



ORIGINAL RESEARCH ARTICLE

Tobacco cigarette smoking exacerbates aortic calcification in an early stage of myocardial infarction in a female mouse model

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Funding information

Université Libanaise, Grant/Award Number: EH 28-1893; Medical Practice Plan, American University of Beirut; Collaborative Research Stimulus 103556 and AUBMC Seed Grant, Grant/Award Number: 100410

Abstract

Despite increased social awareness, marketing restraints, tobacco taxation, and available smoking cessation rehab programs, active and passive smoking remain a worldwide challenging epidemic and a key risk factor for cardiovascular diseases development. Although cardiovascular (CV) protection is more pronounced in women than in men due to estrogenic effects, tobacco cigarette smoking exposure seems to alter this protection by modulating estrogen actions via undefined mechanisms. Premenopausal cigarette smoking women are at higher risk of adverse CV effects than non-smokers. In this study, we investigated the impact of cigarette smoking on early CV injury after myocardial infarction (MI) in non-menopausal female mice. Aortic arch calcification, fibrosis, reactive oxygen species, and gene expression of inflammatory and calcification genes were exaggerated in mice exposed to cigarette smoke (CS). These findings suggest that aortic injury following MI, characterized by vascular smooth muscle cells transdifferentiation, calcification, inflammation, and collagen deposition but not cardiac dysfunction is exacerbated with CS exposure. The novel findings of this study highlight the importance of aortic injury on short and long-term prognosis in CS-exposed MI females. Linking those findings to estrogen alteration is probable and entails investigation.

KEYWORDS

aortic calcification, cigarette smoking, females, inflammation, myocardial infarction

Abbreviations: BP, blood pressure; CS, cigarette smoke; CVD, cardiovascular disease; DHE, dihydroethidium; ER, Estrogen receptor; ERR, Estrogen related receptor; LV, left ventricle; LVEDV, left ventricular end-diastolic volume; MI, myocardial infarction; MMP, matrix metalloproteinase; ROS, reactive oxygen species; VSMC, vascular smooth muscle cells.

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1 | INTRODUCTION

Active tobacco cigarette smoking increases the risk of coronary artery disease up to four folds while passive cigarette smokers are at 30% risk of developing heart disease and stroke (Centers for Disease Control and Prevention, 2014). In the context of the vasculature, cigarette smoking is considered an important risk factor in the development of atherosclerosis and peripheral artery diseases. In fact, coronary arteries and aortic calcification, loss of aortic elasticity, and subsequent aortic stiffening has long been studied and linked to CS exposure in the absence or presence of underlying diseases, such as hypertension, and is directly associated with increased risk of mortality from cardiovascular (CV) events (Jayalath, Mangan, & Gollidge, 2005; Jiang et al., 2009; Madhavan et al., 2014).

In the context of the heart, the impact of CS on cellular and molecular cardiac remodeling in the presence or absence of injuries, such as myocardial infarction (MI), could be summarized in four overlapping and interchangeable mechanisms termed RIMD, which include reactive oxygen species (ROS), inflammation, metabolic impairment, and cellular death (A. Kaplan et al., 2017). Cardiac cellular and molecular modifications can lead to structural and functional alterations, subsequently increasing the risk of ischemic and nonischemic cardiomyopathy and heart failure development (A. Kaplan et al., 2017). Ostensibly, both MI and cigarette smoking constitute an auspicious combination toward an adverse remodeling of the aortic arch, given their vast impact on boosting multiple hostile factors including inflammation and ROS generation (Alta et al., 2015; A. Kaplan et al., 2017).

Combined in a population, one would expect a worsened aortic calcification and stiffness as a complex outcome of the combined processes of vascular smooth muscle cells (VSMCs) transdifferentiation into osteoblast-like cells, oxidative stress, inflammation, elastin degradation, and other factors potentiated by cigarette smoking and MI. However, this anticipation is challenged in a gender-biased manner with premenopausal women expressing pronounced CV protection when compared to men, mainly due to estrogen production (Iorga et al., 2017; J. R. Kaplan & Manuck, 2017). Although endogenous estrogen exerts CV protective effects, several *in vitro* studies showed that the constituents of CS alter the effect of estrogen (Baron, La Vecchia, & Levi, 1990; Raza, Reinhart, & Movahed, 2004; Sathish et al., 2015; Tanko & Christiansen, 2004).

