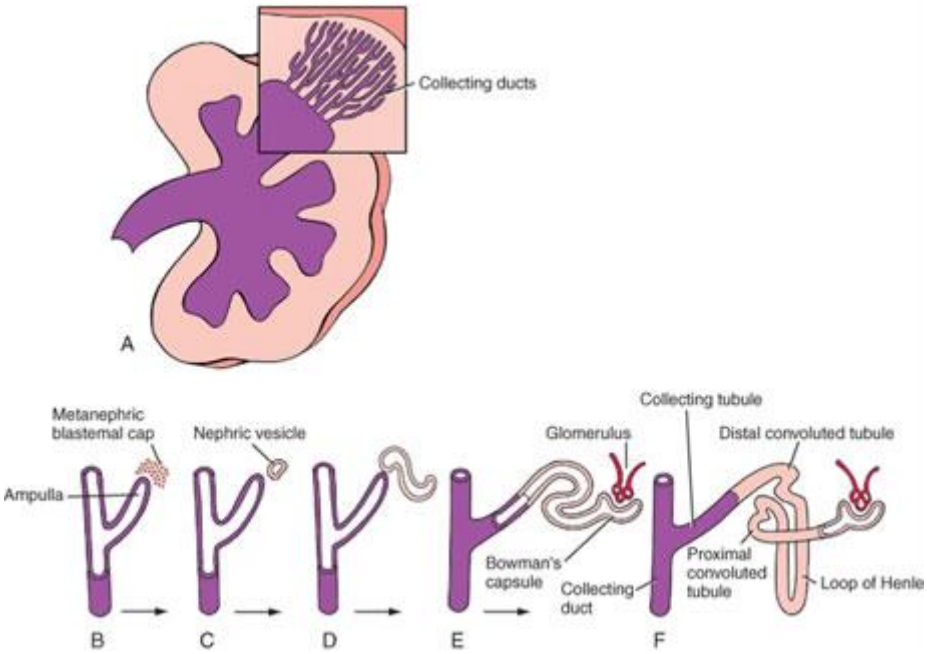


**Figure 2.4**: Bifurcations of the calyx into renal collecting system (A); Differentiation of the nephron (B-F) (Sadler, 2012).

The nephron originated from the metanephric mesoderm with the tubule being covered by a metanephric tissue cap. The uteric bud proliferates and secretes growth factors which enter the metanephric mesoderm and cause it to differentiate into nephron (Ludwig and Landmann, 2005; Krause et al., 2015).

Each newly formed collecting tubule is covered at its distal end by a metanephric tissue cap which differentiates into a renal vesicle. The renal vesicle gives rise to a small S-shaped tubules (figure 2.4D). The small S-shaped tubules will then expand at the end and form a structure known as the Bowman's capsule which has a tuft of capillaries that later forms the glomerulus. The other part of the S-shaped tubule fuses with the ureteric duct lumina and becomes a continuous lumina (Davidson, 2008). Continuous lengthening of the uriniferous tubule results in formation of proximal convoluted tubule, loop of Henle and the distal convoluted tubule (figure 2.4F). Formation of nephrons continues until birth until there is approximately 1 million in each kidney

### 2.3 Kidney Histology

The nephrons are the functional units of the kidney and there are around 1 million nephrons in each kidney. There are two types of nephrons: (i) Cortical nephrons (renal corpuscles in the outer cortex of Henle' loop short) (ii) Juxtamedullary nephrons: (%15) (composed of the renal corpuscle-deep in the cortex; Henle' loop long which extends deep into the medulla to the papilla) (Pocock and Richards, 2006). In short, each nephron is composed of several parts. (Figure 2.5).

1. **Renal corpuscle**
2. **Proximal tubule:** Long convoluted part; in the cortex; pars convolute. The shorter straight part ; enters into the medulla ; pars recta
3. **Thin loop of Henle** (in the medulla)
4. **Distal tubule**: Straight part; pars recta; enters the cortex; and the convoluted part which is in the cortex;

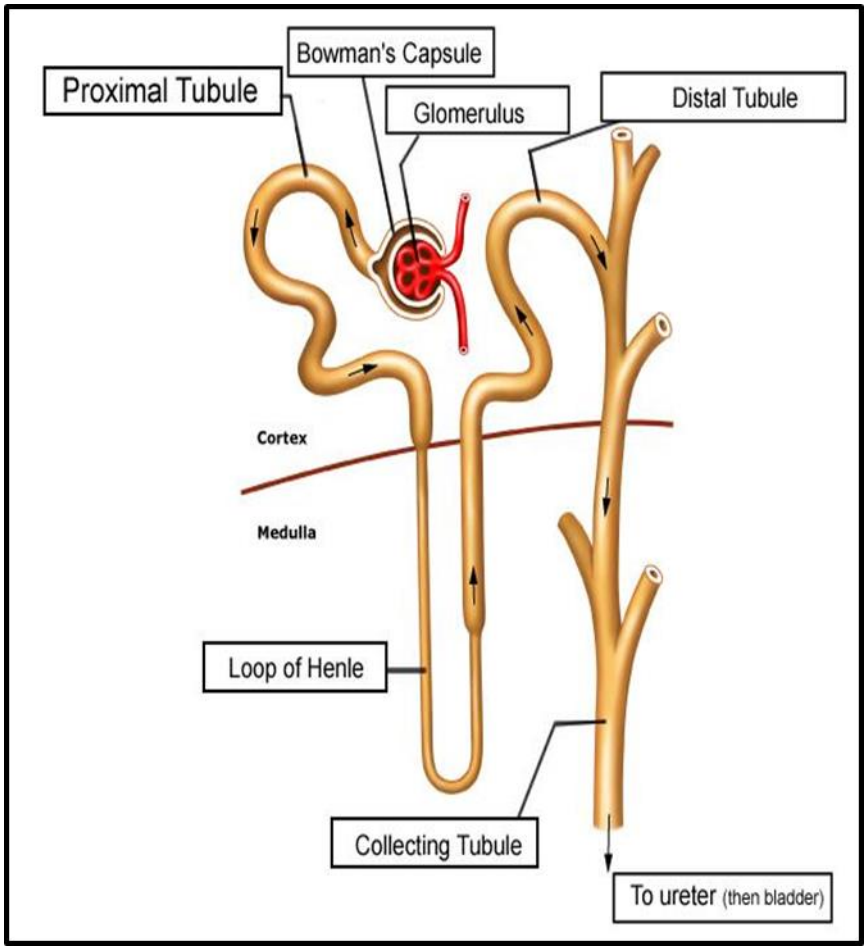


**Figure 2.5:** Illustration of Nephron. Adapted from Short et al., 2014.

### 2.3.1 Renal corpuscle

A round structure in the renal corpuscle, with a diameter of about 200 μm, is a glomerulus consisting of a tuft of capillaries enclosed by the capsule of Bowman. (Rodrigues-Díez et al., 2017). They are afferent and efferent aeterioles. The mesangium containing mesangial cells and its matrix is supported by the capillary tuft (Pocock and Richards, 2006). The capsule of Bowman consists of a parietal layer of a clear layer of epithelial squamous cells and a visceral layer of podocytes. The mesangial cells contain actomyosin to administer the filtration rate and to modulate it. It has prominent foot (pedicles) processes that cover the capillary walls (Figure 2.6). Small gaps, called filter slits, form a natural filter and the adjacent foot processes. Bowman's capsule comprises a parietal layer of squamous epithelial cells known as parietal (PECs) (Gagliardini et al., 2010).

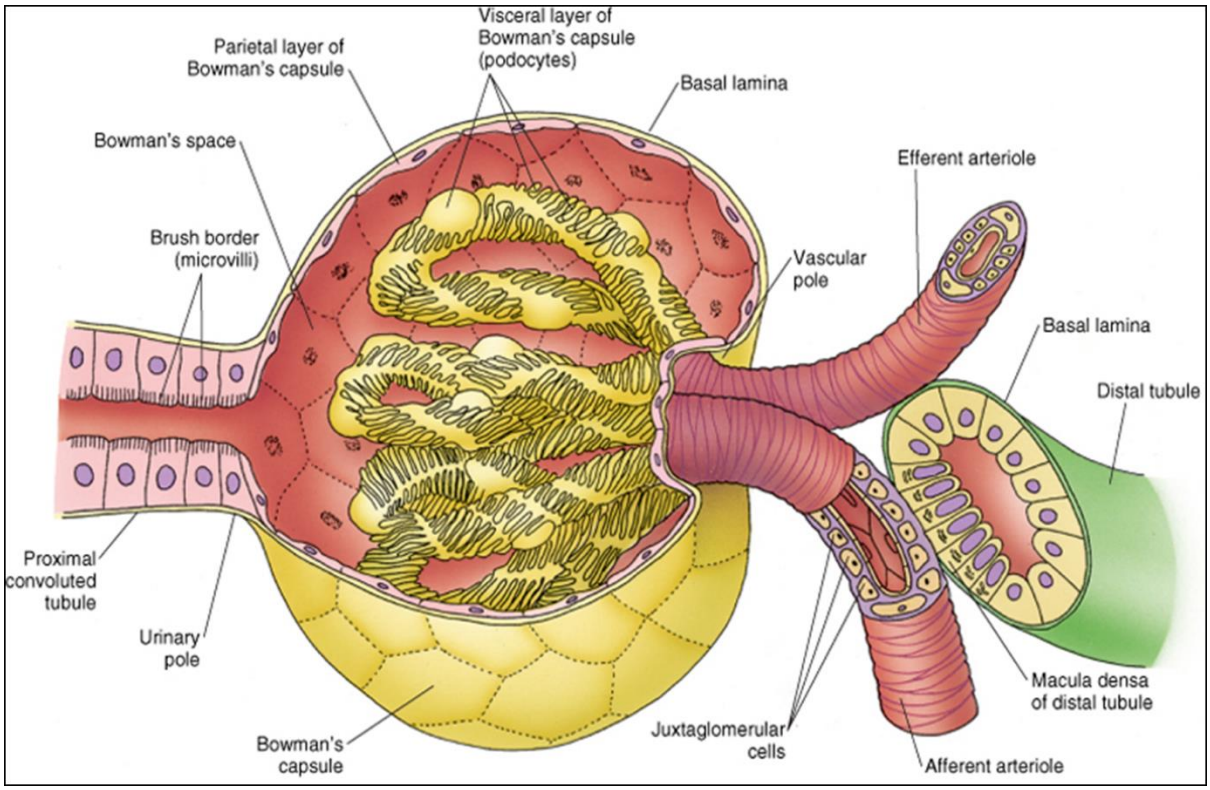


**Figure 2.6:** Illustration of Bowman's capsule (Pocock and Richards, 2006).

The glomerulus comprises a tuft of capillaries between the afferent and the efferent arterioles. As a consequence, ultrafiltrate pressure (through filtration barrier) passes into the space of the bowman from the glomerulus in the afferent arteriole. The capillaries are called fenestra and broader than other capillaries in the glomerular. 70-90 nm hence the pores are not passable for blood cells and platelets (Figure 2.7).

