2024

STREPTOZOTOCIN-INDUCED DIABETES INCREASES KIDNEY TOXICITY IN RATS EXPOSED TO OCHRATOXIN A

Belkys Gonzalez

STREPTOZOTOCIN-INDUCED DIABETES INCREASES KIDNEY TOXICITY IN RATS EXPOSED TO OCHRATOXIN A

A dissertation submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

to the faculty of the

DEPARTMENT OF PHARMACEUTICAL SCIENCES

of

COLLEGE OF PHARMACY AND HEALTH SCIENCES

at

ST. JOHN'S UNIVERSITY
New York
by
Belkys Gonzalez

Date Submitted 11/27/2023 Date Approved 12/14/2023

Belkys Gonzalez

Dr. Sue M. Ford

ABSTRACT

STREPTOZOTOCIN-INDUCED DIABETES INCREASES KIDNEY TOXICITY IN RATS EXPOSED TO OCHRATOXIN A

Belkys Gonzalez

Due to the rising occurrence of diabetes in the population and the prevalence of OTA exposure via ingestion of contaminated foodstuffs, there is a need to assess the possible renal interaction of a diabetic disease state in concurrence with OTA exposure.

Sprague Dawley rats were treated with streptozotocin to induce experimental diabetes and then orally exposed to 0.5 mg/kg body weight OTA once a day for 21 days. Renal biomarkers and light microscopy were evaluated to assess the interaction between diabetic nephropathy and OTA exposure. LLC-PK_1 proximal tubule cells were used as an *in vitro* model to assess the potential mechanism involved in this interaction. Numerical data were analyzed with t-test or ANOVA with Tukey's post-hoc. All indications of significance were at $p \leq 0.05$.

Histopathological findings of diabetic animals treated with OTA showed increased inflammation, tubular degeneration, glomerular degeneration, interstitial fibrosis, necrosis and cast formation. Classic and novel biomarker assessment indicated that the interaction between DN and OTA exposure exacerbated renal damage and significant tubular damage. These assessments support that the interaction between diabetic nephropathy and OTA exposure exacerbated renal damage and inflammation.

The LLC-PK_1 porcine proximal tubule cell line was used to assess the interaction of glucose levels and OTA exposure on cell viability. LC_{50} of the high glucose groups

(17.5 mM glucose, 30 mM glucose, and 5 mM/30 mM glucose) were significantly lower from that of the 5 mM group indicating enhanced toxicity of OTA.

The effects of the combination treatment (STZ/OTA) were greater than for animals exposed to either treatment alone. These findings were supported by biomarker profiles which reveal greater effects on serum creatinine, serum protein, KIM-1, and NGAL in the STZ/OTA-treated animals compared to those treated with STZ or OTA alone. These results indicate that exposure to environmental mycotoxins such as OTA may represent an increased risk to diabetics compared to non-diabetic individuals. The demonstration that kidney cells grown in high glucose media were more sensitive to OTA suggests that increased glucose levels of plasma and/or urine may contribute directly to such interactive toxicity of diabetes and OTA exposure.

ACKNOWLEDGMENTS

First and foremost, I would like to sincerely thank Dr. Sue M. Ford for her many years of guidance, mentoring, and knowledge. I am grateful for having been her student and having the privilege to witness what it takes to be a great scientist, person and mentor. Without her, I would not have discovered the love and dedication I have for this discipline. I am also indebted to Dr. V. Korlipara, Chairman of the Department of Pharmaceutical Studies, who has been supportive of my goals and who actively worked to provide me with the tools and skills to successfully complete this study.

I would also like to extend this gratitude to Dr. Louis Trombetta, Dr. Raymond Ochs, Dr. Blase Billack and Dr. Xingguo Cheng for being members of my committee. Their support, wisdom, and professional guidance have made a profound impact on the completion and direction of this study. Genuine gratitude is extended to the Pharmaceutical Sciences Department and by extension St. John's University for providing the financial support and opportunity to serve as a Graduate Fellow.

I am also grateful for lab mates and colleagues, past and present. I am humbled by the unending support of my friends who were there many late nights and always there to lend a thought and a hand whenever it was needed. They were all a pleasure to work with.

My family and partner have been the sources of my motivation to complete this journey. I am forever indebted to them for their unconditional love and support. There are no words to express my love and appreciation for all the sacrifices, late nights and inspiration.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1- INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species that causes nephrotoxicity and renal tumors in a variety of animal species (Chen & Wu, 2017). OTA exposure mainly occurs via contaminated foodstuffs like oats, wheat, maize, barley, raisins, and dried vine fruits. It is present at all stages of the food chain. It is among 20 mycotoxins monitored in animal feed and human food by the FDA and the World Health Organization (Kőszegi & Poór, 2016). OTA is classified as a 2B, possible human carcinogen by the International Agency for Research on Cancer based on the evidence of carcinogenicity in several animal studies. In the kidney, the main nephrotoxic effect of OTA is lesions on the proximal tubule where membrane integrity is lost and severe damage to the brush border and interstitial fibrosis were observed (Malir et al., 2016). Despite the many studies conducted on OTA, the mode of action and attenuation of toxicity is only partially understood. In addition to OTA being well-known and widely-spread all over the world, several epidemiological studies suggest an association between human kidney disease and OTA exposure (Chen & Wu, 2017).

Renal excretion is one of the most important and vital routes of xenobiotic elimination in the body, and for some drugs the main route of elimination (Dresser, 2001). The renal organic anion and cation transport systems (OAT and OCT, respectively) are two of the primary transport systems in the kidney (Dresser et al., 2001). Some of the substrates transported by the OAT and OCT systems include ochratoxin A, probenecid and para-aminohippurate (PAH). Over the past several decades, both the mechanism of these transport systems and the associated transport proteins have been studied extensively both *in vivo* and *in vitro* However, the crystal structures have not yet

been determined (Roth et al., 2012). These proteins remain partly characterized and the underlying regulation of these transporters is not completely understood (Riedmaier et al., 2012). The past few decades have shown the importance of clarifying OAT and OCT transport system complete function and development within the body and their roles in disease states in addition to their response to therapeutic treatments (Riedmaier et al., 2012).

The kidney is not only susceptible to damage by OTA, but according to the National Kidney Foundation, diabetes is the leading cause of kidney failure. About 1 out of 4 adults with diabetes has kidney disease. Risk factors for developing diabetes include being overweight, inactive lifestyle, age 45 or older, family history, high blood pressure, abnormal blood fats, and being of African-American, Native American, Alaskan American, Hispanic, Pacific Islander, or Asian descent. Diabetes impairs the renal function of removing wastes, leading to an accumulation of waste products which can lead to other health issues. Both OTA exposure and diabetes frequently lead to chronic kidney disease.

Due to the rising occurrence of diabetes and the high prevalence of OTA exposure via ingestion, there is a need to assess the possible synergistic effects of a diabetic disease state in concurrence with OTA toxicity with regards to nephrotoxicity (Bui-Klimke & Wu, 2015; Chen & Wu, 2017). The present study was conducted to investigate the interaction where Sprague Dawley rats were treated with streptozotocin (Figure 1) to induce experimental diabetes and then orally exposed to 0.5mg/kg bodyweight OTA once

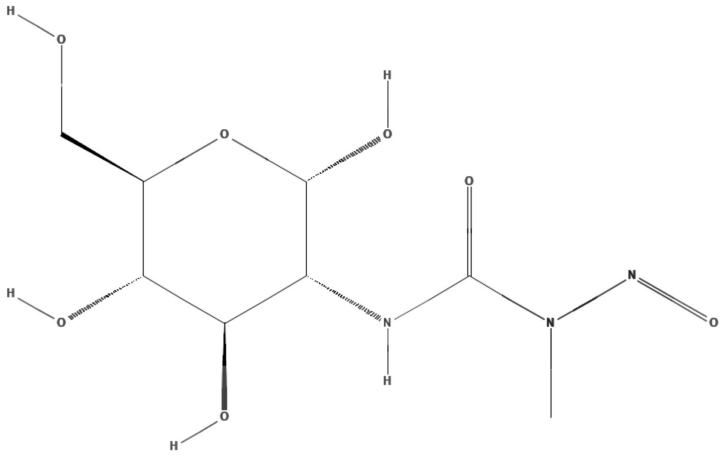

Figure 1. Streptozotocin. Structure of Streptozotocin, (Source: PubChem)

a day for 21 days. Renal cortical slices taken from treated animals were used to assess the transport of radiolabeled OTA. In addition to the functional transport assays, serum biomarkers were assessed, and light microscopy was conducted.

1.1 Mycotoxins

Mycotoxins are toxic secondary metabolites produced by certain fungi (Alshannaq & Yu, 2017). Mycotoxins are natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals (Bennett, 1987). Mycotoxins are a large group of several hundred chemically diverse fungi products which include aflatoxin, ergot alkaloids, penicillic acid, ochratoxins and citrinin. Mycotoxin producing fungi can grow on a variety of different foodstuffs including cereals, nuts, spices, dried fruits, apples and coffee beans usually during warm and humid conditions (WHO, 2018). Contamination with these fungi can occur before, during, or after harvesting and during storage, and these fungi are not always readily visible. Mycotoxin exposure can occur by directly consuming the contaminated foodstuff or through the consumption of animals that were fed contaminated feed. Mycotoxin contamination of the food supply is a global issue that is still occurring despite proper agricultural and processing practices and with no existing functioning solutions (Alshannaq & Yu, 2017)

Oral exposure to mycotoxins have resulted in acute symptoms of severe illness which manifest quickly. Some symptoms include vomiting, abdominal pain, pulmonary edema, convulsions, coma and in rare cases death (Yu, 2023). In particular, mycotoxins have been associated with nephrotoxicity including but not limited to interstitial fibrosis, tubular atrophy, and karyomegaly primarily in the proximal tubules (Heussner & Bingle,

2015). The most common way to be directly exposed is through maize, the crop that is known to be the most affected (Alshannaq & Yu, 2017). Most mycotoxins are chemically and thermally stable during processing (cooking, boiling, frying, pasteurization). Contamination by the mycotoxin producing fungi can also develop from the soil, decaying vegetation and rotting grains (Karlovsky, 2016).

Although mycotoxin contamination is a concern, the approach to solve this contamination problem is not clear-cut. Many authorities including the US Food and Drug Administration (US FDA), World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), and the European Food Safety Authority (EFSA), are implementing strict guidelines for the major mycotoxin classes in food and animal feed. However, not all mycotoxin classes have set limits for both the US FDA and European Union (EU) standards (Alshannaq & Yu, 2017). Two major approaches for providing guidance to reduce health risks and economic costs are increasing awareness for producers and handlers along the food supply chain and encouraging the adoption of good agricultural practices (GAP) and good manufacturing practices (GMP) (Karlovsky, 2016).

1.2 Ochratoxin A

Ochratoxins are a group of well-known global secondary metabolites produced by the micro fungi *Penicillium* and *Aspergillus*. Ochratoxin A (OTA) is the most toxic of the ochratoxins and was first found in the Balkan region but can be detected globally (Ringot et al., 2006). The mean dietary exposure estimates for adults have an upper limit of 5.09 ng/kg bodyweight per day and a lower limit of 2.41 ng/kg bodyweight per day. However,

children have been shown to surpass that TDI with an upper limit of 10.78 ng/kg bodyweight per day and a lower limit of 5.03 ng/kg bodyweight per day. (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2020)

The World Health Organization has recommended a tolerable daily intake (TDI) of 5 ng/kg body weight/day as it has been found in human blood and breast milk. This recommendation comes in addition to reducing the levels of OTA in food and animal feed because OTA has high heat stability. Cooking contaminated food is therefore not likely an appropriate method to remove OTA from food (Kőszegi & Poór, 2016). Decontamination or detoxification methods can be used to remove or reduce OTA levels. These removal/reducing methods can be physical, chemical, or biological methods like sorting and peeling to remove the contaminated fractions, using ammonium to destroy OTA, or using sorbents as additives that serve to absorb the OTA and reducing the bioavailability of OTA (Abrunhosa et al., 2010). However, the final product can be impacted and not be usable after these types of removal treatments.

Epidemiological studies have indicated a statistically significant difference of disease rates in those exposed to OTA compared to those who were not (Chen & Wu, 2017). Wafa and colleagues conducted a case-control study in 1998 which linked kidney disease (end-stage renal disease or nephritic syndrome) with high levels of urinary OTA as compared to reference groups. This group concluded OTA may be involved in the progression of kidney disease into end-stage renal disease (ESRD) and/or nephritic syndrome (Wafa, 1998). Other studies found similar results where OTA is suspected of playing a role in patients with diabetes developing nephropathies compared to other groups not exposed to OTA (Hsieh, 2004).

The proximal tubule has been shown to be one of the main targets of OTA in the nephron. OTA has been implicated to cause kidney lesions which manifest as epithelial cell toxicity where membrane integrity was lost and brush-boarder was damaged (Malir et al., 2016). Glomerular filtration of OTA is heavily limited because it is 99% protein-bound, and therefore relies mostly on tubular secretion for OTA excretion into the urine (Anzai et al., 2010).

OTA is considered cumulative since it is absorbed in the gastrointestinal tract (stomach and jejunum), heavily binds to serum proteins, and is minimally eliminated through the biliary or urinary routes of excretion (Abrunhosa et al., 2010; Pyo et al., 2021). The oral bioavailability in humans is about 93%, 60% in pigs, and much lower in rodents. The serum half-life varies across species and is dependent upon affinity and degree of protein binding which can range from a few days to one month (Kőszegi & Poór, 2016). Oral OTA half-life in humans is 35.5 days, 21 days in monkeys, 72-120 hours in pig, 55-120 hours in rats, and 40 hours in mice (Abrunhosa et al., 2010). In the kidney, glomerular filtration of OTA is limited due to the high binding affinity of OTA to albumin with small fractions of filtered and secreted OTA being reabsorbed. (Ringot et al., 2006). This high affinity binding can contribute to the large half-life in some species as it limits the transfer from the serum to the hepatic and renal cells for excretion and elimination (Abrunhosa et al., 2010).

The kidneys are highly vascularized and receive 25% of the cardiac output and filter the equivalent volume (Pyo et al., 2021). The structure of renal microcirculation is essential for renal function and glomerular filtration. Disruption of blood flow leaves the kidneys vulnerable to injury (Krishnan et al., 2021). High renal blood flow per tissue

weight results in a large amount of OTA reaching the renal tissue and resulting in accumulation (Pyo et al., 2021). OTA acts on differently on each nephron section to affect functions such as glomerular filtration, blood flow, and tubular activity and integrity (Petrik et al., 2003). Acute OTA toxicity has been considerably studied in animals and has defined the main acute effect to be multifocal hemorrhage the targeted organs (Jessica Gil-Serna et al., 2019). Purchase and colleagues documented renal necrosis after a high dose of OTA with lower doses producing tubular dilation between days 4-10 post dosing. Apparent tubular blockages were attributed to medullar tubular blockages in addition to more severe lesions being due to multiple blocked nephrons in the same area of tissue (Purchase & Theron, 1968). Exposure to chronic low doses of OTA has shown to lead to accumulation in various tissues, with proximal tubule being the highest (Petrik et al., 2003). Accumulation, regardless of acute or chronic exposure, is believed to occur due to the high rate of reabsorption by the proximal tubule (70%) in addition to 2/3 of OTA filtered by the glomerulus is also reabsorbed by the proximal tubule (Zingerle, 1997).

The high levels of accumulated OTA have been associated with effects such as protein synthesis inhibition, lipid peroxidation, and diminished cell membrane integrity. Chronic renal effects of OTA have been investigated by Kanisawa et al. in 1977 which indicated their findings to include tubular degeneration, renal necrosis, and epithelium deterioration of the proximal tubule leading to 8ubule-interstitial fibrosis.

OTA is associated with interstitial nephritis related to inhibition of protein synthesis, interference of metabolic pathways involving phenylalanine, disruption of calcium homeostasis, promotion of membrane lipid peroxidation, inhibition of

mitochondrial respiration, and DNA damage and the modulation of gene expression (Veiga-Matos et al., 2020). The mechanism of OTA renal toxicity is still yet to be fully clarified.

Ochratoxin A has been shown to be mostly unchanged, however OTA is able to undergo biotransformation by both phase I and phase II enzymes. The resulting metabolites show no or low toxicity (Figure 2) (Kőszegi & Poór, 2016). Hydrolysis of OTA occurs via proteolytic enzymes to ochratoxin A-α (OT-α). Opening of the OTA lactone ring under alkaline conditions results in a highly toxic metabolite (OTA-OA), however this highly toxic metabolite only occurs in rodents (Kőszegi & Poór, 2016). An additional hydroxyl metabolite of OTA is also observed in different species. Phase II conjugations of OTA, including glucuronide, glutathione, sulfonation, have been observed in tissues, blood, and urine of animals and humans. Although the mechanism of OTA toxicity is not fully clarified, transcriptome sequencing (RNA-seq) results indicate metabolism of OTA by CYP1A1 and CYP1A2 results in excess ROS production which ultimately leads to apoptosis and epithelial to mesenchymal transition (Pyo et al., 2021).

The mechanism of OTA-induced nephrotoxicity was investigated in rats treated with 250 or 500 μg OTA/kg bodyweight/day for 7 days induced oxidative stress, decrease in glucose transporter expression and alterations in proximal tubular structure (Peraica et al., 2010). In an *in vitro* study using rat proximal tubule cells and LLC-PK_1 (porcine proximal tubule cells) treated with OTA, showed a concentration-dependent increase in the ROS levels with a concurrent decrease in cellular glutathione (GSH) levels (Schaaf et al., 2002). Both *in vivo* and *in vitro* studies have indicated OTA exposure results in overproduction of free radicals.