This is the first study to investigate the impact of cigarette tobacco smoke exposure on aortic calcification after acute cardiac injury in CS-exposed female mice. Although females are now known to undergo a less extensive left ventricular (LV) remodeling, with less dilation, and better preserved LV systolic function and ventricular-aortic coupling post-MI than males, coronary and aortic calcification in premenopausal women strongly correlates with CS exposure (Kuller, Matthews, Sutton-Tyrrell, Edmundowicz, & Bunker, 1999; Matthews, Kuller, Chang, & Edmundowicz, 2007; Wu, Nasser, Bloch, Picard, & Scherrer-Crosbie, 2003).

The presence of CS with its well-known impact on estrogen alteration, preventing subsequently the potential estrogen-mediated vascular protective effects following MI has not been assessed before

and is evaluated in this study. The key functional and molecular parameters including blood pressure (BP), cardiac hemodynamics, inflammation, oxidative stress, calcification, and elasticity markers are evaluated in a CS-exposed MI female murine model.

2 | MATERIALS AND METHODS

2.1 | Animals

This study was approved by the Institutional Animal Care and Use Committee of the American University of Beirut (#18-2-469). Female C57BL/6J mice (20–25 g; 20 weeks-old) were obtained from the animal facility of the American University of Beirut and Charles River Laboratories (Ecully, France) and were housed five per cage with cotton cocoon as enrichment environment in temperature- and humidity-controlled rooms, kept on a 12-hr light–dark cycle, and provided with food and water *ad lib* in the animal facility of the American University of Beirut. Body weight and food intake were monitored daily throughout the study period.

2.2 | Experimental protocol

All surgical procedures were performed under inhaled isoflurane. Two groups of 6–9 female mice were subjected during the 3-weeks-study period to MI (left anterior descending [LAD] ligation for 7 days) or smoking (20 cigarettes per day for 14 days) followed by 7 days of CS. The mice were then killed. Sham surgery was performed on sex- and age-matched C57BL/6J mice. Non-MI mice were also exposed to CS under the same conditions as the mice subjected to MI.

2.3 | Cigarette smoking exposure

After a brief acclimatization period in the smoking exposure room, age-matched C57BL/6J female mice were exposed to cigarette smoke (CS) as described previously (Kobeissy et al., 2017). In brief, the mice received 10 cigarettes twice daily (7 days/week) for 2 weeks followed by MI and a 1 week of similar cigarettes exposure. Previously, plasma cotinine level after 1-day exposure was determined by competitive chemiluminescent immunoassay (Siemens Healthcare Diagnostics, Llanberis, UK) in the smoke group to be 76 ± 7.6 ng/ml (Husari et al., 2016).

2.4 | BP measurements

Basal mice BP was measured non-invasively using tail cuffs and volume pressure recording sensor technology and CODA[®] high-throughput monitoring (Kent Scientific, Torrington, CT), performed as described previously (Kobeissy et al., 2017). Mice were trained for 7 days by measuring BP daily, after which BP recordings were made.

2.5 | MI surgery and echocardiography

MI was induced by permanent ligation of the LAD coronary artery as described (A. Kaplan et al., 2017; Kobeissy et al., 2017). Occlusion of LAD was confirmed by the appearance of a pale color in the anterior wall of the left ventricle (LV) and sinus tachycardia elevation on the electrocardiogram using Indus Mouse Monitor system after ligation. Sham animals were prepared identically without coronary occlusion. Transthoracic echocardiography was performed using Vevo 2100™ High-Resolution Imaging System (Visual Sonics, Toronto, Canada). For image acquisition, mice were anesthetized with 2% isoflurane in an oxygen mix chamber and placed on an electrically heated platform. M-mode and B-mode images of LV were acquired from the parasternal long axis view in supine position. LV ejection fraction and LV end-diastolic volume (LVEDV) from B-mode images and fractional shortening from M-mode images were calculated as previously described (Wu et al., 2003). Body temperature, heart rate, and respiratory rate were continuously monitored throughout the procedure via Indus MouseMonitor Heated Surgical Platform and the depth of anesthesia was adjusted accordingly.

