AMERICAN UNIVERSITY OF BEIRUT

The EFFECT OF CIGARETTE SMOKING ON THE HEART IN MALE MICE AND IN OVARIECTOMIZED AND NON-OVARIECTOMIZED FEMALE MICE

by
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ABSTRACT
OF THE THESIS OF

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Major: Pharmacology and Therapeutics

Title: The Effect of Cigarette Smoking on the Heart in Male Mice and in Ovariectomized and Non-Ovariectomized Female Mice

Cigarette smoking (CS) is a predominant risk factor for cardiovascular disease progression worldwide. There is ample evidence of gender-based disparities in CVDs, with premenopausal females being less susceptible to the burden, given the cardioprotective effects of the female sex hormone, estrogen. Nonetheless, no study has examined gender differences in cardiac remodeling post-CS and the role played by estrogen. Thus, in this study, we aimed at investigating the CS-induced deleterious remodeling between males and females and its correlation with estrogen activity. Therefore, age-matched C57BL/6J male and female mice were allocated into five groups as follows: control female and male groups (FCTRL and MCTRL respectively), smoking female and male groups (FCS and MCS, respectively), and an ovariectomized smoking group (FOVX). Cardiac function was assessed using 2-dimensional B-mode and M-mode echocardiography, and the heart was subjected to histological and molecular analysis two days after 8 weeks of CS exposure. The cardiovascular hemodynamic assessment revealed enhanced cardiac contractility post-CS in the ovariectomized CS females only as evidenced by a significant rise in cardiac output (CO), blood pressure (BP), stroke volume (SV), and heart rate (HR). The CS female group witnessed a considerable increase in ejection fraction and heart rate. Whereas in males, cardiac systolic function and blood pressure weren't significantly affected with CS. At the structural and histological levels, CS was associated with increased left ventricular mass (LVM) in both FCS and FOVX groups, with no change in the MCS group. Besides, the cardiomyocytes' cross-sectional area (CSA) showed a noticeable increase in both males and females CS groups. This increase in CSA and LVM reflects CS-induced cardiomyocyte hypertrophy. Molecularly, no inflammation was detected in all CS groups. Nevertheless, CS was associated with enhanced fibrosis and apoptosis as evidenced by the rise in the mRNA expression levels of the profibrotic markers α-smooth muscle actin (α-SMA) and connective tissue growth factor (CTGF), as well as the apoptotic regulatory genes such as Bcl-2-associated X protein/B-cell lymphoma 2 ratio (Bax/BCL2) in the CS females only. In contrast, CS in males and ovariectomized females didn't affect these apoptotic and fibrotic pathways. In conclusion, CS females, but not males, maintained pronounced cardiac systolic function with an increased BP in ovariectomized females only, 2 days after the last CS exposure. Additionally, CS non-ovariectomized females seems more prone to CS-induced cardiac injury potentially due to the alteration of estrogen activity/metabolism in the presence of CS.

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ABBREVIATIONS

CVD	Cardiovascular Disease
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
COPD	Chronic Obstructive Pulmonary Disease
TSNAs	Tobacco-Specific Nitrosamines
PAHs	Polycyclic Aromatic Hydrocarbons
BBB	Blood-Brain-Barrier
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
PAD	Peripheral Artery Disease
MI	Myocardial Infarction
CS	Cigarette Smoke
MMPs	Matrix Metalloproteinases
MAPK	Mitogen-activated Protein Kinase
LVEDD	Left Ventricular End-Diastolic Diameter
LVESD	Left Ventricular End-Systolic Diameter
LVFS	Left Ventricular Fractional Shortening
SV	Stroke Volume
CO	Cardiac Output
LVSV	Left Ventricular Systolic Volume (LVSV)
EF	Ejection Fraction
FS	Fractional Shortening

LVM Ventricular Mass

HW/BW Heart Weight-to-Body Weight

CSA Cross-Sectional Area

ROS Reactive Oxygen Species

GSSG Glutathione disulfide

GSH Glutathione

SOD Superoxide Dismutase

FA Fatty Acid

NF-B Nuclear Factor-B

ERKs Extracellular Signal-Regulated Kinases

JNKs c-Jun-NH2-terminal kinases

TNF- α Tumor Necrosis Factor-alpha

IL-8 Interleukin-8

AP-1 Activator Protein-1

DAMPs Danger-Associated Molecular Patterns

hs-CRP High-sensitivity C-Reactive Protein

MCP-1 Monocyte Chemotactic Protein-1 (MCP-1)

mPTP Mitochondrial Permeability Transition Pore

ECM Extracellular Matrix

ER Estrogen Receptor (ER)

GPR30 G Protein-Coupled Receptor 30

SEM Standard Error of the Mean

WT Wild type

ACF AUB animal care facility (ACF)

IACUC The Institutional Animal Care and Use Committee

TPM Total Particulate Matter

FCTRL Female Control Group

FCS Female Smoking Group

FOVX Female Ovariectomized group

MCTRL Male Control Group

MCS Male Smoking Group

VPR Volume Pressure Recording

H&E Hematoxylin and Eosin

RT-qPCR Reverse Transcription Quantitative Polymerase Chain Reaction

IL-1β Interleukin-1 beta

IL-4 Interleukin-4

IL-13 Interleukin-13

α-SMA Alpha-Smooth Muscle Actin

BAX B-Cell Lymphoma-Associated X

BCL2 B-cell Lymphoma 2

MMP-2 Matrix Metalloproteinase-2

MMP-9 Matrix Metalloproteinase-9

Nox-4 NADPH Oxidase 4

CTGF Connective Tissue Growth Factor

GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase

CHAPTER 1

INTRODUCTION

1.1. Chronic Cigarette Smoking (CS)

1.1.1. Epidemiology

Tobacco consumption is among the most serious public health issues worldwide. Almost 1-in-4 adults smoke tobacco globally. Millions of individuals suffer from inadequate health as a result of smoking[2]. In the twentieth century, tobacco led to the death of 100 million individuals, the majority of which occurred in industrialized nations. On the assumption that ongoing smoking modes endure, around 1 billion humans will be killed owing to smoking[3]. The World Health Organization (WHO) and Institute for Health Metrics and Evaluation, approximate that annually, almost 8 million deaths occur prematurely because of smoking, equivalent to about 22,000 deaths daily. It has been reported that smoking is responsible for 15% of all global mortalities. Over 7 million of such fatalities are correlated to direct tobacco usage. Nearly 1.2 million of these losses are non-smokers who died due to second-hand smoke exposure[2, 4]. The gender-based disparity shows that the number of male smokers is more than female smokers. Statistical data reveal that one-third of men in the world smoke, whereas one out of every ten women does[5]. Men account for the vast majority (71 %) of the mentioned premature deaths[6]. Considering smoking-attributable mortality by age, it is noticed that the old population is the most vulnerable one. The death toll is substantially greater among persons over the age of 70, followed by those aged fifty to sixty-nine (50 to 69). Indeed, tobacco in all

aspects and kinds is dangerous. Cigars, waterpipe tobacco, smokeless tobacco, and cigarillos are some examples of tobacco products. However, cigarette smoking (CS) is by far the most prevalent type of tobacco consumption in the world. Not only the pervasiveness of smokers among the different populations governs the extent of smoking, but also the frequency of smoking does. Around 20 to 25 cigarettes are consumed by each smoker across the globe on a daily basis[7]. The WHO approximates that of the 1.3 billion tobacco consumers, more than 80%, reside in third-world countries. Age-standardized estimates of current tobacco use, tobacco smoking, and cigarette smoking data by country for 2022, show that 42.6% of the total population in Lebanon are smokers. This survey was conducted by the WHO, and categorized Lebanon as the 6th among the ten countries with the greatest smoking rates [8]. As reported by the Centers for Disease Control and Prevention (CDC), over 16 million Americans suffer from a smoking-associated illness [9]. Cancer, diabetes, lung and heart disease, chronic obstructive pulmonary disease (COPD), and immune system drawbacks, are all outcomes of smoking. Therefore, smoking is thought to be detrimental to multiple systems in our bodies[10].

1.1.2. Definition:

Cigarette smoke is a death-dealing combination of over 7000 chemical constituents [11]. At the minimum, 250 of these ingredients are extremely deleterious, counting ammonia, carbon monoxide, and hydrogen cyanide. Of these 250 components, 69 are carcinogenic such as cadmium, tobacco-specific nitrosamines (TSNAs), phenol, polycyclic aromatic hydrocarbons (PAHs), and tar [12]. Roughly 50% of the chemicals making up tobacco smoke are from natural sources, specifically found in the green tobacco leaf, whereas the remaining 50% results from the combustion of tobacco [13].

Nicotine's pharmacologic actions are principally liable for tobacco addiction, even though the major toxic impacts of smoking are attributed to the other elements of cigarette smoke [14].

1.2. CS and Cardiovascular Disease

1.2.1. Definition and Risk Factors

By definition, cardiovascular diseases are a variety of disturbances that adversely affect the cardiovascular system, comprising the heart and the blood vessels. In fact, heart disease encompasses numerous distinct conditions, such as coronary artery disease (CAD) which is also designated as coronary heart disease (CHD), peripheral artery disease (PAD), atherosclerosis, pulmonary embolism, congenital heart disease, and vein thrombosis [15]. Touching upon the CAD, it is a condition that compromises the blood vessels nourishing the heart and is associated with altered myocardial perfusion as well as ischemia. This may bring forth angina, myocardial infarction (MI), and in severe cases heart failure [16]. Multiple risk factors underlying cardiovascular disease have been identified by researchers[17] [18]. Hypertension, dyslipidemia, and diabetes mellitus are all well-known determinants of heart disease. Significantly, behavioral risk factors, comprising sedentary lifestyle, obesity, and smoking have a remarkable impact on conventional risk factors, as well as on new risk pathways such as inflammatory processes, oxidative stress, endothelial function, arrhythmia, and thrombosis [19] [20].

1.2.2. Epidemiology

Cardiovascular diseases (CVD) are the world's leading cause of mortality and their prevalence is continuously escalating in both developed and underdeveloped countries [21]. According to the WHO, around 17.9 million annual deaths are attributed to CVDs thus accounting for 32% of all global fatalities, the majority of which are caused by myocardial infarction (MI) and strokes (85%) [22]. By 2030, it is estimated that nearly 23.6 million individuals would die from CVDs [23]. The World Health Federation estimates that smoking causes almost 10% of all CVD cases and is considered to represent the second leading cause of CVD after hypertension [24]. Indeed, coronary artery disease (CAD) is increased by 2 to 4 folds as a result of chronic tobacco smoking, which contributes to around 17% of total CVD mortalities annually (the equivalent of more than 3 million individuals) [24] [25]. Additionally, non-smokers' probability of developing CVD is raised by 25-30% when second-hand smoke is inhaled [23] [25].

1.3. Impact of CS on Cardiac Remodeling

The term "cardiac remodeling" refers to a set of molecular, cellular, and interstitial changes that present clinically as modifications in the heart's size, mass, shape, and function following injury or cardiac load [26] [27] [28]. Cardiac remodeling can be brought on by myocardial infarction (MI), myocarditis, pressure overload, dilated cardiomyopathy, volume overload (valvular regurgitation), as well as cigarette smoke exposure [26] [29].

In fact, the hemodynamics of the cardiovascular system are well documented to change significantly as a result of cigarette smoking [29] [30]. Consequently, cardiac

remodeling and compromised heart function will ensue such as cardiac chamber expansion, myocardial hypertrophy, and ventricular malfunction [29] [31]. Potential processes underlying these modifications encompass oxidative stress, hemodynamic and neurohormonal disturbances, nitric oxide bioavailability, inflammation, matrix metalloproteinases (MMPs), and mitogen-activated protein kinase (MAPK) activation [29] [32].

As previously stated, pathological stimuli can cause cardiac remodeling. Following a pathogenic stressor, combined molecular and cellular variations can induce ventricular hypertrophy and/or dilatation which ultimately manifest in diastolic and/or systolic dysfunction.

1.3.1. Structural and Functional Level

1.3.1.1. Cardiac Hemodynamic' Alteration

In the absence of comorbidities, Gvozdjakova et al. established the term "smoke cardiomyopathy" to characterize metabolic and anatomical modifications in the rabbit myocardium following chronic cigarette smoking [33] [34]. As demonstrated by various studies, both short-term and long-term smoking adversely impact ventricular systolic and diastolic function in both animals and humans [35] [36] [37] [38] [39] [40] [41]. Clinical and experimental studies have demonstrated that exposure to CS has both direct and indirect deleterious impacts on the myocardium [42]. Direct effects have been reported, including myocardial ischemia, myocardial fiber edema, necrosis and fibrosis, localized myocarditis, coronary vasocontraction, functional and structural changes in myocardial mitochondria [42]. In contrast, elevated blood pressure, alteration of the plasma

cholesterol level, increased platelet clumping, disrupted T-cell performance, and elevated inflammatory profile are examples of indirect outcomes of CS on the heart [42].

A preclinical study on healthy human smokers demonstrated that the enhanced sympathetic outflow to the vascular system and the heart was accountable for the CS-induced elevation in blood pressure and heart rate, respectively [43]. Significantly, rodents exposed to smoke have been reported to experience alterations in their hearts' functionality and hemodynamics throughout a range of timescales. For instance, in their five weeks CS- exposed Sprague-Dawley rats, Lianzhi Gu and colleagues documented significant increases in the Left Ventricular End-Diastolic Diameter (LVEDD) and Left Ventricular End-Systolic Diameter (LVESD) accompanied with a major decrease in the Left Ventricular Fractional Shortening (LVFS) as opposed to their control counterpart [44]. In addition, upon comparing the cardiac parameters of mice exposed to cigarette smoking for 32 weeks with control mice, the end-diastolic volume was considerably lower and the LV wall was thicker [45]. As a result, stroke volume (SV) and cardiac output (CO) in CS-exposed animals were significantly lower than in control mice [45]. In a different research, an echocardiographic examination of Wistar rats subjected to 8 weeks of CS, revealed left atria enlargement, elevated left ventricular systolic volume (LVSV), impaired systolic function, reduced ejection fraction (EF), and fractional shortening (FS) [46].

