Daily Intake of Grape Powder Protects Kidney Function in Obese ZSF-1 Rats

by

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Abstract

Metabolic syndrome (diabetes, hypertension, obesity and hypercholesteremia) increases the risk of high-mortality chronic diseases including chronic kidney disease, which accounts for 50% of end-stage renal disease (ESRD) in the developed world. Over 1/3 of the world's adult population have metabolic syndrome. Oxidative stress plays a central role in metabolic syndrome pathophysiology. Grape is one of the broadly studied natural anti-oxidants. Literature demonstrates grape antioxidant's significant protective effects on metabolic syndrome, however, not yet on metabolic syndrome-related kidney disease. This study evaluates the effect of whole grape on kidney disease associated with metabolic syndrome.

Material and methods: Preclinical model of metabolic syndrome-related kidney disease, Obese ZSF-1 rats, ingested whole grape powder (5% of daily diet) for 6 months. Blood and urine samples were analyzed monthly to assess renal function parameters including 24-hour urine volumes, proteinuria, and urine protein to creatinine ratio (PCR). Rats' kidney tissue histopathology and PCR array studies were conducted. In vitro kidney cell death was examined in cultured podocytes using flow cytometry.

Results: Here, collective data from 6-month preclinical study showed chronic kidney disease consistent with an early stage diabetic nephropathy picture in both experimental and control groups. Renal function in rats of the experimental group was significantly enhanced compared with those of the control group, indicated by less 24-hour urine volumes (34.79 ± 15.77 mL vs. 55.8 ± 20.27 mL, $p = 0.0147$) and less proteinuria (8.56 ± 5.71 g vs. 24.01 ± 37.51 g, $p = 0.0412$) in the experimental group. Urine PCR was significantly lower in the experimental group versus

control (3.42 ± 1.289 vs. 9.722 ± 9.156, p = 0.0084). Histopathology and PCR array analysis showed less oxidative stress picture in experimental group versus control. In vitro antioxidant assays showed significantly reduced H2O2-induced cell death in podocytes treated with grape extract versus control.

Conclusion: This pilot study indicates that daily intake of whole grape powder has a protective effect on the kidney in obese ZSF-1 rats, suggesting the potential of grape antioxidants as a prevention strategy for reducing kidney disease progression in metabolic syndrome patients. Further investigations are required to support this preliminary study.

Preface

This thesis has been written in partial fulfillment of the requirement for the degree of Master of Science in Experimental Medicine. I have written this thesis under the direction and supervision of Dr. Caigan Du and Dr. Carolyn Gotay from September 2014 to October 2016. Dr. Caigan Du reviewed this thesis. All the experimental works and analysis of this research project was performed by Salwa Almomen in Dr. Caigan Du Lab at the Vancouver Prostate Centre with technical skills supervision of lab manager, Qiunong Guan.

The BC Cancer Agency (BCCA) lab has conducted the processing of kidney tissues into slides and staining with H&E (Hematoxylin and Eosin) stain for histopathological analysis done by Salwa Almomen. The chemical study methodology (section 2.3.1) was performed and results (section 3.3.1) were produced by Kaidi Yang. Later, the chemical experimental methodology was re-performed and results were re-produced by Salwa Almomen. The results presented in this thesis are the original production of Kaidi Yang. Dr. Adeera Levin has overviewed the abstract presented in this thesis and edited its conclusion.

This research was presented in part as a poster in the Natural Health Products Research Society of Canada (NHPRS) in May 2016. Also, this research in whole was presented as an oral presentation in the Vancouver Prostate Centre (VPC) Seminar in October 2016.

The Certificate Number of the Ethics Certificate obtained to conduct the animal study in this project is (A14-0168).

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Acknowledgements

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I would like to give many thanks to Dr. Carolyn Gotay, member of my supervisory committee, for her interest in this project and my work and for the time and effort she has invested in providing me with enlightening feedback.

Special thanks are owed to Kaidi Yang whose contribution to this project has been of great value and to Ghida Aldairi whose emotional support was positively impactful at all difficult times.

Dedication

This degree is dedicated to my family who has been a source of all kinds of support throughout my lifetime academic journey. My overwhelming gratitude is forever owed to my dear parents, Muhamad and Ayesha, and my precious husband, Walid, for their providing of constant inspiration, motivation and most valued prayers that are truly the essence of any success and accomplishments of mine. Finally, the most refreshing moments that has always recharged me with warmth and positivity were provided by my three-year old son, Ahmed, whose smiling face is my ultimate happiness.

Chapter 1: Introduction

1.1 Metabolic syndrome (MetS)

1.1.1 History

The initial understanding of the chronic health condition known as Metabolic Syndrome (MetS) was rather a concept than a medical diagnosis [1]. This concept has evolved throughout the past century originating in 1920 when a Swedish physician, Kylin, described the association of high blood pressure (hypertension), high blood glucose (hyperglycemia), and gout [2]. Following this in 1947, it was demonstrated by Vague, that visceral obesity was found to often associate with the metabolic abnormalities seen in cardiovascular disease (CVD) and type 2 Diabetes Mellitus (T2DM) [3]. Hypertension, hyperglycemia and obesity were together again described as comprising a syndrome in an abstract presented by Avogaro and Crepaldi in 1965 at the European Association for Diabetes annual meeting [4]. The concept was progressed significantly when insulin resistance was introduced to the concept by Reaven in 1988 who described it as "a cluster of risk factors for diabetes and cardiovascular disease" and named it "Syndrome X" [5]. Surprisingly, obesity or visceral obesity was missed from his definition, which was later added as an important abnormality. There was a renaming of the syndrome by Kaplan in 1989 as "The Deadly Quarter", pointing to the combination of the four components; upper body obesity, glucose intolerance, hypertriglyceridemia, and hypertension [6]. Additionally, in 1992, it was renamed "The Insulin Resistance Syndrome" [7].

1.1.2 Definitions

Not only had the understanding of metabolic syndrome evolved throughout the years, but the definition of it as well. Attempts in providing a diagnostic criteria to clinically identify MetS

have been made by multiple groups [8]. World Health Organization (WHO) made the first attempt to develop a definition of MetS in 1998 [9]. A year later, the definition was modified by the European Group for the study of Insulin Resistance (EGIR) [6], which in turn was followed by a definition released by the National Cholesterol Education Program Adult Treatment Panel (NCEP/ATP) in 2001 [10]. In response, the American Association of Clinical Endocrinologists (AACE) shared its views about defining the syndrome [11]. Clearly, the proliferation of the syndrome definitions required a consensus to unify or standardize them into a single definition. [12]. As a result, a new definition of MetS was released in April 2005 by the International Diabetes Federation (IDF) [13].

MetS is defined as a collection of interconnected physiological, biochemical, clinical, and metabolic factors that directly increases the risk of atherosclerotic cardiovascular disease (ASCVD), T2DM, and all-cause mortality [14, 15]. This group of medically concerning body measurements and laboratory test results include hypertension, diabetes (glucose intolerance), dyslipidemia, prothrombotic and proinflmmatory states, all of which lead to atherogenic events resulting in micro and macrovascular changes. While multiple definitions of MetS have been provided, the current and most commonly used diagnostic criteria are those offered by World Health Organization (WHO) [9], the International Diabetes Federation (IDF) [13], the European Group for the study of Insulin Resistance (EGIR), the American Association of Clinical Endocrinologists (AACE) [11] and National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III) [10]. All of the diagnostic criteria proposed by these leading scientific committees include parameter measurements of insulin resistance, serum glucose, body weight, serum lipids and blood pressure [16].

1.1.3 Pathophysiology

When genetic and environmental risk factors co-exist, susceptibility to the syndrome increases as a complex interplay between those factors lead to chronic low grade inflammation resulting in the manifestations of MetS. Factors that magnify the risk of MetS include genetic susceptibility, hyperglycemia, hypertension, smoking, sedentary lifestyle, visceral obesity and atherogenic dyslipidemia (Figure1) [16].

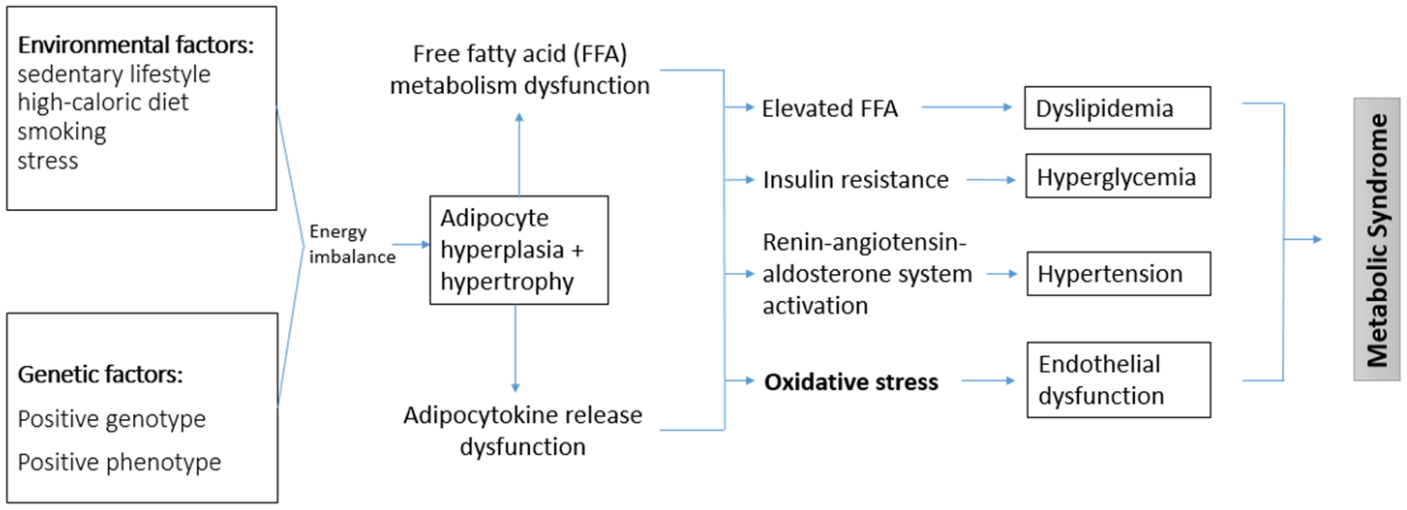


Figure 1- Pathophysiology background of metabolic syndrome [16].

Alongside elevated blood pressure and hyperglycemia, visceral obesity (abdominal obesity) is a major culprit in the constitution of MetS. Like all epidemics, abdominal obesity has emerged as a global critical health condition affecting wide demographics that consist of male, female, adults and children as well. In principle, the reason behind this increased body mass is increased intake of high caloric food and reduced physical activity which collectively drive the rapid and dynamic response by adipose tissue resulting in adipocytes hypertrophy and hyperplasia. Adipose tissue is

composed of a heterogeneous mix of adipocytes, immune cells, stromal cells and endothelial cells [17]. Since adipocytes generate adipocytokines and free fatty acids (FFA), they both are liable to disturbances in response to adipocytes hypertrophy and hyperplasia [16].

Adipocytokines: Progressive enlargement of adipocyte tissue may cause reduction in the blood perfusion of adipocytes and hypoxia, consequently [18]. It has been proposed that through necrosis and macrophage infiltration into the adipose tissue both attributed to hypoxia, an over production of adipocytokines takes place. Adipocytokines are biologically active metabolites including glycerol, free fatty acids (FFA), proinflammatory mediators [tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6)], plasminogen activator inhibitor-1 (PAI-1), and C-reactive protein (CRP) [19]. These events lead to a localized inflammation in adipose tissue that consequently launches the overall systemic inflammation involved in the development of obesity related comorbidities [20]. Adipocytokines possess a broad spectrum of systemic effects since they integrate endocrine, autocrine, and paracrine signals. These systemic effects are reflected in the activation of multiple crucial biological processes including oxidative stress [21], insulin sensitivity [22], energy metabolism, blood coagulation, and inflammatory responses [23] and are believed to be the attributors to progressive atherothrombosis, atherosclerosis and plaque rupture. Therefore, the importance of adipose tissue lies not only in its functions in the storage and transportation of lipids, but also in the release of numerous cytokines as an endocrine organ.

Free fatty acids (FFA): The majority of the circulating FFA is produced by upper body subcutaneous adipocytes, while splanchnic FFA levels have been associated with intra-abdominal fat composition, to which fatty liver is often attributed [24]. The pancreas and skeletal

muscles are two sources of FFA that have been identified as contributors to insulin resistance and, thus, hyperglycemia [25]. Increased levels of FFA in the pancreas causes the impairment of pancreatic β-cell function, while exposure of skeletal muscles to elevated FFA levels induces insulin resistance through inhibiting insulin-mediated glucose uptake [25].

Both disturbed FFA metabolism and adipocytokines release rise the levels of oxidative stress and consequent endothelial dysfunction through related enzymes and factors such as Protease Inhibitor 1 (PAI-1). PAI-1 is a serine protease inhibitor and is released by intra-abdominal adipocytes, endothelium and platelets [19]. It acts as an inhibitor of the tissue plasminogen activator (tPA) [26], the reason why it is considered as an atherothrombosis and impaired fibrinolysis marker. In conditions of abdominal obesity [27] and inflammatory states [28], plasma PAI-1 levels are elevated and so is the risk of intravascular thrombosis and adverse cardiovascular events [29]

In summary, Alterations in FFA metabolism and the release of adipocytokines as a result of adipose tissue hypertrophy and hyperplasia lead to elevated levels of specific factors and related enzymes including Protease Inhibitor 1 (PAI-1). The latter causes increased oxidative stress and, therefore, endothelial dysfunction which in turn results in systemic inflammation, thrombosis hypercoagulable state and eventually Metabolic Syndrome.

Further elaboration on the details of the involvement of oxidative stress as an underlying mechanism in the pathophysiology of metabolic syndrome is found in (section 1.4.4.1 as described below).

1.1.4 Epidemiology

Epidemiological surveillance of worldwide prevalence of MetS shows variability in ranges that can reach to as high as 84% or as low as 10%. This variability is largely dependent on multiple factors affecting the targeted population including their age, gender, race and ethnicity. Not only individuals' physical composition affects the prevalence of MetS, but also which region in the world and what environment (rural vs. urban) they live in. The complexity of the factors influencing the risk of MetS and therefore its prevalence includes sedentary lifestyle, diet, smoking, family history of diabetes and education [30]. Moreover, the definition of MetS used in epidemiological studies play as well a role in dictating the estimated prevalence [31,32]. However, in light of the multiple estimated prevalences of MetS, a general conclusion by the IDF states that one-quarter of the world's adult population is diagnosed with MetS [13].

As established through literature, the more components of MetS present, the higher the risk of the disease [33]. As far as weight is concerned, National Health and Nutrition Examination Survey (NHANES) observed the prevalence of the MetS as up to 60% among obese subjects, 22% among overweight subjects and 5% among normal subjects [34]. Additional evidence supports body weight effects on MetS as a report by a Framingham Heart Study demonstrate that a weight increase of ≥2.25kg over a 16-year duration was correlated with an increased risk of MetS that reaches up to 45% [35], while another study by Palaniappan et al. indicated that a waist circumference (WC) increase of 11 cm is followed with an adjusted risk of developing the syndrome reaching to as high as 80% within 5 years [36].

1.1.5 Management

Clinical recognition of MetS is important to the planning of an adequate and comprehensive approach to treat the disease and reduce the risks of subsequent diseases [37] such as chronic kidney disease (CKD). Disease prevention strategies proven effective are primarily lifestyle modifications including physical activity, diet and weight loss. In cases of difficult or unsuccessful implementation of these preventive measures, an appropriate pharmacological therapy is then considered to reduce specific risk factors of the syndrome [38]. Since MetS is a multifactor disease, the clinical therapeutic approach of it should be through targeting those multiple underlying rick factors. While there is no identified single method of treatment that can improve the syndrome as a whole [5], physicians target each component of MetS separately. The emphasis in selecting the choice of treatments strongly relies on which component is most amenable to drug treatment. Realistically, the expectations and outcomes from the attempts to alter lifestyles of affected individuals in the hope of not only eliminating modifiable risk factors, but also sustaining the desired low levels of blood pressure, glucose and triglycerides, is unfortunately low. On the other hand, it is faster and easier to prescribe a drug to target those risk factors than executing a long-term plan to change people's unhealthy lifestyles.

The aim of MetS therapy is to reduce its short-time and lifetime risk, which should be clinically assessed for the affected individual. If MetS is present, this alone indicates a higher lifetime risk. The standard Framingham algorithm is commonly used for a 10-year risk of the coronary heart disease (CHD) estimation. It has been known as a practical method for MetS patients without diabetes or ASCVD to estimate absolute short-term CHD/CVD risk [39]. This risk assessment equation takes in consideration most of CVD risk in MetS patients and include total cholesterol,

high density lipids (HDLD), blood pressure, cigarette smoking and age. While there is no need to do Framingham risk scoring for patients with ASCVD or diabetes as they are already in a high risk category, standard Framingham algorithm place patients into three categories on the basis of CHD 10-year risk;

1) 10-year risk (≥20%): High risk.
2) 10-year risk (10% to 20%): Moderately high risk
3) 10-year risk (≤10%): Moderate or low risk

After patients have their CVD risk assessment done, current treatment guidelines addressing their risk that are followed by physicians include the American Heart Association(AHA)[14], the American Diabetes Association (ADA) [40], the National Institute of Health Obesity Initiative[41], National Cholesterol Education Programme(NCEP) [39] and the seventh Joint National Commission (JNC-VII) for blood pressure treatment [42].

Clinical management strategies for MetS patients with CKD should include a plan for reducing weight, increasing physical activity and modifying dietary intake. All of which must be synchronized with targeted specific CVD risk factors (hyperglycemia, hypertension and dyslipidemia) therapy plan that conform to the appropriate national guidelines.

1.1.6 Systematic effects

As explained in the pathophysiology of MetS, chronic low grade inflammation is largely involved in its etiology and systemic effects. Profound complications of MetS include:

-Renal dysfunction (e.g. proteinuria/microalbuminuria, CKD, glomerulomegaly, impaired filtration and glomerulosclerosis) [43].

- Cardiovascular system (e.g. thrombosis and stroke, myocardial infarction and coronary heart disease) [45].

- Hepatic (non-alcoholic fatty liver disease, hepatic fibrosis and cirrhosis) [46].

- Skin (Acanthosis nigricans, systemic lupus erythematosus, lichen planus, psoriasis and genetic alopecia [47].

- Productive system (e.g. erectile dysfunction, hypogonadism and polycystic ovarian syndrome) [48].

- Eye (e.g. non-diabetic retinopathy, primary open angle glaucoma and oculomotor nerve palsy) [49].

- Respiratory (e.g. obstructive sleep apnea) [50].

- Cancer (e.g. pancreas, breast and prostrate) [51].

Further elaboration on the association of metabolic syndrome and kidney disease is found in (section 1.3.5 as described below).

1.2 The kidney

1.2.1 The renal system

All of the following structure and function information regarding the renal system is adopted from Human physiology/ The renal system wikibook 2014 [51].

The renal system, also known as urinary system, consists of a collection of organs that are mainly responsible for the filtration of excess fluid and other substances from the body. Urine is the liquid end product that contains the filtered-out substances composed of excreted excess minerals, vitamins and blood corpuscles. The production of urine originates at the kidney where it is generated to pass through the ureters into the collecting bladder to be eventually expelled through the urethra. The urinary system is composed of 2 kidneys, 2 ureters, a bladder and a urethra (Figure 2) [51]. Maintaining homeostasis is achieved by the cooperation of the urinary system with the other systems in the body. The main organ concerned with homeostasis is the kidney as it functions to control both the water-salt balance and the acid-base balance in the body.

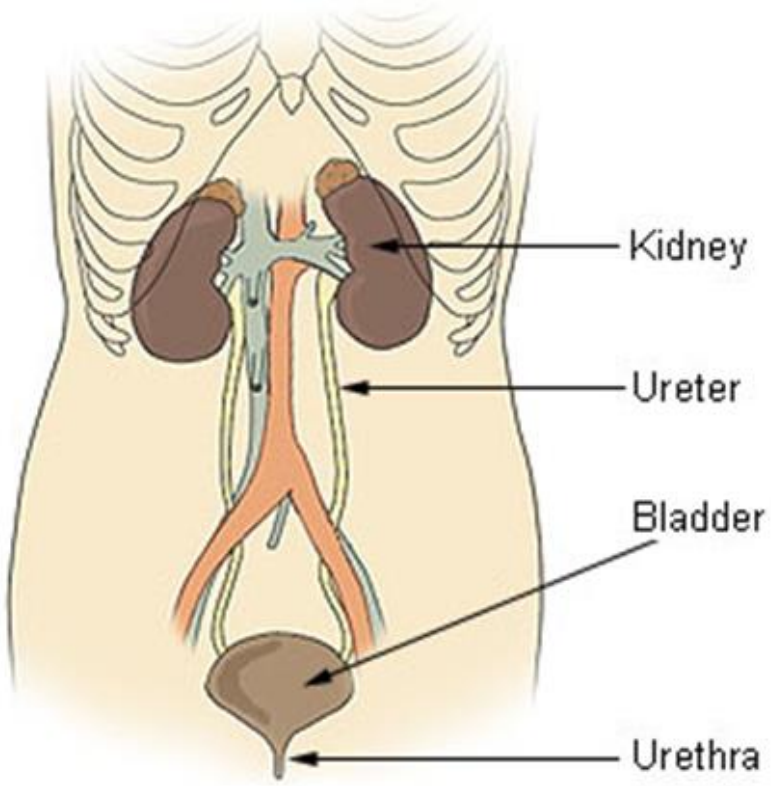


Figure 2- Structure of the renal system [51].

1.2.1.1 Organs structure and function

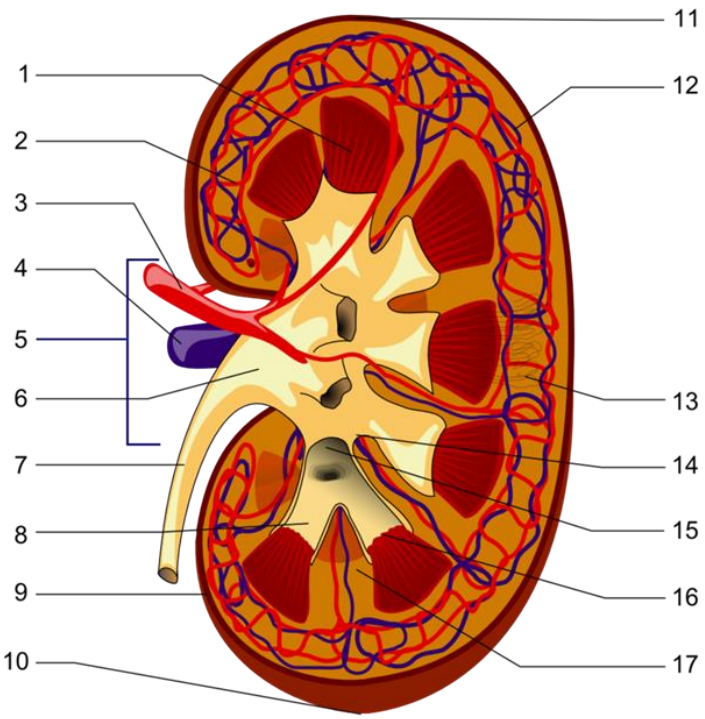


Figure 3- Structure of the kidney [51].

1. Renal pyramid 2. Interlobar artery 3. Renal artery 4. Renal vein 5. Renal hylum 6. Renal pelvis 7. Ureter 8. Minor calyx 9. Renal capsule 10. Inferior renal capsule 11. Superior renal capsule 12. Interlobar vein 13. Nephron 14. Minor calyx 15. Major calyx 16. Renal papilla 17. Renal column.

- The Kidney: The kidneys are located behind the peritoneum and, therefore, are considered retroperitoneal. They are located just above the waistline at the posterior wall of the abdominal cavity where they are protected by the rib cage. The kidneys are described as a couple of brown bean-shape organs measuring 10-12 cm long, approximately the size of one's fist. A tough cover made of fibrous connective tissue

serves as the kidney's capsule, while two layers of fat attach to the kidney's surface as protective cushions. The depression in the kidney where the renal artery enters while the renal vein and ureter exit, is located at the concave side of the bean shaped kidney. Looking into the kidney, it is divided into three major regions that layer the kidney from the outside towards the inside: renal cortex, renal medulla and the renal pelvis. The renal cortex is the granulated outer layer stretching down between the renal medulla which is a radially striated inner layer. Contained in this part, the renal pyramids are made of pyramid shaped tissue and are separated by renal columns. In the middle region of the kidney, the ureters are centered as they are continuous with the renal pelvis.

- The renal veins: drain the kidneys connecting them to the inferior vena cava (IVC) which carries the blood that is leaving the kidney back to the heart. The left renal vein is longer that than the right renal kidney simply because the IVC runs along the right side of the body rather than at the middle. There is a left gonadal vein (left testicular and left ovarian veins in male and female, respectively) and a left suprarenal vein draining into the left renal vein.
- The renal arteries: perfuse the kidneys carrying blood from the abdominal aorta, approximately one third of the cardiac output, to be filtered. Opposite to the veins, the right renal artery is generally longer than the left renal artery due to the locations of aorta, inferior vena cava and the kidneys.
- The ureters: are two, around 10-inch long, muscular tubes sending urine in small spurts out of the kidneys and into the bladder. A stretchable pouch at the anterior wall of the renal pelvis allows urine to be stored there before going down the ureters.

- The urinary bladder: The ureters enter the bladder in a diagonal direction at the dorsolateral sides of the bladder through a triangular area known as the trigone. This area is located on the kidney wall postero-inferiorly and at the triangle's lowest point is where the urethra exits. The bladder is an elastic muscular organ that is hollow and stretchable. It is positioned on the pelvic floor with the pubis symphysis lying on its anterior border. In males, the prostate lies inferiorly and the rectum posteriorly. In females, the vagina lies posteriorly.

  Since small folds in the bladder mucosa serve as valves to prevent backflow of the urine, the bladder can store around 500-350 ml of urine. The desire to urinate is, however, usually when urine volume reaches to about 150-200 ml. When the bladder is approximately half full, stretch receptors send signals up the spinal cord to the brain which subsequently sends back a nerve impulse to the sphincter at the neck of the bladder causing the relaxation reflex that allows urine to pass into the urethra. The Internal urethral sphincter is under both involuntary and voluntary control as it is a partly a learned response to relax the sphincter.
- The urethra: is a muscular tube that measures ~3.8 cm (in females) and ~20 cm (in males) connecting the bladder to the outside of the body where it discards of the urine.