2.6 | Histological staining

Von Kossa and Alizarin Red stainings were carried out on 4- μ m frozen sections of the aortic arch to assess calcification as described previously (Fakhry et al., 2018). Masson's Trichrome staining was performed on 4- μ m frozen aortic arch sections to detect collagen. Morphometric pixel analysis was performed on 5–7 nonoverlapping randomly chosen fields (magnification: $\times 200$ for Von Kossa and Masson's Trichrome and $\times 400$ Alizarin Red) per section per mouse using the ImageJ software (NIH). The total pixel in the positively stained area was corrected to the total stained area section of the vessel for Van Kossa, Alizarin Red, and Masson's Trichrome stainings. Transverse 4 μ m sections of the heart embedded in paraffin were also stained with Masson's Trichrome staining (magnification: $\times 20$).

2.7 | Dihydroethidium staining

Superoxide generation in aortic arch was visualized using dihydroethidium (DHE) fluorescence (Calbiochem, Darmstadt, Germany). Briefly, 10 μ M DHE was applied to frozen aortic sections and incubated in a dark humidified chamber for 30 min at 37°C. ProLong™ Gold Antifade Mountant with DAPI (P36935; Thermo Fisher Scientific) was added. Images were acquired using Microscope Zeiss Axio (Leica Microsystems, Cambridge, UK), 488 nm excitation and 585 nm emission. Zeiss Zen Software was used to quantify the intensity of the Fluorescence of DHE. A total of 10–17 images per section were acquired.

2.8 | α -Smooth muscle actin immunohistochemistry

Frozen aortic sections were fixed with pure ethanol and α -smooth muscle actin (α -SMA) expression was performed as described previously (Habib et al., 2018). Mouse monoclonal anti- α -SMA antibody (Sigma; A254; clone 1A4) was used at a 1/10,000 dilution. Five fields (magnification: $\times 400$) from three mice per group were quantified with ImageJ software. Total pixels were corrected to the total vessel area. Results were expressed as fold of MI. No staining was observed when the primary antibody was omitted.

2.9 | RNA isolation and real-time polymerase chain reaction

RNA was extracted from frozen aortic tissues using QIAzol (QIAGEN; 79306). One microgram of total RNA was reversed transcribed using iScript cDNA Synthesis Kit (00407363; Thermo Fisher Scientific). Real-time polymerase chain reaction (RT-PCR) was carried out on CFX384 cyclor using iQ SYBR Green Supermix Kit (Bio-Rad). Gene expression was normalized to the house-keeping gene 18S ribosomal RNA. All primers were synthesized by Eurofins Genomics (Ebersberg, Germany). Sequences were used based on (Habib et al., 2018) and others are listed in S1 table. Experiments were performed using six mice per group repeated twice for MI and MI+CS and three to six mice per group for sham and sham+CS. RT-PCRs were performed on RNA extracted from five to nine aortic arches per MI groups and three to six for sham. Results were expressed as mean \pm the standard error of the mean.

2.10 | Data analysis

The ImageJ software was used for the quantification of the histology staining and α -SMA immunohistochemistry. Statistical analysis was performed using GraphPad Prism 5 (GraphPad, San Diego, CA). Statistical comparisons were performed using the Mann-Whitney test for RT-PCR and the unpaired *t* test for staining experiments. The *p* values **p* < .05, ***p* < .01, and ****p* < .001 are considered as statistically significant.

3 | RESULTS

3.1 | Cigarette smoking has no impact on systolic arterial pressure before MI

No difference in systolic BP was noticed between CS exposed and nonexposed mice groups before MI (Figure 1a). These findings suggested that any differences observed at the level of the aorta are potentially mediated by direct rather than a global impact of CS, with respect, at least, to BP differences.

