HISTOLOGICAL ASSESSMENT OF KIDNEY DEVELOPMENT FOLLOWING INTRAUTERINE EXPOSURE TO CAFFEINE IN WISTAR DAMS

BY

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A PROJECT SUBMITTED TO THE DEPARTMENT OF ANATOMY, SCHOOL OF BASIC MEDICAL SCIENCES, UNIVERSITY OF BENIN, BENIN CITY.

IN PARTIAL FULFILLMENT FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN ANATOMY OF THE UNIVERSITY OF BENIN, BENIN CITY

OCTOBER, 2023

DECLARATION

I declare that:

- This project report is based on the experimental work undertaken by me in the Department of Anatomy, University of Benin, under the supervision of DR. VITALIS C. EZEUKO.

- This work has not been previously submitted for the award of a degree elsewhere.

- All ideas and views are essentially based on this research and where the views of others have been expressed, such words were duly acknowledged.

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CERTIFICATION

This is to certify that this research work titled "**HISTOLOGICAL ASSESSMENT OF KIDNEY DEVELOPMENT FOLLOWING INTRAUTERINE EXPOSURE TO CAFFEINE IN WISTAR DAMS**" for the award of a degree of Bachelor of Science (B.Sc.) in Anatomy was carried out by **IRABOR NATALIE ONOSETALE** under the supervision of **DR. V. C. EZEUKO**. All literatures used in this study have been acknowledged and properly referenced.

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EXTERNAL EXAMINER **DATE**

DEDICATION

This is dedicated to the ALMIGHTY GOD, for His infinite grace and mercy in the completion of this project work. My parents PASTOR & EVANGELIST (MRS.) PAUL, IRABOR.

ACKNOWLEDGMENT

My immense gratitude goes to God Almighty for His grace, strength, focus and faith, that has accompanied me over the years, and that has not allowed me to give up.

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ABSTRACT

Kidney development involves a complex series of molecular events, including cellular interactions, genetic signaling, and tissue differentiation, which collectively establish the foundation for these essential organs' functionality. However, this intricate developmental process can be sensitive to external influences. Given the kidneys' vital role in maintaining the body's balance and their susceptibility to developmental disruptions, it is crucial to investigate the potential consequences of prenatal Caffeine exposure. The impact of early-life exposure to toxins is increasingly recognized as having long-lasting effects that can contribute to various health issues throughout an individual's lifespan. Considering the widespread use of Caffeine among pregnant women, it becomes imperative to assess its effects on fetal kidney development. The aim of this study is to assess the histology of kidney development following intrauterine exposure to Caffeine in Wistar dams. In this study, thirty (30) adult Wistar rats weighing between 170 g and 180 g were used. The animals were paired overnight at the estrous cycle with sexually active males in the ratio of 2:1. Estrous cycle was confirmed by vaginal lavage. The presence of vaginal plug and/or sperm in the vaginal smear was GD0. The pregnant rats were divided into two groups (A and B) with fifteen (15) rats per group. Group A served as Control and was administered with a single intraperitoneal injection of 1ml of normal saline on GD 11, in addition to free access to feed and water. Group B served as the treated group and was administered a single intraperitoneal injection of 150mg/kg/day from GD11. On each gestational day (GD15, GD17, and GD19), five (5) animals were sampled from each group and sacrificed. The uterine horns were exteriorized and incised at the greater curvature of the horns. Fetal kidney tissues were harvested from each group for histological assessment. Histological studies showed that the group treated with Caffeine presented hypoplasia of the glomerulus on GD 17. Hypoplasia of the glomerulus and incomplete canalization of the renal tubules were what characterized the kidney on GD 19. In conclusion, caffeine has teratogenic potential against kidney development in Wistar rats.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Coffee plants are probably indigenous to the Ethiopian region and were introduced into Arabia and the rest of the East by the fourth century (Purseglove, 1976). In the mid-fifteenth century, the Sufis of Yemen used coffee to stay awake during prayers. In the sixteenth century, there were coffee houses in Istanbul, Cairo, and Mecca, and in the mid-seventeenth century coffee houses opened in Europe (Horowitz, 1989). Tea has been consumed in China for thousands of years, where it has been purported to have been discovered by the Chinese emperor Shen Nung in 2737 B.C.E. Traditional stories tell that monks drank tea to stay awake during meditation practice (Purseglove, 1976; Horowitz, 1989).

Guarana' and yerba mate' are plants indigenous to South America and it is presumed that the use of both of these plants by ancient peoples such as the Guarani tribesmen, from whom the guarana plant was named, started before any recorded history of this area (Purseglove, 1976; Roehrs and Roth, 2008). Cola nuts are indigenous to West Africa and have been chewed by local people possibly for thousands of years. Cola has been traded to other countries as a valuable commodity since probably before the fourteenth century. The nuts have been used as a stimulant by African Islamic people who use them instead of alcohol, which is forbidden (Roehrs and Roth, 2008).

Cacao in the form of a chocolate beverage has been traced to the early Maya about 2,600 years ago. In 1519 Hernando Cortes entered Mexico and reported that cocoa was being consumed in large quantities by the Aztec leader Montezuma (Huntley and Juliano, 2012). Guarana beverages are made from the seeds of the plant *Paullinia cupana* that have been roasted, ground to a paste, and dissolved in water. This paste is also used to make medicines or to flavor foods.

Guarana seeds contain larger amounts of caffeine than coffee beans, with reported levels as high as 80 mg per gram of seed (Raintree Nutrition 2006; de Mejia and Ramirez-Mares, 2014).

Often considered one of the most delicious sources of caffeine is chocolate. This is obtained from the seeds of the cacao plant, Theobroma cacao. These seeds are processed to make cocoa, chocolate, and cocoa butter. Cacao seeds have only a small amount of caffeine, with 2.5 mg/g. A typical serving of a milk chocolate bar (28 g) has about 20 mg of caffeine (Roehrs and Roth, 2008). Cola (kola) nuts are a natural source of caffeine that was once used as the sole source of caffeine in the first Coca-Cola beverages. There are about 40 species of the cola plant, with *Cola nitida*, and *C.acuminata* being the most common commercial species. Cola nuts contain up to 25 mg of caffeine per gram (Muehlbacher *et al.,* 2020).

Yerba mate' (Ilex paraguensis) is a tree that grows in South America; its leaves are used to make a caffeine-containing tea. The flavor and aroma of the leaves of the wild trees are considered by some to be much better than the cultivated ones. The level of caffeine in the leaves is about 20 mg/g (Huntley and Juliano, 2012). Most modern soft drinks that contain caffeine rely on purified caffeine as the sole source. Some sports or energy drinks have very high levels of this stimulant. Red Bull has about 100 mg of caffeine per serving. Soft drinks like Coca-Cola contain 23 mg per 8oz (Coca Cola 2006; Willson, 2018) and Pepsi One contains 36 mg per 8oz (Pepsi 2005; Willson, 2018).

The impact of maternal factors on fetal development has garnered significant attention in the field of reproductive biology and developmental toxicology. In this context, intrauterine exposure to various substances, including caffeine, has emerged as an area of interest due to its potential influence on the developing organs of the fetus. Caffeine, a widely consumed psychoactive compound found in coffee, tea, energy drinks, and certain medications, has raised concerns regarding its effects on embryonic and fetal development (Muehlbacher *et al.,* 2020).

Numerous studies have investigated the potential risks associated with caffeine consumption during pregnancy. While caffeine readily crosses the placental barrier, its effects on fetal development have been a subject of debate. Research has indicated that maternal caffeine intake may impact various aspects of fetal growth and organogenesis, with a focus on neurological and cardiovascular outcomes. However, despite the growing body of literature on caffeine's effects, its impact specifically on kidney development remains relatively understudied (Knutti *et al.*, 1982; Qian *et al.*, 2020).

The kidneys play a pivotal role in maintaining homeostasis, regulating fluid and electrolyte balance, and filtering waste products from the blood. Given the kidneys' essential functions and their vulnerability during development, understanding how maternal caffeine exposure might influence kidney development is of paramount importance. A thorough assessment of this phenomenon can shed light on potential implications for the offspring's renal health, both during early life and possibly into adulthood (Bolignano *et al.*, 2007; Peerapen and Thongboonkerd, 2018).

Previous research has suggested that caffeine's vasoconstrictive properties might influence fetal blood flow, potentially affecting nutrient and oxygen delivery to developing organs, including the kidneys (Murphy *et al.*, 2006). Moreover, caffeine's role as an adenosine receptor antagonist could perturb crucial developmental processes, including cell proliferation, differentiation, and apoptosis. These mechanistic insights provide a rationale for investigating whether intrauterine exposure to caffeine could disrupt normal kidney development and subsequently impact the functional capacity of the kidneys.

1.2 STATEMENT OF RESEARCH PROBLEM

Maternal behaviors and exposures during pregnancy play a pivotal role in shaping fetal development and long-term health outcomes (Muehlbacher *et al.,* 2020). Among these exposures, maternal caffeine consumption has been a subject of growing concern due to its widespread use and potential impact on fetal growth and organ development (Willson, 2018). While considerable research has explored the effects of caffeine on various aspects of pregnancy, including birth weight and preterm birth (Vitti *et al.*, 2018), a critical gap remains in our understanding of how maternal caffeine intake might specifically influence the developing fetal kidneys.

This study aims to address the research problem of whether maternal caffeine consumption during pregnancy has a discernible impact on fetal kidney development. Despite caffeine's ability to cross the placenta and enter the fetal bloodstream (Eteng, *et al.*, 1977), its specific effects on nephrogenesis and kidney growth have not been comprehensively investigated. The potential consequences of altered kidney development due to caffeine exposure hold significant implications for both short-term pregnancy outcomes and the long-term health of offspring.

This research problem arises from the need to bridge the gap in our understanding of how maternal caffeine consumption could influence fetal kidney development, particularly during the critical periods of organogenesis (Bolignano *et al.*, 2007). While previous studies have explored caffeine's impact on various other fetal organs, including the brain and cardiovascular system, the kidneys remain relatively understudied in this context. As the kidneys play a fundamental role in regulating fluid balance, electrolyte levels, and waste elimination, disturbances during their development could have profound implications for an individual's health throughout their lifespan (Bolignano *et al.*, 2007; Vitti *et al.*, 2018).

The research problem is compounded by the prevalence of caffeine consumption among pregnant individuals. Caffeine is a commonly consumed psychoactive substance found in various beverages and foods (Grosso and Bracken, 2005). Despite guidelines recommending limited caffeine intake during pregnancy, many expectant mothers continue to consume it, sometimes in quantities that may exceed recommended limits (Grosso and Bracken, 2005; Roehrs and Roth, 2008).

1.3 AIM AND SPECIFIC OBJECTIVES OF THE STUDY

The aim of the study was to assess kidney development following intrauterine exposure to caffeine in Wistar Dams. The specific objective of this study is to assess the effect of intrauterine caffeine exposure on the fetal histology of the kidney in Wistar rats.

1.4 JUSTIFICATION OF THE STUDY

The developmental phase within the womb is a crucial period that significantly shapes an individual's lifelong health and well-being (Maggi *et al.,* 2010). During this intricate journey, numerous external factors can potentially influence the trajectory of organ development, with lasting implications for the individual's overall health. One such factor that has garnered substantial attention is maternal caffeine consumption, which has raised questions about its potential impact on fetal organ development, including the delicate and intricate development of the kidneys (Bolignano *et al.,* 2007).

Caffeine, a widely consumed psychoactive substance found in various foods and beverages, readily crosses the placental barrier, exposing the developing fetus to its effects. The fetal kidney, a complex and multifunctional organ, plays a pivotal role in regulating electrolyte balance, fluid volume, and waste elimination. Considering the high consumption rates of caffeine among pregnant individuals, it becomes paramount to investigate whether caffeine's

presence in the fetal environment could exert any influence on kidney development (de Mejia and Ramirez-Mares, 2014; Muehlbacher *et al.,* 2020).

While previous research has focused on the general effects of caffeine on adult kidneys, a significant gap remains in our understanding of how caffeine might impact the intricate processes of nephrogenesis, the formation of nephrons, and overall kidney growth during the fetal stage. The potential effects of caffeine on angiogenesis, cellular differentiation, and cellular migration within the fetal kidneys remain largely unexplored. Given that nephrogenesis occurs during a limited window of gestational development, it is crucial to examine whether maternal caffeine intake during this period could potentially disrupt the finely tuned processes of nephron formation and impact the long-term functionality of the fetal kidneys.

Moreover, the association between maternal caffeine consumption and adverse pregnancy outcomes, such as preterm birth and low birth weight, adds a layer of complexity to the investigation (Roehrs and Roth, 2008). Understanding whether these outcomes are linked to caffeine's direct influence on fetal kidney development or occur through other mechanisms is essential for unraveling the intricate web of caffeine's effects on the developing fetus.

Therefore, this study aims to address these critical gaps by assessing fetal kidney development following exposure to Caffeine. The findings of this study have the potential to shed light on whether caffeine poses a developmental risk to the fetal kidney and whether strategies to mitigate these effects can be implemented to safeguard the long-term health of individuals from early life stages.