1.2.1.2 Renal system function

Even though excretion in the body is carried out not only by the kidneys, but also by other organs, the kidney remains the main and most important excretory organ. In general, maintaining homeostasis of the body (the internal environment balance) is the primary and ultimate function of the kidneys. Excretion is the action that serves this function. It is the elimination of

unnecessary and harmful materials such as waste products of the body's metabolism. This is achieved as the kidneys filter out urea, mineral salts, toxins and other metabolites from the blood stream. To keep a healthy life, at least one kidney should be functioning properly. The collective roles of the kidneys are found as follows:

1) Regulating electrolyte balance consisting of plasma ionic composition and levels of bicarbonate, sodium, magnesium, calcium, potassium and phosphates.
2) Regulating plasma osmolarity through direct control of excreted ions and water.
3) Regulating plasma volume through controlling water excretion. Plasma volume affects total blood volume and therefore blood pressure.
4) Regulating plasma (pH) (acid-base balance) through controlling hydrogen ion concentration by the excretion of hydrogen ions and reabsorption of bicarbonate ions as required. The kidneys are joined by the lungs in keeping an optimal overall acid-base balance in the body.
5) Clearance of waste products (metabolites) and harmful substances from the plasma. Nitrogenous waste, primarily urea, is one of the most important excreted waste. Urea is generated in the liver as ammonia (amino acids metabolite) and carbon dioxide combine. Urea is much less toxic than ammonia, which is the reason why it is generated by the liver. Alongside urea, some ammonia, uric acid and creatinine are excreted in the urine as well. Uric acid is the product of nucleotides breakdown while creatinine is the product of breaking down of creatine phosphate produced by muscles. Accumulation of insoluble uric acid in the blood leads to the formation and deposition of crystals in the joints, a condition known as gout. Creatinine, not exclusively but mainly excreted by the kidney, is a toxic substance that is used as an indicator of renal dysfunction.

6) Hormones secretion. Beside the endocrine system, the release of hormones is also a function of the kidneys. Kidneys secrete renin, which leads to aldosterone secretion by the adrenal cortex of the adrenal glands seated on top of each kidney. Aldosterone induces the reabsorption of sodium (Na+) ions by the kidney. Moreover, erythropoietin is a hormone secreted by kidneys when the blood's oxygen carrying capacity is decreased, and it functions by stimulating red blood cells (erythrocytes) production. Kidneys also promotes the activation of Vitamin D absorption from the skin, which is required for the absorption of Calcium (Ca+) from the digestive tract.

1.2.2 The nephron

All of the kidney's excretory functions are executed by its functional unit, the nephron (Figure 4). It is named after the Greek word (nephrons) which means kidney. The main function of nephrons is blood filtration by excreting undesirable substances while reabsorbing required elements. Generally, the overall functions of the nephron include volume and pressure regulation as well as electrolyte and acid-base balance. The endocrine system controls nephrons function through a group of hormones including aldosterone, antidiuretic hormone and parathyroid hormone.

1.2.2.1 Structure and function

Blood supply of each nephron consists of two capillary regions (glomerulus) originating from the renal artery to supply the nephron. The primary composition of each nephron starts with the initial filtration part called the renal corpuscle, followed by the reabsorption and secretion part called the renal tubules. The latter receive water and small solutes from the renal corpuscle after

it is filtered out of the large solutes. Further modification of the passed solution is carried out by the renal tubules (Figure 3) [51].

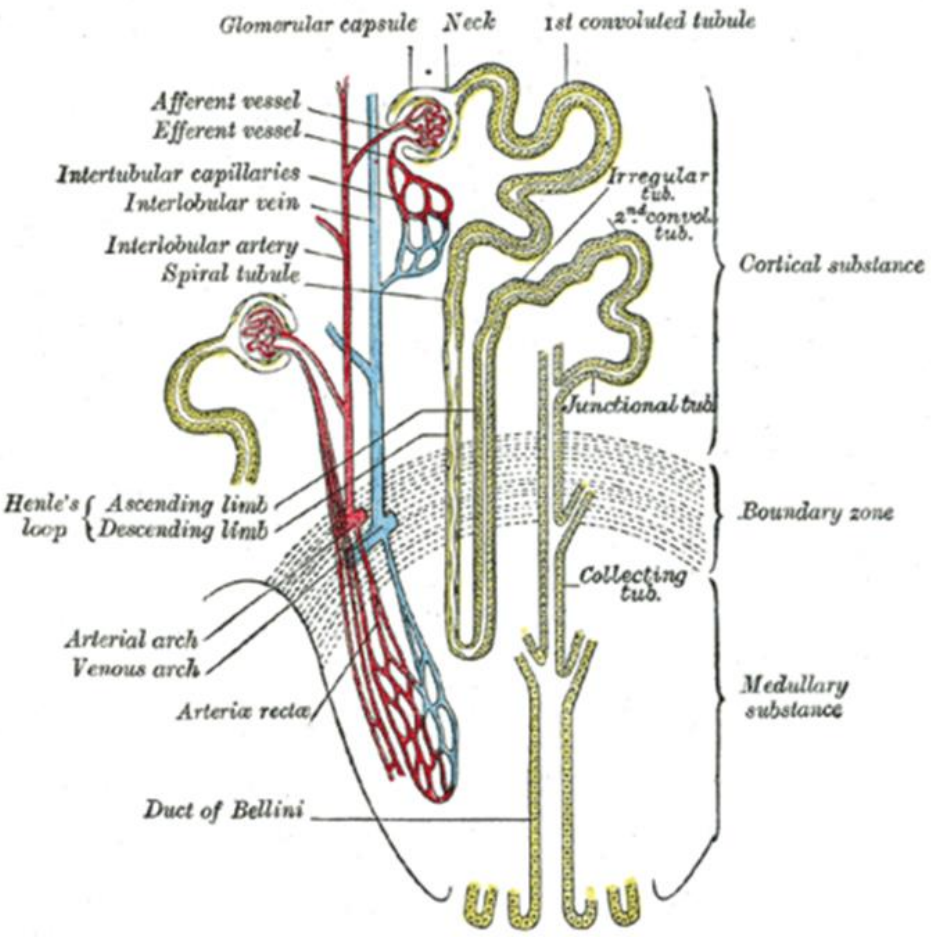


Figure 4- The stucture of the nephron [51].

- <u>The glomerulus:</u> It is a capillary tuft and is perfused with blood by an afferent arteriole branching from the renal arteries. The blood pressure of the glomerulus is the force that drives the fluid and solutes into the space between the glomerulus and the bowman's capsule to be filtered out of the blood. The efferent arteriole receives the remainder of the blood that was not filtered, and passes it on to the vasa recta where it meets with reabsorbed

substances. Vasa recta are capillaries that intertwine with convoluted tubules throughout the interstitial space. The fluid in the vasa recta is combined with efferent venules leaving other nephrons back to the renal vein and the main bloodstream.

- Afferent/Efferent Arterioles: Afferent arterioles carry blood into the glomerulus while efferent arterioles carry the blood out of the glomerulus. Juxtaglomerular cells are specialized cells surrounding the afferent arterioles as they enter the renal corpuscle. Other specialized cells called macula densa lie between the two arterioles. Together, macula densa and juxtaglomerular cells form the juxtaglomerular apparatus, the location of the formation and storage of the enzyme renin. Renin secretion is triggered by sympathetic stimulation of nerve receptors (beta-adrenergic) located on the juxtaglomerular apparatus, decreased afferent arterioles blood pressure and decreased sodium chloride levels in the distal convoluted tubule. Renin is required for Angiotensin I and Angiotensin II formation, both of which promote the release of aldosterone by the adrenal cortex.

- Glomerular Capsule or Bowman's Capsule: This tissue is made of two layers that encapsulate the glomerulus; an outer (parietal) layer and an inner (visceral) layer, both consisting of simple squamous epithelial cells. The visceral layer also consists of podocytes surrounding the glomerular thick basement membrane. These are cells with branching foot-shaped processes that extends over the length of the glomerulus creating a barrier through which blood is filtered into Bowman’s capsular space and from there to the renal tubules. Podocytes’ foot-like processes or projections run across the spaces between the processes of other podocytes to form filtration slits lined by diaphragms. Restriction in the filtration

system is caused by the small size of those slits which prevents large molecules from passing through and thus prevents their loss in urine. Examples of large molecules are platelets, albumin and red blood cells. Examples of small molecules that can pass through (roughly 30 kilodaltons or smaller) are water, amino acids, urea, sodium chloride (salt) and glucose. Additionally, another factor that contributes to restricted filtration is the glycocalyx coat secreted by the foot processes which is a negatively charged repelling negative-charged molecules such as albumin.

In summary, the glomerular ultrafiltration is achieved by three components: the glomerular thick basement membrane, the filtration slits diaphragm and podocytes' glycocalyx coat. In contrast, the visceral layer made up of only a single squamous epithelium layer does not play a role in filtration.

- Bowman's space or capsular space: This space occupies the area between the visceral and parietal layers. After the filtrate moves across podocyte slits, it enters this space and then runs through the proximal convoluted tubule. The renal corpuscle is the glomerulus and Bowman's capsule combined.

    - Proximal Tubule (PT): PT is composed of two anatomical regions, the proximal convoluted tubule and the proximal straight tubule. Histologically (base on cell's appearance), the proximal convoluted tubule is made of S1 and S2 segments and the proximal straight tubule, S3 segment. The nephron has only one region of simple cuboidal epithelial cells located in the lumen of the proximal convoluted tubule. This

single layer of cells possesses millions of microvilli which increase reabsorption surface area.

After the glomerular filtrate leave the capsular space into the proximal convoluted tubule surrounded by the peritubular capillaries, around two-thirds of the filtered water and salt are reabsorbed as well as all organic solutes (mostly amino acids and glucose). The Na+/K+ ATPase in the epithelial cells basement membrane causes the transport of sodium out the lumen into the blood, creating the driving force of the filtrate reabsorption through the non-selective tight junctions between the cells passing out large amounts of water and solutes. The osmotic properties of the fluid taken up by the proximal convoluted tubule are the same as that of the original filtrate, therefore, the absorption of the solutes is isotonic. Additionally, the reabsorption of amino acids, inorganic phosphate and glucose occurs by a secondary active transport via cotransport channels which drives out the sodium gradient from the nephron.

- <u>The loop of Henle:</u> This tissue is a U-shaped tube that receives the filtrate passing down the proximal convoluted tubule. The main role of it is to concentrate the salt in the surrounding tissue, the interstitium. The loop consists of a descending limb originating in the cortex and extending into the medulla, as well as an ascending limb leaving the medulla back to the cortex and leading its contents to the distal convoluted tubule.

  The descending and ascending limbs contrast in terms of their permeability creating a countercurrent exchange machinery in the loop. While the descending limb is permeable to water but not salt, the ascending limb is permeable to sodium but not water. The descending limb runs deep into the medullary hypertonic interstitium moving water out by osmosis and

thus indirectly contributing to the concentration at the interstitium. On the other hand, active pump at the ascending limb filters sodium out of the filtrate forming the hypertonicity of the interstitium and therefore prompting the counteract exchange by the descending limb until the filtrate and interstitium tonicity balance is reached. The filtrate grows hypotonic while passing up the ascending limb as it loses most of its sodium in the medullary interstitium. Next, this filtrate moves on to the distal convoluted tubule located in the renal cortex.

- Distal Convoluted Tubule (DCT): The structure and function of the distal convoluted tubule is similar to that of the proximal convoluted tubule. Active transport is carried out by the lining cells due to the numerous mitochondria present that provide the required energy by ATP. The endocrine system regulates the transport of the majority of ions.
  The parathyroid hormone promotes the reabsorption of calcium and the excretion of phosphate by the distal convoluted tubule and atrial natriuretic peptide induces more excretion of sodium. Moreover, hydrogen and ammonium are secreted by the tubule to regulate pH. At the end of the filtrate travel through the distal convoluted tubule, only 3% of the water is left and negligible salt content remains. It is estimated that most of the water in the glomerular filtrate join the convoluted tubules and collecting ducts via osmosis (~97%).

- Collecting ducts: The first segment of the collecting ducts is the connecting tubule which receives the filtrate coming from the convoluted tubule. Starting at the renal cortex, the collecting duct travels down the medulla where the interstitium is hypertonic due to the exchange machinery of the loop of Henle. Normally, the collecting ducts are impermeable to water, unless permeability is induced by the antidiuretic hormone (ADH) which can drive the

reabsorption of water by osmosis to up to 75%. ADH levels ,therefore, regulate the concentration or dilution of urine and are affected by conditions of dehydration and water sufficiency. In addition, the collecting duct is permeable to urea at its lower portion where some of it goes into the medulla playing a role in keeping the required hypertonicity of that interstitium. After urine travels down the medullary collecting ducts, it enters the renal papilla and passes on to the renal calyces emptying into the renal pelvis where it escapes the kidney to reach the bladder through the ureter.

1.2.2.2 Urine formation overview

The development of urine consists of three processes respectively: filtration, reabsorption and secretion.

1) Filtration: The blood flowing in the afferent arteriole to enter the glomerulus has both filterable (e.g. urea, water,salts "ions"..etc.) and non-filterable contents (e.g. albumin, blood cells, platelets). While filterable contents proceed to the inside of the glomerulus, non-filterable contents escape the filtration process as they exit in the efferent arteriole. At this point, filterable contents are in the form of a plasma-like fluid known as glomerular filtrate. Since most of the glomerular filtrate composition is reabsorbed while travelling down the renal tubules, the consistency of the filtrate is not the same as that of the urine.
2) Reabsorption: Ions and molecules reabsorption into the blood takes place within the peritubular capillary network surrounding the renal tubules. Compared to the glomerular filtrate, the blood osmolarity is increased due to the reabsorbed sodium chloride which leads water to pass out of the filtrate into the bloodstream. Other important filtrate contents that are also reabsorbed back to the blood are glucose and amino acids. At the renal tubules,

glomerular filtrate is divided into reabsorbed and non-reabsorbed filtrate, known as tubular fluid, that further travels to the collecting where it is processed to form the urine.

3) Secretion: Some of the urine substances (Creatinine, hydrogen and drugs) are cleared through the peritubular capillary network out of the blood and into the distal convoluted tubule or collecting duct.

In summary, urine is a collection of substances that were filtered at the glomerulus, not reabsorbed at the renal tubules and finally secreted by the nephron.

1.3 Chronic kidney disease (CKD)

1.3.1 Definition

In 2002, the National Kidney Foundation (NKF) Kidney Disease Outcomes Quality Initiative (KDOQI) introduced guidelines for the defining and classifying of chronic kidney disease (CKD), which received minor modifications and was adopted by the international guideline group Kidney Disease Improving Global Outcomes (KDIGO) in 2004 [52,53]. These CKD guidelines approved by the leading scientific organizations in this field, highlighted the consideration of the concept of kidney disease as a common chronic condition bearing a spectrum of severity that previously may had been viewed as an uncommon condition to be addressed by nephrologists. Rather, CKD is a common life-threatening condition that requires to be collectively tackled by general internists, early detection and better yet prevention strategies [54,55].

CKD is identified based on the level of kidney function and presence of kidney damage, regardless of the underlying cause or type of the disease.

Based on the definition by (Kidney Disease Improving Global Outcome, 2012) guidelines, CKD is defined as the presence of kidney damage for ≥3 months, indicated by:

1) Structural or functional abnormalities of the kidney (markers of kidney damage), with or without decreased glomerular filtration rate (GFR), including;

    Proteinuria or Albuminuria (protein or Albumin in urine)

    Urine sediment abnormalities

    Electrolyte and other abnormalities due to tubular disrorders

    Histological abnormalities

    Structural abnormalities detected by imaging

    History of kidney transplant

    Or

2) Glomerular filtration rate (GFR) <60 mL/min/1.73m2 for a duration of ≥3 months, with or without kidney damage.

Another definition of CKD is progressive loss of renal function over time based on declining values of GFR and creatinine clearance. The diagnosis of CKD is indicated as [57]:

1. Kidney function decline for 3 months or more

   AND

2. Evidence of kidney damage (e.g. albuminuria or abnormal biopsy) OR GFR <60 mL/min/1.73 m2.

1.3.2 Staging

The intention of CKD staging system is primarily to provide physicians with assistance in the management of the disease by identifying patients with:

- Severe condition (at later disease stages) who are, therefore, at greatest risk for rapid progression and complications.
- Less severe condition (at earlier disease stages) who could be recognized and controlled to prevent progression to later stages.

Staging systems for CKD use multiple methods based on the cause, GFR and albuminuria or proteinuria (albumin or protein in urine). This facilitates a more comprehensive risk description for the major complications of CKD [52,53].

-GFR: Since GFR is considered an indicator of overall kidney function, it is viewed as the hallmark of declining kidney function [57]. The most commonly approved threshold that defines a decreased GFR is less than 60 mL/min per 1.73 m, on the basis of creatinine clearance values in healthy people and in kidney disease patients. Direct GFR measurement using exogenous filtration markers clearance such as inulin or iothalamate [58] are impractical and difficult to carry out. Clinically, typical measurements of GFR use estimated GFR (eGFR) from and endogenous filtration marker, serum creatinine concentration.

In healthy individuals, there is a variability in eGFR measurements inflicted by factors such as dietary protein intake, age, sex and possibly by race-ethnicity. GFR can be affected by multiple

conditions including arterial pressure, afferent and efferent arteriole constriction. Also, GFR can be affected by plasma protein concentration and colloidal osmotic pressure [59].

-Albuminuria (albumin loss in urine): Albuminuria is a type of proteinuria (loss of plasma proteins in urine). Albumin is a large plasma protein that can be normally found in small amounts in the urine. Large amount of albumin in the urine indicates kidney disease. Regardless of the etiology or the cause of an injury to the kidney filtration unit, the nephron, the resulting loss of nephrons and/or hemodynamic effects lead to increased glomerular permeability that translates in the loss of plasma proteins such as Albumin in the urine. Further details of the underlying mechanism of proteinuria are illustrated in (section 1.3.4 as described below).

The critical role of proteinuria in causing accelerated progression of renal dysfunction to end-stage kidney disease has been proven by consistent scientific evidence. Pathways through which proteinuria carries out its deleterious effect on the kidney include complement activation and induction of tubular chemokine expression. In response, the renal interstitium is infiltrated with inflammatory cells where sustained fibrogenesis take place. Not only proteinuria is widely considered as a marker of CKD severity, but it is also considered as a prediction tool of future GFR decline making it a sensitive renal function parameter. Nevertheless, in diabetic and non-diabetic CKD patients, decreasing proteinuria level is invariably reflected as protection from further decline in renal function [60].

One method to quantify proteinuria is by collecting a 24-hour urine volume, measuring a sample from that volume and multiplying that value by the total volume collected. Estimating

albuminuria is done by a urine dipstick test which can measure a rough approximation value. This test uses bromphenol blue agent which is specific to albumin, the most dominant protein in the plasma. Despite the multiple measurement methods of albuminuria, the "spot" untimed urine measurement of the albumin-to-creatinine ratio (ACR) is advantaged. Generally, the threshold widely accepted for abnormally increased ACR is 30 mg/g (3.4 mg/mol) or greater [61, 62]. Clinically, the most common assessed marker of kidney damage is albuminuria. Due to albumin's large size, albumin loss in urine reflects increased macromolecule glomerular permeability [63]. Specifically, albuminuria may reflect generalized endothelial dysfunction such as the case in diabetes, hypertension, dyslipidemias, obesity and smoking. All of which are components and risk factors of metabolic syndrome [61,62].

It has been indicated that albumin to creatinine ratio (ACR) is not an adequate source from which total proteinuria can be predicted properly and results from the literature suggest to be careful using ACR with non-diabetic CKD patients. As for the prediction of clinically relevant proteinuria, some evidence indicate that total protein to creatinine ratio (TPCR or PCR) is more sensitive as a screening test than albumin to creatinine ratio (ACR) [64]. Using either diagnostic tests, the age and gender should be considered when interpreting results as these factors can cause variability in the measured values [64].

<u>The GFR staging system is based on the original CKD classification scheme as [65]:</u>

Stage 1: GFR > 90 mL/min per 1.73 m^2 → Kidney damage with normal or increased GFR

Stage 2: GFR 60 to 89 mL/min per 1.73 m^2 → Kidney damage with mild decrease in GFR

Stage 3a: GFR 45 to 59 mL/min per 1.73 m^2 → Moderate decrease in GFR

Stage 3b: GFR 30 to 44 mL/min per 1.73 m² → Severe decrease in GFR

Stage 4: GFR 15 to 29 mL/min per 1.73 m² → Severe decrease in GFR

Stage 5: GFR < 15 mL/min per 1.73 m or treatment by dialysis → Kidney failure

The albumin to creatinine (ACR) staging system is based on the familiar definitions of normal, moderate and severe proteinuria [65]:

Stage 1: ACR < 30 mg/g (<3.4 mg/mmol)

Stage 2: 30 to 299 mg/g (3.4 to 34.0 mg/mmol)

Stage 3: ACR ≥ 300 mg/g (>34.0 mg/mmol)

1.3.3 CKD in rats

The use of animal models in studying human diseases has been of tremendous value. Fortunately, not only some species share similar anatomical and histological characteristics with humans but also those species can be utilized to reproduce and simulate human diseases pharmacologically, surgically and genetically. At present, experimental studies remain the best evidence provider for diseases etiology through which clinical science and treatments are developed. It is commonly not possible, unethical and too risky to use human subjects in experimentation which creates the urgency of utilizing animal models that are crucial to the advancement in significant scientific and medical knowledge of diseases. For the replication of human diseases, a number of different animal models may be used. The selection of the most appropriate species or model is dependent on experiment requirements and goals [66].

Details of the justifications of our choice of animal model for this study are found in the chapter of methods and materials (section 2.1). Since rats were chosen to be our experimental model of MetS with renal dysfunction, it is relevant and informative to refer to the parameters of progressive kidney disease in rats from the literature.

In this study, the animal model for metabolic syndrome with kidney disease was obese ZSF-1 rat that were around 8 weeks at the start of the in vivo study. According to the literature, the baseline ranges for renal functions in these rats at that age include [67]:

- Urine volume (ml/kg/day) → 164.8 ± 14.5
- Proteinuria (mg/kg/day) → 307 ± 23
- Protein to creatinine ratio (PCR) → 5.98 ± 0.41

1.3.4 Pathophysiology

Two main components of MetS, diabetes and hypertension are as well the most common causes of CKD, respectively. Other causes of CKD include renal pathologies such as glomerulonephritides, interstitial nephritis and pyelonephritis and obstructive nephropathy. Also, untreated acute kidney injury (AKI) can lead to CKD. Examples of AKI causes include medications, infections and heavy metal toxicity (e.g. lead) [68-69]. Most importantly, once there is an injury to the kidney leading to the loss of its functional unit, the nephron, progression of the dysfunction cascade is similar regardless of the etiology or the cause behind it (Figure 5) [56]. Initially, loss of injured nephrons in the kidney causes increased glomerular filtration by other functional nephrons as an adaptive compensatory mechanism. This results in increased glomerular permeability to contents of the blood including plasma proteins leading to the loss of

those proteins. Elevated protein levels in the urine, a condition known as proteinuria, is an early sign of renal dysfunction and kidney disease. At later stages, persisting renal dysfunction leads to decreased GFR and urine output [56].

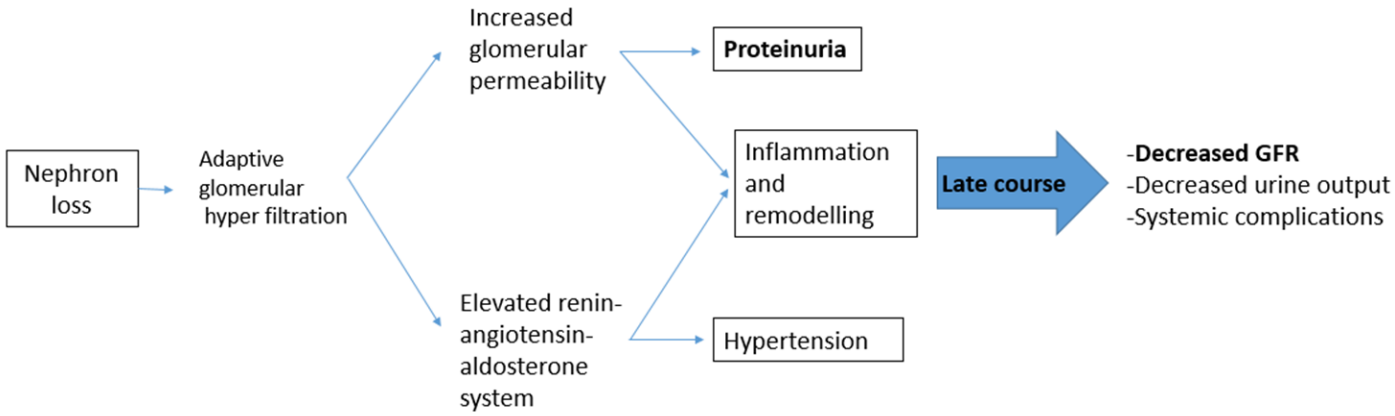


Figure 5- Pathophysiology background of Chronic kidney disease [56].

1.3.5 CKD association with metabolic syndrome

The chronic condition of MetS is known as a selection of prevalent risk factors that are related to cardiovascular disease (CVD). Clearly, the somewhat ambiguous definition of MetS does not obscure the view of its strong association with CKD markers such as proteinuria/albuminuria, decreased GFR and hitstopathological changes (e.g. tubular atrophy) [57]. The sequential details of the relationship between MetS and CKD however remain not thoroughly understood. The complexity and bidirectional nature of their relationship leave room for interpretations. The most comprehensive interpretation is viewed as while both conditions are common chronic diseases, MetS may be an intervening contributor along the course of CKD [86].

Both MetS and CKD share etiological backgrounds in terms of their risk factors and components. It has been indicated that the components of MetS are not only risk factors for CVD but also for CKD down the course of the disease. Moreover, these risk factors (hyperglycemia, hypertension and dyslipidemia) are associated with increased incidence and progression of CKD [70-72]. It has been clinically observed that MetS patients carry a higher risk of CVD at all CKD stages starting at early kidney insufficiency and all the way to end-stage kidney disease or renal failure, increasing physicians' and researchers' attention towards the association of MetS with CKD [73-75]. The risk of MetS in CKD progression has been identified by multiple population-based studies. At CKD stage 3 or 4, MetS presence was related to a high hazard ratio for end-stage renal disease (ESRD) over just a 2-3 year follow up period in a cohort study of more than 15000 patients. Particularly, elevated blood pressure, hyperglycemia and hypertriglyceridemia are were identified to be related to increased risk of ESRD [76].