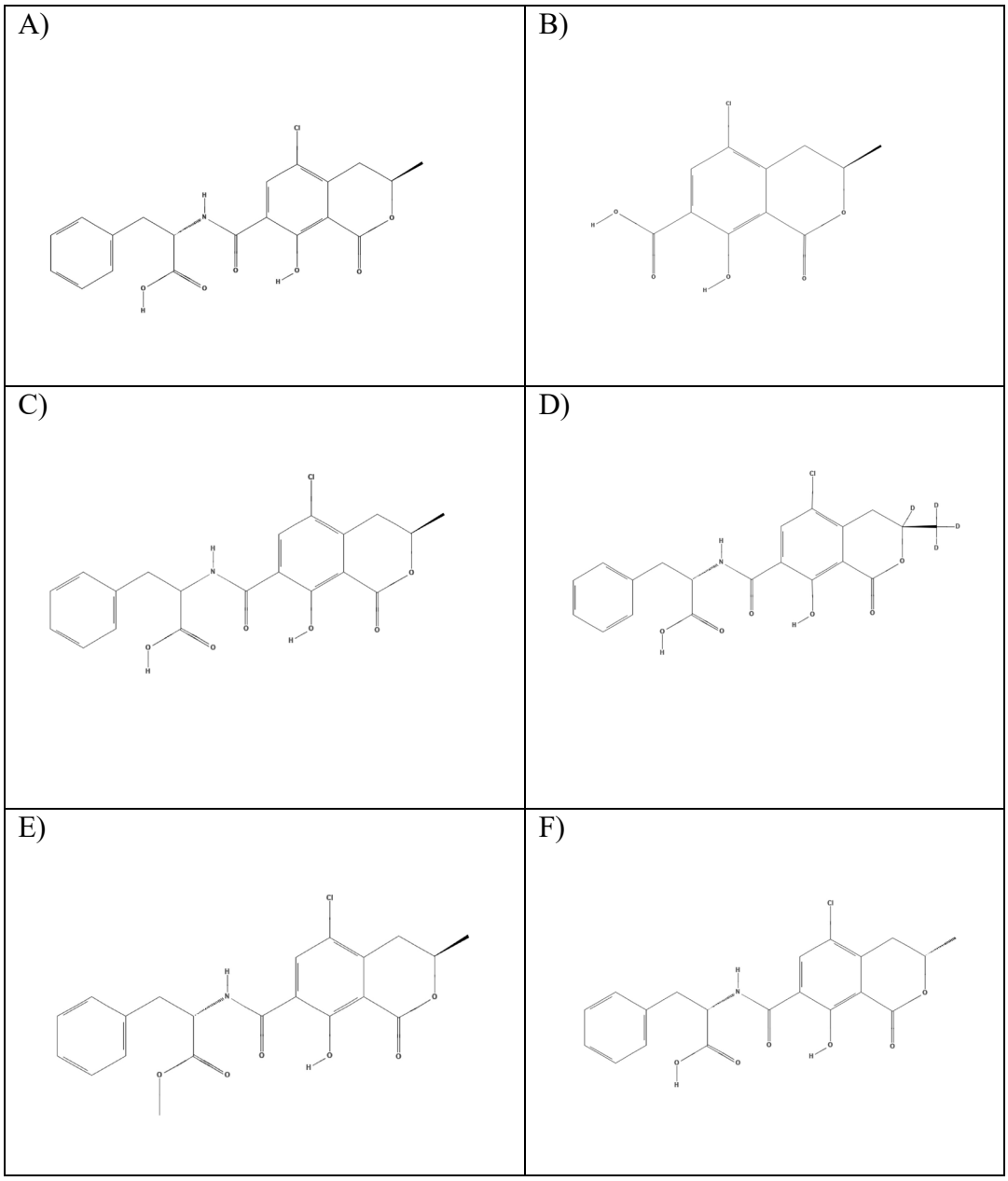

Figure 2. Ochratoxin A and metabolites.

(A) Ochratoxin A, B) Alpha Ochratoxin, (C) Phenylalanine Ochratoxin (D) Ochratoxin A-D4 (E) Ochratoxin A Methyl Ester (F) 3-epi-Ochratoxin A (Source: PubChem)

OTA is believed to form a phenoxyl radical with peroxidase activation which is then converted back to OTA in the presence of GSH at the expense of the formation of a superoxide radical (Yanfei Tao & Yuanhu Pan, 2018). Reactive oxygen species (ROS) are produced by incomplete reduction of molecular oxygen. ROS includes the superoxide anion, hydrogen peroxide, hydroxyl radical, and the single oxygen. As these molecules are highly reactive, the unpaired electron is reacting with other free electrons which then causes the creation of more free radicals and therefore begin a chain reaction. These reactive molecules and subsequent chain reactions are then able to degrade the glomerular basement, alter glomerular and tubular function (Baud & Ardaillou, 1986). The main sites of ROS production in living organisms include mitochondrial electron transport chain, peroxisomal fatty acid, cytochrome P-450, and phagocytic cells. The kidney is highly sensitive to ROS damage due to the abundance of long-chain polysaturated fatty acids present. In the past decade ROS damage has been associated with being one of the mechanisms involved with diabetes-meillitus-induced nephropathy because of uncontrolled blood glucose levels (Ozbek, 2012). Under normal physiological conditions, ROS are maintained at low levels by reduction-oxidation homeostasis and serve as signal molecules for cellular proliferation, growth factor signaling, immune responses, differentiation, and autophagy. Enzymes that aid in the maintenance of ROS base levels include superoxide dismutase (SOD), catalase, peroxidases, glutathione and thioredoxin. In clinical cases where the antioxidant enzymes are depleted, amino acids like N-acetylcysteine (NAC), (an acetylated variant of L-cysteine) is a source of sulfhydryl groups which has been used to replenish the activity of antioxidant enzymes (Kaushal et al., 2019). OTA was also found to initiate free radical formation and increase

Nrf2 mRNA in the kidney in a dose and duration dependent manner (Ferenczi et al., 2020). As stated previously, excess ROS production will ultimately result in apoptosis and EMT, which is heavily associated with fibrosis.

Epithelial to mesenchymal transition (EMT) is a key event in renal fibrosis, which is known to be initiated by transforming growth factor-beta1 (TGF-β1) (Nagavally et al., 2021). EMT is a step which occurs during tissue repair where the surviving epithelial cells are able to transition and migrate with a mesenchymal phenotype, losing all epithelial cell type characteristics (Christiansen & Rajasekaran, 2006). In an experiment conducted with HK-2 cells exposed to OTA, downregulation of E-cadherin and upregulation in fibronectin was confirmed with RNA-seq and qRT-PCR which confirms OTA induces EMT via AhR-Smad2/3 pathway. Further studies show in addition to the induction of EMT, the suppression of Smad2/3 also indicates apoptosis is also induced via Smad3 pathway (Pyo et al., 2021). Additional studies using a nontoxic concentration of OTA with glomerular mesangial cells and renal tubular cells indicated OTA was found to exacerbate cytotoxicity of Adriamycin and cyclosporin A by intensifying renal fibrosis via activation of TGF-ß1/SMAD2/3 (Du et al., 2022).

In addition to investigating apoptosis progression and EMT in OTA exposure, hypoxia as a mechanism to OTA exposure was also assessed. Hypoxia was reported in clinical and laboratory studies to have occurred in both acute and chronic kidney disease. Hypoxia occurs due to microcirculation disruption and hypofusion. HK-2 cells were used to assess the involvement of hypoxia in OTA exposure. Hypoxia-inducible factor-1α (HIF-1α) was shown to suppress the procession of apoptosis, EMT, and kidney injury (Pyo et al., 2021).

Although the complete mechanism of OTA in the kidney has not been elucidated, supporting evidence of the involvement of ROS inducing EMT and apoptosis triggered by OTA exposure. Thus, the attenuation and prevention of OTA induced renal toxicity can be better understood.

1.3 Diabetes

Diabetes can be a chronic condition that regardless of the underlying cause, results in excess blood glucose. Chronic Diabetes includes type 1 and type 2. According to the CDC and United States National Institute of Health (NIH), type 1 diabetes is thought to be caused by an autoimmune reaction, genetic factors, and/or environmental factors all of which result in insulin no longer being produced due to the loss of the pancreatic islet β-cells. Type 1 diabetes involves the overall destruction of islet β-cells and hyperglycemia due to the lack of insulin availability. Insulin dysregulation leads to serious damage of main organ systems (kidney, neurological, hematological) (WHO, 2021).

Diabetic symptoms include the following: increased thirst and urination, increased hunger, blurred vision, fatigue, unexplained weight loss, and diabetic ketoacidosis. However, the primary complication of diabetes is diabetic nephropathy (DN). DN is the leading cause of end-stage renal failure (ESRF) and according to WHO ESRF is the ninth leading cause of death with an estimated 1.5 million cases. DN is known to also cause cardiovascular mortality which can increase damage to the heart, blood vessels, eyes, kidneys, and nerves (WHO, 2021). DN is characterized by glomerular hypertrophy, thickness of membranes (basement, tubular, glomerular), and accumulation of extracellular matrix which leads to renal fibrosis.

Pathways involved in the development of DN have yet to be fully investigated. As a results, the role of inflammation in the pathogenesis of DN has yet to be completely clarified. Studies have demonstrated that a combination of expression of cell adhesion molecules, growth factors, chemokines and pro-inflammatory cytokines are increased in the renal tissues of diabetic patients which are all correlated with increased albuminuria (Duran-Salgado, 2014). Renal function and structural changes are pathological features of DN which are caused by accumulation of extracellular matrix (ECM) proteins and epithelial-to-mesenchymal transition (EMT). The resulting extent of renal fibrosis is used as a predictor of for the cause of DN mortality. Although glomerular structural and functional damage are hallmarks of DN, recent studies conducted in streptozotocin (STZ)-induced diabetes mellitus mouse model have indicated the proximal tubules are the initial pathological target which is linked to DN progression and is a potential therapeutic target for the clinical treatment of DN (Haraguchi et al., 2020). Cellular morphological changes in the proximal tubules are early symptoms of DN and progressive tubule-interstitial fibrosis plays a critical role in the progression from DN to ESRF and can more prominently reflect the progression of disease (Haraguchi et al., 2020)

1.4 Kidney

The kidney is divided into three distinct regions called the renal cortex, renal medulla and renal pelvis with the nephron as its functional unit. The nephron is responsible for performing all the functions of the kidney in conjunction with the capillary vessels throughout the tissue that aid in the excretion, secretion, and reabsorption solutes. The major target organs of ochratoxin A includes the kidneys and

to a lower extent the liver, skeletal muscle, fat tissue, and brain. OTA is known to accumulate in proximal tubule cells and then initiates cellular damage through oxidative stress, DNA damage, apoptosis and an inflammatory response. The alteration caused by OTA affects tubular reabsorption and the balance between fluid and solutes, one of its main functions. In the kidney, OTA mainly impairs the proximal tubule functions causing glucosuria, enzymuria, and a decrease in transport function (organic anion transport system) (Anzai et al., 2010).

Renal transporter systems throughout the nephron are responsible for uptake and efflux of endobiotics and xenobiotics. As OTA highly binds to plasma proteins, in the kidney glomerular filtration of OTA is limited. OTA can undergo tubular elimination via carrier-mediated transport proteins. Organic Anion transporters (OAT) 1/3 was indicated to move OTA across the basolateral membrane of the proximal tubule from the plasma into the cell, while OTA is excreted into the lumen via carrier-mediated transport protein (OAT4, MRP2) across the apical membrane. The transport systems responsible for uptake of OTA into the proximal tubule are more efficient than the efflux transport systems, which leads to tubular damage via accumulation in the tubular cell (Veiga-Matos et al., 2020). Apical OAT4 is also suspected to be responsible for urinary reabsorption of OTA, which contributes to accumulation in the kidney tubule cell. (Figure 3). The half-life of OTA is also impacted by the reabsorption of OTA via both

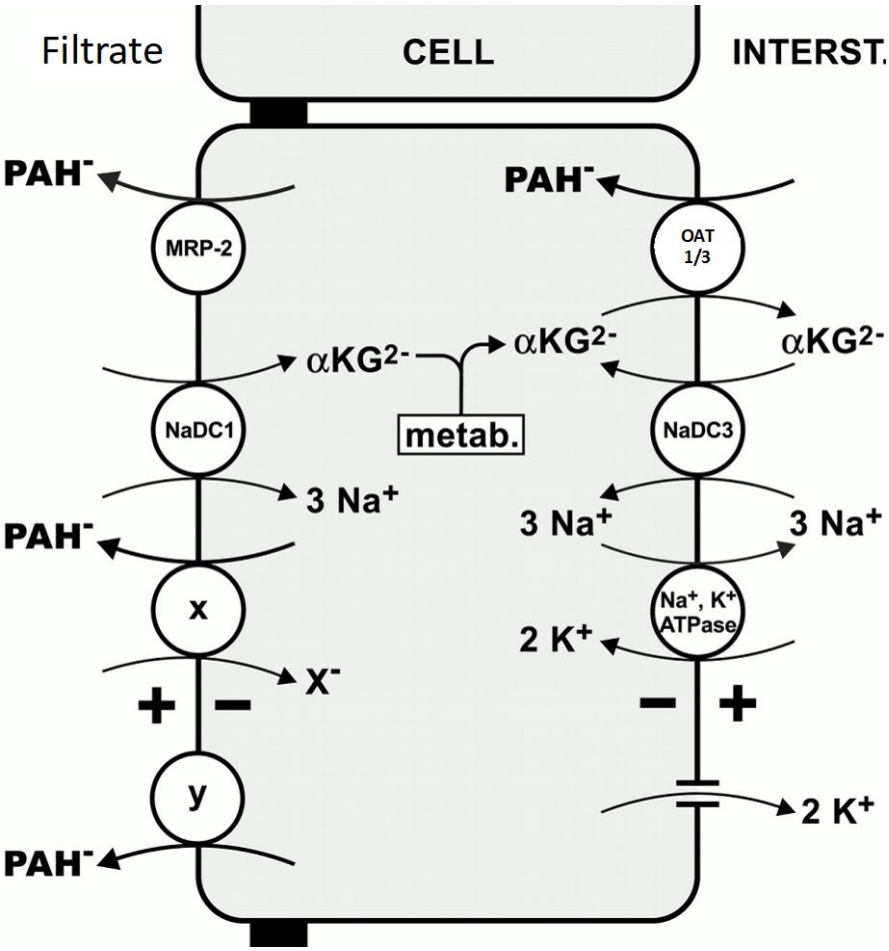

Figure 3. Picture representation of postulated proximal tubular OTA secretion.

Proximal tubule cell is shown with the left side facing the lumen (apical membrane) and the right side facing the interstitial fluid (basolateral membrane). O denotes transporters and the channels by =. Upward-bent arrows notate transport against concentration gradient and/or electrical gradients; downwardly bent arrows denote energetically downhill transport. Abbreviations: αKG^{2-}, α-ketoglutarate; metab., metabolism; MRP-2, multidrug resistance protein 2; NaDC1 and NaDC3, Na^{+}-dicarboxylate cotransporters 1 and 3; X^{-}, anion antitransporter of unknown molecular identity.

facilitated diffusion and carrier-mediated transport, which can occur throughout all sections of the nephron (Kőszegi & Poór, 2016).

1.5 LLC-PK$_1$

Using a cell culture system as an experimental model for investigating the interaction between ochratoxin A exposure and high levels of glucose allows for simplicity. LLC-PK$_1$ is a well-established epithelial cell line derived from male Hampshire pig kidney proximal convoluted tubule (PCT) cells (Hull et al., 1976). These cells are capable of transporting electrolytes, endogenous molecules, xenobiotics and water. LLC-PK$_1$ cells form monolayers of highly polarized cells with desmosomes and basolateral infoldings with apical microvilli (Gstraunthaler et al., 1985). LLC-PK$_1$ cells have also been used to study other PCT cells features. Some of these features include sodium dependent sugar transport (Mullin et al., 1980; Rabito & Ausiello, 1980; Sunilkumar & Ford, 2019), sodium dependent phosphate transport (Brown et al., 1984; Noronha-Blob et al., 1984), responsiveness to vasopressin and calcitonin (Goldring et al., 1978), capacity to perform gluconeogenesis (Gstraunthaler & Handler, 1987) and enzymatic activities found in the brush boarder membranes such as alkaline phosphatase and leucine aminopeptidase (Gstraunthaler et al., 1985). The presence of these functions and features makes LLC-PK$_1$ a very good model for studying renal physiological systems.

The advantage LLC-PK$_1$ has over the primary culture includes the ability to be split multiple times, because the cell line is immortalized, and the fact that this cell line is more easily available than the primary culture. This cell line also has the advantage of having been spontaneously immortalized without having to have any of the existing

transport systems transfected into the cell line. A commonly used proximal tubule cell line is HK-2 (human renal epithelial cells). Researchers Jenkinson et al. (2012) have shown that HK-2 cells have limited transporter expression and therefore showed the lowest accuracy when compared to primary or LLC-PK_1 cell line (Bajaj et al., 2018; Jenkinson et al., 2012). Studies have shown that LLC-PK_1 cells possess the OAT transport system, one of the primary suspected transporters of ochratoxin A in the proximal tubule.

OAT1 is expressed on the basolateral side of LLC-PK_1 cells. You and coworkers (2000) demonstrated the potential to modulate OAT1 using LLC-PK_1 as an in vitro model (You et al., 2000). They amplified OAT1 function by transfecting LLC-PK_1 cells with mouse OAT (mOAT) and grew the transfected cells on Transwell ® filter inserts. Their transport study used radioactive PAH as a substrate. Their results not only proved para-aminohippurate to be a dependable substrate of OAT1, but they were able to illustrate, at least in part, the modulation of PAH transport via OAT1 by okadaic acid and protein kinase C (PKC) activity. These advantages and features make LLC-PK_1 cells a good *in vitro* experimental model to study the interaction of ochratoxin A and high glucose conditions.

1.6 Aim of the Study

Despite the many studies conducted on OTA, the mode of action and attenuation of toxicity is only partially understood. OTA's main nephrotoxic effect is lesions on the proximal tubule where membrane integrity is lost, severe damage to the brush border, and interstitial fibrosis (Malir et al., 2016). In addition to OTA being well-known and widely-

spread all over the world, several epidemiological studies suggest an association between human kidney disease and OTA exposure (Chen & Wu, 2017).

Renal excretion is one of the most important and vital routes of OTA elimination in the body (Dresser et al., 2001). The kidney is not only susceptible to damage by OTA, but according to the National Kidney Foundation, diabetes is the leading cause of kidney failure. Diabetes impairs the kidneys' ability to remove wastes from the body, leading to an accumulation of waste products and can lead to other health issues. Both OTA exposure and diabetes frequently lead to chronic kidney disease. Due to the rising occurrence of diabetes and the prevalence of OTA in food, there is a need to assess the possible synergistic effects of a diabetic disease state in concurrence with OTA toxicity with regards to nephrotoxicity. The purpose of this study is to assess the nephrotoxic effects of ochratoxin A in a rodent diabetic model. To assess this interaction *in vivo,* Sprague Dawley rats were treated with streptozotocin and orally exposed to 0.5mg/kg bodyweight OTA once a day for 21 days. To effectively study this mechanism on a molecular level, pig kidney proximal tubule cell line (LLC-PK_1) was used as an *in vitro* model.

1.6.1 Hypothesis

The hypothesis of the research program is that ochratoxin A exacerbates experimental diabetes mellitus induced nephropathy using *in vivo* and *in vitro* models.

To test this hypothesis the following was assessed:

a) *Histopathological changes in renal tissue of diabetic rats treated with ochratoxin*
b) *Renal function in renal tissue of diabetic rats treated with ochratoxin A using serum biomarkers*

c) *Renal transport activity of ochratoxin A in renal tissue of diabetic rats treated with ochratoxin A*

d) *The effect of glucose concentration on cytotoxicity of OTA in porcine renal proximal tubule cells*

CHAPTER 2- MATERIALS AND METHODS

2.1 Treatment of Sprague Dawley Rats

This study investigated the toxic effect of ochratoxin A on the kidneys of diabetic rats to determine if diabetes exacerbates the nephrotoxic effects of ochratoxin A. The animals were separated into four treatment groups: control (CON), experimental diabetes (STZ), ochratoxin A treated (OTA), experimental diabetes with OTA treatment (STZ/OTA). The dose used in this study, 0.5 mg/kg/day, was based on studies found in the literature (Pastor et al., 2018).