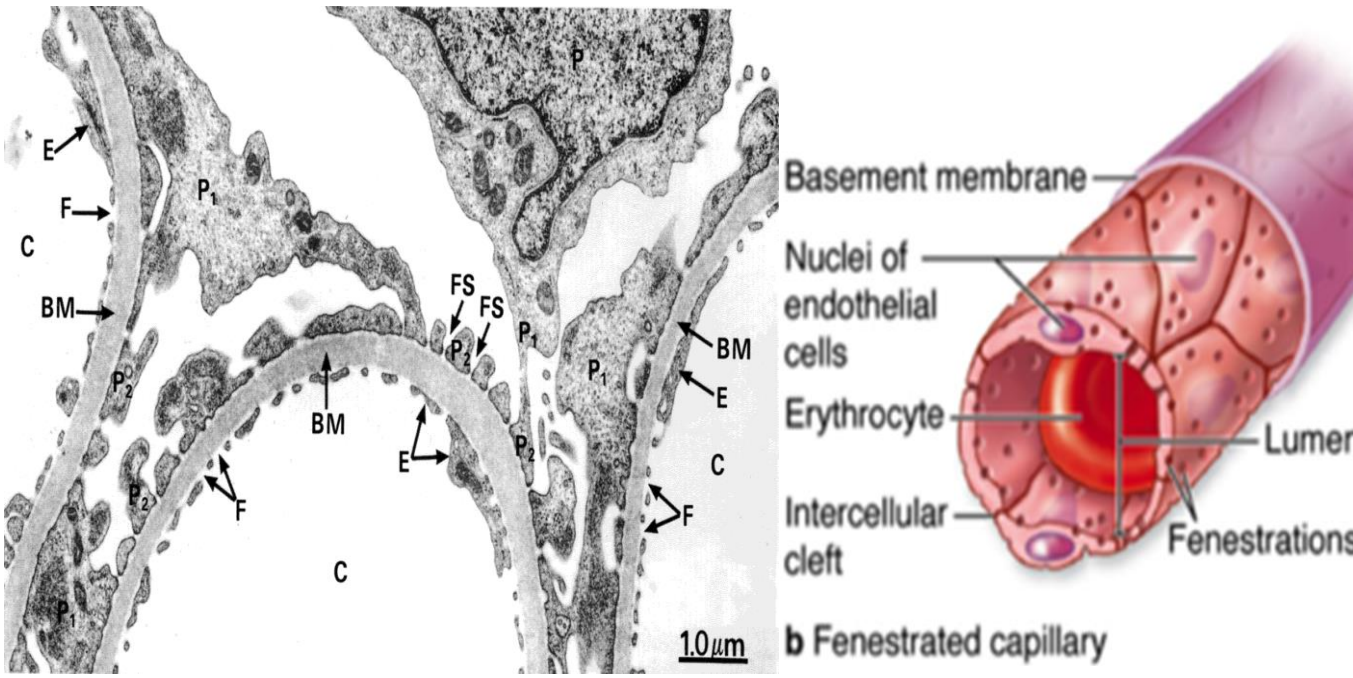


**Figure 2.7:** Illustration of Fenestrated capillary (Pocock and Richards, 2006)

FS: Filtration slits; BM: Basement membrane; F: Fenestrations; C: Capillary;$P_1$: Primary process; $P_2$: Secondary process; E: Endothelium

The visceral layer consisting of the podocytes is the largest nephronic cells. The podocytes ' basal lamina is fused to form the glomerular capillaries ' basal lamina, which permits filter exchanges for the podocytes ' cytoplasms and the glomerular capillaries ' cytoplasms (Figure 2.8).

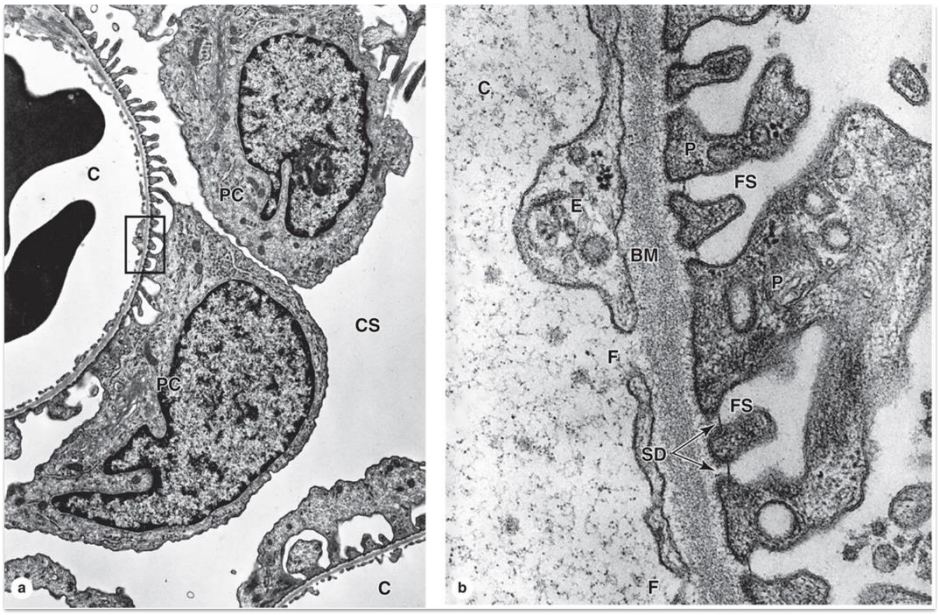


**Figure 2.8:** Ultrastructure of the glomerular filtration barrier. Triangle represents were the podocytes and glomerular filtrate have fused together. (Adopted from Jeffrey 2011)

FS: Filtration slits; BM: Basement membrane; F: Fenestrations; C: Capillary; E: Endothelium;

Filtration slits and Slit Diaphragms Bridge the slit pores between the pedicels. They have been modified and specialized tight junction which are zipper-like. Nephrons, other glycoproteins and proteoglycans create an electrochemical gradient which forms a negatively charged surface (Mescher, 2018). Therefore it does not allow the passage of small proteins and negatively charged molecules (organic anions) from the blood to the ultrafiltrate (Figure 2.9).

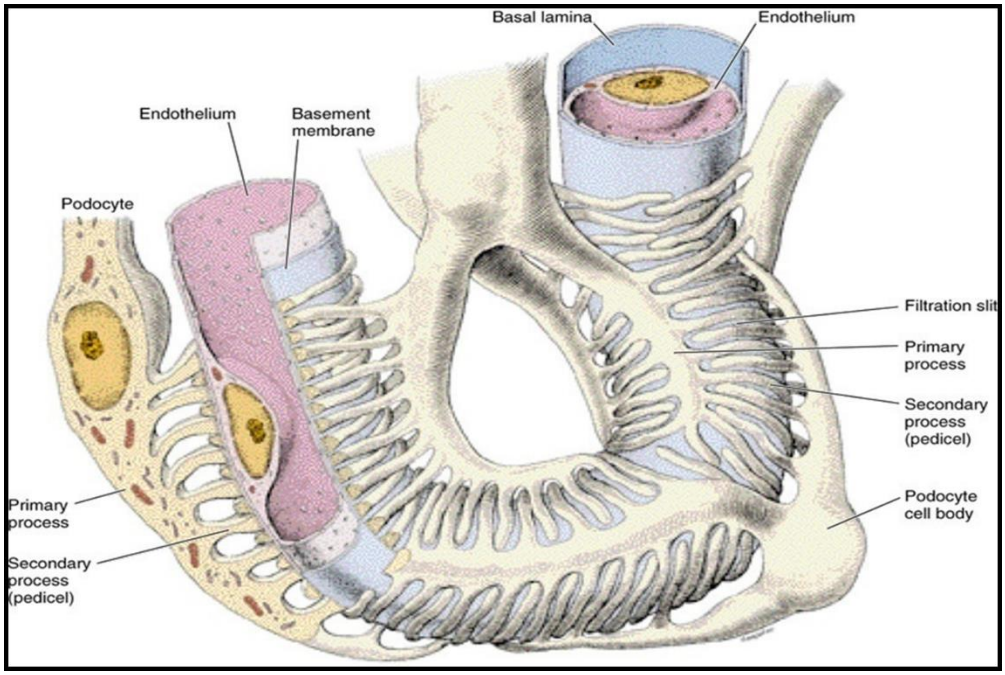


**Figure 2.9:** Filtration slits and Slit diaphragms (Tortora and Gerard, 2010)

Intraglomerular mesangial cells fill the spaces between the glomerular capilleries. They lie in the basal lamina of the capillaries. They provide physical support of glomerular capillaries. They resemble vascular pericytes which contract thereby controlling the amount of blood reaching the glomerulus (figure 2.10). They are also capable of phagocytosis by aggregating the proteins that trapped in the glomerular basal lamina during filtration. They are also responsible for secretion of some cytokines like prostaglandins and other factors for immune defense and repair of the glomerulus (Mescher, 2018).

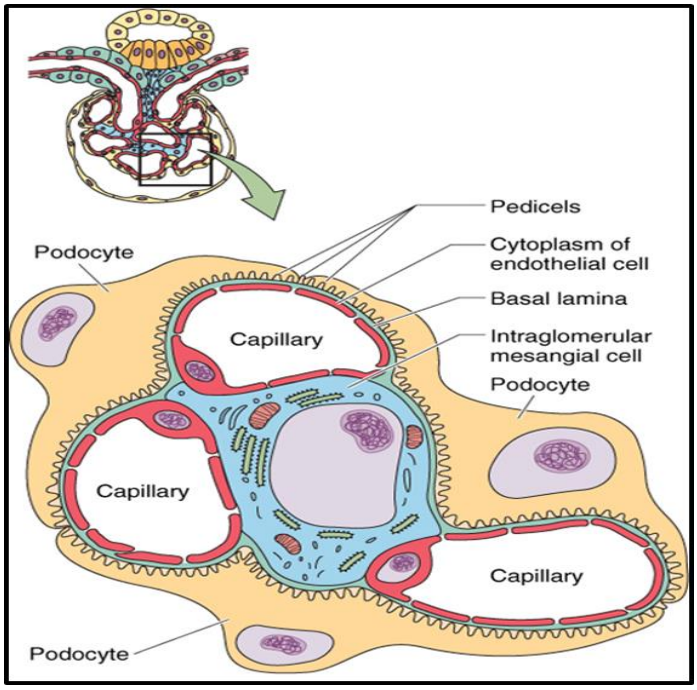


**Figure 2.10:** Intraglomerular mesangial cells (Tortora and Gerard, 2010)

### 2.3.2 Proximal tubule

The primary glomerular filtrate enters the proximal tubules (PTs). It consists of a convoluted part, being a continuation of the Bowman's capsule, followed by a straight part located in the medullary rays and in total measures around 14 mm. It lies in the cortex. It is composed of simple cuboidal epithelium. Numerous basal infoldings and lateral interdigitations are formed between them (Figure 2.11). They contain numerous mitochondria and the cells have long microvilli (brush border) which contain high numbers of aquaporin 1, a water channel, allowing for water reabsorption (Young et al., 2013).

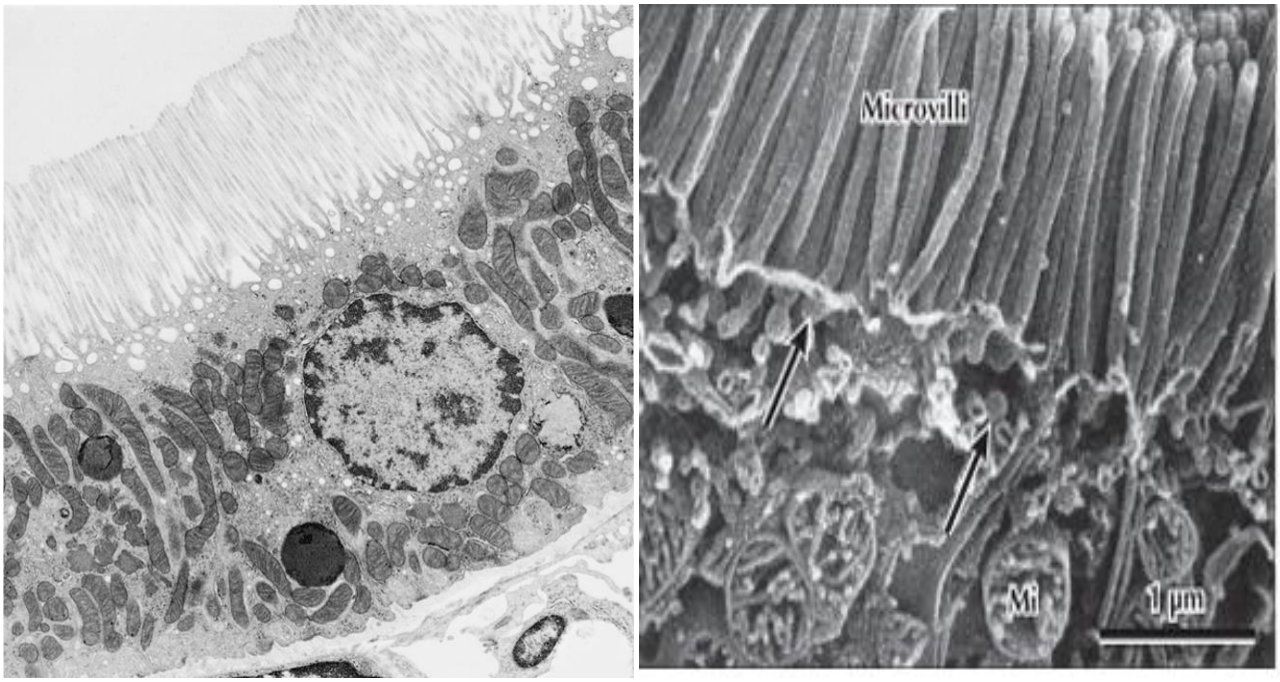


Figure 2.11 Electron micrograph of the wall of a proximal tubule (Young et al., 2013).