Important renal associations with MetS have been described by many studies. Those associations include proteinuria and/or microalbuminuria [73,77,78,79,80], eGFR [75,81], ultrasound abnormalities (increased intra-renal resistive indices) [82] and histopathological changes (tubular dilation, arterial sclerosis, interstitial fibrosis) [83].

Capturing MetS and CKD relationship from a mechanistic point of view is more convincing than just the association between them. Hence, the search for start points that lead MetS to CKD or vice versa is encouraged. It is suggested in the literature the possibility that it is a one linear mechanism where MetS and CKD are parallel to each other and therefore co-existing. Another

possibility is that the mechanisms are multiple distinct yet inter-connected and displayed by MetS while functioning simultaneously to result in significant renal dysfunction [84]. Although maybe less likely in this context, the speculation that two widely common chronic diseases may co-exist by chance is not excluded. It could be a “perfect recipe” of risk factors such as hypertension, insulin resistance, dyslipidemia and inflammation of which the production is the increased expression of fibrotic factor [84]. According to the literature, a described mechanism of renal disease in metabolic syndrome indicate that insulin resistance alongside the release of inflammatory cytokines result in impaired podocytes, mesangial expansion inside the glomeruli, basement membrane thickening and decreased slit diaphragm integrity [85]. In addition, factors further contributing to renal dysfunction include oxidative stress endothelial dysfunction, activated renin-angiotensin-aldosterone system (RAAS) and increased plasminogen-activator-inhibitor-1 (PAI-1). All of these changes eventually lead to tubule-interstitial injury and glomerulosclerosis [85]. Another pointer to the close relationship between MetS and CKD is that renal functions parameters were included in the proposed diagnostic criteria of MetS in 1998 [9].

Whether both conditions share similar causative factors or the mechanistic processes of one lead to the other, the accumulating indications of the association between MetS and CKD are apparently concerning and further investigations are needed to better understand the details of that relationship. At present, it is supported by most studies that the relationship is directed from MetS to CKD and not the opposite, yet this still needs to be confirmed [86].

1.3.6 Epidemiology: incidence and prevalence of MetS-related CKD

Despite the multiple estimated prevalence of MetS, IDF has concluded a general estimate stating that one quarter of the world's adult population is diagnosed with MetS [13]. MetS cases have become increasingly common [87-89], unfortunately not only in adults but also in adolescents [90].

In addition, epidemiologic studies support the link between MetS and an increased risk of the early indicator of renal injury and endothelial dysfunction, microalbuminuria [91–95]. Hoehner et al. conducted a cross-sectional survey where a non-diabetic population was controlled for demographic and social comorbidity factors, demonstrating an 80-130% more likelihood of developing microalbuminuria in patients with 2 or 3 components of MetS, respectively, compared with those without MetS [92]. Individual components of MetS were all described to exhibit a hazard ratio for end stage renal disease (ESDR) and all-cause mortality, of which hypertension showed the highest hazard ratio followed by hyperglycemia. In fact, the incidence (over 4 years) of ESDR in individuals with or without metabolic syndrome was also described in the same previous study demonstrating a much higher incidence rate of ESDR with metabolic syndrome (p-value= <0.001) [96].

In summary, CKD is a global health condition with enrollment of ESRD patients in Medicare-funded program reaching up to 10,000 benefeciaries in 1973 and increasing to 661,648 as of 2013 [97,98], which creates financial burdens that cannot be dismissed. Previous clinical under-recognition of early stages of CKD or populations demographic changes could partially explain the growing number of ESRD, however, the exact reason is unknown [99-102]. Despite the

growing amount of financial and medical resources serving the management of ESRD and the substantial improvements in the quality of dialysis, the mortality and morbidity rates among these patients remain significant.

1.3.7 CKD manifestations

CKD is known as an initially silent condition where clinical manifestations are usually under-reported at its early stages. Whenever the signs and symptoms of CKD are present, they appear to be non-specific and poorly indicative of the severity of the condition. Additionally, typical manifestations of uremia are mostly not reported in early stages (stages 1-3), instead, those signs and symptoms develop and start erupting only at later stages [103].

-Examples of signs and symptoms of early stage CKD include: edema, pale skin, weakness, increased blood pressure, decreased appetite, nausea, urinary symptoms (polyuria, nocturia), blood in urine, foam or bubbles in urine, pain in the loin region [103].

-Examples of signs and symptoms of late stage CKD include [103]:

1) General symptoms (fatigue, edema, uremic fetor, decreased mental acuity).

2) Skin (sallow appearance, pruritic excoriations, uremic frost).

3) Cardiovascular (congestive heart failure).

4) Pulmonary (dyspnea, pulmonary edema).

5) Gastrointestinal (nausea and vomiting, stomatitis, unpleasant taste in the mouth).

6) Endocrine-metabolic (amenorrhea, impotence).

7) Neuromuscular (muscular cramps, peripheral sensory and motor neuropathies, hyperreflexia, encephalopathy, seizures, coma).

8) Hematologic (anemia).

1.3.8 Management

To be able to properly evaluate and address renal dysfunction, nephrologists closely monitor the disease progression and plan an adequate management strategy accordingly. An appropriate assessment of renal function in patients should be undergone through estimating GFR and additional evaluation of other kidney function parameters considered in the definition of CKD including proteinuria, kidney ultrasound for structural deformities and renal biopsy for histological analysis. Typically, the referral to a nephrologist should be considered if the albumin to creatinine ratio is (>300 mg/g), if there are red blood casts in the urine and if there is a rapid declining renal function [104].

The scope of this study's interest is in regards to the prevention and slowing down of CKD early stages progression in patients with MetS by assessing a potential dietary recommendation. Therefore, the background knowledge of the overview CKD management goals is rather more relevant to this introduction than specific pharmacological therapies targeting individual CKD risk factors or complications. The clinical management actions required in the stages of CKD is presented in (Figure 6) [105].

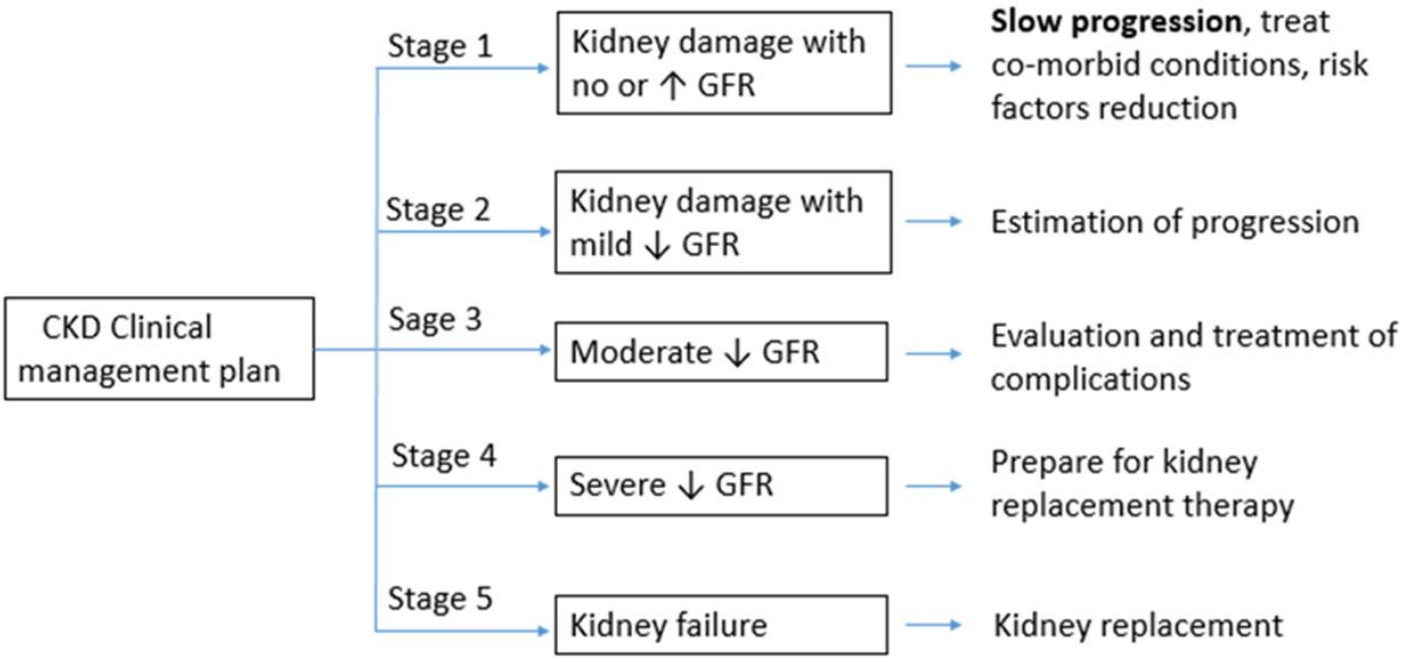


Figure 6- Management action plan for chronic kidney disease [105].

By the time serum creatinine shows abnormally increased level and subsequent abnormal eGFR value is detected, it has been estimated that 50% or more of the kidney function had already been lost [106]. Inversely, early detection of CKD with appropriate management may reduce the otherwise foreseeable renal function deterioration or better yet reverse it [107]. This sheds the light on the tremendous impact of early stages management main directions to slowing down the progression of the disease (Figure 6) highlighting the importance of prevention strategies that include lifestyle modifications and dietary recommendations.

1.4 Oxidative stress

1.4.1 Background

Oxygen molecule possesses a unique characteristic, known as reactivity, that has both a positive benefit to biological systems and a negative side effect that could damage it. Reactivity is a chemical property that gives oxygen the ability to participate in transferring high-energy electrons. Through oxidative phosphorylation, this reactivity results in generating large amounts of adenosine-5-triphosphate (ATP) inside the mitochondria. Reactive oxygen species (ROS) serve as second messengers in many intracellular signaling events that target the maintenance of the cell's homeostasis with its surrounding environment. Even though the reactivity of oxygen is important for a multicellular organism evolution, it is the cause of oxygen liability to harm biological molecules within that organism such as proteins, lipids or DNA [108]. High levels of ROS can lead to unbiased damage to biological molecules, leading to loss of cellular functions or even cellular death. As a result, the body undergoes a state of continuous oxidative stress inflicted by ROS. Consequently, antioxidant complex defense systems exist in order to function as an opposing force keeping oxidative stress influence balanced. However, this defense system can be disrupted on occasions resulting in unopposed overwhelming oxidative stress [109].

1.4.2 Definition

Due to the variety and diversity of the effects of oxygen toxicity on the cells, oxidative stress is ought to be defined in generally inclusive terms as disturbances in the pro-oxidant-antioxidant balance favoring the pro-oxidant state and leading to potential damage to the body [109]. In fact, oxidative stress is now considered as a key player in the background mechanisms of numerous

and various conditions, including metabolic syndrome [16] and its systemic effects such as CVD and CKD [119-122].

The reasons behind the importance of the concept of the pro-oxidant–antioxidant balance in understanding oxidative stress include three major considerations. First, it clarifies that disrupted pro-oxidant-antioxidant balance may be a result of changes on either opposing components of the equilibrium such as abnormally elevated ROS levels or insufficiency in the antioxidant defenses. Second, it emphasizes the homeostatic levels of ROS. Third, the balance concept highlights that in cases of oxidative stress, a graded response takes place. This explains the likely development of homeostatic reactions at several levels in response to minor disturbances in the surrounding environment. In contrast, irreversible injury and cell death may be the response to more major disturbances. This makes the boundary separating pathological insults from normal physiological changes hardly distinguishable. Therefore, the provided definition of oxidative stress is necessarily broad and its outcome partially depending on the cellular environment in which ROS are produced [109].

1.4.3 Reactive oxygen species (ROS)

ROS terminology is used to describe both free radicals as well as their non-radical intermediates. The definition of free radicals is species that possess one or more unpaired electrons conferring their high reactivity. In biological systems, oxygen and nitrogen are the most important elements generating free radicals (Figure 7).

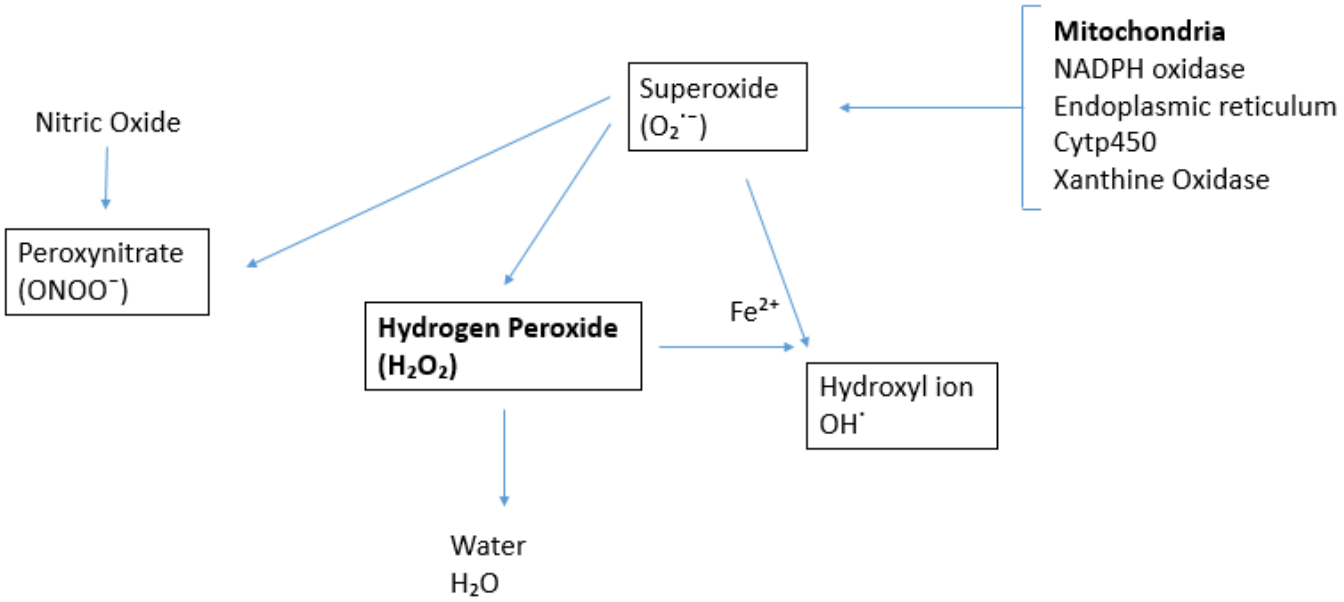


Figure 7- Reactive oxygen species (ROS).

Physiologically, superoxide anion (O2•−) is the most common free oxygen radical which is mainly produced by the mitochondria [110]. Leakage of electrons to oxygen leads to the formation of O2•−, when transferring electrons along the enzymes of the respiratory chain in the mitochondria is insufficient. Thus, the rate of O2•− formation depends on the number of electrons present on the chain, and, therefore, is increased in conditions of elevated oxygen levels as well as elevated glucose, as in diabetes [110].

Normally, 2% of consumed oxygen is transformed to O2•− in the mitochondria and remains within it because of its charge, which renders it membrane impermeable [110].

1.4.3.1 Hydrogen peroxide (H2O2)

Superoxide dismutase (SOD) enzymes detoxify O2•− to hydrogen peroxide (H2O2). Although H2O2 is not a free radical and thus its reactivity is less than that of O2•−, it is included under the

ROS term since it is closely involved in the generation of free radicals. Then, enzymes catalase and glutathione function to detoxify H2O2 to water. Unlike O2•−, H2O2 is membrane permeable because of its non-polarity. It can diffuse through the membranes of the cells and its organelles serving broadly as a second messenger in signaling cascades. The synchronicity of antioxidant enzymes (i.e. SOD and catalase) functions is necessary to maintain balanced O2•− concentrations, which if disturbed, could result in generating the far more notorious hydroxyl ion OH•. Free ferrous ions are responsible for catalyzing this reaction. The hydroxyl ion acts in the immediately surrounding environment reacting with any biological molecules with a lifespan estimated to be 9-10 seconds [109]. There is no recognized scavenger of OH• due to its high reactivity.

It is well established that oxidation is a vital process in living organisms despite the deleterious effects of its excessive amounts. These effects involve protein, lipid and DNA oxidation and can promote the injury and death of cells [111]. Beside the effects of the oxidation of biological molecules, oxidative stress can induce neuro-inflammatory reactions [112], increase intracellular Ca2+ levels [113] and promote apoptotic pathways. When these processes take place, H2O2 is produced in large amounts by different types of cells. Owing to its high permeability to cellular membranes, H2O2 is not only toxic to the generating cells but also to the surrounding ones. Hence, H2O2 is commonly used as an oxidant in the purpose of mimicking oxidative stress-induced injury in cell culture (in vitro) [114]. In addition, it has been shown by many studies that the drive of H2O2-induced oxidative is triggered mitochondrial dysfunction associated with the changes of apoptosis cascade members of Bcl-2 family proteins, cytochrome C release and caspases activation [115]. Through activation of transcription factors sensitive to oxidation such

as activator protein-1 (AP-1) and nuclear factor-κappaB (NF-κB), mitogen-activated protein kinases (MAPKs) which regulate signaling events related to cell growth and apoptosis [116] can alter cellular signaling in the nucleus [117]. Therefore, in regards to treatment of oxidative stress-induced diseases, designing therapeutics that function through strategies of inhibiting ROS-induced apoptosis could be an effective strategy in the prevention of these conditions.

1.4.4 Oxidative stress in chronic diseases: pathophysiology

While aging, it is expected to find accumulation of progressive adverse changes throughout the body as a production of prolonged free radical reactions. In fact, it has been postulated that aging-induced changes are a result of processes in which oxidative stress play a central role [118]. These changes are often considered normal in the correlating age of the body. However, they are not alone as there are superimposed various environmental and genetic patterns which modulate free radical injury. These are factors that play a central role in determining the manifestations of diseases at certain ages including atherosclerosis and cancer, two silent free-radical related diseases that are major causes of increased morbidity [118]. Furthermore, oxidative stress is believed to be a culprit on tremendously wide spectrum of diseases. It has been distinguished as a significant contributor to all ischemic diseases (e.g. heart diseases, stroke) inflammatory disorders (e.g. vasculitis, lupus erythematous, arthritis, glomerulonephritis, adult respiratory diseases syndrome), neurological disorder (e.g. Parkinson's disease, Alzheimer's disease, muscular dystrophy) as well as gastric ulcers, emphysema, hemochromatosis, hypertension and preeclampsia, acquired immunodeficiency syndrome, smoking-related diseases, and many others [118]. The structure and function of proteins, lipids and other biological molecules can be disturbed in the presence of high oxidative stress levels as they

become oxidized leading to all sorts of disorders. For example, studies have indicated that the injury of the endothelial cells lining the blood vessels is caused partially by oxidation of diet-derived lipids by free radicals releasing peroxides and other damaging substances that lead to the atherosclerotic changes, the hallmark of cardiovascular diseases [118].

1.4.4.1 Oxidative stress in MetS and CKD

ROS are by-products of oxygen metabolism. They play vital roles in various physiological processes including signal transduction and gene expression. In MetS, the unbalanced oxidative stress is developed by elevated oxidant capacity as well as lowered antioxidant capacity. In a healthy state unlike MetS, the balanced oxidative state is largely regulated by enzymatic activities of (e.g. glutathione, SOD and catalase) and non-enzymatic antioxidants. Thus, the pathophysiology underlying MetS progression and its components involve increased oxidative stress as a unifying key factor. As in other chronic diseases, oxidized lipids, proteins and DNA as well as induced pro-apoptotic pathways are mechanisms by which elevated ROS damage cellular structure and function in MetS and its systemic effects including micro- and macrovascular complications, consequent CVD and CKD [119– 122]. In fact, endothelia dysfunction is involved throughout the whole course of MetS not only as complication of MetS, but also as the channel through which oxidative stress plays its key role in the underlying pathophysiology of MetS, as illustrated in the outline of the fundamental players in MetS causative mechanism (Fig. 1) [16].

It has been hypothesized that what occurs in muscle and fat cells takes place as well in other cells, in specific, beta-cells in the pancreas and endothelial cells. These cells are believed to be

influenced by increased feeding and do not depend on insulin for glucose uptake but on facilitated diffusion [123]. This means that they are not able to downregulate the overflow of nutrients through insulin resistance in cases of increased food supplements. Instead, they are prone to a directly proportional increase of intracellular concentrations of nutrients to the influx of nutrition. Thus, it has been suggested by many studies that chronic subjection of beta cells to increased FFA levels and/or glucose levels result in cellular dysfunction [123]. Hence, the hypothesis of response-to-injury regarding atherosclerosis indicates that the original damage to endothelial cells of the arteries lead to the dysfunctional arterial endothelium [124]. Moreover, current evidence confirms the association of oxidative stress to endothelial dysfunction that predicts cardiovascular disease [125, 126]. As illustrated in (Fig. 1), co-existing of genetic risk factors such as thrifty genotype and environmental risk factors such as smoking, hyperplasia and hypertrophy of adipose tissue take place leading to altered FFA metabolism and released adipokines. These events cause rising levels of oxidative stress which in turn causes damage to the endothelial cells lining the blood vessels and subsequently micro- and macrovascular dysfunction occur. Downstream systemic events including pro-inflammatory, prothrombotic and hypercoagulable states are key elements of atherosclerotic changes that are largely triggered or exacerbated by oxidative stress in MetS leading to its systemic complications such as CVD and CKD [16].

Regarding renal dysfunction, elevated oxidative stress is considered one of the mechanisms of renal injury in CKD alongside overproduction of proinflammatory cytokine and fibrotic factor, increased connective tissue growth, increased microvascular injury and renal ischemia [127]. A recent comprehensive review of MetS published in the World Journal of Nephrology in 2014

[16] demonstrated a list of the recognized causative factors leading to CKD associated with MetS, identifying oxidative stress as one of these causes [128,129]. Other causes include glomerular expansion and podocyte hypertrophy [130], increased pro-inflammatory cytokines [131] connective tissue overgrowth and/or fibrosis factors [132,133-135], triglyceride- and free-fatty acid induced injury [136] ischemia and microvascular injury [137,138] and hyperuricemia [139,140].

In conclusion, a puzzle of complex interplaying evidence-based factors suggest that overproduction of ROS may be described as a key element in the development of MetS components such as diabetes and complications such as CVD and CKD [141].

1.5 Antioxidants

1.5.1 Background

Originally, the description that was used to refer to an antioxidant was an oxygen consumption preventing chemical. In early biological research on the function of antioxidants, the emphasis was on their potential in inhibiting the oxidation of unsaturated lipids that lead to organic decomposition [142]. By simply measuring the rate of oxygen consumption in a closed chamber containing fat and oxygen, antioxidants activity could be measured. The recognition of vitamins (A,C and E) as antioxidants, however, was a revolutionary discovery in biology that has shaped the realization of antioxidants vitality in living organisms biochemistry [143,144]. The exploration of the possible mechanisms of antioxidants functions was initiated upon the recognition that substances are capable of conducting antioxidant activity, and that those substances are likely to be readily oxidized themselves [145]. Scientific investigations on the mechanisms of lipid peroxidation prevention by vitamin E has set the path to the recognition of

antioxidants characteristic as reducing agents that inhibit oxidative reactions commonly through ROS scavenging [146].

1.5.2 Definition

An antioxidant is defined as a stable molecule capable of donating an electron and neutralizing an active free radical reducing its capacity to cause damage. Scavenging of free radicals is a main feature that antioxidants exhibit to prevent or delay cellular injury [147]. Antioxidants are low-molecular-weight substances that are able to safely interfere with free radicals and arrest the chain reaction before damage is inflicted on valuable molecules. Glutatione and ubiquinol are examples of enzymatic antioxidants generated in the body during normal metabolism [148]. In addition, lighter non-enzymatic antioxidants present in dietary sources include vitamin C (ascorbic acid), vitamin E (α-tocopherol), and β-carotene. These micronutrients are the chief antioxidants in the body although several ROS scavenging enzymes are present [149]. Non-enzymatic antioxidants must be supplied in the diet as they are not produced by the body.

1.5.3 Antioxidant defenses: mechanism of action

ROS is detoxified by both enzymatic and non-enzymatic antioxidants existing in both intracellular and extracellular environments. They serve through numerous activities such as radical scavenging, peroxide decomposer, electron donor, hydrogen donation, metal-chelating, enzyme inhibition, gene expression regulation and many other functions [150,151]. It has been proposed that antioxidants function by two major mechanisms: the chain-arresting mechanism through antioxidant donation of an electron to a free radical, and the elimination of ROS inducers through quenching chain- induction catalyst [152].

There are four levels at which antioxidant defense system operates; preventive, radical scavenging, repair and adaptation [153]:

1) The first line is the preventive antioxidants defense that suppresses the development of free radicals. Even though it is not well described yet how and where exactly free radicals are formed in the body, H2O2 and hydroperoxides metal-induced derivatives must be some of the important sources. An example of antioxidants acting at this level is glutathione peroxidase.
2) The second line is the ROS scavenging antioxidants defense that suppresses oxidant chain initiation and/or disrupt the progression of pro-oxidant chain reactions. Examples of antioxidants acting at this level are vitamins such as vitamin E.`
3) The third line is the repair and de novo antioxidants defense that serves to identify, break down, and eliminate oxidized proteins preventing their accumulation as well as DNA repair. Various kinds of enzymes that can repair the damaged DNA are identified. Among antioxidants acting at this level are proteolytic enzymes and proteases found in the mitochondria and cytosol of mammalian cells. An example of DNA repair enzymes are glycosylases and nucleases.
4) The final line is the adaptation. Signals that act to cause free radicals to be produced also activate synchronicity to induce the appropriate antioxidants formation and transportation to the proper site.

1.5.4 Dietary antioxidants

1.5.4.1 Background: the French paradox

In 1986, the term “French Paradox” was introduced for the first time by the newsletter “The Letter” of the International Organization of Vine and Wine. Next in 1989, this term was used again by Professor George Riley Kernodle of the University of Arkansas in a chapter of his book “Theatre In History” which was separately republished as an academic paper [154]. Dr. Serge Renaud from Bordeaux University in France, who is considered now the father of the “French Paradox” phrase exhibited his scientific results in 1991 from his investigation into the actual data behind the term [155]. This was further presented to the world as it was broadcasted as a public documentary on the American CBS News television program, 60 Minutes. This phrase basically describes the apparent paradoxical findings of an epidemiological observation suggesting that the French population show relatively low rate of coronary heart disease (CHD) despite their consumption of a diet relatively rich in saturated fats [156], which apparently contradicts the vastly adopted belief that the consumption of such diet is a risk factor for CHD. In the case of the association of saturated fat with CHD being valid, it would be expected that the French people show higher incidence of CHD in comparison with other populations that have lower consumption levels of such fatty diet. Consequently, two possibilities may be implied from the French Paradox; the first possibility is a speculated validity of the fats relationship with CHD and the second possibility is the involvement of additional factors (e.g. diet or lifestyle) that modify the outcome of that relationship. Therefore, the latter possibility may suggest that if those modifying factors can be recognized and implemented (e.g. dietary or lifestyle modifications) in other populations, the expected outcome would be similar to that of the French population, which is lower rates of CHD. In addition, there were speculations regarding the validity of the French

Paradox conclusions in terms of data collection and other factors that could have affected the interpretation of results [157]. The scientific dilemmas that have emerged from that epidemiological paradoxical observation lead to the generation of not only substantial media attention, but also scientific interest in further investigating the matter.