1.3.1.2. Cardiac Hypertrophy

Cardiac hypertrophy is a typical kind of cardiac remodeling that arises when the heart is overloaded or following an insult [47]. In the remodeling process, myocytes are presumed to perform a pivotal role with cardiomyocytes receiving the most focus of all

cardiovascular wall components owing to their capacity to contract and contribute to mass of the heart [26] [48] . In actuality, myocyte counts decline following an insult, and surviving ones undergo hypertrophy as the first stage of a compensatory and adaptive mechanism to preserve stroke volume, minimize wall stress and sustain output after contractile tissue loss [26] [47]. Nevertheless, ultimately with time, these beneficial aspects are diminished and ventricular function deteriorates frequently resulting in heart failure [47]. To demonstrate, a preclinical study showed that 4-weeks cigarette smoke exposure led to a rise in heart-to-body weight ratio (H/W) which is indicative of cardiac hypertrophy in Sprague-Dawley rats [49]. Likewise, prior findings concluded consistent outcomes of myocardial hypertrophy as a result of cigarette smoking [29] [45]. Notably, it was revealed by Talukder et al. that CS had an evident impact on the cardiac mass where 32 weeks CS-exposed animals had considerably greater left ventricular mass (LVM) and heart weight-to-body weight ratio (HW/BW) versus control mice [45]. Additionally, Santos et al. quantified cardiomyocyte hypertrophy using the immunofluorescence approach [46]. Their histologic analyses revealed an increase in the cross-sectional area (CSA) of myocytes in rats exposed to CS for two months, indicating myocyte hypertrophy [46]. It should be noted that these hemodynamic alterations in cardiac function and structure were examined with respect to the changes assessed at the cellular and molecular levels. The latter will be addressed further below.

1.3.2. Molecular and Cellular Level

Following cigarette smoking, myocardium cellular and molecular damage is directly correlated to at least four interchangeable mechanisms, designated RIMD, which

comprise oxidative stress (R), inflammation (I), metabolic impairment (M), and cell death (D) [50] [51] [52] as well as fibrosis

1.3.2.1. Oxidative Stress

When the generation of reactive oxygen species (ROS) and the intrinsic cell's antioxidant defense systems are imbalanced, oxidative stress arises [53] [54]. Under physiologic conditions, aerobic or oxygen metabolism and normal cell activity produce ROS as natural by-products [55]. Normally, many intracellular signaling cascades, aimed at preserving the cell's equilibrium with its natural environment, use reactive oxygen species (ROS) as second messengers [53] [56]. At elevated rates, the cellular redox equilibrium is disrupted, causing uncontrolled detrimental damage to biological components, DNA strand breaks, and lipid peroxidation; thus resulting in function loss and even induction of apoptosis [57] [58].

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 [50] [59]. The cellular oxidative stress is exacerbated by the interaction of free radicals like superoxide and NO, which lowers NO availability and also produces peroxynitrite [60] . As a consequence of the oxidative stress and hemodynamic burden, inflammatory pathways activation, MMP engagement, proliferation of cardiac fibroblasts, and promotion of intrinsic remodeling, CS-triggered heart remodeling is aggravated notably myocardial injury and damage to vascular endothelium [60] [45] [31]. Talukder et al. examined the ROS production in white blood cells of newly obtained entire blood from normal and CS-treated mice to assess if 32

weeks of CS treatment promotes activation of leukocytes and oxidative stress [45]. It was demonstrated that blood cells CS-exposed animals for 32 weeks produced more hydrogen peroxide and superoxide than their control counterparts, resulting in systemic oxidative stress and moderate hypertrophic heart in mice who were not prone to illness [45].

Additionally, the impact of CS on ventricular remodeling post MI in rat models was evaluated by Duarte et al. The results showed a considerable rise in glutathione disulfide (GSSG) and a reduction in glutathione (GSH) as well as GSH/GSSG ratio, confirming that CS aggravated LV remodeling post-MI [61]. Santos and colleagues found comparable pathologies in rats exposed to CS for 8 weeks. Their examination showed direct toxicity to the heart which is explained by reduced GPx and SOD function, diminished fatty acid (FA) oxidation, a concomitant rise in ROS production, impairment of the mitochondria and lipotoxicity [46]. This data point to compromised antioxidant defense mechanisms which corroborate the oxidative influence of smoking on cardiac alterations and remodeling.

1.3.2.2. Inflammation (I)

ROS generation causes inflammation by directly affecting both innate (neutrophils and macrophages) and adaptive immunity, which promotes pro-inflammatory cytokines production (IL-8, TNF-α, IL-1) [62]. Numerous intracellular signaling pathways that are activated by reactive oxygen species (ROS), are essential for the initiation and progression of the inflammatory response. This includes, but is not limited to, the activation of mitogen-activated protein kinases (MAPKs), regulation of transcriptional activity via nuclear factor-B (NF-B), as well as other ROS-sensitive cascades [63]. The MAPK cascade ultimately culminates in the engagement of

downstream effectors that play a key role in triggering inflammation and inducing apoptosis [63]. As a response, inflammatory gene expression is activated and chronic immune cell recruitment occurs, boosting the production of proinflammatory cytokines such as TNF- α and IL-8 [63] [64].

Alongside the induction of MAPKs signal transduction cascades, prior research focused on the role of activator protein-1 (AP-1) in inflammation by promoting IL-8 secretion from macrophages and monocytes [63] [65]. Likewise, preliminary studies highlighted the influence of oxidative injury in generating danger-associated molecular patterns (DAMPs), which induce immune responses and consequent inflammation [66].

Zhou et al. examined the inflammatory profile in two distinct trials following four months of cigarette smoke administration in rats. They discovered increased cardiac gene expression of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β as well as the levels of circulating high-sensitivity C-reactive protein (hs-CRP), monocyte chemotactic protein-1 (MCP-1), IL-6, and IL-1 in the blood implying systemic inflammation [67] [68]. Other investigations confirmed the downregulation of anti-inflammatory cytokines like IL-10 and the elevation of other proinflammatory cytokines including IL-8, IL-2, GM-CSF , and IFN-γ [69] [70].

The findings of other investigations, however, were contradictory. Cardiac levels of diverse cytokines such as TNF-α, IFN- γ, ICAM-1, and IL-10 were unaffected by cigarette smoking as reported by Santos et al. and Duarte et al. [71] [72]. The discrepancies between studies regarding CS exposure time and concentration might be an explanation for these conflicting results.

1.3.2.3. Cellular and Metabolic Impairment (M)

Another cause of oxidative stress that fosters the inflammatory response following CS is cellular metabolic impairment. Excessive oxidants destroy cellular components irreversibly, causing alterations in cellular functioning or apoptosis [73]. This damage is primarily caused by provoking mitochondrial injury through the attenuation of mRNA transcripts encoded by mtDNA, modification in the production of mitochondrial protein, and reduction of the levels of ATP, as well as redox potential of the mitochondria [74]. In cardiomyocytes, cellular energy generation and regulatory mechanisms may be altered by the mitochondria due to their significance in cell signaling and apoptosis [75]. Thus, prolonged oxidant exposure may impede mitochondrial signal transduction, resulting in decreased energy production and cell death [75]. After CS, animals showed morphological and functional alterations in their cardiac mitochondria, including enlargement, lipid accumulation, external membrane modification, generation of ROS, decreased rate of oxidative phosphorylation, and opening of the mitochondrial permeability transition pore (mPTP) [34] [76] [77] [78] [79].

On a separate study, Yamada et al. came to the conclusion that during an ischemic simulation, scientifically relevant levels of cigarette smoke extracts lead to elevated levels of cardiomyocyte mitochondrial Ca2+ as well as the vulnerability of mPTP opening, resulting in apoptosis [80]. Additionally, as per Santos et al., alterations in metabolic activities and energy homeostasis, such as lipotoxicity, impaired mitochondrial respiration, elevated triglyceride density in cardiomyocytes and oxidative stress, were evidenced in their two-month CS exposure study of rats [46].

As previously demonstrated in this part, metabolic impairment following CS exposure plays a significant role in cellular malfunction by contributing to the destructive cycle of CS-induced RIMD.

1.3.2.4. Cell Death (D)

As discussed in the previous section, CS induces high levels of ROS which triggers inflammation and metabolic impairment, ultimately leading to cardiomyocyte dysfunction and death [50] [46] [67] [81] [82]. Das et al. demonstrated the stimulation of both endogenously-mediated (p53 phosphorylation, caspase 3 activation, and elevated ratio of Bax/Bcl-2) and exogenously-mediated (induction of caspase 8 and upregulation of Tumor Necrosis Factor) apoptotic mechanisms in the myocardium following cigarette exposure in guinea pig model [50].

Additionally, in their CS rat model, Zhou and colleagues documented an elevated rate of apoptosis in cardiomyocytes [67]. In separate research, Zhou et al. demonstrated that CS exposure enhanced JNK and P38 MAPK signaling pathways while inhibiting PI3K/AKT pathways in the rat heart, noting that these routes have been associated with cellular apoptosis [82] [83] [84]. Smoking-induced left ventricular systolic dysfunction has also been linked to autophagy in addition to apoptosis, according to publications [82] [85].

1.3.2.5. Fibrosis

Cardiac fibrosis is described as an imbalanced mechanism in the formation and degradation of extracellular matrix (ECM) components, particularly collagen, ultimately contributing to cardiac malfunction in various cardiac pathophysiologic disorders [86]

[87]. In fact, it is considered as a natural, adaptive and protective mechanism that is critical for tissue repair [88] [89]. However, when cardiac fibrosis advances uncontrollably, it leads to irreversible ventricular remodeling, permanent stiffening of the heart tissue, and markedly compromised heart function [88] [90]. Cigarette smoke exposure is one of the numerous factors that induce cardiac fibrosis [91] by promoting collagen deposition [92] [93]. It has been shown through a number of in-vivo fibrosis models that nicotine activates TGF-1 and CTGF, thus inducing fibroblast activity in an autocrine manner [94].

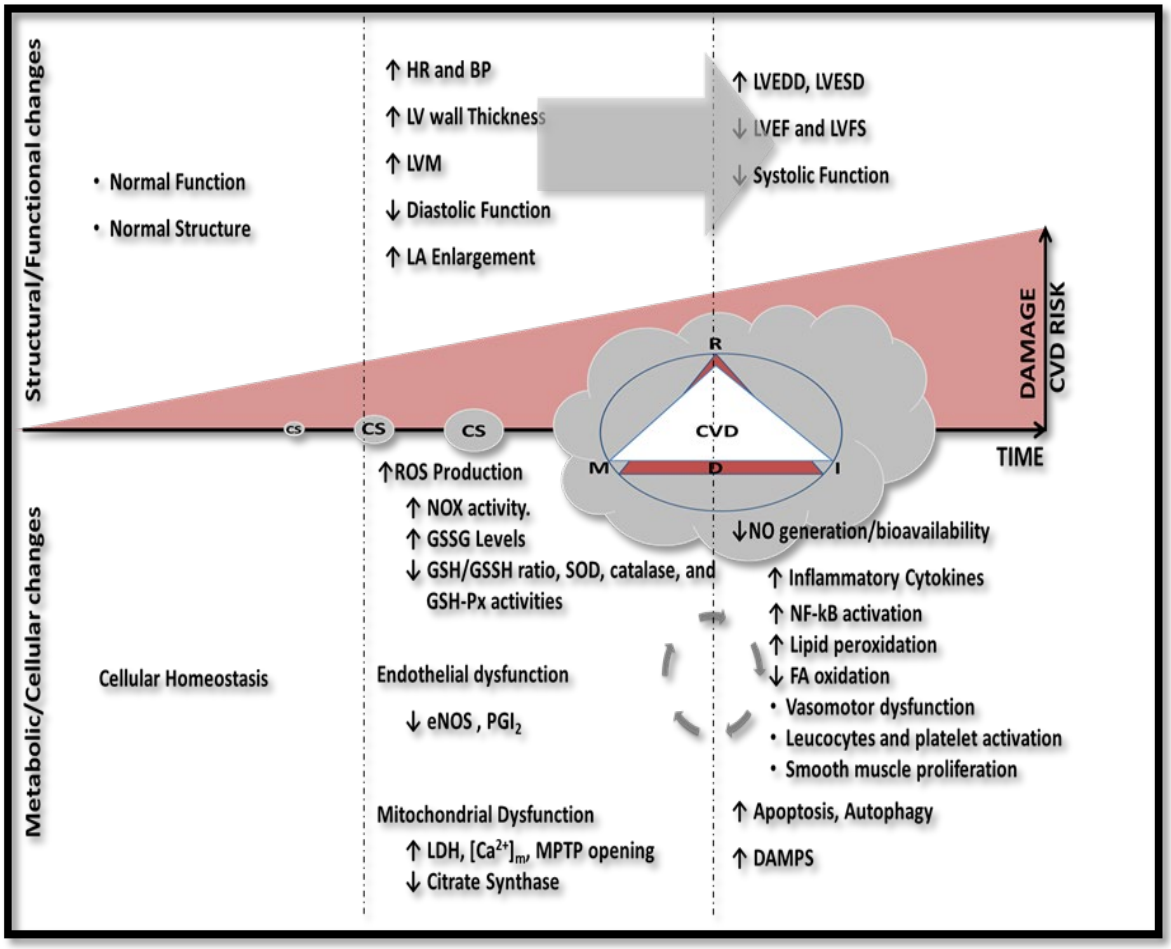

Figure 1: Illustration of the effect of persistent smoking on cardiovascular events as well as cardiac structural, functional, and cellular damage. RMID induces metabolic and cellular damage, altering heart structure and function and increasing the risk of CVD and myocardial injury [95].