### 2.3.3 Thin loop of Henle

The loop of Henle that penetrates deep into the medulla where it doubles back in a hair pin loop. The descending thick portion of the Henle's loop comes from the straight portion of the proximal tubule and descends into the medulla and medullary rays. They have simple cuboidal epithelium cells that do not possess any microvilli. They facilitate in the active reabsorption of various electrolytes (Young et al., 2013). The thin portion of the Henle's loop lies in the medulla and contains simple squamous epithelium cells that have a few mitochondria. They facilitate in the Passive reabsorption of Na+ and Cl-. The Straight portion of the distal tubule which passes through the medulla and medullary rays consists of simple cuboidal epithelium with no microvilli. They function in active reabsorption of various electrolytes.

### 2.3.4 Distal convoluted tubule

The distal tubule is the next segment of the nephron connecting to the collecting duct. It lies in the cortex and consists of simple cuboidal epithelium cells which are smaller than the proximal convoluted (figure 2.12). They have a larger lumen and short microvilli and basolateral folds. Their main function is reabsorption of electrolytes.

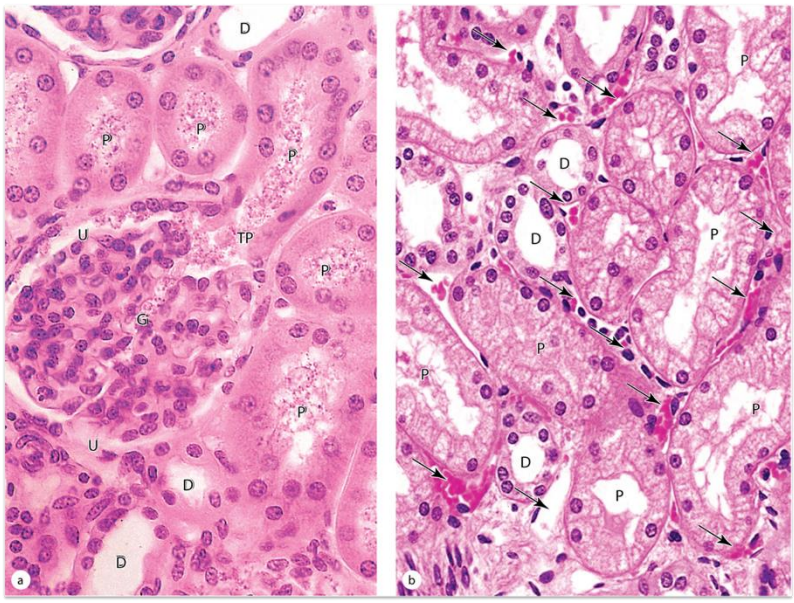


**Figure 2.12:** Illustration of Distal convoluted tubule adopted from (Young et al., 2013)

G: Glomerulus; P: Proximal convoluted tubules; D: Distal convoluted tubule

## 2.4 Kidney Physiology

Creatine, ammonia, uric acid and contaminants are regulated from the kidney, and water reabsorption and essential nutrients are also regulated through filtration of blood to extract metabolic waste products for excretion. The filtration of the blood in the glomerulus begins and results in urinary production (Rodrigues-Diez et al., 2017). Filtration barrier that divides blood and the space of Bowman is formed by 3 layers: foot processes from the diaphragm pores, basement membrane of the glomerular and the endothelium (Satchell and Braet, 2009).

Also molecules with smaller molecular dimensions than albumins (68kDa) pass through the filter and enter space of the bowman under physiological conditions and form the glomerula. The glomerula passes through the sequential parts of the renal pipe, where the heavily controlled reabsorption and secretion procedures are modified. The ultra-filtrate also contains useful metabolic solutes which require reabsorption together with toxic products and waste.

The largest part of the ultra-filtrate reuptake (65 to 70 percent) occurs in the proximal tube that can return to the bloodstream through the capillaries via water, electrolytes, amino acids, glucose, vitamins and others. Those involve creatine, amino acids, phosphate, lactate, and citrate, on sodium co-transporters (membrane proteins that bind the passage of two or more different solutes together)

that transfer sodium down its electrochemical gradient through epithelial tubule cells (Rodrigues-Diez et al., 2017). The filtrate then joins the Henle loop (descending and rising limbs), which is responsible for separating or diluting the tubular fluid using a multiplying mechanism called countercurrent. At a concentration that retains body fluid homeostasis, the distal convoluted tubule and accumulating ducts are then primarily responsible for the reabsorption of water as needed to manufacture urine (Subramanya and Ellison, 2014).

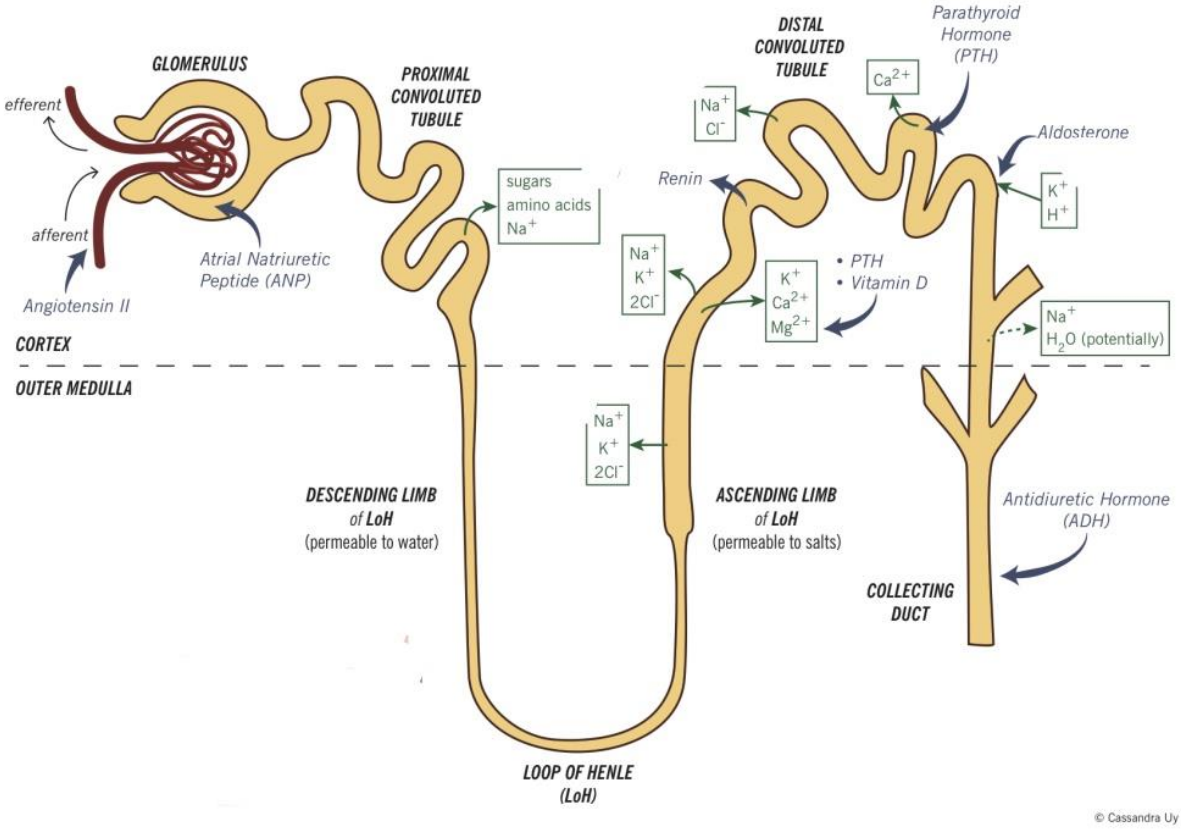


**Figure 2.13:** Illustration of the nephron and the mechanisms of reabsorption. (Adopted from http://www.pathophys.org/diuretics/mpr-nephron/)

The kidneys play a major role in regulation of blood pH, amount of water and electrolyte control. Homeostasis is also important. Kidneys can also control blood pressure by producing the renin proteolytic enzyme secreted which controls the nephron by means of juxtaglomerular cells of the apparatus. Renin causes blood pressure to rise in response to hypertension by activating the renin-

angiotensin pathway, a hormonal cascade that regulates homeostatic arthriology. (Rodrigues-Diez et al., 2017).

The kidney also has several endocrine functions. Erythropoietin and calcitriol are the two main hormones produced by the kidney. The first is a peritubular interstitial fibroblast-secreted glycoprotein that activates red blood cell development to respond to anemia. It can also make vitamin D through the proximal tubular cells. The involvement of Calcitriol in distal convoluted tubules and collection ducts has been shown to have been involved both in suppressing the development of the renin and preserving calcium homeostasis (Wang et al., 2012; Freundlich et al., 2008).

## 3.1 Materials and Methods

### 3.1.1 Animals

Eight-week-old Wister male rats with a body weight of between 150–200 g at the beginning of the study were used in studies. The rats were kept at room temperatures (22-25 °C) with 12 h light/12 h dark cycles and they received a standard pellet diet and had free access to water throughout the study period. Permission to perform animal experiments was sort from the Institutional Animal Ethics Committee. (Near East University Animal Ethics Committee 2020/109)

### 3.1.2 Diabetes Induction

Diabetes was induced by injecting the potent alkylating agent streptozotocin intraperitoneal. The rats were fasted for four hours and then induced for diabetes by injecting with STZ-Na-Citrate solution which was freshly prepared and injected. One rat was anesthetized and when its breathing had slowed down it was removed and injected with STZ solution at a dosage of 60mg/kg rat (Jelodar et al., 2007). After induction the rats were awoken and put back into the cage and supplied with 10% sucrose water overnight to avoid sudden hypoglycemia post injection. The rats were tested for sufficient levels of hyperglycemia two days after injection and those with glucose levels above 250 mg where termed severely diabetic.

### 3.1.3 Experimental design:

The rats which were diabetic at three week days were then randomly divided into the following groups for treatment:

- Group I (control): this will be normal rats (n=6).
- Group II (DM): animals that received STZ injection (60mg/kg) (n=6)
- Group III (DM+PCA): PCA for 3 weeks (n=6)
- Group IV (PCA): PCA for 3 weeks (n=6)

### 3.1.4 Data collection.

All rats were fasted before scarification. Ketamine- xylazine (90:10 mg/kg BW) was injected into the animals as anesthesia. Blood collected was collected through cardiac puncture and centrifuged for plasma collection. Tissues and blood samples were collected.

### 3.1.5 Histological Preparation

The kidneys were cut along the mid dorsal plane into small sections of 2mm and were immediately fixed in 10% neutral formalin for preservation. The tissue were further washed by running tap water for about 2 minutes and then dehydrated in ascending grades of ethyl alcohols, cleaned in xylene and finally embedded in paraffin wax at 60 C° using (LEICA EG1150 H tissue processor). The paraffin sections were cut to a size of about 5-6 µm thick using (digital microtom (LEICA BM225).


**Figure 3.1:** LEICA EG1150 H tissue processor on the right used for embedding and the LEICA BM225 used for sectioning.

### 3.1.6 Periodic Acid Schiff (PAS) Stain (Bio-Optica- 04-130808-Milan-Italy)

1. The PAS stain was done using the method developed by Bio-Optica as shown below:
2. Section was rinsed using distilled water.
3. 10 drops of reagent A (Periodic Acid Solution) were put on the slide and left for 10 minutes for the reaction to act.
4. The slide with the section was washed in distilled water.