The link of the French Paradox to the rationale of this study is the suggestions that has resulted from studies attempting to solve the paradox, proposing that red wine consumption is a principle factor in the French equation that leads to the observed lower rate of CHD. Again, this hypothesis was explained in a broadcast by the "60 Minutes" television program in 1991. This has led to not only a large increase in the demand for red wines in North America but also a large increase in the scientific attention on antioxidants research, particularly grape antioxidants. A group of antioxidants (polyphenols), oligomeric procyanidins, is believed to exert a strong protective effect on human vascular cells and was identified by Professor Roger Corder and his team. Interestingly, a greatest concentration of these antioxidants tested from 165 wines was found in European red wines of particular regions correlating with observed longevity in those regions [158]. Moreover, it was indicated that polyphenols present in wine decrease malondialdehyde absorption found implicated in diabetes, atherosclerosis, cancer and other diseases.

1.5.4.2 Grape antioxidants health effects

Since it has been widely demonstrated that dietary patterns could play a vital role in the prevention of chronic diseases, the focus of a healthy eating plan presented by the 2010 Dietary Guidelines for Americans is the consumption of fruit and vegetables. Biological protective

effects in chronic diseases were exerted by a broad spectrum of phytochemicals such as phenolics, isoflavonoids, flavonoids, ascorbic acid and many other antioxidants, all of which are contained in fruits and vegetables. One of the most widely cultivated and popularly consumed fruits in the world, grapes, are rich in antioxidants (phytochemicals). Grapes contain a various group of powerful phytochemicals that mainly include phenolic acids, anthocyanins, proanthocyanidins, proanthocyanidins and stilbenes. The composition of phytochemicals differs largely among the grape varieties [159].

Regarding the mechanistic background of the beneficial health effects of the grape, many studies have revealed underlying processes by which the antioxidant activities of the grape are exerted. Some of these antioxidants effects and involved mechanisms that have been published include:

1) decreasing the oxidated LDL in plasma (grape seeds) [160].
2) oxidative stress reduction in serum (grape juice) [161].
3) Protection against aortic fatty streak accumulation in hypercholestremic hamsters (wine) [162].
4) protection against H2O2-induced oxidation of Saccharomyces cerevisiae membrane (wine) [163].

In a clinical context, a review study on grape antioxidants health effects has documented findings from many studies over the past decade that indicate grape polyphenols numerous protective effects in various chronic conditions [164]. In fact, these conditions are components and risk factors of MetS. Grape health effects, listed below, are findings from in vivo as well as clinical studies using various types of grape products:

1) Hypertension: - Decreased renin-angiotensin enzyme activity (wines), 2003 [165].

- Decrease systemic blood pressure in rats (red wine), 2001 [166].

- Improved aortic elasticity and blood pressure in rats (polyphenol extract), 1999 [167].

- Decreased blood pressure in humans (grape juice), 2004 [168].

2) Diabetes: - Anti-hyperglycemic effects in rats (seed extract), 2007 [169].

- Increased insulin sensitivity in mice (Resveratrol), 2007 [169].

3) Hypercholesterolemia: - Decreased cholesterol levels in hamsters (wine), 2005 [170, 171].

- Increased low-density lipoprotein receptor expression and activity (grape juice), 2006 [172].

- Decreased cholesterol and increased high-density lipoprotein levels, 2006 [170].

Established effective health benefits in preventing chronic diseases include modifications of risk factors and causative mechanisms of neurodegenerative diseases, cancer, diabetes and heart diseases (Figure 8) [173]. Systemic biological effects of grape on the body include: antiproliferative activity, inhibition of platelet aggregation, inhibition of lipid oxidation and anti-inflammatory activity, as well as its effects on cell cycle, apoptosis and related signal transduction [159].

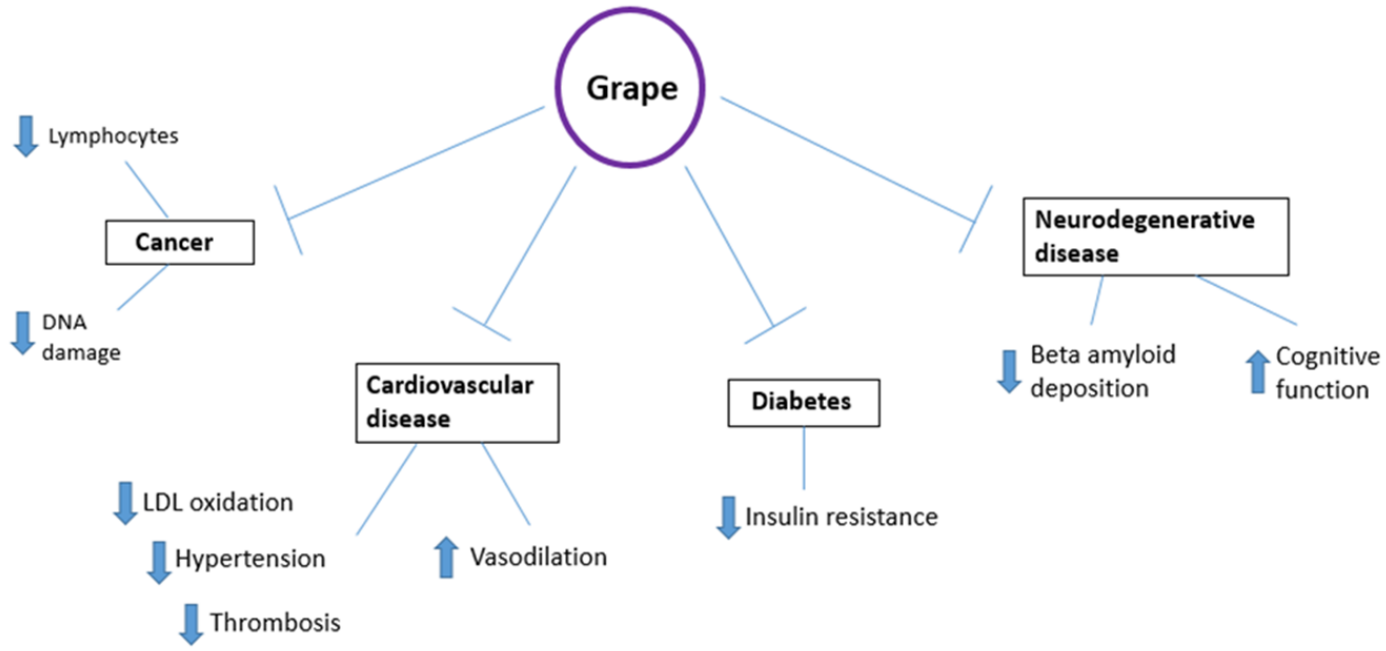


Figure 8- Grape health effects [173].

1.6 Thesis project rationale and objectives

Based on our background knowledge (as illustrated in this introduction chapter) of;

1) The association, possibly a cause and effect relationship, that forms the impression of MetS not only leading to CKD but also worsening its prognosis.

2) Oxidative stress is a common underlying factor in the pathophysiology of MetS and CKD.

3) Grape antioxidants well-established health effects as protective against a number of chronic diseases especially MetS components such as diabetes.

4) Grape antioxidants show protective effect on cardiovascular diseases, a MetS complication. While antioxidants generally showed positive effects on diabetic nephropathy, another MetS complication, grape antioxidant in particular has not been evaluated on kidney disease yet.

This pre-clinical pilot study was designed to evaluate the effect of whole grape intake on kidney function in a picture of metS. Here is a brief overview of the study:

-**Hypothesis:** Consumption of grape might have protective effect against progression of CKD in individuals with MetS.

-Aim: to investigate whether the whole grape has protective effect on the kidney in subjects with MetS.

-Objectives:

1) **In vivo:** evaluate renal function progression in MetS+CKD model (obese ZSF1 rats) with daily ingestion of whole grape *vs*. control through conducting;

- Blood/urine chemistry
- Renal histopathology
- PCR array analysis of oxidative stress in kidney tissues

2) **In vitro:** study the effects of whole grape extract on kidney cells (HSMP) under oxidative stress through;

- Cell survival using flow cytometric analysis
- Cell viability using MTT assay

3) **Chemical study:** evaluate antioxidative properties of the whole grape through;

- Measure antioxidants activity using FRAP, ABTS, DPPH assays

Chapter 2: Materials and methods

2.1 In vivo:

The objective of this study is to evaluate renal function progression in MetS with CKD animal model by daily ingestion of whole grape *vs*. control through conducting;

a) Blood/urine chemistry

b) Renal histopathology

c) PCR array analysis of oxidative stress-related gene expression

Here is an outline of the experimental plan carried out in this animal study (Figure 9):

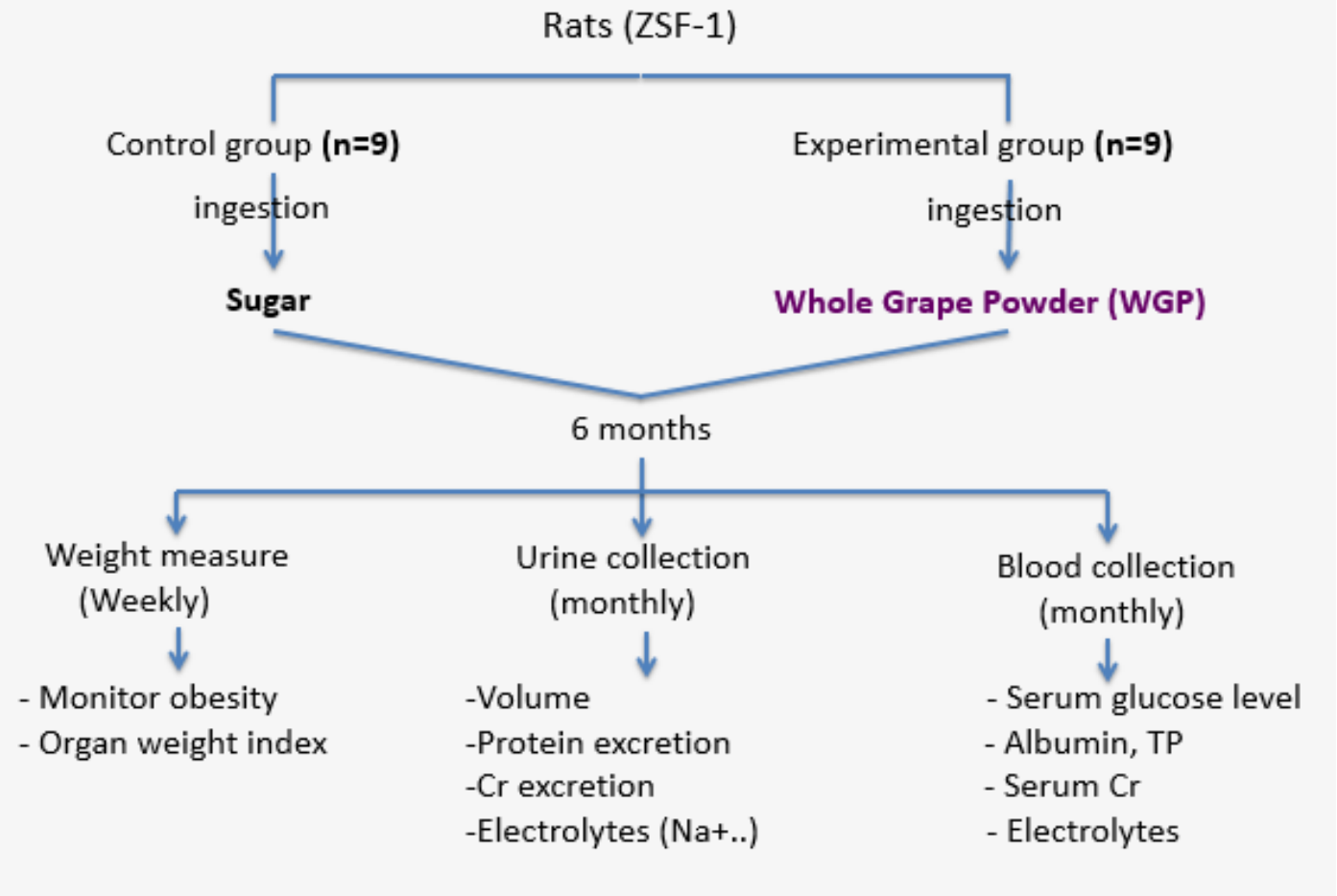


Figure 9- In vivo study experimental plan.

2.1.1.1 Experimental model choice

The animal model that we found to be the most suitable for the aim and requirements of this study is the obese ZSF-1 rat. The obese diabetic ZSF1 rats (Charles River, Wilmington, MA, USA) are a genetically developed hybrid model of type-2 diabetes with renal dysfunction produced by crossing the obese female Zucker diabetic fatty rat (ZDF) with the lean male spontaneously hypertensive heart failure rat (SHHF) (Tofovic & Jackson 2003). Both lean and obese ZSF-1 rats carry the hypertension gene from the paternal SHR strain and therefore have elevated blood pressure. However, only obese ZSF1 rats develop renal sclerosis and fibrosis

alongside dyslipidemia and hyperglycemia (Tofovic et al. 2000, 2002, Zhang et al. 2007, Rafikova et al. 2008). Some of the complications common in the parental strains such as overt congestive heart failure in SHHF rats and hydronephrosis in ZDF rats could compromise results of renal function and structure studies. Fortunately, it was determined that obese ZSF1 rats indeed do not develop these complications (McCune et al. 1990, Vora et al. 1996, Heyen et al. 2002, Marsh et al. 2007, Baynes & Murray 2009). Griffin et al. (2007) have recently demonstrated that the kidney disease developed in the ZSF1 rat model is mostly independent of hypertension and/or hypertensive nephropathy. Therefore, obese ZSF1 rat model allows for the attribution of renal pathophysiology strictly to rather obesity and hyperglycemia and not to the changes due to hypertension which makes it an appropriate model for the purposes of investigating MetS-related kidney disease or diabetic nephropathy (DN) [174].

Obese ZSF-1 rats could develop DN based on characterization of kidney disease similar to the renal changes in the light of DN recently established criteria in rodents (Brosius et al. 2009) as well as by the classification of DN in humans recently published (Tervaert et al. 2010) suggesting that ZSF1 rats can be considered a model of MetS, diabetes, and chronic renal disease. MetS, diabetes and early signs of renal disease (proteinuria, renal hypertrophy, glomerular collagen IV deposition, and tubule-interstitial inflammation) are developed in these rats at 8 weeks of age [175]. By 32 weeks of age, the renal histopathology findings (mesangial expansion, tubular dilation and atrophy, glomerulosclerosis, tubule-interstitial inflammation and fibrosis, arteriolar thickening) consistent with DN is developed [175]. Obese ZSF1 rat demonstrate currently approved criteria for experimental progressive diabetic kidney disease in rodents, indicating the possible consideration of this model as the best available for simulation of human DN [175] and these findings were supported by a study that examined obese ZSF1 rats as

a model for diabetic nephropathy indicating its representation of DN according to histopathological findings [176]. A comparison summary of the characteristics of related animal models on which our experimental model was selected is illustrated in (Figure 10).

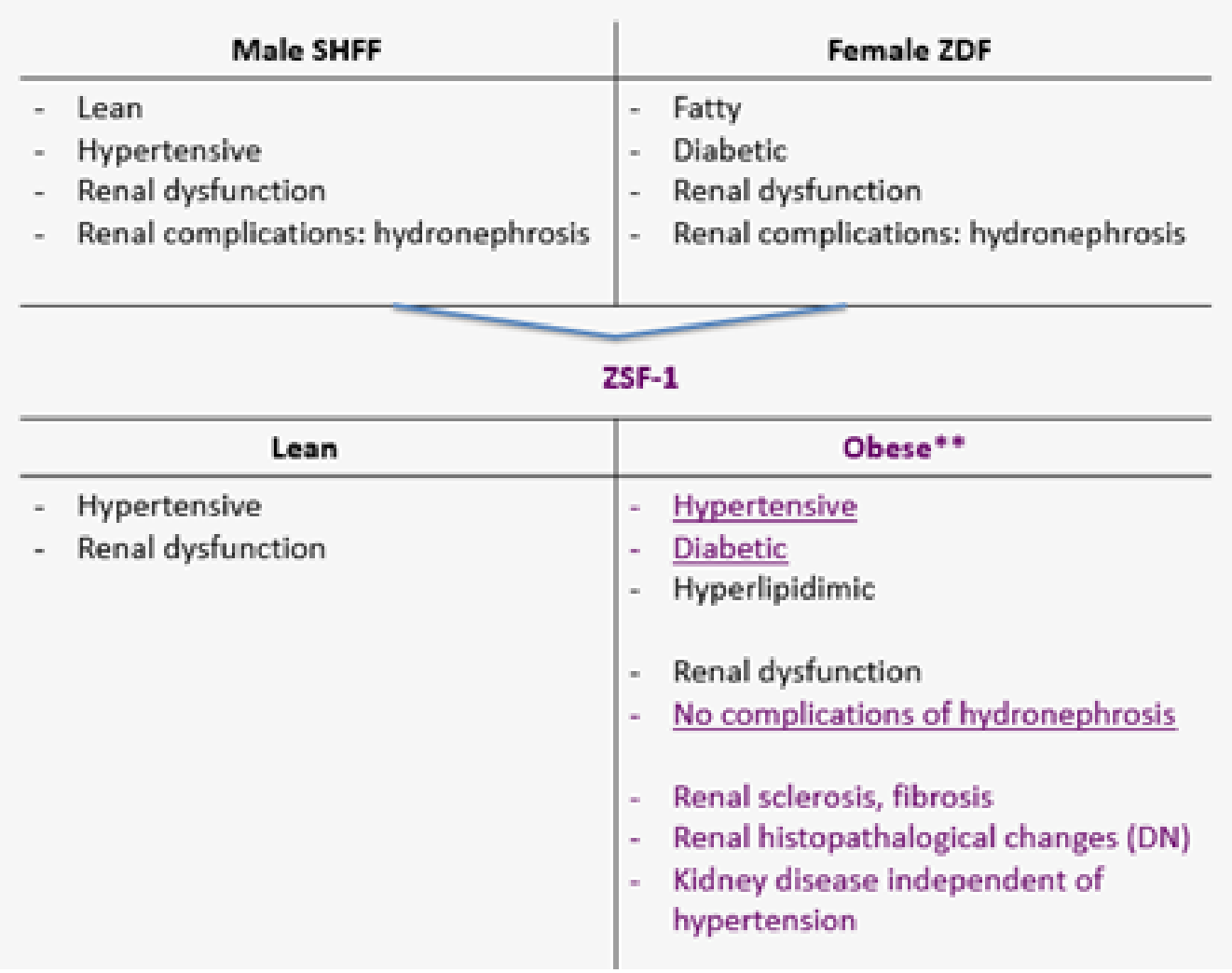


Figure 10- Basis of the choice of animal model (ZSF-1 rat).

2.1.1.2 Animal care and study design

A total of 32 male obese ZSF-1 rats (8 weeks old) were purchased from the (Charles River in Wilmington, MA, USA) and housed at the Jack Bell Research Centre (JBRC) animal facility in Vancouver, Canada. After they had gone through a quarantine period of around 3 weeks, rats were placed in cages (2 rats per cage) in conventional rooms. The animals were divided into two groups; Grape (experimental) group n=16, and Sugar (control) group n=16. The experimental group was fed daily with normal rat food (chow) containing whole grape powder (5%, w/w), while the control group was fed with the chow containing sugar control (glucose: 2.25%; fructose: 2.25% - the same amount of the sugar in the whole grape powder). One of the rats in the grape group suffered a urinary tract infection (UTI), diagnosed early during the second month of the study and followed up by the facility veterinary, which resulted in its sacrifice due to severity of the disease, therefore, the grape group total subjects number was n=15). Research animal care, UBC Animal Care Committee guidelines, were implemented and monitored by trained and certified lab staff and animal facility personnel throughout the period of the study (6 months), to ensure the delivery of the care quality approved by the guidelines.

2.1.1.3 Food preparation

Rats daily food was composed of regular rat food (chow) supplemented with whole grape powder (for the Grape group) or the amount of sugar in grape powder (for the Sugar group). The whole grape powder was provided by [California Table Grape Commission (CTGC) Fresno, California, USA]. The following information below was adopted from CTGC analysis report (Year 2011) and guidelines for usage in research studies and presented here to clarify the nature of the grape product used in this study.

-Fresh grapes are composed of about 82% water, 12-18% sugar, and 0.2-0.8% acid, mainly tartaric and malic acid. Most importantly, grapes contain numerous amounts of antioxidants. Grape antioxidants include tremendous forms primarily phenolic compounds including phenolic acids, flavonoids, flavans, flavonols, anthocyanins and stilbenes. Fresh grapes standard serving size is approximately 3/4 cup (126 grams).

-Whole grape powder (WGP): Basically, this powder is freeze-dried grape developed for the usage of research purposes only. Based on consumers' actual consumption patterns, it is made to be composed of seeded and seedless varieties of fresh green, red and black California grapes which have been initially frozen then ground with food-quality dry ice. Good Manufacturing Practices was used for freeze-drying and re-grounding of food products. Processing and storage of the powder was done for preserving the integrity of the fresh grapes' biologically-active compounds found. Similar to fresh grapes, this grape powder contains resveratrol, flavans (including catechin), flavonols (including quercetin), anthocyanins and simple phenolics. Moisture content of the grape should be accounted for in order to estimate the represented amount of fresh grape by the powder. The powder was stored at (-70ºC) in moisture-proof containers because of its hygroscopic nature. Here are some relevant additional facts about the WGP used in this study:

- It is not an extract and prepared solely from whole California grapes. Therefore, direct attribution should be made to grape consumption regarding findings that result from feeding grape powder in studies.
- This powder was developed specifically for research purposes to be relied on for solid reproducible data, and was not made for commercial use or sale.

- The dose of this WGP equivalent to a standard serving of fresh grapes (3/4 cup or 126 g), was quantified to be 23 g.
- When conducting animal studies using this WGP, it should be mixed into the animal diet to be able to control the amount consumed. A recommended method is to incorporate it into a powder diet fed to the animals. In this study, chow was grinded to semi powder that can be mixed with WGP.
- **When designing for studies using grape-containing animal diets, the controls should be matched for sugar type of the used grape in the specified percentages**. Since ripened grapes is composed of around half glucose and half fructose (not sucrose) and the grape preparation is composed of around 90% sugar, the recommended dosage range for animal studies is 3 to 5% grape powder incorporated in the diet. This means a 100 g of the final diet should contain from 3 to 5 g of grape powder.

Based on CTGC guidelines for usage of WGP, the food preparation for both Grape group and Control group was done as follows: for Grape group (950 g chow + 50 g WGP) and for Sugar group (955 g chow + 22.5 g glucose + 22.5 g fructose). Required amounts of ground chow (chow powder), WGP, glucose and fructose were weighed and mixed as indicated above, using water to produce a dough-like mixture that was cut in cubes and left to dry in the hood. Prepared food was stored in the cold room (4° C). Rat cages were supplemented with food and water daily and weight of consumed food was monitored to estimate the ingested dose of WGP.

2.1.1.4 Body weight monitoring

All rats were weighed once a week to monitor weight gain/loss for evaluation of obesity, overall health and calculations of organ weight index.

2.1.1.5 Urine collection

Monthly collection of 24-hour urine was done for evaluating urine volumes required in renal function monitoring and calculations of renal parameters such as proteinuria and protein to creatinine ratio (PCR). Urine protein and creatinine measurements were done for monthly collected samples in the same time at the end of the study to minimize technical errors due to variability in sample handling. Rats were placed in metabolic cages supplemented with food and water. After 24 hours the rats are removed back to their cages and metabolic cages were de-assembled, washed and re-assembled to accommodate the next rat. Urine volumes collected by the metabolic cages are measured, samples were centrifuged (~3000 rpm for 15 mins) just enough to separate food scraps that fall into urine collecting tube as rats feed. Collected centrifuged urine samples were stored in – 20° C to be used at the end of the study 6-month duration for urinary protein and creatinine measurements.

2.1.1.5.1 Measurement of protein in urine

The principle and procedure information are adopted from Bio-Rad Colorimetric Protein Assay protocol manual (tech note 1069).

Principle:

The Colorimetric Bio-Rad Protein Assays, such as used in this study, depend on the observation of the shift in the absorbance maximum as the dye Coomassie Brilliant Blue G-250 is mixed and

then react with protein (Fazekas de St. Groth et al. 1963, Reisner et al. 1975 Sedmak and Grossberg 1977). Similar to all colorimetric protein assays, the color response varies among different proteins. The color response resulting from the dye binding and its variations all are attributed to the 3 absorbing species of the dye: red cationic species, green neutral species and blue ionic species. At the pH of the assay, the red cationic dye form represents doubly protonated dye molecules. The blue anionic dye detected at 595 nm represent the binding of the dye to stabilized protein. It has been indicated that Coomassiee brilliant blue G-250 binds mainly to basic and aromatic amino acid residues (Compton and Jones 1985). Therefore, the color response variations are based on the protein contents of active basic or aromatic residues. The only substances able to carry out this stabilization are large protein (> 8-9 amino acid).

Procedure:

The protein measurement was done for a "spot" test of the 24-hour urine collected from all rats. The calculation for total protein concentration in the urine was done based on the corresponding 24-hour urine volume. In a 1-ml tube, 500µl of the urine sample was mixed with 500µl of 10% Trichloroacetic acid (TCA) by a shaker before standing for 10 minutes at room temperature. This was done from all rats urine collections. Then, 1-ml tubes were centrifuged at 10,000 rpm for 10 minutes. Supernatant were discarded and ~50µl of 3% sodium hydroxide (NaOH) was added to the pellets (volume of added 3% NaOH depends on the pellet sizes, all tested tubes must have the same added 3% NaOH volume). All tubes were mixed by rigorous pipetting. Next, Bio-Rad dye preparation was done by 1:10 adding 1 ml of dye to 9 ml of distill water (dH2O) for example. In a 96-well plate, 200µl of prepared Bio-Rad dye in each well is mixed with 1µl of prepared urine samples (pellets with 3% NaOH), the color will turn blue. Two or three with only the dye as

background wells are required for calculations later on. The optical density (OD) numbers for all wells were read at 595 nm. After subtracting the OD number of the background wells, the protein concentration (mg/ml) was determined using a standard curve. The determined protein concentration for each sample was multiplied by the corresponding collected 24-hour urine volume to measure total 24-hr urinary protein concentration.