1.4. CS and Gender Bias

Men and women are believed to manage their smoking behavior differently [96]. In general, men tend to use all tobacco products more frequently than women to reinforce nicotine effects [97]. According to WHO estimates, almost 40% of males are smokers compared to nearly 9% of women, and the rate of tobacco consumption peaks between the ages of 45-54 for men and 55-64 for women [7]. To further elaborate, current smokers in the world are 942 million males and 175 million females aged 15 and above [98]. In terms of mean adult male smoking prevalence, developed and developing nations are comparable (30.1% & 32%, respectively). Contrarily, industrialized countries have a substantially greater female smoking prevalence than developing nations (17.2% vs. 3.1%) [14] [99] [100].

Moreover, as per the World Bank report, 2.15 million of the 8.71 million annual cigarette fatalities are women with 71% living in low- and middle-income countries [101] [102]. As previously stated, smoking continues to be a significant contributor to the leading risk factors for cardiovascular disease in 2022, with a significant association between tobacco use and cardiovascular risk reported for both men and women [103]. According to studies, males who smoke have a risk of developing myocardial infarction (MI) that is nearly five times greater than that of women, and rises with cigarette consumption [104]. This gender disparity has been mostly linked to the female hormones' beneficial protective impact on the cardiovascular system [104]. Several decades of research have revealed a differential trend of CVD prevalence dependent on sex. Furthermore, the most recent epidemiological report indicated that, younger women have a reduced risk of developing CVD, the gender difference vanishes between the ages of

60 and 79, and that women surpass men at the age of 80 [105], i.e., young premenopausal women are protected against CVDs, but this protection ceases post menopause. As a result, the cardioprotective influence of the female hormone estrogen has been recognized as a primary factor contributing to the gender difference in CVD incidence [106].

It has been reported in contradictory research that women who smoke are more likely than males to experience negative health outcomes [107]. This has been attributable to a range of aspects. Women's undesirable health outcomes may be due to genetic and biological determinants, hormonal variables, personal lifestyle, work overload, socioeconomic factors, and second-hand smoke exposure [108].

Given these distinctions, it is reasonable to anticipate that the female heart will respond differently to the same extent of damage as the male heart. Eventually, the documented sex discrepancies in cardiovascular disease, notably in ventricular remodeling, have generated considerable speculation about the underlying etiology, with the function of circulating sex hormones undoubtedly playing a role [109].

1.5. Estrogen and Estrogen Receptors in CVD

1.5.1. Estrogen and Estrogen Receptors

Estrogen is the principal female sex hormone; it is vital in the development and physiology of numerous organ systems, including the cardiovascular system [110]. Estrogen exists in three major types: estrone (E1), estradiol (E2, also known as 17-estradiol), and estriol (E3) [111]. In the premenopausal phase of a woman's life, 17-β estradiol or estrogen E2 is the most prevalent type of circulating estrogen with the most potent estrogenic characteristics, therefore it has the strongest biological activity [112].

E2 is mostly synthetized and secreted by the ovaries until menopause [113]. Estrogen activity and physiological functions are modulated significantly through the estrogen receptor (ER), a member of a broad superfamily of nuclear receptors that operate as ligand-activated transcription factors [114].

The ER has been categorized into two distinct subtypes, alpha (ER-α) and beta (ER-β) [115]. In addition, G protein-coupled receptor 30 (GPR30) mediates estrogen functioning [116] [117]. ER-α, ER-β, and GPR30 are abundant in cardiac cells [118] [119] [120]. When estradiol (E2) binds to these receptors, it can act in two main ways: genomic (or nuclear) and non-genomic (or cytoplasmic) signaling pathways, as illustrated in figure 2 [121] [1].

In the direct genomic signaling pathway, when estradiol interacts with the cytoplasmic ER-α or ER-β, a conformational alteration occurs resulting in the receptor dimerization [122]. This complex subsequently relocates to the nucleus and attaches to chromatin at estrogen response elements (ERE) sequences [123]. Additionally, estradiol can influence the transcriptional activation of numerous genes lacking EREs in their promoter sequences (such as the vascular endothelial growth factor, VEGF). These processes through which estrogen regulates gene expression without directly binding DNA, are termed as "indirect genomic signaling" or "transcriptional cross-talk," and are dependent on estrogen receptors activating gene expression [124] [125].

Addressing the non-genomic route, it entails the stimulation of signal-transduction pathways with the subsequent generation of intracellular second messengers, thus ultimately modifying gene expression indirectly [126]. For example, when E2 binds to ER or GPR30, MAPK is activated, resulting in the enhanced expression of eNOS, which is a vasodilator.

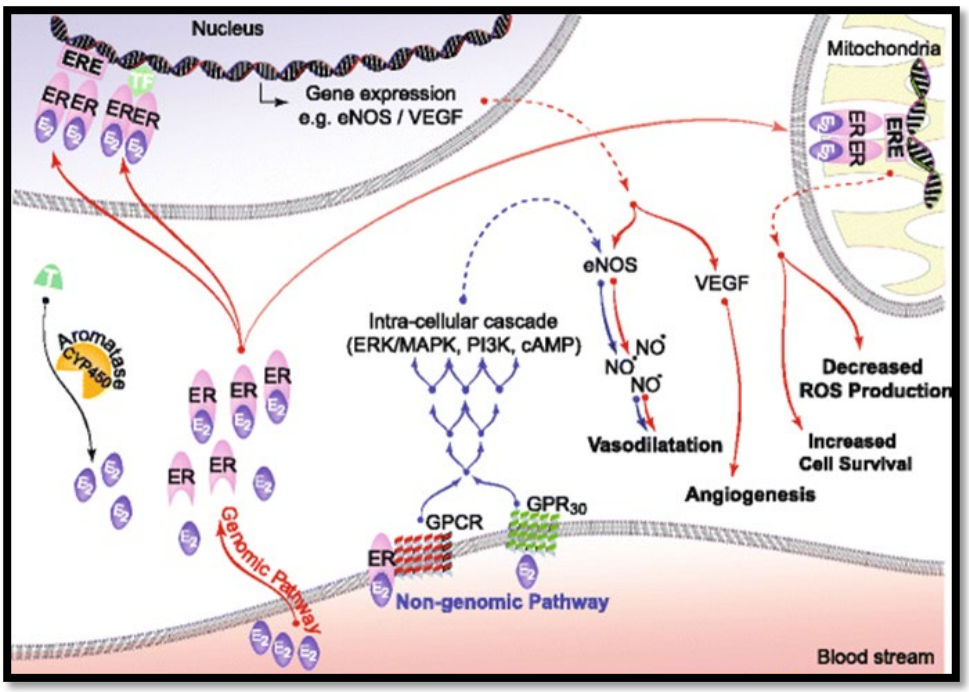

Figure 2*:* Nuclear and cytoplasmic signaling pathways of Estradiol [1]

1.5.2. Cardio Protective Effects of Estrogen

The fact that the gender disparity in the occurrence of cardiovascular events decreases after menopause, elucidates the assumption that estrogen performs an important protective function on the cardiovascular system, which is assumed to be mediated by ER. There is evidence that estrogen stimulates the fast efflux of NO from endothelial cells through a non-genomic mechanism, relaxing the smooth muscle of the vascular system, accompanied by an increase in intracellular calcium levels. [127] [128] . E2 has been found to lower MMP-2 gene expression by enhancing its phosphorylation via the MAPK signaling pathway [129], since it has been demonstrated that there is a correlation between systolic dysfunction and high

expression of MMP-2 [130]. GPR30 also exerts a cardioprotective role by restricting the proliferation of cardiac fibroblasts through anti-fibrotic pathways [131].

Further evidence that estrogen may have an anti-fibrotic effect comes from the actuality that estrogen and ERs perform a significant function in controlling MMP/TIMP activity [132]. Additionally, the absence of estrogen promotes the upregulation of genes that exacerbate cardiac oxidative stress and modulates inflammation-related genes especially TNF-α [133].

To corroborate these findings, research demonstrated that inhibited ERs aggravated cardiac remodeling and functional abnormalities that were formerly experienced in ovariectomized rats, supporting the hypothesis that estrogen has a crucial protective role on the cardiovascular system [132]. For instance, several studies have demonstrated a correlation between certain functions of estrogen in the cardiovascular system and the local oxidative stress reduction [134]. This is due to estrogen's ability to regulate the production of reactive oxygen species enzymes which can enhance the elimination of ROS and thereby decreasing oxidative stress [134]. Moreover, recent research showed that 17β-estradiol offers a protection against fibrosis in the heart as well as against the adverse remodeling of the extracellular matrix [121]. This is achieved through the regulation of the fibroblast proliferation and the suppression of pro-fibrotic genes [121]. To add, E2 also mediates it protection in the heart by stimulating angiogenesis and vasodilation. Thus, via these mechanisms, estrogen is able to curtails the remodeling of the heart and to mitigate cardiac remodeling [121].

CHAPTER 2

AIMS AND HYPOTHESES

CS has been established as a key risk factor for CVD [25], and CS exposure promotes smoke cardiomyopathy in both men and women in the absence of comorbidities. It has been demonstrated that CAD in males may be more prominent than in age-matched premenopausal women, although these gender-based discrepancies appear to be restricted at menopause [135]. Furthermore, the onset of atherosclerosis escalates when estrogen production ceases, whether naturally or as a result of surgery, and in women with compromised ovarian function [136] [137]. In fact, estrogen is known to have a protective effect against CVD in premenopausal females [110] [138]. The main objective of this project is to compare the CS-induced deleterious remodeling between males and females and its correlation with the estrogen hormone. We hypothesize that premenopausal females are less prone to adverse cardiac remodeling post-CS exposure than males and that endogenous estrogen may be a contributing factor in this process.

Specific Aims:

1. To assess the impact of smoking on the cardiac systolic performance, function and hemodynamics, 2-dimensional M-mode and B-mode echocardiography was performed. We assessed ejection fraction (EF), left ventricular end systolic diameter (LVESD), left ventricular end diastolic diameter (LVEDD), heart rate (HR), stroke volume (SV), and cardiac output (CO). Moreover, non-invasive

blood pressure system (CODA-2) was used to measure the systolic blood pressure.

2. In order to evaluate smoking effect on the heart structure, cardiomyocyte cross-sectional area was assessed to detect if cardiomyocyte hypertrophy took place. Moreover, left ventricular mass was measured for the detection of cardiac hypertrophy secondary to smoking.
3. Different biomarkers and cytokines were assessed by RT-qPCR and western blot to rule out smoking consequences at the molecular level in the heart:
    - ❖ We assessed inflammation in terms of the pro-inflammatory cytokines IL-1β protein expression levels and tumor necrosis factor alpha (TNF-α) mRNA expression levels. Moreover, protein expression levels of anti-inflammatory cytokines IL-4 and IL-13 were assessed.
    - ❖ We investigated the changes in the protein expression levels of Alpha Smooth Muscle Actin an indicator of fibroblasts to myofibroblasts differentiation, the mRNA levels of CTGF which is considered an important mediator of tissue fibrosis, and the ratio of proapoptotic BAX mRNA levels to the antiapoptotic BCL2. Moreover, we examined the expression of gelatinases MMP-2 and MMP-9 which are associated with ECM degradation. These were performed to detect the effect of smoking on some markers related to fibrosis and apoptosis.
    - ❖ Since smoking can have an immediate effect on oxidative stress, we measured the mRNA expression levels of NOX4 and SOD-1.

CHAPTER 3

MATERIALS AND METHODS

3.1. Study Input

3.1.1. Animals

The experiments outlined in this research study were conducted using four months old wild-type (WT) C57BL/6J male and female mice weighing 20-25 grams. Animals were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at the AUB animal care facility (ACF) under pathogen-free conditions with constant temperature and humidity control. They were kept in ideal circumstances including a 12-hour light/12-hour dark cycle and unrestricted access to typical chow and water. Male mice were split into two subgroups, as shown in the figure below: a control group (MCTRL) and a chronic smoking (MCS) group (8 weeks of CS exposure). However, the females were divided into three subgroups: a control group (FCTRL), a chronic smoking group (FCS), and an ovariectomized chronic smoking group (OVX+CS) (ovariectomy prior to 8 weeks of CS exposure). The Institutional Animal Care and Use Committee (IACUC # 18-2-RN560) approved all animal experiments in accordance with the 8th edition of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Of note, an ovariectomized control group without exposure to cigarette smoking was used at the cardiac functional level. Unfortunately, at the molecular and structural level, the tissues of this group were not good to use, and this was a limiting factor in our study.

