AMERICAN UNIVERSITY OF BEIRUT

# THE EFFECT OF CHRONIC CIGARETTE SMOKING ON THE KIDNEYS IN MALE MICE AND IN OVARIECTOMIZED AND NON OVARIECTOMIZED FEMALE MICE

by
TANIA HUSSEIN EL MOKDAD

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Submitted in partial fulfillment of the requirements
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# ABSTRACT

# OF THE THESIS OF

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Title: THE EFFECT OF CHRONIC CIGARETTE SMOKING ON THE KIDNEYS IN MALE MICE AND OVARIECTOMIZED AND NON OVARIECTOMIZED FEMALE MICE

Chronic Cigarette Smoking (CS), a well-known risk factor for numerous diseases, has been shown to play a significant influence in renal disorders in both females and males. The progression of chronic kidney disease (CKD) may change depending on gender. According to national US reports, men died from CKD at a higher rate than women in most states. In accordance, premenopausal women are less prone to CKD due to the presence of estrogen. Estrogen is known to be nephroprotective and exerts its effect on several aspects. The objective of this study is to examine the effects of CS on the kidneys and to assess the involvement of estrogen in that regard. Age-matched C57BL/6J female and male mice were divided into 6 groups: 1) Male control group; 2) Male CS group; 3) Female control group; 4) Female CS group, and 5) Ovariectomized female CS group. Of note, there was also an ovariectomized control group but unfortunately it didn't work on the molecular level. Cardiac systolic function was evaluated, and kidneys were subjected to histological and molecular analysis. Hemodynamic cardiac assessment revealed an enhanced heart workload characterized by a marked elevation in blood pressure and cardiac output in ovariectomized female group. Our renal findings indicate that females are more prone to morphological alterations and fibrosis following CS as evidenced by a significant increase in bowman's capsule area, a remarkable retraction in the glomerular capillary area, and a remarkable decrease in the proximal tubule area in both CS females and CS ovariectomized groups. On the other hand, these parameters weren't affected following CS in males. Moreover, CS female and CS ovariectomized female groups exhibited a significant increase in renal fibrosis paralleled with a decrease in renal mRNA expression of MMP-2 and MMP-9 levels. These expressions were not observed in CS male group. In addition, there was a significant decrease in α-smooth muscle actin (α-SMA) protein level, a renal pro-fibrotic marker, in males. However, renal inflammatory assessment showed that CS males are more prone to inflammation with a marked decrease in the anti-inflammatory IL-4 protein levels and a substantial rise in the pro-inflammatory TNF-α mRNA expression levels. In Conclusion, following 8 weeks of CS, the female kidneys seem to experience prominent morphological alterations while males seem to have an imbalanced inflammatory profile. The ovariectomized CS females were hemodynamically affected two days post-CS cessation in contrast to cigarette smoking females group. However, at the structural and molecular level of the kidneys, the estrogen effect seems to be masked in the presence of CS as such results were more likely consistent with the ovariectomized CS female.

TABLE OF CONTENTS

# ILLUSTRATIONS

Figure

# TABLES

Table

# CHAPTER I

# INTRODUCTION

## A. Renal Physiology

The renal system plays several vital functions in the maintenance of body homeostasis. It filters about 200 liters of fluid each day and controls electrolyte composition by eliminating toxins, metabolic waste products, and excess ions from the blood. Besides its role in regulating plasma osmolarity, the kidneys regulate blood pressure by producing renin, a vital enzyme of the renin-angiotensin system (RAS) [1] [2]. It also produces erythropoietin, a stimulator of red blood cell production, and ensures long-term acid-base equilibrium. The renal system however is not an independent system. Normal functioning of the kidneys is interconnected with cardiac physiology. The heart, by regulating end-organ perfusion, and the kidneys, through their effect on body fluid control hemodynamic stability. For instance, any change in renal perfusion (i.e. drop in cardiac output) will be noticed and adjusted by renal feedback mechanisms [3]. The key component of blood pressure homeostasis is the renal sodium excretion's control over extracellular fluid volume. Indeed neurohormonal activity, which includes numerous physiological systems such as the natriuretic peptide (NP) system, the autonomic nervous system (ANS), and the renin-angiotensin system (RAS), is one of the most essential contributors to hemodynamic body stability [3]. In terms of arterial pressure, it is known that it is regulated by two main compensation processes known as short and long term [4]. The short term regulatory response involves the baroreceptor reflex, which

immediately responds to changes in arterial pressure via baroreceptors to trigger appropriate systemic response. These baroreceptors are found in the aortic arch and carotid sinus walls [5]. Long-term control, on the other hand, is dependent on the interaction of hormones [3]. Elevating low arterial pressure, for example, is accomplished by the stimulation of both sympathetic activity and the renin-angiotensin system while decreasing atrial natriuretic peptide secretion [4]. The renin-angiotensin system (RAS) is a potent regulator of blood volume and systemic vascular resistance [6]. Renin, a glycoprotein hormone, is secreted by the renal afferent arteriole of the juxtaglomerular cells (JC). Renin secretion is regulated by neuronal renal baroreceptors and salt transport to the macula densa. Consequently, renin can be released immediately in response to a drop in blood pressure and activation of sympathetic nerve cells to the the $\beta$1-adrenoreceptors, or indirectly in reaction to a low sodium condition in the distal convoluted tubule recognized by the macula densa [7]. Renin converts angiotensinogen, a protein generated by the liver, into angiotensin I (AgI) [8]. In turn, AgI will be transformed into angiotensin II (AgII) by a circulating plasma enzyme known as angiotensin converting enzyme (ACE) [9] [10]. Angiotensin type-1 receptor (AT1R) and angiotensin type-2 receptor (AT2R) are two types of receptors involved. When AgII binds to AT1R, it causes vasoconstriction of the efferent arteriole, salt retention, and aldosterone production from the adrenal gland [11]. Antidiuretic hormone vasopressin is produced by the hypothalamus and stored in the posterior pituitary gland. It gets secreted in response to low blood volume to maintain extracellular fluid [12] [13]. Natriuretic peptides, on the other hand, suppress ADH release centrally while peripherally shutting down the renin-angiotensin-aldosterone pathway, promoting natriuresis and vasodilation [14].

## B. Tobacco Smoking

Tobacco smoking, a well-known global health concern, contributes to a range of diseases and early mortality. Admittedly, tobacco, in all of its forms, is hazardous [15], and cigarette smoking is by far the most common kind of tobacco use in the globe. Smoking has a significant impact on the renal, cardiovascular, pulmonary, and genitourinary systems [16, 17]. Smokers grow addicted to smoking as a result of their physical and psychological reliance [18]. The pathological and physiological effects that affect the body through a variety of pathways that lead to addiction are primarily due to nicotine [19] [20] [21]. Indeed, nicotinic receptors have been demonstrated to influence organ systems, cell division, and apoptosis. In addition to that, nicotine can enhance reactive oxygen species release, inflammation, and fibrosis [19] [22]. Tobacco consists about 4,000 gases and particles, some of which are hazardous to the kidneys [15].

### *1. Epidemiology*

Tobacco usage is one of the most serious public health challenges facing the globe today. About 1 in 4 adults use tobacco globally [23]. There is an estimation that the average daily cigarette smoking ranges between 20 and 25 cigarette per day [24]. According to the World Health Organization (WHO), over than 80% of the 1.3 billion current smokers, reside in third-world countries. In the $20^{th}$ century, the estimate death of tobacco-related diseases was 100 million people, the bulk of which happened in industrialized countries [25]. According to evaluations from the World Health Organization and Institute for Health Metrics and Evaluation, smoking causes an estimated 8 million premature deaths yearly, or 22,000 fatalities each day. Smoking is responsible for 15% of all global fatalities. For instance, 7 million of these deaths are

directly connected to tobacco use. On the other hand, almost 1.2 million of these deaths are non-smokers who died as a result of second-hand smoke exposure [23] [26]. According to the CDC, nearly 16 million Americans suffer from a smoking-related illness [27]. Smoking is damaging to almost all of our bodies' processes [27]. When smoking-attributable mortality by age is considered, it is shown that the elderly group is the most vulnerable. The mortality toll is significantly higher among individuals above the age of 70, followed by those aged 50 to 69 [28]. According to age-standardized estimations of current tobacco use data by nation for 2022, 42.6% of Lebanon's entire population smokes. Moreover, Lebanon ranked sixth among the ten nations with the highest smoking rates.

### C. **Cigarette Smoking Associated Kidney Damage**

Chronic kidney disease (CKD) is a worldwide health burden, due to its high rates of morbidity, mortality, and medical costs [29]. Patients with CKD are at substantial risk of acquiring end-stage renal disease as well as a number of concomitant conditions, including a 20–30 fold elevation in cardiovascular disease risk. The systemic ramifications of oxidative stress, inflammation, and the accumulation of uremic toxins all are factors in the serious complications associated with CKD diagnosis [30]. Studies done on humans by Júnior et al. and Burton et al. revealed that smokers who smoke more than 15 cigarettes per day, are at a high risk of developing chronic kidney disease [31] [32]. This is due to several features which include oxidative stress that is prevalent in smokers and in patients having early onset of CKD [33].

D. **Indirect Impact of Cigarette Smoking on the Kidney**

In view of the vital importance of blood pressure on the advancement of renal disease, the impact of smoking on blood pressure is quite interesting to nephrologists. For instance, smoking causes an immediate rise in heart rate and blood pressure that is accompanied by direct increase in plasma catecholamines (i.e epinephrine and norepinephrine), or indirect mediated increase in local angiotensin II concentrations, which is thought to be the result of adrenergic nerve system stimulation [34] [35]. Reduced norepinephrine reuptake, adrenal gland stimulation, and decreased clearance of catecholamine contribute to the acute hemodynamic effect of smoking [36]. Vasoconstriction has been observed in various arterial beds. It has been determined that smoking causes renal afferent arterioles vasoconstriction in healthy individuals, likely shielding the glomerulus from the transient elevation in blood pressure [37]. Meanwhile, there is a concurrent spectacular drop in central sympathetic nerve activity through the activity of the baro-reflex [38]. It was anticipated that smoking may cause overt sympathetic activation in people with reduced baroreflex function [39]. In contrast, chronic smoking causes long –term endothelial cell dysfunction. This is reflected as a reduction in endothelial cell-dependent vasodilation, nitric oxide availability, and intimal cell hyperplasia [40]. Smoking is linked to lower eGFR or a higher chance of developing chronic kidney disease (CKD) in healthy individuals [41]. Due to the functional abnormalities of the renal vasculature after the elevation in plasma endothelin concentrations, Gambaro et al. demonstrated that in chronic smokers there was a lower renal plasma flow than in non-smokers [42]. This renal hypoperfusion is the one responsible for the development of acute kidney injury (AKI). Prerenal AKI may progress into cell injury known as ischemic acute tubular necrosis. As a compensatory mechanism,

this will result in glomerular hypertrophy and hyperfiltration. After injury, epithelial cells undergo structural alterations or cell death that lead to epithelial cells infiltration releasing inflammatory mediator and endothelial activation [43]. Mitochondria are principal generators of reactive oxygen species in the states of ischemia and reperfusion [44]. Ischemic damage also increases the manifestation of proinflammatory cytokines and attracts phagocytes, which produce ROS. ROS, in turn, affect renal vascular resistance and vasoconstriction [45]. In contrast, S Ljungman et al. revealed that GFR remained the same during the rise in blood pressure due to the autoregulatory mechanism of the kidney. Consequently, this led to a reduced renal blood flow and an elevation in renal vascular resistance [46].