2.1.1.5.2 Measurement of creatinine in urine

Principle and procedure adopted from Creatinine Colorimetric/Fluorimetric Assay by BioVision protocol manual (Catalog # K625-100).

Principle: Creatinine is the end metabolite of creatine sulphate produced by the muscles of the body during contraction. It is toxic to the body and is therefore removed from the blood primarily by the kidneys through glomerular filtration into the urine. Based on this, creatinine levels decrease in the urine while increasing in the blood when the renal function is compromised due to kidney disease. Thus, creatinine clearance has been one of the gold markers of deteriorating glomerular filtration rate. The simple and direct procedures to measure creatinine concentrations in biological systems, particularly in the urine, have been popularly utilized in research. This assay works as it converts creatinine in the sample to creatine by the enzyme creatininase. Creatine is in turn converted to sarcosine that is particularly oxidized and produces a product which generates a red color (OD 570 nm) by reacting with a probe. This assay unlike other assays is more suitable for biological samples such as urine and serum.

Procedure:

1) In a 96-well plate, 10µl of urine was mixed with 40µl of assay buffer (provided in the kit) in wells for each collected urine sample.

2) A mixture containing 42µl assay buffer, 2µl of each of creatinase, creatininase enzyme mix and probe (all provided in the kit) was made as each one of these volumes was multiplied by the number of urine samples to be tested.

3) In each well of the 96-well plate containing urine and assay buffer prepared in the first step, 50µl of the mixture prepared in the second step was added. The plate was placed in (37° C) incubator for 1 hour.

4) OD numbers for all wells were measured at 570 nm. Using a standard curve ($y = 0.1264x + 0.0477$, $R^2 = 0.9983$), the total creatinine concentration (mg/dl) in the 24-hr collected urine volume was calculated.

2.1.1.6 Blood collection

Blood collection was done every three months (monthly collection could be too distressful on rats affecting their overall health and therefore the parameters required for the study). Blood samples (~ 400µl) were withdrawn from rats' tail vein after when they had been anesthetized using isoflurane. Blood samples placed in special tubes containing heparin to prevent clotting were sent to the chemistry lab at the JBRC animal facility for blood chemical analysis. The measured blood parameters are primarily related to both renal function and liver function.

2.1.1.7 Animal euthanization and organ harvesting

At the end of the 6-month duration of feeding, all rats were sacrificed using the anesthetic agent Isoflurane and carbon dioxide. A cut along the midline of the chest and abdomen was made to expose the underlying organs. Harvested organs (the heart, lungs, liver, spleen and kidneys) weights were recorded. Small pieces of each organ were cut, placed in a 1-ml tube and kept in liquid nitrogen followed by storage at (-80° C) for RNA extraction, which was required for PCR array analysis. All organs were fixed in formalin (10% formaldehyde) and stored at room temperature for the histopathology study.

2.1.2 Renal histopathology

This terminology refers to examining tissues microscopically for the purpose of studying disease characteristics. Particularly, from a medical prospective, histopathology refers to the pathologist examination of biological specimens acquired from patients. Typically, specimens are processed and histological sections are mounted on glass slides to be stained with a specific dye (i.e. hematoxylin and eosin) and viewed through a microscope or as scanned microscopic views uploaded in a computer system analysis system.

Processing kidney tissues: kidneys fixed in formalin were cut in half (horizontal cut at the renal pelvis level). One half of each kidney was placed in blocks immersed in formalin and sent to British Columbia Cancer Agency (BCCA) lab where kidney tissues were processed into slides, mounted on glass slides and stained.

Staining: In histology and experiment labs, organ tissues are stained with a numerous selection of stains for the purpose of identifying and labelling the structural features of the tissue such as blood vessels, matrix, functional cells, inflammatory reactions, substance depositions etc. The

choice of stain depends on the structures and substances required to be identified for examination and analysis. Our kidney tissue slides were stained with two stains separately, hematoxylin and eosin (H&E) stain and Periodic Acid Schiff (PAS) stain. Tissue staining with H&E was done by the BCCA lab and sent to our lab while PAS stain was done in our lab. In this study, H&E stain was required for identifying general anatomical structures of the tissues that allows some of the expected histopathological changes related to be recognized such as tubular atrophy (atrophied renal tubules). PAS stain was required for identifying some of the expected structural changes that would not be clearly visible with H&E stain, such as mesangial expansion (enlarged matrix cells).

PAS stain procedure:

1) In a fume hood, kidney slides were deparaffinized and hydrated with water by immersing the slides for 5 minutes in each xylene 10%, xylene 100%, xylene 1:1, ethanol 100%, ethanol 95%, ethanol 75%, water, respectively.

2) Next, tissue slides were oxidized using drops of 0.5% Periodic Acid covering the slides for 5 minutes before they were rinsed with water.

3) The slides were then covered with Schiff reagent drops for 15 minutes (color of the slides turned pink). Using luke warm water, the slides were rinsed for 5 minutes (color turned dark pink).

4) Following that, the slides were covered with drops of Mayer's Hematoxylin for 1 minute to stain the nucleus. Right after, slides were rinsed with water for 5 minutes then checked under the microscope to confirm staining of the nucleus.

5) The slides were dehydrated by repeating the first step in a backwards direction immersing slides first in ethanol 75% all the way to xylene 10%. Unlike with each part of this step, slides should not spend 5 minutes in ethanol 100% but only be immersed twice or three times.
6) Finally, tissue slides were sealed with a coverslip using mounting medium, left to dry in the fume hood for overnight before they were scanned and uploaded to a computer imaging system for histological analysis.

Tissue analysis:

The kidney tissues randomly selected for analysis were 6 tissues (made into slides) out of 16 tissues from each Grape group (n=15) and Sugar group (n=16). From each tissue (slide), 8-10 views were randomly selected from each slide for analysis. Later, the selected rat proteinuria parameters were compared to the whole group proteinuria parameters to find out where in each group's full spectrum of values the selected rats lie. The randomly selected kidney tissues (6 out of 16 tissues) are **representative** of typical histological changes that could be seen in correlation with the observed renal functions of those rats, and are **reflective of the spectrum** (severity levels) of the renal function decline indicated by proteinuria (protein in urine) measured for rats of both group. The following table shows the kidney function of the randomly selected rats from each group for tissue analysis.

The proteinuria (g/24 hr) measurements of rat groups and randomly selected rats for histopathology are as follows:

1) Range of proteinuria measurements of rats in Sugar (control) group: minimum 129 to maximum 1487.5

➔ Proteinuria of each selected rat from Control group: 129, 225, 318, 486, 1015, 1487.5

2) Range of proteinuria measurements of rats in Grape group: minimum 72 to maximum 268

➔ Proteinuria of each selected rat from Grape group: 72, 99, 133, 143, 205, 241

Control versus Grape: $p = 0.0306$ (one-tailed t-test, $n = 6$)

Histopathological examination of kidney tissues from both groups revealed a number of structural abnormalities including primarily tubular atrophy, tubular proteinaceous material, glomerular atrophy and mesangial expansion. Two tissue analysis systems were designed to evaluate and quantify these changes were:

1) Tally system for tubular protein casts and glomerular atrophy: by counting the total number of these changes in a view, calculating the average of total counts from all views of each group (Grape vs. Control) and finally comparing these averages between the two groups.

2) Scoring system for tubular atrophy and mesangial expansion: by giving a score to the percentage of these changes of a view (as seen in the table below), calculating the average of scores from all of the views of each group and comparing these averages between the two groups (Table 1).

Score	% Tubular atrophy in view	Score	% PAS stain in glomerulus
1	0-24 %	1	0-24 %
2	25-49 %	2	25-49 %
3	50-74 %	3	50-74 %
4	75-100 %	4	75-100 %

Table 1- Scoring system for tubular atrophy and mesangial expansion in rat kidney tissues.

In order to rule out potential bias in data interpretation, a blinding fashion of the tissue histological analysis was implemented by obscuring the slides identities and assigning the analysis to be done by another student that was not involved in the study. This way, the slides reader is neither involved in the study nor aware of the slides identities (which group they belong to) when analyzing.

2.1.3 Tissue PCR array: oxidative stress

Principle:

In molecular biology, the polymerase chain reaction (PCR) is a method used to amplify a piece of DNA single copy or a few copies to generate thousands or even millions of copies of that particular DNA sequence. This technology provides an easy DNA amplifying tool that is useful in studying genetic diseases by analyzing the function of targeted DNA segment as well as other various medical and scientific uses [177]. The PCR technique is based on employing the DNA polymerase ability to synthesize a new DNA strand complementary to an available template strand. First, the original double-strand DNA is separated to its single strands using high

temperature. Since DNA polymerase requires a pre-existing 3'-OH group onto which it adds a nucleotide, it needs as well a primer that the first nucleotide can be added to. This makes delineating a specific region of template sequence that is needed to be amplified amplify possible. Billions of copies (amplicons) from a particular sequence can be produced at the end of the PCR reaction. At present times, PCR is considered not only a common but also often an unreplaceable technique used in clinical and research laboratories for a wide variety of applications [178,179].

There is a wide variety of assay technologies optimized to provide solutions in oxidative stress studies. These methods include PCR array, mutation analysis, DNA methylation, and protein expression products. One of the PCR methods is rat Oxidative Stress RT² Profiler PCR Array which profiles the expression of 84 genes involved in biological oxidative stress. Some of the genes included in this PCR array are those involved in the metabolism of reactive oxygen species (ROS) such as oxidative stress response genes and superoxide metabolism genes. In this study, real-time PCR was used to analyze the expression of these genes in attempt to explore the effect of grape intake on oxidative stress present in the kidney tissues of our rats.

Procedure:

Rat Oxidative Stress RT² Profiler PCR Array kit was purchased form Qiagen for gene analysis of our rats' kidney tissues. The PCR Array used was a set of optimized real-time PCR primer assay on 384-well plate disc for rat oxidative stress genes. It is capable of performing gene expression analysis with real-time PCR sensitivity as well as multi-gene profiling. Total 4 kidney tissues were randomly selected from each group (Grape versus Control) for this analysis. In brief, total

RNA was isolated from each tissue sample, and was reversed to cDNA. The cDNA sample was mixed with the appropriate ready-to-use PCR master mix provided in the kit. Equal volumes were aliquoted to each well of the PCR Array, followed by running real-time PCR cycling program. The following illustration outlines the procedure steps (adopted and modified from Qiagen protocol chart) (Figure 11).

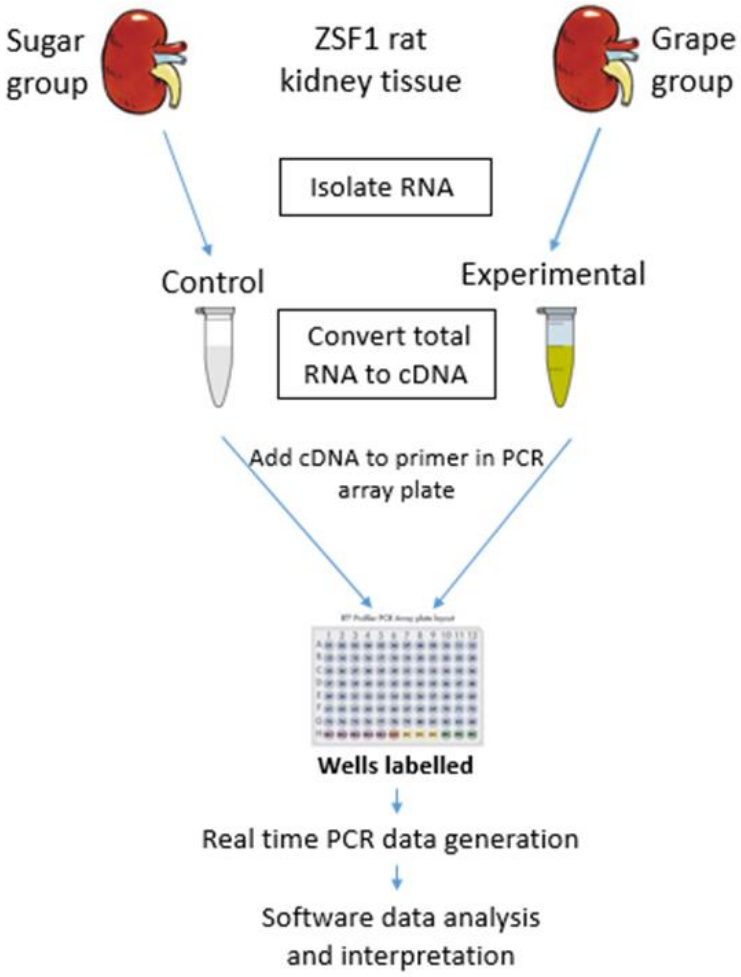


Figure 11- Procedure of PCR array.

2.2 In vitro:

The objective of this study is to examine the effects of whole grape extract on kidney cells (HSMP) under oxidative stress through;

- Cell survival using Flow cytometry
- Cell viability using MTT

Here is the outline of this study experimental plan (Figure 12):

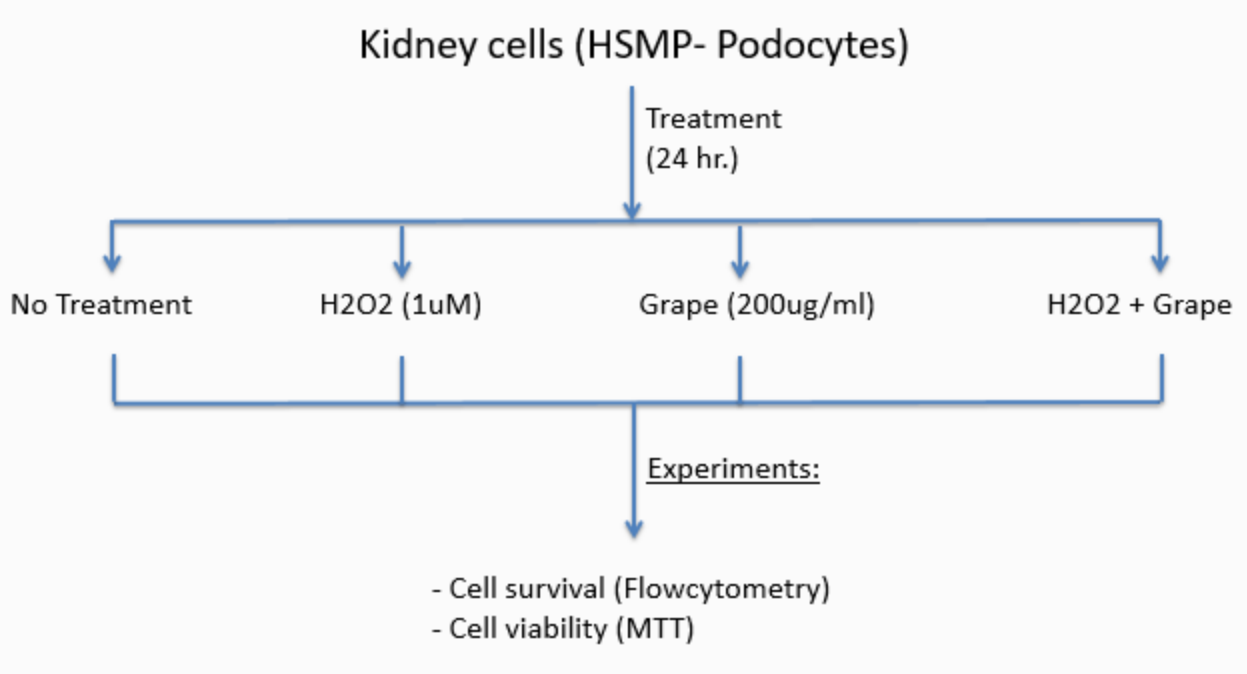


Figure 12- In vitro study experimental plan.

2.2.1 Cell culture

The type of cells that was chosen based on the rationale of this study was podocytes (heat-sensitive mouse podocytes: HSMP), a kind gift from Dr. Stuart Shankland (University of Washington School of Medicine, Seattle, WA, USA). Podocytes, as explained in the introduction

chapter (section 1.2.2.1), are the renal cells known to be in the first line of the glomerular filtration process. Compromised filtration through these cells result in loss of plasma proteins in urine as the spaces (slits) through which substances are filtered enlarge. The larger the filtration slits are due to injury of podocytes, the larger the substance lost in urine that is otherwise not normally found there, such as albumin [51]. With kidney disease where proteinuria is found, there is usually an expected injury to podocytes. Stored HSMP cells in -80°C were thawed, cultured in petri dish using the appropriate media for this type of cells which was prepared as a mixture of 500ml of sterilized 10% RMPI media, 50ml of fetal bovine serum (FBS), 5ml of penicillin, and 1µl of interferon gamma IFN-γ. Cells were sub cultured three times (3 passages) or more before experiments were carried out to approach cells fitness for treatments. The appropriate incubating temperature for HSMP cells is 33°C.

-Sub culture procedure: After sucking out media in the culture plate, trypsin-EDTA solution was added to cells adherent on the plate (enough volume to cover cells) to be placed in the incubator (33°C) for a few minutes to allow suspension of the adherent cells. The plate was then checked under the microscope for suspended cells which appear floating in the medium. Some media or washing buffer was added to the plate to collect the cells into a tube using a pipette. Following that, cells collected in the tube was centrifuged (2000 rpm for 4-5 minutes) to separate the cells from the media in a form of a pellet at the bottom of the tube. After that, supernatant was discarded and pellet was suspended in ~ 1 ml of culture media using a pipette (volume of media depends on pellet size). Depending on how much cells we needed to subculture (pass), a volume of the media with suspended cells in the tube (~300-500 µl) was added in a new culture dish to culture media (enough volume to generously cover the plate). The plate was kept in the incubator

(33°C) and monitored for circumcision of the cells (growing cells covering the plate bottom area), the cells then were ready for another passaging done by repeating this sub culturing procedure.

2.2.2 Cell treatment

-Oxidative stress: Hydrogen peroxide (H2O2) was used in the experiments to mimic a biological oxidative stress environment. According to the literature, H2O2 is an abundant oxidant in the body that is commonly used in cell culture studies as a simulation system of biological oxidative stress as it is a primary and key element in that environment. The suitability of H2O2 as an oxidative stress simulator is presented in the introduction chapter (section 1.4.3.1).

- Antioxidant defense: Whole grape powder (WGP) was used to attempt antioxidant effect on cells under H2O2 oxidative stress. According to CTGC guidelines for usage of the grape powder in cell studies, the powder form has to be made into an extract as 90% of grape powder consist of sugars which should be separated from other contents (antioxidants) for cell culture treatments.

2.2.2.1 WGP- extract preparation

An extract was prepared from WGP to produce the required grape antioxidants treatment for cell culture as the following:

1) In a flask with a stirring magnet inside, 4 g of WGP was dissolved in 200 ml of methanol and left to stir for ~72 hours in room temperature.

2) WGP-methanol solution was distributed to 50-ml tubes and centrifuges (3000 rpm for 15 minutes). Supernatant was collected in new (weighed) 50-ml tubes and then the solution was weighed (to document the WGP-methanol volume generated from the amount of WGP used).

3) Collected supernatant in 50-ml tubes was left to dry out into a concentrate in a fume hood. The concentrate was then transferred in weighed 1-ml tubes which were placed in a speed vacuum machine to turn the concentrate into an extract by evaporating the remaining methanol.
4) Tubes with extracts were weighed to calculate extract weight (to document the weight of the extract yielded from the amount of WGP used). Around 3.3 g of WGP-methanol extract was produced from 4 g of WGP using 200 ml of methanol.
5) A sterilized WGP-extract stock concentration of 5 mg/ml was made to be used for treatment dose preparation.

2.2.2.2 Optimized doses for treatment

In order to reach an appropriate dosage for the treatment of cells with an optimal outcome where the percentage of surviving cells allows valid recognition and interpretation of the treatment effect (grape antioxidant effect against H2O2 oxidative stress), optimization of the H2O2 and Grape extract doses were required. This was done through a series of testing a range of doses on cells to decide the optimal concentration of grape extract treatment that is not toxic to the cells (~80% live cells) and the dose of H2O2 that is not too toxic (20-30% of dead cells). This allows the effect of grape extract against oxidative stress to be appreciated. After determining the optimal dosages for treatment, cells cultures were divided into 4 groups as:

1) No treatment (cells in culture media only)
2) Grape extract alone (optimized concentration is 200 μg/ml)
3) H2O2 (optimized concentration is 1 μM)
4) H2O2 (1μM) + Grape extract (200 μg/ml)

Preparation of treatment doses was as:

1) 1 ml of Grape extract treatment dose (200 µg/ml) was prepared using a grape extract stock concentration of (5 mg/ml) as 40µl from the grape extract stock added to 960µl of culture media.
2) 1 ml of H2O2 treatment dose (1 µM) was prepared using an H2O2 stock concentration of 10 µM as 10 times dilution of the stock adding 100µl from H2O2 stock to 900µl of culture media.

2.2.2.3 Cell survival experiment: flow cytometric analysis

-Principle:

A sophisticated instrument, flow cytometry, measures multiple physical characteristics such as a single cell size and granularity in a simultaneous manner as a suspension of cells flow through a measuring device. Dyes or monoclonal antibodies target either molecules found on the cell surface (extracellular molecules) or found inside the cell (intracellular molecules). These dyes drive the light scattering characteristic of the investigated cells on which this method depend. This feature is a powerful tool of flow cytometry that allows for comprehensive analysis of complex populations in a short time duration [180].

Modern flow cytometers have the ability to analyze “in real time” thousands of particles each second, and also isolate particles with specified properties. A flow cytometer serve similarly to a microscope with the exception of its ability to develop an image of the cell. This way, flow cytometry provides a "high-throughput" automated measurement of a selection of parameters for a large number of cells. A specialized type of flow cytometry is known as Fluorescence-activated cell sorting (FACS). It functions as it sorts out a mixture of heterogeneously characterized cells

one at a time into two or more compartments based on the fluorescence and the specific light scattering features of each cell. Its usefulness is in providing rapid and objective quantitative recording of fluorescent signals from each cell while carrying out physical isolation of cells of a specific interest. The principles of FACS operation are as the following steps:

1) Fluorescent dye labelled cells in a suspension are directed into a thin stream in a way that all cells pass in a single file. A monoclonal antibody is coupled to the dye binding to the cells carrying the antigen for which the antibody is specific.

2) This stream is broken down into 40,000 droplets every second which may contain cells as it passes through a vibrating nozzle at ~ 40,000 cycles per second.

3) Just before the stream transforms into particles, a laser beam is directed at it and as every labelled cell is subjected to the beam it fluoresces and that fluorescence is received by two detectors.

4) The stream is given an electrical charge (+ or -) in the case that signals from the two detectors meet the criteria set for fluorescence and size. These charged droplets are electromagnetically separated according to their charges. Droplets that do not contain cells or contain cells that do not meet the described criteria are uncharged and therefore passed out into a third compartment that is later discarded.

In order to examine the effect of grape extract on cell survival under the oxidative stress, the stains used for flow cytometry in this study were Annexin V and 7-Aminoactinomycin D (7-AAD) which are affinity assays that quantify the number of cells undergoing apoptosis and necrosis. Both Annexin V and 7-AAD function as they label apoptotic and necrotic cells of which the numbers are then quantified using FACS. The following information about Annexin V

and 7AAD cell death detection and data interpretation is adopted from (BD Biosciences data sheets of PE Annexin V Apoptosis Detection Kit).

Apoptosis is programmed cell death that is believed to occur in most biological systems from human and animal bodies to plant cells. Apoptosis result in distinct morphological and biological changes in the life cycle of cells. The loss of plasma membrane integrity and asymmetry is an early event in the apoptotic pathway. Later, this results in the translocation of phospholipid phosphatidylserine (PS) located in the inner leaflet of the cell membrane into the outer leaflet exposing it to external cellular environment. Since Annexin V is a calcium-dependent protein with a high affinity to bind exposed PS, when it is tagged with a fluorescent label, such as FITC, it is often used in flow cytometry measurement of apoptosis. Because Annexin V detects the loss of membrane integrity which occurs in later stages of cell death as a result of either apoptosis or necrosis, staining with Annexin V-FITC is typically used in conjunction with a live/dead dye such (7-AAD) in order to identify early apoptotic cells (7-AAD negative, Annexin V-FITC positive) from late apoptosis (7-AAD positive, AnnexinV-FITC positive) and necrosis (7-AAD positive, Annexin V-FITC negative). Live cells are (Annexin V-FITC and 7-AAD negative).

-Procedure:

Using cell count, HSMP cells were set up in a 24-well plate as a cell density of approximately 0.22 million cells per well. Cells were kept in the incubator (33°C) to grow. The next day cell cultures were treated differently as explained above (No treatment, Grape extract, H2O2, H2O2+Grape extract) using the doses specified above. A 24-well plate had around 400µl total volume of treatment in each well. Treated cells were then kept in the incubator (33°C) for around 24 hours before flow cytometry was carried on as the following:

1) Plate with treated cells was removed from the incubator after 24 hours of treatment and checked under the microscope to visually evaluate the effect of treatments on the cells before collecting both live and dead cells.

2) Dead cells were collected by collecting the media in the wells into 1-ml tubes.

3) Live cells were collected by adding trypsin- EDTA solution to the wells, incubating to detach adherent cells, collect detached live cells with washing buffer into the tubes with collected dead cells from step 2. Tubes with both dead and live cells were centrifuged (~8000 rpm for 2-3 minutes).

4) Supernatant was discarded and cells pellet was suspended in a mixture of (25µl of 1xbinding buffer, 1.25µl Annexin V, 1.25µl 7-ADD) that was added to each tube. This step stains the cells for apoptosis/necrosis reading and analysis by FACS machine.

5) Tubes were standing for 15 minutes in the dark to allow enough time for staining. Following that, around 300µl of 1x binding buffer was added to each tube and cells were transferred into glass tubes that are used in the FACS machine.

6) Analysis of apoptotic, necrotic and live cells percentages and distributions was generated by the FACS software as seen in

(Figure 13).