Half of the animals were injected once via IP with streptozotocin (STZ) dissolved in 10 mM citrate buffer (pH 4.5) to induce experimental diabetes. Blood glucose levels were monitored daily via initial tail snip (< 2 mm) and subsequent removal of scab using commercially available glucometer. Levels were monitored on day 0, 7, 14, 21, and 28. If the initial wound healed, an additional (< 2 mm) snip was taken and the scab removed. Animals had a lidocaine ointment dip on the tip of the tail to prevent animal from damaging its wound. Animals having blood glucose over 300 mg/dL at the end of 28 days post injection were considered diabetic, and those animals were included in studies. Animals injected with STZ that were not deemed diabetic were euthanized. All animals used in the study were orally gavaged with either the vehicle (corn oil) or 0.5 mg/kg b.w. once every day for 21 days. The control animals received corn oil while the other groups received a mixture of corn oil and ochratoxin A (0.5 mg/kg b.w.). The animals were provided food and water ad libitum.

At the end of day 21, the animals were placed in wire-bottom metabolism cages over night for no more than 12 hours. The animals were euthanized the following

morning through decapitation via guillotine. This method was used to prevent alterations in blood glucose levels using other forms of euthanasia. Anesthetic agents were omitted from this procedure to prevent their possible interference with the outcome of the renal cortical transport study. Anesthesia is known to interfere with renal transport that is being assessed using the renal cortical slices. Previous studies by Hirsch & Hook (1970) in rat did not use anesthesia prior to decapitation due to the need of viable, intact, and non-compromised renal tissue. Previously cited articles indicated using CO_2 asphyxiation, isoflurane, or sevoflurane will compromise the tissue by disrupting the necessary transport systems that maintain ion gradients. Trunk blood was collected. Both kidneys were collected and used for light histology and renal cortical slice uptake study. The treatments are summarized in Table 1.

2.2 Light Microscopy

Histopathological studies for the animals entailed microscopy for the observation of alterations in tissue indicative of toxicity. One kidney per animal was decapsulated and dissected using a longitudinal cut with a razor blade and placed in either cold Sorensen's phosphate buffer at pH 7.4 or 0.9% saline solution. The tissue was placed in 10% normal buffered formalin for 3 days on a shaker. The samples were dehydrated using 30% ethanol, 50% ethanol, 70% ethanol and then embedded using STP-120 carousel-type tissue processor. Sections of paraffined tissue samples at 3 μm thickness were made with Reichert-Jun 2030 microtome. Sections were stained with either hematoxylin and eosin or Masson's Trichrome staining procedure. Masson's trichrome procedure included

Groups	Pre-treatment	28 days	21 days of gavage	Day 22
		Glucose monitoring		
Control	Saline, i.p.		Corn Oil	•Kidneys for histology, renal cortical function •Trunk blood for biomarker assessment •Urine for biomarker assessment
Experimental Diabetes	STZ, i.p.	*	Corn Oil*	
Ochratoxin A	Saline, i.p.		OTA	
Experimental Diabetes + Ochratoxin A	STZ, i.p.	*	OTA*	

Table 1. Experimental design of diabetic rats treated with ochratoxin A.

Sprague Dawley rats were pre-treated with streptozotocin and allowed experimental diabetes to be established for 28 days. The animals were then treated with either 0.5 mg/kg b.w./day or corn oil via oral gavage for 21 days. Animals were then euthanized via guillotine decapitation on day 22 where kidneys, trunk blood, and urine were collected for analysis. (*) Indicated animals received insulin daily to control blood glucose levels to levels below 300 mg/dL as needed after STZ treatment.

deparaffinizing and rehydrating the sections using 100%, 95% and 70% ethanol. Sections were washed in distilled water, fixed in Bouin's fixative followed by nuclear staining with Weigert's iron hematoxylin. Sections were stained with Biebrich Scarlet Acid fuschin which stains cytoplasm, muscle and collagen. Collagen fibers in sections were then decolorized with phosphotungstic phosphomolybdic acid and followed by aniline blue. The stained sections were dehydrated and mounted for imaging.

2.3 Point-counting

Point-counting was conducted to quantify interstitial fibrosis in renal cortex micrographs of animals treated with ochratoxin A for 21-days. Point-counting consisted of applying the same size grid (10 x 10) to same size and magnification micrograph. If a cross-section point intersect within the grid landed on area of interest, in this case a collagen deposit blue or deep purple areas, it was considered a positive result. Calculations were conducted as follows (# positive points/# of total points) * 100 = X%. A total of 10 micrographs were point-counted per group.

2.4 Renal Biomarkers

Blood and urine were used to assess kidney damage by measuring renal biomarkers. Urine was collected using metabolism cages prior to euthanizing for a period of 12 hours. Trunk blood was collected after decapitation of each animal. Serum was attained after collection in serum collection tubes. The blood was left at room temp for 45 minutes to clot then spun at 1,800 x g for 10 minutes at 4° C. Serum and urine samples were stored at -80° C until used.

Protein levels in serum were assessed using the Bradford dye-binding assay according to Thermo Fisher protocol (Cat. #J61522-K). Glucose levels in urine and

serum were assessed using Cayman Glucose assay kit (Cayman Chemical 100095822), a colorimetric assay. This assay was based on the glucose oxidase-peroxide reaction. Glucose is oxidized to d-gluconolactone with reduction of the flavin adenine dinucleotide (FAD)-dependent enzyme glucose oxidase. The reduced form of glucose oxidase was regenerated to its oxidized form by molecular oxygen to produce hydrogen peroxide. The colorimetric product (pink dye) was produced by the reaction of hydrogen peroxide with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine.

Urea levels in serum were assessed using QuantiChrom™ Urea Assay Kit II (Bioassay Systems DUR2-100). This colorimetric urea assay was based on the conversion of urea to ammonium and carbon dioxide by urease. The pH of the reaction was visualized by a chromogen and the intensity of the reaction product was directly proportional to the urea concentration in the sample.

Serum creatinine was assessed using Cayman Chemical Creatinine (serum) Colorimetric Assay Kit (Cayman Chemical 7004602). These colorimetric assays used the Jaffe reaction where creatinine reacts with picric acid in an alkaline solution to form a reddish colored complex which was then solubilized in an acidic pH.

Novel biomarkers were also assessed. KIM-1 was assessed using Abcam Rat enzyme-linked immunosorbent assay (ELISA) kit (Abcam ab119597) serum. NGAL (lipocalin-2) was also assessed serum using Rat ELISA kit (ab119602). All kits used the Promega Glomax Multi+ detection system.

2.5 Renal Cortical Slices

One kidney per animal included in this study was used to make renal cortical slices. Transport studies using renal cortical slices were used to compare the

accumulation of ochratoxin A between the various treatment groups. The kidneys were excised from the animal carcass immediately after decapitation and placed into 0.9% NaCl and placed on ice. Renal cortical slices of about 0.3 to 0.4mm thickness were cut using one half of a double-edged razor blade over ice.

About 100 mg of renal cortical slices per animal per were placed in Griffin beakers (10 mL) and 2.7 mL of Cross and Taggert phosphate buffer. The Cross and Taggert transport buffer (pH 7.4) (appendix) was made up with 0.125 μCi of H^3-OTA with either 20 nM or 520 nM OTA (non-radiolabeled). The griffin beakers containing the slices and buffer were then placed in a Dubanoff shaker at 25°C at 100 RPM in 100% oxygen for 90 minutes.

The slices were then manually homogenized using a Dounce homogenizer with 3 mL of 10% trichloroacetic acid and then rinsed with 2 mL ultra-pure water and decanted for a total of 5 mL. A 1 mL sample of incubation medium from each sample was diluted with 1.5 mL of 10% trichloroacetic acid and 2.5 mL ultra-pure water for a total of 5 mL. Samples were analyzed via liquid scintillation counter (Perkin Elmer tri-count) using 100 μL of sample and 3 mL of liquid scintillation cocktail. The slice to medium (S:M) ratio was calculated by dividing the concentration of OTA in the cortical slices by the concentration of OTA remaining in the medium to give an index of the ability of the tissue to transport OTA into the proximal tubule cells.

2.6 Cell Culture

LLC-PK_1 clone 5 was isolated by Dr. S. Ford in 1990 from cells obtained from ATCC. The growth medium was a custom formula based on SFFD (50:50 DMEM : Ham's nutrient F12 with 15mM HEPES) lacking phenol red (GIBCO custom formula, F#

91-5173 EL). It was reconstituted with ultra-pure water and the appropriate components to pH 7.4 and the addition of 3% fetal bovine serum (FBS) (Hyclone Thermo Scientific; defined FBS cat # SH30070.03). Antibiotics were not used. Bionique mycoplasma testing was conducted routinely and found to be negative. The LLC-PK_1 cells were maintained in an incubator at 37°C in an atmosphere of 95% air and 5% CO2. The medium in the tissue culture plate was changed every three days after inoculation and the cells were subcultured on the fourth day after inoculation. Subculturing was done by trypsinizing the cells with trypsin-EDTA (Gibco by Life Technologies; ref # 15400-054) and split at a ratio of 1:5 or 1:10. The cells used in the experiments were passages 224 to 270.

2.7 Cell viability Assay

LLC-PK_1 cells were seeded on 35 mm tissue culture plates at a density of 4 x 10^4 cells per ml of cell suspension with 2 mL per plate, grown for 4-5 days with a change of medium every 48-72 hours containing either 5 mM glucose or 17.5 mM glucose. The plates were treated with the test media compounds when plates were at confluence. Test media consisted of 5 mM, 17.5 mM or 30 mM glucose with increasing concentrations of ochratoxin A (0.01, 0.1, 1, 10, 100, 100, 1000 uM). The 30 mM glucose treatment group consisted of cell monolayers grown in 5 mM glucose media and were then treated with 30 mM glucose test media. The 5 mM and 17.5 mM glucose treatment groups were conducted in cells grown in their respective glucose media. Viable and non-viable cells were counted by trypan exclusion method after 24 ± 1 hour of treatment.

The trypsinization was done with 1 ml of 0.05% trysin-EDTA (Gibco by Life Technologies, catalog # 15400-054). After complete cellular detachment the trypsin was neutralized with 1 ml of cell culture medium with 3% FBS. A cell suspension was

obtained and 100 µL was diluted in 100 µL PBS and 200 µL trypan blue. The number of cells that appeared colorless were counted. The counts were done in duplicate and the mean was used to determine the number of live cells for that plate. The percent viability was plotted as % V.C. on the Y-axis and the concentration of the corresponding compound was plotted on the X-axis.

$$\frac{\text{\# of viable cells in the treatment plate}}{\text{\# of viable cells in the control plate}} \times 100$$

Using this graph, the LC-50 was determined for each of the three aforementioned compounds.

2.8 Statistical Analysis

Differences among treatment groups were analyzed using one-way ANOVA followed by Tukey's Post Hoc Test with Graphpad Prism 10.0. The criterion for statistical significance was $p < 0.05$.

CHAPTER 3- RESULTS

3.1 Daily body weight of Sprague Dawley rats

Sprague Dawley rats were weighed daily and treated via oral gavage with either corn coil or ochratoxin A at 0.5 mg/kg/day once daily for 21 days (Figure 4). The animals were separated into four treatment groups with 4 animals in each group: control (CON), experimental diabetes control (STZ), ochratoxin A (OTA), and ochratoxin A in experimental diabetes (STZ/OTA). Body weights for each group were not significantly different from each other.

3.2 Urine Volume of animals treated with ochratoxin A

Animals were placed in wire-bottom metabolism cages at the end of day 21 of treatment over night for no more than 12 hours. The urine and serum (from trunk blood) were collected and used to run panels assessing kidney function utilizing renal biomarkers. Urine volume collected over 12 ± 1 hours showed no significant difference between any of the treatment groups (Figure 5).

3.3 Pathohistological assessment

Renal histopathological assessment of diabetic Sprague Dawley rats with treated with ochratoxin A for 21 days with resulted in significantly increased renal tissue damage when compared to control group. Histopathological assessment using light microscopy of H&E stained renal tissue sections from animals in the STZ/OTA group indicated an increased occurrence of inflammation, tubular degeneration, glomerular degeneration, necrosis, and cast formation (Figures 6-8). These histopathological results are consistent

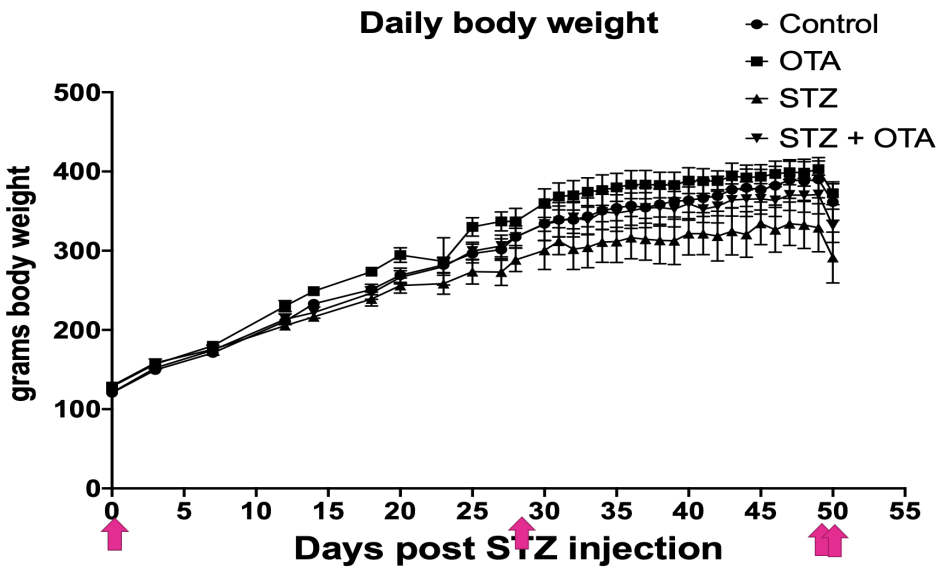

Figure 4. Daily body weight of Sprague Dawley rats treated with ochratoxin A.

Body weights recorded throughout study. Arrows indicate initial streptozotocin injection, start of ochratoxin A treatment, start of metabolism cage and day of sacrifice. Body weights were not significantly different at end of study.

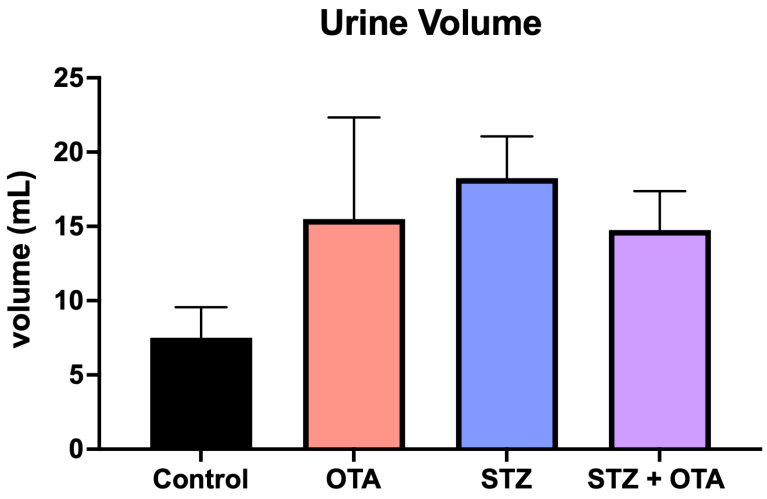

Figure 5. Urine volume of animals treated with ochratoxin A.

Urine volume of animals treated with ochratoxin A for 21-days collected over 12 ± 1 hour. No significant difference between the treatment groups. The data is shown as the mean ± SEM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test.

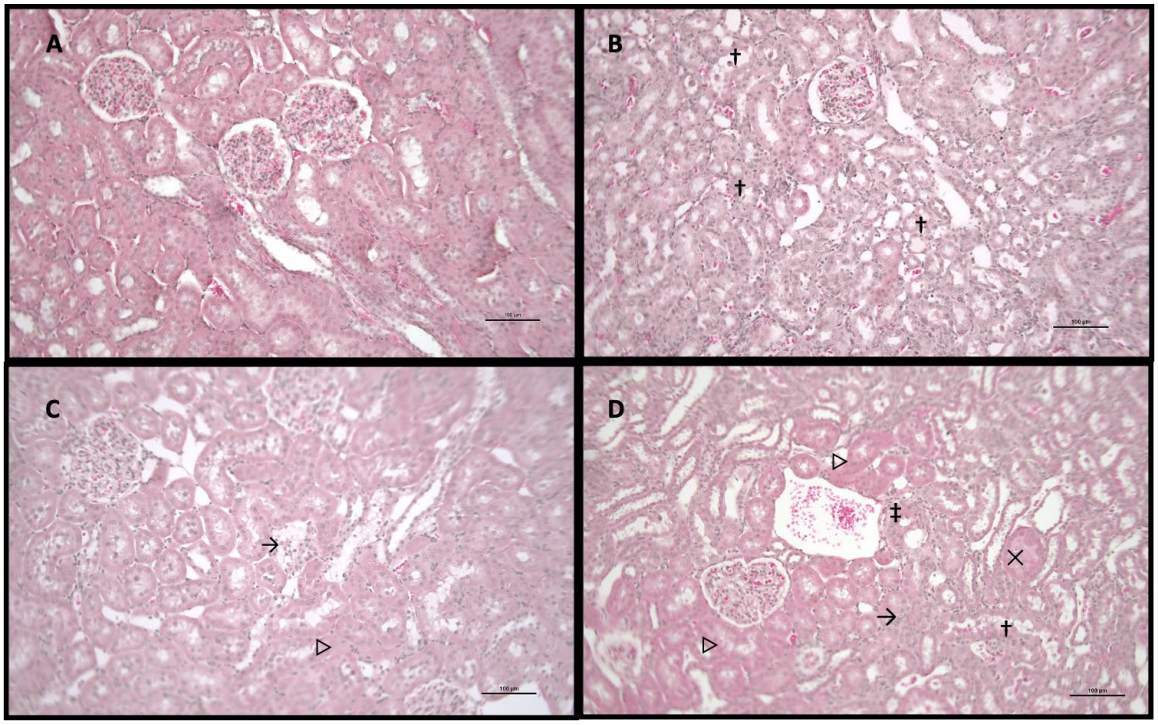

Figure 6. H&E 20x representative micrograph summary

Histopathological appearance of the renal cortex after 21-day OTA exposure period. (A) CON group, Normal histology using Hematoxylin and Eosin; (B) OTA group, (▷) inflammation, (†) tubular degeneration (C) STZ group, (→) Necrosis , (▷) inflammation (D) STZ/OTA group (‡) Glomerular degeneration, (▷) inflammation, (†) tubular degeneration, (→) Necrosis

.