### *E. **Direct Impact of Cigarette Smoking on the Kidney***

#### 1. ***Cellular and Molecular Level***

##### *a. **Oxidative Stress***

During physiological and pathological conditions, mitochondria are primarily responsible for the production of reactive oxygen species (ROS) as a byproduct of oxygen metabolism [47] [48]. Mitochondria are the most prolific generators of oxygen radicals, which makes kidneys more vulnerable to oxidative stress-induced damage [49]. Normal physiologic levels of ROS present inside the kidney cells are needed to act as a signaling molecules to adapt to the stress and are required for basic biological processes such as proliferation, growth, and cell survival [50]. On the other hand, under pathological conditions, excess production of ROS and suppression of antioxidant mechanism results in oxidative damage [51] [52]. For instance, exogenous and endogenous stimuli contribute to a great increase in ROS production which in turn result in protein alteration,

DNA damage, cellular senescence, apoptosis, and further types of intracellular damage that are harmful to cell structure and function [50] [53].

The composition of cigarette smoke is complex, comprising an extensive array of chemical compounds, estimated to be more than 4000 in total. Some of which include free radicals, long-lived radicals, and other oxidants that impair intracellular antioxidant systems in addition to inducing intracellular ROS [54]. Free radicals react with superoxide and NO leading to a decrease in NO availability (i.e. antioxidant) along with generating peroxynitrite. This Excess ROS can reduce tubules Na reabsorption, alter gene expression, increase glomerular basement membrane permeability, and induce cellular death.

According to a study conducted on smokers, passive smokers, and nonsmokers, it was shown that a decline in antioxidant capacity in smokers is connected with an increase in oxidant and free radical generation [55]. Rezonzew et al. showed in a study done on eight week old male Sprague-Dawley rats that nicotine via binding to the 7nAChR subunit of renal nicotinic receptors, oxidative stress increases [56].

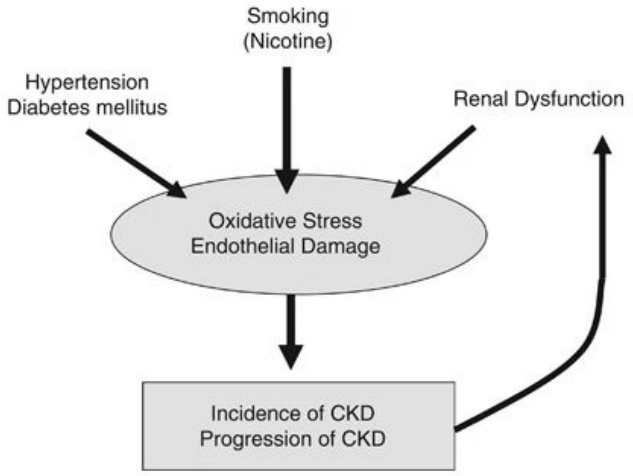



**Figure 1.** *Relationship between smoking and CKD [33].*

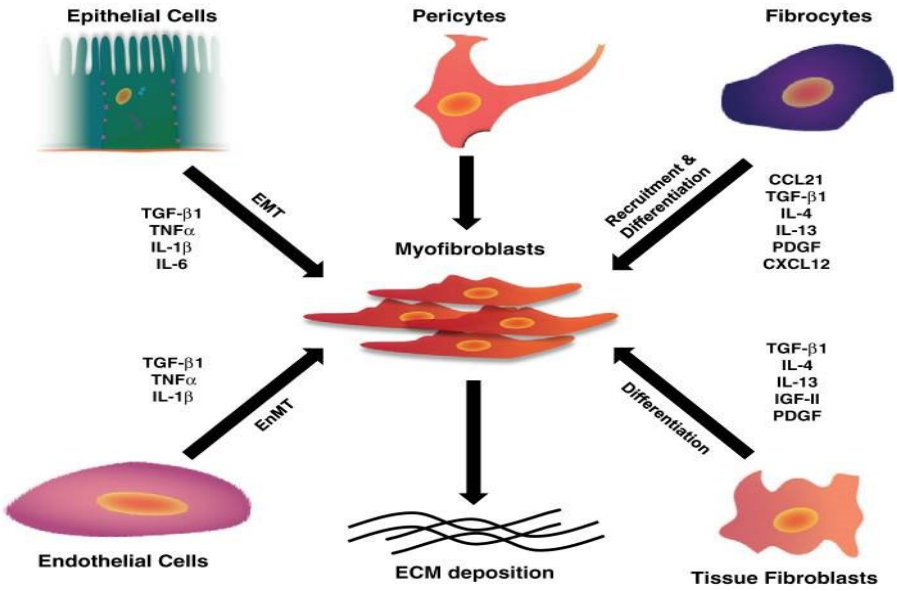
b. Inflammation

Irrespective of the underlying mechanisms, chronic renal disease is frequently associated with an inflammatory response. Cigarette smoking with its different constituents, can carry immunosuppressive and immunostimulatory effects. Indeed cigarette smoke is divided into two phases based on particle size: the vapor phase and the particulate phase; however, prolonged intake of the vapor phase has no effect on immunological response [57]. As a result, the particulate phase of cigarette smoke plays an essential role in the immunosuppression caused by cigarette smoking. Sopori et al. showed that nicotine is the substance that is responsible for the suppression of both adaptive and innate immune response [58]. In accordance with Sopori's study, others confirm his findings by stating that chronic cigarette smoke exposure in mice and rats alters T-cell responsiveness. This could explain why people and other animals exposed to cigarette smoke exhibit lower T-cell proliferative and T-dependent antibody responses [59]. Cigarette smoking augments the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF- α), interleukin (IL)-1, IL-6, and IL-8 in healthy active smokers [60]. Higher levels of C-reactive protein (CRP) have been also demonstrated in current smokers relative to nonsmokers. The serum levels of TNF- positively correlated with tobacco smoke exposure [61]. These pro-inflammatory effects are primarily linked to the activation of the NF-kB pathway. One study has revealed a significant increase in renal endothelin-1, and C-reactive protein in the streptozotocin (STZ) rat model of diabetes when exposed to 4 weeks of exposure to cigarette smoking (1h daily for 6 d/week) [62].

c. Fibrosis

Fibrosis result from interchangeable events that include increased inflammation, oxidative stress, and neurohormonal activation, resulting in renal stiffness and loss of function. Primarily, the outcome of all progressive kidney diseases is represented by interstitial fibrosis. Infiltration of inflammatory cells such as macrophages monocytes, lymphocytes, and dendritic cells, as well as the production of danger chemicals such as ROS and profibrotic cytokines, provide a platform for the activation and proliferation of matrix-producing cells, mostly fibroblasts [63]. Nicotine causes damage to the epithelium and the endothelium, is pro-inflammatory and pro-oxidant, and activates fibroblasts in numerous organs [35]. In a rodent nephrectomy model, cigarette smoking exposure has been shown to accelerate tissue kidney injury. This is in accordance with enhanced expression of fibrotic markers and suppressed expression of the anti-fibrotic microRNA miR-29b-3 [64]. Many mechanisms vital to kidney fibrogenesis are damaged by smoking, including oxidative stress, endothelial function, activation of growth factors such as endothelin-1 and angiotensin-II, altered insulin resistance, and lipoprotein metabolism.

**Figure 2.** *The origin of the myofibroblasts during fibrosis [65].*



d. Apoptosis

A delicate balance between proliferation and death determines the number of cells in each organ. Apoptosis, a programmed cell death process, plays a central role in the physiological processes underlying kidney growth and remodeling. Indeed, it is controlled by members of BCL-2 protein family which controls the penetrability of the outer mitochondrial membrane. For example, BAX, a member of the BCL-2 family, is the one responsible for apoptosis initiation after attaching to the mitochondrial outer membrane, releasing apoptotic proteins, and changing its permeability [66]. The balance between the anti-apoptotic BCL-2 protein and the pro-apoptotic BAX protein is crucial for commencing the apoptotic pathway. BAX increases and enables organisms to eliminate surplus or faulty cells [67]. As a result, apoptosis is mostly prevalent in tissues that are growing, and it may be important for the recovery period of inflammatory diseases like glomerulonephritis [68]. Of note, proximal tubular epithelial cells are extremely vulnerable to cell death contributing to renal failure. A study done by Seong Kim et al. on human proximal epithelial (HK-2) cells that were treated with 200 micrometer of nicotine. HK-2 cells contain nicotine acetylcholine receptors (nAChRs) that were examined along with intracellular levels of ROS and downstream signaling pathway [56]. As a result, nicotine induced cell death in HK-2 cells via nAChRs after contributing to an increase in ROS levels and activation of downstream signaling pathways. This change causes an increase in a dose dependent manner in BAX/BCL-2 ratio [67].

## F. **CS and Gender Bias**

The behavior of men and women in smoking differs and is reflected in several ways [69]. Despite variations in general incidence rates throughout time, men tend to use tobacco products more than women in order to reinforce effects of nicotine [70] [71]. In 2015, it was shown that the prevalence of current smoking is almost 16.7% in men compared to 13.6% in women [72]. WHO estimates that about 9% of women smoke compared to over 40% of men, and that tobacco use peaks for both men and women between the ages of 45 and 54 and 55 and 64, respectively [23]. About 35% of males in affluent countries and 50% of men in developing countries smoke, totaling approximately one billion. In contrast, a total of 250 million women in the world smoke including 22% in developed countries and 9% in developing countries [73] [74] [75]. Furthermore, according to the World Bank, women account for 2.15 million of the 8.71 million annual cigarette fatalities, with 71% living in low- and middle-income countries [76]. As previously mentioned, smoking is a significant contributor for the progression of CKD [77]. Multiple years of studies have demonstrated a sex-dependent trend in CKD prevalence. According to Silbiger and Neugarten, the fundamental processes for gender disparity include the differences in glomerular shape, glomerular hemodynamics, diet variance, synthesis and activity of local cytokines and hormones [78]. Many clinical and experimental investigations have been conducted to investigate the positive effects of estrogen on the kidney [79]. Shin Young et al. demonstrated in a recent study that women have a slower progression into end renal stage diseases than men [80]. However, postmenopausal women aged 50 years and above become more prone than men due to the wearing off estrogen's protective effect [81]. For that, the nephro-protective effect of

the estrogen hormone has been primarily designated to the gender disparity in the development of CKD [82].