CHAPTER TWO

LITERATURE REVIEW

2.1 CAFFEINE

2.1.1 Description and Properties of Caffeine

Caffeine, a purine alkaloid and trimethyl xanthine, functions as a stimulant for the Central Nervous System (CNS). It is represented by the chemical formula $C_8H_{10}N_4O_2$ and is known by various names such as methyl theobromine, 1,3,7-trimethylxanthine, 7-methyl theophylline, guaranine, or theine. This white crystalline purine, devoid of odor, possesses a bitter taste (Huntley and Juliano, 2012). Found naturally in the seeds, leaves, and fruits of plants and trees indigenous to Africa, East Asia, and South America, caffeine is a methylxanthine alkaloid. Its synthesis can be accomplished through the reaction between dimethylurea and malonic acid. Notably, caffeine is derived from purine xanthine, present in over sixty plants, including coffee, tea, and cocoa, and stands as one of the most widely consumed psychoactive substances worldwide (Sun and Hou, 2008).

When consumed, caffeine interacts with adenosine receptors in the CNS, obstructing adenosine binding. This interplay between caffeine and adenosine yields both positive and negative health effects. Prolonged daily usage can lead to mild drug dependence, resulting in withdrawal symptoms like drowsiness, headaches, and irritability upon cessation of habitual consumption (Bothe and Cammenga, 1979). The structure of caffeine resembles that of the purine ring. Specifically, caffeine is a trimethylxanthine comprised of two fused rings – pyrimidinedione and imidazole. These rings are heterocyclic in nature; the pyrimidinedione ring features six members with two nitrogen atoms, while the imidazole ring consists of five members with two nitrogen atoms (Bertrand *et al.*, 2014).

Caffeine, a methylxanthine alkaloid, is naturally present in the seeds, leaves, and fruit of various plants and trees native to Africa, East Asia, and South America. It can be synthesized through reactions involving dimethylurea and malonic acid. Additionally, caffeine can be generated by treating theobromine with methyl iodide and sodium methoxide or by subjecting uric acid to alkaline solutions and further reactions with phosphoryl chloride ($POCl_3$) and hydrogen iodide (Azam *et al.,* 2003).

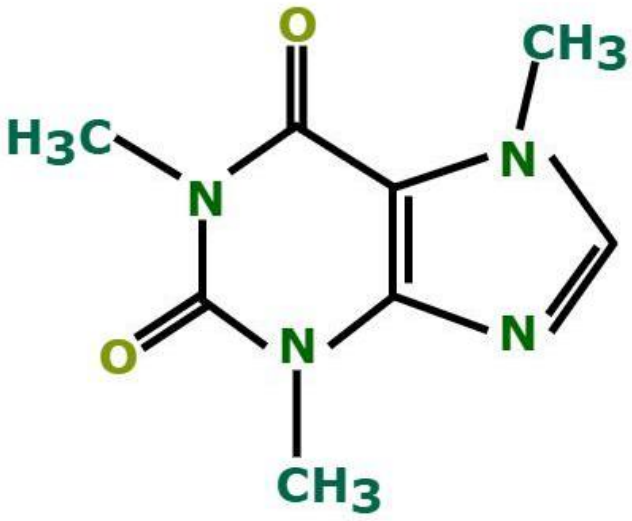

Fig. 2.1: Structure of Caffeine (Azam *et al.,* 2003)

This compound is also a by-product of the decaffeination process used in the production of decaffeinated coffee, where caffeine is extracted from its natural sources, such as coffee (Azam *et al.,* 2003). Caffeine is characterized by its properties: it appears as white crystals with an odorless nature and a bitter taste. It has a density of 1.23 g/cm³ and a melting point ranging from 235 to 238 °C in its anhydrous form. The boiling point of caffeine is 178 °C. It exhibits slight solubility in water and organic solvents while being moderately soluble in ether (Bertrand *et al.,* 2014).

2.1.2 Sources of caffeine

In nature, caffeine is found in varying concentrations along with other xanthine alkaloids such as theophylline and theobromine, which are also stimulants. The world's primary source of caffeine is the coffee bean (the seed of the coffee plant), from which coffee is brewed. There are many species of the genus *Coffea* whose caffeine content varies widely. There are many factors affecting the caffeine content of a cup of coffee including the type of bean, the roasting method, and the method of preparation used, but in general, one 8 oz. serving of coffee has about 100 milligrams (mg) of caffeine. Darker roasts of coffee have less caffeine than lighter roasts since the roasting process reduces the caffeine content of the beans. Arabica coffee beans average 24 mg/gram (g) of caffeine whereas the *Robusta* variety averages 13 mg/g (Casal *et al.*, 2000).

Tea, another common source of caffeine, is produced by brewing leaves of the tea plant (Camellia sinensis), which has hundreds of varieties. The amount of oxidation that the plucked leaf undergoes determines whether it is classified as white, green, oolong, or black; where white has the least amount of oxidation of the leaf and black tea has the most. More oxidation results in higher levels of caffeine. In black tea, caffeine was found to be 25 mg/g of tea leaf, whereas in green tea the caffeine level was 15 mg/g of leaf (Khokhar *et al.*, 2002).

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2.1.3 Effects of Caffeine on the Body

Caffeine functions as a stimulant targeting the central nervous system to enhance mental alertness when combating drowsiness. This leads to improved cognitive agility, heightened focus, and enhanced coordination. Particularly sensitive to caffeine's effects is the sleep-wakefulness cycle, believed to be regulated by brain areas including the locus ceruleus, raphe nuclei, and reticular formation. Consequently, caffeine extends sleep onset latency and reduces sleep duration. It prompts augmented blood flow to the kidneys and escalates urine production, concurrently inhibiting sodium and water tubular reabsorption, leading to diluted urine (Smith, 2002).

Caffeine's vascular impact results in brain blood vessel constriction but peripheral dilation. It briefly heightens heart rate, cardiac output, and contraction force. Doses exceeding 250 mg can induce extra beats, rapid heart rate (tachycardia), and significant ventricular arrhythmias. Studies indicate caffeine-induced dose-dependent elevation of systolic and diastolic blood pressure and skin temperature. Skeletal muscles are invigorated, bolstering contraction strength and diminishing fatigue while encouraging glycogen and lipid breakdown to enhance endurance (Nawrot *et al.,* 2003).

In caffeine-naive individuals, this substance elevates plasma levels of epinephrine, norepinephrine, and renin, instigating the angiotensinogen/angiotensin cascade for blood pressure elevation (Noever *et al.,* 1995). Neurotransmitter turnover in the brain, particularly acetylcholine, and monoamines like norepinephrine and dopamine, is accelerated by caffeine, enhancing dopamine-releasing cells' activity in the prefrontal cortex. However, unlike common drugs of abuse, caffeine has no impact on dopamine release in the nucleus accumbens.

Caffeine is occasionally combined with analgesics to heighten their efficacy, such as with ergotamine for migraine and cluster headaches, or with pain relievers like aspirin and acetaminophen. It can counteract antihistamine-induced drowsiness. In premature infants, citrated caffeine is employed for treating apnea (Hoeger *et al.,* 2002). Excessive consumption of caffeine has been correlated with nervousness, insomnia, jitteriness, and shallow sleep. Prolonged use may result in stomach ulcers, dependence, and withdrawal symptoms (Hoeger *et al.,* 2002). While relatively safe for humans, caffeine is significantly more toxic for certain animals due to inadequate metabolization ability (Noever *et al.,* 1995).

2.1.4 Caffeine Absorption and Metabolism

Caffeine absorption is initiated by consuming caffeine-containing products like coffee, tea, energy drinks, and soft drinks. After ingestion, caffeine moves through the stomach's acidic

environment without significant breakdown due to its stability. As it reaches the alkaline small intestine, caffeine's solubility improves, and passive diffusion enables it to enter the bloodstream from higher to lower concentrations, aided by continuous stomach delivery (Aranda *et al.,* 2010; Alsabri *et al.,* 2018). Crossing the intestinal lining, caffeine spreads to various tissues and organs, including the brain due to its blood-brain barrier penetration.

Caffeine absorption is influenced by solubility in water and lipids, stomach and small intestine pH levels, gastric emptying rates affected by diet, and genetic variations in metabolism. The form of caffeine intake matters, as liquids are absorbed more swiftly than solids, potentially leading to quicker absorption from beverages (dePaula and Farah, 2019).

Upon oral consumption, caffeine is readily absorbed by the body, with a remarkable 99 percent absorption occurring within a window of 15 to 120 minutes. This rapid uptake results in the attainment of peak plasma levels of 5 to 25 micrograms per milliliter (ml) when a dose of 250 mg is administered. Notably, the effectiveness of caffeine hinges on achieving a plasma concentration ranging from 6 to 13 micrograms/ml (dePaula and Farah, 2019). The intricate process of caffeine metabolism unfolds as it traverses the body, revealing both its rapid distribution and its capacity to permeate barriers such as the placenta and blood-brain barrier. Furthermore, it merits mention that caffeine enters breast milk, albeit in small quantities (Nehlig, 2018).

Continued consumption of caffeine fosters a phenomenon known as drug tolerance, wherein the body adapts to its presence. However, upon cessation of caffeine intake, the body exhibits heightened sensitivity to adenosine, which consequently triggers a drastic drop in blood pressure, manifesting as headaches and other associated symptoms (Bonati *et al.,* 2012).

The complex metabolic journey of caffeine primarily unfolds within the liver, orchestrated through processes of demethylation and oxidation. This intricate choreography is carried out

by a specific cytochrome P450 enzyme system recognized as 1A2 or CYP1A2. As caffeine encounters this metabolic pathway, it undergoes transformation into three distinct dimethylxanthines:

- 1,7-dimethylxanthine, also referred to as paraxanthine, accounts for approximately 80 to 84 percent of the metabolites.
- 3,7-dimethylxanthine, known as theobromine, constitutes approximately 10 to 12 percent of the metabolites.
- 1,3-dimethylxanthine, recognized as theophylline, comprises around 4 percent of the metabolites (Nehlig, 2018).

Subsequently, each of these metabolites undergoes further processing and subsequent excretion via urine, predominantly as methylated urates and methylated xanthines. Remarkably, approximately one percent of caffeine perseveres in its unchanged form and enters the urine as such. This intricate web of metabolic transformations and urinary excretion serves as the culmination of caffeine's journey within the body (Bonati *et al.*, 2012; dePaula and Farah, 2019).

Caffeine elimination is the body's process of removing caffeine and its byproducts from the bloodstream and eventually excreting them. This occurs primarily through the liver and urinary system. After metabolism, caffeine metabolites undergo further changes to become more water-soluble, aiding excretion through urine (Nehlig, 2018).

Metabolites enter the bloodstream and are filtered by the kidneys, with some actively secreted into renal tubules for enhanced elimination. This concentration in urine streamlines excretion. Metabolites are eventually expelled through urination, traveling through the urinary tract (dePaula and Farah, 2019).

2.1.5 Mechanism of Action of Caffeine

At the heart of caffeine's dynamic interaction with the human body lies its multifaceted mechanism of action. The key premise centers around the pivotal role of caffeine in obstructing adenosine receptors (A1 and A2a) found on the surfaces of cells within the central nervous system (CNS). Adenosine, a compound comprising adenine and ribose, acts as a neuromodulator influencing the release of neurotransmitters from nerve cells. Leveraging its structural similarity to adenine, caffeine adeptly binds to adenosine receptors without triggering their activation, thus effectively preventing their engagement by adenosine molecules. This intricate dance of binding and inhibition reshapes cellular responses. By blocking these receptors, caffeine instigates a ripple effect, which manifests as secondary consequences on a myriad of neurotransmitters including acetylcholine, gamma amino butyric acid, serotonin, dopamine, and noradrenaline. Delving deeper, caffeine orchestrates shifts in neurotransmitter turnover, most notably affecting neurotransmitters like 5-hydroxytryptamine and dopamine (Fredholm 1999).

While caffeine's role as a competitive inhibitor of cyclic AMP-phosphodiesterase has been explored, its therapeutic impact outstrips this enzyme-related effect. Notably, the levels of caffeine necessary to elicit blood pressure increases remain significantly below those needed for cyclic AMP-phosphodiesterase inhibition (Chawla *et al.,* 2006), thus debunking this as the sole explanatory mechanism.

Consideration extends to caffeine's metabolites, which contribute significantly to its physiological effects. Theobromine, for instance, emerges as a vasodilator, enhancing blood vessel dilation. This vasodilation, in turn, augments blood flow, ushering in heightened oxygen and nutrient delivery to the brain and muscles. Theophylline enters the fray as a smooth muscle relaxant, with a pronounced impact on bronchioles. In addition to this, theophylline propels heart rate elevation and efficiency. Paraxanthine, or 1,7-dimethylxanthine, steps forward as a

catalyst in the breakdown of triglycerides, thus liberating glycerol and fatty acids into the bloodstream (Dews *et al.*, 1984).