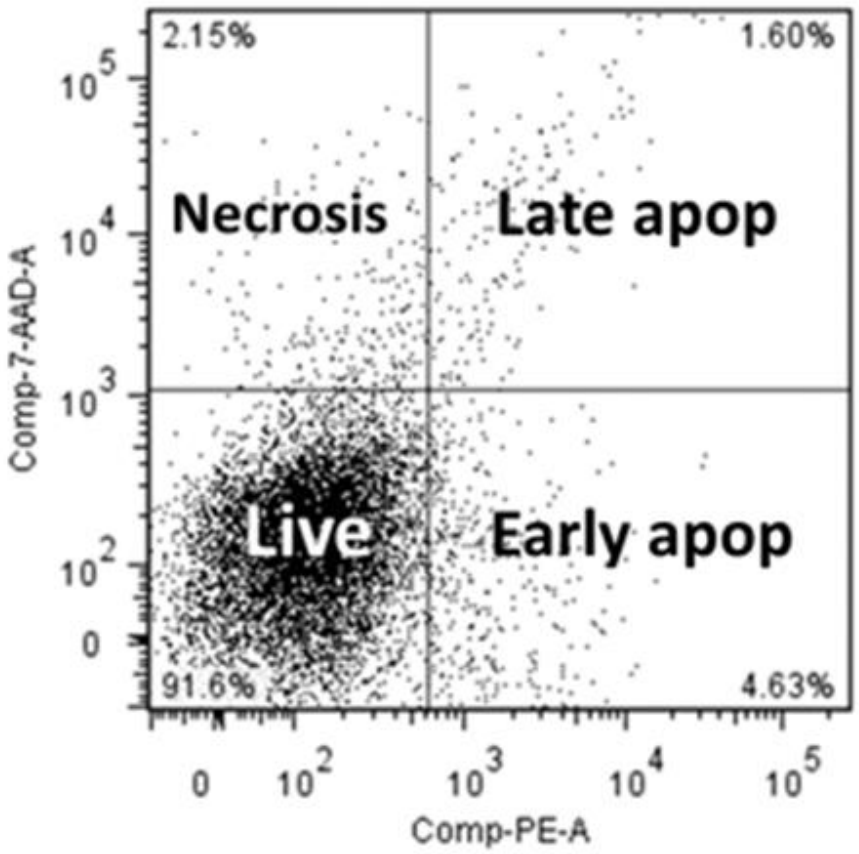


Figure 13- FACS data interpretation of apoptosis.

2.2.2.4 Cell viability experiment: MTT

-Principle:

Well-functioning mitochondria (metabolism) are vital for viable cells. Among the various roles of mitochondria, the most prominent functions include energy production in the form of ATP (phosphorylated ADP) achieved through respiration. Another prominent function is to operate cellular metabolism [14]. The metabolism-operating set of reactions is collectively known as the Krebs cycle which is involved in ATP production. In addition to ATP production, mitochondria

are a key player in many other metabolic tasks, such as signaling through ROS [181] and apoptotic cell death [182].

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its insoluble formazan, which has a purple color [183].

Procedure:

Using cell count, HSMP cells were seeded in 96-well plates with two cell densities of approximately 3000 and 2000 cells per well (to compare final results). Cells were kept in the incubator (33°C) to grow. The next day cells were treated differently as explained above (No treatment, Grape extract, H2O2, H2O2+Grape extract) using the doses specified above. A 24-well plate had around 100ul total volume of treatment in each well. Treated cells were then kept in the incubator (33°C) for around 24 hours before MTT experiment was carried on as the following:

1) 10µl of MTT solution was added in each well on top of existing treatment with gentle shaking to ensure mixing of solution in wells. Then, the plate was kept in the incubator (33°C) for 4 hours.

2) After 4 hours in the incubator, the fluids in the wells were removed and 100 µl of DMSO was added to each well.

3) Optic density (OD) number was measured at 562 nm. The averages of OD for each treatment group was calculated and interpreted as the OD measurement is directly proportionate to the viability of the cells. The higher the OD number is, the more viable "healthier" the cells are.

2.3 Chemical study

The objective of this study is to evaluate antioxidant properties of the whole grape through;

- Measuring antioxidants activity using FRAP, ABTS, DPPH assays

Here is an outline of the experimental plan of this study (Figure 14):

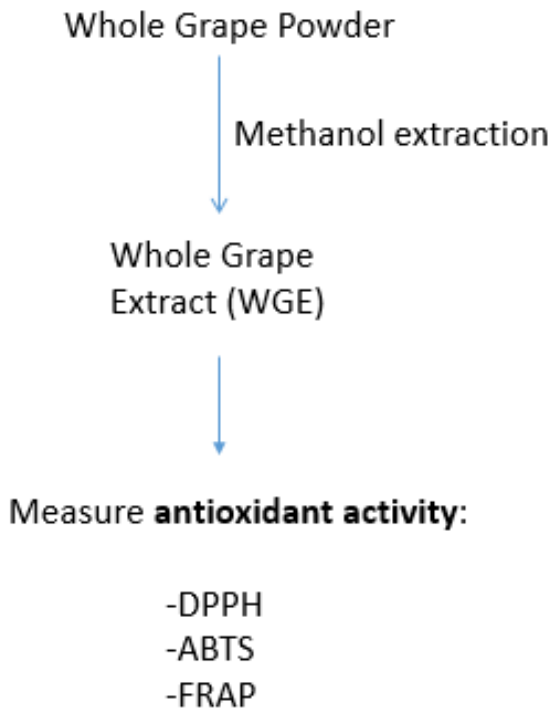


Figure 14- Chemical study experimental plan.

The form of grape used in this study was the WGP-methanol extract explained in (section 2.2.2.1).

2.3.1 Antioxidant activity measurement

As normal aerobic metabolism take place, a release of potentially harmful ROS results as a consequence. In normal healthy conditions, these ‘‘free radicals’’ are usually eliminated or inhibited by a group of antioxidants. Therefore, any absolute or partial deficiency in the antioxidant defense system may result in a state of oxidative stress which has been found to be a major contributor to the development of various diseases. An antioxidant defense setting involves various forms of individually contributing specific oxidant combatting processes and activities which collectively develop the end result of oxidative stress reduction. For example, some antioxidants function through metal chelating activity while others function through scavenging ROS. Many tests have been developed and utilized in the research to measure “antioxidant power” or antioxidant activity of substrates such as fruit extracts [184]. In this study, three antioxidant activity assays were employed to evaluate the antioxidant potential of the WGP used in this study; FRAP assay, DPPH assay, and ABTS assay.

2.3.1.1 FRAP assay (ferric reducing ability of plasma)

-Principle:

The FRAP assay is based on plasma ability to reduce ferric. This assay measures the antioxidants as ferric reductants where ferric is transformed to ferrous at low pH causes the formation of blue-colored ferrous-tripyridyltriazine (TPTZ) complex. FRAP values are quantified by comparison of the absorbance change at 593 nm in test samples with those of a mixture containing known concentrations of ferrous ions using a (standard curve). Absorbance changes curve is linear over

a broad range of antioxidant mixutres concentrations, including plasma, and as well with one antioxidant purified form solutions [184].

-Procedure: [185]

The grape extract prepared by WGP-methanol extraction (section 2.2.2.1) was used in this assay. FRAP assay was done as the following steps:

1) FRAP reagent was prepared by mixing 25 ml of (300 mM acetate buffer) + 2.5 ml of (10 mM TPTZ) + 2.5ml of (20 mM Ferric chloride). Acetate buffer (300 mM) was prepared as 0.31 g Sodium acetate.3H2O + 1.6 ml glacial acetic acid + 100 ml dH2O. TPTZ (10 mM) must be prepared in the same as 0.031 g TPTZ in 10 ml of 40 mM HCl (prepared as 1.46 ml concentrated HCl in 1 L dH2O). Ferric chloride (20 mM) was prepared as 0.0324 g ferric chloride in 10 ml dH2O made fresh on the same day.

2) 300 µl of freshly prepared FRAP reagent was warmed to 37° C. Using 96-well plate, the OD number of 100 µl FRAP reagent in triplet wells was read at 593 nm, labelled as M593.

3) 10 µl of grape extract was added to 30ul water and 100ml FRAP reagent in triplet wells. After 4 minutes, OD number was read and labelled as S593. Ascorbic acid of 0.1 mg/ml concentration was used as positive control (reference).

4) Calculations were done for grape extract = (S593 – M593). Results were compared to a ferrous sulfate standard curve tested in parallel.

2.3.1.2 DPPH assay (2,2-diphenyle-1-picrylhydrazyl)

The following principle information adopted from protocol data sheet [(15.3b Natural Product Screening: Anti-oxidant Screen for Extracts] by Dr. Marsha J. Lewis, 2012].

-Principle:

This assay is popularly used in the antioxidants studies due to its sensitivity and simplicity. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is one of limited selection of commercially available stable organic nitrogen radical. DPPH Assay is based on the theory that hydrogen donation is a characteristic of antioxidants by which they function. Therefore, this assay measures radical scavenging activity of an antioxidant as DPPH radical accepts hydrogen. The diminishing of DPPH radical in test samples is directly proportional to the antioxidant effect of that sample. DPPH radical can be measured by absorption at 517 nm showing strong absorption (purple colored). As DPPH radical accepts hydrogen donation from antioxidants, the purple color turns yellow.

-Procedure: [185]

The grape extract prepared by WGP-methanol extraction (section 2.2.2.1) was used in this assay. DPPH assay was done as the following steps:

1) DPPH (0.135 mM) solution was prepared as 1.064664 mg DPPH in 20 ml methanol.

2) 250 µl of grape extract was added to 250 µl of DPPH solution. Using 96-well plate, 100 µl of that mixture was added in triplet wells and kept in dark for 30 minutes at room temperature. Ascorbic acid of 0.1 mg/ml concentration was used as (reference). Methanol was used as control.

3) OD number was read at 517 nm and labelled as OD control for methanol and OD sample, including ascorbic acid for sample test.

4) DPPH radical scavenging activity (%) = [(OD control – OD sample) / OD control] x 100.

2.3.1.3 ABTS assay [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)]

The following principle information is adopted from manufacturer's protocol (Zen Bio Antioxidant Assay Kit Cat# AOX-1, Instruction manual ZBM0034.03).

-Principle:

Hydrogen peroxide and metmyoglobin cause the formation of a ferryl myoglobin radical which has the ability to oxidize ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] that leads to the generation of a radical cation green in color measured by absorbance at 405 nm, known as ABTS. This radical is suppressed by antioxidants through electron donation radical scavenging which inhibits the green colored ABTS radical formation. Hence, the ABTS radical formation is inversely proportional to the test sample antioxidants.

-Procedure: [185]

The grape extract prepared by WGP-methanol extraction (section 2.2.2.1) was used in this assay. ABTS assay was done as the following steps:

1) ABTS (7 mM) solution was prepared as 0.038409 mg ABTS in 10 ml dH2O. Ammonium persulfate (2.4 mM) was prepared as 0.0111818 g ammonium persulfate in 20 ml dH2O.

2) Working solution was prepared by mixing equal amounts of freshly prepared ABTS and ammonium persulfate (e.g. 10 ml of each) and kept in dark for 12-16 hours at room temperature.

3) Diluted working solution by mixing ~500 µl with 6ml methanol and OD number was read at 734 nm to be 0.70 plus or minus 0.02.

4) 250 µl of each urine sample was added to 250 µl of diluted ABTS solution. Using 96-well plate, 100 µl of that mixture was added in triplet wells and kept in dark for 7 minutes at room

temperature. Ascorbic acid of 0.1 mg/ml concentration was used as a reference. Methanol was used as background control.

3) OD number was read at 734 nm and labelled as OD control for methanol and OD sample, including ascorbic acid for the sample test.

4) DPPH radical scavenging activity (%) = [(OD control – OD sample) / OD control] x 100.

Chapter 3: Results

3.1 In vivo study

3.1.1 Rats blood and urine analysis

3.1.1.1 Obesity and organ weight index

-Daily intake of WGP shows no effect on bodyweight gain or obesity:

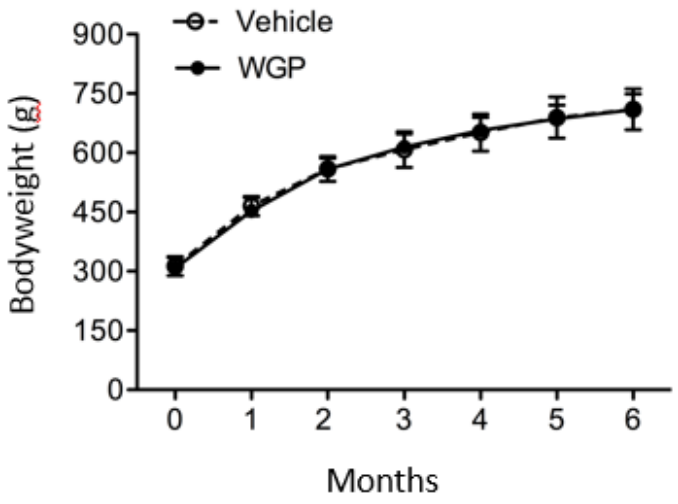


Figure 15- The changes of the body weights in WGP group compared with control (Vehicle). Data are presented as mean ± standard derivation (SD) of each group (WGP: n = 15; Vehicle: n = 16)

This figure shows the body weights (g) of rats in WGP group versus those in control group over 6 months, demonstrating a complete overlap between these two obese groups. This indicates no effect of grape intake on weight gain or obesity. The increase in body weights of the two groups is identical, p = 0.077 (2-way ANOVA).

- Daily intake of WGP shows significant effect on organ weight index of both kidneys and Livers:

	Kidneys	Liver	Spleen	Heart	Lung
Vehicle (n = 16)	0.0070 ± 0.00072	0.0627 ± 0.00418	0.00135± 0.00010	0.00289 ± 0.00115	0.00356 ± 0.00096
WGE (n = 15)	0.0063 ± 0.00069	0.0544 ± 0.00546	0.00129 ± 0.00001	0.00251± 0.00022	0.00371 ± 0.00103
P-value (t-test)	0.0107	< 0.0001	0.1210	0.2319	0.6907

Table 2- Organ weight index (organ weight/body weight) at the end of 6 months. Data are presented as mean ± SD of each group (WGP: n = 15; Vehicle: n = 16).

This table highlights the effect of grape daily intake on the weight indexes of the kidneys and livers (organ weight/bodyweight). In comparison to the rats in control group (n=16), both the kidneys and the livers in the WGP rats have decreased weight index. The difference is statistically significant as demonstrated by the p-values in the table. There is no difference between these two groups regarding other organs weight indexes.

3.1.1.2 Diabetes

Daily-intake of WGP may have beneficial effect on the reduction of blood glucose:

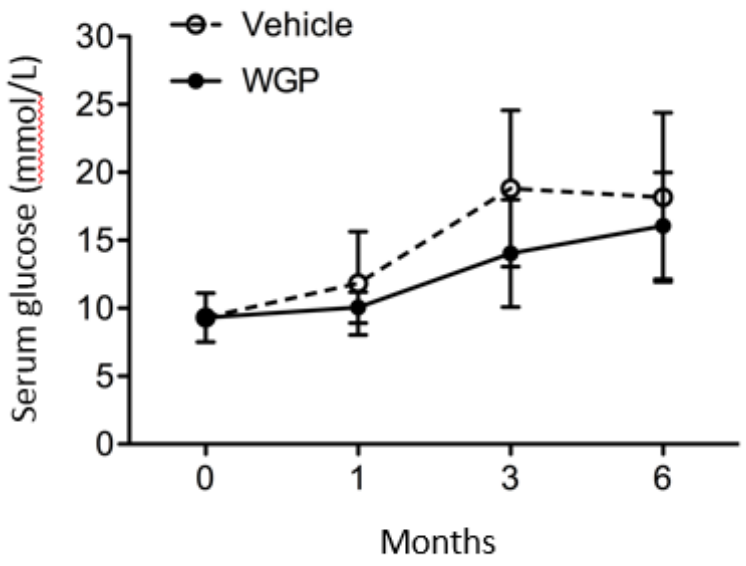


Figure 16- Serum glucose (mmol/L) of rats in WGP group compared to control (Vehicle) over 6 months. Data are presented as mean ± SD of each group (WGP: n = 4 - 10; Vehicle: n = 4 - 11).

This figure shows the overall serum glucose levels of the rats fed with WGP being lower than those in the control group, which is more significant in the 3rd month with a p-value of 0.0312 (WGP vs. Control, n = 8 - 9, one-tailed t-test) than at the beginning or end of the six months where both groups curves meet. This indicates that grape may potentially affect blood glucose levels in these diabetic subjects, however, the overall difference between these two groups is not statistically significant in this limited number of animals, p-value= 0.0709 (2-way ANOVA). Also, there is an overlap of standard deviations between the two groups.

3.1.1.3 Liver parameters

- Daily intake of WGP may have beneficial effect on the maintenance of blood albumin (produced by the liver):

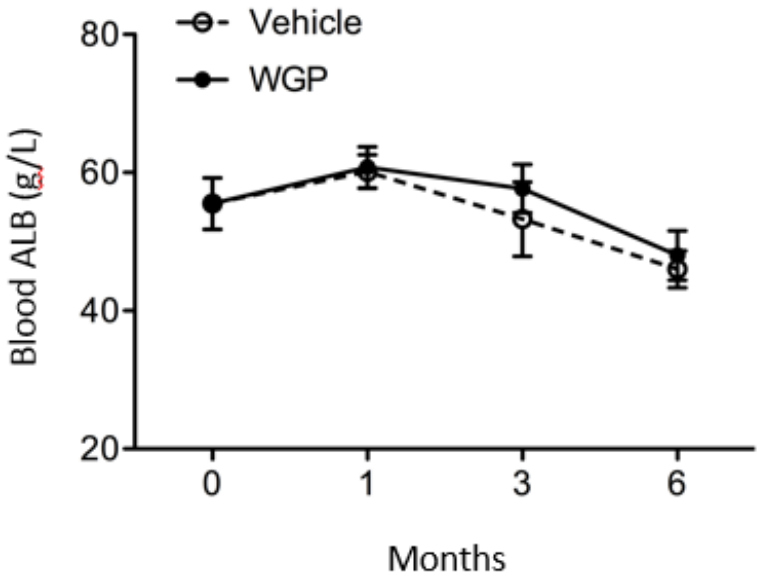


Figure 17- Serum albumin (g/L) of rats fed with rats fed with WGP compared to rats in control group (Vehicle) over 6 months. Data are presented as mean ± SD of each group (WGP: n = 4 - 10; Vehicle: n = 4 - 11).

This figure shows the overall serum albumin (ALB) levels difference between the two groups over 6 months of treatment. The ALB starts decrease at the 3rd month and appears significantly higher in WGP group (57.67 ± 3.5 g/L, n = 9) than in control group (53.25 ± 5.37 g/L, n = 8) with $p = 0.0300$ (one-tailed t-test), however, diminishing at the sixth month with an overall p-value=0.0714 (2-way ANOVA) indicating a low overall significance with overlapping standard deviations. The trajectory of serum albumin levels in both groups is declining as there may be loss in the urine due to CKD.

-Daily-intake of WGP affects serum total protein (TP) levels:

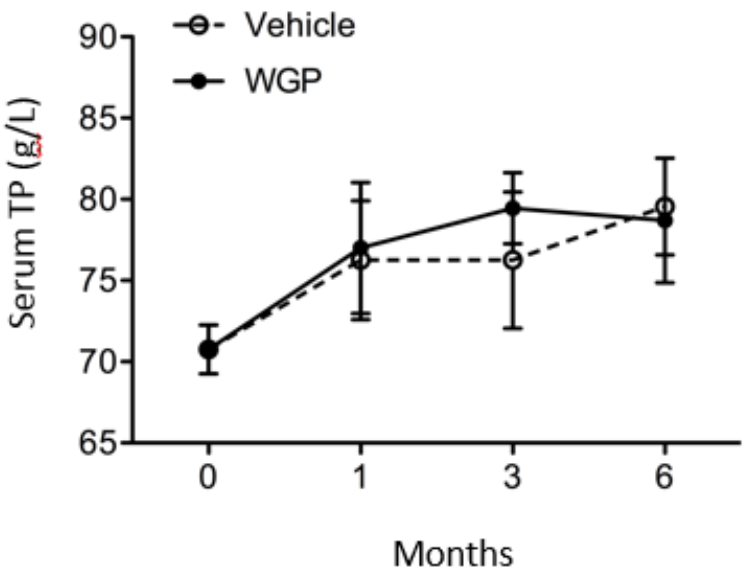


Figure 18- Serum total protein (TP) levels (g/L) in WGP group compared with control (Vehicle) over 6 months. Data are presented as mean ± SD of each group (WGP: n = 4 - 10; Vehicle: n = 4 - 11).

This figure shows the difference in TP between these two groups indicating that at the third month of treatment, the TP in WGP are significantly higher than those in control group (p = 0.0321, one-tailed t-test, n = 8 – 9) however, the data from the whole 6 months of treatment show the overlapping SD with no significant grape effect p = 0.3983 (two-way ANOVA).

-Daily intake of WGP has no significant effect on serum total bilirubin (TBIL) levels (increases with liver disease):

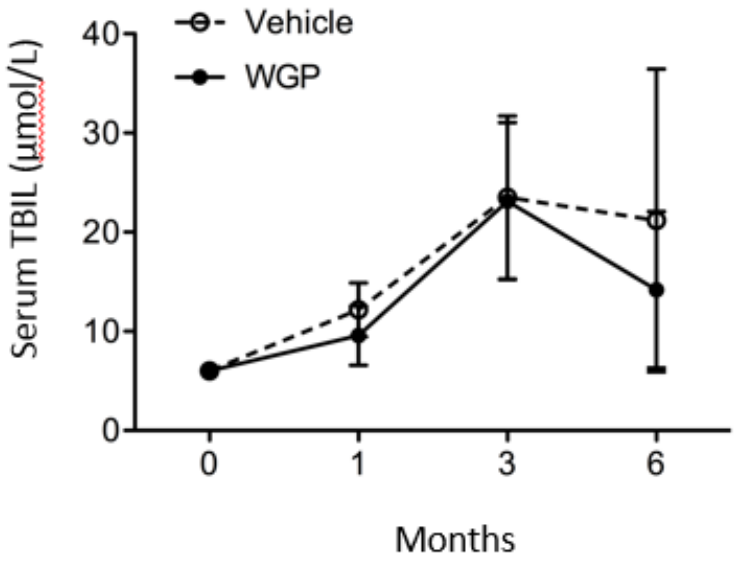


Figure 19- Serum total bilirubin (umol/L) in WGP compared with control (Vehicle) over 6 months. Data are presented as mean ± SD of each group (WGP: n = 4 - 10; Vehicle: n = 4 - 11).

This figure shows no significant difference in serum total bilirubin levels between these two groups as the curves overlap. The bilirubin levels in WGP group appear progressively lower after the third month until the sixth month, however, overlapping SD and p-value of 0.3179 indicate no significance.

3.1.1.4 Renal function

-Daily intake of WGP shows no beneficial effect on the reduction of blood creatinine:

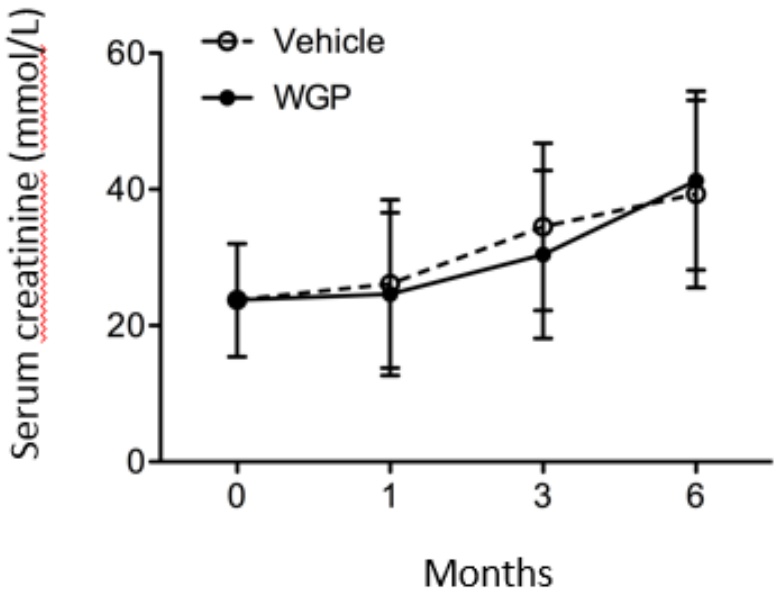


Figure 20- Serum creatinine (mmol/L) in WGP group compared with control (Vehicle) over 6 months. Data are presented as mean ± SD of each group (WGP: n = 4 - 15; Vehicle: n = 4 - 16).

This figure shows no effect of grape daily intake on serum creatinine levels as both curves overlap throughout the whole six-month period with a p-value of 0.7858 (two-way ANOVA), indicating no significance. Serum creatinine levels are increasing throughout the 6 months of treatment in both groups.

-Daily intake of WGP shows no beneficial effect on the reduction of blood urea nitrogen (BUN):

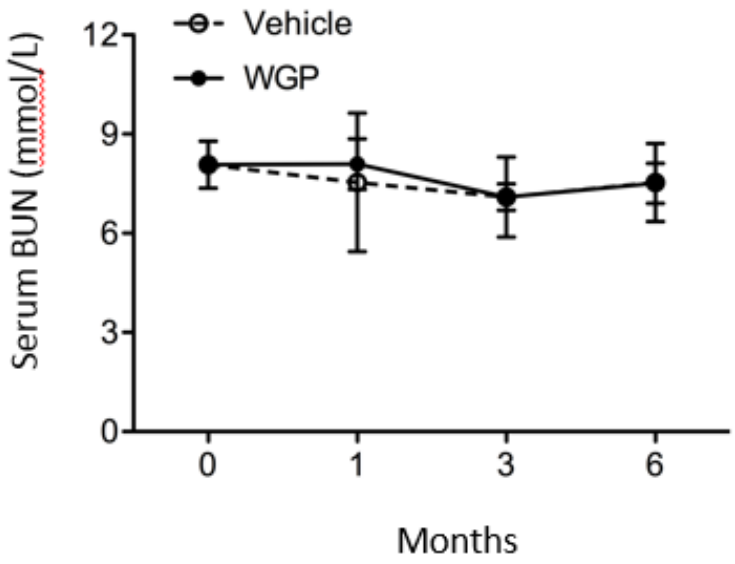


Figure 21- Serum BUN (mmol/L) of WGP group compared with control (Vehicle) over 6 months. Data are presented as mean ± SD of each group (WGP: n = 4 - 15; Vehicle: n = 4 - 16).

This figure shows no effect of grape daily intake on serum urea and nitrogen levels as both curves overlap throughout the six months with a p-value of 0.6457 (two-way ANOVA) indicating no significance. BUN levels are in a steady state throughout the whole 6-month period in both groups.