## G. **Estrogen**

Estrogen is the principal female sex hormones which performs critical effects in reproductive and non-reproductive systems [83]. To elaborate more, it is essential in the development and physiology of numerous organ systems, including the, cardiovascular, endocrine, reproductive, respiratory, and neurological systems [84]. In addition, estrogen is known to be found in three major types including estrone (E1), 17β-estradiol (estradiol or E2), and estriol (E3). Each estrogen type delivers a different result from cholesterol via a series of events that occur throughout estrogen production. Estradiol (E2) is the most potent estrogen during a woman's premenopausal era that is produced and secreted by the ovaries [85] [86] [87] [88] . Furthermore, extragonadal sites have been shown to take up and transform circulating precursor chemicals such dehydroepiandrosterone sulfate (DHEAS) and estrone sulfate (E1S) to dehydroepiandrosterone (DHEA) and estrone (E1) [89] [90] [91].

### *1. **Estrogen Receptors***

In physiological and pathophysiological conditions, estrogen exerts its activity through the nuclear estrogen receptor (ER) which are ligand-dependent transcriptional regulators [92]. These receptors can be also found in the cytoplasm and mitochondria [93]. Estrogen receptors are divided into two subtypes including ER-α and ER-β [94] [95]. Both receptors share 56% and 95% homology in their ligand binding domains and DNA binding domain, respectively. This will lead to different biological effects [85] [96].

The two primary mechanisms through which estradiol can act through ERs are genomic (nuclear) and nongenomic (cytoplasmic) signaling, as illustrated in figure 3[97] [98] [99]. Regarding genomic signaling, estrogen cytoplasmic receptors ER-α or ER-β , where they dimerize and bind to specific response elements called estrogen response elements (EREs) found in the promoters of target genes to carry out ER action after translocating to the nucleus [100] [101] . Usually this pathway takes hours to occur [102]. In addition to that, they bind to transcription factors for instance vascular endothelial growth factor (VEGF) to activate its transcription. As for the non-genomic pathway, that takes place within minutes are dependent on estrogen receptors activating gene expression [103]. One of the primary estrogen-sensitive receptors known as GPR30 [104]. Once E2 binds to its receptors, MAPK pathway is activated leading to enhancement of eNOS gene expression. This ultimately indirectly modifies gene expression [103].

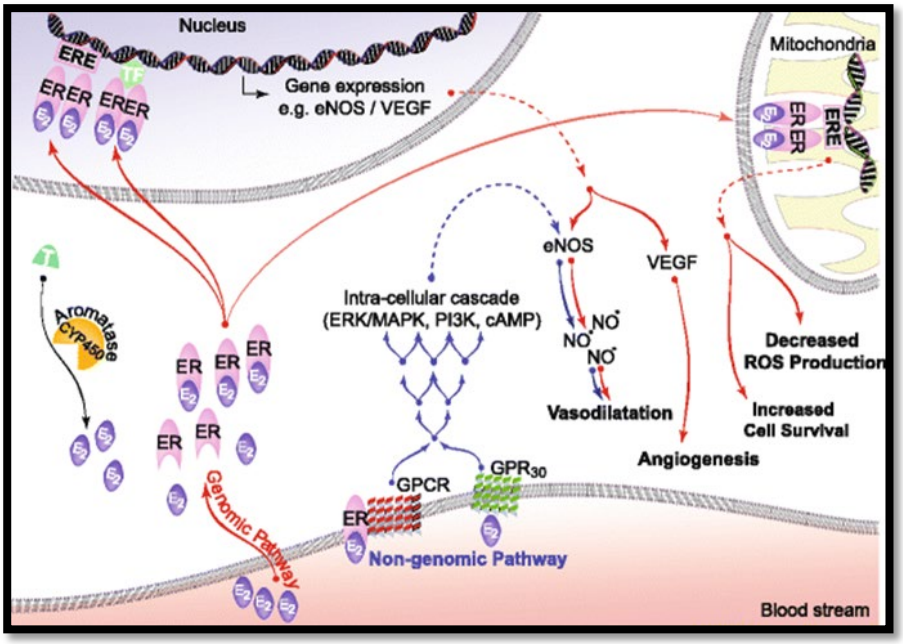



**Figure 3.***Genomic and non-genomic pathways of E2 [105].*

## *2. Estrogen's Reno-protective Effects*

Threats to global health from acute kidney injury (AKI) and chronic kidney disease (CKD) are serious. Several physiological processes in the kidney are influenced by estrogen and estrogen receptors (ERs). As a result, altered or dysregulated estrogen/ERs signaling pathways may contribute to a number of kidney illnesses. These include diabetic kidney disease (DKD), AKI, CKD complications IgA nephropathy (IgAN), lupus nephritis (LN), and others [106].

Estrogen protects against mitochondrial dysfunction inflammation and via the ERa/SIRT1 pathway [107]. 17-beta estradiol protects the vasculature by inhibiting the processes that mediate vascular remodeling [108]. This is achieved by stimulating nitric oxide synthesis by glomerular endothelial cells and thus protecting the kidney from glomerulosclerosis [82] [109]. The estrogen receptor alpha regulates renal sodium and potassium balance as well as the renin angiotensin system [110] . In fact, studies done on animals and humans demonstrated that estrogen has an anti-hypertensive effect through the activation of the vasodilator pathway and the inhibition of the vasoconstrictor pathway [111]. This is mediated through nitric oxide and sympathetic nervous system, respectively. Strong vasoconstrictors including angiotensin II (Ang II), endothelin-1, and catecholamines are less likely to be produced when estradiol is present [112]. By increasing angiotensinogen synthesis and decreasing renin and angiotensin-converting enzyme (ACE) synthesis, estrogen may also have kidney-protective effects [113]. In addition, estradiol inhibits the transforming growth factor 1 (TGF- 1), which causes apoptosis [107].

# CHAPTER II

# AIM and HYPOTHESIS

Cigarette smoking (CS) is a risk factor for the development and progression of chronic kidney disease (CKD) [114]. Kidney damage was evaluated in both men and women after CS exposure in the presence of other comorbidities such as CVD [115]. It has been established that although females are more prone for CKD development, the prognosis and mortality rate are higher in males [116]. However, studies looking into kidney damage development attributable to CS without pre-existing disease is lacking. Estrogen is known to have a nephro-protective effect in premenopausal women and the onset and progression of CKD increases when estrogen levels ceases (i.e postmonepause) [80] [81]. The main objective of this project is to investigate the cigarette smoking induced kidney damage in males and females and its potential correlation with estrogen hormone in females. We hypothesize that premenopausal females are less prone to kidney damage post-CS exposure than males and that endogenous estrogen may play a key role in this process.

## A. Specific Aims

1- In order to assess the effect of smoking on the cardiac systolic performance, function and hemodynamics, 2-dimensional M-mode and B-mode echocardiography was performed. Due to the inter-relationship between the heart and the kidney cardiac output was assessed. In addition to blood pressure measurement was taken using non-invasive blood pressure system.

2- In order to evaluate smoking effect on the kidney structure, total glomerular area, glomerular capillary area, bowman's capsule area, and proximal convoluted tubule cross-sectional area were assessed using periodic acid Schiff stain. These were utilized to detect if glomerular hypertrophy took place along with the assessment of kidney filtration. Moreover, total renal fibrosis was assessed using Masson's trichome stain.

3- At the molecular level, for assessing different biomarkers and cytokines, RT-qPCR and western blot were used to evaluate smoking consequences on the kidney:

- For evaluating kidney inflammation, pro-inflammatory cytokines IL-1β protein expression levels and tumor necrosis factor alpha (TNF-α) mRNA expression levels were assessed. However, protein expression levels of anti-inflammatory cytokines IL-4 and IL-13 were evaluated.
- For evaluating kidney fibrosis, protein expression levels of Alpha Smooth Muscle Actin which is an indicator of fibroblasts to myofibroblasts differentiation was examined. In addition, we measured the expression of gelatinases MMP-2 and MMP-9 which are responsible in extracellular matrix degradation. On the other hand, for assessing apoptosis, the ratio of proapoptotic BAX mRNA levels to the antiapoptotic BCL2 were measured.
- For evaluating effect of smoking on oxidative stress, we measured the mRNA expression levels of NOX4.

# CHAPTER III

# MATERIALS AND METHODS

## A. Study Input

### *1. Animals*

In the Animal Care Facility at the American University of Beirut, several experiments were conducted on four months old wild-type (WT) C57BL/6J male and female mice weighing 20-25g for the stated aim in this pilot study. Mice were exposed to 12light/12 dark hours cycle along with unrestricted access to ordinary chow and water. As shown in the figure 4, female mice were categorized into three groups: a control group, a chronic smoking (CS) group being exposed for 8 weeks to CS, and an ovariectomized chronic smoking group (8 weeks of CS exposure preceded by ovariectomy). Male mice were categorized into two groups: a control group, and CS group being exposed for 8 weeks to CS. The Institutional Animal Care and Use Committee (IACUC # 18-2-RN560) authorized all animal studies in conformity with the 8$^{th}$ edition of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Of note, an ovariectomized non-smoking group was utilized in assessing the cardiac hemodynamics. However, due to the fact that tissues were damaged, this was a limiting factor for assessing this group at the structural and molecular levels.