As a conductor of remarkable transformations within the body, caffeine unfurls an astonishing capacity to elevate physical endurance. A landmark study conducted in 1979 illuminated a remarkable 7 percent surge in cycling distance over two hours in subjects administered caffeine, as opposed to control groups (Ivy *et al.*, 1979). Subsequent explorations unveiled caffeine's potential to revolutionize endurance among trained runners. With a dosage of 9 milligrams per kilogram of body weight, athletes enjoyed a staggering 44 percent increase in "race-pace" endurance, accompanied by a remarkable 51 percent surge in cycling stamina (Graham and Spriet 1991). Building on this foundation, another investigation illuminated that a caffeine dosage of 5.5 milligrams per kilogram of body mass extended cycling performance during high-intensity circuits by a remarkable 29 percent (Trice and Hayes 1995). These remarkable findings collectively underscore caffeine's intricate engagement with human physiology, reshaping endurance thresholds and redefining the boundaries of human performance.

2.1.6 Caffeine Toxicity

Extensive research has been conducted to investigate the effects of caffeine on human health. The Food and Drug Administration (FDA) established its safety for consumption as early as 1958. Recent reviews have further indicated that caffeine's inclusion in carbonated beverages is unlikely to yield adverse health effects. The American Medical Association (AMA) also underscores the safety of consuming moderate amounts of coffee and tea, assuring minimal concerns about caffeine's impact on health (Price and Fligner, 2000). Notably, the potential lethal dosage of caffeine is estimated to range from 150 to 200 mg/kg of body weight, accompanied by symptoms like nausea, vomiting, diarrhea, and, in severe cases, seizures

(Sauer, 1994). Tragically, instances of death resulting from intentional caffeine pill overdosing have been recorded.

Excessive and prolonged caffeine consumption can lead to various physical and mental conditions. As outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), caffeine-induced psychiatric disorders encompass intoxication, anxiety disorder, sleep disorder, and unspecified caffeine-related disorders. Overdosing on caffeine can manifest as intoxication or poisoning, exhibiting both physiological and psychological symptoms. These include restlessness, insomnia, increased heart rate, muscle twitching, and gastrointestinal discomfort, among others (Price and Fligner, 2000).

The potential for caffeine to induce conditions mirroring organic mental disorders, such as panic disorder and bipolar disorder, has raised concerns about misdiagnosis and unnecessary medication. Experts suggest that caffeine-induced psychosis can be challenging to differentiate from other psychoses and that the remedy lies in ceasing further caffeine consumption (Trice and Hayes 1995). Caffeine's impact on stomach acid production and relaxation of the gastro-esophageal sphincter can lead to issues like peptic ulcers, erosive esophagitis, and gastroesophageal reflux disease (GERD) when consumed excessively over time (Beauchamp *et al.*, 2017). Additionally, certain individuals with a variant of the cytochrome P450 1A2 (CYP1A2) enzyme, known as "slow metabolizers," are believed to face an elevated risk of nonfatal myocardial infarction (Sardao *et al.*, 2002).

Excessive caffeine consumption is rarely fatal, with estimated lethal doses for adults at 10 g/person. Symptoms of caffeine toxicity range from nervousness and insomnia to severe vomiting and agitation. Prolonged caffeine exposure is linked to various health issues. Caffeine works by blocking adenosine receptors, enhancing neurotransmitter release, and increasing catecholamine circulation (Beauchamp *et al.*, 2017).

Daily caffeine intake over 500-600 mg is considered risky, with prolonged misuse leading to 'caffeinism', showing various symptoms. High caffeine intake (>400 mg/day) increases the risk of bladder instability, especially for those with preexisting bladder problems (Price and Fligner, 2000). Caffeine's impact on bone metabolism is complex, with effects on urinary calcium excretion and bone density. High caffeine and low calcium intake patterns negatively affect women's bones, especially below 400 mg/day (Beauchamp *et al.*, 2017).

2.1.7 Teratogenic Effect of Caffeine on the Kidney

Caffeine, a central nervous system stimulant found in various foods and beverages, is a widely consumed psychoactive substance globally. Its stimulating properties, commonly sought for their ability to enhance alertness and reduce fatigue, are not without controversy. The consumption of caffeine during pregnancy has been a subject of concern due to its potential teratogenic effects, especially on fetal organ development (Price and Fligner, 2000).

Although caffeine is primarily recognized for its effects on the central nervous system, studies have explored its potential teratogenic impact on various organs, including the kidneys. The kidneys, vital organs responsible for filtering waste products and maintaining electrolyte balance, undergo a complex developmental process during pregnancy. Renal development is characterized by the formation of nephrons, the functional units of the kidney. Nephrogenesis begins early in fetal life and continues until shortly after birth. Disruption of this intricate process can lead to congenital kidney anomalies (Sardao *et al.*, 2002; Beauchamp *et al.*, 2017).

The potential teratogenic effects of caffeine have led to concerns about maternal caffeine consumption during pregnancy. Caffeine readily crosses the placenta and enters the fetal bloodstream, where its metabolism differs from that in adults. This altered metabolism can result in prolonged caffeine exposure for the developing fetus, raising questions about its impact on fetal organ development, including the kidneys (Beauchamp *et al.*, 2017).

Animal studies have provided valuable insights into the teratogenic potential of caffeine on the kidneys. Researchers have observed alterations in renal development, such as changes in nephron number and morphology, associated with maternal caffeine consumption. These studies suggest that caffeine exposure can interfere with the proper formation of nephrons, leading to structural and functional abnormalities. A study by Imai et al. (2006) found that caffeine exposure during pregnancy caused kidney defects in mice. The researchers also found that caffeine exposure caused a decrease in kidney weight and a decrease in the number of nephrons, which are the functional units of the kidney. Another study by Park et al. (2013) found that caffeine exposure during pregnancy caused kidney defects in rats. The researchers also found that caffeine exposure caused a decrease in kidney function.

While human studies exploring the direct link between maternal caffeine consumption and congenital kidney anomalies are limited, evidence from related research is instructive. Epidemiological studies have associated caffeine intake during pregnancy with an increased risk of various congenital anomalies, some of which could indirectly impact kidney development. A study by Liu et al. (2020) found that caffeine exposure during pregnancy was associated with an increased risk of urinary tract infections in infants. A study by Zhang et al. (2021) found that caffeine exposure during pregnancy was associated with an increased risk of kidney stones in children. A study by Li et al. (2022) found that caffeine exposure during pregnancy was associated with decreased kidney function in adults.

2.2 ORGAN OF STUDY: Kidney

2.2.1 Gross Anatomy of the Wistar Rat Kidney

The gross anatomy of the kidney in the Wistar rat reveals a complex and intricately organized structure that plays a vital role in the rat's physiological processes. The kidney, a bean-shaped organ, is situated within the abdominal cavity, adjacent to the vertebral column. It is

characterized by its smooth surfaces, possessing both convex and concave borders that contribute to its distinctive appearance (Guan *et al.,* 2000).

Each kidney consists of dorsal and ventral surfaces, as well as medial and lateral borders. The lateral border exhibits a convex contour, while the medial border showcases a concave curve. The kidneys possess distinct upper and lower poles, with an indented hilus that serves as the point of entry and exit for various structures, including blood vessels, nerves, and the ureter. Adipose tissue surrounds the hilus and sides of the kidney, providing a protective cushion. This layer of fat not only safeguards the delicate structures of the kidney but also aids in maintaining its proper positioning within the abdominal cavity (Braidy *et al.,* 2011; Olukole, 2021).

Fig. 2.2: Wistar Rat Kidney (Braidy *et al.,* 2011).

Anatomically, the right kidney is positioned more cranially than the left kidney. It is closely related to the liver, while the left kidney is in proximity to other organs such as the stomach, pancreas, descending colon, spleen, and small intestine. This spatial arrangement highlights the intricate relationships between the kidney and neighboring structures, underscoring its role in various physiological processes, including filtration, regulation of electrolyte balance, and waste elimination (Braidy *et al.,* 2011). The kidneys lie between the diaphragm and the pelvis, just below the adrenal glands. The size of the kidneys can vary based on factors such as age, sex, and overall health. In adult Wistar rats, the average length of the kidney typically ranges from 2 to 3 cm (Braidy *et al.*, 2011).

A thin fibrous connective tissue layer known as the renal capsule envelops each kidney, providing structural support and protection to the underlying renal tissue. This layer maintains the integrity of the kidney's architecture and helps in preventing damage from external factors. Upon dissection, the kidney can be divided into two main regions: the outer renal cortex and the inner renal medulla. The renal cortex appears lighter in color due to the presence of numerous renal corpuscles (glomeruli) and convoluted tubules. In contrast, the renal medulla, with its triangular-shaped renal pyramids, appears darker. The base of each pyramid faces the cortex, while the apex, known as the renal papilla, points towards the renal pelvis (Golalipour *et al.,* 2009). The renal pelvis, situated at the center of the kidney, serves as a collecting chamber for urine. It collects urine formed within the kidney and funnels it into the ureter, a muscular tube responsible for transporting urine to the urinary bladder for eventual excretion (Golalipour *et al.,* 2009).

Blood supply to the kidneys is rich and vital for their function. The renal artery branches into smaller arteries and arterioles within the kidney, ultimately forming the intricate network of capillaries known as the glomerulus. The glomerulus is a fundamental unit involved in the filtration of blood to produce urine. The filtered blood is subsequently drained by renal veins, ensuring the removal of waste and excess substances from the body (Braidy *et al.,* 2011; Eshetu *et al.,* 2016; Golalipour *et al.,* 2009).

2.2.2 Development of the Wistar Rat Kidney

The embryology of the kidney in the Wistar rat traces a fascinating journey of organ development, from its inception as a primitive structure to its intricate formation and functional maturation. This complex process involves the orchestration of multiple cellular and molecular events that culminate in the establishment of a fully functional renal system (Martins and Neuhaus, 2007).

The development of the rat kidney initiates during the early stages of embryogenesis. At around embryonic day 10 (E10), the intermediate mesoderm gives rise to the nephrogenic cord, a precursor structure from which the kidneys will develop. The nephrogenic cord extends along the posterior body axis, eventually segmenting into distinct units termed nephrotomes. Subsequently, specialized structures known as nephric vesicles emerge within the nephrogenic cord. Around E12-E13, these vesicles undergo a mesenchymal-to-epithelial transformation, giving rise to the renal vesicles. These renal vesicles serve as the primordial renal tubules and are the foundation for the development of nephrons, the functional units of the kidney (Hollenberg, 2004; Li *et al.*, 2010).

As development progresses, the renal vesicles undergo a series of intricate morphological changes. By approximately E14-E15, some renal vesicles invaginate to form S-shaped bodies. These structures are crucial for the development of proximal and distal convoluted tubules as well as the loop of Henle. The continued growth and differentiation of these S-shaped bodies lead to the establishment of the various components of the nephron, including Bowman's capsule, glomerulus, proximal tubule, loop of Henle, and distal tubule (Braidy *et al.,* 2011; Olukole, 2021).

Around E16-E17, the collecting duct system begins to form as the nephric duct elongates and connects with the S-shaped bodies. The interaction between the elongating nephric duct and the developing nephrons is crucial for the establishment of proper connections and drainage pathways. The collecting duct system eventually branches and fuses with the ureteric bud, an outgrowth from the nephric duct, to form the complex tubular structures that collect and transport urine (Martins and Neuhaus, 2007; Braidy *et al.,* 2011; Olukole, 2021).

As the rat embryo develops further, the differentiation and maturation of nephrons and associated structures continue. By E18-E19, the nephrons become functional, with glomeruli

capable of filtering blood and forming urine. The gradual development of the renal corpuscle, tubules, and connecting structures contributes to the refinement of the kidney's filtration, reabsorption, and secretion capabilities (Martins and Neuhaus, 2007).

The intricate process of vascularization also occurs during embryonic kidney development. Blood vessels begin to invade the developing kidney, providing the necessary oxygen and nutrients for continued growth and maturation. The integration of the vasculature with the nephrons ensures the efficient filtration and transport of substances throughout the developing renal system (Olukole, 2021).

The development of the kidney is a complex process that begins in the fourth week of embryonic life and continues until birth. The kidney is derived from the intermediate mesoderm, and its development is divided into three stages: the pronephros, the mesonephros, and the metanephros.

The pronephros is the first rudimentary kidney to form in the embryo. It appears at the cranial level of the intermediate mesoderm in the fourth week of gestation. The pronephros consist of a series of epithelial buds that form tubules. These tubules are connected to a duct called the pronephric duct, which drains into the cloaca. The pronephros is functional for a short period of time in early embryonic development, but it degenerates by the end of the fourth week (Gilbert, 2013; Larsen, 2014). The mesonephros is the second rudimentary kidney to form in the embryo. It appears at the thoracic and lumbar levels of the intermediate mesoderm in the fifth week of gestation. The mesonephros consists of a series of epithelial buds that form tubules and a duct called the mesonephric duct. The mesonephric tubules are connected to the Bowman's capsule of the glomerulus, which is a cluster of blood capillaries. The mesonephros is functional for a longer period of time than the pronephros, and it is the main kidney in the embryo from the fifth to the tenth week of gestation. However, the mesonephros also degenerates by the end of the tenth week of gestation (Sadler, 2012).