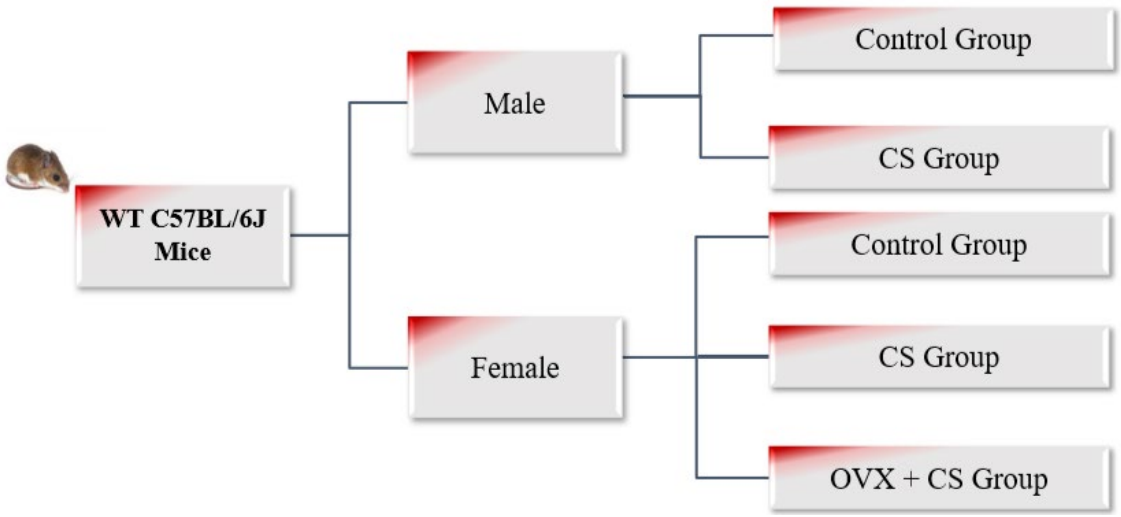

Figure 1: Study input. CS: cigarette smoking; OVX: ovariectomy

3.1.2. CS Exposure Protocol

Mice were subjected to 90-minute smoking sessions, with 12 cigarettes per session, twice a day for 8 weeks using Oro-Nasal and Respiratory Exposure Systems device (ONARES, CH Technologies, U.S.A). This apparatus comprises a smoke generator with a mixing chamber as well as a rodent exposure carousel with a "nose only" mode. To distinguish the impacts of smoking, conscious and immobilized mice were exposed to smoke using 3R4F scientifically manufactured cigarettes (University of Kentucky, Lexington, KY, U.S.A.) having restrained dosages of toxins and molecules administered to the mice through the respiratory system. The cigarettes, positioned in the cigarette puffer, were set to generate puffs at a consistent frequency of 2 puffs every 50 seconds with a duration of 2 seconds/puff, thus resulting in a total particulate matter (TPM) concentration of approximately 100 mg/cm3/mouse/session.

3.1.3. Experimental Design and Timeline

The study design consisted of conducting baseline echocardiography on the ovariectomized group prior to, and post-ovariectomy. Similarly, baseline echocardiography was also performed on the male and female smoking groups prior to cigarette smoke exposure. For estrogen to clear before CS exposure, smoking was initiated after four weeks of ovariectomy. All mice were exposed to 8-weeks of cigarette smoke inhalation, followed by a final echocardiography assessment. Systolic blood pressure was measured at baseline, after ovariectomy, and at two days after the last smoked cigarette Upon completion of the exposure period, mice were sacrificed and the heart was collected for histological and molecular analysis.

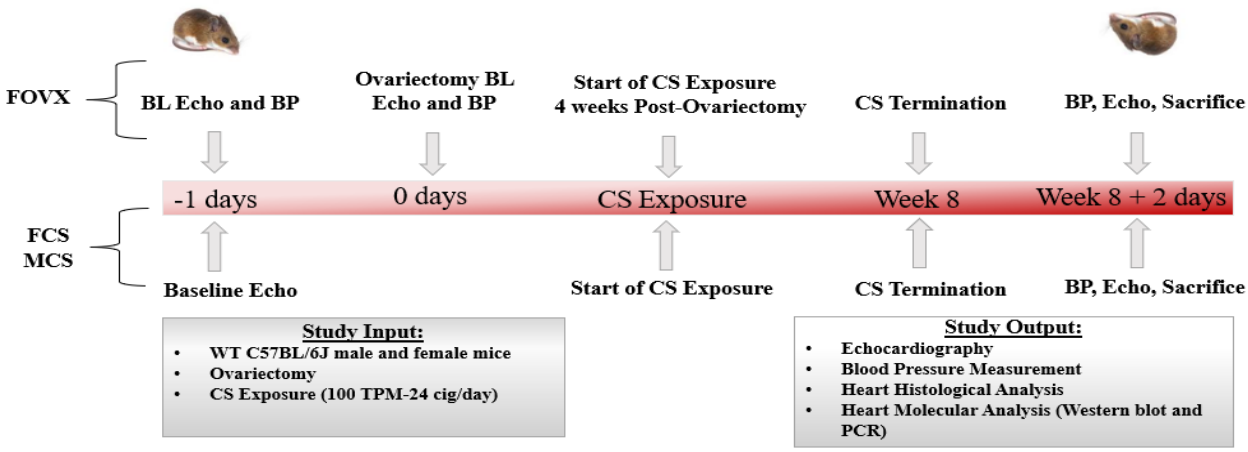

Figure 4: Study design and timeline

3.2. Blood Pressure Measurements:

Blood pressure was measured in unconscious anesthetized mice according to the Institutional Animal Care and Use Committee (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 at two days after the last smoked cigarette.

3.3. Surgical Procedures

3.3.1. Ovariectomy

Chlorhexidine solution was used to disinfect the skin after shaving hair off the flank area (between the last rib and above the pelvis). Note that the work was carried out in an aseptic environment. An incision was made on the right side of the skin and curved-tip scissors were used to separate the musculature. Following that, the ovarian fat pad was carefully removed from the incision, and the area below the ovary was firmly constricted using hemostatic tweezers. To delimit the region to be removed, two knots were tied using sterile thread, and then the ovary was removed. Similarly, the same steps were repeated on the left side. Tramadol was administered subcutaneously to finalize the surgery and ovariectomized mice were positioned on a heating pad until they recovered. A 1.4 mL solution of acetaminophen was added to 300 mL of water (final concentration, 0.47 mg/mL) and kept at libitum for three days. The animals were monitored regularly on a daily basis, for any symptoms of inflammation and the surgical site was examined for any signs of pain or discomfort.

3.3.2. Echocardiography

Echocardiography was done in accordance with the American Society of Echocardiography Guidelines using the Visual Sonics Echo system (Vevo 2100, High-Resolution Imaging System, VisualSonics, Inc., Toronto, Canada) equipped with a 22–55 MHz (MS550D) linear transducer (VisualSonics). Preceding echocardiography, mice from each group were anesthetized via 3-4% isoflurane diluted in oxygen before being positioned on the temperature-controlled platform to preserve the optimal body temperature at 37 °C. This was followed by chest hair removal and the application of ultrasonic gel to the heart area. In the parasternal long and short-axis views, the probe was put on the left thorax and the ultra-sound beam was oriented at the mid-papillary muscle level to produce B-mode and M-mode echocardiogram images. For the male and female smoking groups, echocardiography was recorded both at baseline and after CS exposure. Whereas for the female ovariectomized group, echocardiography was performed at baseline (before ovariectomy), after ovariectomy, and after CS exposure.

3.3.3. Necropsy

First, the mice received an intraperitoneal injection of 100 µl Heparin 15 minutes before being sacrificed for blood collection. Following this, 3% isoflurane vapor (Forane®) diluted with O2 was used to induce anesthesia. A cardiac puncture was performed to evacuate the blood from the left ventricle, which was then centrifuged at 2200 rpm for 10 min. Plasma was isolated, then combined with protease inhibitors, flash-frozen in liquid nitrogen, and preserved at -80°C for further analysis. The apex and base of the heart were collected in cryotubes, snap-frozen in liquid nitrogen, and maintained at -80°C for molecular analysis. However, the mid-section of the heart was stored in 10%

formalin for histological examination. Animals were sacrificed two days after the last smoked cigarette.

3.4. Histology

3.4.1. Hematoxylin & Eosin (H&E) Staining

Cardiomyocytes hypertrophy was assessed by using H&E staining. Hematoxylin is a basic blue-purple dye that stains nucleic acids. Eosin, on the other hand, is an acidic pink dye that stains protein. In brief, formalin-fixed, paraffin-embedded heart sections were deparaffinized and rehydrated with a gradient percentage of ethanol (100%, 95%, and 70%). The sections were subsequently rinsed in distilled water and stained with Weigert's iron hematoxylin solution for 10 min. Then, the slides were washed before being examined under light microscopy. Images were obtained at 20X magnification and hypertrophy was evaluated via Image J software (https://imagej.nih.gov/ij/).

3.5. Molecular Analysis

3.5.1. Protein Extraction and Western Blot

Heart tissues were placed in a 200 µl extraction buffer (RIPA) after being crushed in liquid nitrogen, and left overnight on a rotary mixer at 4°C. After collecting the supernatant, the protein concentrations were quantified using a Detergent Compatible Assay (DC protein assay kit, Bio-Rad catalog# 5000112). Samples were heated in Laemmli buffer for 10 minutes at 95°C and stored at -20°C. Protein samples (30µg) were loaded into a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) wells, then electroblotted into nitrocellulose membranes at 90 volts inside the cold room (at 4ºC) for 90 minutes. Membranes were blocked with 5% non-fat dry milk in 0.1% TBST (Tris buffer saline with 0.1% Tween 20) for one hour at room temperature. Afterward, the membranes were probed overnight with primary antibodies against Interleukins (IL-1β, IL-4, IL-13) and α-SMA (alpha-smooth muscle actin) diluted in 0.1% TBST. The next day, TBST 0.02% was used to wash the membranes four times before being incubated with the Goat pAb to Rb IgG (HRP) secondary antibody (1/5000) at room temperature for one hour. After washing the membranes twice with TBST 0.02% and then twice with TBS 1x, the bands were visualized with an enhanced chemiluminescence kit (Biorad) using the chemidoc MP imaging System-Bio-Rad machine. By incubating the membrane with the reversible total protein stain (Ponceau Red) for few minutes, the protein expression level was normalized to total protein to establish equal loading. Image J software was used to analyze the bands (https://imagej.nih.gov/ij/).

Primary Antibody	**Dilution**
Anti-IL-1β (Abcam, catalog#)	1/500
Anti-IL-4 (Abcam, catalog#)	1/1000
Anti-IL-13 (Abcam, catalog#)	1/500
Anti-α-SMA (Abcam, catalog# ab5694)	1/200

Table 1. Western blot analysis antibodies

3.5.2. RNA Extraction and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Frozen heart tissues were used to extract total RNA by using Trizol as directed by the manufacturer's instructions (Thermo Fisher Scientific, Grand Island, NY, USA) and the NanoDrop® ND-1000 UV-Vis Spectrophotometer was used to measure RNA concentrations. The 260:280 absorbance ratio validated the purity of the extracted RNA. cDNA was synthesized from 1 µg RNA using Revert Aid 1st Strand cDNA synthesis kit (Thermo, USA) and the CFX96 real-time PCR system (Bio-Rad, Germany) was then used to analyze the mRNA expression. To quantify the expression of the genes listed in table 2, RT-qPCR was performed in duplicate with a final volume of 10µl using SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and gene-specific primers. These genes are: BCL (B Cell Lymphoma)-Associated X (BAX), B-cell lymphoma 2 (BCL2), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), NADPH oxidase 4 (Nox-4), tumor necrosis factor-alpha (TNF-α), connective tissue growth factor (CTGF) and superoxide dismutase 1 (SOD1). 4 µl cDNA, 0.9µl DNase free water, 5µl SYBR green, and 0.05µl of the forward and reverse primers were used for each 10µl PCR mixture. Cardiac gene expression between different samples was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and results were reported as $2^{-\Delta\Delta Ct}$ values. The following primers were obtained from Macrogen (Seoul, South Korea):

Primer	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
GAPDH	TGT GTC CGT GGA TCT GA	TTG CTG TTG AAG TCG CAG GAG
BAX	ATC CAA GAC CAG GGT GGC T	CCT TCC CCC ATT CAT CCC AG

BCL2	AGT ACC TGA ACC GGC ATC TG	TAT GCA CCC AGA GTG ATG CAG
TNF-α	TGT GCT CAG AGC TTT CAA CAA	CTT GAT GGT GGT GCA TGA GA
NOX-4	ACC AAA TGT TGG GCG ATT GTG	GGC TAC ATG CAC ACC TGA GA
MMP-2	AGA TGC AGA AGT TCT TTG GGC TGC	AGT TGT AGT TGG CCA CAT CTG GGT
MMP-9	ACC ACA GCC AAC TAT GAC CAG GAT	AAG AGT ACT GCT TGC CCA GGA AGA
CTGF	GTG GAA TAT TGC CGG TGC A	CCA TTG AAG CAT CTT GGT TCG
SOD-1	ACT GGT GGT CCA TGA AAA AGC	AAC GAC TTC CAG CGT TTC CT

Table 2. Primers used for RT-PCR

3.6. Statistical Analysis

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

CHAPTER 4

RESULTS

4.1. Effect of CS on Ejection Fraction (EF)

In the male smoking group (MCS) the ejection fraction (EF) remained unchanged compared to the male non-smoking group (MCTRL) (Figure 5A).

Nevertheless, this parameter showed a significant increase in the female smoking group (F+CS) when compared to its control non-smoking group (F-CS). However, the ovariectomized female smoking group (FOVX+CS) showed similar EF when compared to its ovariectomized female non-smoking group (FOVX-CS) (Figure 5B).

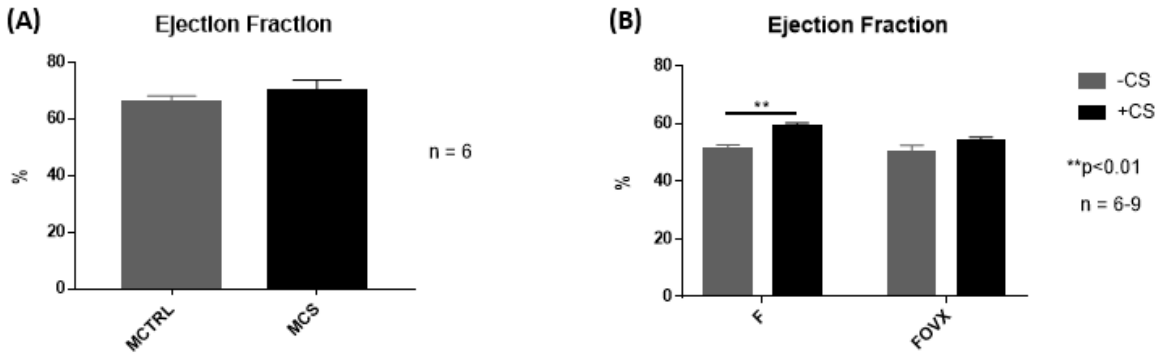

Figure 5: Effect of CS on the EF. (B) EF showed a remarkable increase in the F+CS group when compared with the F-CS group. However, it remained unchanged in the MCS group compared to the MCTRL group (A) and in the FOVX+CS group compared to the FOVX-CS group (B). F: female; FOVX: ovariectomized female; MCTRL: male control; MCS: male smoking; -CS: before cigarette smoking; +CS: after cigarette smoking; EF: ejection fraction. Two-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B) and males (A), respectively. All bars represent mean ± SEM (**P<0.01).