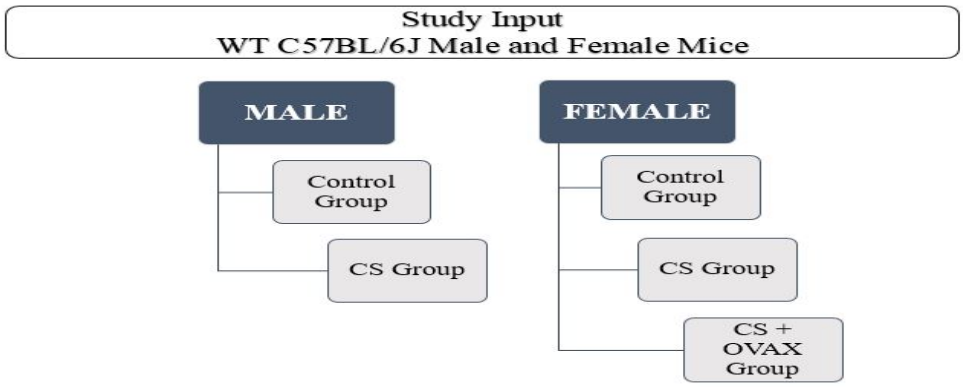



**Figure 4.** *Study Input Design.*

### *2. Study Design and Timeline*

Baseline echocardiography was recorded in all mice and after 2 days following 8 weeks of CS exposure. Female mice randomly allocated to the FOVX (CS ovariectomized females) group underwent baseline echocardiography after ovariectomy. For estrogen to clear before CS exposure, smoking was initiated after four weeks of ovariectomy and extended to eight weeks. Systolic blood pressure was measured at baseline, after ovariectomy, and at two days after the last smoked cigarette after which the mice were sacrificed. During the sacrifice, kidney tissues were collected for histological and molecular investigations.

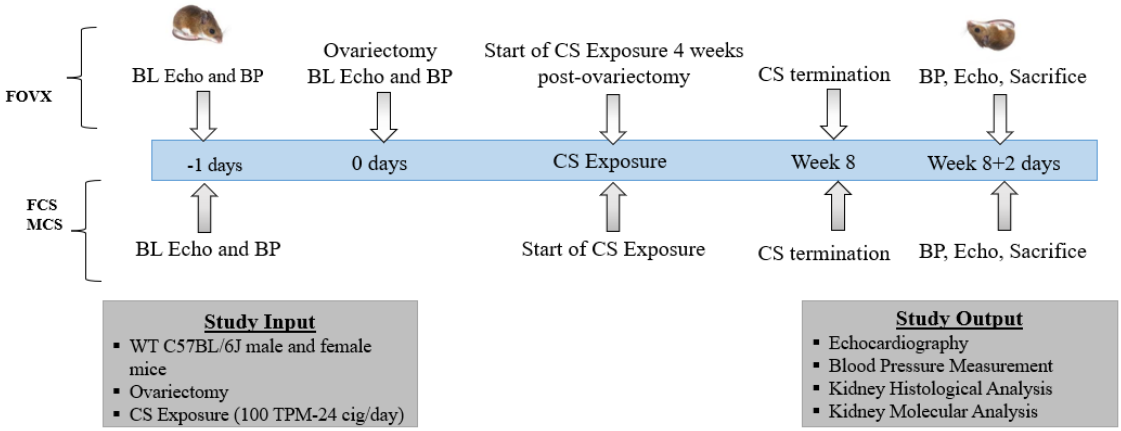



**Figure 5.** *Study design and timeline.*

### *3. CS Exposure Protocol*

Mice were subjected to two cigarette smoking sessions on a daily basis up to 8 weeks by applying an Oro-Nasal and Respiratory Exposure Systems (ONARES, CH Technologies, U.S.A). During each session, 12 cigarettes were used over a period of 90 minutes. This “nose only” apparatus involves a smoke generator along with a mixing chamber and a rodent exposure carousel. Scientifically customized cigarette 3R4F (University of Kentucky, Lexington, KY, U.S.A.) were used to expose conscious restrained mice to smoking. These consist of controlled delivery of chemicals and toxins to mice through the respiratory tract. In a cigarette puffer, the cigarettes were positioned to produce two puffs/50s over a duration of 2s/puff, producing a total particular matter (TPM) concentration almost 100 mg/cm$^3$/mouse/session.

## B. Surgical Procedures

### *1. Ovariectomy*

In an aseptic area, the hair of the mice was shaved off the flank area followed by chlorhexidine solution to disinfect the skin. An incision was performed on the right side of the abdomen and using a curved tip scissors, a musculature was dissected. After that, the ovarian fat pad was removed cautiously and the region under the ovary was tightly clamped by a tweezer hemostatic. Following that, using a sterile thread, two knots were formed in order to delimitate the area to be removed. For the left side, same steps were done. At the end of the procedure, ovariectomized mice were injected subcutaneously with tramadol and positioned on a heating pad for their recovery. For 3 days, 1.4mL of acetaminophen solution was added to 300mL of water (final concentration, 0.47 mg/mL)

and maintained at libitum. The animals were monitored for any inflammatory signs on daily basis, and the surgical wound was checked for any symptom that suggests pain.

### *2. Echocardiography*

Vevo 2100™ (VisualSonics, Inc., Toronto, Canada), a high-resolution imaging system, was utilized to perform echocardiography in conformity with the American Society of Echocardiography guidelines. Mice were anesthetized by (3-4%) isoflurane diluted with oxygen and then secured to the animal-heating platform in order to preserve body temperature at 37 °C. This was followed by chest hair removal along with ultrasonic gel application on the heart area. Recording of the 2-dimensional B-mode echocardiogram was obtained along the parasternal long-axis noting that the probe was located on the left thorax and the ultrasound beam was oriented on the mid-papillary muscle.

### *3. Blood Pressure Measurement*

Blood pressure was measured in unconscious anesthetized mice according to IACUC guidelines using the CODA mouse tail-cuff system (CODA-2, Kent Scientific, Torrington, CT), a non-invasive blood pressure system. The apparatus design was made to allow accurate blood pressure measurement in mice by **volume Pressure Recording (VPR)** sensor technology, and tracked as well in real-time. BP was measured at baseline and at two days after the last smoked cigarette.

### *1. Necropsy*

An intraperitoneal injection of 100µl Heparin (1,000 units/ml) was first given to the mice 15mins prior to the surgery for blood collection. After that, 3% isoflurane (Forane®) diluted with $O_2$ was given to induce anesthesia. A cardiac puncture was used to evacuate blood from the left ventricle which was centrifuged at 2200 rpm for 10 minutes in order

to separate plasma. Plasma was then collected, mixed with protease inhibitors, flash-frozen in liquid nitrogen, and stored at −80°C. For kidney collection, right kidney was collected into a cryotube placed in liquid nitrogen and preserved at −80°C for molecular analysis. Whereas, left kidney was collected into 10% formalin tubes for histological examination. Animals were sacrificed two days after the last smoked cigarette.

## C. Histology

### *1. Massons' trichrome (MTC) Stain*

Massons' trichome staining was used to evaluate renal fibrosis. Paraffin-embedded tissues were dewaxed, rehydrated, and then immersed for 1 hour in Bouein's solution at 56 °C. After that, tissues were washed and rinsed for 5-10minutes with distilled water. Weigert's iron hematoxylin solution, a nuclear dye, was applied to the tissues for 10 minutes. After being washed and rinsed with water, tissues were incubated in Biebrich scarlet-acid fuchsin solution in order to stain the acidophilic tissue elements with red. Collagen fibers were decolorized for discrimination using phosphomolybdic-phosphotungstic acid solution for 10 minutes and was stained by transferring them directly to aniline blue solution. Under a light microscope (Olympus CX41 microscope), images were taken at 10X magnification and fibrosis was measured using image-j software (https://imagej.nih.gov/ij/).

### *2. Periodic Acid Schiff (PAS) Staining*

The total glomerular area, Bowman's capsule area, and glomerular capillary area were measured by PAS for indicating if hypertrophy has occurred at the level of the kidney. After dewaxing and rehydration, xylene was utilized to clear 4µm thick kidney sections and then was rinsed in increasing concentration of ethanol. Afterwards, kidney sections

slides were soaked in 0.5% PAS for 10 minutes, rinsed in distilled water, shielded with Schiff's reagent for 15 minutes, and then washed for 5 minutes. Under a light microscope (Olympus CX41 microscope), images were taken at 40X magnification and areas were measured using Image J software (https://imagej.nih.gov/ij/).

## D. Molecular Analysis

### *1. Protein Extraction and Western Blots*

Kidney tissues were crushed in 400µl extraction buffer (RIPA) and left on a rotary mixer at 4°C overnight, the supernatant was then collected and protein was quantified using a detergent compatible assay (DC protein assay kit "bio-rad catalog# 5000112"). Samples were then heated in Laemmli buffer for 10 minutes at 95°C and stored at -20°C. Protein samples (100µg) were loaded in the wells of 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then electroblotted into nitrocellulose membranes at 100 volts for 1 hour in the cold room. Membranes were blocked with 5% non-fat dry milk in 0.1% TBST (Tris buffer saline with 0.1% Tween 20) for 1 hour at room temperature. Membranes were probed overnight with primary antibodies against interlukins (IL-1β, IL-4, IL-13) and alpha-SMA diluted in 0.1% TBST. Using TBST (0.02%), membranes were washed four times and were then incubated with goat pAb to Rb IgG (HRP) secondary antibody (1/5000) at room temperature for 1 hour. After washing the membranes twice with TBST (0.02%) and twice with TBS (1X), bands were visualized using the chemidoc MP imaging system- Biorad machine with an intensified chemiluminescence kit (Biorad). In order to validate equal loading, the protein expression level was normalized to total protein by incubating the membrane with the reversible total

protein stain (Ponceau Red). Bands were analyzed using Image J software (https://imagej.nih.gov/ij/).

| Primary Antibody | Dilution Factor |
|---|---|
| **Anti IL1β (Abcam, catalog#ab 9722 )** | 1/500 |
| **Anti IL4 (Abcam, catalog#ab 9728)** | 1/1000 |
| **Anti IL-13 (Abcam, catalog#ab 106732)** | 1/500 |
| **Anti-Alpha SMA (Abcam, catalog#ab 5694)** | 1/200 |

**Table 1.** *List of antibodies studied using western blot.*

### *2. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)*

According to producer's instructions (Thermo Fisher Scientific, Grand Island, NY, USA), trizol was utilized to extract total RNA from frozen kidney tissues. Using NanoDrop® ND-1000 UV-Vis Spectrophotometer, RNA concentrations were determined and then their purity was confirmed by the 260:280 absorbance ratio. Afterwards, starting with 1 µg RNA, cDNA was produced using Revert Aid 1st Strand cDNA synthesis kit (Thermo, USA) and mRNA expression was then analyzed in a CFX96 real-time PCR system (Bio-Rad, Germany). To measure the expression of the genes listed in **Table 2**, RT-qPCR was conducted in duplicate using SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and gene specific primers with a final volume of 10 µl. The genes are as follow: BCL (B Cell Lymphoma)-Associated X (BAX), B-cell lymphoma 2 (BCL2), matrix metalloproteinase-2 (MMP2), matrix metalloproteinase-9 (MMP9), NADPH Oxidase 4 (NOX-4), and Tumor Necrosis Factor alpha (TNF-alpha). A total of 10µl PCR mixture consisted of 4 µl cDNA, 5µl SYBR green, 0.9µl DNase free

water, and 0.05µl of the forward and reverse primers renal genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized to normalize kidney genes expression and results were presented as $2^{-\Delta\Delta Ct}$ values.

| Primer | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| **GAPDH** | TGTGTCCGTGGATCTGA | TTGCTGTTGAAGTCGCAGGAG |
| **BAX** | ATCCAAGACCAGGGTGGCT | CCTTCCCCCATTCATCCCAG |
| **BCL2** | AGTACCTGAACCGGCATCTG | TATGCACCCAGAGTGATGCAG |
| **MMP-2** | AGATGCAGAAGTTCTTTGGGCTGC | AGTTGTAGTTGGCCACATCTGGGT |
| **MMP-9** | ACCACAGCCAACTATGACCAGGAT | AAGAGTACTGCTTGCCCAGGAAGA |
| **NOX-4** | ACCAAATGTTGGGCGATTGTG | GGCTACATGCACACCTGAGA |
| **TNF-Alpha** | TGTGCTCAGAGCTTTCAACAA | CTTGATGGTGGTGCATGAGA |

**Table 2.** *List of Primers studied using RT-PCR.*

### E. Statistical Analysis

GraphPad Prism 7 was used for statistical analysis. Data are expressed as mean ± standard error of mean (SEM). statistical analysis was performed using an unpaired t test followed by parametric tests for non-Gaussian distributions. Two-Way-Anova and One-Way-Anova were also used when appropriate. P-values of $p < .05$ (*), $p < .01$(**), and $p < .001$(***) were considered statistically significant.