The metanephros is the permanent kidney. It begins to form in the fifth week of gestation, but it does not become functional until the twelfth week of gestation. The metanephros develops from two parts: the ureteric bud and the metanephric blastema. The ureteric bud is an outgrowth of the mesonephric duct, and the metanephric blastema is a mass of mesenchymal cells that surrounds the ureteric bud (Gilbert, 2013).

The ureteric bud grows into the metanephric blastema and branches repeatedly to form the collecting system of the kidney (Larsen, 2014). The metanephric blastema differentiates into the nephrons, which are the functional units of the kidney. Each nephron consists of a glomerulus and a tubule system. The glomerulus is a cluster of blood capillaries that filters the blood. The tubule system reabsorbs and secretes substances from the filtrate to form urine. The development of the metanephros is a complex process that is regulated by a variety of genes and signaling molecules. The metanephros is not fully functional until the third trimester of pregnancy (Sadler, 2012; Gilbert, 2013).

The histological changes during kidney development can be divided into three stages: the early stage, the middle stage, and the late stage. The early stage of kidney development is characterized by the formation of the ureteric bud and the metanephric blastema. The ureteric bud is a simple epithelial tube that branches repeatedly to form the collecting system of the kidney. The metanephric blastema is a mass of mesenchymal cells that surrounds the ureteric bud (Sadler, 2012; Larsen, 2014). The middle stage of kidney development is characterized by the formation of the nephrons. The nephrons are the functional units of the kidney, and they consist of a glomerulus and a tubule system. The glomerulus is a cluster of blood capillaries that filters the blood. The tubule system reabsorbs and secretes substances from the filtrate to form urine (Gilbert, 2013). The late stage of kidney development is characterized by the maturation of the nephrons and the collecting system. The nephrons become more complex

and develop the ability to filter blood and produce urine. The collecting system also matures and becomes able to transport urine to the ureter (Sadler, 2012, Gilbert, 2013; Larsen, 2014).

2.2.3 Histology of the Wistar Rat Kidney

The histology of the kidney in the Wistar rat unveils a highly organized and intricate microarchitecture that underpins its vital functions within the organism. Comprising an array of specialized cellular structures and functional units, the rat kidney's histological composition reflects its role in maintaining fluid and electrolyte balance, eliminating waste products, and regulating blood pressure (Danielson *et al.*, 2006; Amini *et al.*, 2012).

At the histological level, the kidney can be divided into distinct regions that collaborate harmoniously to fulfill its multifaceted roles. The renal cortex constitutes the outermost layer and harbors key components such as renal corpuscles, convoluted tubules, and proximal and distal convoluted tubules. The renal corpuscles, consisting of glomeruli and Bowman's capsules, are focal points of blood filtration. The glomeruli are intricate networks of capillaries enveloped by Bowman's capsules, where the initial stage of urine formation occurs through the filtration of blood plasma (Danielson *et al.*, 2006; Dardouri *et al.*, 2016).

The proximal convoluted tubules, characterized by their densely packed brush-border epithelial cells, reabsorb essential substances like glucose, ions, and amino acids from the filtrate back into the bloodstream. This segment plays a pivotal role in maintaining the body's electrolyte and nutrient balance. Following this, the filtrate progresses to the loop of Henle, which consists of descending and ascending limbs. The loop of Henle serves to establish an osmotic gradient within the kidney, contributing to the concentration of urine (Danielson *et al.*, 2006; Palipoch and Punsawad, 2013). Adjacent to the loop of Henle, the distal convoluted tubules continue to fine-tune the composition of the filtrate. Here, specialized cells regulate the secretion and

reabsorption of ions, including sodium, potassium, and hydrogen ions, crucial for electrolyte balance and acid-base regulation (Amini *et al.,* 2012; Palipoch and Punsawad, 2013).

The renal medulla, deeper within the kidney, contains renal pyramids, which consist of collecting ducts and connecting tubules. These structures play a pivotal role in concentrating urine by responding to the body's hydration status through the secretion of antidiuretic hormone (ADH). As the filtrate passes through the collecting ducts, water reabsorption can be precisely controlled, resulting in either concentrated or diluted urine production (Amini *et al.,* 2012). Furthermore, the juxtaglomerular apparatus, situated at the junction of the distal convoluted tubule and the afferent arteriole, plays a pivotal role in regulating blood pressure and sodium balance. Specialized cells within this region, known as juxtaglomerular cells, release the enzyme renin in response to changes in blood pressure, initiating the renin-angiotensin-aldosterone system that acts to restore blood pressure and fluid balance (Dardouri *et al.,* 2016).

CHAPTER THREE

MATERIALS AND METHOD

3.1 EQUIPMENT

The following equipment were used for the study:

- Electronic weighing balance (manufactured by Excell Precision Co., Ltd., Taiwan)
- Centrifuge 412B (Techmel and Techmel, U.S.A)
- Rotary microtome (Bright B5143, Huntington, England)
- LABO® trinocular microscope (manufactured by Labo Microsystems GmbH, Baecker Strasse, 21244 Buchholz, Germany)
- Camera (OMAX Company Limited, Korea)
- Carbon fiber composite digital caliper (Entatial, China)
- Refrigerator (UV MCO-80IC Panasonic, MDF-U3386S)
- Water bath
- Paraffin dispenser
- Dissecting set
- Polypropylene cages with wire gauze
- Microscopic slides
- Oven
- 1ml pipette

3.2 CAFFEINE

Caffeine (manufactured by Macfarlan Smith Ltd, Edinburgh) was purchased from Emmytex Biomedical Chemicals Store, 112 Ugbowo-Lagos Road, Benin City, Edo State, Nigeria.

3.3 COMPUTER SOFTWARES

The following computer software was used for the analyses in the study:

- ToUpView software version x64, 3.7.71.49 (manufactured by Hangzhou ToupTek Photonics Co., Ltd, Block 1, 3#, Xiyuan 9 Road, Hangzhou, 310030, Zhejiang, P.R, China; released in 2016)
- IBM Statistical Package for Social Sciences, Version 23 (manufactured by International Business Corporations {IBM}; released in 2015).

3.4 EXPERIMENTAL DESIGN

Thirty (30) adult Wistar rats weighing between 170 g and 180 g were used for this study. The animals were bred at the Animal House, Department of Anatomy, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City, Edo State, Nigeria. They were kept in polypropylene cages at room temperature with 12 hours' light and dark cycle. The animals were fed with pelleted feed (manufactured by Premier Feed Mills Company Limited, 1 Eagle Flour Road, Lagos/Ibadan Expressway Toll point, Ibadan, Oyo State, Nigeria) and clean tap water. They were weighed weekly before commencement and throughout the duration of the experiment using a digital weighing scale calibrated in grams and recorded to the nearest whole number. Animals were paired overnight at the estrous cycle with sexually active males in the ratio of 2:1. Estrous cycle was confirmed by vaginal lavage. The presence of a vaginal plug and/or sperm in the vaginal smear is GD0 as this indicates successful mating. The pregnant rats were divided into two groups (A and B) with fifteen (15) rats per group. Group A served as the Control that was administered with a single intraperitoneal injection of 1ml of normal saline on GD 11, in addition to free access to feed and water. Group B served as the treated group and was administered a single intraperitoneal injection of 150mg/Kg body weight of caffeine from GD11 as previously described by Richard *et al.* (2012). On each gestational day (GD15, GD17, and GD19), five (5) animals were sampled from each group.

The uterine horns were exteriorized and incised at the greater curvature of the horns. Fetal kidney tissues were harvested from each group for histological assessment. The harvested kidneys were fixed with 10% neutral buffered formalin before being taken to the laboratory for histological assessment.

3.5 HAEMATOXYLIN AND EOSIN STAINING PROCEDURE

Tissue sections were deparaffinized in two changes of xylene for two minutes in each change and passed through two changes of absolute alcohol for four minutes each. They were hydrated using a series of descending grades of alcohol until water was used. Procedures of Haematoxylin and Eosin adopted in the sections were described by Drury and Wallington (1980) and Scheehan and Hrapchak (1980). The sections were:

- Dewaxed in two changes of xylene for two minutes in each change;
- Rehydrated in descending grades of alcohol (absolute II, absolute I, 95%, 90%, 70%, and 50% ethanol) for two minutes each;
- Rinsed in distilled water for three minutes
- Stained in hematoxylin for 15-20 minutes
- Excess hematoxylin stain was removed by rinsing well in running tap water for two to three minutes (sections were examined microscopically at this stage to confirm the sufficient degree of staining);
- Differentiated in acid alcohol (0.5% HCl in 70% ethanol for two to three minutes;
- Rinsed well in running water for 10-15 minutes;
- Counterstained in 1% aqueous eosin for two to four minutes;
- Excess stain was washed off in running water and examined under a microscope;

- Dehydrated rapidly in ascending grades of ethanol (50% through absolute ethanol), cleared in xylene, and mounted in a synthetic resin medium (DPX).

3.6 STATISTICAL ANALYSIS

Data was analyzed using the IBM statistical package for social sciences. Results were presented as mean ± standard error of the mean (mean ± SEM). The parameters for all the groups were compared using students' t-test (two-tailed, assuming equal variance). Differences in mean were considered statistically significant at a 95% confidence level (that is when probability would be less than 0.05 {$P<0.05$}).

3.7 PHOTOMICROGRAPHY

The processed slides were captured with a LABO® trinocular microscope (Labo Microsystems GmbH, Germany) on which was mounted an Omax 9.0 MP USB Digital Microscope Camera (made in Korea). The camera features 9 megapixel (3488 X 2616 pixel) high-resolution color digital camera and a 0.5X reduction lens. It was connected to a laptop on which ToUpView software (version x64, 3.7.71.49; built-in 2016) was installed. A panoramic view of the slides was captured using X4 and X10 objective lenses.

CHAPTER FOUR

RESULTS

4.1 HISTOLOGICAL FINDINGS

The pregnant Wistar rats in groups A and B were used for this assessment. The findings were demonstrated in plates 4.1A, 4.1B, 4.2A, 4.2B, 4.3A, 4.3B, 4.4A, 4.4B, 4.5A , 4.5B, 4.6A and 4.6B.

Plate 4.1A: Photomicrograph of a section of the kidney of the control group at gestational day 15 showing developing glomerulus (DG), and branching renal tubules (BR) (H&E; 400X; scale bar = 100 µm)

Plate 4.1B: Photomicrograph of a section of the kidney of the control group at gestational day 15 showing developing glomerulus (DG), and branching renal tubules (BR) (H&E; 400X; scale bar = 25 µm)

Plate 4.2A: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 15 showing edematous interstitial space (E) (H&E; 400X; scale bar = 100 µm).

Plate 4.2B: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 15 showing edematous interstitial space (E) (H&E; 400X; scale bar = 25 µm).

Fig. 4.3A: Photomicrograph of a section of the kidney of the control group at gestational day 17 showing developing glomerulus (DG) and canalizing renal tubules (CR) (H&E; 400X; scale bar = 100 µm).

Fig. 4.3B: Photomicrograph of a section of the kidney of the control group at gestational day 17 showing developing glomerulus (DG) and canalizing renal tubules (CR) (H&E; 400X; scale bar = 25 µm).

Fig. 4.4A: Photomicrograph of the kidney of the group treated with caffeine at gestational day 17 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 100 µm).

Fig. 4.4B: Photomicrograph of the kidney of the group treated with caffeine at gestational day 17 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 25 µm).

Fig. 4.5A: Photomicrograph of a section of the kidney of the control group at gestational day 19 showing developed glomerulus (GL) and renal tubules (RT) (H&E; 400X; scale bar = 100 µm).

Fig. 4.5B: Photomicrograph of a section of the kidney of the control group at gestational day 19 showing developed glomerulus (GL) and renal tubules (RT) (H&E; 400X; scale bar = 25 µm).

Plate 4.6: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 19 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 100 µm).

Plate 4.6: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 19 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 25 µm).