-Daily intake of WGP shows no beneficial effect on glomerular filtration rate (GFR):

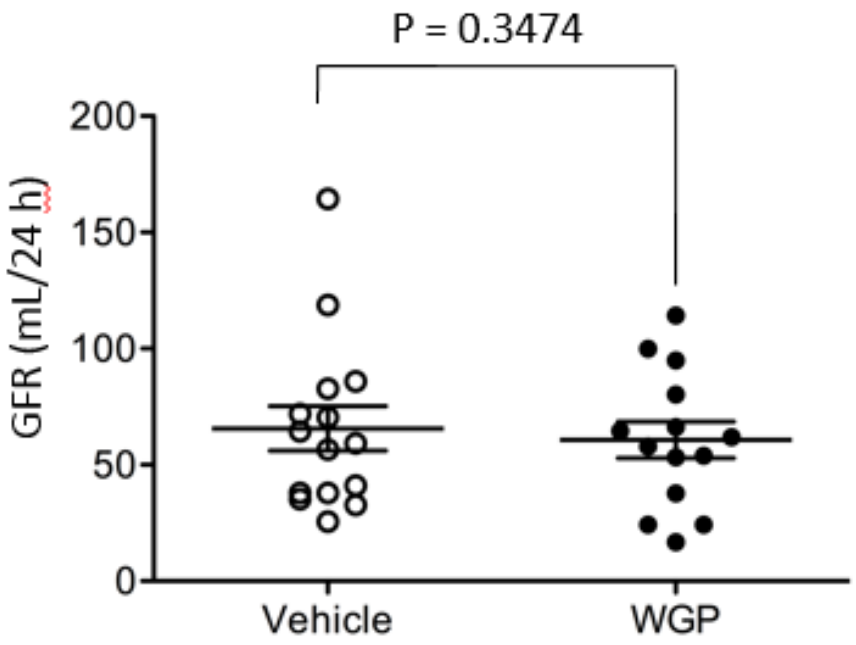


Figure 22- The GFR of WGP group and control (Vehicle) at the end of 6th month.

This figure shows no significant difference of GFR between these two groups with a p value of 0.3474 (t-test) but it is noted to be higher in the rats of control group.

-Daily intake of WGP seems to significantly decrease 24-hour urine volume at the end of 6 months of treatment:

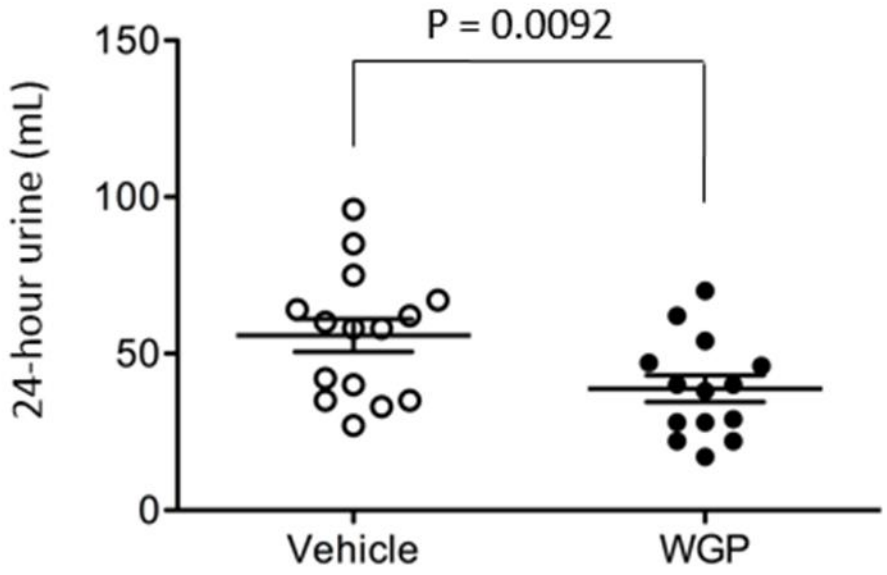


Figure 23- 24-hour urine volumes (ml) of WGP group (n = 14) compared with control (Vehicle) (n = 15) at the end of 6 months.

This figure shows the significant effect of grape daily intake on decreasing urine output in which is normally increased due to diabetes. Statistical analysis indicated significant less urine produced by rats fed with WGP compared with control p = 0.0092 (one-tailed t-test).

-Daily intake of WGP seems to significantly decrease proteinuria (urinary protein):

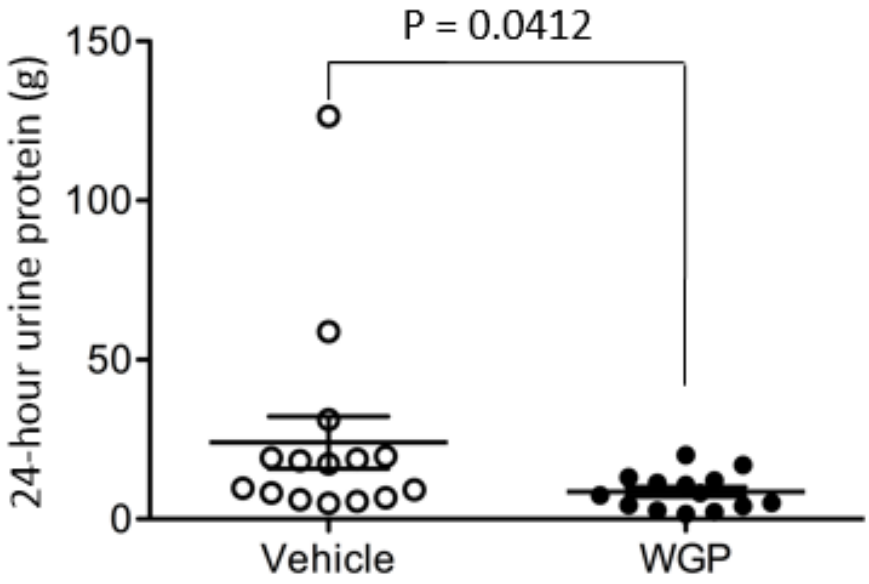


Figure 24- Urine total protein (g/day) of WGP group (n = 14) compared with control group (Vehicle) (n = 15) at the end of 6 months.

This figure shows the significant effect of grape daily intake on decreasing protein loss in urine caused by CKD. Statistical comparison of these two groups shows the, p-value of 0.0412 (one-tailed t-test).

-Daily intake of WGP seems to significantly decrease urine protein to creatinine ratio (PCR):

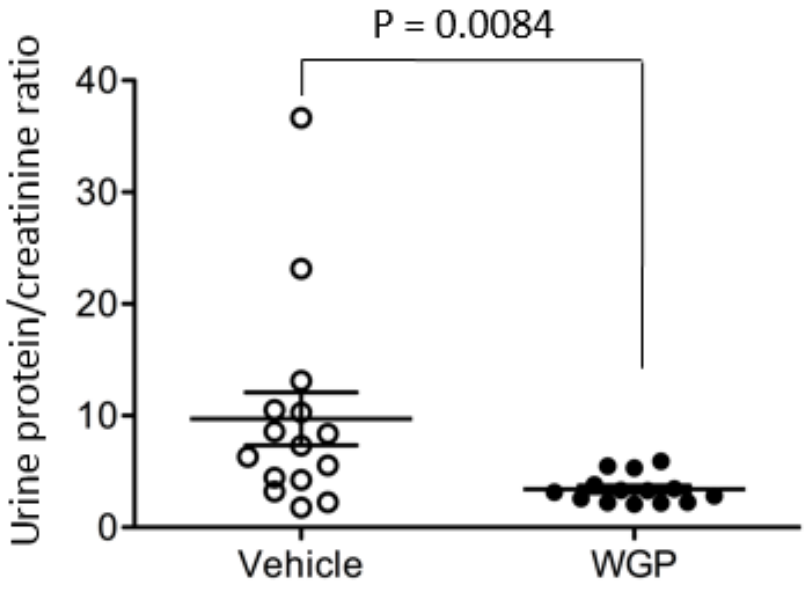


Figure 25- Urine protein to creatinine ratio (PCR) of WGP P group (n = 14) compared with control (Vehicle) group (n = 15) at the end of 6 months.

The PCR is determined to further confirm the data of proteinuria. This figure shows the significant effect of grape daily intake on decreasing urine protein to creatinine ratio (PCR) which increased in CKD. The statistical analysis indicate the, p-value of 0.0084 (one-tailed t-test) in comparison of the two groups.

3.1.2 Renal histopathology

3.1.2.1 Tubular protein casts

-Daily intake of WGP seems to significantly decrease tubular protein casts (caused by renal dysfunction):

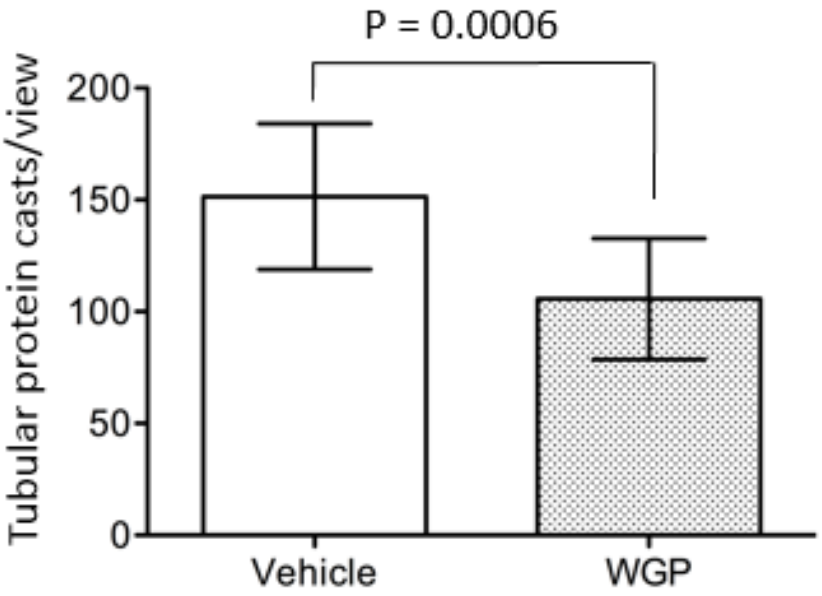


Figure 26- Averages of tubular protein casts in rats fed with WGP compared to rats in control group at the end of 6 months.

This figure shows the grape effect on the deposition of proteinaceous material in renal tubules caused from the loss and trapping of serum proteins in the kidney. The average of numbers of protein casts found in rats fed with WGP appear significantly lower compared to rats in control group, p-value of 0.0006 (2-tailed t-test).

3.1.2.2 Tubular atrophy (dilation)

-Daily intake of WGP seems to significantly decrease tubular atrophy (caused in renal dysfunction):

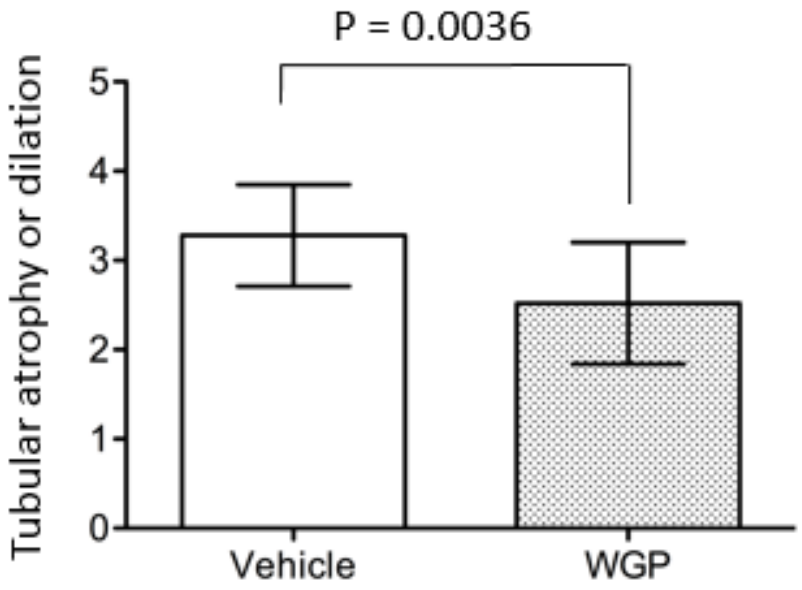


Figure 27- Average scores of tubular atrophy in WGP group compared with control.

This figure shows the grape effect on tubular cells as the average percentage of renal tubule atrophy (dilation) in the WGP-fed rat kidney tissue views significantly lower compared with Sugar rats, p-value of 0.0036 (2-tailed t-test).

3.1.2.3 Glomerular atrophy

-Daily intake of WGP seems to significantly decrease glomerular atrophy (caused by renal dysfunction):

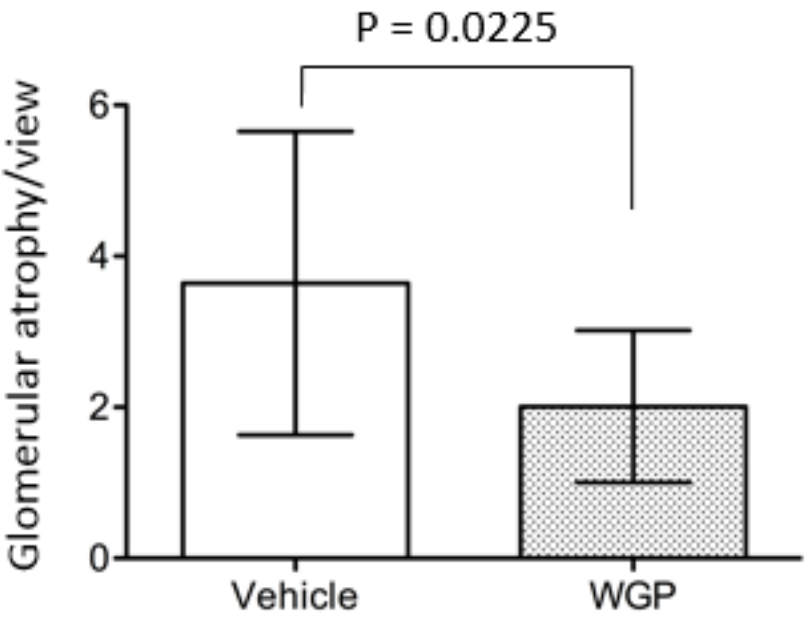


Figure 28- Averages of atrophied glomerulus in WGP group compared with control.

This figure shows the grape effect on glomerular cells. The average total number of atrophied glomeruli (dilated capsular space) due to loss of glomerular blood vessels "tuft" appear significantly lower in the WGP-fed rats compared to control rats, p-value of 0.0225 (2-tailed t-test).

3.1.2.4 Mesangial expansion

-Daily intake of WGP seems to significantly mesangial expansion (caused in renal dysfunction):

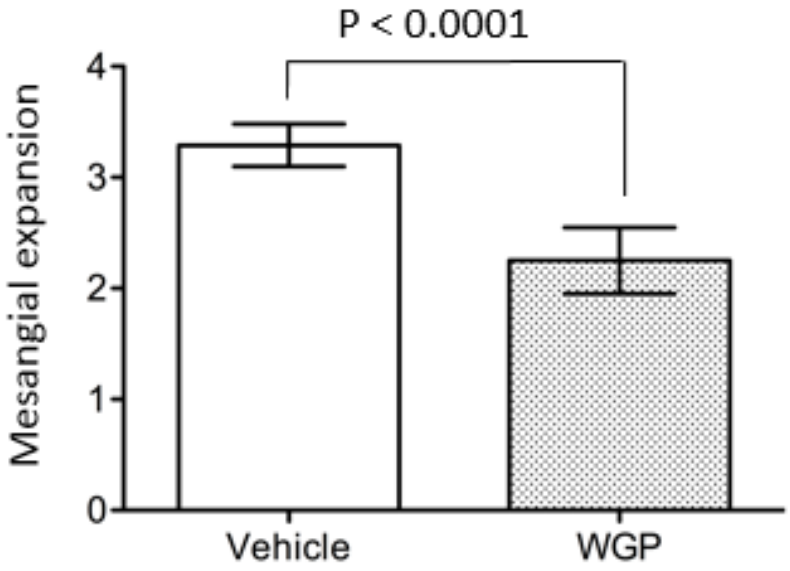


Figure 29- Average scores of mesangial expansion in glomeruli of WGP group compared with control.

This figure shows the grape effect on the glomerular cells composition (mesangial cells) as the concentration of these cells supporting glomerular blood vessels "tuft" appear significantly less in the WGP-fed rats compared with control rats, p-value <0.0001 (2-tailed t-test).

3.1.2.5 Microscopic views of kidney tissue

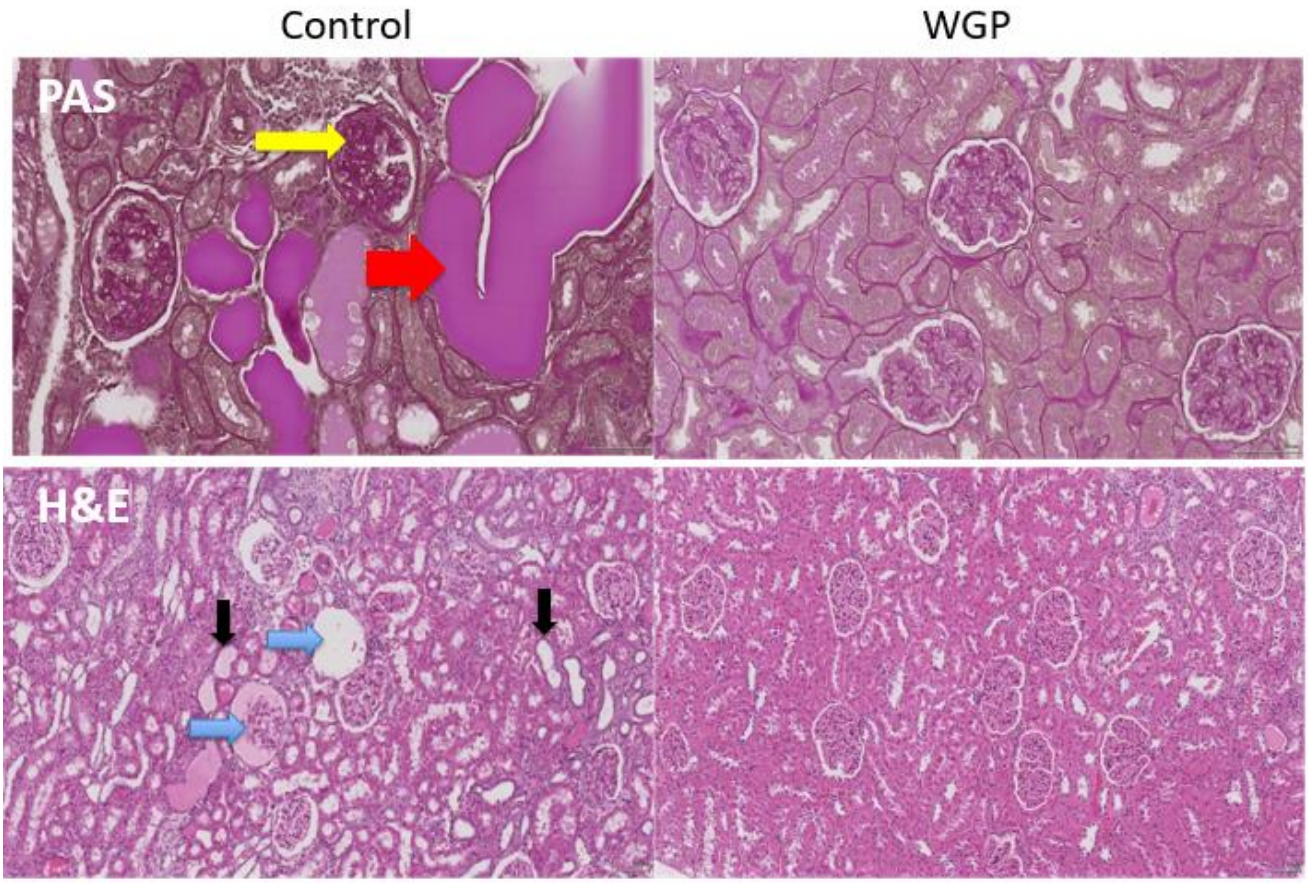


Figure 30- Typical histology microscopic views of rat kidney tissues.

This figure shows the typical changes seen in the analysis of renal histopathology (PAS and H&E stains) of kidney tissues taken from WGP-fed rats and control rats. Four diabetic-nephropathy changes were observed to be significantly less after 6 months of grape intake, as seen WGP kidney tissues in comparison with Control where tubular protein casts (red arrow), mesangial expansion (yellow arrow), glomerular atrophy (blue arrow) and tubular atrophy (black arrow) appear in higher magnitude.

3.1.3 Kidney tissue PCR array analysis of oxidative stress-related genes

-PCR array significant genes:

	Gene symbol	Gene name	P-value	Fold change	Function
1.	Dhcr24	24-dehydrocholesterol reductase	0.00222	4.265	**H2O2 scavenger**, H2O2-induced cell death protection
2.	Cyba	cytochrome b-245, alpha polypeptide	0.00875	4.215	NADPH oxidase subunit, optimizing immunity
3.	Gstk1	Glutathione S-transferase kappa 1	0.01279	1.1475	Cellular detoxification (lipid persoxide detoxification)
4.	Hmox1	Heme oxygenase (decycling) 1	0.01283	-101.533	Heme degradation→ carbon monoxide production
5.	Prdx2	Peroxiredoxin 2	0.02746	1.7625	**H2O2** and Alkyl hydroperoxide **antioxidant**
6.	Ercc6	Excision repair cross-complementing rodent repair deficiency, complementation group 6	0.02908	-3.9575	Damaged DNA repair
7.	Sod2	Superoxide dismutase 2, mitochondrial	0.02983	2.3375	Limit ROS detrimental effect during ischemia-reperfusion injury, moderate ROS release for ischemic preconditioning.
8.	Park7	Parkinson disease (autosomal recessive, early onset) 7	0.03723	1.24	Redox-sensitive chaperone
9.	Gpx4	Glutathione peroxidase 4	0.03756	1.96	**H2O2**, lipid peroxide and hydroperoxide **reduction**
10.	Gstp1	Glutathione S-transferase pi 1	0.0405	-22.4875	Cellular detoxification
11.	Txnip	Thioredoxin interacting protein	0.04436	-37.8825	Increase ROS production
12.	Gpx1	Glutathione peroxidase 1	0.04614	3.235	**H2O2 antioxidant** (one of most important antioxidant enzymes)

Table 3- PCR array analysis of rat oxidative stress-related genes significantly upregulated (green) or downregulated (brown) in Grape rats' kidneys vs. Sugar rats' kidneys.

This table lists 12 rat oxidative stress-related genes of which 8 genes were significantly upregulated (numbers in green) and 4 genes were significantly downregulated (numbers in red) in the kidneys kidneys in WGP group compared to the expression of these genes in the controls kidneys with p-values <0.05. The up-regulated genes are mostly part of the response to H2O2.

-PCR array "almost" significant genes ($p < 0.05 - 0.1$) in this limited number of samples ($n = 4$):

	Gene symbol	Gene name	P-value	Fold change	Function
1.	**Cygb**	Cytoglobin	0.05231	-6.15	Protection from hypoxia
2.	**Psmb5**	Proteasome (prosome, macropain) subunit, beta type 5	0.05309	0.165	Antioxidant, protection from oxidative damage
3.	**Fth1**	Ferritin, heavy polypeptide 1	0.06321	-1.27	Iron storage
4.	**Prdx4**	Peroxiredoxin 4	0.06649	-0.7075	Cpmpartment-specific H2O2 sensor
5.	**Prdx1**	Peroxiredoxin 1	0.06705	0.8075	Peroxide reduction, thought to regulate **H2O2** intracellular concentrations
6.	**Ccs**	Copper chaperone for superoxide dismutase	0.06717	-0.13	oxidative stress sensor
7.	**Vim**	Vimentin	0.07027	1.875	Cytoskeletal stabilization, protein detoxification
8.	**Vimp**	Selenoprotein S	0.0703	1.6175	Degradation of misfolded ER proteins
9.	**Ucp2**	Uncoupling protein 2 (mitochondrial, proton carrier)	0.07522	-1.5775	Sterol sensor, induction of sterol biosynthesis upon sterol depletion
10.	**Txn1**	Thioredoxin 1	0.08049	-4.9725	Redox-sentitive cyteine residue, oxidative stress sensor, antioxidant
11.	**Sod1**	Superoxide dismutase 1, soluble	0.08135	-0.2225	Superoxide radicals break down, detoxification
12.	**Srxn1**	Sulfiredoxin 1 homolog (S. cerevisiae)	0.0856	-4.7	Antioxidants metabolism

Table 4- PCR array analysis of rat oxidative stress-related genes "almost" significantly upregulated (green) or downregulated (brown) in the kidneys in WGP group compared vs. control kidneys.

This table lists 12 rat oxidative stress-related genes of which 4 genes were significantly upregulated (numbers in green) and 8 genes were significantly downregulated (numbers in red) in the WGP kidneys compared to the expression of these genes in the control kidneys with p-values 0.05 - 0.085.

3.2 In vitro study

3.2.1 Flow cytometry: FACS apoptosis

- 24-hour Grape extract treatment seems to significantly increase HSMP (podocytes) cell survival in the response to H2O2 treatment::

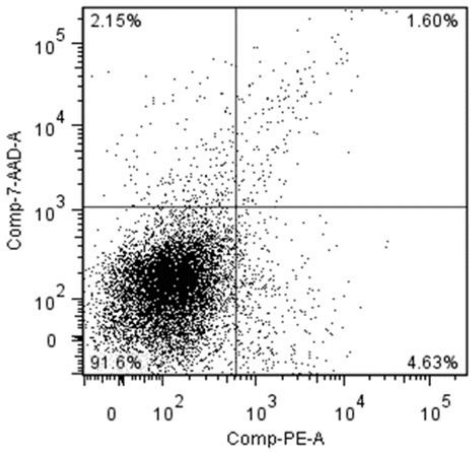
FACS experiment	1uM H2O2	1uM H2O2 + 200ug Grape
1	68	82
2	72.35	75.75
3	84.45	85.5
4	80.85	89.8
5	84.9	95.4
6	66	74
7	67.9	78
8	70.7	84
Average	74.39	83.05

Table 5- FACS live cells (%) in HSMP cells under 24-hr treatment with H2O2 (oxidative stress) and in cells treated with H2O2+Grape extract (oxidative stress+antioxidants).