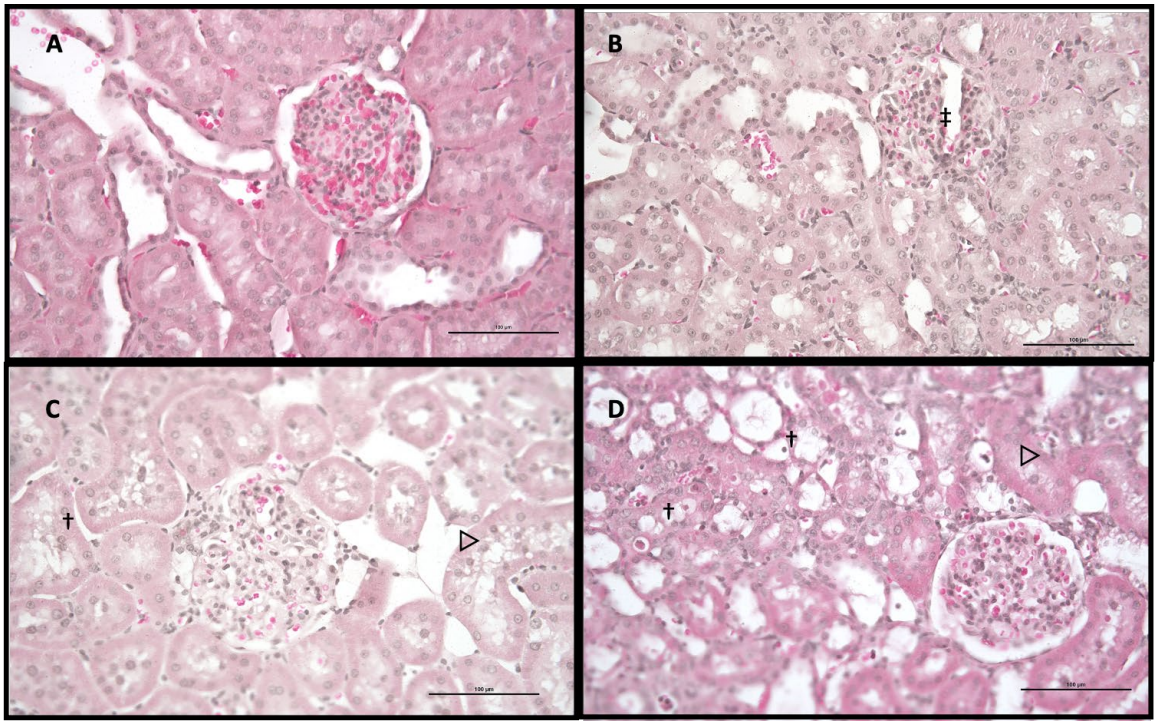

Figure 7. H&E 40x representative micrograph summary of the outer cortex.

Histopathological appearance of the renal cortex after 21-day OTA exposure period. (A) CON group, Normal histology using Hematoxylin and Eosin; (B) OTA group, (‡) Glomerular degeneration (C) STZ group, (▷) inflammation, (†) tubular degeneration (D) STZ/OTA group, (▷) inflammation, (†) tubular degeneration

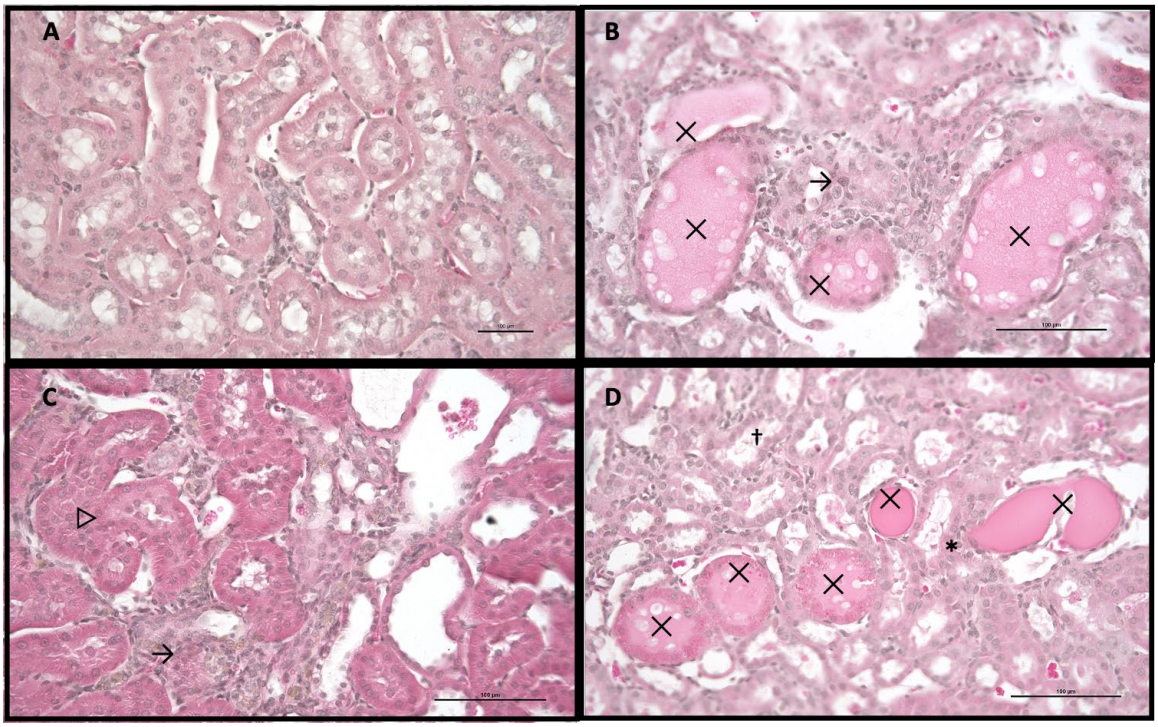

Figure 8. H&E 40x representative micrograph summary of the inner cortex.

Histopathological appearance of the renal cortex after 21-day OTA exposure period. (A) CON group, Normal histology using Hematoxylin and Eosin; (B) OTA group, (✕) cast formation, (→) Necrosis (C) STZ group, (→) Necrosis, (▷) inflammation (D) STZ/OTA (†) tubular degeneration, (✕) cast formation (✱) interstitial fibrosis

with nephrotoxic effects caused by ochratoxin A alone and diabetic nephropathy alone.

Literature has shown OTA and diabetes to individually cause tubular degeneration, interstitial fibrosis, tubular cysts/casts formation, cytoplasmic alterations, inflammation, glomerular degeneration and necrosis (Aydin et al., 2003; Boorman et al., 1992; Jain, 2012; Nishi et al., 2000). The histopathological results of the STZ/OTA group in our study show the nephrotoxic effects are greater in the STZ/OTA group than those in any of the other treatment groups (CON, STZ or OTA).

3.4 Renal interstitial fibrosis

Renal interstitial fibrosis was assessed using light microscopy and Masson's trichrome tissue stains. Histopathological assessment of diabetic rats treated with OTA indicated increased renal tissue damage and areas of fibrosis indicated by collagen deposits (Figures 9-12). Sections from diabetic rats treated with OTA showed increased levels of tubular and glomerular degeneration, inflammation, cast formation and interstitial fibrosis.

Interstitial fibrosis was quantified using the point-count method previously described (Figure 13A). The results showed STZ/OTA rats was significantly increased from all other treatment groups (Figure 13B). The CON group was found to be 7.33 ± 1.33 % positive points, OTA group 20.80 ± 2.08 % positive points, STZ 13.00 ± 1.79 % positive points and STZ/OTA 31.60 ± 3.14 % positive points. OTA is known to cause renal interstitial fibrosis (Aydin et al., 2003; Boorman et al., 1992) and was seen in both the histopathology of the OTA and the STZ/OTA groups. These data show the combination of DN and OTA significantly induces renal interstitial fibrosis.

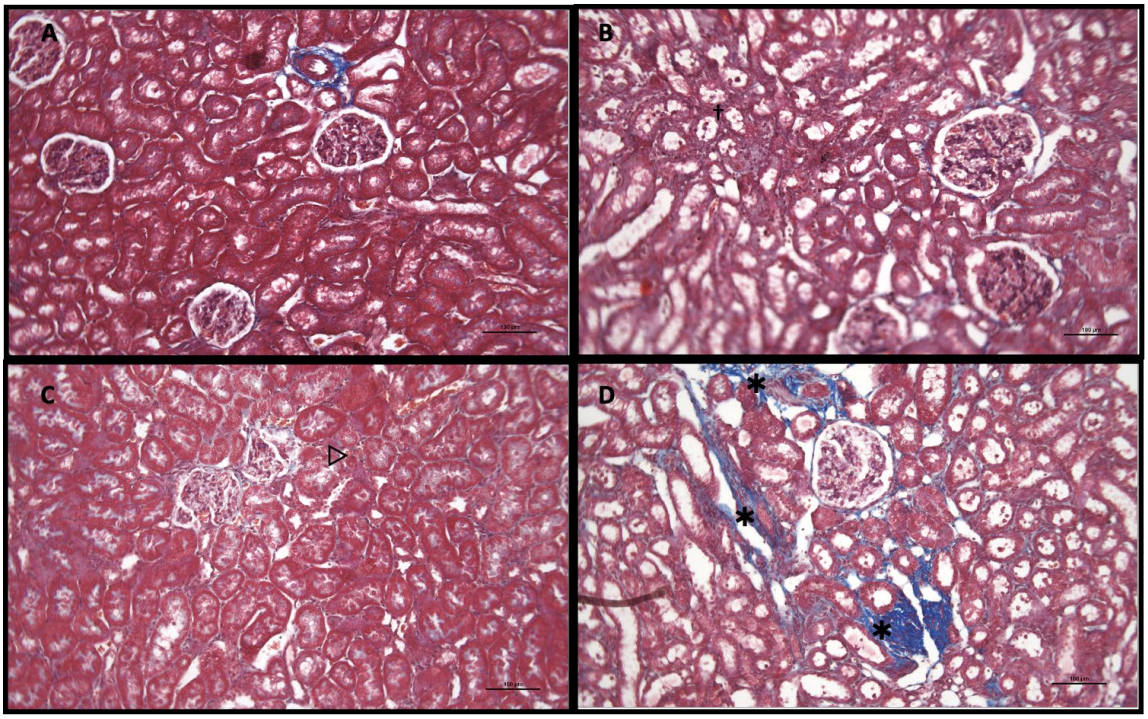

Figure 9. Masson's Trichrome 20X representative micrograph summary of the outer cortex region. Histopathological appearance of the renal cortex after 21-day OTA exposure period. (A) CON group, Normal histology using Masson's Trichrome; (B) OTA group, (†) tubular degeneration (C) STZ group, (▷) inflammation (D) STZ/OTA (✱) interstitial fibrosis (†) tubular degeneration

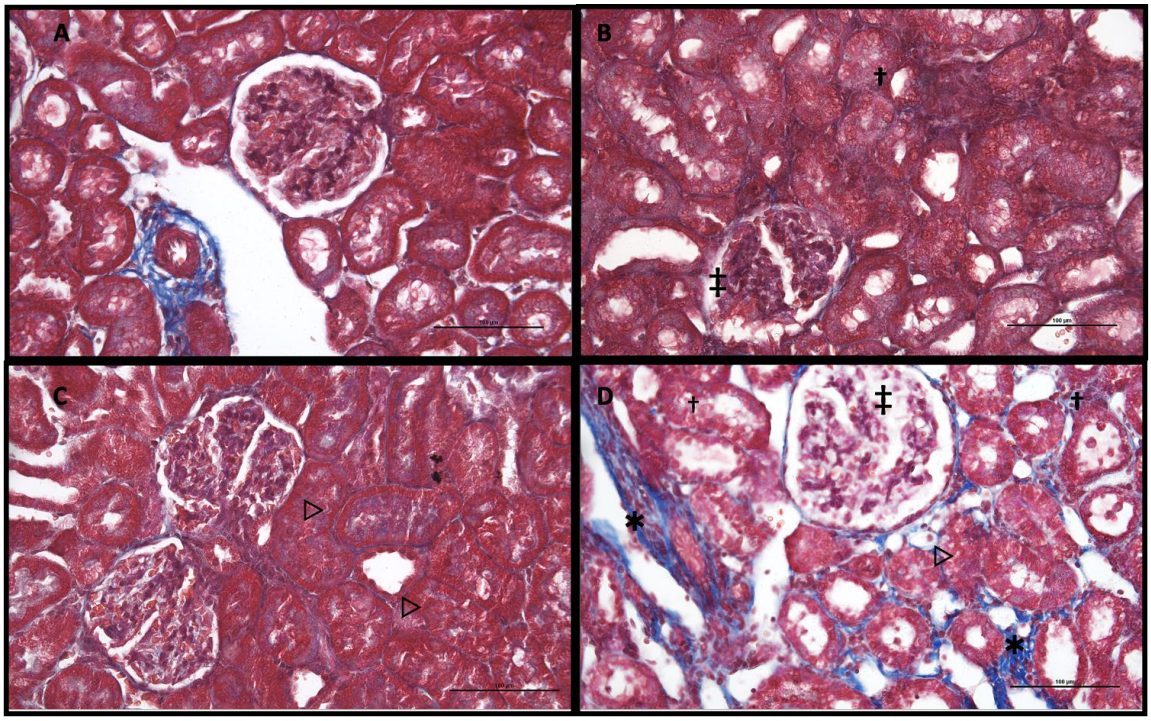

Figure 10. Masson's Trichrome 40X representative micrograph summary of the outer cortex region. Histopathological appearance of the renal cortex after 21-day OTA exposure period. (A) CON group, Normal histology using Masson's Trichrome; (B) OTA group, (†) tubular degeneration, (‡) Glomerular degeneration (C) STZ group, (▷) inflammation (D) STZ/OTA group (‡) Glomerular degeneration (✱) interstitial fibrosis (†) tubular degeneration

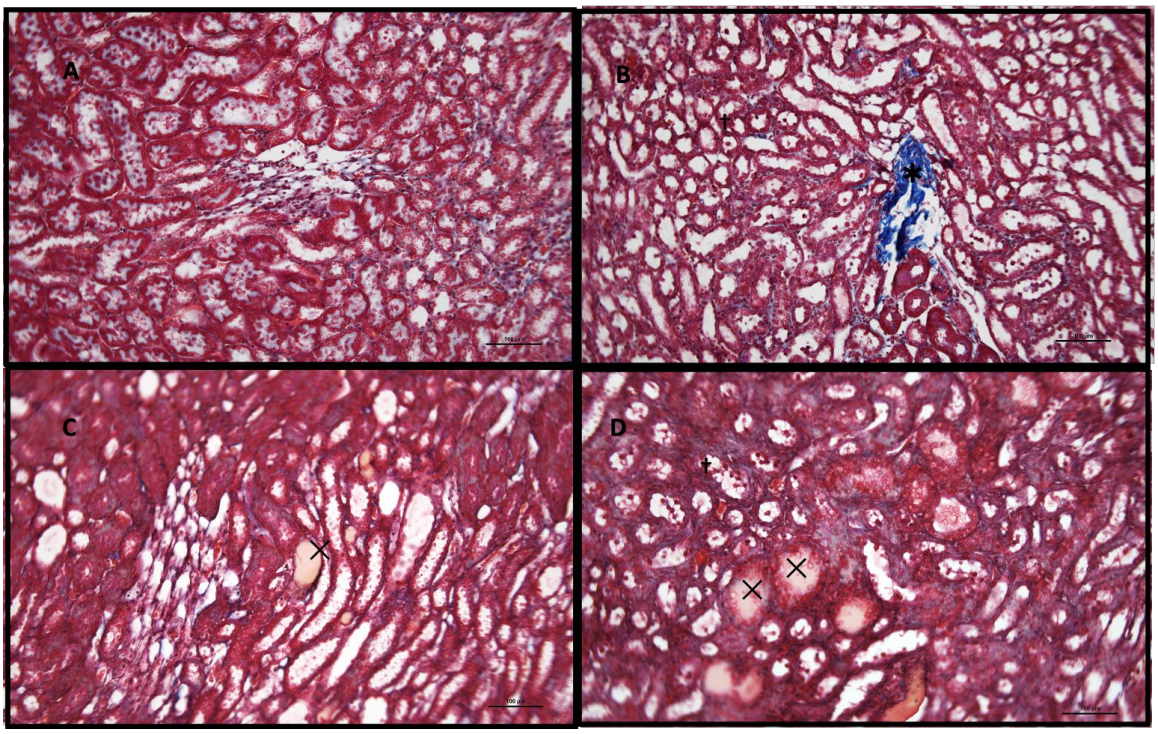

Figure 11. Masson's Trichrome 20X representative micrograph summary of the inner cortex region. Histopathological appearance of the renal cortex after 21-day OTA exposure period. (A) CON group, Normal histology using Masson's Trichrome; (B) OTA group, (✱) interstitial fibrosis (C) STZ group, (✕) cast formation (D) STZ/OTA group (✕) cast formation (†) tubular degeneration

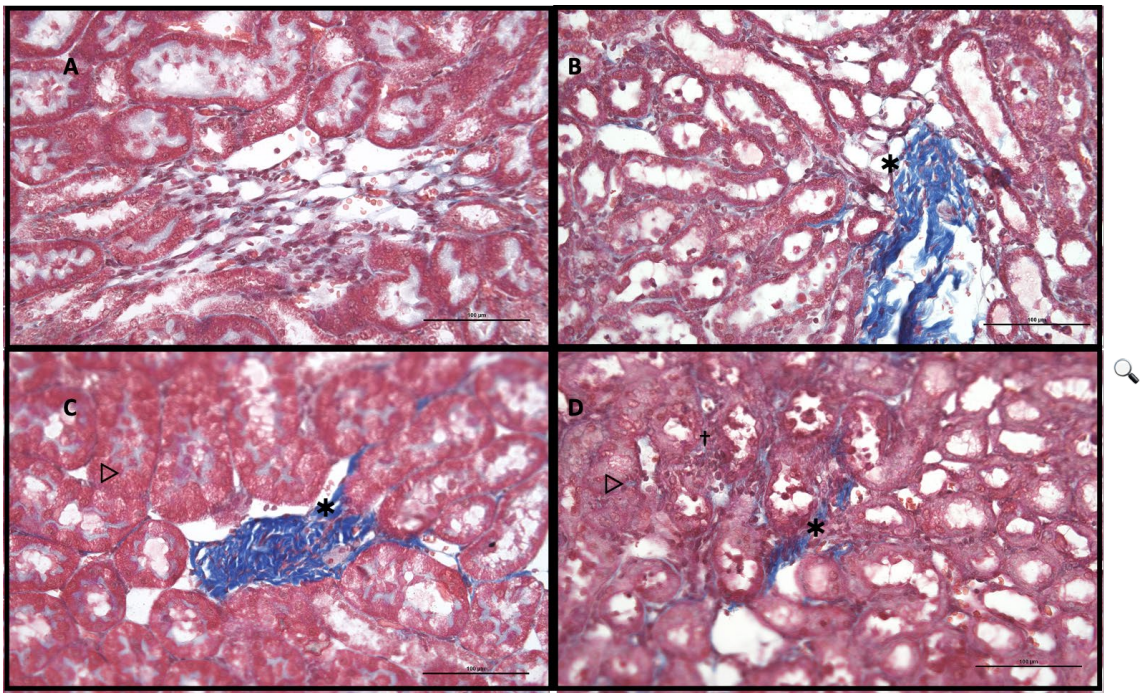

Figure 12. Masson's Trichrome 40X representative micrograph summary of the inner cortex region. Histopathological appearance of the renal cortex after 21-day OTA exposure period. (A) CON group, Normal histology using Masson's Trichrome; (B) OTA group, (†) tubular degeneration, (✱) interstitial fibrosis (C) STZ group, (▷) inflammation, (✱) interstitial fibrosis (D) STZ/OTA group (✱) interstitial fibrosis (†) tubular degeneration, (▷) inflammation