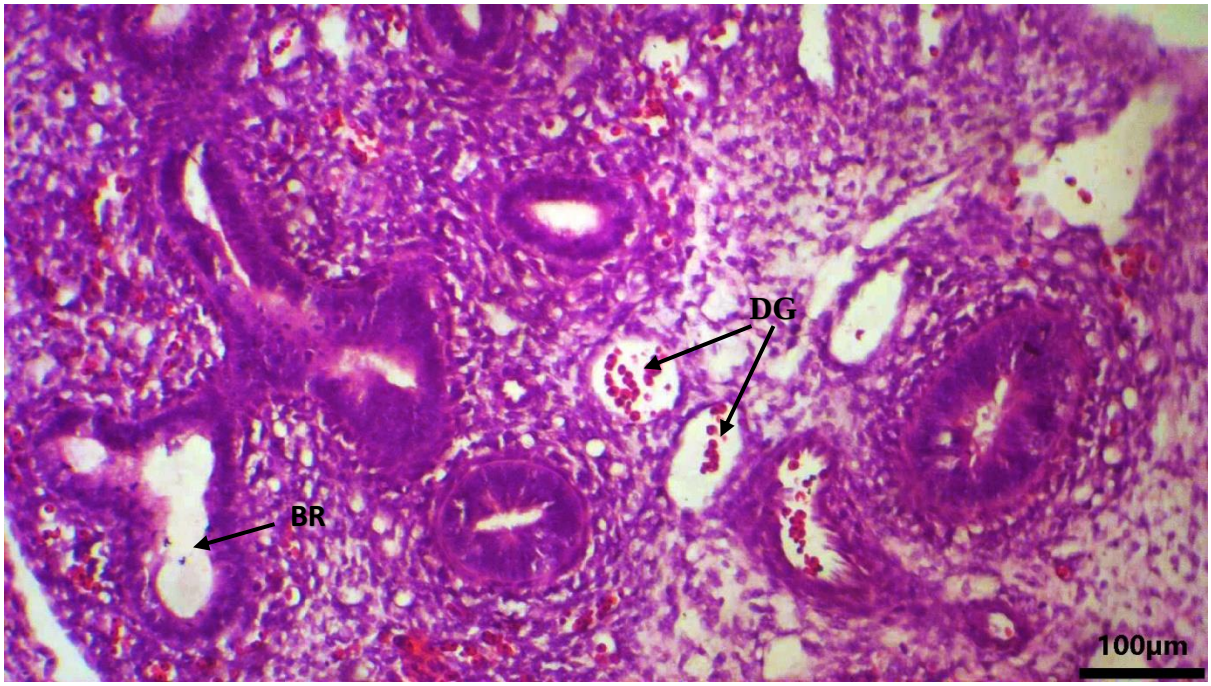

Plate 4.1A: Photomicrograph of a section of the kidney of the control group at gestational day 15 showing developing glomerulus (DG), and branching renal tubules (BR) (H&E; 400X; scale bar = 100 µm)

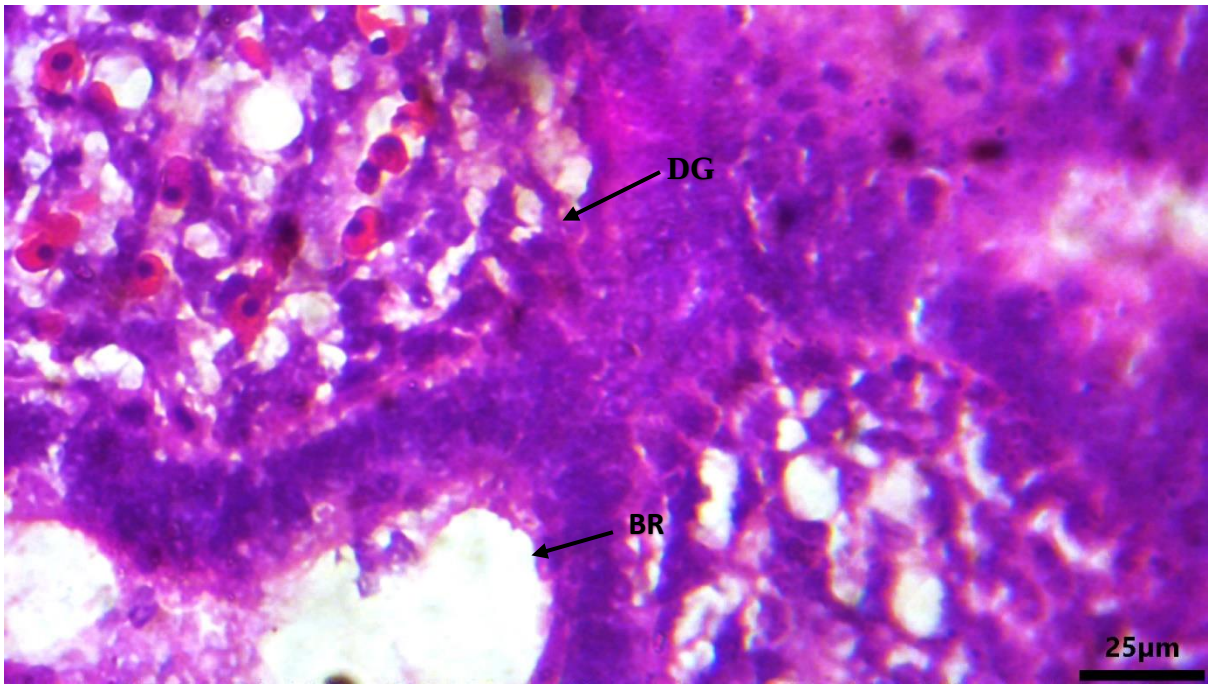

Plate 4.1B: Photomicrograph of a section of the kidney of the control group at gestational day 15 showing developing glomerulus (DG), and branching renal tubules (BR) (H&E; 400X; scale bar = 25 µm)

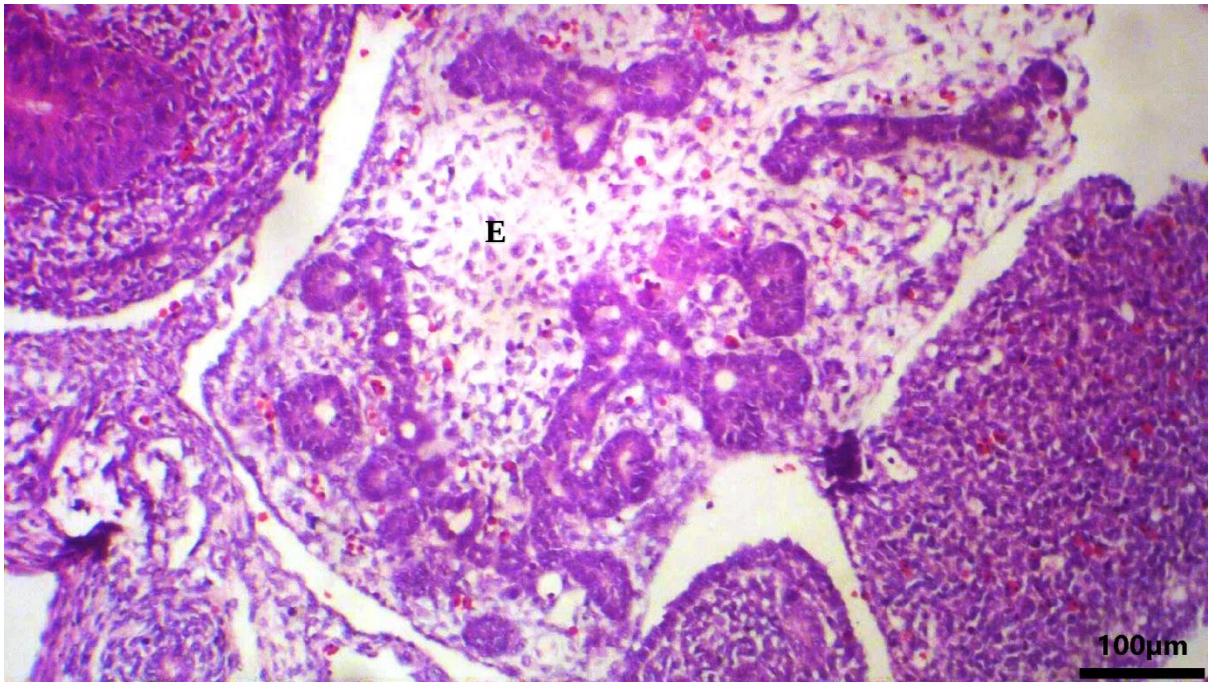

Plate 4.2A: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 15 showing edematous interstitial space (E) (H&E; 400X; scale bar = 100 µm).

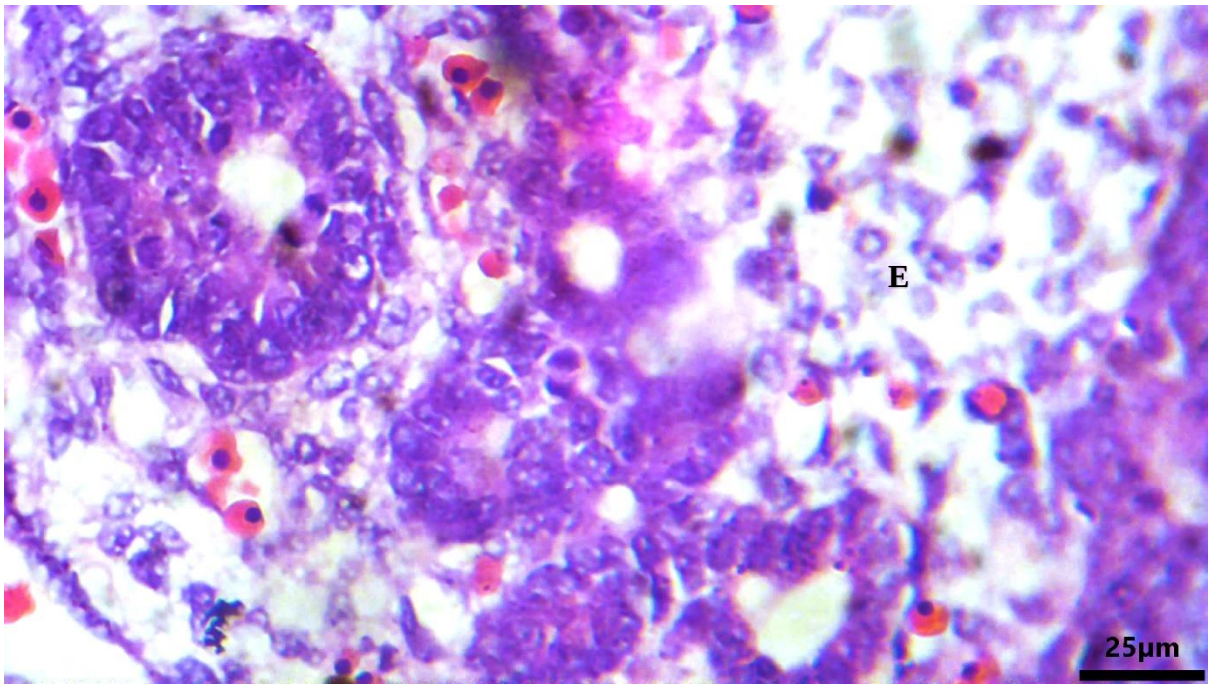

Plate 4.2B: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 15 showing edematous interstitial space (E) (H&E; 400X; scale bar = 25 µm).

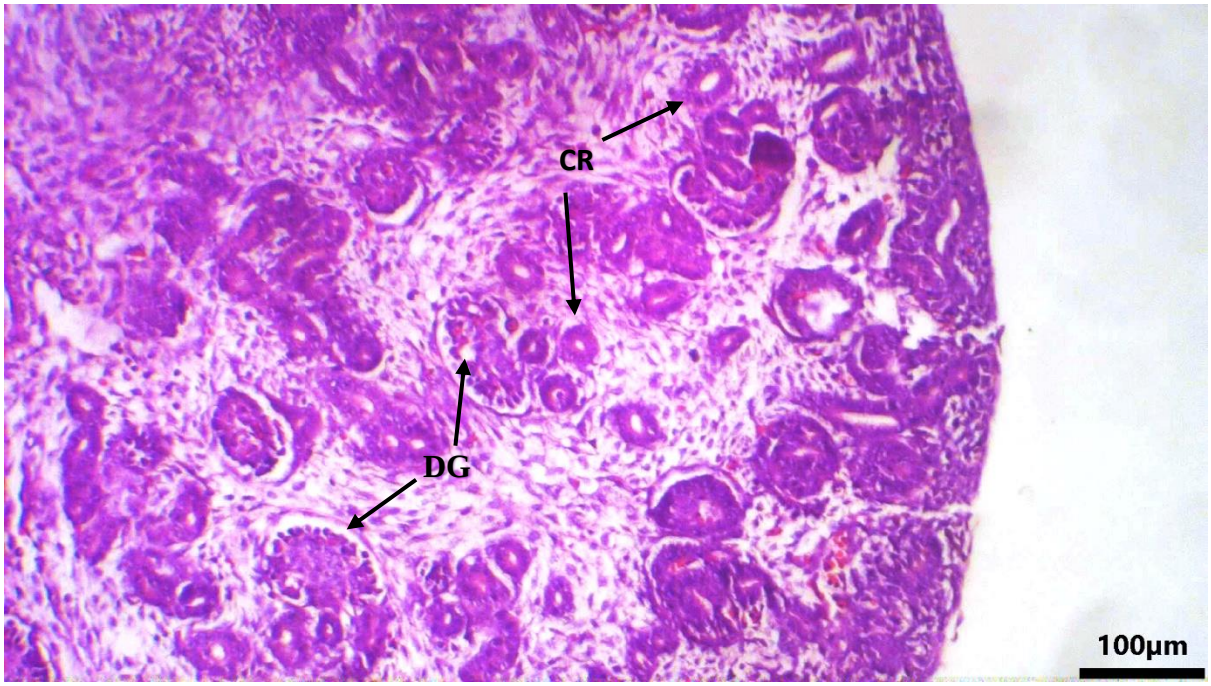

Fig. 4.3A: Photomicrograph of a section of the kidney of the control group at gestational day 17 showing developing glomerulus (DG) and canalizing renal tubules (CR) (H&E; 400X; scale bar = 100 µm).