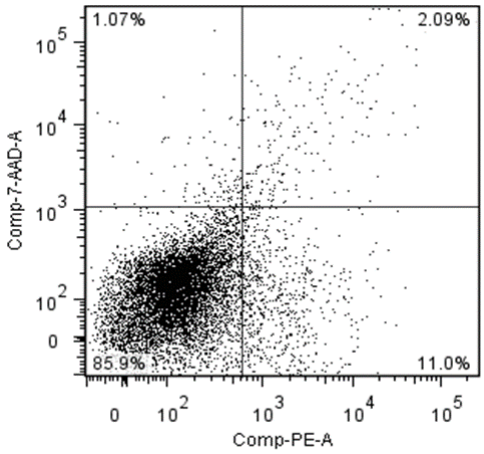
This table shows the averages of live cells percentages from 8 separate experiments in which a group of HSMP cells are treated for 24-hours with oxidative stress only and another group treated with oxidative stress plus grape extract (antioxidants). Grape extract treatment significantly increases the percentage of live cells with a p-value of 0.0185 (one-tail t-test).

- A representative graph of FACS analysis- 24-hour Grape extract treatment seems to significantly increase HSMP (podocytes) cell survival:

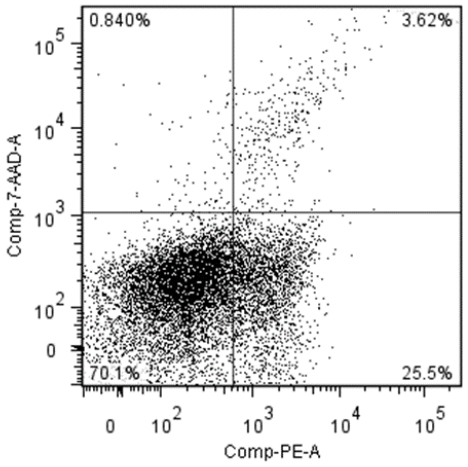
1) No Treatment: 91.6% Live cells



2) 200 µg grape extract: 85.9% Live cells



3) 1 µM H2O2: 70.1% Live cells



4) 1 µM H2O2 + 200 µg grape extract: 82.2% Live cells

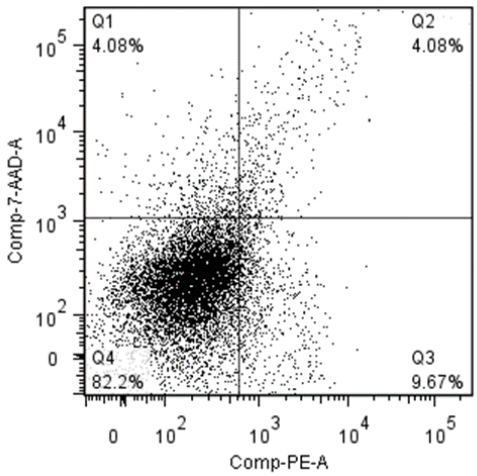


Figure 31- A representative FACS graph showing apoptosis or live HSMP cells after 24-hr treatment with: 1) No treatment. 2) Grape extract. 3) H2O2. 4) /combination of H2O2 and grape extract.

These are typical FACS graphs showing the significant antioxidant effect of grape extract treatment on the survival of HSMP (podocytes) undergoing H2O2-oxidative stress.

3.2.2 MTT assay (mitochondrial activity) results

- 24 hour of grape extract treatment seems to significantly increase HSMP (podocytes) cell viability:

MTT experiment	Cell density	Average H2O2 OD	Average H2O2+Gr OD	P=value
1	3000/well	0.1152	0.1253	0.00057 (t-test, 2 tail)
2	4000/well	0.1198	0.1539	0.00014 (t-test, 2 tail)
3	3000/well	0.1435	0.1524	0.018 (t-test, 2 tail)
4	4000/well	0.1422	0.1602	0.00029 (t-test, 2 tail)
5	3000/well	0.1093	0.1131	0.0007 (t-test, 2 tail)

Table 6- The averages of measured optical density (OD) of HSMP cells treated for 24 hours with 1 µM H2O2 and cells treated with combination of 1 µM H2O2 and 200 µg grape extract.

This table shows the significant antioxidant effect of grape extract treatment on the viability of HSMP (podocytes) undergoing H2O2-oxidative stress. The p-values highlighted in the table show the statistically different.

3.3 Chemical study

3.3.1 Antioxidant activity measurement

3.3.1.1 FRAP assay

-The grape product used in this study has antioxidant activity through iron chelation:

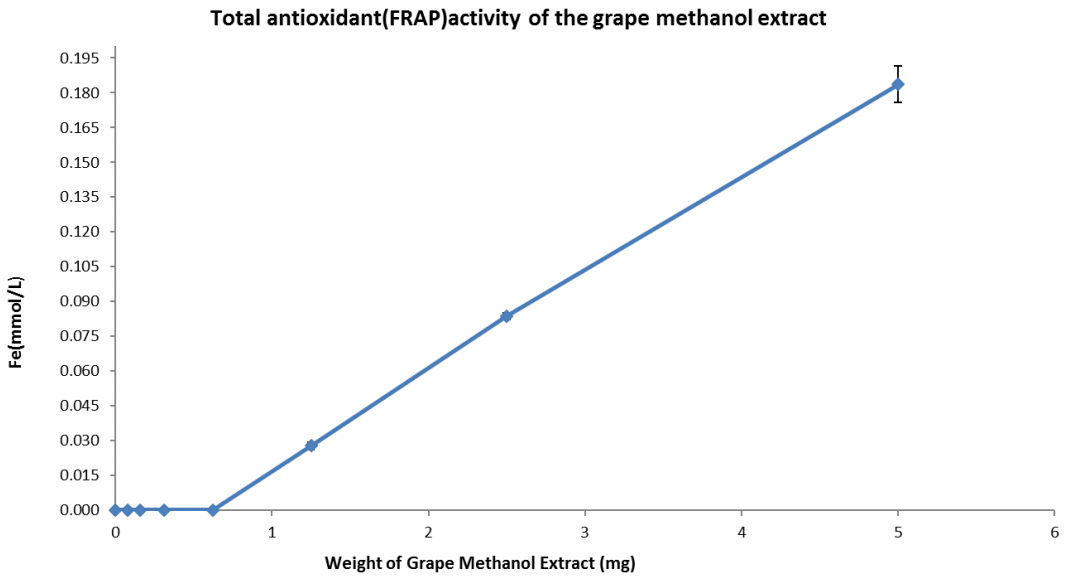


Figure 32- Antioxidant activity of Grape extract measured by FRAP assay.

This figure shows the grape methanol extract (antioxidants) activity measured by its ability to reduce toxic ferric to ferrous (mmol/L).

Volume of ascorbic acid used in assay	Absolute weight of Ascorbic Acid (mg) in 50ul from stock conc. (0.1mg/ml)	Fe (mmol/L)		
50µl	0.1 x 0.05= 0.005mg/ml= 5µg	0.045	Average	**0.0472**
		0.046	SD	0.0027
		0.050		

Table 7- Ascorbic acid FRAP antioxidant activity

This table shows the ascorbic acid antioxidant activity measured by its ability to reduce toxic ferric to ferrous (mmol/L).

3.3.1.2 DPPH assay

-The grape product used in this study has antioxidant activity through nitrogen radical scavenging:

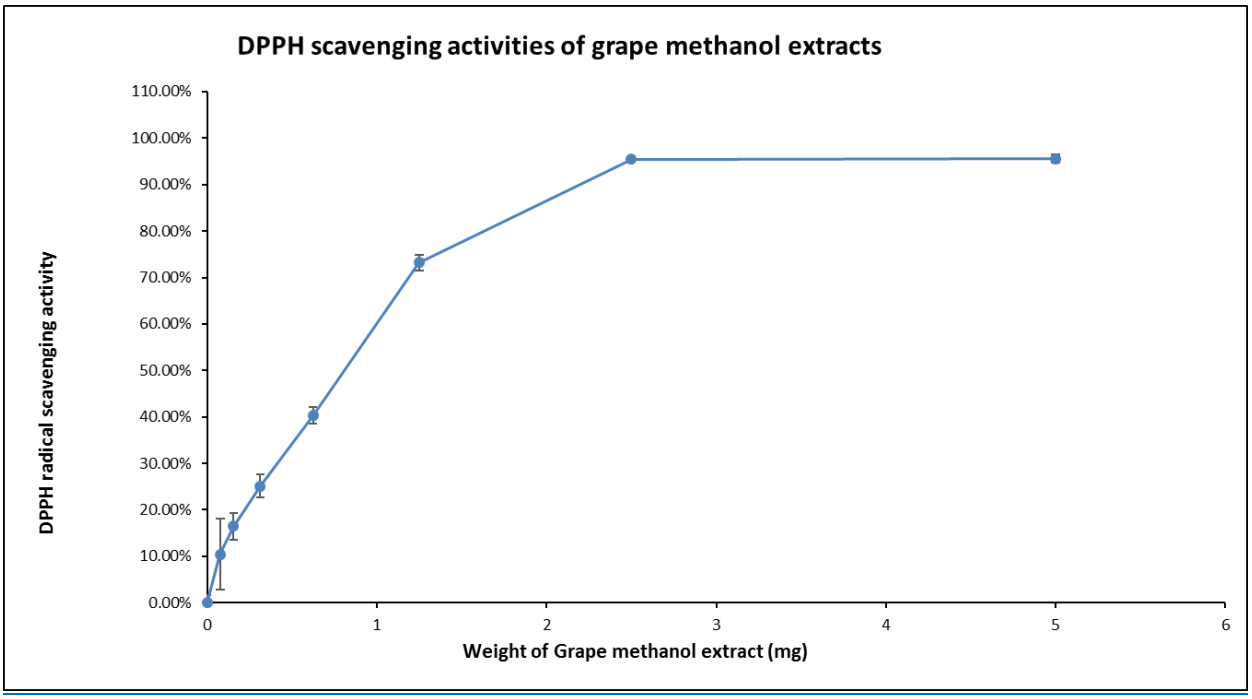


Figure 33- Antioxidant activity of Grape extract measured by DPPH assay.

This figure shows the grape methanol extract (antioxidants) activity measured by its ability to scavenge DPPH which is a nitrogen radical compound found in oxidative stress.

Volume of ascorbic acid used in assay	Absolute weight of Ascorbic Acid (mg) in 50 µl from stock conc. (0.1mg/ml)	DPPH scavenging %		
50 µl	0.1 x 0.05= 0.005 mg/ml= 5µg	89.28%	Average	**89.07%**
		88.87%	SD	0.002062
		89.07%		

Table 8- Ascorbic acid DPPH antioxidant activity

This table shows the ascorbic acid antioxidant activity measured by its ability to scavenge DPPH which is a nitrogen radical compound found in oxidative stress.

3.3.1.3 ABTS assay

-The grape product used in this study has antioxidant activity through scavenging of an H2O2-related radical:

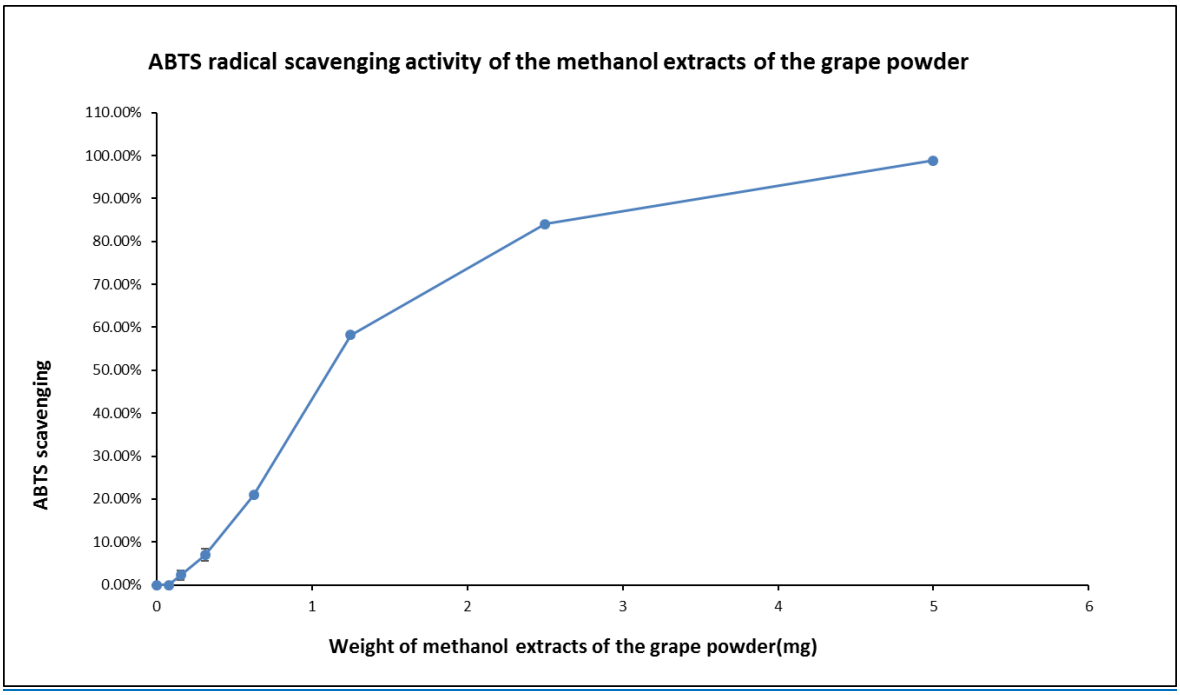


Figure 34- Antioxidant activity of Grape extract measured by ABTS assay.

This figure shows the grape methanol extract (antioxidants) activity measured by its ability to scavenge ABTS which is a ferryl myoglobin radical formed from metmyoglobin and H2O2.

Volume of ascorbic acid used in assay	Absolute weight of Ascorbic Acid (mg) in 50 µl from stock conc. (0.1mg/ml)	ABTS scavenging %		
50 µl	0.1 x 0.05= 0.005 mg/ml= 5µg	56.27%	Average	**54.60%**
		51.86%	SD	0.031169

Table 9- Ascorbic acid ABTS antioxidant activity

This table shows the ascorbic acid antioxidant activity measured by its ability to scavenge ABTS which is a ferryl myoglobin radical formed from metmyoglobin and H2O2.

Chapter 4: Discussion

4.1 In vivo:

This pilot pre-clinical study is to investigate the preventive effect of WGP on kidney disease progression associated MetS using obese ZSF-1 rats. The experiment has lasted for a duration of six months. Rats' urine and blood parameters, kidney tissue histopathological analysis and oxidative stress-related genes analyzed from rats' kidney tissue were performed. Details on the choice of our animal model and its suitability for this study purposes is presented in the methodology chapter (section 2.1.1.1).

In order to determine the daily dose of WGP ingested by our animal model of MetS with renal dysfunction (male obese ZSF-1 rat), we have done an estimation of the WGP dose each rat consumes per day using fresh grape standard serving size (3/4 cup or 126 g) as a reference. The amount of prepared food consumed from each cage in ~24 hours measured to around 500 g which contains 25 g of WGP based on the formula used to prepare the food. Each cage has two rats, assuming rats consume the food in approximately equal amounts, each rat would consume roughly 12.5 g of WGP per day. The standard serving size of fresh grape (126g) correlate to 23 g of WGP. Upon this, the estimated dose of WGP each rat ingests per day is approximately half a standard serving size of fresh grape.

4.1.1 Renal function

According to the literature, it has been established that obese ZSF-1 rats develop signs of CKD by the age of (8 weeks) and a picture of diabetic nephropathy at the age of (32 weeks) histology based on histopathological change [175].

Our data analysis of the blood and urine parameters of the rats reveals significant improvements in renal function at the end of 6-month duration of daily grape ingestion, observed through analysis of a set of parameters including GFR, 24-hour urine volumes, proteinuria and protein to creatinine ratio (PCR). There was no observation of significant improvements in eGFR levels ($p = 0.3474$) which may suggest that a clinical decline in GFR has not been reached yet as seen in an early stage of CKD where signs of kidney damage can present before GFR deterioration is appreciated [105]. In fact, decreased eGFR measurements often appear at relatively later disease stages when around 50% of nephrons has been lost [106]. Based on these findings and the most recent CKD guidelines, these obese ZSF-1 rats may show signs of early stages of CKD where an evidence of kidney damage such as proteinuria is present with normal eGFR [52]. While grape ingestion showed no effect on eGFR, protein to creatinine ratio (PCR) often used in clinics as a marker of decreased filtration was significantly decreased ($p = 0.0084$) which may suggest that grape intake actually enhances renal filtration despite not significantly appreciated GFR improvement. In fact, it has been indicated in the literature that PCR is more sensitive than GFR kidney damage marker in diabetic-related dysfunction suggesting a diabetes-induce kidney disease in our rats [64]. In addition, GFR in the early stage of CKD (Stage 1 in GFR staging system) could appear increased or normal, as a result of glomerular hyperfiltration in response to nephron loss [65], which probably explains its low relativity in reflecting decreased renal function in early stage of CKD that can be otherwise appreciated by PCR or proteinuria measurements.

In diabetes, smaller sizes of protein pass through glomerular filtration in the early stages of the disease, a condition called microalbuminuria. Therefore, it has been suggested in the literature

that albumin to creatinine ratio (ACR) is more specific as a screening test for renal dysfunction in diabetics compared to protein to creatinine ratio (PCR) since it is more specific to increased levels of albumin which may be missed with (PCR) [64]. On the other hand, it has been indicated that PCR is more sensitive that ACR as a screening test for predicting clinically relevant proteinuria [64]. Protein loss in urine known as proteinuria is a sign of kidney damage as the filtration slits between podocytes enlarge due to cellular atrophy and therefore large substances such as plasma proteins pass through and be lost in urine [51]. In this study, proteinuria measurement in 24-hour urine collections was significantly decreased by grape intake ($p = 0.0412$) suggesting improved renal injury in WGP-fed rats vs. rats in control group. Another finding pointing to the diabetic nephropathy picture of the early stage kidney disease in these rats is the significant decrease in 24-hour urine output ($p = 0.0092$) caused by grape intake. Urine output is abnormally increased in diabetes due to increased glucose in urine (glycosuria) that carries out water in urine, a symptom of diabetes called osmotic diuresis or polyuria [186]. Also, nephron loss in early stages of CKD causes a compensatory hyperfiltration in functioning nephrons leading to increased urine volume while at later stages when there is severe nephron loss urine volume is decreased consequently (Figure 5) [56].

Moreover, the histopathological analysis of structural changes in the kidney tissues of these rats denote a renal injury consistent with that of diabetic nephropathy (DN) [176] which is one of CKD types that appear as early stages of the disease [176]. These histopathological findings include renal tubular atrophy, renal protein casts, glomerular atrophy and mesangial expansion. Renal tubular atrophy caused by loss of the tubular cellular lining, and deposition of protein casts in these tubules caused by protein loss and trapping in tubular spaces were found to be

significantly less in grape-ingesting rats vs. control rats with ($p = 0.0036$) and ($p = 0.0006$) respectively. Glomerular atrophy that result from the loss of the glomerular blood vessels "tuft", and the mesangial expansion due to matrix cells enlargement in the glomerulus which fills the glomerulus with non-filtration cells were found to be significantly less in grape-ingesting rats v. control rats with ($p = 0.0225$) and ($p < 0.0001$) respectively. Mesangial expansion in the kidney lead to enlarged kidney size and hence increased organ weight index which appear in early stages of diabetic related CKD in contrast to smaller kidney sized with decreased organ index due to atrophy and loss of renal cells in more severe and later stages [103,105]. In this study, kidneys organ indexes of control rats were significantly higher than that of grape-ingesting rats ($p = 0.0107$) indicating a more severe injury. This finding may suggest that grape intake slows down progression of kidney disease in these rats.

Regarding the PCR array oxidative stress-related gene analysis of our rats' kidney tissues, the expression of 12 genes was found significantly affected ($p < 0.05$) and another 12 genes were "almost" or less significantly affected (p ranges from 0.05 to 0.085) by grape intake. Among the significantly affected genes, 8 were upregulated and 4 were downregulated. While 4 genes were upregulated and 8 were downregulated among the near significant genes. In other words, we may say 12 genes were upregulated and another 12 genes were downregulated aside from the level of significance. These genes have various functions including oxygen radicals scavenging, oxidant radical reduction, DNA damage repair as well as other functions illustrated in the results (section 3.1.3). The overall functions of the upregulated genes were involved in the antioxidant defense system while those of the downregulated genes were involved in the pro-oxidative stress system.

Particularly, a total of 6 genes were found to generally play distinctive roles in combatting H2O2-induced oxidative stress.

4.1.2 Other parameters

4.1.2.1 Liver

Apart from renal function parameter, one parameter found significantly affected by grape intake. Among all other parameters analyzed in this study, organ weight index of control rats were significantly higher compared to those of grape-ingesting rats ($p < 0.0001$) denoting larger and/or heavier livers which appear in fatty liver disease due to fat deposition in the liver tissue. Even though this finding seem to be highly significant, other liver function parameters were not found significantly affected by grape intake in this study. These parameters include serum total protein, serum bilirubin and serum albumin levels. However, serum albumin which is produced by the liver appear significantly higher in grape-ingesting rats around the third month (p value = 0.0300) as well as overall almost higher levels compared to control rats ($p = 0.0714$) indicating better liver function. The trajectory of serum albumin throughout the study duration show decreasing levels towards the 6^{th} month which may reflect the loss of albumin in the urine due to renal dysfunction (albuminuria). However, serum total protein levels do not show decreasing levels. It is not known whether this may be caused by a compensatory mechanism by the liver to produce more protein in response to its loss in the urine or/and other factors contribute to the maintenance of total protein levels in the serum. This finding is out of the scope of our study and requires further investigation regarding liver and other related organs functions.

As for serum bilirubin (TBIL) levels, it appears to be on the lower side in grape-ingesting rats vs. control rats especially towards the end of the study at the 6^{th} month which may indicate enhanced

liver function since TBIL is increased in liver disease, however, the effect is not significant ($p = 0.3179$).

4.1.2.2 Diabetes and obesity

The effect of grape intake on reducing serum blood glucose appears significant using one-tailed t-test ($p = 0.0312$) and close to significant using 2-way ANOVA ($p = 0.0709$) with overlapping SD indicating a somewhat low effect of grape-intake during 6- month duration. However, the overall lower level in grape-ingesting rats and the other significant improvements in diabetic nephropathy-like renal picture may suggest that grape could significantly reduce diabetes perhaps in a longer study duration and/or a larger sample size. According to the literature, Grape antioxidants shows significant diabetes reduction [169].

In regards to grape intake effect on obesity, it is found there in no difference between the two groups in terms of weight gain. In fact, body weights of the two groups are identical suggesting that grape consumption does not cause weight loss in obese ZSF-1 rats during the 6 months of this study.

4.2 In vitro

An insufficient glomerular filtration lead to the renal dysfunction signs such as those observed in this study including proteinuria, increased PCR and deposition of tubular protein casts. This faulty in the filtration system is largely attributed to compromised podocytes as they are responsible for the first steps of the filtration process where substances should selectively pass through to urine.

In this project, we were interested in supporting the in vivo study with a cell culture study to further investigate the antioxidant effects of whole grape in the kidney. Hence, this in vitro study was designed to examine not only the survival of podocytes cells (HSMP) but also their viability. We believe as importantly as the prevention of cell death, the cell viability status denoting a healthy well-functioning cell and reflecting the stamina of podocytes affects the overall filtration performance. Therefore, flow cytometry measuring apoptosis was done to evaluate cell survival while MTT assay measuring cells metabolism through mitochondrial activity was done to evaluate cell viability.

HSMP cells subjected to H2O2-induced oxidative stress were treated for 24-hours with whole grape extract representing antioxidants. According to the literature, the application of H2O2 in mimicking biological oxidative stress in cell cultures has been often effectively used in studies (Min Liu et. al., 2014). The H2O2 and grape treatment doses were 1 μM of H2O2 and 200 μg/ml of grape extract achieved based on optimizations using a range of doses to determine the optimal doses that are not too toxic to cells and could show appreciated effect with grape treatment. Detailed explanation of the optimization is presented in the methodology chapter (section 2.2.2).

In this study, a set of 8 FACS experiments were conducted all of which resulted in an average of ~ 10% significant increase in live cells population after 24-hour treatment with combination og grape extract and H2O2 *v*s. H2O2 alone ($p = 0.0185$, one-tailed t-test).

Mitochondrial involvement in the mechanism through with H2O2 inflicts its role in inducing cellular damage and death may suggest a more relativity and suitability of MTT assay as for measuring cell viability of cells under H2O2-induced oxidative stress as in this study, in

comparison with other cell viability measuring assays. In this study, a set of 5 MTT experiments were repetitions of 2 cell densities undergoing H2O2-induced oxidative stress and treated for 24 hours with whole grape extract. Two cell densities were used to compare and confirm results which were similar showing significant increase in metabolic (mitochondrial) activity of combination of grape extract and H2O2 treated cells *v*s. H2O2 alone in all experiments (2-tailed t-test p-value of one experiment = 0.018 and of other experiments < 0.001).

4.3 Chemical study

In order to further support the observed antioxidant effect of the whole grape used in the in vivo and in vitro studies of this project, evaluation of its antioxidant performance was examined chemically using ascorbic acid as a reference. Ascorbic acid is often used in antioxidant activity measuring assays as a reference because of its established very high antioxidants activities profile. In this study, measurement of antioxidant activity of whole grape powder was done using three assays and findings were as:

1) FRAP assay measuring the iron chelating ability of whole grape showed that FRAP antioxidant activity expressed in units of (Fe 0.045 mmol/l) is reached at 1.7 – 1.8 mg of whole grape methanol extract which is equivalent to the 5 μg of ascorbic acid used as a reference.

2) DPPH assay measuring nitrogen radical reduction ability of whole grape showed that 89.07 % antioxidant activity is reached at 2 mg of whole grape methanol extract which is equivalent to the 5 μg of ascorbic acid.

3) ABTS assay measuring H2O2-related radical scavenging ability of whole grape showed that 54.06 % antioxidant activity is reached at 1.2 mg of whole grape methanol extract which is equivalent to the 5 μg of ascorbic acid.

Chapter 5: Conclusion

Based on our findings from this pilot in vivo study, we may conclude that daily intake of whole grape for six months seems to have a protective effect on kidney function in a metabolic syndrome animal model (obese ZSF-1 rats) through slowing down renal injury in Diabetic nephropathy (early stages of CKD) indicated by decreased proteinuria (protein loss in urine), urine protein to creatinine ratio (PCR), diabetic uremia (increased urine volumes), tubular and glomerular injury as well as affecting the expression of genes related to antioxidant defense.

According to our in vitro study results, we may conclude that whole grape extract seems to have a protective effect on kidney cells (podocytes- HSMP) from H2O2-induced oxidative stress indicated by not only increased cell survival observed by FACS apoptosis analysis but also enhanced cell viability (health) observed by elevated metabolic activity measured by MTT assay.

The chemical examination of the antioxidant activity of the whole grape product used in this study reveal its ability to participate in oxidative stress reduction through metal chelating measured by FRAP, nitrogen radical scavenging measured by DPPH and H2O2-related radical scavenging measured by ABTS.

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