# CHAPTER III

# RESULTS

## A. Effect of CS on Systolic Blood Pressure (SBP)

In non-ovariectomized females, the SBP was comparable at baseline before CS and after CS. In ovariectomized females, the SBP was comparable at baseline before ovariectomy and after ovariectomy. However, following the exposure to CS, the SBP significantly increased. **(Figure 6a, 6b)**

In males, the SBP was comparable at baseline before CS and after CS. (**Figure6c)**

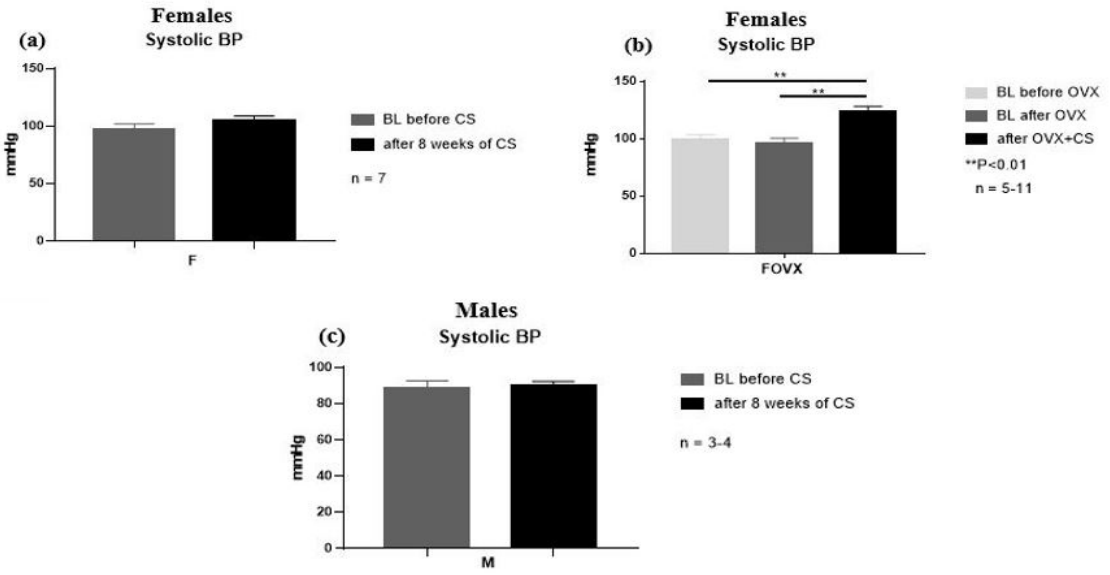



**Figure 6.** *Impact of CS on SBP.*

(a) Comparable SBP in CS females at BL and after CS. (b) Increased SBP in FOVX following CS. (c) Comparable SBP in males at BL and after CS. F: female; M: male; OVX: ovariectomized; CS: cigarette smoking; BL: baseline; BP: blood pressure. The significance of the data was determined using Two-way ANOVA in females (n=5-11) and unpaired T-test in males (n=3-4). Results are stated as ±SEM. (**: P<0.01)

## B. Effect of CS on Cardiac Output (CO)

In **Figure7a**, CO tends to increase in female CS group when compared to the control female group**.** A significant increase in CO was shown in ovariectomized CS female group in comparison to non-smoking ovariectomized female group.

In **Figure 7b**, CO was comparable between male CS group and relative control group.

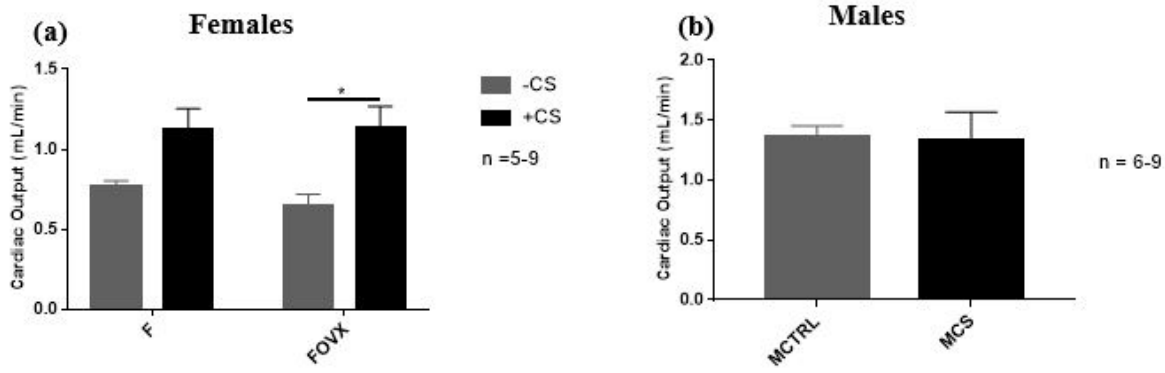



**Figure 7.** *Impact of CS on CO*
(a) Increased CO in FOVX (b) Comparable CO in males. F: female; FOVX: female ovariectomized; MCTRL: male control; MCS: male cigarette smoking; CS: cigarette smoking. The significance of the data was determined using Two-way ANOVA. in females (n=5-9) and unpaired T-test in males (n=6-9). Results are stated as ±SEM. (*: $P<0.05$)

## C. Effect of CS on Total Glomerular Area, Glomerular Capsule Area, and Glomerular Capillary Area

The total glomerular area, Bowman's capsule area, and glomerular capillary area were assessed using PAS. **Figure 8a** shows no remarkable change in total glomerular area in the female groups. **Figure 8c** shows a marked elevation in the Bowman's capsule area in female CS group and in ovariectomized CS female group when compared with their relative control group. Conversely, **Figure 8e** reveals a significant decrease in

glomerular capillary area in in female CS group and in ovariectomized CS female group when compared with their relative control group.

**Figure 8b, 8d and 8f** shows no significant change in total glomerular area, bowman's capsule area and total capillary area, respectively, in the male CS group compared with its relative control.

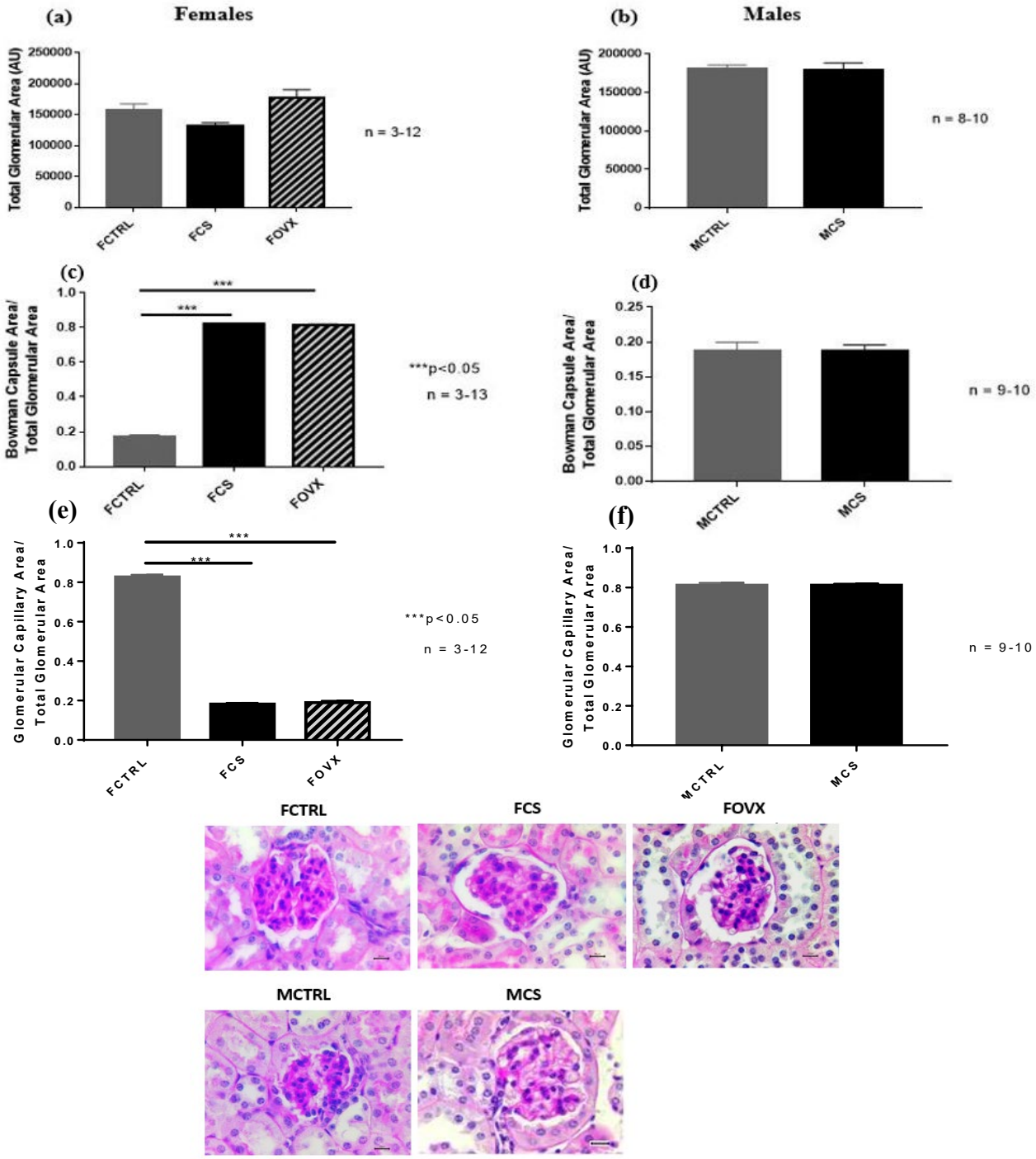


**Figure 8.** *Impact of CS on Total Glomerular Area and Bowman's Capsule Area, and Glomerular Capillary Area*
(a) Comparable total glomerular area in females. (b) Comparable total glomerular area in males. (c) Increased bowman's capsule area in FCS and FOVX. (d) Comparable bowman's capsule area in males. (e) decreased glomerular capillary area in FCS and FOVX. (f) Comparable glomerular capillary area in males. FCTRL: female control; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3-13) and unpaired T-test in males (n=8-10). Results are stated as ±SEM. (***: P<0.001)

**D. Effect of CS on Proximal Tubule Cross Sectional Area (CSA)**

**Figure 9a** shows a significant decrease in proximal tubules CSA in CS female group and in ovariectomized CS female group when compared with their relative control. Nevertheless, the proximal tubule CSA was significantly larger in the ovariectomized CS female group than that of CS female group.