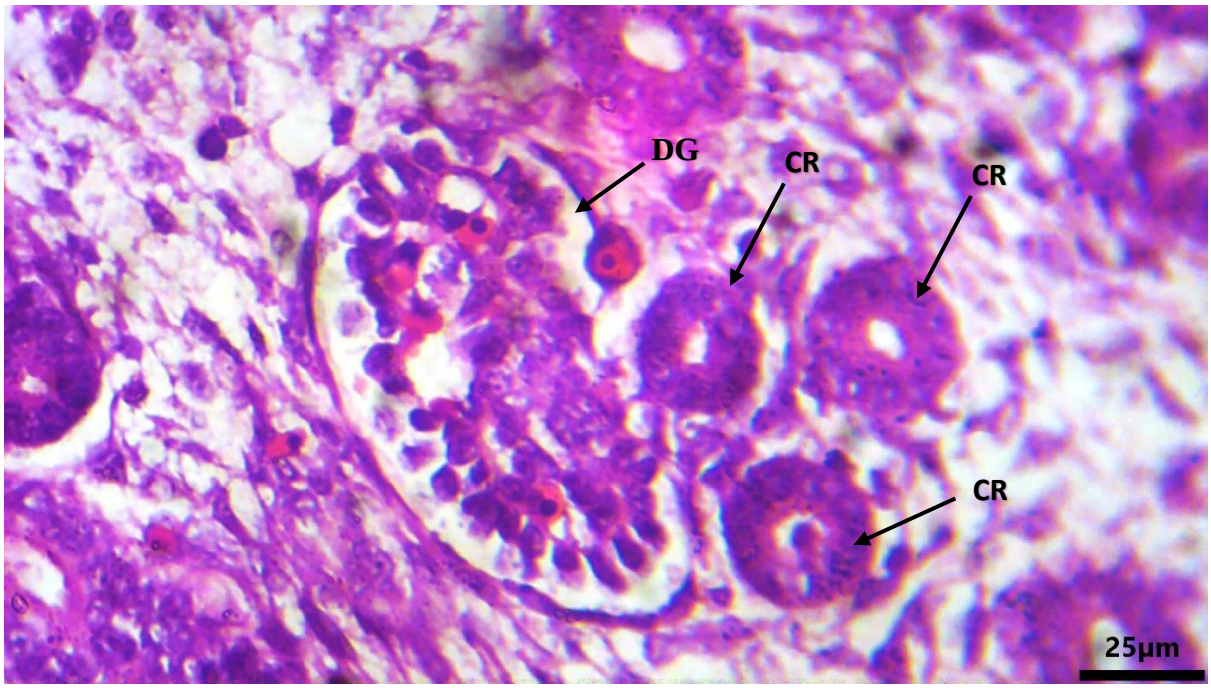

Fig. 4.3B: Photomicrograph of a section of the kidney of the control group at gestational day 17 showing developing glomerulus (DG) and canalizing renal tubules (CR) (H&E; 400X; scale bar = 25 µm).

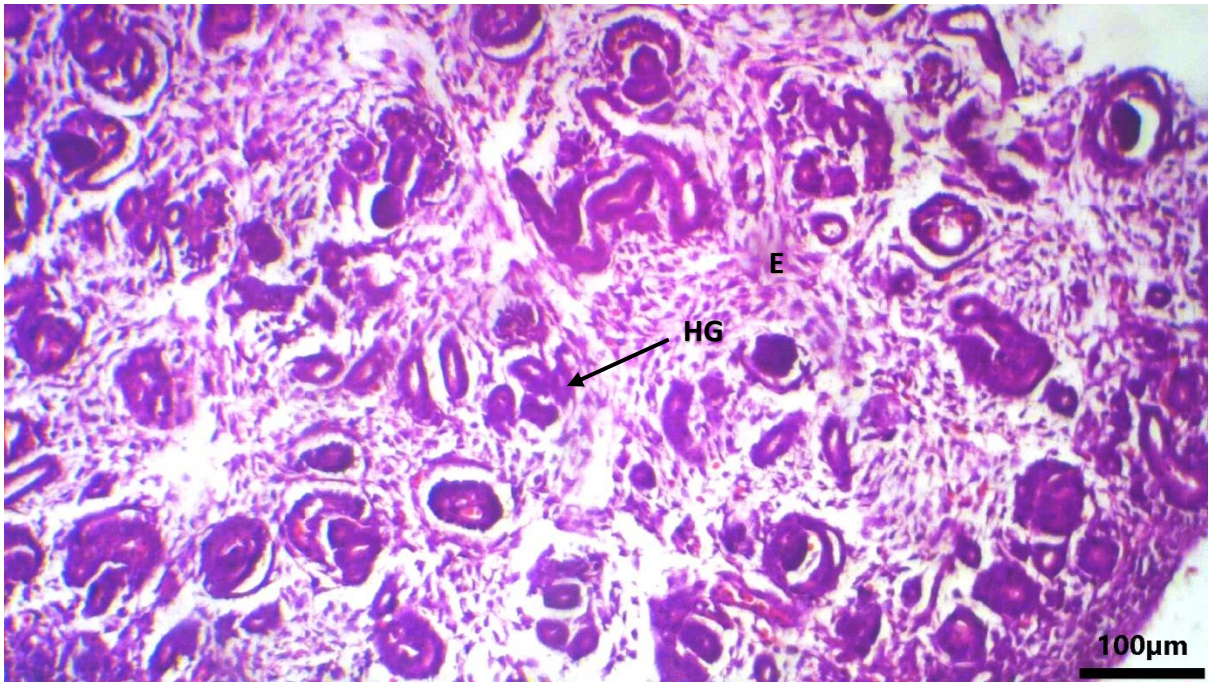

Fig. 4.4A: Photomicrograph of the kidney of the group treated with caffeine at gestational day 17 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 100 µm).

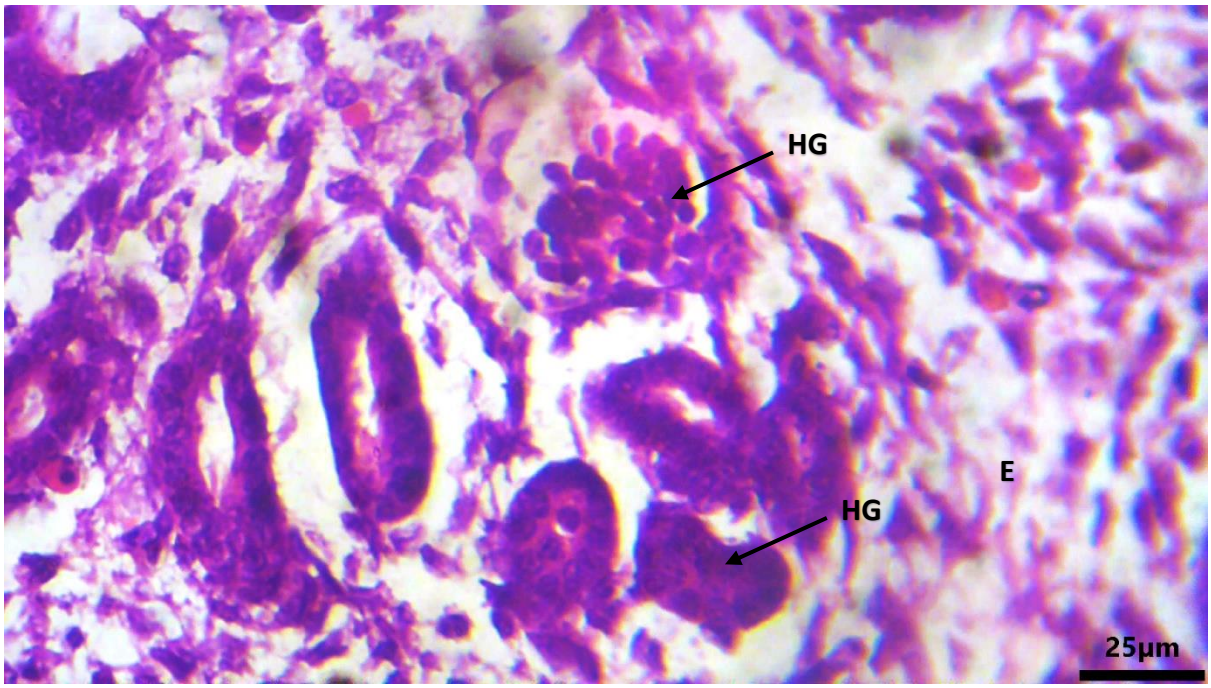

Fig. 4.4B: Photomicrograph of the kidney of the group treated with caffeine at gestational day 17 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 25 µm).

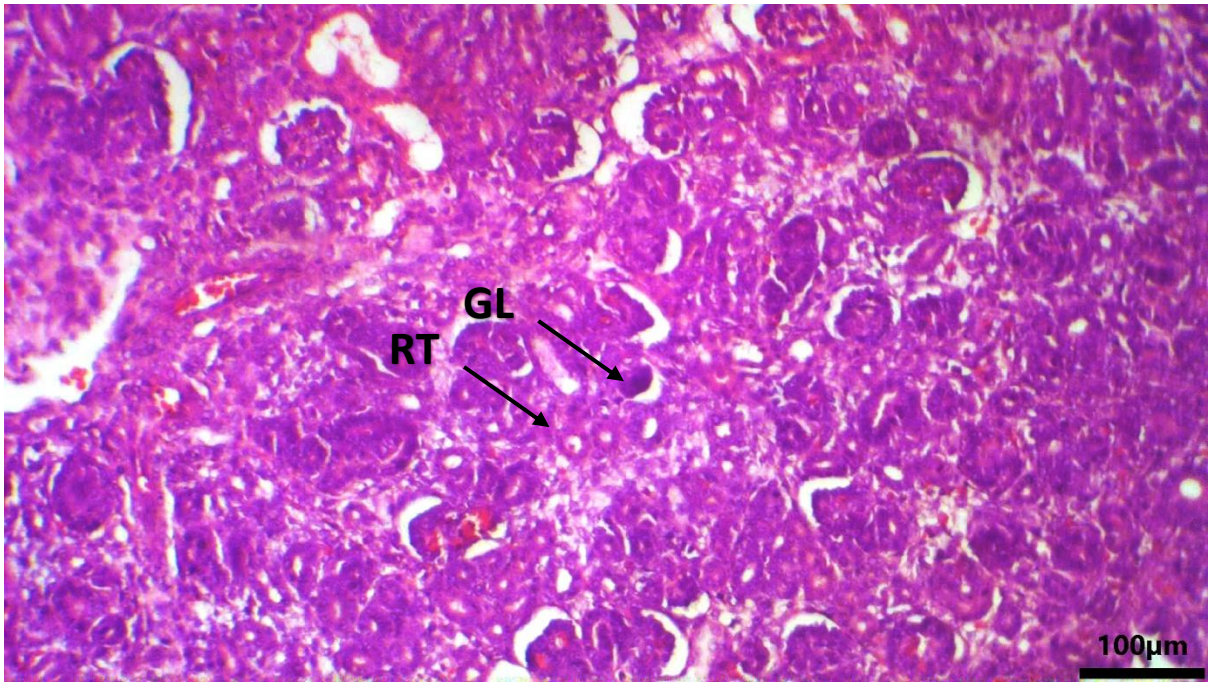

Fig. 4.5A: Photomicrograph of a section of the kidney of the control group at gestational day 19 showing developed glomerulus (GL) and renal tubules (RT) (H&E; 400X; scale bar = 100 µm).