5. 10 drops of reagent B (Schiff reagent Hotchkiss McManus) was poured and left to act for 10 minutes.
6. Section was rinsed using distilled water.
7. 10 drops of reagent C (Potassium metabisulphite solution) was added and left to act for 5 minutes.
8. Slides were drained and 10 drops of reagent D (Fixative Solution) was added and left to act for three minutes.
9. Section was rinsed using distilled water.
10. 10 drops of reagent E (Mayers Hemalum) was poured and left to act for three minutes.
11. The slide with the section was washed in tap water.
12. The slide was dehydrated by applying a series of ascending alcohols and then cleared with xylene before mounting.

### 3.1.7 Masson Trichrome stain (Bio-Optica 04-010802-Milan-Italy)

The Masson Trichrome stain was done using the method developed by Bio-Optica as shown below:

1. The section was rinsed using distilled water before the staining procedure.
2. 6 drops of reagent A (Weigert's iron hematoxylin A solution) and 6 drops of reagent B (Weigert's iron hematoxylin - B solution) were dispensed onto the slides with sections and left for 10 minutes.
3. The slides were then drained without washing and 10 drops of reagent C (Picric acid alcoholic solution) was added on the section and incubated for four minutes.
4. The slides were then washed with distilled water and 10 drops of the reagent D (Ponceau acid fuchsin according to Mallory) was poured and left to act for four minutes.
5. The section was rinsed under distilled water and 10 drops of the reagent E (Phosphomolybdic acid solution) was poured and left to act for ten minutes.
6. The slides were then drained without washing and 10 drops of reagent F (Masson aniline blue) was added and left to act for five minutes.

7. The slide with the section was washed in distilled water
8. The slide was dehydrated by applying a series of ascending alcohols and then cleared with xylene before mounting.

### 3.1.8 Hematoxylin Eosin Staining (Hematoxylin: HX69657153, Eosine: HX69574839, Merck, Germany)

The method employs the use of two staining rapid solutions: the hematoxylin No.3, nuclear staining, and the Eosin alcoholic solution, a cytoplasmic and connective tissue staining.

1. Tissues were incubated for 90 minutes in an oven at 60 degrees Celsius for drying tissue
2. The tissues were kept in xylol for 1 minute at room temperature.
3. After incubation in xylol, tissues were further kept in 100% (v/v) alcohol for 1 minute.
4. The slides were then transferred to 80% (v/v) alcohol and left for 1 minute.
5. After the alcohol treatment the tissues were kept in distilled water for 1 minute.
6. The slides were then taken to the haematoxylin solution and kept in for 1 minute.
7. Acid alcohol was then added for 30 seconds to remove excess stain.
8. Tissues were then transferred to 80% (v/v) alcohol.
9. The slides were then taken to the Eosin solution and kept in for 1 minute.
10. The slides were first transferred to 100% (v/v) alcohol for 30 seconds and then into xylol before mounting.

### 3.1.9 Light Microscopy

After the staining procedure the kidney sections were viewed under the microscope at x10 and X40 magnifications.

### 3.1.10 Data Analysis

The data extracted was analyzed using Statistical Package for Social Sciences (IBM SPSS version 21) and a P value less than 0.05 was considered as statistically significant. SPSS is a Windows based program that can be used to perform data entry and analysis and to create tables and graphs.

This program (SPSS) performs statistical calculations automatically given the researcher has correctly entered the variables under study. The data was analysed using Kruskal Wallis test and where there was a difference a Mann Whitney U test was used.

## 4.1 RESULTS

### 4.1.1 Biochemical Analysis

A significant elevation of blood urea nitrogen (BUN) was found in the DM group. The average mean was 82.8 which was twofold above the control group. There was a significant increase observed in the serum creatine levels of the diabetic rats compared to the control group and the PCA group as the creatine level for DM group was 0.77 whilst that for PCA and control group were 0.31 and 0.32. The rats showed a significant improvement in the DM+PCA treatment group when compared with the DM group as the urea levels fell down to 42.6 and creatine was 0.48 (Table 4.1).

**Table 4.1: Biochemical values in groups**

| Experimental Groups | Urea | Creatine |
|---|---|---|
| Control (n:6) | 34.1±3.86 | 0.32± 0.05 |
| PCA (n:6) | 31.8±4.70 | 0.31± 0.03 |
| DM (n:6) | 82.8±7.67* | 0.77± 0.10* |
| DM + PCA (n:6) | 42.6±6.34*# α | 0.48± 0.05*# α |

Values are mean ± SD of six rats from each group

*values when compared to control statistically significant p<0,05 .

α values when compared to DM statistically significant p<0,05.

# values when compared to PCA statistically significant p<0,05.

Abbreviations: PCA, Protocatechuic acid; DM, Diabetes Mellitus; DM+PCA, diabetic treatment group with Protocatechuic acid. Values are means ± SD (n=6)

The urea and creatine levels are indicators of renal function and should be monitored to determine the status of a healthy kidney. In our study a close relation was observed in the groups between urea and creatine levels. The results are displayed in figure 4.1 and figure 4.2.

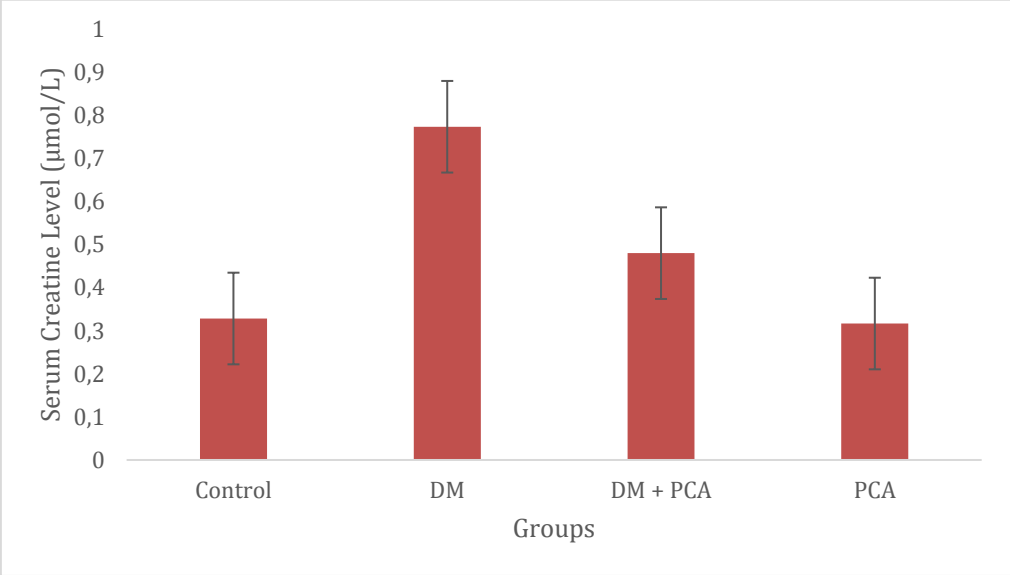


Figure 4.1: Serum creatine level as expressed as means and Standard error bars.

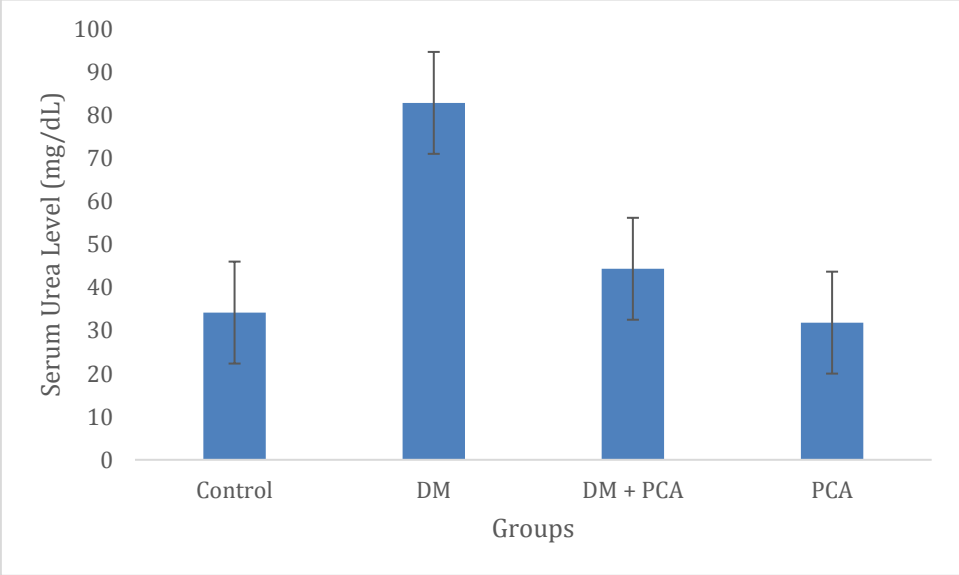


Figure 4.2: Serum Urea Level expressed as means and standard error bars.

### 4.1.2 Statistical Analysis of Biochemical results.

#### 4.1.2.1 Kruskal Wallis test Analysis

The Kruskal Wallis test was performed to see whether there was a difference between the biochemical parameters of the groups. The mean ranks for urea and creatine readings showed that DM group had the highest mean rank of 21.50 for urea and creatine followed by that of DM+PCA

which was 14 for urea and 15.42 for creatine. The PCA group had a mean rank of 6.17 for urea and 5.83 for creatine. The control group mean rank was 8.33 for urea and 7.25 for creatine.

**Table 4.2: Mean ranks data analysis**

***RANKS***

| | Groups | N | Mean Rank |
|---|---|---|---|
| ***UREA*** | Control | 6 | 8.33 |
| | PCA | 6 | 6.17 |
| | DM | 6 | 21.50 |
| | DM+PCA | 6 | 14.00 |
| | Total | 24 | |
| ***CREATINE*** | Control | 6 | 7.25 |
| | PCA | 6 | 5.83 |
| | DM | 6 | 21.50 |
| | DM+PCA | 6 | 15.42 |
| | Total | 24 | |

**Table 4.3: Kruskal Wallis Analysis**

| | **UREA** | **CREATINE** |
|---|---|---|
| **CHI-SQUARE** | 16.9 | 19.4 |
| **DF** | 3 | 3 |
| **SIGNIFICANCE LEVEL** | 0.001 | 0.000 |

### 4.1.2.2 Mann-Whitney U test

The Mann-Whitney U test was used to determine in which groups the significant difference occurred. If the $P < 0.05$ we would reject the null hypotheses and if we found that $P \geq 0.05$ we don't reject the null hypothesis. The null hypothesis states that there is no significant difference in the biochemical parameters whilst the alternative hypothesis states that there is a significant difference in the biochemical parameters.

Comparison of the control group and the PCA group there was no significant difference in the urea and creatine levels since the calculated significance for both urea (p=0.39) and creatine (p=0.589) were above $p \geq 0.05$ indicating that results were similar for both groups. The non-parametric tests for comparing the control group and the DM group showed that there was a significance difference in the urea and creatine levels as the calculated significance was for urea (p=0.002) and creatine (p=0.002) which is less than $p < 0.05$ and shows that the biochemical parameters are significantly different.

The control group and the DM+PCA showed that the difference observed was statistically significant in values obtained in urea and creatine levels as the calculated significance was for urea (p=0.041) and creatine (p=0.002) which is less than $p < 0.05$ indicating that there was a good improvement in the treatment improving the levels. The PCA and DM+PCA showed that there was a significant difference in the biochemical parameters. There was an improvement in the treatment as the calculated p value for urea was p=0.026 and that for creatine was p=0.002. The comparison between DM and DM+PCA showed that for both urea and creatine levels the p calculated value was 0.002 which indicates a significant difference showing that treatment with PCA improved the biochemical parameters.

### 4.1.3 Histological examination

Normal structure glomerulus and tubules are seen in Figure 4.3. There is no thickening of the proximal and distal tubule basement membranes and Bowman membrane (Figure 4.4) in the kidney tissue of the control group. Bowman membrane and basal membrane around the tubules (arrow) appear in normal thickness. With a compact cell structure the morphology of the renal tubules, interstitium and glomerulus remained regular. The collector, proximal and distal tubule parts in the medulla (Figure 4.5) are normal. There is no increase in connective tissue seen in both figure 4.5 and 4.6. Glomerulus and tubules appear normal structure.