**Figure 9b** shows no significant change in the proximal tubules CSA in the male groups.

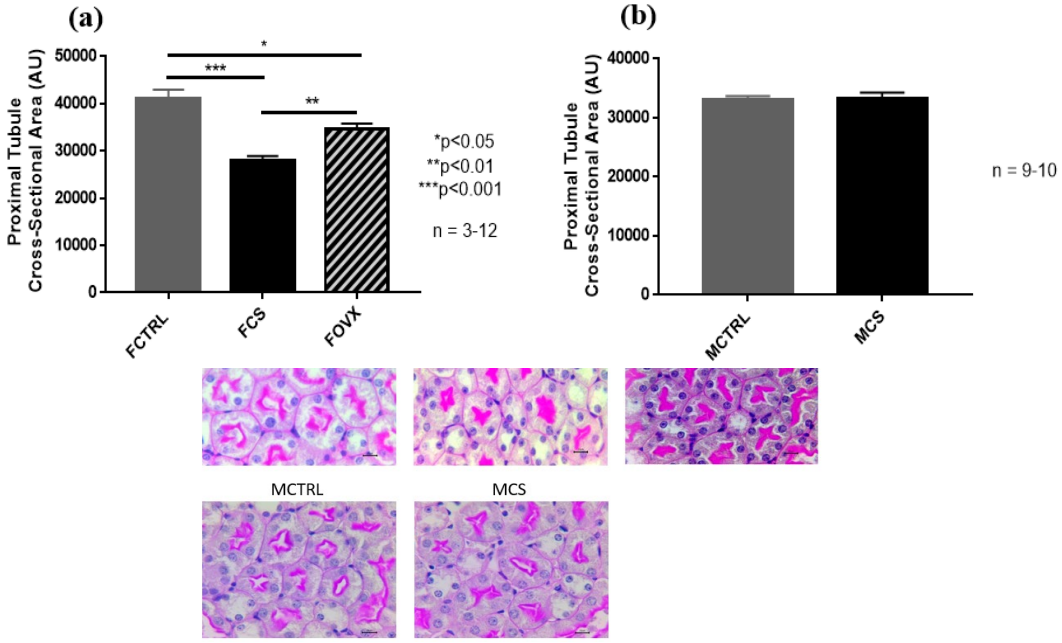



**Figure 9.** *Impact of CS on Proximal Tubules Cross-sectional Area*
(a) Decreased Proximal Tubule CSA in FCS and FOVX. h) Comparable Proximal Tubule CSA in males. FCTRL: female control; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two way ANOVA in females (n=3-13) and unpaired T-test in males (n=8-10). Results are stated as ±SEM. (***: P<0.001; **: P<0.01)

## E. Effect of CS on Renal Fibrosis

Using Masson stain, the total renal fibrosis was evaluated. **Figure 10a** shows a substantial increase in total renal fibrosis in female CS group and ovariectomized CS female group when compared with female control group. In **Figure 10b**, the total renal fibrosis tended to decrease in male CS group in comparison to male control group.

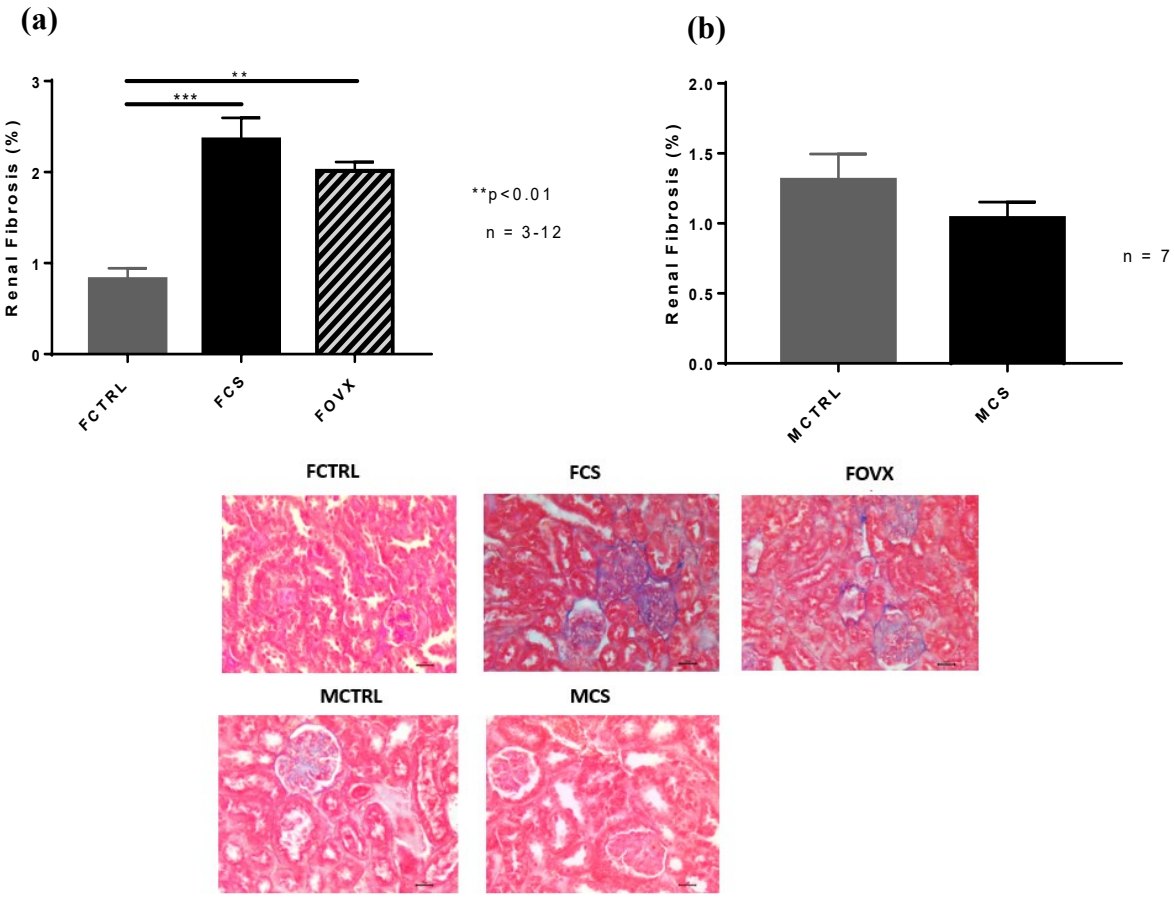



**Figure 10.** *Impact of CS on renal fibrosis*
(a) Increased total renal fibrosis in FCS and FOVX. (b) Comparable total renal fibrosis in males. FCTRL: female control; FCS: female smoking; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3-12) and unpaired T-test in males (n=3-7). Results are stated expressed as ±SEM. (*: P<0.05, **: P<0.01, ***: P<0.001)

## F. Effect of CS on Renal Protein Expression levels of α-SMA and mRNA expression levels of MMP2 and MMP9

**Figure 11a**, shows no considerable change in the protein expression levels of renal α-SMA among the female groups. Whereas, a marked decrease in mRNA expression levels of MMP-2 and MMP-9 in both the CS and ovariectomized CS female groups compared to the control group was observed **(Figures 11c, 11e).**

**Figure 11b** reveals a remarkable decrease in renal alpha smooth muscle actin (α-SMA) protein levels in the male CS group in comparison to male control group. However, in **Figure 11d and 11f** a marked rise in mRNA expression levels of MMP-2 and MMP-9 in male CS group was shown compared to male control group.