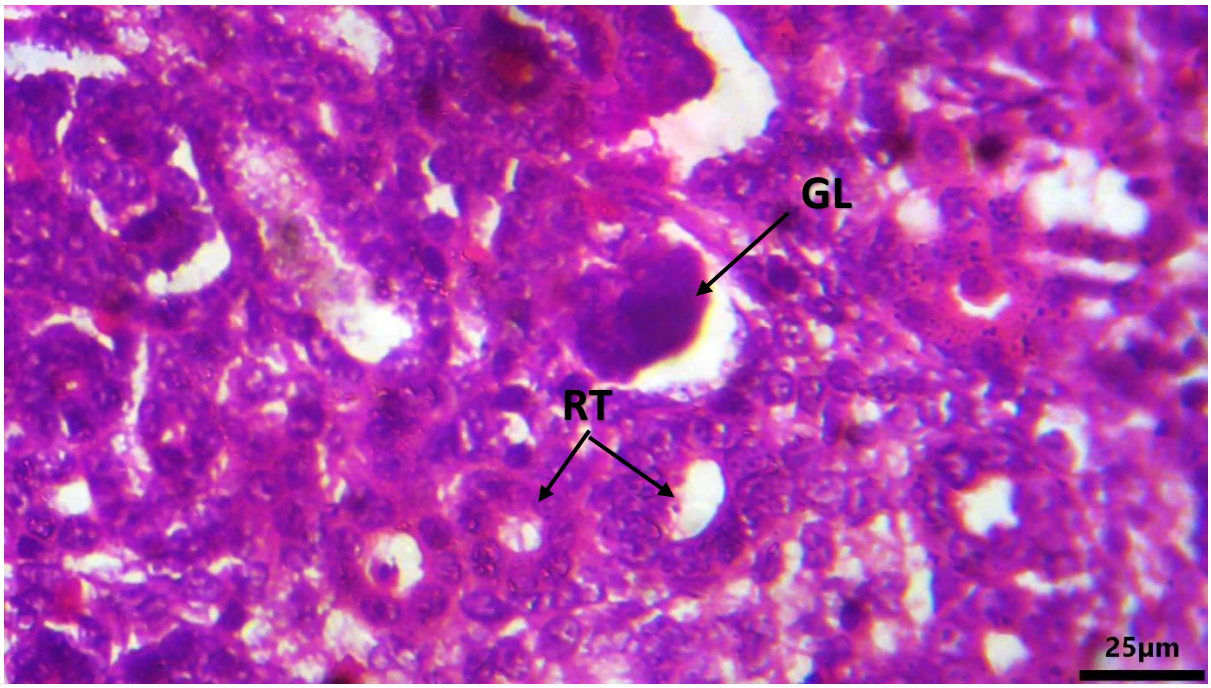

Fig. 4.5B: Photomicrograph of a section of the kidney of the control group at gestational day 19 showing developed glomerulus (GL) and renal tubules (RT) (H&E; 400X; scale bar = 25 µm).

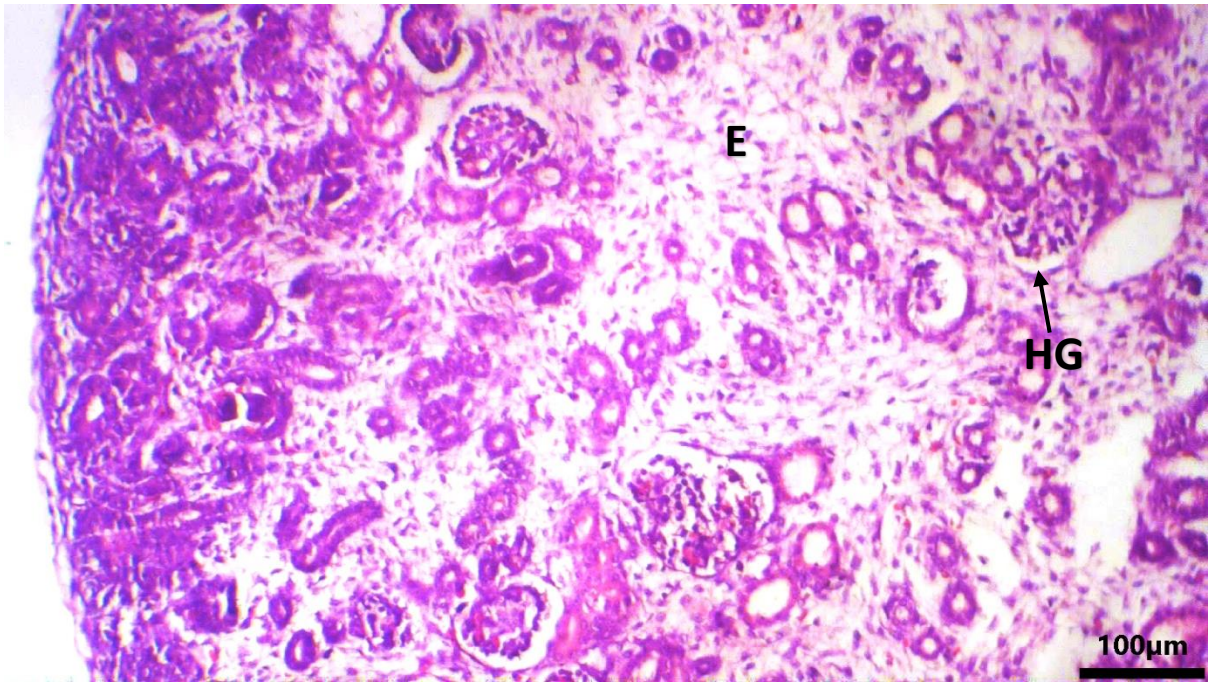

Plate 4.6: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 19 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 100 µm).

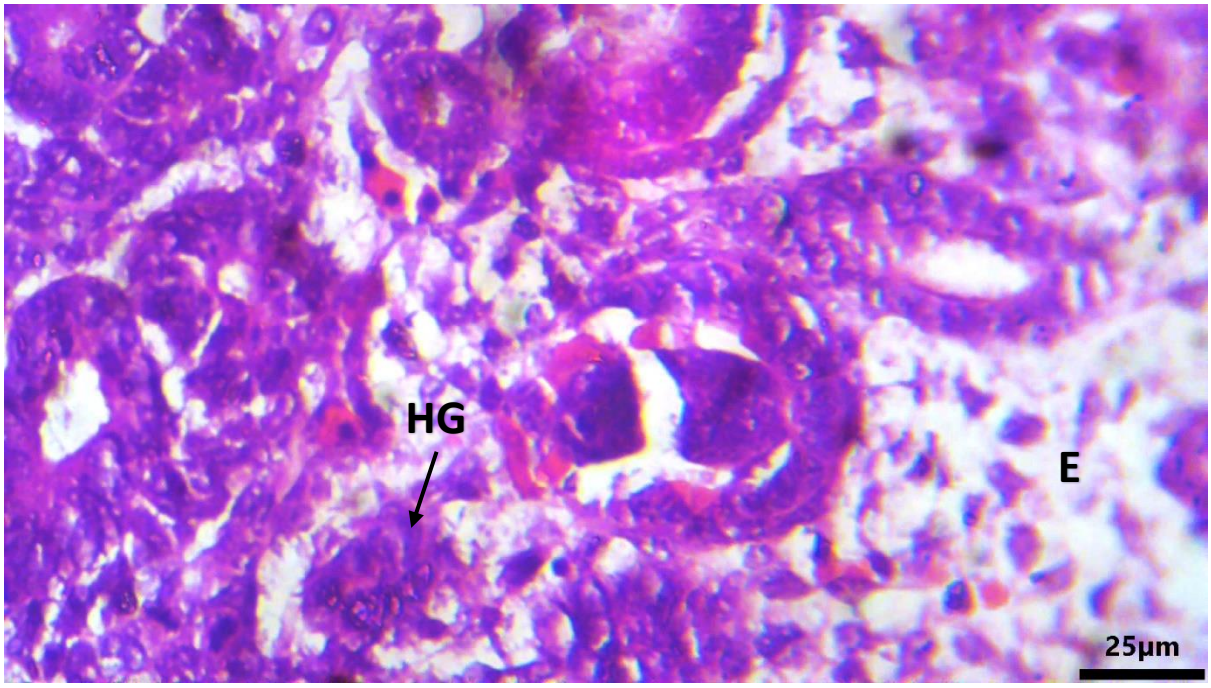

Plate 4.6: Photomicrograph of a section of the kidney of the group treated with caffeine at gestational day 19 showing edematous interstitial space (E) as well as hypoplasia of the glomerulus (HG). (H&E; 400X; scale bar = 25 µm).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

Caffeine is a widely consumed substance, but it is important to be aware of the potential risks of caffeine exposure during pregnancy. Caffeine can cross the placenta and reach the developing fetus, where it can interfere with growth and development. The placenta serves as a lifeline between the mother and the developing fetus, providing nutrients and oxygen essential for the growth and well-being of the unborn child (Muehlbacher *et al.,* 2020). While the placenta acts as a remarkable filter, safeguarding the fetus from a multitude of potentially harmful substances, caffeine is one of the exceptions that can traverse this protective barrier. Once caffeine gains access to the fetal environment, it has the capacity to exert profound effects on the developing fetus (Bolignano *et al.,* 2007).

The present study investigated the effects of intrauterine caffeine exposure on kidney development in Wistar rats. The results showed that caffeine exposure caused significant alterations in kidney morphology at GD 15, 17, and 19.

Findings from this study show that while the developing glomerulus and branching renal tubules are clearly visible in the control group on GD15, the caffeine-treated rats at GD15 showed severe interstitial congestion with fetal blood. Edematous interstitial space is a condition in which the interstitial space of a tissue is filled with excess fluid. This can occur due to a variety of factors, such as inflammation, infection, or trauma. In the present study, the interstitial congestion observed in the caffeine-exposed kidneys is likely due to the C. Caffeine causes vasodilation, which leads to an increase in blood flow to the kidneys. This increased blood flow can put stress on the delicate blood vessels of the developing kidney, leading to congestion and rupture. This is consistent with the findings by Ogunwole *et al* (2015), who

reported that Caffeine-treated dams showed interstitial congestion and hence adversely affected reproductive indices in male offspring of Wistar rats.

Findings from the study show that the developing glomerulus and canalizing renal tubules are clearly visible in the control group on GD17. However, the caffeine-treated rats at GD17 showed Edematous interstitial space as well as hypoplasia of the glomerulus. Hypoplasia is a condition in which an organ or tissue is underdeveloped. In the present study, the hypoplasia of the glomerulus observed in the caffeine-exposed kidneys is likely due to the disruptive effects of caffeine on cell growth and differentiation. Caffeine can interfere with a variety of cellular processes, including DNA replication, protein synthesis, and cell signaling. These disruptions can lead to impaired growth and development of the glomerulus, which is a critical component of the kidney filtration system. This finding is consistent with the findings of a study by Pacifici (2014), who reported mild congestion as well as evidence of disrupted cell growth in rats exposed to caffeine.

On GD19, findings from the study showed that the developing glomerulus and canalizing renal tubules were clearly visible, in the control group. However, mild interstitial congestion, as well as hypoplasia of the glomerulus, were evident in the caffeine-treated rats.

The findings of the present study are consistent with previous studies that have reported adverse effects of intrauterine caffeine exposure on kidney development. For example, a study by Onodera *et al.* (1993) found that caffeine exposure during pregnancy caused a decrease in the number of glomeruli in rat pups. Another study by Joaquim *et al.* (1996) found that caffeine exposure during pregnancy caused a delay in the maturation of the renal tubules in rat pups.

The findings of the present study suggest that intrauterine caffeine exposure can have a significant impact on kidney development. The observed alterations in kidney morphology could lead to functional impairments in renal function later in life.

5.2 CONCLUSION

In conclusion, the findings from this study show that Caffeine has teratogenic potential against kidney development in Wistar rats. The present study provides further evidence that intrauterine caffeine exposure can have a significant impact on kidney development. The observed alterations in kidney morphology could lead to functional impairments in renal function later in life.

5.3 RECOMMENDATION

Following the findings from the study, the following are the recommendations

1. Further research is needed to investigate the long-term effects of intrauterine caffeine exposure on kidney function and health.
2. Further research is necessary to understand the exact mechanisms through which caffeine affects kidney development and to determine whether these effects are reversible or permanent.
3. Cautious caffeine consumption during pregnancy is advised. Pregnant women should be aware of the potential risks of caffeine exposure during pregnancy and limit their caffeine intake to no more than 200 mg per day.

REFERENCE

Alsabri, S. G., Mari, W. O., Younes, S., Elsadawi, M. A., & Oroszi, T. L. (2018). Kinetic and dynamic description of caffeine. *Journal of Caffeine and Adenosine Research*, *8*(1), 3-9.

Amini, F. G., Rafieian-Kopaei, M., Nematbakhsh, M., Baradaran, A., & Nasri, H. (2012). Ameliorative effects of metformin on renal histologic and biochemical alterations of gentamicin-induced renal toxicity in Wistar rats. *Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences*, *17*(7), 621.

Aranda, J. V., Gorman, W., Bergsteinsson, H., & Gunn, T. (1977). Efficacy of caffeine in treatment of apnea in the low-birth-weight infant. *The Journal of pediatrics*, *90*(3), 467-472.

Azam, S., Hadi, N., Khan, N. U., & Hadi, S. M. (2003). Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. *Medical science monitor: international medical journal of experimental and clinical research*, *9*(9), BR325-30.

Beauchamp, G., Amaducci, A., & Cook, M. (2017). Caffeine toxicity: a brief review and update. *Clinical Pediatric Emergency Medicine*, *18*(3), 197-202.

Bertrand, B., Stefan, L., Pirrotta, M., Monchaud, D., Bodio, E., Richard, P., ... & Casini, A. (2014). Caffeine-based gold (I) N-heterocyclic carbenes as possible anticancer agents: synthesis and biological properties. *Inorganic chemistry*, *53*(4), 2296-2303.

Bolignano, D., Coppolino, G., Barillà, A., Campo, S., Criseo, M., Tripodo, D., & Buemi, M. (2007). Caffeine and the kidney: what evidence right now?. *Journal of Renal Nutrition*, *17*(4), 225-234.

Bonati, M., Latini, R., Galletti, F., Young, J. F., Tognoni, G., & Garattini, S. (2012). Caffeine disposition after oral doses. *Clinical Pharmacology & Therapeutics*, *32*(1), 98-106.