It is seen that basal membrane structure is similar to the control group only in the group with PCA. Only in the group with PCA, kidney structures were similar to the control group (Figure 4.7, 4.8 and 4.9). It is seen that there is no increase in connective tissue between the tubules in the PCA-only group (figure 4.9). In the PCA-only group the kidney structures were similar to the control group.

In the diabetic group significant morphological changes were observed. The enlargement of the kidney cortex in a large number of tubules was observed (Figure 4.10). In the diabetic group the renal tissues exhibited major morphological alterations in the glomerulus and the proximal tubules. Irregularities in some proximal tubular epithelium and cell rash in the lumen, disfigured glomeruli (Figure 4.11), there is apoptotic bodies (Figure 4.12). In the diabetic group, congestion is observed in glomeruli capillaries and inter tubular capillaries (Figure 4.13). Connective tissue increased and fibrosis are not observed. In Figure 4.14 no connective tissue increase was observed in the cortex and medulla.

In diabetic and PCA-treated group kidney tissue, it was determined that the enlargements in the tubules are reduced (Figure 4.15) and inter-tubular congestion decreased (Figure 4.16). Restored function of the morphological structure of glomerulus and distal convoluted tubules was observed. There was no increase in intraglomerular and extraglomerular connective tissue (Figure 4.17) in the diabetic group treated with PCA.

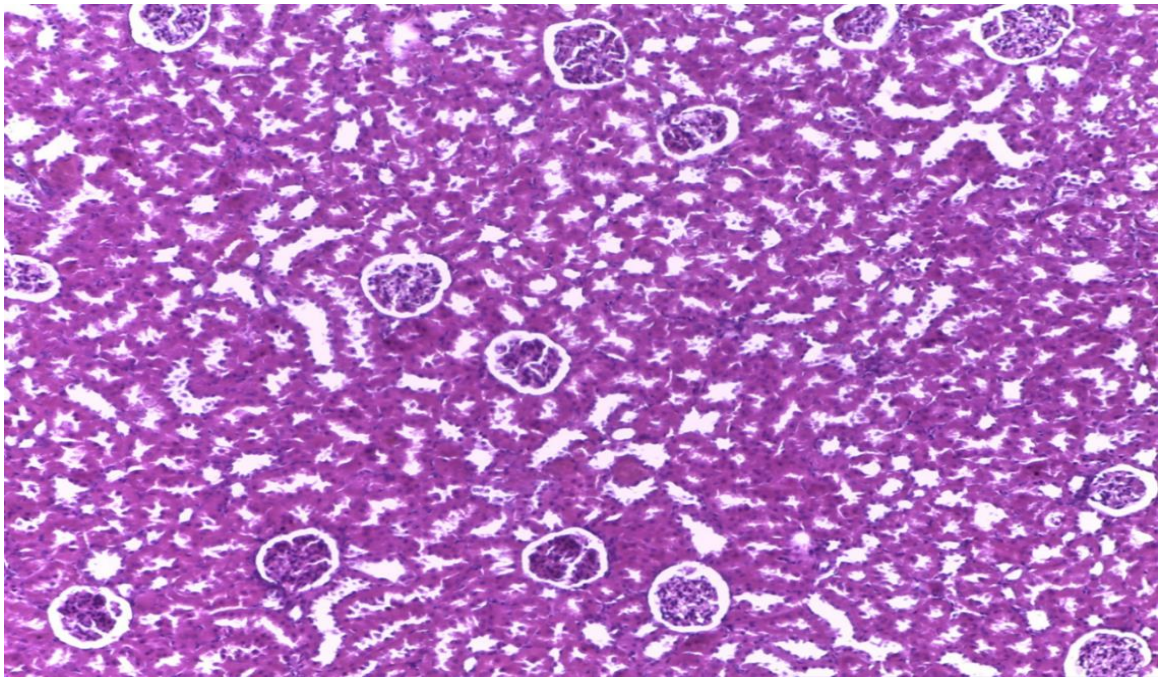

Figure 4.3: Control kidney tissue. Normal structure glomerules and tubules. HE staining x10

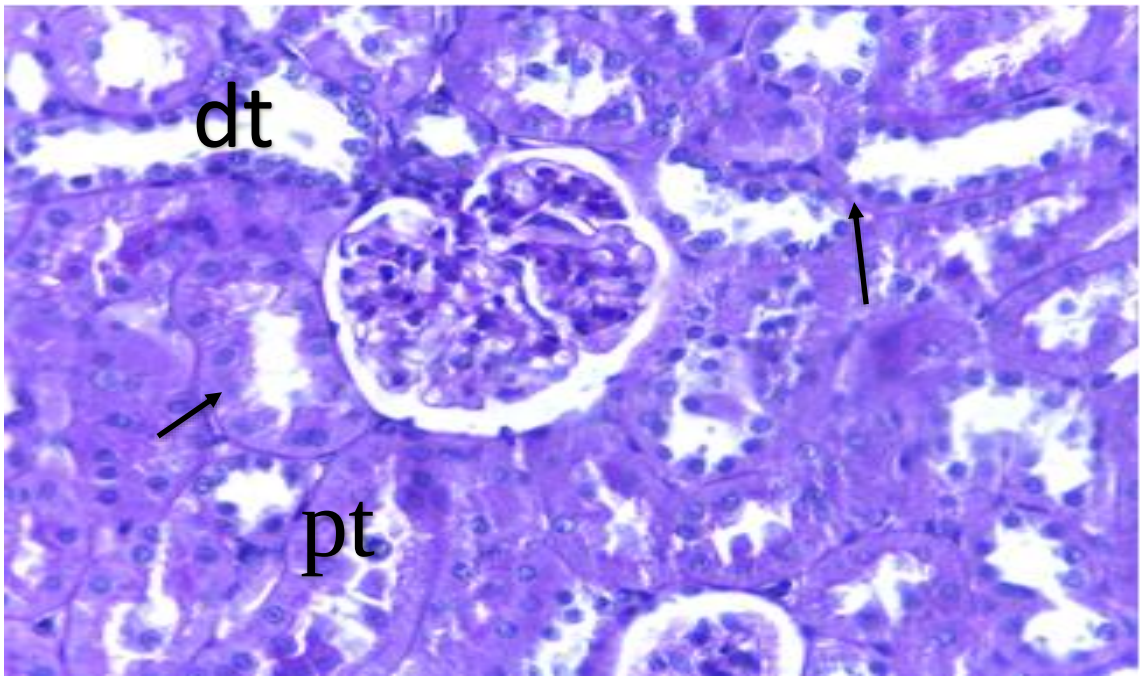


Figure 4.4. Kidney cortex tissue of the control group. Bowman membrane and basal membrane around the tubules (arrow) appear in normal thickness. Proximal tubules (pt), distal tubules(dt). PAS staining x40.

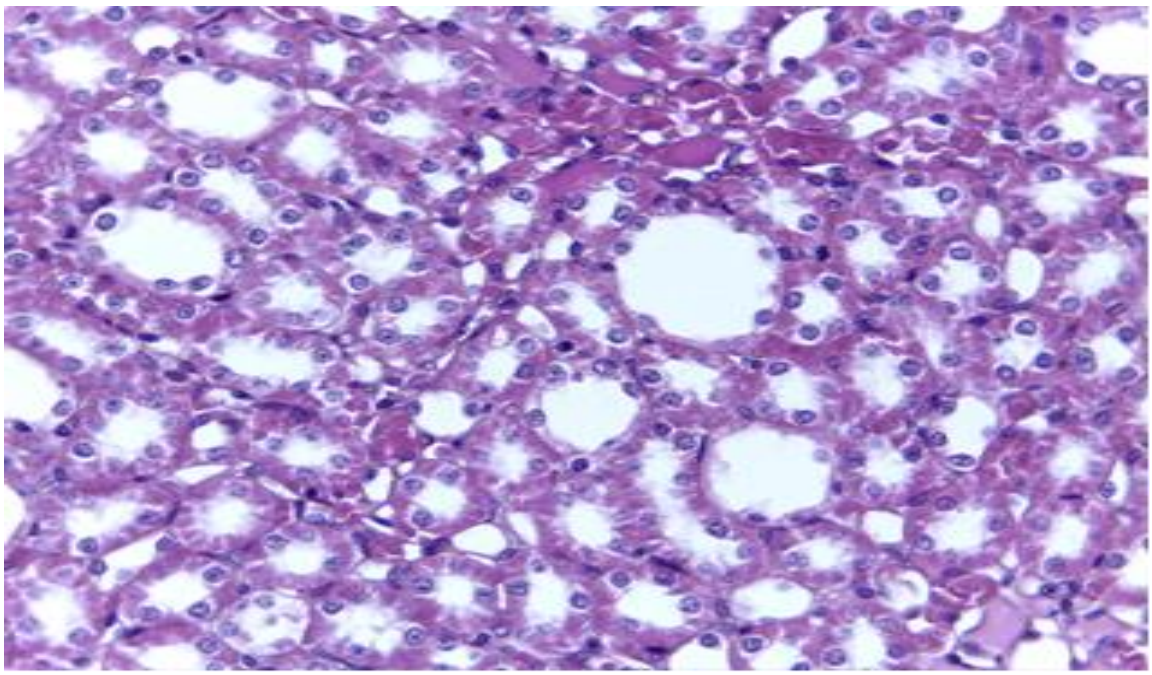

Figure 4.5. Kidney medulla tissue of the control group. HE staining x40.

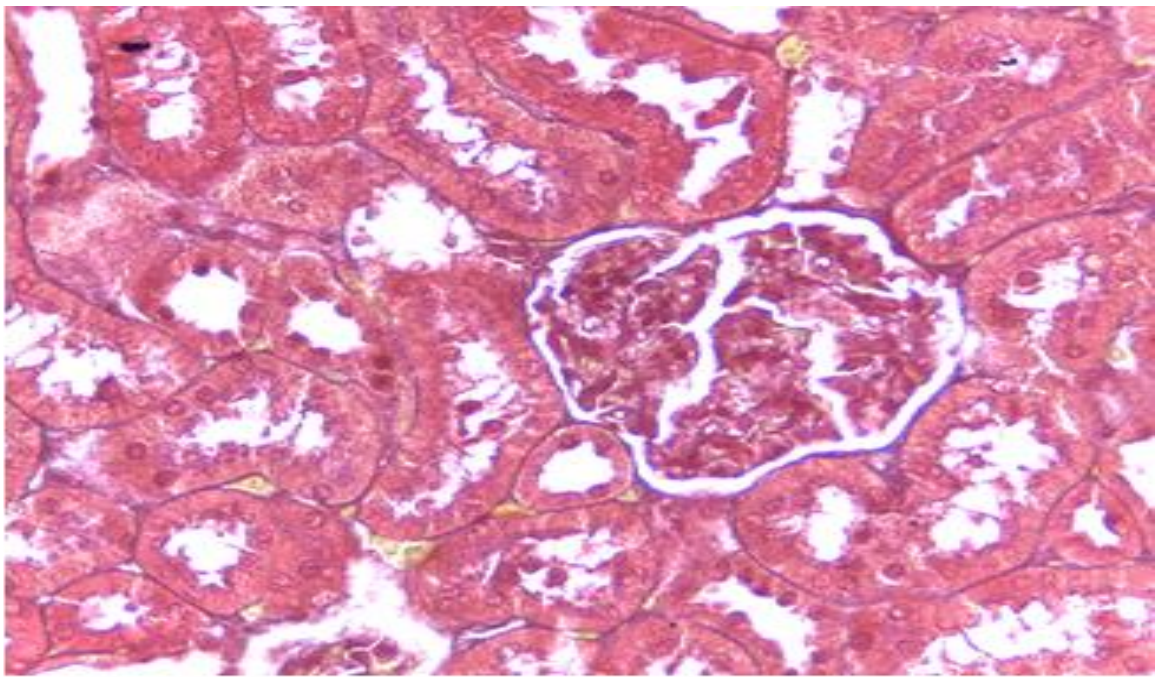

Figure 4.6. There is no increase connective tissue in the kidney cortex tissue of the control group. Masson trichrome staining x40.