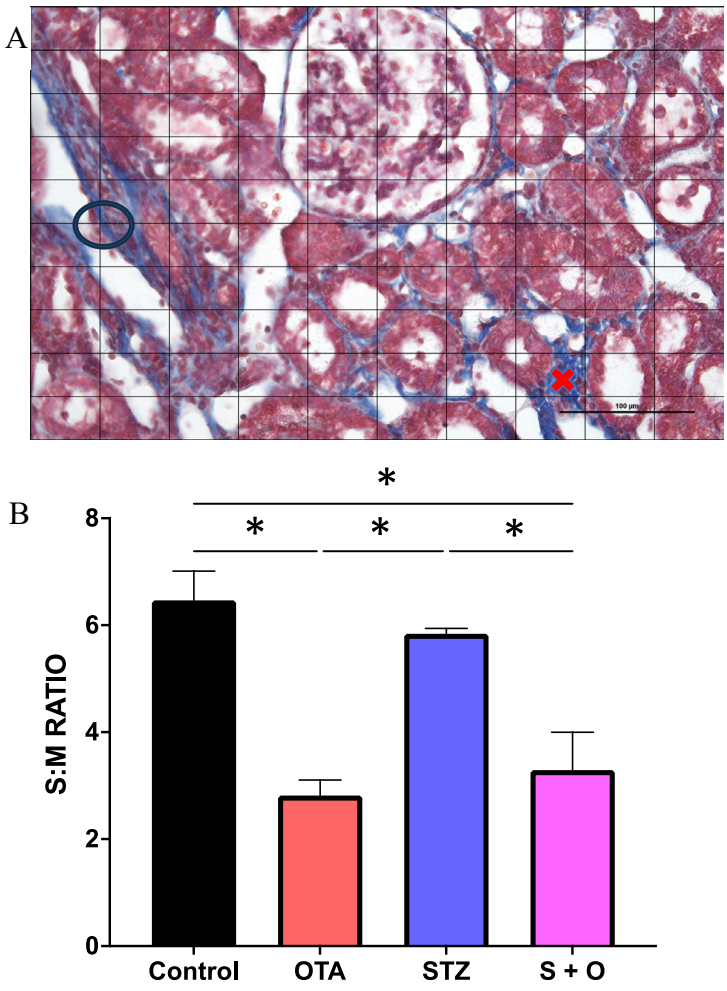

Figure 13. Point counting in micrographs of animals treated with ochratoxin A. Point-counting of interstitial fibrosis in renal cortex micrographs of animals treated with ochratoxin A for 21-days. Notable statistical increase in interstitial fibrosis in STZ/OTA group. (A) Point-counting procedure. 10 x 10 grid overlapped on micrograph. Circle denotes a positive result where the X denotes a negative. (B) Levels of positive point-counts of interstitial fibrosis. The data is shown as the mean ± SEM of 10 micrographs per group. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$.

3.5 Classic Biomarker Assessment

Trunk blood and urine were collected from the animals at the end of the study period to assess the following classic biomarkers: glucose, protein, urea and creatinine.

3.5.1 Urine and Serum Glucose

Serum glucose levels at the end of the study were found to be CON group 283.8 ± 107.10 mg/dL, STZ 1798 ± 430.40 mg/dL, OTA group 363.1 ± 76.80 mg/dL and STZ/OTA group 1089 ± 424.50 mg/dL (Figure 14). The STZ treated group is significantly increased from the control group and the OTA treated group. Urine glucose levels were found to be CON group 9.78 ± 2.63 mg/dL, STZ 26740 ± 12900 mg/dL, OTA group 15.21 ± 4.48 mg/dL and STZ/OTA group 666.4 ± 618.30 mg/dL. These data, both serum and urine, are not indicative of renal damage but are used to confirm the establishment of the experimental diabetes criteria (>300 mg/dL) for the STZ and STZ/OTA animals to be included in the study were met.

3.5.2 Serum Protein

Serum protein levels were found to be CON group 23.46 ± 2.90 mg/mL, STZ 186.07 ± 22.63 mg/mL, OTA group 278.73 ± 71.27 mg/mL, and STZ/OTA group 413.69 ± 27.77 mg/mL (Figure 15). The OTA treated group and the STZ/OTA groups were significantly increased from the control group. In addition, the STZ/OTA group is significantly increased from the STZ group. Serum protein levels are not specific as a renal biomarker. Serum protein levels are used to aid in the screening of a liver, kidney or digestive disorders. Hyperproteinemia is associated with dehydration, chronic inflammation, liver and/or kidney disease (Cardon et al., 1943). The histopathology

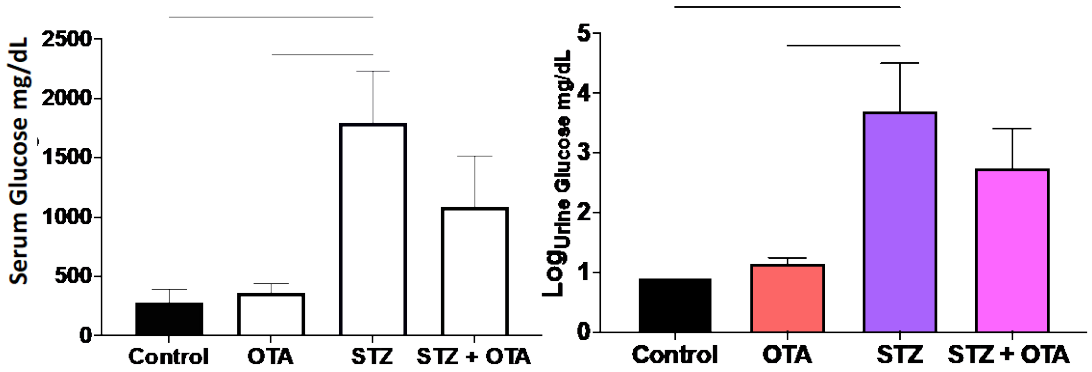

Figure 14. Glucose levels in serum or urine of animals treated with ochratoxin A. Glucose levels in urine and serum were assessed using Cayman Glucose assay kit (Cayman Chemical 100095822). Serum glucose levels were found to be CON group 283.8 ± 107.1 mg/dL, STZ 1798 ± 430.4 mg/dL, OTA group 363.1 ± 76.80 mg/dL and STZ/OTA group 1089 ± 424.5 mg/dL. Urine glucose levels were found to be CON group 9.78 ± 2.634 mg/dL, STZ 26,740 ± 12,900 mg/dL, OTA group 15.21 ± 4.480 mg/dL, and STZ/OTA treated 666.4 ± 618.3 mg/dL. Data indicates both STZ and STZ/OTA treated groups fulfilled the experimental diabetes criteria (>300 mg/dL). All results were expressed as mean ± SEM, Statistical analysis with one-way ANOVA with Tukey's post-hoc test, *P < 0.05

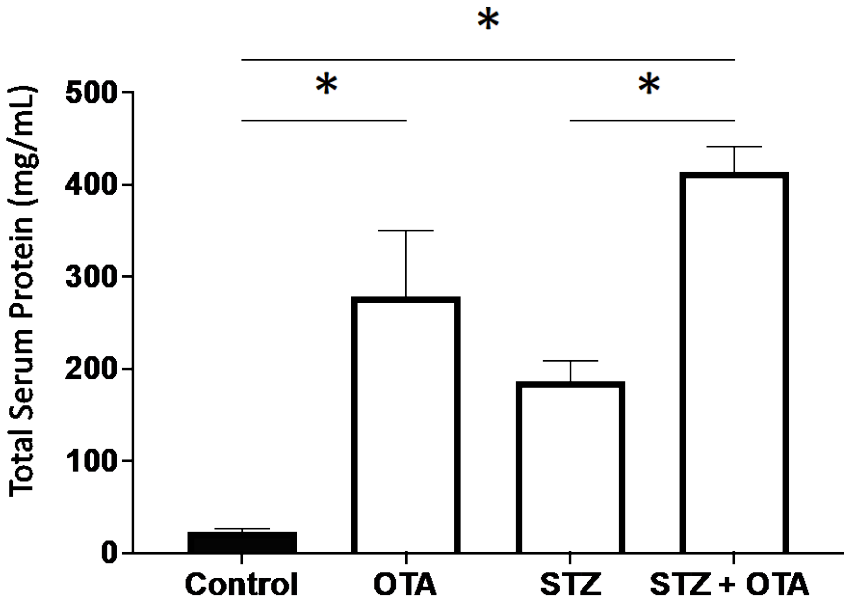

Figure 15. Serum protein levels of animals treated with ochratoxin A.

Serum protein levels of animals treated with ochratoxin A for 21-days. STZ, OTA and STZ/OTA data are indicative of hyperproteinemia. The data is shown as the mean ± SEM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$

results support the presence of inflammation in the renal tissue and renal damage, both of which can explain the significant hyperproteinemia seen in the OTA and STZ/OTA groups.

3.5.3 Serum Urea

Serum urea (SUr) levels were found to be the following CON group 44.68 ± 12.97 mg/dL, STZ group 160.85 ± 53.10 mg/dL, OTA group 54.00 ± 8.14 mg/dL and STZ/OTA group 119.50 ± 38.67 mg/dL (Figure 16). These data do not show any significant differences in SUr and was attributed to lack of biomarker sensitivity.

3.5.4 Serum Creatinine

Serum creatinine (SCr) levels were CON group 60.18 ± 5.23 mg/dL, STZ 8.00 ± 4.12 mg/dL, OTA group 29.95 ± 7.01 mg/dL and STZ/OTA group 9.19 ± 2.88 mg/dL (Figure 17). All groups were significantly decreased from the control group. Additionally, the diabetic group was significantly decreased from the OTA treated group as well. In a clinical review of patient cases, a similar decrease in SCr was noted and attributed to dehydration, muscle loss and chronic kidney disease (CKD) (Thongprayoon et al., 2016). In cases of CKD, literature noted that creatinine secretion increases which may explain the significant decrease in SCr (Ellam, 2011).

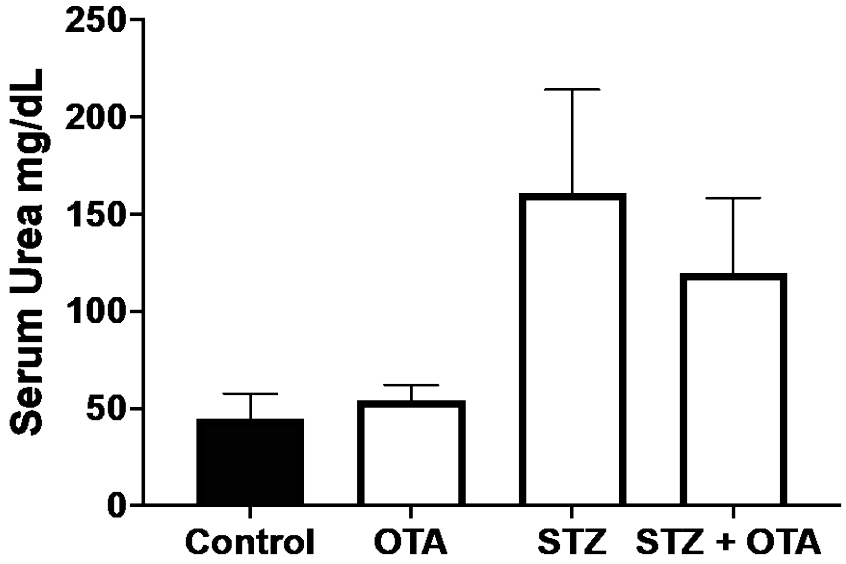

Figure 16. Serum urea levels of animals treated with ochratoxin A.

Serum urea (SUr) levels of animals treated with ochratoxin A for 21-days. There were no significant differences in SUr between the treatment groups. The data is shown as the mean ± SEM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$

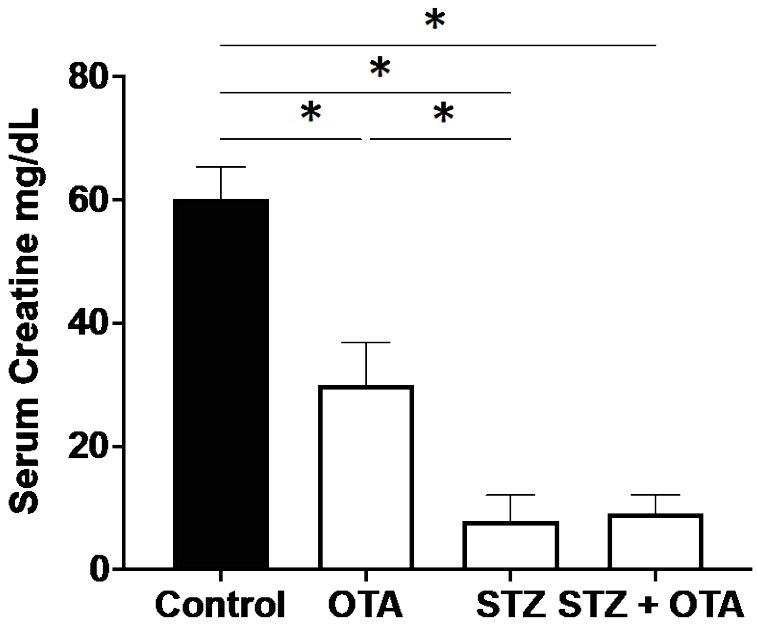

Figure 17. Serum creatinine levels of animals treated with ochratoxin A.

Serum creatinine levels of animals treated with ochratoxin A for 21-days. OTA, STZ and STZ/OTA groups were significantly decreased from the CON group. The STZ group was significantly decreased from the OTA treated group. SCr decrease can be attributed to CKD, of which can be supported by the tissue damage seen in the histopathology assessment. The data is shown as the mean ± SEM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$

3.6 Novel Biomarker assessment

3.6.1 Serum KIM-1

In addition to the classic renal biomarkers, novel markers were also included in the biomarker profile. Serum KIM-1 is a proximal tubule specific marker that is elevated only in cases of proximal tubule damage. Serum KIM-1 levels were found to be CON group 8.9 ± 3.45 pg/mL, STZ 141.78 ± 38.66 pg/mL, OTA group 122.45 ± 28.29 pg/mL and STZ/OTA group 354.39 ± 70.45 pg/mL (Figure 18). The STZ/OTA group is significantly increased from all other groups which indicates significant proximal tubular damage.

3.6.2 Serum NGAL

In addition to KIM-1, another novel biomarker included in the biomarker profile was neutrophil gelatinase-associated lipocalin (NGAL). NGAL was site specific to distal tubules. Serum NGAL levels were found to be CON group 5.51 ± 0.257 ng/mL, STZ 194.61 ± 11.69 ng/mL OTA group 289.29 ± 45.78 ng/mL and STZ/OTA group 564.16 ± 87.60 ng/mL (Figure 19). The STZ/OTA group was significantly increased from all other groups. In addition, the OTA treated group is significantly different from the control group. NGAL was significantly elevated in the OTA group when compared to the CON group, which indicates OTA plays a significant role in the distal tubular damage. The significant increase of the STZ/OTA group compared to all other groups suggests severe distal tubular damage was due to the simultaneous combination of DN and OTA exposure.

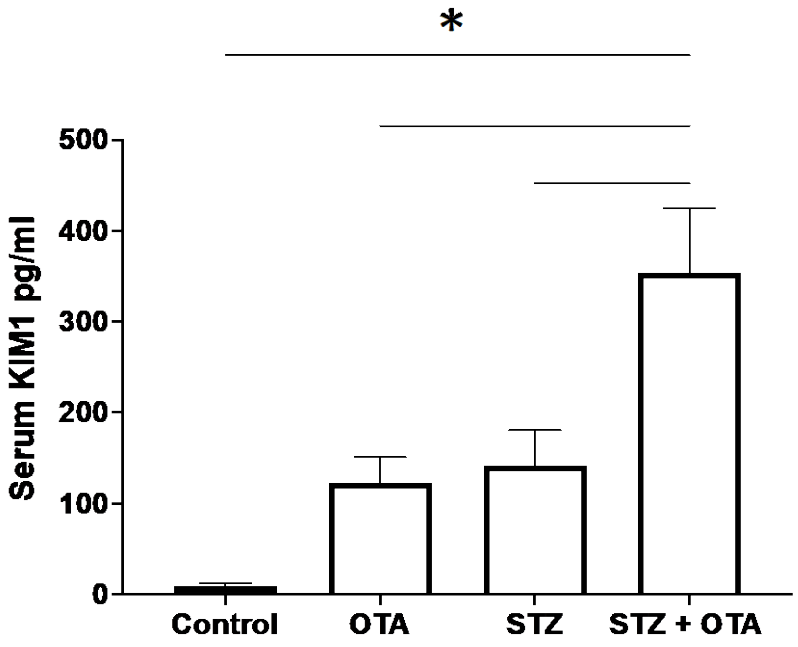

Figure 18. Serum KIM-1 levels of animals treated with ochratoxin A.

Serum KIM-1 levels of animals treated with ochratoxin A for 21-days. OTA, STZ and STZ/OTA groups were significantly increased when compared to CON group. Significant elevation of KIM-1 indicates proximal tubular damage. The data is shown as the mean ± SEM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$

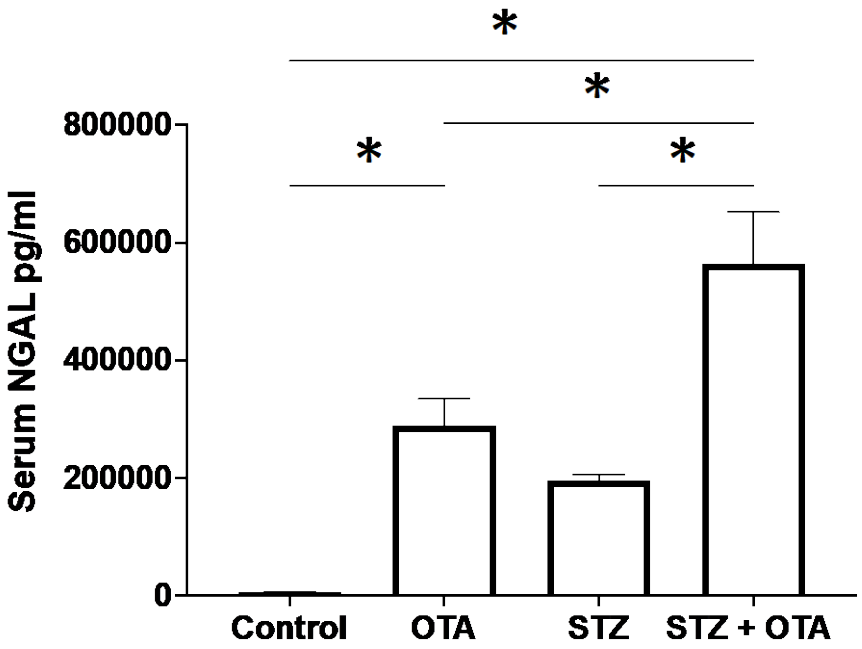

Figure 19. Serum NGAL levels of animals treated with ochratoxin A.