Bothe, H., & Cammenga, H. K. (1979). Phase transitions and thermodynamic properties of anhydrous caffeine. *Journal of Thermal Analysis and Calorimetry*, *16*(2), 267-275.

Braidy, N., Guillemin, G. J., Mansour, H., Chan-Ling, T., & Grant, R. (2011). Changes in kynurenine pathway metabolism in the brain, liver and kidney of aged female Wistar rats. *The FEBS journal*, *278*(22), 4425-4434.

Bryant, C. M., Dowell, C. J., & Fairbrother, G. (2002). Caffeine reduction education to improve urinary symptoms. *British Journal of Nursing*, *11*(8), 560-565.

Bulger, R. E., Siegel, F. L., & Pendergrass, R. (1974). Scanning and transmission electron microscopy of the rat kidney. *American Journal of Anatomy*, *139*(4), 483-501.

Casal, S., Oliveira, M. B. P. P., Alves, M. R., & Ferreira, M. A. (2000). Discriminate analysis of roasted coffee varieties for trigonelline, nicotinic acid, and caffeine content. *Journal of agricultural and food chemistry*, *48*(8), 3420-3424.

Chawla, J., Lorenzo, N., & Suleman, A. (2006). Neurologic effects of caffeine. *International Journal of Work-Integrated Learning*, *22(4), 463.*

Danielson, L. A., Welford, A., & Harris, A. (2006). Relaxin improves renal function and histology in aging Munich Wistar rats. *Journal of the American Society of Nephrology*, *17*(5), 1325-1333.

Dardouri, K., Haouem, S., Gharbi, I., Sriha, B., Haouas, Z., El Hani, A., & Hammami, M. (2016). Combined effects of Cd and Hg on liver and kidney histology and function in Wistar rats. *Journal of Agricultural chemistry and Environment*, *5*(4), 159-169.

de Mejia, E. G., & Ramirez-Mares, M. V. (2014). Impact of caffeine and coffee on our health. *Trends in Endocrinology & Metabolism*, *25*(10), 489-492.

dePaula, J., & Farah, A. (2019). Caffeine consumption through coffee: Content in the beverage, metabolism, health benefits and risks. *Beverages*, *5*(2), 37.

Dews, P. B. (1984). Behavioral effects of caffeine. In *Caffeine: Perspectives from recent research* (pp. 86-103). Berlin, Heidelberg: Springer Berlin Heidelberg.

Drury, R. A. B., & Wallington, E. A. (1980). Carleton's histological technique 5th ed. New York: Churchill Livingstone.

Eshetu, N., Afework, M., Makonnen, E., Debella, A., Ergete, W., & Tolesssa, T. (2016). Evaluation of the acute and sub-chronic toxic effects of aqueous leaf extracts of artemisia afra on liver, kidney and some blood parameters in Wistar Rats. *Advances in Bioscience and Bioengineering*, *4*(1), 1-9.

Eteng, M. U., Eyong, E. U., Akpanyung, E. O., Agiang, M. A., & Aremu, C. Y. (1997). Recent advances in caffeine and theobromine toxicities: a review. *Plant foods for human nutrition*, *51*, 231-243.

Frary, C. D., Johnson, R. K., & Wang, M. Q. (2005). Food sources and intakes of caffeine in the diets of persons in the United States. *Journal of the american dietetic association*, *105*(1), 110-113.

Fredholm, B. B., Bättig, K., Holmén, J., Nehlig, A., & Zvartau, E. E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacological reviews*, *51*(1), 83-133.

Gilbert, S. F. (2013). Postembryonic Development: Metamorphosis, Regeneration, and Aging in Developmental Biology 10 th ed (ed. Gilbert SF) Ch. 16, 549–590.

Golalipour, M. J., Azarhoush, R., Ghafari, S., Davarian, A., & Fazeli, S. A. (2009). Can formaldehyde exposure induce histopathologic and morphometric changes on rat kidney?[¿ la exposición al formaldehído puede inducir cambios histopatológicos y morfométricos sobre el riñón de rata?]. *International Journal of Morphology*, *27*(4), 1195-1200.

Graham, T. E., & Spriet, L. L. (1991). Performance and metabolic responses to a high caffeine dose during prolonged exercise. *Journal of applied physiology*, *71*(6), 2292-2298.

Grosso, L. M., & Bracken, M. B. (2005). Caffeine metabolism, genetics, and perinatal outcomes: a review of exposure assessment considerations during pregnancy. *Annals of epidemiology*, *15*(6), 460-466.

Hinds, T. S., West, W. L., Knight, E. M., & Harland, B. F. (1996). The effect of caffeine on pregnancy outcome variables. *Nutrition reviews*, *54*(7), 203-207.

Hoeger, W. W., Turner, L. W., & Hafen, B. Q. (2002). *Wellness: Guidelines for a healthy lifestyle*. Brooks Cole.

Hollenberg, N. K. (2004). Aldosterone in the development and progression of renal injury. *Kidney international*, *66*(1), 1-9.

Horowitz, E. (1989). Coffee, coffeehouses, and the nocturnal rituals of early modern Jewry. *ajs Review*, *14*(1), 17-46.

Huntley, E. D., & Juliano, L. M. (2012). Caffeine Expectancy Questionnaire (CaffEQ): construction, psychometric properties, and associations with caffeine use, caffeine dependence, and other related variables. *Psychological assessment*, *24*(3), 592.

Huntley, E. D., & Juliano, L. M. (2012). Caffeine Expectancy Questionnaire (CaffEQ): construction, psychometric properties, and associations with caffeine use, caffeine dependence, and other related variables. *Psychological assessment*, *24*(3), 592.

Imai, K., Nakamura, S., Kaneko, S., Yamasaki, K., & Hirose, M. (2006). Caffeine exposure during pregnancy causes kidney defects in mice. Toxicology Letters, 163(1), 51-58.

Ivy, J. L., Costill, D. L., Fink, W. J., & Lower, R. W. (1979). Influence of caffeine and carbohydrate feedings on endurance performance. *Pulse*, *1620*(16.18), 1693.

Joaquim, J., Costa, J., Ribeiro, F., & Correia, J. (1996). Effects of caffeine exposure during pregnancy on the development of the kidney in rat pups. Pediatric Nephrology, 10(6), 736-740.

Khokhar, S., & Magnusdottir, S. G. M. (2002). Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *Journal of agricultural and food chemistry*, *50*(3), 565-570.

Knutti, R., Rothweiler, H., & Schlatter, C. (1982). The effect of pregnancy on the pharmacokinetics of caffeine. In *New Toxicology for Old: A Critique of Accepted Requirements and Methodology* (pp. 187-192). Springer Berlin Heidelberg.

Kuczkowski, K. M. (2009). Caffeine in pregnancy. *Archives of gynecology and obstetrics*, *280*(5), 695-698.

Larsen, W. J. (2014). Human embryology (4th ed.). Churchill Livingstone.

Li, L., Wu, Y., & Zhang, W. (2010). PAX2 re-expression in renal tubular epithelial cells and correlation with renal interstitial fibrosis of rats with obstructive nephropathy. *Renal Failure*, *32*(5), 603-611.

Li, Y., Zheng, Y., Xu, X., Wang, X., & Hu, Y. (2022). Association between caffeine intake during pregnancy and kidney function in adulthood: A nationwide prospective cohort study. Nutrients, 14(15), 3085.

Liu, Y., Liu, S., Sun, Y., Wang, X., & Hu, Y. (2020). Association between maternal caffeine intake during pregnancy and urinary tract infection in infants: A systematic review and meta-analysis. *Frontiers in Nutrition*, *7*, 599985.

Maggi, S., Irwin, L. J., Siddiqi, A., & Hertzman, C. (2010). The social determinants of early child development: an overview. *Journal of paediatrics and child health*, *46*(11), 627-635.

Muehlbacher, T., Gaertner, V. D., & Bassler, D. (2020, December). History of caffeine use in neonatal medicine and the role of the CAP trial. In *Seminars in Fetal and Neonatal Medicine* (Vol. 25, No. 6). Elsevier.

Murphy, V. E., Smith, R., Giles, W. B., & Clifton, V. L. (2006). Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. *Endocrine reviews*, *27*(2), 141-169.

Nawrot, P., Jordan, S., Eastwood, J., Rotstein, J., Hugenholtz, A., & Feeley, M. (2003). Effects of caffeine on human health. *Food Additives & Contaminants*, *20*(1), 1-30.

Nehlig, A. (2018). Interindividual differences in caffeine metabolism and factors driving caffeine consumption. *Pharmacological reviews*, *70*(2), 384-411.

Noever, D. A., Cronise, R. J., & Relwani, R. A. (1995). Using spider-web patterns to determine toxicity. *NASA Tech Briefs*, *19*(MFS-28921).

Ogunwole, E., Akindele, O. O., Oluwole, O. F., Salami, S. A., & Raji, Y. (2015). Effects of oral maternal administration of caffeine on reproductive functions of male offspring of Wistar rats. *Nigerian Journal of Physiological Sciences*, *30*(1-2), 51-58.

Olukole, S. G. (2021). Morphometric analysis of the kidneys of the adult domesticated African great cane rat (Thryonomys swinderianus). *European Journal of Anatomy*, *13*(3), 117-120.

Onodera, S., Ohta, H., & Ikeda, S. (1993). Effects of caffeine exposure during pregnancy on the kidney of rat pups. Journal of Toxicological Sciences, 18(2), 107-116.

Pacifici, G. M. (2014). Clinical pharmacology of caffeine citrate in preterm infants. *MedicalExpress*, *1*, 243-250.

Palipoch, S., & Punsawad, C. (2013). Biochemical and histological study of rat liver and kidney injury induced by cisplatin. *Journal of toxicologic pathology*, *26*(3), 293-299.

Park, E. K., Hong, Y. S., Kim, J. Y., Kim, J. H., & Kim, S. R. (2013). Adverse effects of caffeine exposure during pregnancy on rat kidney development. *PLoS ONE*, *8*(11), e80018.

Peerapen, P., & Thongboonkerd, V. (2018). Caffeine in kidney stone disease: risk or benefit?. *Advances in Nutrition*, *9*(4), 419-424.

Price, K. R., & Fligner, D. J. (2000). Treatment of caffeine toxicity with esmolol. *Annals of emergency medicine*, *19*(1), 44-46.

Purseglove, J. W. (1976). The origins and migrations of crops in tropical Africa. *Origins of African plant domestication*, 291-310.

Qian, J., Chen, Q., Ward, S. M., Duan, E., & Zhang, Y. (2020). Impacts of caffeine during pregnancy. *Trends in Endocrinology & Metabolism*, *31*(3), 218-227.

Roehrs, T., & Roth, T. (2008). Caffeine: sleep and daytime sleepiness. *Sleep medicine reviews*, *12*(2), 153-162.

Sadler, T. W. (2012). *Langman's Medical Embryology* (12th ed.). Wolters Kluwer Health.

Sardao, V. A., Oliveira, P. J., & Moreno, A. J. (2002). Caffeine enhances the calcium-dependent cardiac mitochondrial permeability transition: relevance for caffeine toxicity. *Toxicology and applied pharmacology*, *179*(1), 50-56.

Sauer, A. H. (1994). "Caffeine." *Clinical Toxicology Review* 17 (2).

Schimpl, F. C., da Silva, J. F., de Carvalho Gonçalves, J. F., & Mazzafera, P. (2013). Guarana: revisiting a highly caffeinated plant from the Amazon. *Journal of ethnopharmacology*, *150*(1), 14-31.

Smith, A. (2002). Effects of caffeine on human behavior. *Food and chemical toxicology*, *40*(9), 1243-1255.

Sun, C. C., & Hou, H. (2008). Improving mechanical properties of caffeine and methyl gallate crystals by cocrystallization. *Crystal Growth and Design*, *8*(5), 1575-1579.

Trice, I., & Haymes, E. M. (1995). Effects of caffeine ingestion on exercise-induced change during high-intensity intermittent exercise. *International Journal of Sport Nutrition and Exercise Metabolism*, *5*(1), 37-44.

Vigevano, F., Vergani, P., Parazzini, F., Ricci, E., & Stronati, M. (2019). Maternal caffeine intake in pregnancy and kidney function in children: A systematic review and meta-analysis. *Pediatric Nephrology*, *34*(12), 2293-2305.

Vitti, F. P., Grandi, C., Cavalli, R. D. C., Simões, V. M. F., Batista, R. F. L., & Cardoso, V. C. (2018). Association between caffeine consumption in pregnancy and low birth weight

and preterm birth in the birth Cohort of Ribeirão Preto. *Revista Brasileira de Ginecologia e Obstetrícia*, *40*, 749-756.

Willson, C. (2018). The clinical toxicology of caffeine: A review and case study. *Toxicology reports*, *5*, 1140-1152.

Zhang, Y., Wang, X., Xu, X., Liu, S., & Hu, Y. (2021). Association between maternal caffeine intake during pregnancy and kidney stone formation in children: A systematic review and meta-analysis. *Nutrients*, *13*(10), 3377.