4.2. Effect of CS on Left Ventricular End Systolic Diameter (LVESD) and Diastolic Diameter (LVEDD)

The LVESD showed a trend to decrease in the male smoking group (MCS) when compared to the male non-smoking group (MCTRL) (Figure 6A).

Additionally, a significant decrease was observed in the LVESD in the female smoking group following CS (F+CS) when compared to its non-smoking counterpart (F-CS). Similarly, a marked decrease was revealed post-CS in the ovariectomized female smoking group (FOVX+CS) when compared to its non-smoking counterpart (FOVX-CS) (Figure 6B).

For the LVEDD, all the male and female groups showed comparable results before and after cigarette smoking (Figure 6C, 6D).

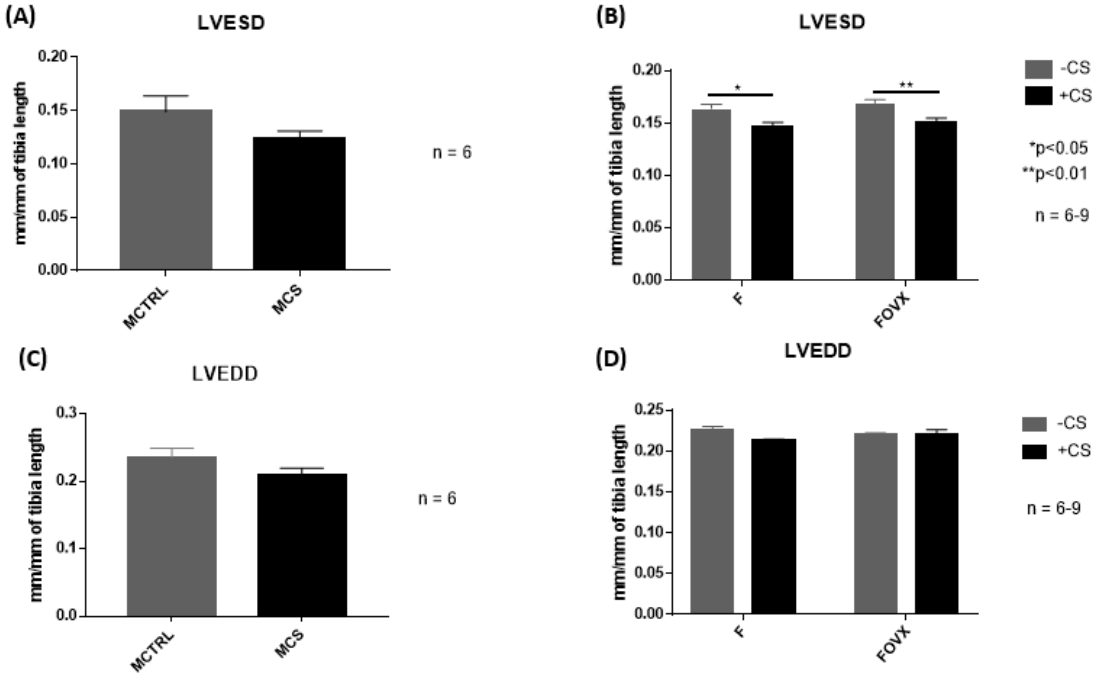

Figure 6: Effect of CS on LVESD and LVEDD. LVESD significantly decreased in the F+CS group when compared with the F-CS and in the FOVX-CS group relative to the FOVX+CS (B). In the MCS group compared to the MCTRL group, LVESD showed a decreasing tendency (A). As for the LVEDD, it remained unchanged in the male and female groups, before and after cigarette smoking exposure (C and D). F: female; FOVX: ovariectomized female; MCTRL: male control; MCS: male smoking; -CS: before cigarette smoking; +CS: after cigarette smoking; LVESD: left ventricular end systolic diameter; LVEDD: left ventricular end diastolic diameter. Two-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B and D) and males (A and C), respectively. All bars represent mean ± SEM (*P<0.05, **P<0.01)

4.3. Effect of CS on Heart Rate (HR)

The heart rate was comparable in the smoking male group (MCS) and non-smoking male group (MCTRL) (Figure 7A).

On the other hand, results showed that the HR in the female smoking group (F+CS) and ovariectomized female smoking group (FOVX+CS) was significantly higher than their non-smoking counterparts, (F-CS) and (FOVX-CS) respectively (Figure 7B).

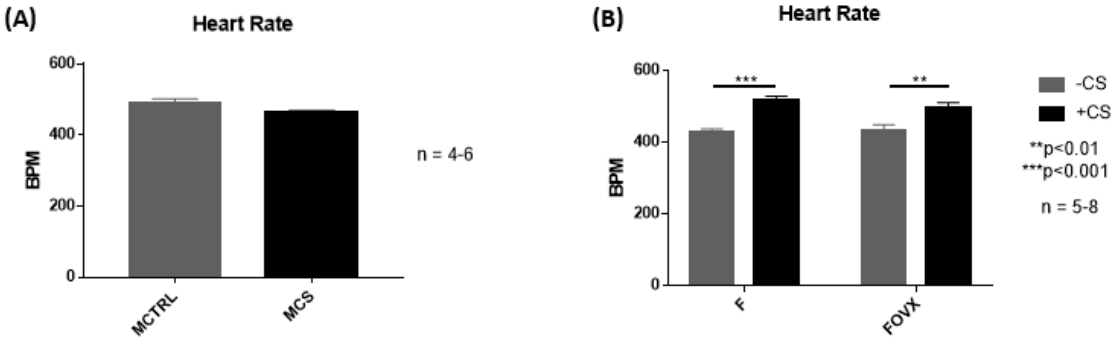

Figure 7: Effect of CS on HR. Heart rate considerably increased in the F+CS and FOVX+CS groups relative to their control counterparts, F-CS and FOVX-CS, respectively (B). In the MCS group, HR remained unchanged when compared to the MCTRL group (A). F: female; FOVX: ovariectomized female; MCTRL: male control; MCS: male smoking; -CS: before cigarette smoking; +CS: after cigarette smoking; HR: heart rate. Two-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B) and males (A), respectively. All bars represent mean ± SEM (**P<0.01, ***P<0.001)

4.4. Effect of CS on Stroke Volume

The stroke volume (SV) was comparable between the male smoking group (MCS) and the male non-smoking group (MCTRL) (Figure 8A).

Similarly, the female smoking group (F+CS) showed no significant change in the SV when compared to its non-smoking counterpart (F-CS). Whereas the SV in the ovariectomized female smoking group (FOVX+CS) was significantly higher than the ovariectomized female non-smoking group (FOVX-CS) (Figure 8B).

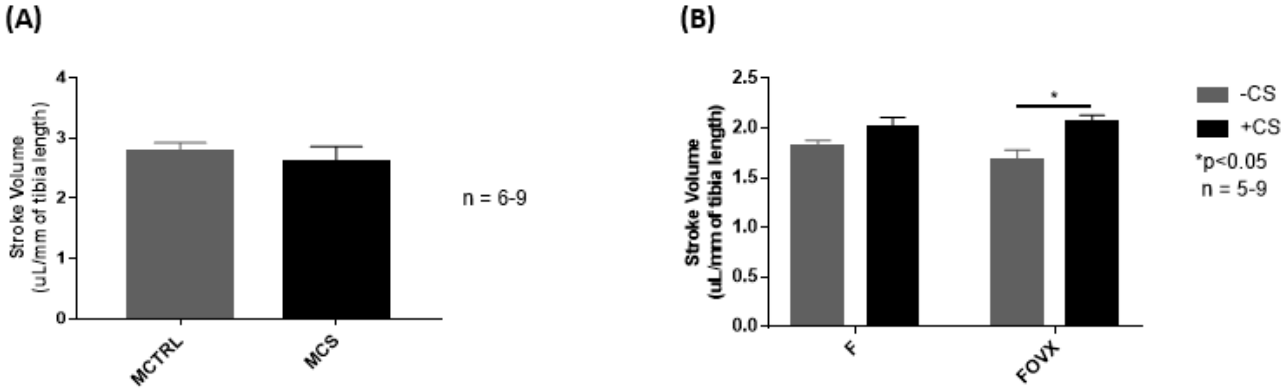

Figure 8: Effect of CS on SV. SV notably increased in the FOVX+CS group when compared to the FOVX-CS group (B). In the F+CS group, SV showed a tendency to increase compared to the F-CS group (B). As for males, SV didn't change post-CS (A). F: female; FOVX: ovariectomized female; MCTRL: male control; MCS: male smoking; -CS: before cigarette smoking; +CS: after cigarette smoking; SV: stroke volume. Two-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B) and males (A), respectively. All bars represent mean ± SEM (*P<0.05)

4.5. Effect of CS on Cardiac Output (CO)

Similar cardiac output was seen between the male smoking group (MCS) and the male non-smoking group (MCTRL) (Figure 9A). The cardiac output in the female smoking group (F+CS) showed a trend to increase when compared to its non-smoking counterpart (F-CS). Added to that, CO was significantly higher in the ovariectomized female smoking group (FOVX+CS) when compared to the ovariectomized female non-smoking group (FOVX-CS) (Figure 9B).

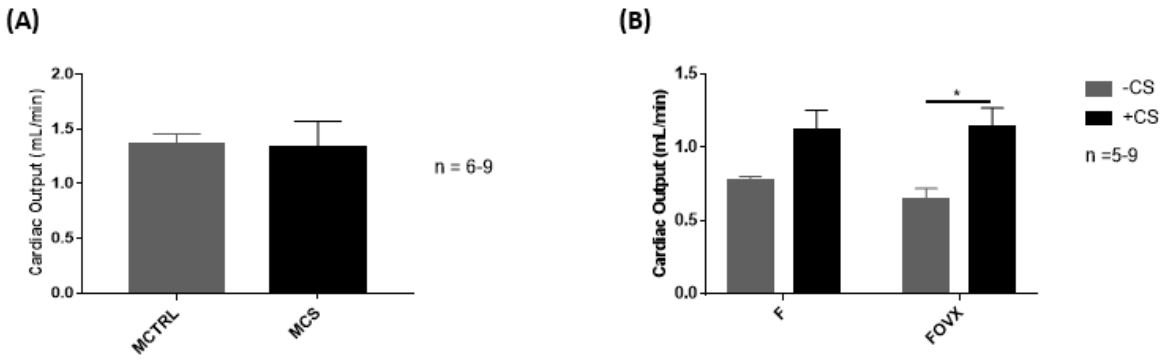

Figure 9: Effect of CS on CO. CO significantly increased in the FOVX+CS group when compared to the FOVX-CS group (B) and revealed a trend to increase in the F+CS group when compared to the F-CS group (B). In males, CO remained unchanged pre- and post-CS (A). F: female; FOVX: ovariectomized female; MCTRL: male control; MCS: male smoking; -CS: before cigarette smoking; +CS: after cigarette smoking; CO: cardiac output. Two-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B) and males (A), respectively All bars represent mean ± SEM (*P<0.05

4.6. Effect of CS on Systolic Blood Pressure (BP)

In the male smoking group (MCS), the systolic blood pressure (SBP) remained unchanged compared to the male non-smoking group (MCTRL) (Figure 10A)

In females, the SBP was comparable between the female smoking group (F+CS) and the female non-smoking group (F-CS) (Figure 10B). However, the BP in ovariectomized smoking female group (FOVX+CS) was significantly higher than that of the ovariectomized group before and after ovariectomy, (FOVX-before OVX) and (FOVX-after OVX), respectively (Figure 10C)

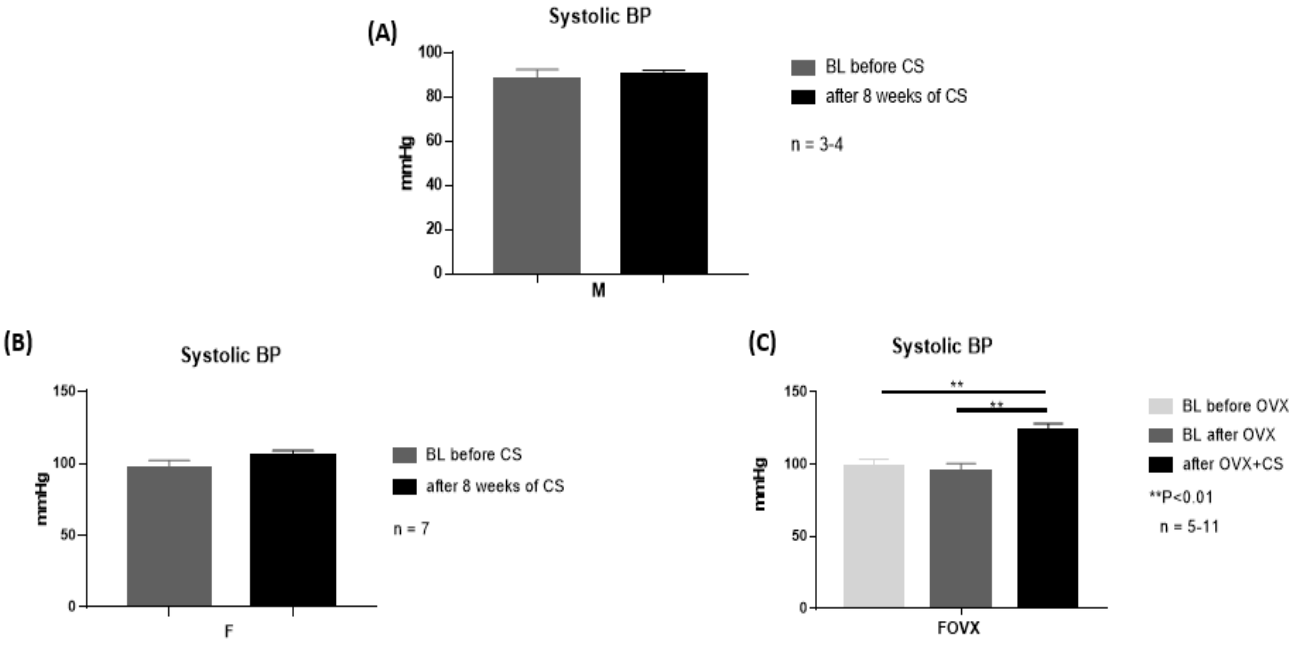

Figure 10: Effect of CS on SBP. Systolic BP revealed a notable elevation in the FOVX+CS group when compared with that of the FOVX-before OVX group and the FOVX-after OVX group(C). SBP in both males and females smoking groups was constant before and after cigarette smoking (A and B). F: female; FOVX: ovariectomized female; M: Male; +CS: after cigarette smoking; BP: blood pressure; BL: baseline; OVX: ovariectomy. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for FOVX group (C) and males and females smoking groups (A and B), respectively. All bars represent mean ± SEM (**P<0.01)

4.7. Effect of CS on Body Weight

The body weight of the male smoking group (MCS) was significantly lower than that of the male non-smoking group (MCTRL) (Figure 11A).