Serum NGAL levels of animals treated with ochratoxin A for 21-days. STZ/OTA were significantly increased when compared to CON, OTA and STZ. OTA was significantly increased when compared to CON. The significant increase in NGAL is indicative of distal tubule damage. The data is shown as the mean ± SEM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$

3.7 Renal Cortical slice uptake of OTA

In additional to the biomarker assessment, functional assessment using cortical uptake of ochratoxin A was determined. The slice-to-medium (S:M) ratio of tritiated OTA in about 100 mg of cortical tissue slices to 2.7 mL of transport buffer was used to calculate the functionality of the cortical tissue (Figure 20). The Cross and Taggert transport buffer contain 0.125 μCi of H^3-OTA with either 20 nM or 520 nM OTA (non-radiolabeled) and was assessed at the 90 minutes time point. The S:M ratio of the CON group was 6.46 ± 0.55, STZ was 5.84 ± 0.10, OTA group was 2.82 ± 0.29 and STZ/OTA group was 3.29 ± 0.71. The STZ/OTA and OTA groups are significantly decreased from the CON group and STZ group suggesting the decrease in cortical tissue functionality is due to the OTA and not the combination of OTA and STZ.

3.8 Cytotoxicity of Ochratoxin A on LLC-PK_1 cells

To assess the potential interactive relationship between high glucose and OTA exposure *in vitro*, LLC-PK_1 cells were treated with increasing concentrations of OTA (0.01, 0.1, 1, 10, 100, 1,000 uM) in either 5 mM, 17.5 mM and 30 mM glucose media. An additional treatment group where the media was switched from 5 mM glucose to 30mM was when the monolayer formed was also included (5 mM/30 mM glucose). Treatment with OTA containing media was conducted for 24 ± 1 hours (Figure 21). The number of viable and dead cells were counted after the incubation time and represented as percentage viable cells against the untreated cells. Cell viability decreased as OTA concentration increased for all glucose conditions, indicating LLC-PK_1 cells are sensitive to OTA exposure.

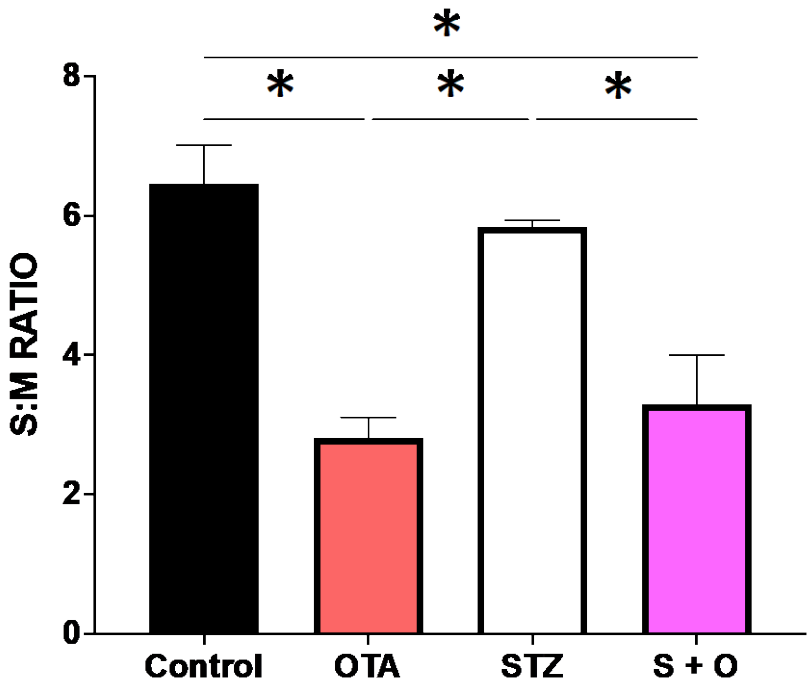

Figure 20. Uptake of tritiated ochratoxin A in renal cortical slices of animals treated with ochratoxin A.

Slice to Medium (S:M) levels in renal cortical slices of animals treated with ochratoxin A for 21-days. STZ/OTA and OTA groups are significantly decreased from the CON group and STZ group indicating the significant decrease in cortical tissue functionality is due to the OTA and not the combination of OTA and STZ. The data is shown as the mean ± SEM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$

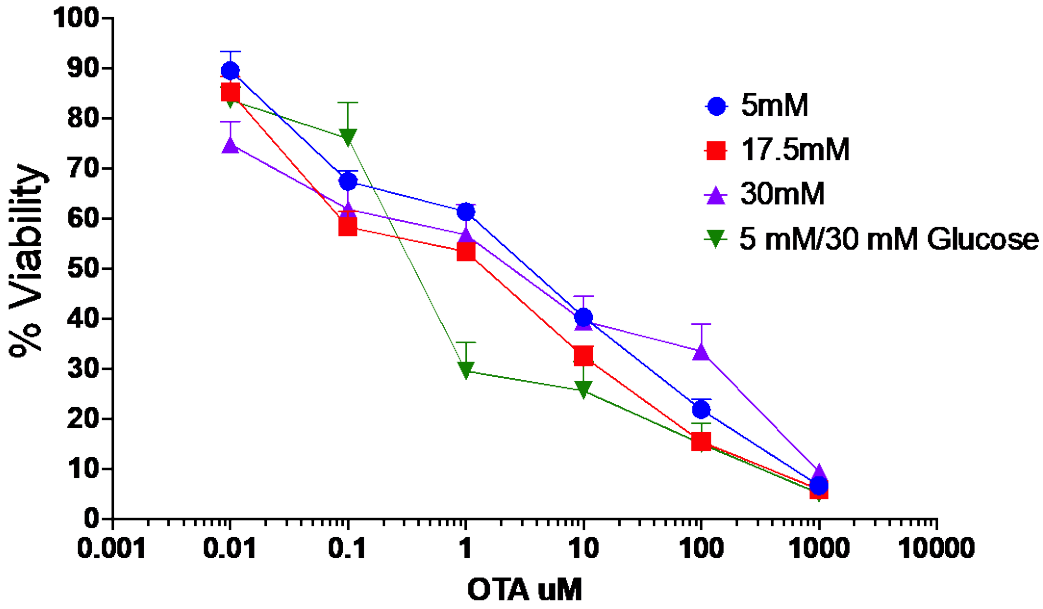

Figure 21. Cytotoxicity of Ochratoxin A on LLC-PK$_1$ cells.

Confluent monolayers of LLC-PK$_1$ were grown in their respective media then treated with media containing 0.001, 0.01, 0.1, 1, 10, 100 and 1000 µM ochratoxin A for 24 hrs. The viability was assessed by trypan blue exclusion. The data were represented as percent viable control:

%VC = (number of live cells in sample)/number of live cells in control) * 100

Results indicated cells grown in high glucose media were significantly more sensitive to OTA as seen by reduced cell viability.

3.9 LC_{50} in LLC-PK_1 monolayers treated with ochratoxin A

LC_{50} for LLC-PK_1 monolayers treated with OTA was determined from the OTA dose-response curves. The LC_{50} for LLC-PK_1 monolayers grown in media with 5 mM glucose was determined to be 3.80 ± 0.52 uM, 17.5 mM glucose media was determined to be 1.58 ± 0.21 uM, 30 mM glucose media was determined to be 2.13 ± 0.30 uM. The LC_{50} for monolayers grown in media with 5 mM glucose then switched to 30 mM glucose was determined to be 0.41 ± 0.12 uM (Figure 21 and 22) (Table 2). The LC_{50} of all groups (17.5 mM glucose, 30 mM glucose, and 5 mM/30 mM glucose) were significantly decreased from that of the 5 mM glucose media group. These data indicated the significantly increased sensitivity of LLC-PK_1 monolayers to OTA as glucose concentrations increase or switched.

CHAPTER 4- DISCUSSION

Both OTA exposure and diabetes individually lead to chronic kidney disease. Due to the rising occurrence of diabetes in the population and the prevalence of successive OTA exposure via ingestion of contaminated food stuffs, there is a need to assess the possible interaction of a diabetic disease state in concurrence with OTA nephrotoxicity (Bui-Klimke & Wu, 2015; Chen & Wu, 2017). The objective of this study was to investigate this interaction using Sprague Dawley rats. The animals were treated with streptozotocin to induce experimental diabetes (Akbarzadeh et al., 2007) and then orally exposed to OTA (0.5 mg/kg bodyweight) once daily for 3 weeks. The dose selected was previously used in a study conducted by Pastor and colleagues in 2018, which is a dose slightly higher than the one used in several OTA carcinogenicity studies (Pastor et al., 2018). The interaction was assessed by evaluation of renal cortical transport activity, renal histopathology, renal biomarker assessment, and evaluation of LLC-PK_1 as an *in vitro* model for this interaction.

The results of this study show significantly increased nephrotoxic effects caused by the interaction of diabetes and OTA exposure. Histopathological findings of diabetic animals treated with OTA showed increased inflammation, tubular degeneration, glomerular degeneration, interstitial fibrosis, necrosis and cast formation throughout (Figures 6 – 12). Histopathological assessment of renal tissue from diabetic Sprague Dawley rats treated with OTA revealed marked increased deposition of extracellular matrix (Figure 13). The histopathological findings of this study in the OTA-exposed groups are consistent with several studies where OTA-treated kidney tissues showed

Media glucose concentration	OTA LC_{50} (μM)
5 mM	3.80 ± 0.52 μM
17.5 mM	1.58 ± 0.21 μM †
30 mM	2.1 ± 0.30 μM †
5 mM/30 mM	0.4 ± 0.12 μM †

Table 2. LC_{50} in LLC-PK_1 monolayers treated with OTA

Confluent monolayers of LLC-PK_1 were treated with 0.001, 0.01, 0.1, 1, 10, 100 and 1000 μM OTA for 24 hr and the viability assessed by trypan blue exclusion. The data were represented as percent viable control: %VC = (number of live cells in sample)/number of live cells in control) * 100

The LC_{50} of all groups (17.5 mM glucose, 30 mM glucose, and 5 mM/30 mM glucose) were significantly decreased from 5 mM glucose media group. These data indicate significant increased sensitivity of LLC-PK_1 monolayers to OTA when glucose concentrations increase. The data is shown as the mean ± SEM of 4 runs. The LC_{50} for the 4 runs in each glucose condition was as follows: 5 mM Glucose = 3.80 ± 0.52 μM, 17.5 mM glucose = 1.58 ± 0.21 μM, 30 mM glucose = 2.1 ± 0.30 μM, and 5 mM/30 mM glucose = 0.4 ± 0.12 μM. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$; † significantly different from 5 mM LC_{50}.

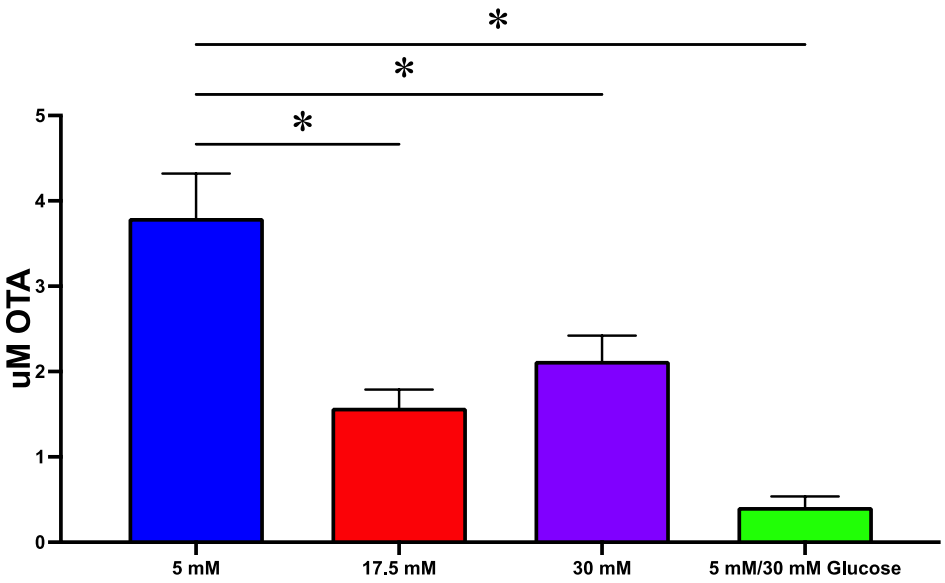

Figure 22. LC_{50} of OTA in LLC-PK_1 monolayers treated with ochratoxin A.

LC_{50} of ochratoxin A in LLC-PK_1 monolayers grown in increasing concentrations of glucose media or switching glucose concentration. The LC_{50} of all groups (17.5 mM glucose, 30 mM glucose, and 5 mM/30 mM glucose) were significantly decreased from 5 mM glucose media group. These data indicate significant increased sensitivity of LLC-PK_1 monolayers to OTA when glucose concentrations increase. The data is shown as the mean ± SEM of 4 runs. Statistical analysis was conducted using a one-way ANOVA with Tukey's post-hoc test, *$P < 0.05$.

tubular degeneration, fibrous tissue, cysts/casts and cytoplasmic alteration (Aydin et al., 2003; Boorman et al., 1992). The histopathological findings of this study seen in the diabetic animals include inflammation, tubular and glomerular degeneration, necrosis and cast formation. These were also consistent with histopathologic changes in diabetic nephropathy (DN) (Jain, 2012; Nishi et al., 2000).

Assessment of the stage of kidney disease is essential in analyzing the interaction between diabetic nephropathy and OTA-exposure. Biomarkers, both traditional and novel, are used to assess a physiological metric. They can be used as a screening or diagnosis tool to monitor disease (Bagshaw & Gibney, 2008). Traditional clinical tools for assessing kidney function such as glomerular filtration rate (GFR) and blood urea nitrogen (BUN) are often not sensitive enough to expose early or mild renal injury or impairment. Renal biomarkers are used to assess either current function or site-specific injury of the kidney (Ford, 2019). An ideal biomarker should generally be non-invasive, easily measured, inexpensive, and provide rapid results using blood or urine. The best type of biomarker is sensitive enough to provide early identification, specific and unaffected by co-morbid conditions. An ideal marker for kidney disease should rapidly reflect any change, condition specific (AKI or CKD), site specific (proximal tubule, distal tubule, glomerulus, loop of Henle), and not interfered with by drugs or endogenous substances (Bagshaw & Gibney, 2008). Both traditional and novel biomarkers were used to assess the renal functional and injury status in diabetic animals exposed to OTA.

The classic biomarkers used in this study include serum creatinine (SCr), serum urea (SUr) and serum protein. Serum creatinine (SCr) and blood urea nitrogen (BUN) have been used to screen and/or diagnose acute kidney injury (Vaidya et al., 2008).

However, serum creatinine and urea levels do not reflect the real-time dynamic changes in glomerular filtration rate (GFR) and none reflect genuine kidney injury (Bagshaw & Gibney, 2008). GFR is the volume of fluid filtered from the glomerular capillaries into the Bowman's capsule per unit time (Lopez-Giacoman & Madero, 2015).

Creatinine is an endogenous amino acid that is nonenzymatically converted from creatine and creatine phosphate and metabolized by the liver and released into the plasma at a relatively constant rate. Creatinine is freely filtered by the glomerulus and not reabsorbed by the tubules or metabolized by the kidney (Bagshaw & Gibney, 2008). Although the levels of creatinine can be highly variable due to production and release, serum creatinine (SCr) levels are used for estimating GFR by using its serum levels and their inversely proportional relationship. Using prediction equations, an increase in SCr levels is generally indicative of a reduced estimate GFR (eGFR), which implies reduced kidney function (Lopez-Giacoman & Madero, 2015). In this study, SCr levels of STZ, OTA and STZ/OTA treatment groups are found to be significantly decreased from CON group (Figure 17). Additionally, the STZ group is significantly decreased from the OTA group. Typically, SCr is expected to be increased when there is kidney damage due to the glomeruli inability to freely filter the creatinine from the serum into the lumen. However, in this study, SCr is significantly decreased. A clinical review using meta-analysis of patient cases was conducted where a similar decrease in SCr was noted (Thongprayoon et al., 2016). The decrease in SCr was attributed to dehydration, muscle loss and chronic kidney disease (CKD). In cases of CKD, it has been noted that creatinine secretion into the lumen increases which may explain the significant decrease in SCr (Ellam, 2011). Augmented renal clearance (ARC) is a noted phenomenon where patients

experience marked increase in functional creatinine clearance in acute illness and is common among intensive care unit patients. The mechanism is not yet clear, however potential mechanisms include increased tubular flow where the decreased nephron number limits the distal tubule reabsorption, disproportionate loss of glomerular function to tubular function, substrate stimulation of the organic cation system and endogenous responses such as systemic inflammatory response with increased cytokines and proinflammatory mediators (Cook & Hatton-Kolpek, 2019; Ellam, 2011). Decreased serum creatinine may be attributed to enhanced tubular creatinine secretion and disproportionate glomerular to tubular function. The cause of decreased SCr can be further supported by the glomerular and tubular damage seen in the pathohistological micrographs of treated animals. Although these classic biomarkers have been assessed individually, an association between urea-to-creatinine ratio and inpatient clinical outcomes has been assessed in an epidemiological study (Brookes & Power, 2022)

In this study, BUN was found to not have any significant differences in SUr concentrations between any treatment groups (Figure 16). Blood urea nitrogen (BUN) is another endogenous biomarker for kidney function. Serum urea (SUr) is expected to be elevated in cases of renal damage and dehydration (Brookes & Power, 2022). Blood urea can also be used to estimate GFR but presents accuracy issues since blood urea levels can be affected by factors outside that of GFR (Bagshaw & Gibney, 2008). Urea production is not stable and can increase when the amount of protein in a diet increases, dehydration, high amount of tissue breakdown (for example, during gastrointestinal hemorrhage), and drugs like corticosteroids or tetracycline (Bagshaw & Gibney, 2008). As BUN levels rise, eGFR declines indicating renal malfunction. BUN and eGFR have a non-linear inverse

relationship (Lopez-Giacoman & Madero, 2015). However, like serum creatinine levels, BUN can vary due to other factors independent of GFR which makes it not an ideal marker on its own as an indicator of kidney function. As indicated in the pathohistological micrographs (Figures 6-12), the glomerular damage seen decreases the filtration of urea and the tubular damage seen impairs the reabsorption of urea. Although these classic biomarkers have been assessed individually, an association between urea-to-creatinine ratio and inpatient clinical outcomes has been assessed in an epidemiological study (Brookes & Power, 2022). Brookes and Power associated higher urea-to-creatinine ratio with greater rates of inpatient mortality and hospital complications and readmissions.