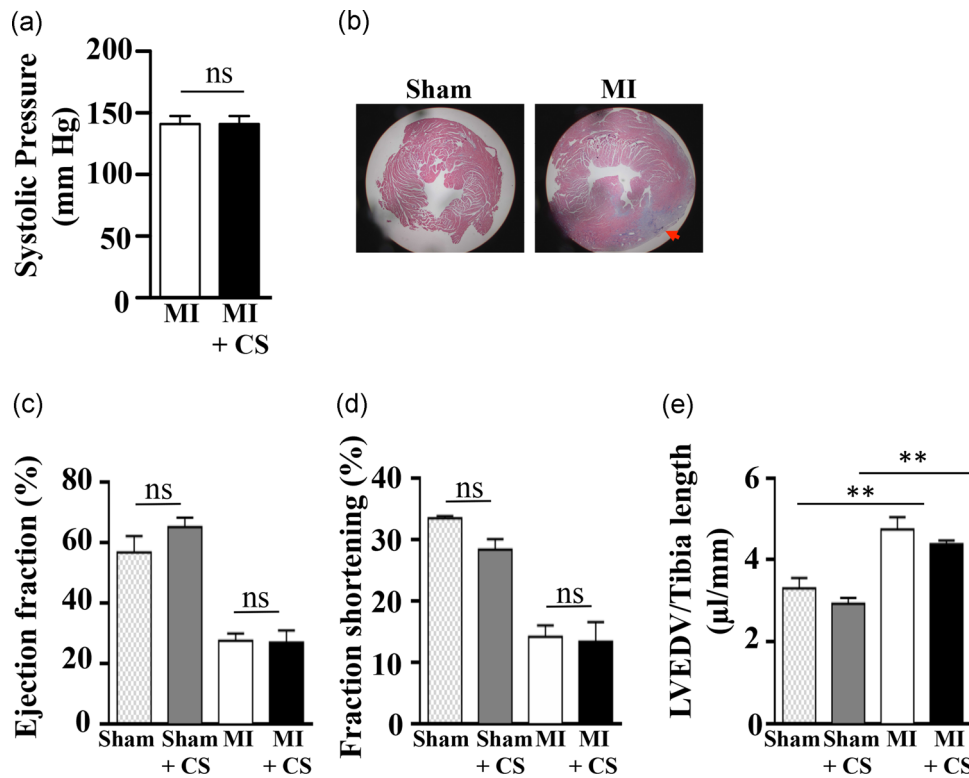


FIGURE 1 Two weeks of smoking exposure had no impact on systolic arterial pressure or cardiac hemodynamics. (a) Both MI and MI+CS groups expressed similar systolic BP measurements before MI induction. (b) MI injury increased fibrosis significantly when compared to sham operated group. Two weeks of smoking exposure followed by MI and another week of CS had no significant impact on (c) ejection fraction, (d) fractional shortening, and (e) LVEDV, when compared with nonsmoking MI groups. Unpaired *t* test was implemented on $n = 6$ per group and revealed no significance between MI and MI+CS groups. BP, blood pressure; LVEDV, left ventricular end-diastolic volume; MI, myocardial infarction

3.2 | Cigarette smoking has no impact on cardiac hemodynamics and fibrosis following MI

First, fibrosis was confirmed in hearts from MI mice compared with the sham mice (Figure 1b). Both MI and MI+CS groups showed similar changes in ejection fraction, fractional shortening, LVEDV 7 days post-MI, and were elevated when compared with the control groups (Figure 1c-e and S2 table). In accordance with the BP finding interpretation, these data imply that any changes seen at the level of the aorta are potentially mediated by the direct impact of CS exposure.

3.3 | Cigarette smoking and MI trigger collagen deposition and fibrosis

We investigated the impact of acute CS exposure on female mice aorta after acute cardiac injury. Aortas from mice subjected to CS after MI displayed increased collagen deposition as detected by the Masson's Trichrome staining (2.5-fold in MI+CS compared to MI; $p < .01$; Figure 2a), and a reduction of α -SMA positive area by 25% ($p < .01$; Figure 2b) suggesting that CS-induced osteocytes differentiation. In addition, CS-induced *Col1a1* gene by 1.9 fold in MI+CS compared to MI alone ($p < .05$; Figure 2c). Similarly, CS increased the expression of two matrix metalloproteinases (MMP) genes, *Mmp2* and *Mmp9*, involved in remodeling and fibrosis, in the MI group with a significant increase when

compared with the MI group (1.8- and 1.7-fold, for *Mmp2* and *Mmp9*, respectively, for MI+CS compared to MI, $p < .05$; Figure 2c), suggesting that CS may contribute to vessel remodeling and matrix deposition and thus to aortic fibrosis. Sham mice exposed to CS showed an increase in *Mmp2* and *Mmp9* gene expression compared with sham alone although the levels were much lower than the MI and MI+CS mice groups (Figure S1). We also examined the expression of two estrogen receptor (ER) genes, *Esr1* and *Essra*, for ER α and ER related (ERR) α receptors. Figure S2 shows an increase in the expression in MI+CS when compared with MI.