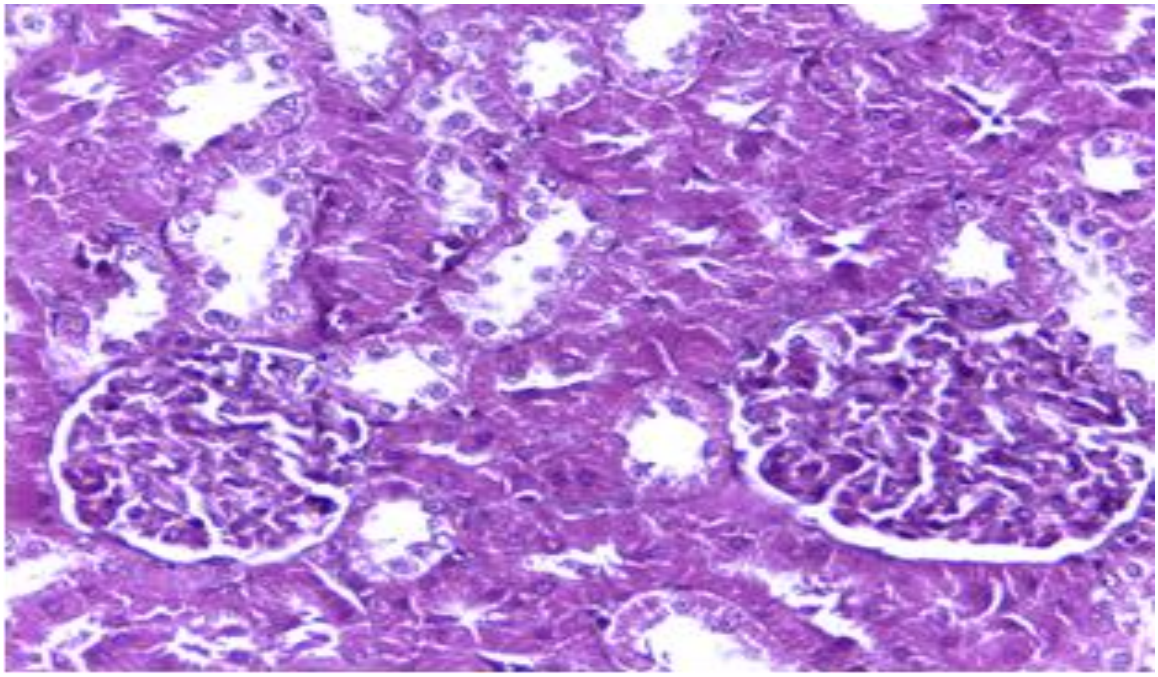

Figure 4.7. Kidney tissue of only PCA treatment group. Glomerulus and tubules appear normal structure. HE staining x40.

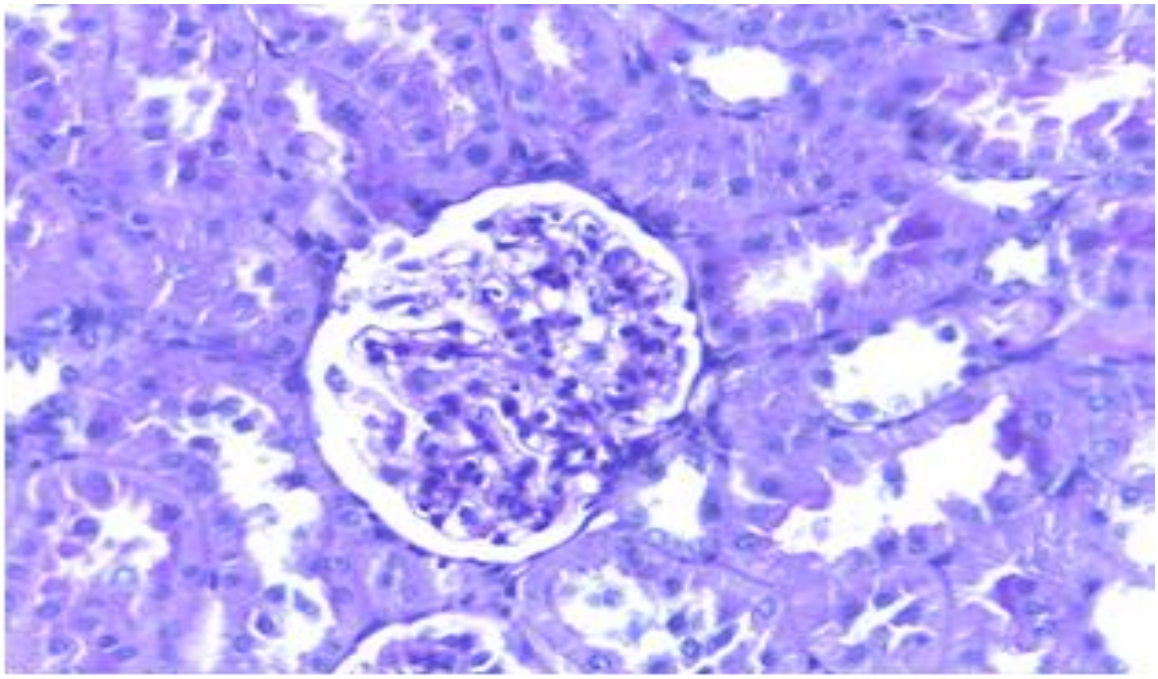

Figure 4.8: Kidney cortex of (PAS staining x40). It is seen that basal membrane structure is similar to the control group only in the group with PCA.

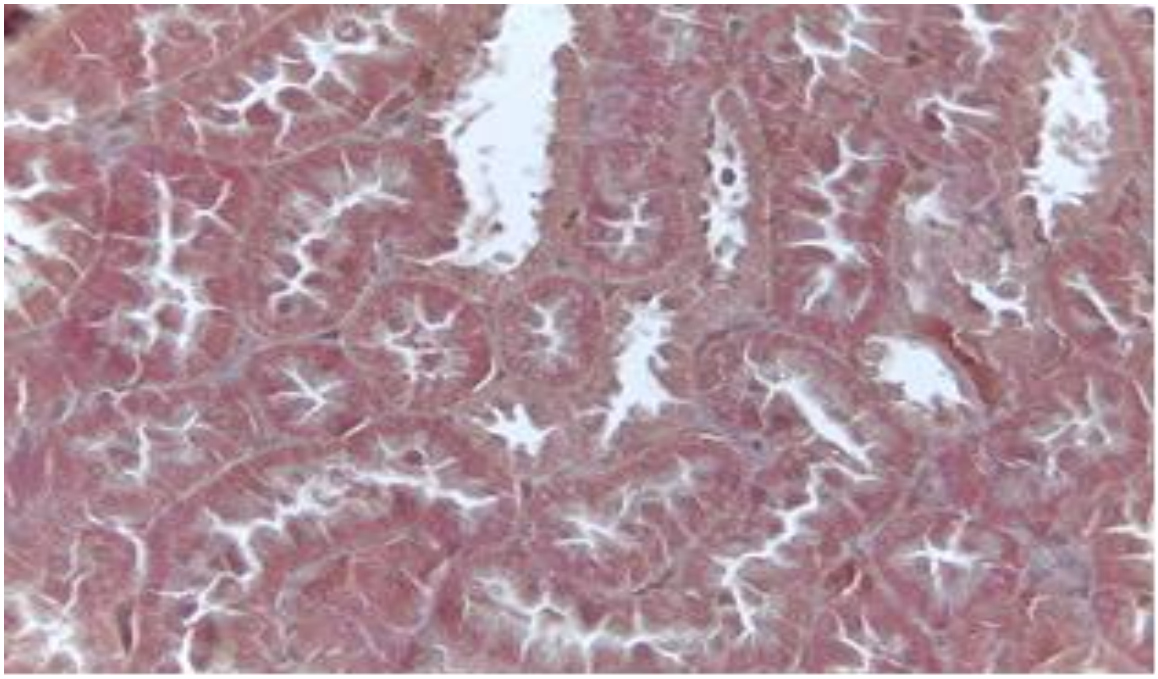

Figure 4.9. It is seen that there is no increase in connective tissue between the tubules in the PCA-only group. Masson Trichrome staining x40.

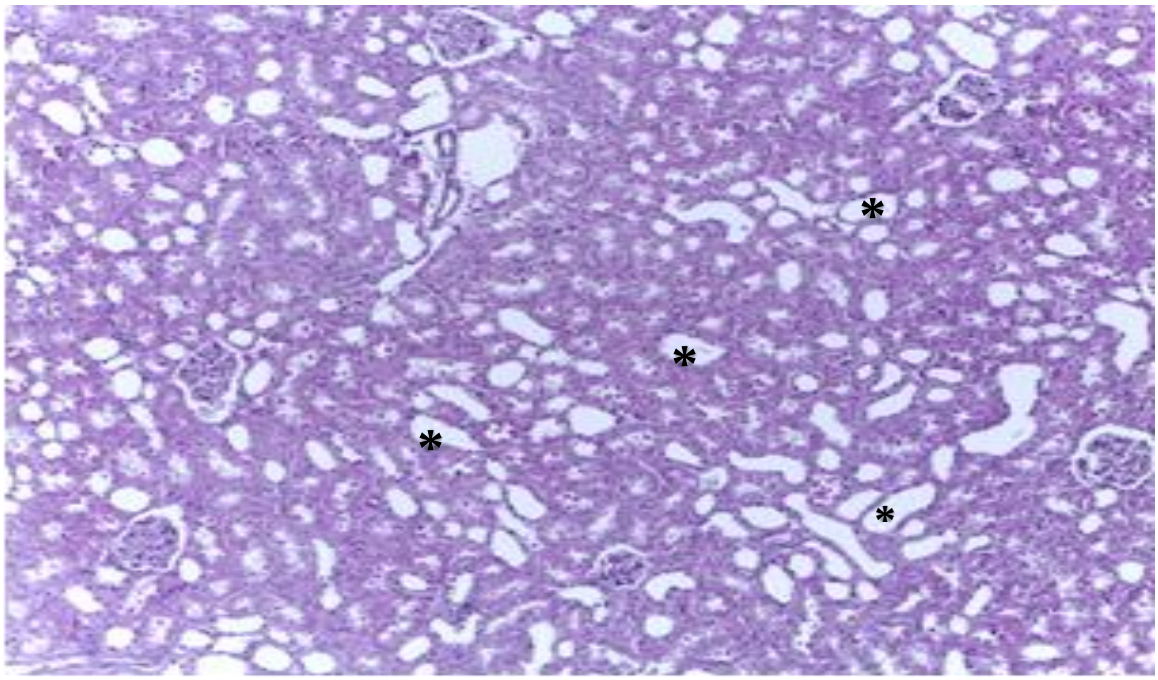


Figure 4.10. Diabetic kidney tissue. Many dilated tubules are seen (*). HE x10

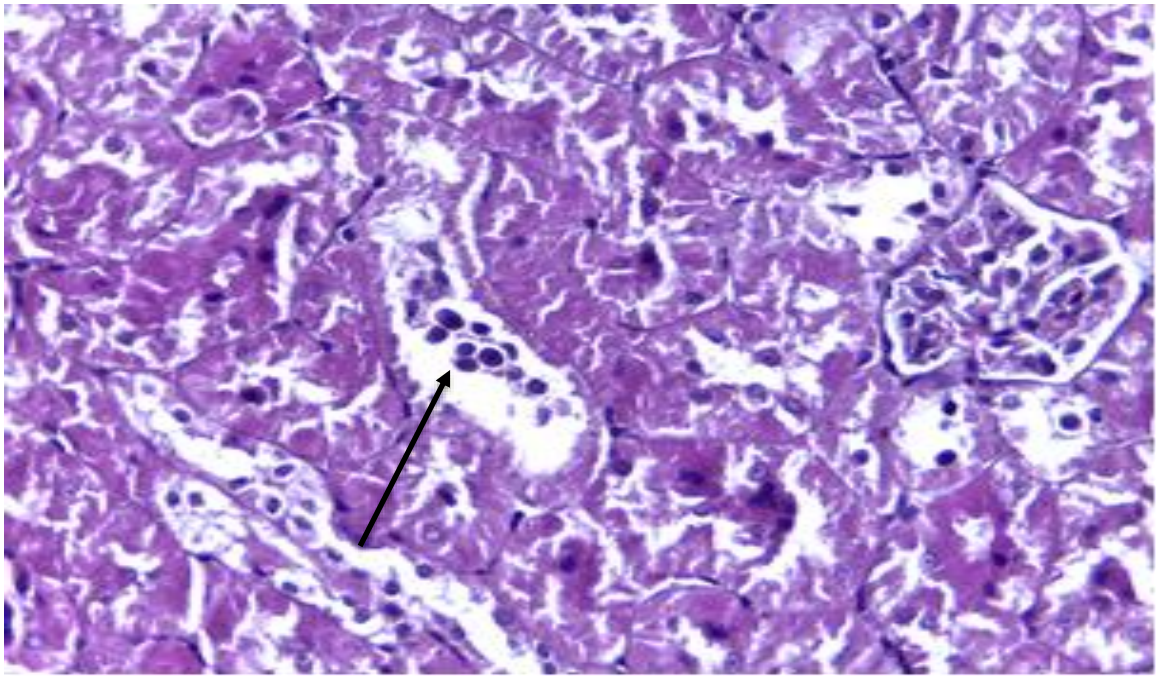

Figure 4.11. Proximal tubular epithelial cells (arrow) poured into the lumen in the diabetic group. HE staining x40.