Serum protein is another typical biomarker used to assess renal function. Total serum protein is measured to assess nutritional, liver and kidney disorders (Henok et al., 2020). In this study, OTA-treated and STZ/OTA-treated animals show significant hyperproteinemia (Figure 15). Hyperproteinemia is associated with dehydration, chronic inflammation, liver and/or kidney disease (Cardon et al., 1943). In addition, DN is also linked to hyperproteinemia which is caused by microvascular damage. Microvascular damage has been documented to result in changes in serum protein composition, particularly albumin depression and globulin elevation. Not only is this DN phenomenon associated with hyperproteinemia, but it is also known to cause increased serum viscosity (McMillan, 1989).

Assessment of the classic renal biomarkers (serum creatinine, serum urea and serum protein) suggests that the interaction between diabetic nephropathy and OTA exposure results in dehydration, renal damage, and chronic inflammation. As seen in the

pathohistological findings, the extracellular matrix deposits and the inflammation are seen all throughout the micrographs at the end of the twenty-one-day study time period. However, classic biomarkers do not offer specificity, high sensitivity or early detection to definitively support the pathohistological findings. To further assess the interaction between diabetes and OTA, two novel biomarkers were used.

Kidney injury molecule-1 (KIM-1) is a transmembrane glycoprotein that serves as an identifying biomarker for proximal tubular toxicity (Vaidya et al., 2008). KIM-1 expression was found to be upregulated after kidney injury in patients with acute tubular necrosis (ATN) and can be seen in urine during the first 12 hours of tubular injury (Lopez-Giacoman & Madero, 2015). KIM-1 has also been established as an *in vitro* marker for PCT damage using NRK-52E (Rached et al., 2008). KIM-1 has been used in ratio with creatinine to evaluate mycotoxin associated renal toxicity in female and male C57BL/6J mice (Oe et al., 2024). KIM-1 was used as CKD biomarker for mycotoxin AA KIM-1. It was proven to be a sensitive and tissue-specific biomarker in rodent and human clinical studies where KIM-1 increased in areas of tubular fibrosis and inflammation (Ráduly et al., 2023). OTA exposure in Wistar rats caused histological changes which were best reflected in the increase of KIM-1 levels, which occured before any changes in traditional clinical parameters were seen (Hoffmann et al., 2010). KIM-1 as a biomarker is more sensitive and site-specific as compared to the typical renal biomarkers. In this study, KIM-1 is significantly increased in the STZ/OTA group (Figure 18). The increase indicates significant tubular damage when compared to all other treatment groups. Proximal tubular damage seen in this study is also supported by the histopathological

findings and correlates with the trends established by the classic biomarkers previously discussed.

In addition to KIM-1, neutrophil gelatinase-associated lipocalin (NGAL) was also used to further assess the interaction between diabetes and OTA. NGAL is a small lipocalin protein and a component of innate immunity against bacterial infection. NGAL is expressed by immune cells, hepatocytes and renal tubules in various disease states (Edelstein, 2017). NGAL has been shown to be site specific to the distal tubule section of the nephron. Serum NGAL has been shown to increase in patients with AKI. It has been shown that NGAL was detected 3.2 days earlier than changes in patient serum creatinine (Edelstein, 2017). NGAL has mainly been studied as a biomarker of cisplatin-induced AKI but has shown promise as a potential biomarker for the detection of nephrotoxicity induced by other agents or causes. In this study, NGAL was significantly increased in the STZ/OTA group when compared to CON, OTA and STZ treatment groups. OTA was significantly increased when compared to CON. The significant increase in NGAL was indicative of significant distal tubule damage (Figure 19). Distal tubular damage is also supported by the histopathological findings and correlates with the trends established by the previously discussed typical biomarkers.

In addition to evaluating histopathology and renal biomarkers for the assessment of the potential interaction between DN and OTA exposure, evaluation of renal cortical tissue function was also assessed. Renal cortical slices used for transport studies has been established as an appropriate and useful *in vitro* technique to evaluate the effects of xenobiotics during short-term or long-term incubations (Rose et al., 1985). Cortical slices can remain metabolically differentiated for at least 24-48 hours (Baverel et al., 2013).

Renal uptake studies using cortical slices were used to compare the accumulation of OTA. Results indicate OTA uptake in OTA and STZ/OTA groups were both significantly decreased when compared to CON and STZ group (Figure 20). However, OTA and STZ/OTA groups are not significantly different. Therefore, these data suggest that the decrease in OTA uptake by cortical slices is due to OTA itself and not the diabetic nephropathy. This assay was conducted 24 hours after the last dose of OTA, and given the half-life of OTA in rat is estimated at 230 hours (Zepnik et al., 2003). In addition to OTA being known to accumulate, it is very possible the short time between assay conduction and last dose of OTA did not allow for sufficient clearance of OTA prior to the start of the cortical uptake assay. These factors may have impacted the assay therefore the results may not truly depict the effects of the interaction of OTA and STZ on the renal transport systems involved.

Significant elevation of KIM-1 and NGAL, hyperproteinemia and decreased serum creatinine in the STZ/OTA group correlates with the histopathological findings of proximal and distal damage. Overall, OTA exposure in animals with diabetic nephropathy results in increased inflammation, proximal and distal tubular degeneration, glomerular degeneration, interstitial fibrosis, necrosis and cast formation. These histopathological findings are supported by the biomarker profile (significantly decreased serum creatinine, significantly elevated serum protein, significantly elevated KIM-1 and NGAL) which support the conclusion of a detrimental interaction between DN and OTA exposure.

The mechanism of this interaction between DN and OTA exposure has yet to be clarified. The LLC-PK_1 porcine proximal tubule cell line was used to assess the

interaction of glucose levels and OTA exposure on cell viability. Monolayers of LLC-PK_1 cells were grown in culture medium with 5 mM glucose (90 mg/dL), 17.5 mM glucose (315 mg/dL), 30 mM glucose (540 mg/dL). An additional group was included where LLC-PK_1 cells grown in 5 mM glucose and then switched to 30 mM glucose media during OTA treatment. LLC-PK_1 dose response curves showed decreased cell viability as OTA concentrations increased (Figure 21). This indicated monolayers were increasingly sensitive to OTA in all treatment groups. The LC_{50} of 5 mM glucose, 17.5 mM glucose, 30 mM glucose, and 5 mM/30 mM glucose media groups were determined from the cytotoxicity curves (Table 2). The LC_{50} of each high glucose group (17.5 mM glucose, 30 mM glucose, and 5 mM/30 mM glucose) were found to be significantly lower from that of the 5 mM group indicating enhanced toxicity of OTA at high glucose levels (Figure 22). These *in vitro* data when taken together indicate LLC-PK_1 cells are an appropriate model to further investigate the mechanism involved in the interaction between high glucose and OTA exposure.

Despite extensive research, the mode of action of OTA is still not clarified. One of the potential focus points of many studies has been the involvement of ROS. *In vitro* studies using kidney microsomes and *in vivo* studies using rats have shown OTA to enhance NADH (nicotinamide adenine dinucleotide)-dependent and ascorbate-dependent lipid peroxidation which impairs the cytoplasmic membrane permeability to calcium and therefore interrupting calcium homeostasis (Longobardi et al., 2022; Rahimtula et al., 1988). In addition, Nrf2 has been shown to be one of the major contributors to OTA-induced ROS in a study using LLC-PK_1 cells resulting in decreased GSH (glutathione)/GSSG (glutathione disulfide) ratio and superoxide dismutase (SOD) activity

(Boesch-Saadatmandi et al., 2009; Longobardi et al., 2022). OTA treatment in HK-2 cells caused oxidative stress related gene expression up-regulation including SOD1, CAT, GSR and KIM-1 (Lee et al., 2023). OTA was found to initiate free radical formation and increase *Nrf2* mRNA in the kidney in a dose and duration dependent manner (Ferenczi et al., 2020). NADH oxidase (NOX), particularly NOX4, is the most abundant isoform which contributes to ROS generation in the kidney by increasing O_2 production in the mitochondria, which then is catalyzed to H_2O_2 and promotes apoptosis by entering the cells via facilitated diffusion (Longobardi et al., 2022). As such, NOX4 is considered one of the main contributors and potential therapeutic targets for oxidative-stress-associated nephrotoxicants like OTA and conditions such as DN which result in CKD or end stage renal disease (Damiano et al., 2020).

The pathogenesis of DN has been better clarified compared to that of OTA, and is directly associated with chronic diabetes, histological lesions and is characterized by albuminuria and reduced eGFR. The complex pathogenesis of DN involved multiple mechanisms, the primary being hyperglycemia (Pelle et al., 2022). A direct effect of high glucose was observed in the present cell culture study, in which case increased glucose in the growth media corresponding to diabetic blood levels resulted in greater cytotoxicity. The morphological changes typical of DN are known to also be affected by advanced glycation end-products (AGEs), which are caused by hyperglycemia, and result in damage of the matrix, glomerular basement membrane alterations and damage to other glomerular components and function (Pelle et al., 2022). These alterations cause hemodynamic abnormalities which in combination with hyperglycemia leads to the hallmarks of diabetic kidney disease including glomerular hyperfiltration, ROS,

endothelial dysfunction, extracellular matrix deposition, mesangial proliferation, podocyte and tubular death, glomerular basement membrane thickness and glomerulosclerosis (Rayego-Mateos et al., 2020). The collagen deposition, podocyte and tubular death were observed in the gross assessment of the histopathological assessment of the STZ animals (Figures 2 and 3). In DN, increased ROS production is caused by the shift from glucose metabolism to non-glycolic metabolism stimulated by hyperglycemia (Pelle et al., 2022). The increased ROS then results in local and systemic inflammation which causes direct and indirect damage to renal cells (Rayego-Mateos et al., 2020).

Hyperglycemia associated inflammation is heavily associated with a positive feedback loop which further enhances renal damage and induces vascular remodeling (Rayego-Mateos et al., 2020). Hyperglycemia activates protein kinase C (PKC), transforming growth factor-β1 (TGF- β1) expression, and angiotensin-II all of which promote fibrotic processes (Pelle et al., 2022). Hyperglycemia has been implicated in proximal tubule glucose toxicity where SGLT2 inhibition caused a reduction partially by way of decreasing oxidative stress (Oe et al., 2024). There is a strong relationship between ROS and inflammation. Proinflammatory factor induction is a major mediating response in DN and can occur through the activation of NF-kB and activator protein-1 (AP-1) by ROS (Rayego-Mateos et al., 2020). A mechanistic study exploring OTA exposure during induced nephropathy explored renal fibrosis via TGF-β1/SMAD2/3 pathway was the primary focus (Du et al., 2022). Fibrosis develops when the synthesis of collagen exceeds the rate at which is it degraded and therefore increases and over time accumulates to lead to the formation of permanent fibrotic scar and loss of tissue function (Wynn, 2008).

Another important pathway in DN is induced intraglomerular hypertension. Glomerular hyperfiltration is a result of hyperglycemia, is responsible for intraglomerular hypertension and is one of the first mechanisms responsible for the onset of albuminuria and reduced eGFR. This process is mediated by the dilation of glomerular afferent arteries through mediators such as TGF- β1, vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), nitric oxide (NO), prostaglandins, and glucagon (Pelle et al., 2022). The resulting renal hypoxia promotes the progression of DN by causing an imbalance in the supply and demand of O_2 due to the loss of peritubular capillaries and interstitial fibrosis and the increased energy requirements from hyperfiltration (Pelle et al., 2022).

The histopathological micrographs and point-counting show evidence of elevated collagen deposition, particularly in the cortical region along with glomerular and tubular damage. Elevated TGF-β1 expression has associated with glomerulosclerosis and tubular fibrosis (Du et al., 2022). Cellular fibrosis is known to be activated and modulated by chemokines, growth factors and cytokines, specifically transforming growth factor β1 (TGF-β1). Under pathological conditions, TGF-β1 induces parenchymal inflammatory and collagen-expressing cells and plays a key role in fibrosis progression (Chen & Raghunath, 2009). α-SMA and Vimentin are well known biomarkers in the progression of renal fibrosis (Wynn, 2008). The results from Du and colleagues (Du et al., 2022) indicated OTA upregulated the expression of the previously mentioned renal fibrosis factors (α-SMA and vimentin). Their results indicated that OTA exposure to humans with CKD would accelerate the development of renal fibrosis which contributes the

progression of CKD to end stage renal disease as a process of renal fibrosis (Du et al., 2022).

There is a great deal of overlap in pathways and mediators of renal injury for both OTA and DN. ROS/RNS, inflammation and fibrosis are major contributors for renal damage. ROS leads to tissue damage and dysfunction which leads to common mediators such as TGF-β1 and NO which triggers pro-fibrotic and oxidative stress processes. Although the direct pathways there may not be the same, as previously noted NOX4 is a major contributor for OTA and hyperglycemia for DN, both OTA and DN progress to renal fibrosis, CKD and ultimately ESRD.

In conclusion, these data, both *in vivo* and *in vitro,* show that the interaction between OTA and diabetic nephropathy have severe nephrotoxic effects and LLC-PK_1 cells are an appropriate model to continue to investigate the mechanism involved in this interaction. Our data demonstrate that the kidneys of diabetic animals are more sensitive to ochratoxin A. The results with the LLC-PK_1 cells suggest that hyperglycemia itself can adversely affect proximal tubule cells. OTA is also known to have an oral half-life in humans of 35.5 days and 55-120 hours in rats (O'Brien & Dietrich, 2005). Such prolonged half-lives can intensify nephrotoxic effects in patients with CKD or DN. Therefore, individuals with impaired renal function due to diabetes can be particularly sensitive to mycotoxins like OTA (Du et al., 2022). Due to the increasing occurrences of both the development of diabetic nephropathies and the prevalence of OTA ingestion, the resulting nephrotoxicity makes it clear this interaction is important to clarify and continue to investigate.

CHAPTER 5- FUTURE DIRECTIONS

It would be useful to re-assess the uptake of OTA in a renal cortical uptake study after sufficient OTA clearance occurs or with a different OAT1/3 substrate, such as para-aminohippurate which shares the same renal transport system. Another area to further investigate is the mechanistic role reactive oxygen species for this interaction. Lastly, using this *in vitro* model to assess epithelial-to-mesenchymal transition as a potential mechanism as the final endpoint of fibrosis after exposure to OTA in high glucose.

REFERENCES

Abrunhosa, L., Paterson, R. R. M., & Venâncio, A. (2010). Biodegradation of Ochratoxin A for Food and Feed Decontamination. *Toxins*, *2*. https://doi.org/10.3390/toxins2051078

Akbarzadeh, A., Norouzian, D., Mehrabi, M. R., Jamshidi, S., Farhangi, A., Allah Verdi, A., Mofidian, S. M. A., & Lame Rad, B. (2007). Induction of Diabetes by Streptozotocin in Rats. *Indian Journal of Clinical Biochemistry*, *22*
.

Alshannaq, A., & Yu, J.-H. (2017). Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. *International Journal of Environmental Research and Public Health*, *14*. https://doi.org/10.3390/ijerph14060632

Anzai, N., Jutabha, P., & Endou, H. (2010). Molecular mechanism of ochratoxin a transport in the kidney. *Toxins*, *2*. https://doi.org/10.3390/toxins2061381

Aydin, G., Ozcelik, N., Cicek, E., & Soyo, M. (2003). Histopathologic changes in liver and renal tissues induced by Ochratoxin a and melatonin in rats. *Human and Experimental Toxicology*, *22*. https://doi.org/https://doi.org/10.1191/0960327103ht354oa

Bagshaw, S. M., & Gibney, R. T. N. (2008). Conventional markers of kidney function. *Critical Care Medicine*, *36*. https://doi.org/10.1097/CCM.0b013e318168c613

Bajaj, P., Chowdhury, S. K., Yucha, R., Kelly, E. J., & Xiao, G. (2018). Emerging Kidney Models to Investigate Metabolism, Transport, and Toxicity of Drugs and Xenobiotics. *Drug Metabolism and Disposition*, *46*. https://doi.org/https://doi.org/10.1124/dmd.118.082958

Baud, L., & Ardaillou, R. (1986). Reactive oxygen species: production and role in the kidney. *American Journal of Physiology-Renal Physiology*, *251*. https://doi.org/10.1152/ajprenal.1986.251.5.F765

Baverel, G., Knouzy, B., Gauthier, C., El Hage, M., Ferrier, B., Martin, G., & Duplany, A. (2013). Use of precision-cut renal cortical slices in nephrotoxicity studies. *Xenobiotica*, *43*. https://doi.org/10.3109/00498254.2012.725142

Bennett, J. W. (1987). Mycotoxins, mycotoxicoses, mycotoxicology and Mycopathologia. *Mycopathologia*, *100*.

Boorman, G. A., McDonald, M. R., Imoto, S., & Persing, R. (1992). Renal lesions induced by ochratoxin A exposure in the F344 rat. *Toxicologic Pathology*, *20*. https://doi.org/10.1177/019262339202000210

Brookes, E. M., & Power, D. A. (2022). Elevated serum urea-to-creatinine ratio is associated with adverse inpatient clinical outcomes in non-end stage chronic

kidney disease. *Scientific Reports*, *12*. https://doi.org/10.1038/s41598-022-25254-7

Brown, C. D. A., Bodmer, M., Biber, J., & Muerer, H. (1984). Sodium-dependent phosphate transport by apical membrane vesicles from a cultured renal epithelial cell line (LLC-PK1). *Biochimica et Biophysica Acta*, *769*(2). https://doi.org/https://doi.org/10.1016/0005-2736(84)90332-8

Bui-Klimke, T. R., & Wu, F. (2015). Ochratoxin A and human health risk: A review of the evidence. *Critical Reviews in Food Science and Nutrition*, *55*. https://doi.org/10.1080/10408398.2012.724480.