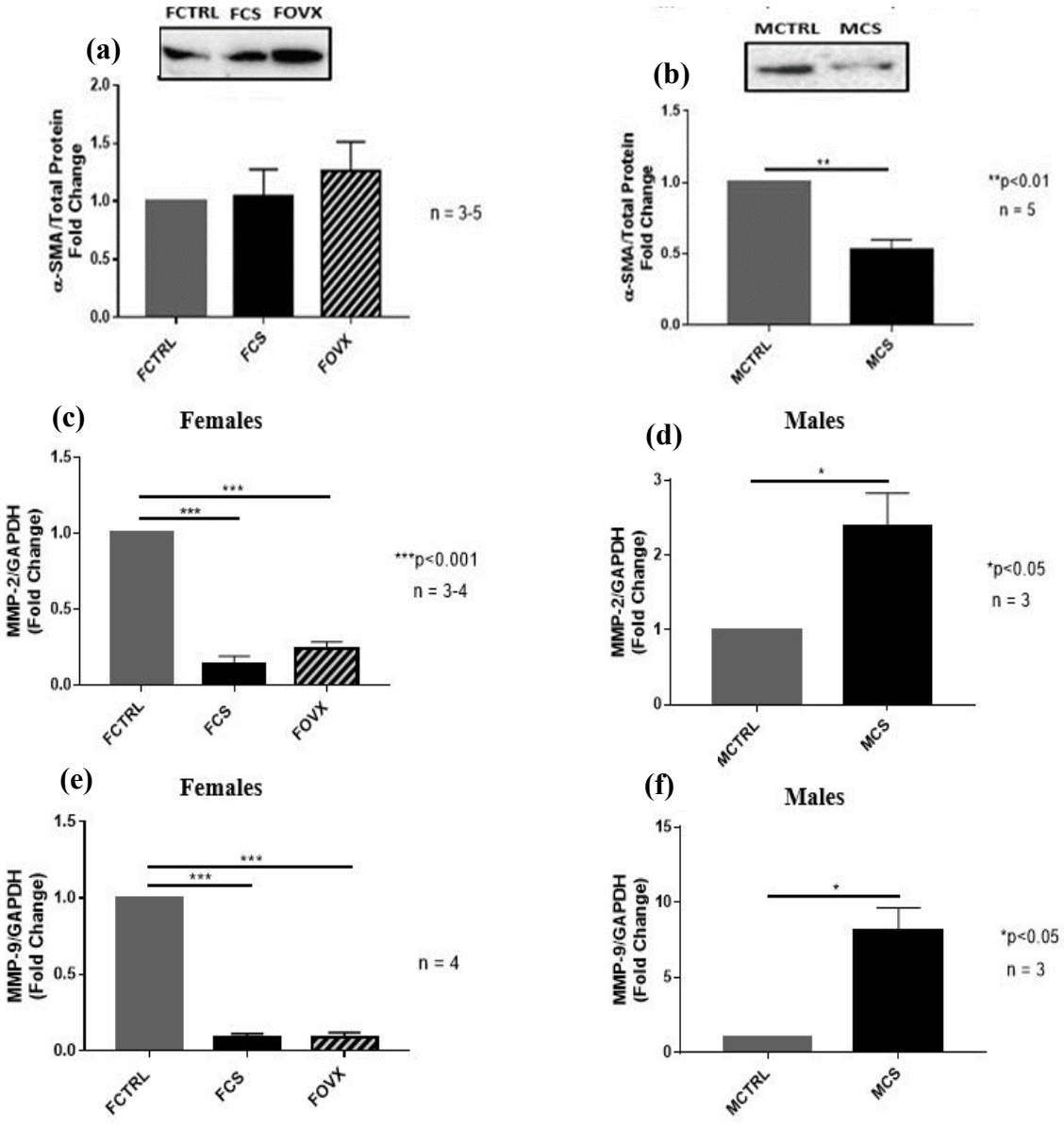


**Figure 11.** *Impact of CS on Expression of α-SMA and MMP-2 and MMP-9*
(a) Comparable α-SMA protein levels in females. (b) Decreased α-SMA protein levels in MCS. (c) Decreased MMP-2 m RNA levels in FCS and FOVX. (d) Increased MMP-2 mRNA levels in MCS. (e) Decreased MMP-9 mRNA levels in FCS and FOVX. (f) Increased MMP-9 mRNA levels in MCS. FCTRL: female control; FCS: female smoking; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3-12) and unpaired T-test in males (n=3-7). Results are stated expressed as ±SEM. (*: P<0.05, **: P<0.01, ***: P<0.001)

## G. Effect of CS on Renal mRNA Expression Levels of TNF-α

**Figure 12a** shows a marked decline in TNF-α mRNA expression levels in CS female group and ovariectomized CS female mice in comparison with their control group.

In **figure 12b,** shows a marked increase in TNF-α levels in CS male mice when compared with their relative group.

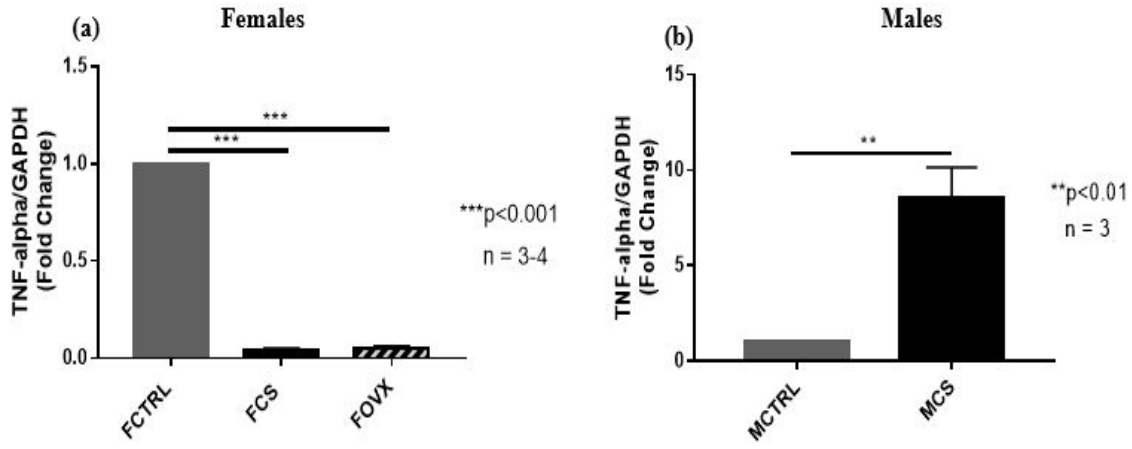



**Figure 12.** *Impact of CS on Renal* TNF-α mRNA Levels
(a) Decreased TNF-α mRNA levels in FCS and FOVX. (b) Increased TNF-α mRNA levels in MCS. FCTRL: female control; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3-4) and unpaired T-test in males (n=3). Results are stated as ±SEM. (**: P<0.01; ***: P<0.001)

## H. Effect of CS on Renal Protein Expression Levels of IL-4 and IL-13

**Figure 13a** shows no change in IL-4 protein expression levels among the female groups. In **Figure 13c**, the protein expression levels of IL-13 tends to increase in the CS and ovariectomized CS female groups when compared to the control group.

**Figure 13b** shows a marked decrease in IL-4 levels in male CS group when compared with the control group. However, the levels of IL-13 between male groups were comparable.(**Figure 13d**)

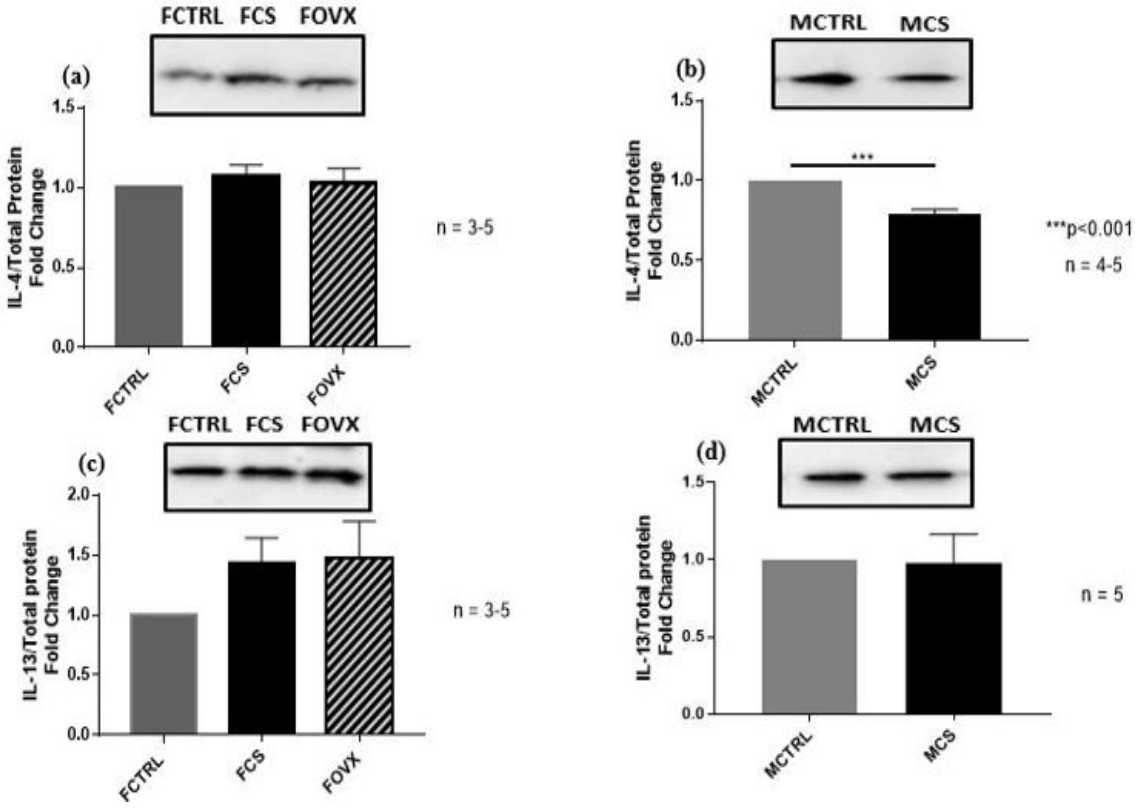



**Figure 13.** *Impact of CS on Renal Protein Expression Levels of* IL-4 and IL-13
(a)Comparable IL-4 protein expression levels in females. (b) Decreased IL-4 protein expression levels in MCS. (c) Increased IL-13 protein expression levels in FCS and FOVX. (d) Comparable IL-13 protein expression levels in males. FCTRL: female control; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3-5) and unpaired T-test in males (n=4-5). Results are stated as ±SEM. (***: P<0.001)

## I. Effect of CS on Renal Protein Expression Levels of IL-1β

**Figure 14a** shows no significant change in the protein expression levels of IL- β in female CS group and ovariectomized CS compared to the control female**.**

**Figure 14b** shows comparable protein expression levels of IL-1β in male CS group and male control group.

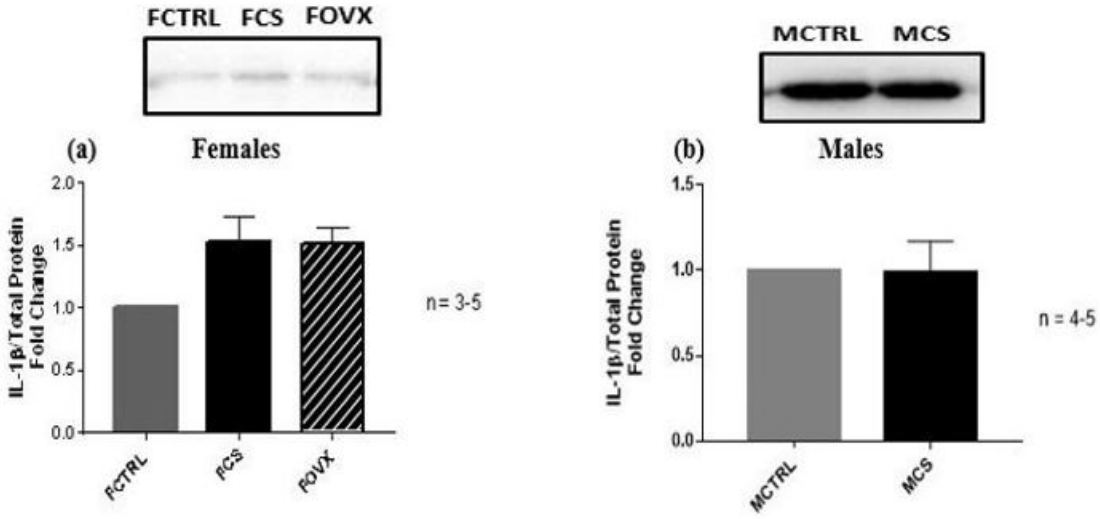



**Figure 14.** *Impact of CS on the Renal Protein Expression Levels of IL- 1β*
(a) No significant change in IL-1β Levels in females. (b) Comparable IL-1β Level in males. FCTRL: female control; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3-5) and unpaired T-test in males (n=4-5). Results are expressed as ±SEM**.**

## J. Effect of CS on the ratio of Renal mRNA Expression levels of BAX/BCL2

**Figure 15a** shows a relatively comparable levels in renal BAX/BCL2 mRNA expression levels ratio in CS female and ovariectomized CS female mice compared to female control group. **Figure 15b,** shows a relatively comparable levels in renal BAX/BCL2 mRNA expression levels ratio in CS males with respect to their relative control group.