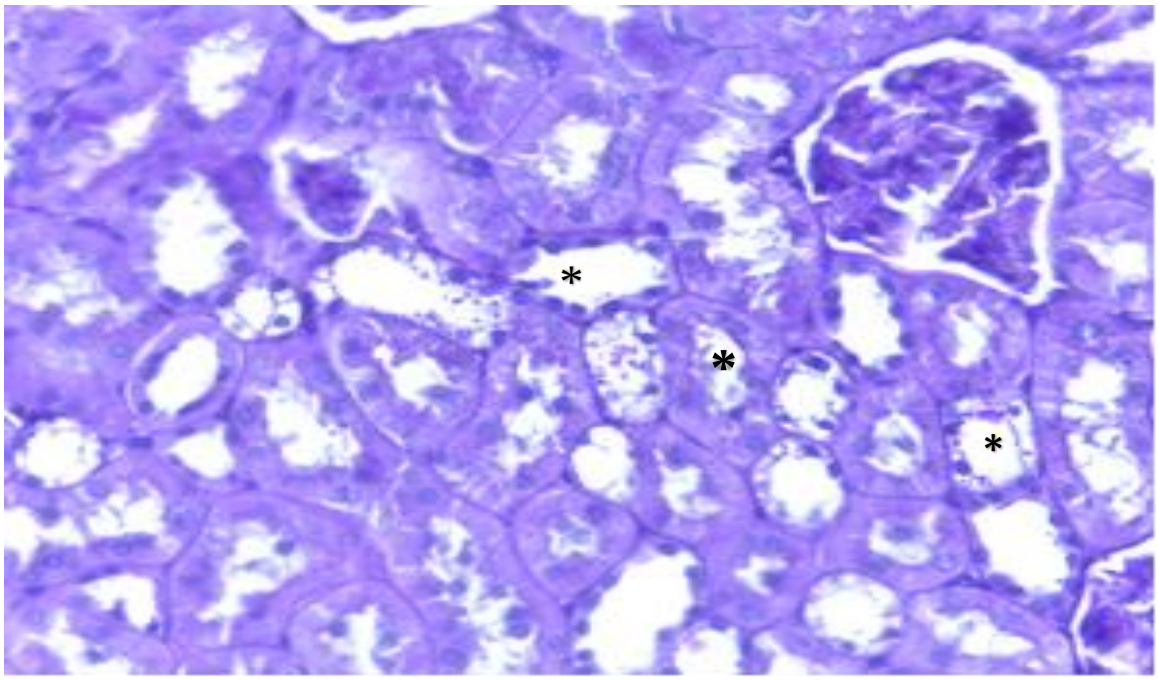


Figure 4.12. In the diabetic group, apoptotic bodies (*) are seen in the tubules. PAS staining x40

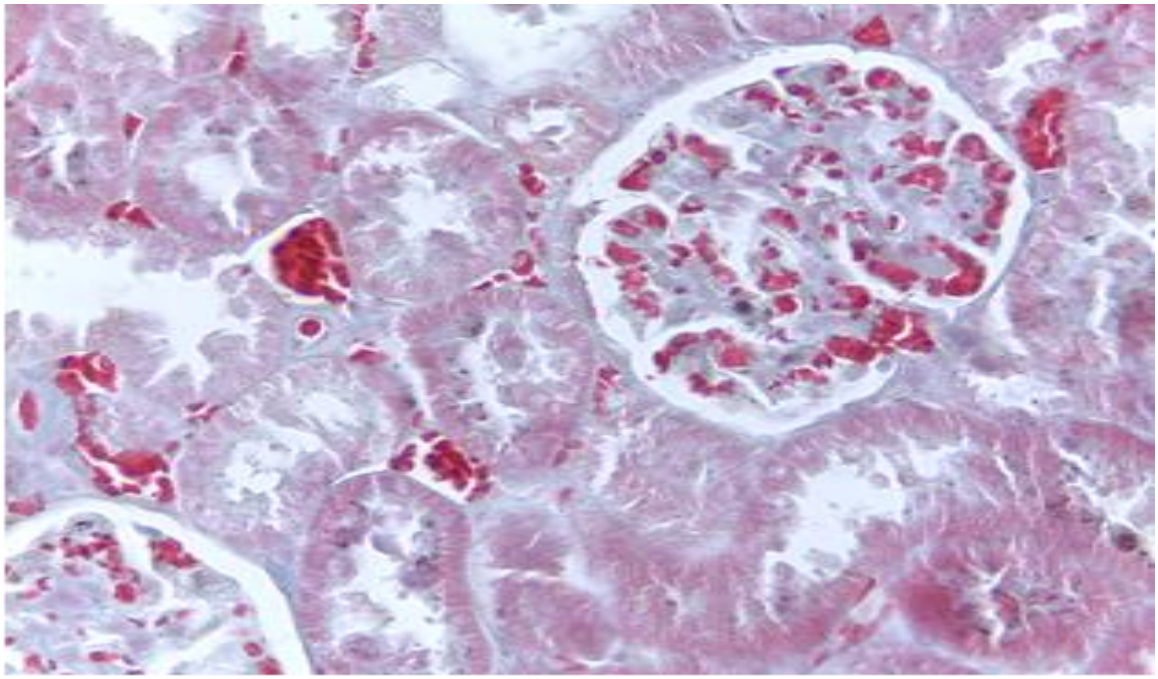

Figure 4.13 In the diabetic group, congestion is observed in glomeruli capillaries and inter tubuler capillaries. Connective tissue increased and fibrosis are not observed. Masson Trichrome staining x40.

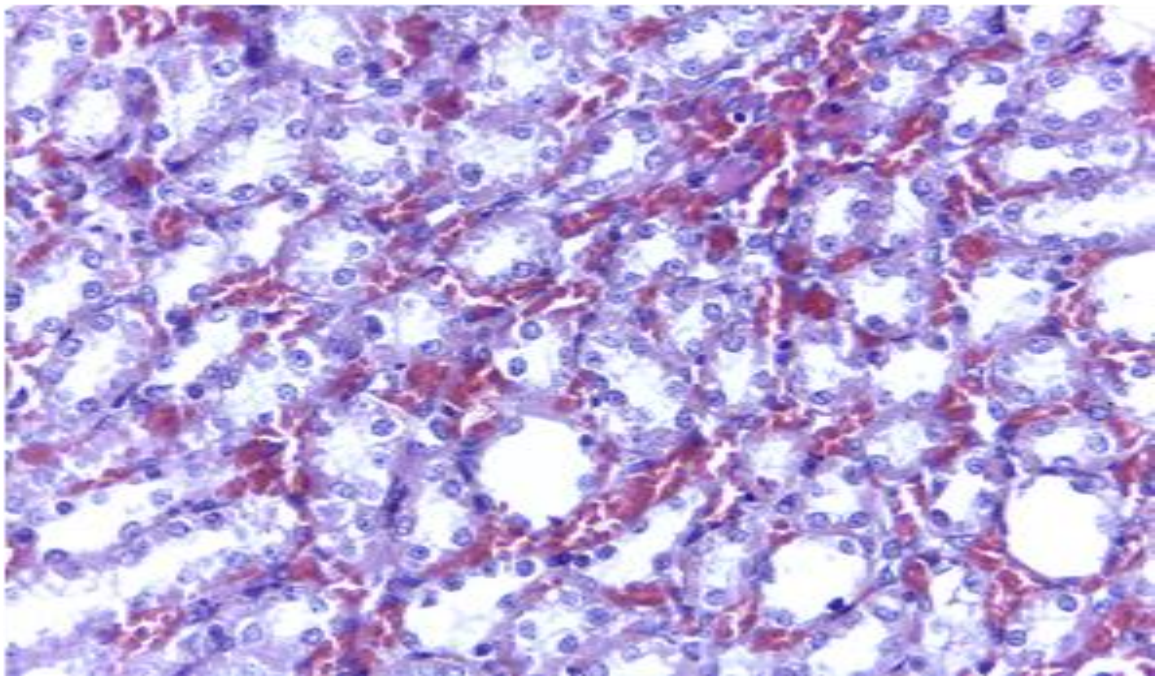

Figure 4.14. Compared to the control group, congestion is common in inter tubules capillaries in the diabetic group. HE staining x40.

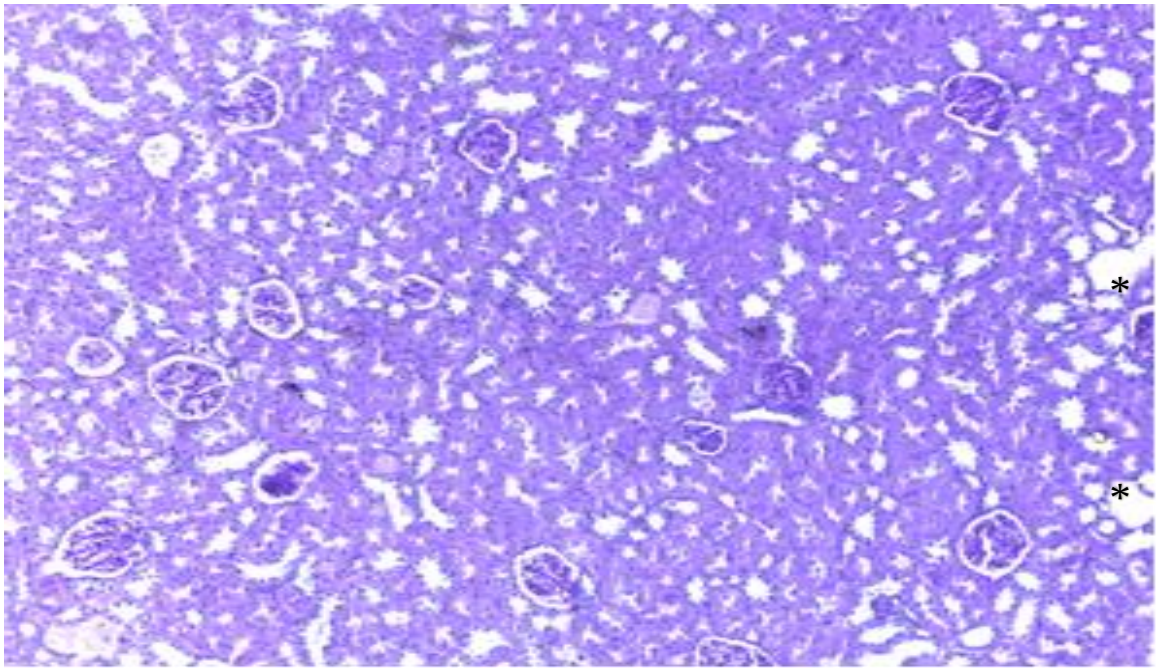


Figure 4.15. In the DM+PCA-treated group the enlargement of tubules (*) appears to be reduced. HE staining x10.