Cardon, L., Atlas, D. H., Brunner, M. J., Aron, E., & Teitelman, S. L. (1943). Incidence and Causes of Hyperproteinemia: A Study of 4,390 cases. *Archives of Internal Medicine*, *71*(3), 377-390. https://doi.org/10.1001/archinte.1943.00210030078007

Chen, C., & Wu, F. (2017). The need to revisit ochratoxin A risk in light of diabetes, obesity, and chronic kidney disease prevalence. *Food and Chemical Toxicology*, *103*. https://doi.org/http://dx.doi.org/10.1016/j.fct.2017.03.001

Chen, C. Z., & Raghunath, M. (2009). Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis- state of the art. *Fibrosis and Tissue Repair*, *2*. https://doi.org/doi:10.1186/1755-1536-2-7

Christiansen, J. J., & Rajasekaran, A. K. (2006). Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Research*, *66*. https://doi.org/10.1158/0008-5472.CAN-06-0410

Cook, A. M., & Hatton-Kolpek, J. (2019). Augmented Renal Clearance. *The Journal of Human Pharmacology and Drug Therapy*, *39*. https://doi.org/10.1002/phar.2231

Damiano, S., Lauritano, C., Longobardi, C., Andretta, E., Elagoz, A. M., Rapisarda, P., Di Iorio, M., Florio, S., & Ciarcia, R. (2020). Effects of a Red Orange and Lemon Extract in Obese Diabetic Zucker Rats: Role of Nicotinamide Adenine Dinucleotide Phosphate Oxidase. *Journal of Clinical Medicine*, *9*(5). https://doi.org/10.3390/jcm9051600

Dresser, M. J., Kaushal Leabman, M., & Giacomini, K. M. (2001). Transporters Involved in the Elimination of Drugs in the Kidney: Organic Anion Transporters and Organic Cation Transporters. *Journal of Pharmaceutical Sciences*, *90*. https://doi.org/https://doi.org/10.1002/1520-6017(200104)90:4<397::AID-JPS1000>3.0.CO;2-D

Du, H., Le, G., Hou, L., Mao, X., Liu, S., & Huang, K. (2022). Nontoxic Concentration of Ochratoxin A Aggravates Renal Fibrosis Induced by Adriamycin Cyclosporine A Nephropathy via TGF-β1/SMAD2/3. *Journal of Agricultural and Food Chemistry*, *70*. https://doi.org/https://doi.org/10.1021/acs.jafc.2c03577

Duran-Salgado, M. B. R.-G., Alberto F. (2014). Diabetic nephropathy and inflammation. *World Journal of Diabetes 5*. https://doi.org/10.4239/wjd.v5.i3.393

Edelstein, C. L. (2017). *Chapter Six- Biomarkers in Acute Kidney Injury* (Seconda ed.). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-803014-1.00006-6

EFSA Panel on Contaminants in the Food Chain (CONTAM), Dieter Schrenk, Laurent Bodin, James Kevin Chipman, Jes us del Mazo, Bettina Grasl-Kraupp, Christer Hogstrand, Laurentius (Ron) Hoogenboom, J.-C. L., Carlo Stefano Nebbia, Elsa Nielsen, Evangelia Ntzani, Annette Petersen, Salomon Sand, Tanja Schwerdtle, Christiane Vleminckx, Heather Wallace, Jan Alexander, Chiara Dall'Asta, Angela Mally, . . . Bignami, M. (2020). Risk Assessment of ochratoxin A in food. *European Food Safety Authority Journal*, *18*. https://doi.org/https://doi.org/10.2903/j.efsa.2020.6113

Ellam, T. (2011). Increased tubular creatinine secretion by remnant nephrons—unexplained but informative? *Nephrology Dialysis Transplantation- Clinical Kidney Journal*, *4*. https://doi.org/10.1093/ndtplus/sfq215

Ferenczi, S., Kuti, D., Cserháti, M., Krifaton, C., Szoboszlay, S., Kukolya, J., Sz˝oke, Z., Albert, M., Kriszt, B., Kovács, K. J., Mézes, M., & Balogh, K. (2020). ffects of Single and Repeated Oral Doses of Ochratoxin A on the Lipid Peroxidation and Antioxidant Defense Systems in Mouse Kidneys. *Toxins*, *12*. https://doi.org/doi:10.3390/toxins12110732

Ford, S. (2019). Conventional and Emerging Renal Biomarkers. In *Biomarkers in Toxicology* (2 ed.). https://doi.org/https://doi.org/10.1016/B978-0-12-814655-2.00015-3

Goldring, S. R., Dayer, J.-M., Ausiello, D. A., & Krane, S. M. (1978). A cell strain cultured from porcine kidney increases cyclic AMP content upon exposure to calcitonin or vasopressin. *Biochemical and Biophysical Research Communications*, *83*(2). https://doi.org/https://doi.org/10.1016/0006-291X(78)91009-4

Gstraunthaler, G., & Handler, J. S. (1987). Isolation, growth, and characterization of a gluconeogenic strain of renal cell. *American Journal of Physiology-Renal Physiology*, *252*(2). https://doi.org/10.1152/ajpcell.1987.252.2.C232

Gstraunthaler, G., Pfaller, W., & Kotanko, P. (1985). Biochemical characterization of renal epithelial cell cultures (LLC-PK1 and MDCK). *American Journal of Physiology-Renal Physiology*, *248*(4). https://doi.org/10.1152/ajprenal.1985.248.4.F536

Haraguchi, R., Kohara, Y., Matsubayashi, K., Kitazawa, R., & Kitazawa, S. (2020). New Insights into the Pathogenesis of Diabetic Nephropathy: Proximal Renal Tubules Are Primary Target of Oxidative Stress in Diabetic Kidney. *ACTA*

HISTOCHEMICA ET CYTOCHEMICA, *53*(2). https://doi.org/https://doi.org/10.1267/ahc.20008

Henok, J. N., Okeleye, B. I., Omodanisi, E. I., Ntwampe, S. K. O., & Aboua, Y. G. (2020). Analysis of Reference Ranges of Total Serum Protein in Namibia: Clinical Implications. *Proteomes*, *8*. https://doi.org/10.3390/proteomes8020007

Heussner, A. H., & Bingle, L. E. H. (2015). Comparative Ochratoxin Toxicity: A Review of the Available Data. *Toxins*, *7*(10). https://doi.org/ https://doi.org/10.3390/toxins7104253

Hoffmann, D., Fuchs, T. C., Henzler, T., Matheis, K. A., Herget, T., Dekant, W., Hewitt, P., & Mally, A. (2010). Evaluation of a urinary kidney biomarker panel in rat models of acute and
subchronic nephrotoxicity. *Toxicology*, *277*, 49-58.

Hsieh, M.-F. C., Hsiao-Ying; Lin-Tan, Dan-Tzu; Lin, Ja-Liang. (2004). Does Human Ochratoxin A Aggravate Proteinuria in Patients with
Chronic Renal Disease. *Renal Failure*, *26*. https://doi.org/10.1081/JDI-200026744

Jain, M. (2012). Histopathological changes in diabetic kidney disease. *Clinical Queries: Nephrology*, *1*(2), 127-133. https://doi.org/https://doi.org/10.1016/S2211-9477(12)70006-7

Jenkinson, S. E., Chung, G. W., van Loon, E., Bakar, N. S., Dalzell, A. M., & Brown, C. D. A. (2012). The limitations of renal epithelial cell line HK-2 as a model of drug transporter expression and function in the proximal tubule. *Pflügers Archiv: European Journal of Physiology*, *464*(6). https://doi.org/10.1007/s00424-012-1163-2.

Jessica Gil-Serna, Covadonga Vazquez, & Patino, B. (2019). *Reference Module in Food Science: Mycotoxins, Toxicology*. https://doi.org/10.1016/B978-0-08-100596-5.22630-9

Karlovsky, P. S., Michele; Berthiller, Franz; De Meester, Johan; Eisenbrand, Gerhard; Perrin, Irène; Oswald, Isabelle P.; Speijers, Gerrit; Chiodini, Alessandro; Recker, Tobias; and Dussort, Pierre. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin Research*, *32*. https://doi.org/10.1007/s12550-016-0257-7

Kaushal, G. P., Chandrashekar, K., & Juncos, L. A. (2019). Molecular Interactions Between Reactive Oxygen Species and Autophagy in Kidney Disease. *International Journal of Molecular Sciences*, *20*. https://doi.org/https://doi.org/10.3390/ijms20153791

Kőszegi, T., & Poór, M. (2016). Ochratoxin A: Molecular Interactions, Mechanisms of Toxicity and Prevention at the Molecular Level. *Toxins*. https://doi.org/10.3390/toxins8040111

Krishnan, S., Suarez-Martinez, A., Bagher, P., Gonzalez, A., Liu, R., Murfee, W., & Mohandas, R. (2021). Microvascular dysfunction and kidney disease: Challenges and opportunities? *Microcirculation*, *28*. https://doi.org/10.1111/micc.12661

Lee, H. J., Kim, H. D., & Ryu, D. (2023). Protective Effect of alpha-Tocopherol Against Ochratoxin A in Kidney Cell Line HK-2. *Journal of Food Protection*, *86*. https://doi.org/https://doi.org/10.1016/j.jfp.2023.100082

Longobardi, C., Ferrara, G., Andretta, E., Montagnaro, S., Damiano, S., & Ciarcia, R. (2022). Ochratoxin A and Kidney Oxidative Stress: The Role of Nutraceuticals in Veterinary Medicine—A Review. *Toxins*, *14*. https://doi.org/https://doi.org/10.3390/toxins14060398

Lopez-Giacoman, S., & Madero, M. (2015). Biomarkers in chronic kidney disease, from kidney function to kidney damage. *World Journal of Nephrology*, *4*. https://doi.org/10.5527/wjn.v4.i1.57.

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J., & Toman, J. (2016). Ochratoxin A: 50 Years of Research. *Toxins*. https://doi.org/10.3390/toxins8070191

McMillan, D. E. (1989). Increased levels of acute-phase serum proteins in diabetes. *Metabolism*, *38*(11), 1042-1046. https://doi.org/https://doi.org/10.1016/0026-0495(89)90038-3

Mullin, J. M., Diamond, L., & Kleinzeller, A. (1980). Effects of ouabain and ortho vanadate on transport-related properties of the LLC-PK1 renal epithelial cell line. *Journal of Cell Physiology*, *105*. https://doi.org/10.1002/jcp.1041050102

Nagavally, R. R. S., Siddharth; , Akhtar, M., Trombetta, L. D., & Ford, S. M. (2021). Chrysin Ameliorates Cyclosporine-A-Induced Renal Fibrosis by Inhibiting TGF-β1-Induced Epithelial-Mesenchymal Transition. *International Journal of Molecular Sciences*, *22*. https://doi.org/10.3390/ijms221910252

Nishi, S., Ueno, M., Hisaki, S., Iino, N., Iguchi, S., Oyama, Y., Imai, N., Arakawa, M., & Gejyo, F. (2000). Ultrastructural characteristics of diabetic nephropathy. *Medical Electron Microscopy*, *33*. https://doi.org/https://doi.org/10.1007/s007950070004

Noronha-Blob, L., Filburn, C., & Sacktor, B. (1984). Phosphate uptake by kidney epithelial (LLC-PK1) cells. *Archives of Biochemistry and Biophysics*, *234*(1). https://doi.org/https://doi.org/10.1016/0003-9861(84)90349-7

O'Brien, E., & Dietrich, D. R. (2005). Ochratoxin A: The Continuing Enigma. *Critical Reviews in Toxicology*, *35*, 33-60. https://doi.org/10.1080/10408440590905948

Oe, Y., Kim, Y. C., Sidorenko, V. S., Zhang, H., Kanoo, S., Lopez, N., Goodluck, H. A., Crespo-Masip, M., & Vallon, V. (2024). SGLT2 inhibitor dapagliflozin protects the kidney in a murine model of Balkan nephropathy. *American Journal of Physiology-Renal Physiology*, *326*, 227-240.

Ozbek, E. (2012). Induction of Oxidative Stress in Kidney. *International Journal of Nephrology*. https://doi.org/10.1155/2012/465897

Pastor, L., Vettorazzia, A., Encisoa, J. M., González-Peñasb, E., García-Jalónc, J. A., Monreald, J. I., & López de Ceraina, A. (2018). Sex differences in ochratoxin a toxicity in F344 rats after 7 and 21 days of
daily oral administration. *Food and Chemical Toxicology*, *111*. https://doi.org/https://doi.org/10.1016/j.fct.2017.11.003

Pelle, M. C., Provenzano, M., Busutti, M., Porcu, C. V., Zaffina, I., Stanga, L., & Arturi, F. (2022). Up-Date on Diabetic Nephropathy. *Life*, *12*. https://doi.org/https://doi.org/10.3390/life12081202

Peraica, M., Flajs, D., Domijan, A.-M., Ivić, D., & Cvjetković, B. (2010). Ochratoxin A contamination of food from Croatia. *Toxins*, *2*. https://doi.org/10.3390/toxins2082098

Petrik, J., Zanic-Grubisic, T., Barisie, K., Pepeljnjak, S., Radic, B., Ferencic, Z., & Cepelak, I. (2003). Apoptosis and oxidative stress induced by ochratoxin A in rat kidney. *Archives Toxicology: Organ Toxicity and Mechanisms*, *77*. https://doi.org/10.1007/s00204-003-0501-8

Purchase, I. F. H., & Theron, J. J. (1968). The Acute Toxicity of Ochratoxin A to Rats. *Food and Cosmetics Toxicology*, *6*(4), 479-480. https://doi.org/https://doi.org/10.1016/0015-6264(68)90138-7

Pyo, M. C., Choi, I.-G., & Lee, K.-W. (2021). Transcriptome Analysis Reveals the AhR, Smad2/3, and HIF-1 Pathways as the Mechanism of Ochratoxin A Toxicity in Kidney Cells. *Toxins*, *12*. https://doi.org/https://doi.org/10.3390/toxins13030190

Rabito, C. A., & Ausiello, D. A. (1980). Na+-dependent sugar transport in a cultured epithelial cell line from pig kidney. *Journal of Membrane Biology*, *54*. https://doi.org/10.1007/BF01875374

Rached, E., Hoffmann, D., Blumbach, K., Weber, K., Dekant, W., & Mally, A. (2008). Evaluation of Putative Biomarkers of Nephrotoxicity after Exposure to Ochratoxin A In Vivo and In Vitro. *Toxicological Sciences*, *103*, 371-381. https://doi.org/doi:10.1093/toxsci/kfn040

Ráduly, Z., Szabó, A., Mézes, M., Balatoni, I., Price, R. G., Dockrell, M. E., Pócsi, I., & Csernoch, L. (2023). New perspectives in application of kidney biomarkers in mycotoxin induced nephrotoxicity, with a particular focus on domestic pigs. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2023.1085818

Rayego-Mateos, S., Morgado-Pascual, J. L., Opazo-Ríos, L., Guerrero-Hue, M., García-Caballero, C., Vázquez-Carballo, C., Mas, S. S., A.B., Herencia, C., Mezzano, S., Gómez-Guerrero, C., Moreno, J. A., & Egido, J. (2020). Pathogenic Pathways and Therapeutic Approaches Targeting Inflammation in Diabetic Nephropathy.

International Journal of Molecular Sciences, *21*(11). https://doi.org/https://doi.org/10.3390/ijms21113798

Riedmaier, A. E., Nies, A. T., Schaeffeler, E., & Schwab, M. (2012). Organic anion transporters and their implications in pharmacotherapy. *Pharmacological Reviews*, *64*. https://doi.org/10.1124/pr.111.004614.

Ringot, D., Chango, A., & Schneider, Y.-J. L., Yvan. (2006). Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-Biological Interactions*, *159*. https://doi.org/10.1016/j.cbi.2005.10.106

Rose, R. C., Bianchi, J., & Schuette, S. A. (1985). Effective use of renal cortical slices in transport and metabolic studies. *Biochimica et Biophysica Acta*, *821*. https://doi.org/10.1016/0005-2736(85)90047-1

Roth, M., Obaidat, A., & Hagenbuch, B. (2012). OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *British Journal of Pharmacology*, *165*. https://doi.org/10.1111/j.1476-5381.2011.01724.x

Schaaf, G. J., Nijmeijer, S. M., Maas, R. F. M., Roestenberg, P., Groene, E. M. d., & Fink-Gremmels, J. (2002). The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. *Biochimica et Biophysica Acta*, *1588*. https://doi.org/10.1016/s0925-4439(02)00159-x

Sunilkumar, S., & Ford, S. M. (2019). Elevated glucose concentration in culture media decreases membrane trafficking of SGLT2 in LLC-PK1 cells via a cAMP/PKA-dependent pathway. *American Journal of Physiology-Cell Physiology*, *316*(6). https://doi.org/10.1152/ajpcell.00433.2018

Thongprayoon, C., Cheungpasitporn, W., & Kashani, K. (2016). Serum creatinine level, a surrogate of muscle mass, predicts mortality in critically ill patients. *Journal of Thoracic Disease*, *8*. https://doi.org/10.21037/jtd.2016.03.62

Vaidya, V. S., Ferguson, M. A., & Bonventre, J. V. (2008). Biomarkers of acute kidney injury. *Annual Review of Pharmacology and Toxicology*, *48*. https://doi.org/10.1146/annurev.pharmtox.48.113006.094615

Veiga-Matos, J., Remião, F., & Morales, A. (2020). Pharmacokinetics andToxicokineticsRolesof Membrane Transporters at Kidney Level. *Journal of Pharmacy and Pharmaceutical Sciences*, *23*. https://doi.org/10.18433/jpps30865

Wafa, E. W. Y., R S; Sobh, M A; Eraky, I; el-Baz, M; el-Gayar, H A; Betbeder, A M; Creppy, E E (1998). Human ochratoxicosis and nephropathy in Egypt: a preliminary study. *Human and Experimental Toxicology*, *17*. https://doi.org/10.1177/096032719801700207

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *Journal of Pathology*, *214*(2). https://doi.org/10.1002/path.2277

Yanfei Tao, S. X., Fanfan Xu, Aimei Liu, Yanxin Wang, Dongmei Chen,, & Yuanhu Pan, L. H., Dapeng Peng, Xu Wang Zonghui Yuan. (2018). Ochratoxin A: Toxicity, oxidative stress and metabolism. *Food and Chemical Toxicology*, *112*. https://doi.org/https://doi.org/10.1016/j.fct.2018.01.002

You, G., Kuze, K., Kohanski, R. A., Amsler, K., & Henderson, S. (2000). Regulation of mOAT-mediated Organic Anion Transport by Okadaic Acid and Protein Kinase C in LLC-PK1 Cells. *Journal of Biological Chemistry*, *275*(14), 10278-10284. https://doi.org/https://doi.org/10.1074/jbc.275.14.10278

Yu, J. P., Ivana Ramos. (2023). Mycotoxins in Cereal-Based Products and Their Impacts on the Health of Humans, Livestock Animals and Pets. *Toxins*, *15*. https://doi.org/https://doi.org/10.3390/toxins15080480

Zepnik, H., Völkel, W., & Dekant, W. (2003). Toxicokinetics of the mycotoxin ochratoxin A in F 344 rats after oral administration. *Toxicology and Applied Pharmacology*, *192*. https://doi.org/10.1016/s0041-008x(03)00261-8

Zingerle, M. S., Stefan; Gekle, Michael. (1997). Reabsorption of the Nephrotoxin Ochratoxin A Along the Rat Nephron In Vivo. *Journal of Pharmacology and Experimental Therapeutics*, *280*.

Vita

Name	*Belkys Gonzalez*
Baccalaureate Degree	*Bachelor of Science* *St. John's University,* *Queens, NY 11439* *Major: Toxicology*
Date Graduated	*May 2010*
Other Degrees and Certificates	*Master of Science* *St. John's University* *Queens, NY 11439* *Major: Toxicology*
Date Graduated	*September 2015*