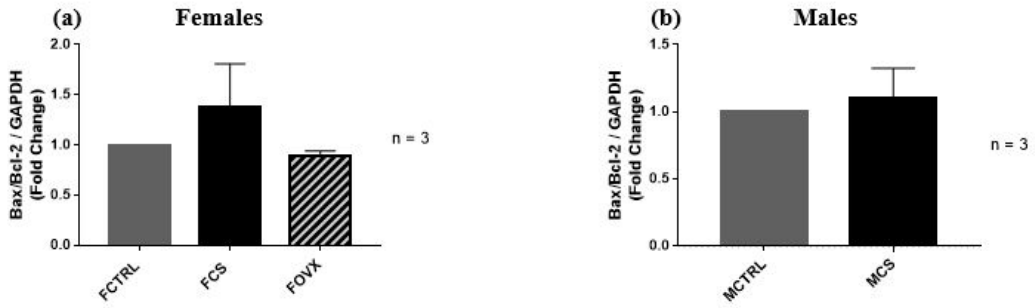



**Figure 15.** *Impact of CS on Renal mRNA Expression levels of BAX/BCL2*
(a)Comparable Renal BAX/BCL2 mRNA levels in females. (b) Comparable Renal BAX/BCL2 mRNA levels in males. FCTRL: female control; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3) and unpaired T-test in males (n=3). Results are stated as ±SEM.

## K. Effect of CS on Renal mRNA Expression levels of NOX-4

In female groups, the mRNA Expression levels of NOX-4 tends to increase in CS female and ovariectomized CS female groups when compared to the control group. **(Figure 16a)** In **Figure 16b**, NOX-4 levels tends to increase in male CS group in comparison to male control group.

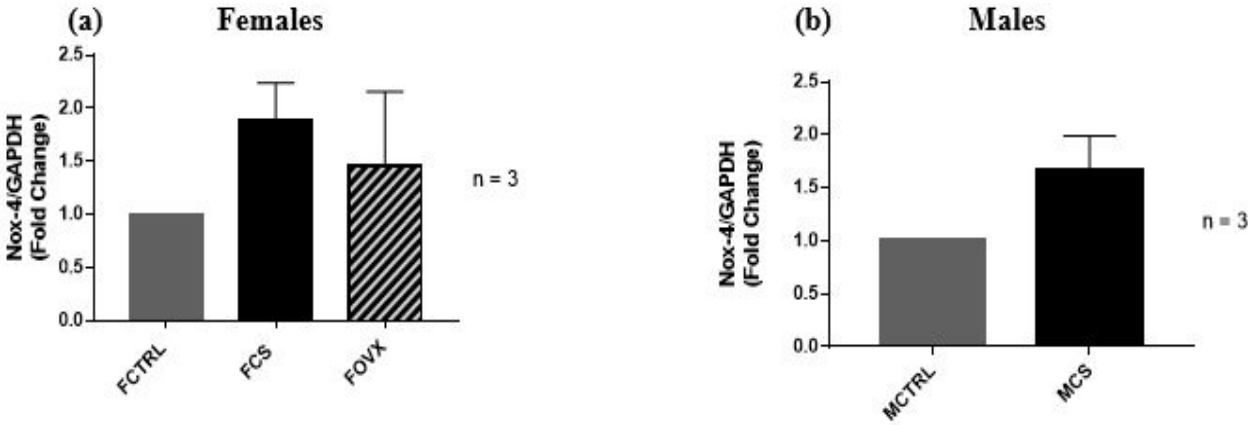



**Figure 16.** *Impact of CS on Renal mRNA Expression levels of NOX-4*
(a)Increased NOX-4 Levels in FCS and FOVX. b) Increased NOX-4 Levels in MCS. FCTRL: female control; FOVX: female ovariectomized; MCTRL: male control; MCS: male smoking. The significance of the data was determined using Two-way ANOVA in females (n=3) and unpaired T-test in males (n=3). Results are stated as ±SEM.

# CHAPTER IV

# DISCUSSION

Cigarette smoking is one of the crucial risk factors for CKD. The wide prevalence of chronic kidney diseases (CKD) [32] [35] [117]. Numerous hemodynamic study findings [3] [118] have demonstrated that chronic smoking causes changes at the structural and molecular level of the kidney in humans and rodent. Gender has been found to have an impact on the rate of advancement of renal illness in both experimental animals and humans [119]. Studies have shown that men have a more pronounced deterioration in renal function than women do in individuals with kidney illnesses [55] [119]. Estrogen is thought to protect the kidney through a variety of methods [110] [120] [121] and the incidence of renal disease increases in women after menopause [108] [122]. The objectives of this study are to investigate CS-induced kidney damage in male and female mice and to determine the role of physiological estrogen in females. Thus, the hemodynamics, structural, ad molecular effects of 8 weeks cigarette smoking on kidney in c57bl/6j male and premenopausal female mice were examined. Additionally, ovariectomized premenopausal female mice were tested to determine the effects of estrogen in that regard.

Our study presents perspectives about how cigarette smoking affects sympathetic and vagal neural activity in healthy habitual smokers. Cigarette smoking aggravates renal damage due to increased activity of the sympathetic nervous system and vasopressin production [12] [15] [39] [123]. This will lead to the elevation in blood pressure and heart rate [124] [125] [126]. In addition, nicotine promotes catecholamine release from sympathetic nerve terminals in the peripheral nervous system and the adrenal medulla through the activation of the renal nicotinic acetylcholine receptors [125] [127] [128]. In

our study, blood pressure and hemodynamic analysis were performed two days after the last cigarette exposure to rule out any potential acute CS effects. CS was associated with the rise in blood pressure in the ovariectomized females only. On the other hand, there was no remarkable change in systolic blood pressure in CS females. Recent findings in human and animal models shed insights into the mechanisms behind estrogen's blood pressure-regulating effects [129] [111] [130]. Interestingly, no effects on cardiac output and blood pressure were observed in the male CS group. These effects of CS, however, seems to be maintained in ovariectomized CS female mice which could indicate the potential development of hypertension in these animals. A continuous telemetry-based blood pressure monitoring system could be used in our future studies to confirm these observations.

At the structural and histological level, our data revealed significant CS effects. These effects include an increase in Bowman’s capsule area, a decrease in the glomerular capillary area, and a decrease in proximal tubule cross sectional area in females CS groups only as opposed to males CS group where no changes were observed. This potentially indicates a decline in the renal function due to decreased renal filtration which resulted from the described alterations at the histological level. Of note, these were accompanied with a no change in the total glomerular area in both females and males CS groups. Renin will initiate the activation of the RAS which will end up in renal efferent arteriole vasoconstriction [131]. Besides, our findings suggested that CS resulted in females’ kidney fibrosis. All these finding could suggest an impairment at the level of blood flow to the glomeruli and potential glomerulosclerosis formation and subsequently a reduction in GFR.

At the molecular level, our findings revealed no significant inflammatory response in CS female groups which was attributed to the decrease in the pro-inflammatory cytokine TNF-α and absence of significant change in the protein expression levels of IL-1β, IL-4 and IL-13. The observed decrease in TNF-α correlates with the decrease found with MMP-2 and MMP-9 expression in females CS groups. In fact, TNF-α is known to participate in the activation of MMP-2 and MMP-9 along with the stimulation of their production [132] [133] [134]. Once activated, MMP-2 and MMP-9 subsequently convert pro-TNF-α to TNF-α which feeds into their own activation [135]. In addition to the absence of inflammation and the decrease in ECM turnover, both α-SMA, a well-known pro-fibrotic marker [136], and the ROS marker NOX-4 showed a tendency to increase in CS female groups without reaching significance. Histologically, renal fibrosis was prominent in the CS female groups. It is not clear whether the inflammatory, ROS, and fibrotic markers are on the slope of decreasing following the two-days of CS cessation, hence assessing their levels during the acute CS phase is warranted to rule in their potential effects on the observed fibrosis. What is clear however, is the significant decrease in ECM turnover as evident by the low levels of MMPs which could ostensibly explain the increased fibrosis in CS female groups.

According to our previous findings at the histological and molecular levels, smoking females, in the presence and absence of estrogen, respond equally to post-eight weeks of cigarette smoking.

Males, on the other hand, responded quite differently to CS exposure. Our findings revealed a significant increase in the pro-inflammatory cytokine TNF-α with no considerable change in IL-1β levels and a significant decrease in both the anti-inflammatory/antifibrotic cytokine, IL-4, and the pro-fibrotic marker, α-SMA. These

findings are in line with the observed fibrosis which showed no significance in males, potentially due to limited fibrosis deposition and to TNF-α activation of MMP-2 and MMP-9 which increases ECM degradation.

Collectively, our findings reveal numerous important findings. Females seem to be more prone to morphological kidney damage than males following 8 weeks of CS exposure. Additionally, no change in the response to the CS-induced damage was observed in the presence or absence of estrogen as evidenced by the same response trends in CS female and CS ovariectomized female groups. The well-known reno-protective and beneficial effects of estrogen are somewhat altered which could be attributed to the anti-estrogenic actions of smoking that are now well-documented [137] [138] [139]. Moreover, in some results, CS ovariectomized female groups, although potentially hypertensive, seems to be more protected than the CS female group at the molecular level. This is because they showed lower level of BAX/BCL2 ratio and less NOX-4 expression levels. All these data could suggest that CS is not only limiting estrogen protective effects, but somehow altering estrogen properties into a detrimental byproduct with potential selective on-target and off-target effects that warrants detailed investigation.

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