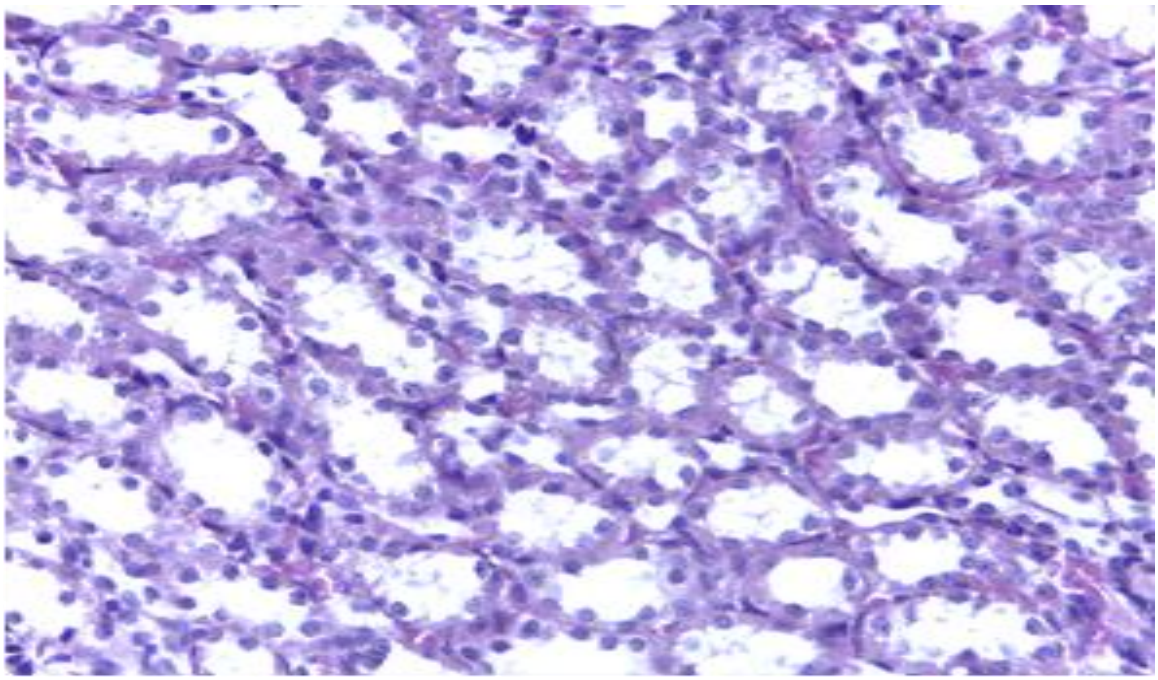

Figure 4.16. In the DM+PCA-treated group, capillary congestion between tubules appears to be significantly reduced. HE staining x40.

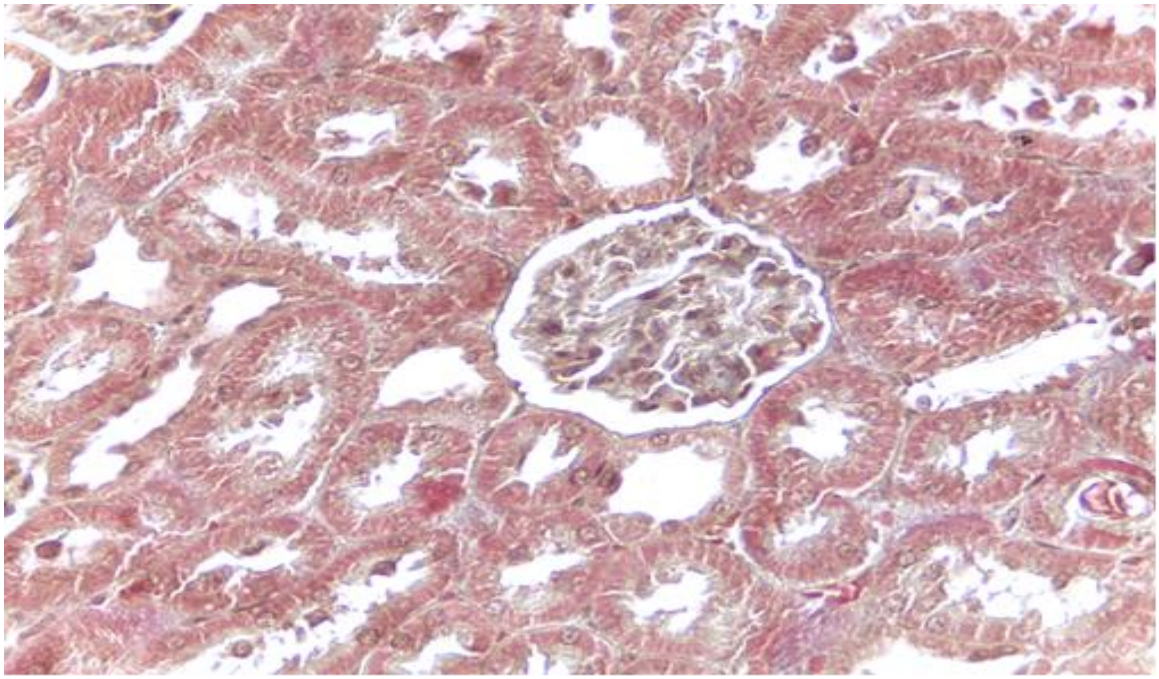

Figure 4.17. There was no increase in connective tissue in the kidney in the DM+PCA treated group. Masson Trichrome staining x40.

## 5 Discussion

Diabetes mellitus is a syndrome with disordered metabolism and inappropriate hyperglycemia due either to a deficiency of insulin secretion or to a combination of insulin resistance and inadequate insulin secretion to compensate (Masharani. 2008). DM often leads to diabetic nephropathy which is a progressive kidney disease and is the main microvascular complication in diabetes. It has been reported that a central role in the progression of diabetic nephropathy is played by oxidative stress, inflammation, and fibrosis. Streptozotocin (STZ)-induced type 1 diabetes is the most frequently used murine model in studies of diabetic nephropathy, and its utilization has revealed several flavonoids that exert important renoprotective effects by various mechanisms.

The key findings of our research are that in STZ-induced chronic diabetic rats, regular treatment with PCA for 3weeks restored renal function. The biochemical parameters showed that urea and creatine levels for the control group and PCA group were not statistically different. This was further supported by the results obtained from the Kruskal wallis that the mean ranks for control and PCA groups were similar. The evidence observed tell us that when we administer PCA orally without any diabetes it will not harm the kidney as the parameters of the normal kidney and that of the PCA group were not different. However, comparison of the urea and creatine levels of the DM group with the PCA and control group there was a significant elevation in the readings suggesting that the kidneys were damaged by STZ. This was supported by Kruskal Wallis test in that the mean rank for the DM group (Table 4.2) was high corresponding with the biochemical analysis. The kidney could not carry out its normal function of regulating the urea and creatine levels that's why the readings observed were high. However, this damage observed was reduced in the DM+PCA group and is supported by the mean ranks value observed.

Histopathological analysis observed showed that the control group together with the PCA had similar structures. The histology analysis showed that both groups morphology of the glomeruli, distal and convulated tubules and arrangement of cells was normal. This evidence therefore supports that if PCA is administered it does not cause any morphological damage to our kidneys and hence is a safe for use. In the diabetic which was induced by STZ there was a significant morphological change observed. The diabetes caused disfigurement of the glomeruli and the arrangement of cells was affected causing congestion in the tubular epithelial cells. This affected the overall function of the kidneys as it could not carry out its normal physiologic roles as evidence

in the elevated biochemical results. Filtration, absorption and reabsorption process could not be carried out due to the blocked glomeruli and blocked tubules.

Diabetes causes discrete structural alterations such as thickening of basement membranes, and progressive glomerular accumulation of extracellular matrix components (Li et al, 2011). However in our study in the diabetic group there was connective tissue increase but no fibrosis was observed in the cortex. Cellular apoptosis is highly significant in kidney injury development (Wu et al, 2016; Havasi and Dong, 2016). We found apoptic bodies in the DM group indicating that the kidney was damaged. Histology analysis of the DM+PCA showed the apoptotic bodies found in the diabetic group were not observed in the treated group. PCA therefore showed that it prevented the cells from dying hence no presence of apoptic thereby supporting its use as a therapeutic compound. The damaged caused by STZ in congesting the glomerular and distal convoluted tubules was not observed in the treatment group. The PCA was able to reverse the morphological damage caused to cells thereby restoring the function of the kidneys. Therefore, PCA was able to ameliorate the damage caused by STZ.

Traditional Chinese medical herbs have been used in China for a long time to treat different diseases. Based on traditional Chinese medicine principle, Chaihuang-Yishen granule (CHYS) was developed and used to treat chronic kidney disease including diabetic nephropathy (DN). The CHYS granule contained 70% PCA and its mechanism of action was investigated in treatment of DN by Zhang et al. (2013). Histopathological analysis showed that there was a significant mesangial expansion, congestion in tubular capillaries and occluded capillaries in rats of DM group. This was however reduced by the treatment as the mesangial expansion was reduced and congested capillaries and inter tubular capillaries was not observed in the DM+PCA.

In a study done by Ma et al. (2017) they showed that high glucose concentration caused the mesangial cells to expand. They studied the effects of PCA on DN and found that PCA was able to protect mesangial cells from damage and that PCA significantly reduced extracellular matrix accumulation. In order to examine the effects of PCA on ECM expression in high glucose (HG) stimulated MCs, the expression of type IV collagen, laminin and fibronectin was measured using western blot assay. HG treatment dramatically up-regulated the protein expression levels of type IV collagen, laminin and fibronectin in MCs, as compared with the normal glucose (NG) group; however, PCA effectively reduced the expression of type IV collagen, laminin and fibronectin

induced by HG, in a dose-dependent manner. The p38 mitogen-activated protein kinase (MAPK) pathway plays an important role in the development of diabetic nephropathy. To gain further insight into the mechanism of the PCA-inhibited MCs proliferation and ECM accumulation, we investigated the effects of PCA on the activation of p38 MAPK signalling pathway in MCs exposed to HG. Their results demonstrated that HG efficiently stimulated p38 MAPK in MCs, compared to the NG group. PCA treatment prevented HG-induced p38 MAPK phosphorylation in MCs. These results suggest that PCA has a potential role in protecting MCs under HG condition through down-regulation of ECM expression hence PCA might be a beneficial agent for the prevention and treatment of diabetic nephropathy.

Previously, *Hibiscus sabdariffa* Linnaeus and its polyphenol extracts were found to possess antioxidative effects and have shown to have more than 70% concentration of PCA in their extracts. Thus Wen-Chi et al. (2009) aimed to investigate the effect of *H. sabdariffa* L. polyphenol extract (HPE) in streptozotocin (STZ) induced diabetic nephropathy. The study findings showed pathological change of HPE on STZ induced diabetic kidneys was evaluated and the renal tissues of the showed an obvious proximal convoluted tubular injury characterized by a vacuolar degeneration of tubular cells and swelling of epithelial cells. HPE significantly ameliorated the changes observed in the DM with the histological analysis similar to the control group. These results are similar to the ones observed in our study and they show that PCA was able to reduce the tubular injury in the distal and proximal convulated tubules.

### 5.1 Study limitations and recommendations

The study could not measure other parameters associated with diabetic nephropathy like cholesterol, antioxidant enzymes levels, biomarkers associated with apoptosis and Immunohistochemical stains association with kidney regeneration. It is highly recommended that future studies will look into parameters as they can give a conclusive evidence as to the effects of PCA on the overall improvement of renal function by using these parameters. Our treatment groups in our study was limited to one. Future research work should also look into using different doses of PCA so as so determine which will be the best treatment by comparing within the treatment groups. We used one treatment concentration of PCA which was a limitation in our study as we could not compare the effect of varying concentrations of PCA on repairing the kidney damage caused by STZ.

## 6 Conclusion

In conclusion our study findings showed that PCA was able to reverse renal dysfunction in rats that had been induced by diabetes. Furthermore, the effects of PCA only on the kidney was investigated and it showed that the kidney cells were not damaged highlighting its safe usage as it does not affect the overall function of cells. The antidiabetic effect of PCA was studied and we found by the third week it was able to reverse and restore normal function supports the administration of PCA as a complementary therapeutic regimen. This study therefore showed that PCA can be used and is safe however further studies are needed for toxicology and the appropriate dosage for safe use. Hence PCA has the potential to be used as an antidiabetic agent

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