Of note, the body weight revealed no significant variation in the female smoking group (F+CS) and ovariectomized female smoking group (FOVX+CS) when compared to their non-smoking counterparts, (F-CS) and (FOVX-CS), respectively. Contrarily, the body weight in the ovariectomized female smoking group (FOVX+CS) was significantly higher than that of the female smoking group (F+CS) (Figure 9B).

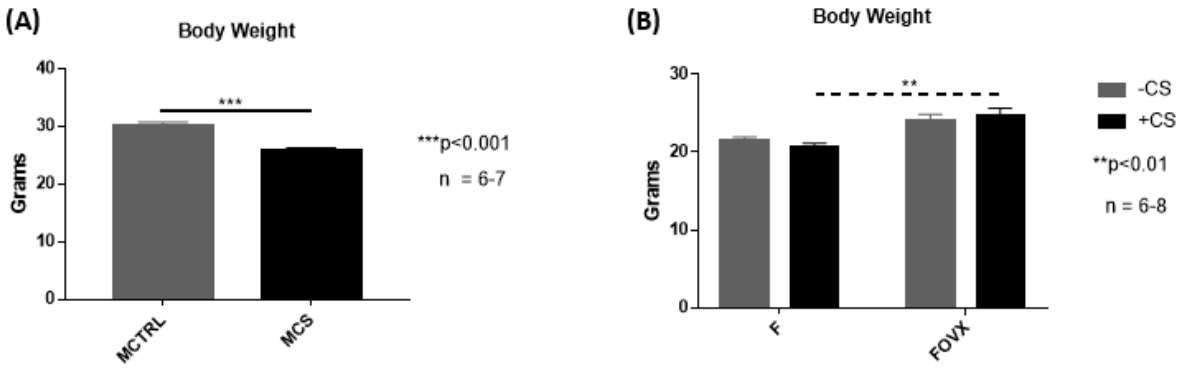

Figure 11: Effect of CS on BW. MCS group showed a significant decrease in body weight when compared to the MCTRL group (A). FOVX+CS group showed a significant increase in body weight when compared to the F+CS group (B). F: female; FOVX: ovariectomized female; MCTRL: male control; MCS: male smoking; -CS: before cigarette smoking; +CS: after cigarette smoking; BW: body weight. Two-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B) and males (A), respectively. All bars represent mean ± SEM (**P<0.01, ***P<0.001)

4.8. Effect of CS on Left Ventricular Mass

In the male smoking group (MCS) similar LV mass was seen in comparison to the male non-smoking group (MCTRL) (Figure 12A).

However, the LV mass of both the female smoking group (FCS) and the ovariectomized female smoking group (FOVX) was significantly higher than that of the female control group (FCTRL) (Figure 12B).

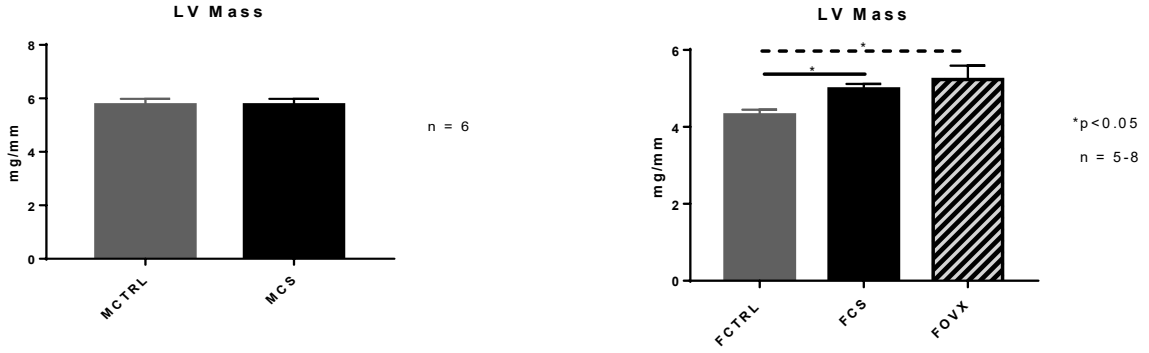

Figure 12: Effect of CS on the LV mass. LV Mass increased remarkably in the FOVX group when compared with both the FCS and FCTRL groups (B). No significant change was observed in the MCS group when compared to the MCTRL group (A). FCTRL: female control; FCS: female smoking FOVX: ovariectomized female smoking; MCTRL: male control; MCS: male smoking; LV: left ventricle. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B) and males (A), respectively. All bars represent mean ± SEM (*P<0.05)

4.9. Effect of CS on Cross-Sectional Area of Cardiomyocytes (CSA)

In the male smoking group (MCS), cardiomyocyte cross-sectional area significantly increased when compared to the male non-smoking group (MCTRL) (Figure 13A).

In the female smoking group (FCS), CSA revealed a trend to increase when compared to the female control group (FCTRL). Moreover, CSA increased significantly in the ovariectomized female smoking group (FOVX) when compared to the female control group (FCTRL) . (Figure 13B)

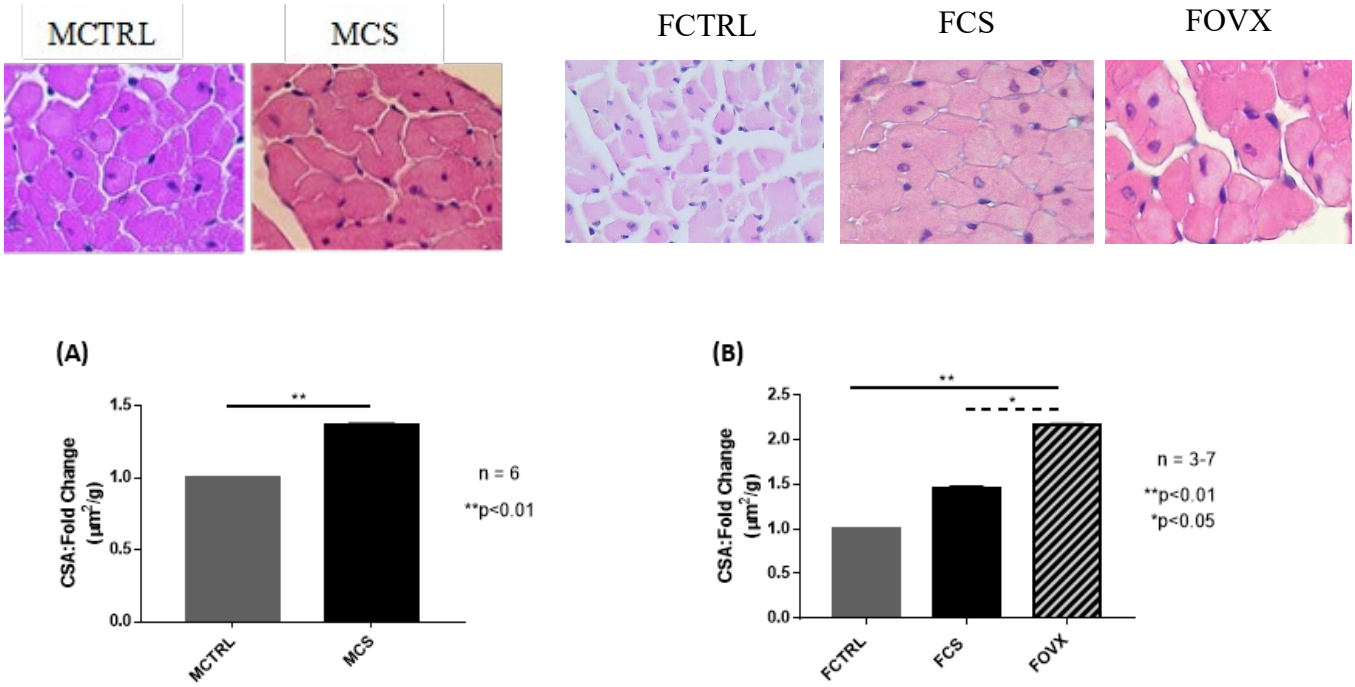

Figure 12: effect of CS on cardiomyocytes CSA. CSA remarkably increased in the MCS group when compared to the male non-smoking group MCTRL (A). In the FOVX group, CSA significantly increased when compared to the FCS and FCTRL groups (B). FCTRL: female control; FCS: female smoking FOVX: ovariectomized female smoking; MCTRL: male control; MCS: male smoking; CSA: cross-sectional area. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B) and males (A), respectively. All bars represent mean ± SEM (*P<0.05, **P<0.01)

4.10. Effect of CS on Cardiac Interleukin-1β (IL-1 β) Protein Levels and Tumor Necrosis Factor Alpha (TNF-α) mRNA Expression Levels

In the male smoking group (MCS), IL-1β protein levels showed no significant change, whereas TNF-α mRNA expression levels showed a tendency to decrease when compared to the male control group (MCTRL) (Figures 14A, 14C)

In the female smoking group (FCS) and the ovariectomized female smoking group (FOVX), IL-1 β protein levels, as well as TNF-α mRNA expression levels revealed a tendency to increase when compared to the female control group (FCTRL) (Figures 14B, 14D).

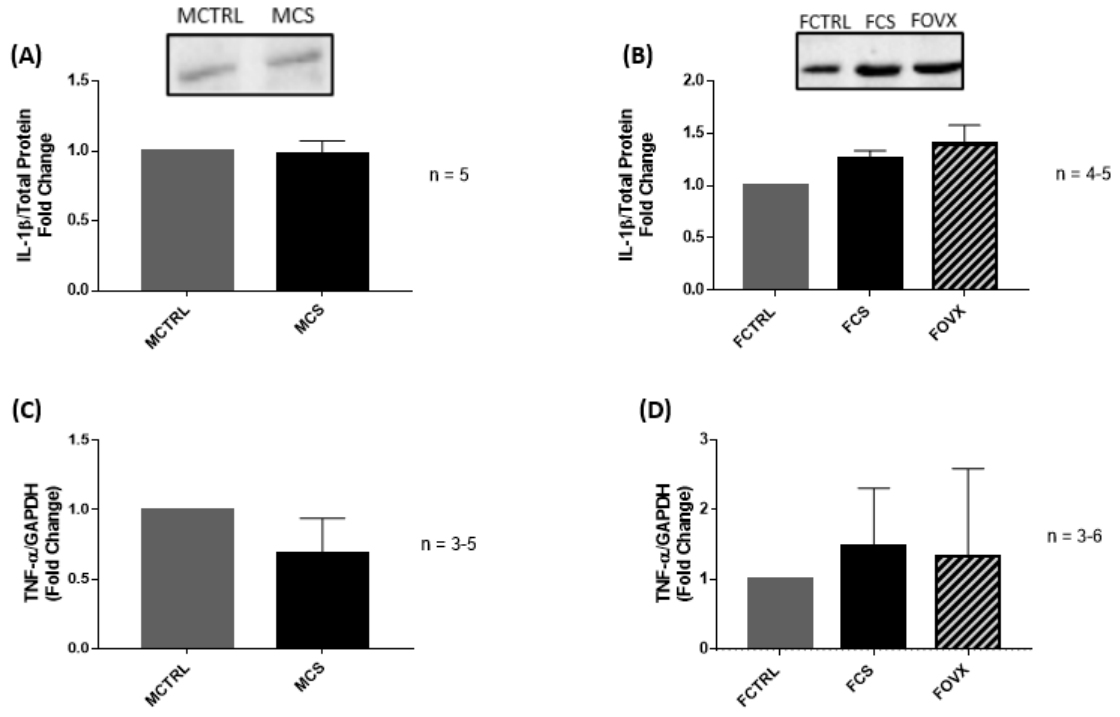

Figure 14: Effect of CS on IL-1β protein levels and TNF-α mRNA expression levels. IL-1β showed comparable results in the MCS and MCTRL groups (A), whereas it showed a trend to increase in the FCS and FOVX groups when compared to the FCTRL group (B). TNFα revealed a trend to decrease in the MCS group when compared to the MCTRL group (C), yet it tends to increase in the FCS and FOVX groups when compared to the FCTRL group. FCTRL: female control; FCS: female smoking; FOVX: ovariectomized female; MCTRL: male control; MCS: male smoking; IL-1β: interleukin-1 beta; TNF-α: tumor necrosis factor alpha. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B and D) and males (A and C), respectively. All bars represent mean ± SEM (**P<0.01)

4.11. Effect of CS on Cardiac α-SMA Protein Levels, CTGF mRNA Expression Levels, and Ratio of BAX/BCL2 mRNA Expression Levels

In the male smoking mice (MCS), cardiac α-SMA protein levels and ratio of BAX/BCL2 mRNA expression levels showed a remarkable decline when compared to the expression detected in the male control group (MCTRL) (Figures 15A, 15E). Similarly, CTGF mRNA expression levels in MCS group showed a trend to decrease when compared to the MCTRL group (Figure 15C).

In the female smoking group (FCS), cardiac α-SMA protein levels and ratio of BAX/BCL2 mRNA expression levels overpassed the levels detected in the female control group (FCTRL) (Figures 15B, 15F). Similarly, there was a tendency for elevated CTGF mRNA expression levels in the FCS group compared to the FCTRL group (Figure 15D). However, BAX/BCL2 ratio significantly decreased in the female ovariectomized female smoking group (FOVX) when compared to the FCS group (Figure 15F). As for the α-SMA and CTGF levels, they exhibited a trend to decrease in the FOVX group when compared to the FCS group (Figures 15B, 15D).

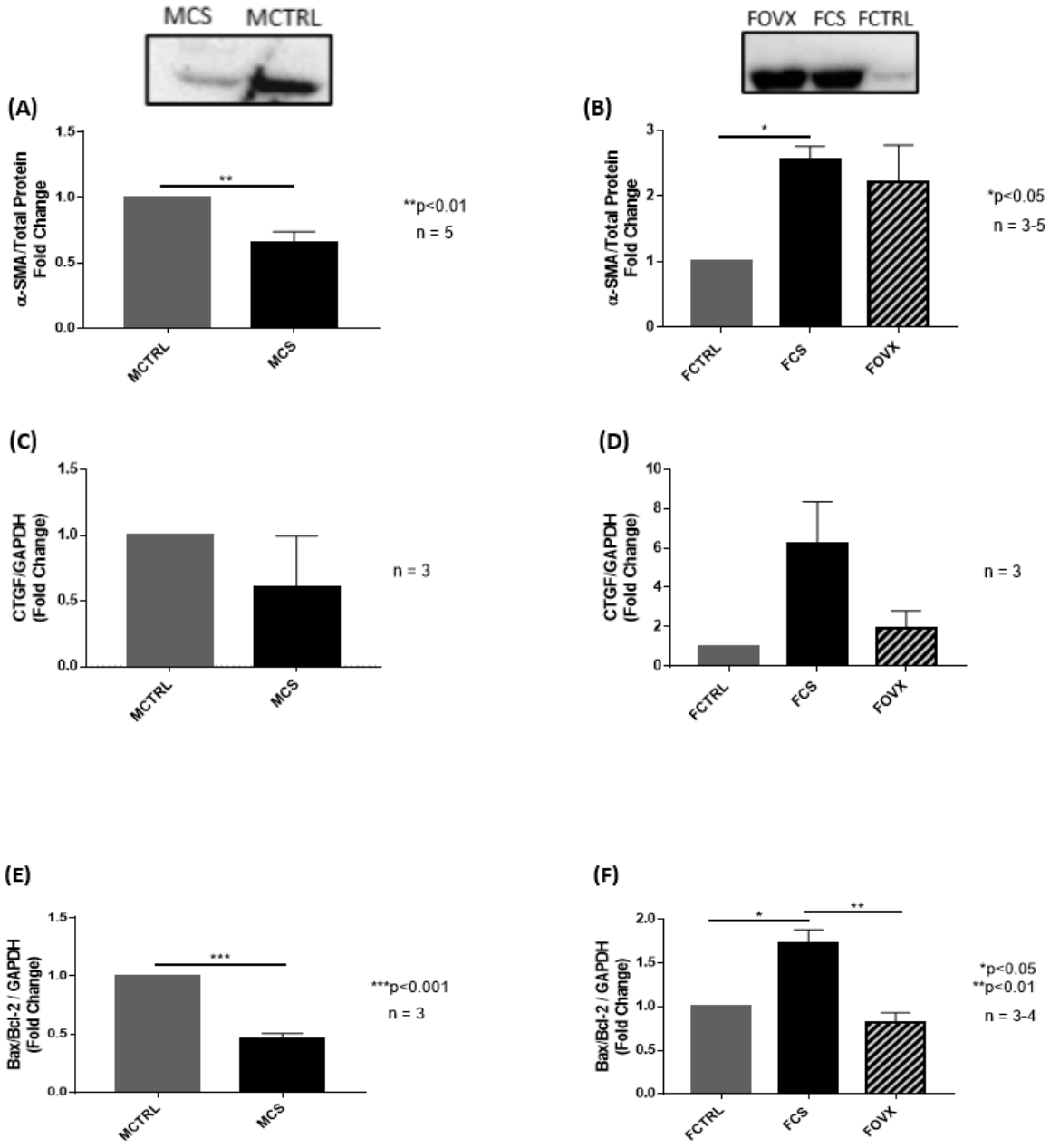

Figure 15: Effect of CS on α-SMA protein levels, CTGF and BAX/BCL2 mRNA expression levels.

α-SMA and BAX/BCL2 ratio levels considerably decreased in the MCS group compared to the MCTRL group (A and E), and CTGF levels showed a trend to decrease (C). α-SMA and BAX/BCL2 significantly increase in the FCS group compared to the FCTRL group (B and F), and CTGF showed a trend to increase (D). BAX/BCL2 ratio declined remarkably in the FOVX group when compared to the FCS group; CTGF and α-SMA exhibited a trend to decrease (B and D. FCTRL: female control; FCS: female smoking; FOVX: ovariectomized female smoking ; MCTRL: male control; MCS: male smoking;

α-SMA: alpha-smooth muscle actin; CTGF: connective tissue growth factor; BAX: B Cell Lymphoma-Associated X; BCL2: B-cell lymphoma 2. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B, D and F) and males (A, C and E), respectively. All bars represent mean ± SEM (*P<0.05, **P<0.01, ***P<0.001)

4.12. Effect of CS on Cardiac IL-4 and IL-13 Protein Levels

The male smoking group (MCS) showed a significant drop in cardiac IL-4 and IL-13 protein levels when compared to the expression detected in the male control group (MCTRL) (Figures 16A, 16C).

Moreover, IL-4 and IL-13 protein levels substantially decreased in both, female smoking group (FCS) and ovariectomized female smoking group (FOVX) when compared to the female control group (FCTRL) (Figures 16B, 16 D).

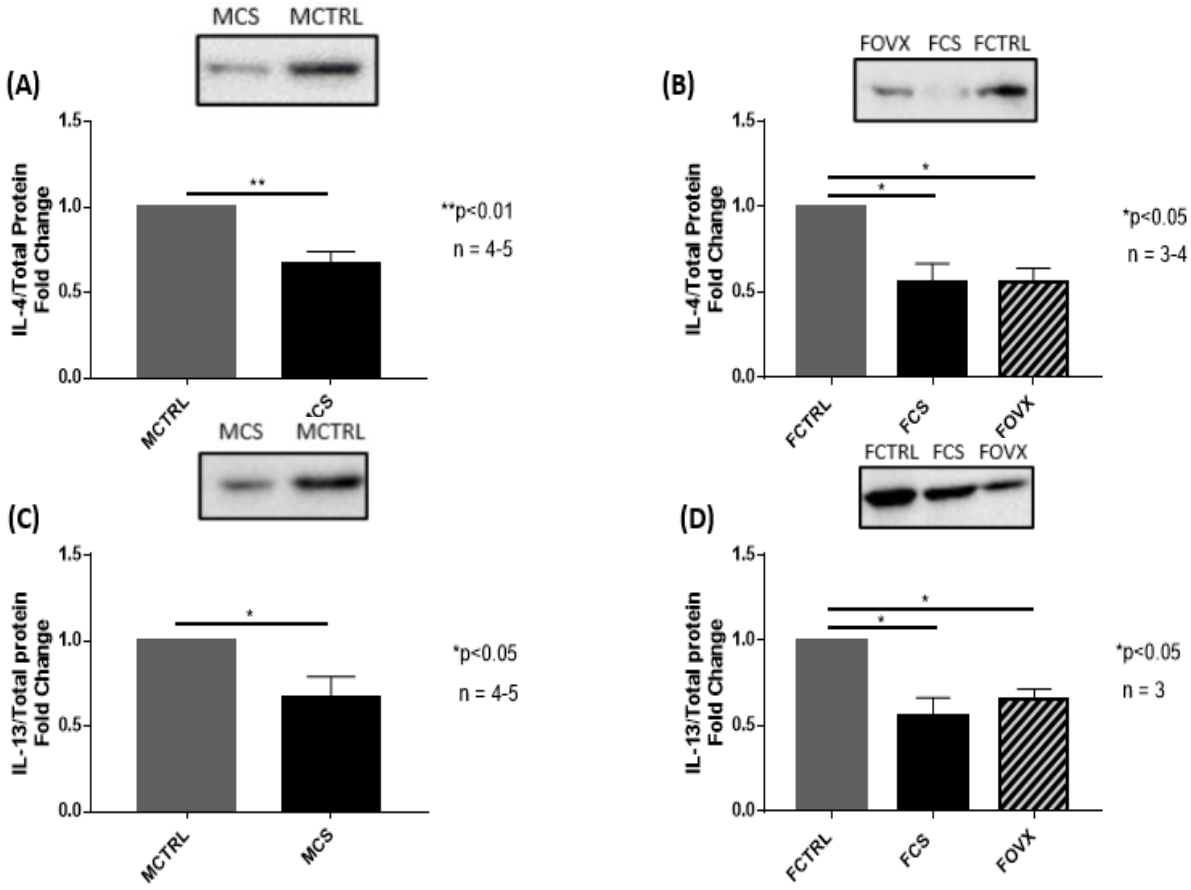

Figure 16: Effect of CS on IL-4 and IL-13 protein levels. IL-4 and IL-13 levels decreased significantly in the MCS group compared to the MCTRL. In the FCS and FOVX groups, IL-4 and IL-13 levels showed remarkable decline when compared to the FCTRL group. FCTRL: female control; FCS: female smoking; FOVX: ovariectomized female smoking; MCTRL: male control; MCS: male smoking; IL-4: interleukin-4; IL-13: interleukin-13. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B and D) and males (A and C), respectively All bars represent mean ± SEM (*P<0.05, **P<0.01)

4.13. Effect of CS on Cardiac Matrix Metalloproteinases MMP-2 and MMP-9 mRNA Expression Levels

In the male smoking group (MCS), MMP-9 mRNA expression levels significantly decreased when compared to the male control group (MCTRL) (Figure 17C). However, upon comparing the MMP-2 levels between MCTRL and MCS, no marked change was observed (Figure 17A).

In the female smoking group (FCS), MMP-2 level revealed a trend to decline whereas it significantly decreased in the ovarictomized female smoking group (FOVX) upon comparing both to the female control group (FCTRL) (Figure 17B). Additionally, MMP-9 levels significantly decreased in both female smoking group (FCS) and ovariectomized female smoking group (FOVX) when compared to the female control group (FCTRL) (Figure 17D).

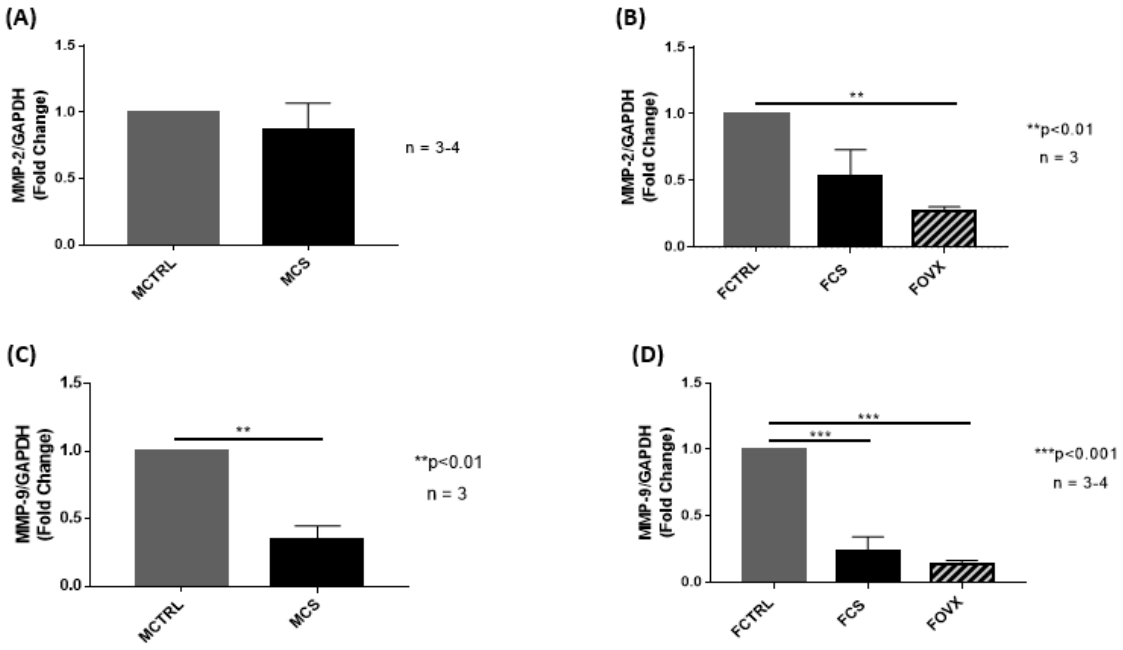

Figure17: Effect of CS of MMP-2 and MMP-9 mRNA expression level. MMP-2 levels showed comparable results in both MCS and MCTRL groups (A), whereas MMP-9 levels decreased remarkably in the MCS group (C). MMP-9 revealed a decline in the FCS and FOVX group when compared to the FCTRL group (D). MMP-2 dropped in the FOVX group compared to the FCTRL group (C). FCTRL: female control; FCS: female smoking: FOVX: ovariectomized female smoking; MCTRL: male control; MCS: male smoking; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B and D), and males (A and C), respectively. All bars represent mean ± SEM (**P<0.01, ***P<0.001)

4.14. Effect of CS on Cardiac NADPH Oxidase 4 (NOX-4) and Superoxide Dismutase 1 (SOD1) mRNA Expression Levels

In the male smoking group (MCS), NOX-4 mRNA expression levels significantly decreased whereas SOD1 levels showed a trend to increase when compared to the male control group (MCTRL) (Figure 18A, 18C).

Moreover, NOX-4 level revealed a trend to decline in both female smoking group (FCS) and ovariectomized female smoking group (FOVX) when compared to the female control group (FCTRL) (Figure 18B). As to SOD1, it substantially increased in the female smoking group (FCS) compared to the female control group (FCTRL). Contrarily, it significantly decreased in the ovariectomized female smoking group (FOVX) compared to the female smoking group (FCS) (Figure 18D).

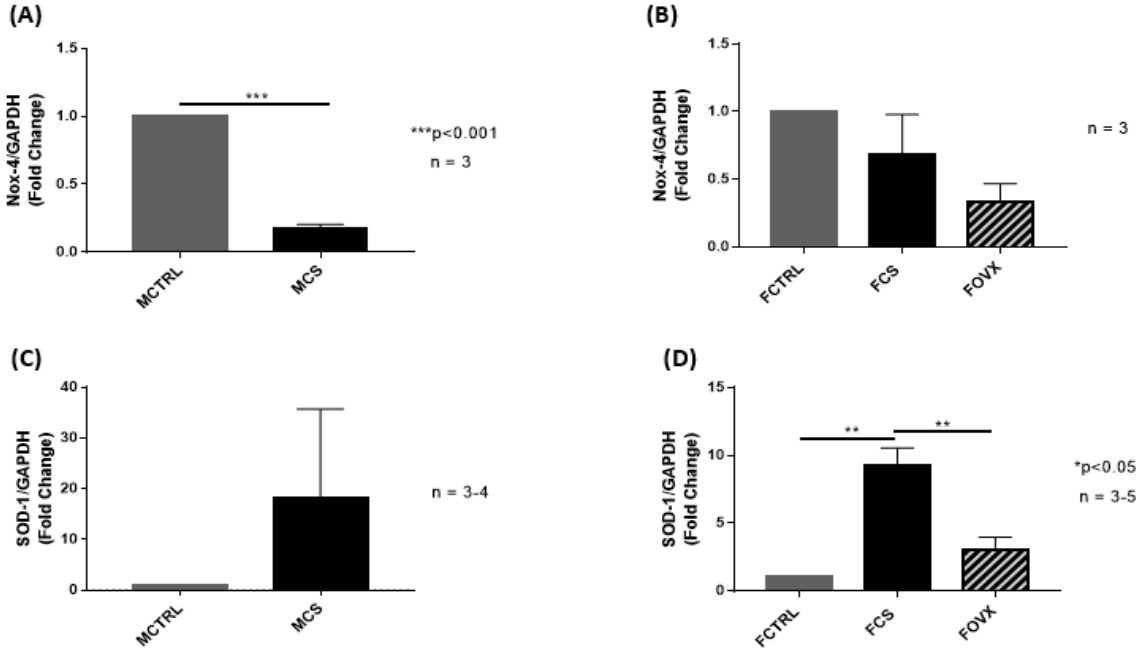

Figure 18: Effect of CS on NOX-4 and SOD1 mRNA expression levels. NOX-4 decreased remarkably in MCS (A), whereas showed a tendency to decline in the FCS and FOVX groups (B). SOD1 revealed a tendency to increase in MCS (C), while significantly increasing in FCS and considerably decreasing in FOVX (D). FCTRL: female control; FCS: female smoking FOVX: ovariectomized female smoking; MCTRL: male control; MCS: male smoking; NOX-4: NADPH oxidase 4; SOD1: superoxide dismutase 1. One-way ANOVA and unpaired t-test were used to analyze the significance of the data for females (B and D) and males (A and C), respectively. All bars represent mean ± SEM (**P<0.01, ***P<0.001)

CHAPTER 5

DISCUSSION

CS is one of the primary causes of CVD [139]. Several hemodynamic studies have proven that prolonged smoking causes ventricular alterations in humans and rodents [44] [45] [35] [36] [37]. When compared to age-matched males, reduced incidence of CVD in premenopausal females is well reported, and the incidence and severity of CVD increase post-menopause. Estrogen is believed to exert, at least in part, a protective effect on the heart through different mechanisms [127] [128] [129] [132] [133]. The current study's purpose is to investigate whether there is a gender-based disparity in CS-induced myocardial damage and to figure out how it relates to females' physiological estrogen levels. Thus, the functional, structural, and molecular impacts of 8 weeks of CS exposure on the heart in age-matched male and female C57BL/6J mice were assessed. Additionally, estrogen's potential impact on the reported results was also assessed using a group of CS ovariectomized female mice.

It appears that sympathetic overactivity is a crucial element in the elevated risk of cardiovascular incidents associated with cigarette smoking [32]. Nicotine elevates heart rate, myocardial contractility, and blood pressure via abrupt boosting of the efferent sympathetic nerve activity [43] [140] as well as norepinephrine and epinephrine secretion [141]. This intracardiac release catecholamine is primarily mediated via beta-adrenoceptors, ultimately leading to an increased myocardial function [142]. This is in accordance with our hemodynamic analysis, which revealed increased cardiac contractility in the female smoking mice, as evidenced by a substantial rise in the ejection

fraction. This might also be described using the following formula: % EF = (SV/EDV) x100, where stroke volume (SV) showed a tendency to increase in smoking females, resulting in greater EF. Furthermore, the remarkable reduction in left-ventricular end-systolic diameters (LVESD) in smoking females, in the presence and absence of ovariectomy, is another parameter reflecting enhanced cardiac contractility and EF.

As was already noted, smoking stimulates SNS and triggers catecholamine release, thus leading to elevation in heart rate [142]. This is in accordance with our functional data that showed significant increase of the heart rate following cigarette smoke in both non-ovariectomized and ovariectomized female groups.

In the FOVX+CS group, the cardiac output (CO) showed a significant increase, which is most likely attributed to the considerable elevation in the heart rate and stroke volume. Of note, CO is also influenced by heart filling from the veins, also known as venous return (VR). Nicotine-activated-SNS causes vasoconstriction and venoconstriction [143], resulting in an increase in the venous return [144] and in turn increase cardiac workload and cardiac output [145]. Another notable finding in the CS ovariectomized females is the elevation in the systolic blood pressure which could be a resultant of nicotine-induced vasoconstriction following prolonged cigarette smoking exposure [146]. Emerging evidence in human and animal models highlighted the mechanisms underlying the regulatory effects of estrogen on blood pressure [147].

On the other hand, smoking didn't cause remarkable changes in cardiac systolic function and blood pressure after cigarette smoking in males. These findings are indicative that in response to 8-weeks of smoking the workload on females' heart and cardiac systolic performance remains triggered, as opposed to males, even after two days of CS cessation. The acute effects on BP seems to be maintained in ovariectomized CS

female mice which could indicate the potential development of permanent hypertension in these animals. A continuous telemetry-based blood pressure and cardiac function monitoring system could be used in our future studies during and post-CS exposure to confirm these observations.

Another well-known impact of smoking is that it lowers body weight by raising energy expenditure and resting metabolic rate while suppressing the expected compensatory increase in caloric intake (i.e. reduction in the appetite) [148] [149]. This was in line with our findings, which revealed a significant body weight decline in the CS males. Nonetheless, body weight loss was not seen in the smoking females. On the contrary, the presence of ovariectomy induced weight gain, which is a well-known effect of estrogen deficiency [150] [151].

On the structural and histological level, our data revealed that CS was associated with an increase cardiomyocytes cross-sectional area in both males and ovariectomized females, implying cardiomyocyte hypertrophy. Consistent with our findings, the histological examination performed by Santos et al revealed CSA increase and cardiac hypertrophy in their 2 months-CS exposed mice [46]. Moreover, the left ventricular mass (LVM) was increased in both female groups, providing additional proof of cardiac hypertrophy occurrence. Accordingly, Talukder et colleagues reported that CS had a considerable influence on cardiac mass, with 32 weeks of CS-exposed animals exhibiting significantly greater left ventricular mass (LVM) versus control mice [45].

At the molecular level, our findings demonstrated that eight weeks of cigarette smoking dampened inflammation, with no substantial changes in the pro-inflammatory cytokines, TNF-α and IL-1β. Additionally, it was shown that CS considerably lowered the anti-inflammatory cytokines, IL-13 and IL-4 in the CS males and females groups.

Even though smoking is known to induce inflammation, this stage does not endure throughout the whole heart injury since it can occur early in the course of cardiac stress [152]. Moreover, persistent inflammation might stimulate more tissue damage and inadequate responses can prolong harmful stimuli [153] [152]. Therefore, the precise timing, duration, and magnitude of inflammatory responses are crucial for optimal healing [154]. As a result, our findings imply that smoking for eight weeks could have enabled the pro-inflammatory reactions, which most likely had already occurred, to resolve and proceed to the reparative phase [155], which entails fibroblast conversion and promoting extracellular matrix (ECM) deposition [153]. This was potentially confirmed through the increase in the pro-fibrotic markers α-SMA and CTGF following CS in female groups only which culminates in rise in fibroblast proliferation and the transition to a myofibroblast phenotype. Myofibroblasts are the major cell type responsible for ECM deposition through the expression of α-SMA [156]. Likewise, CTGF is commonly linked to fibrosis in cardiac remodeling contexts which showed tendency to increase in both CS females groups and to a larger extend in the CS non-ovariectomized group [157]. Additionally, our data indicated that CS was associated with a reduction in extracellular matrix (ECM) turnover, which was accounted for by CS males and females having considerably lower levels of the matrix metalloproteinases MMP-2 (CS females only) and MMP-9. Moreover, IL-1β stimulation has been documented to augment protein levels of MMP-2 and MMP-9 in fibroblasts [158] [159] [160]. In accordance IL-1β levels showed an increasing trend in CS female groups. However, It is not clear whether the inflammatory 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 pro-fibrotic markers.

To add, a growing body of evidence proved the apoptotic impacts of cigarette smoke on the cardiomyocytes [161]. Coherently, the CS non-ovariectomized females' BAX/BCL2 ratio demonstrated a significant rise, inferring that post eight weeks of smoking apoptosis had occurred. Indeed, the BAX/BCL2 ratio controls the mitochondrial outer membrane permeability and thus is categorized as an apoptotic regulator [162]. In accordance, a prior investigation involving mice found that CS caused a noticeable elevation of the pro-apoptotic marker BAX as well as the BAX/BCL2 ratio, confirming apoptosis [163]. For ROS assessment, NOX-4 exhibited a reduction in all CS groups but only reached significance in the male CS group. NOX-4 expression was potentially countered by the observed increase in SOD1. The extent of SOD1 expression was significantly higher in the CS non-ovariectomized female group only suggesting a higher ROS production in these animals.

Collectively, our data implies that both ovariectomized and non-ovariectomized females are more prone to cardiac dysfunction than aged-matched males following 8 weeks of CS exposure. CS non-ovariectomized females however exhibited pronounced cardiac fibrosis and apoptosis with more active anti-oxidant systems denoting that higher oxidative stress evoked defensive mechanisms. These findings were translated into a heightened but impaired cardiac function in this group as evidence by an increased LVM in the absence of proper CSA based cardiomyocytes hypertrophy, which could suggest an impaired myocytes to fibrosis ratio and the likelihood of a reparative rather than reactive hypertrophy. Moreover, CS ovariectomized female groups, although potentially hypertensive, seems to be more protected than the CS female group. This could imply that cigarette smoking combined with estrogen had a stronger detrimental effect on the heart than in the absence of estrogen. Moreover, the protective and beneficial role of

estrogen on the cardiovascular system was not detected in our study. While the gender-specific cardioprotective benefits of female hormones have been extensively documented, it is vital to note that smoking females appear to lose their natural protection against CVD due to the enhanced estradiol metabolism by smoking [164]. Not only are estrogen's benefits minimized, but the long-term effect of potentially hazardous CS-induced estrogen metabolites cannot be excluded either. Smoking can alter estradiol metabolism causing the range of estrogenic effects to shift in distinctive ways [165]. Indeed, prior studies have demonstrated that smoking produces A ring metabolites, which can then be converted into other secondary reactive metabolites, particularly quinones [166] [167]. These compounds exhibit severe reactivity even at trace amounts and have been linked to toxic outcomes [166]. Additionally, it has been shown that some components of cigarette smoke leads to the induction of CYP1A1 enzyme. This in turn results in the metabolism of the cardioprotective 17β-estradiol to the toxic hydroxyestradiol metabolites [168].

In conclusion, our findings point out that eight weeks of CS exposure resulted in a more detrimental impact on the cardiovascular function and molecular remodeling in female subjects, as compared to male subjects. This may be a result of more effective defensive and coping mechanisms in males against the detrimental effects of cigarette smoke, or it may require a higher dose or longer duration of exposure to CS for the negative effects to manifest in males. Additionally, females who are estrogen-positive are more prone to CS-induced molecular distress, and this is potentially attributable to the arising detrimental activity of estrogen in the context of smoking. These data suggest that while estrogen may exert protective effects on the heart in non-smoking subjects, its presence in combination with cigarette smoke may increase the risk of cardiovascular

damage. Additional experiments are warranted for us to build on the drawn conclusions. For instance, assessing markers of fibrosis, inflammation, cell death, and ROS during the acute CS phase could give us a better idea on the observed structural and functional changes between the CS groups. Identifying the molecules that are hindering estrogen activities following CS exposure, being byproduct of estrogen metabolism or alteration in estrogen or estrogen receptors moieties is necessary.

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