3.4 | Cigarette smoking and MI induce the expression of proinflammatory cytokines and ROS production in female mice aortas

Since CS is known to increase inflammation, we investigated whether CS exacerbates inflammation after MI. Figure 3a shows that the expression of *Il6* and *Il1b* genes increased in the CS+MI group compared to the MI group (1.9- and 3-fold for MI+CS compared with MI, respectively, $p < .05$). In sham mice, RT-PCR was assessed with a large amount of complementary DNA (>100 ng) and revealed an absence of detection of *Il6* gene and no significant difference for *Il1b* in the absence or presence of CS (Figure S1). The levels of ROS

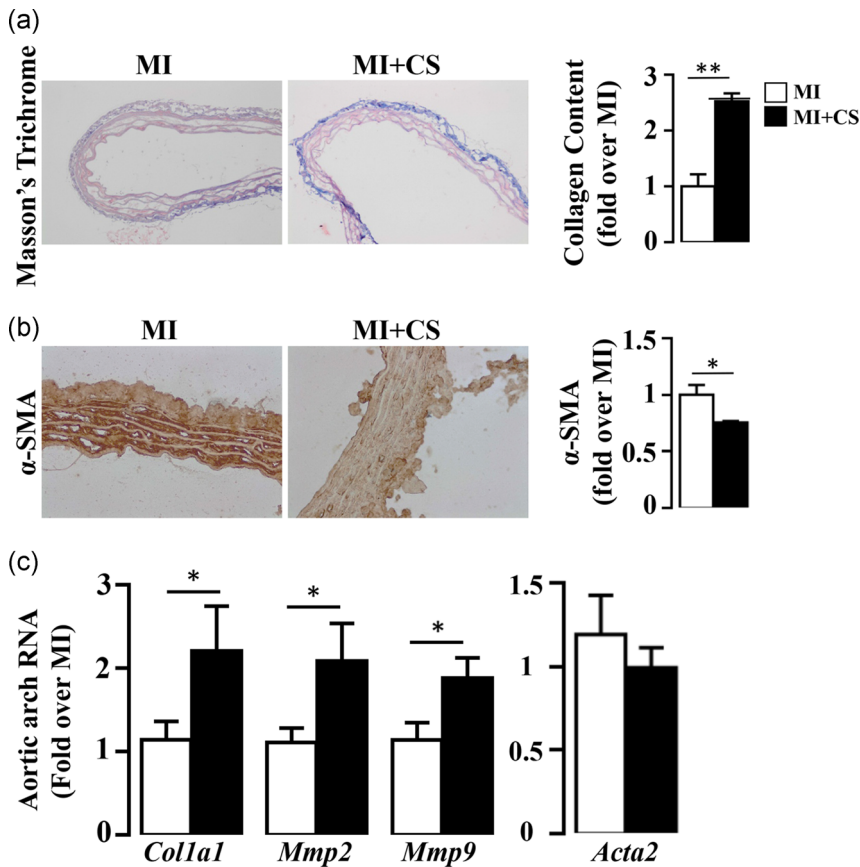


FIGURE 2 CS exposure induces fibrosis in mice subjected to MI. (a) Representative aortic arch section stained with Masson's Trichrome, quantification of the fibrosis by the ImageJ software. The intensity of the positive area was corrected to the total area and expressed as fold of MI alone. Magnification: $\times 200$. Unpaired t test, $**p < .01$ ($n = 3$). (b) Immunohistochemistry of α -SMA, quantification by ImageJ, integrated density values are corrected to total area and expressed as fold of MI; magnification: $\times 400$. $*p < .05$ ($n = 3$), unpaired t test. (c) Gene expression of vascular fibrosis and remodeling ($n = 6-9$); $*p < .05$, Mann-Whitney test. MI, myocardial infarction; α -SMA, α -smooth muscle actin; *Acta2*, gene of α -SMA

assessed by DHE were also elevated in aortic arch sections of MI+CS compared with the MI group (1.5-fold; $p < .01$; Figure 3b).

3.5 | Cigarette smoking and MI induce calcification and transdifferentiation of VSMCs into osteochondrocyte-like cells

To determine the effect of cigarette smoking on calcification in the aorta of mice subjected to MI, tissue calcium deposition was assessed by Alizarin Red and Von Kossa stainings in the aortic arch. Figure 4a,b show an increase in the aortic calcification in the MI+CS group compared to the MI group by 2- and 1.5-fold, respectively, for Alizarin Red and Von Kossa, respectively ($p < .01$). The gene expression of osteogenic and chondrogenic differentiation markers was also determined by RT-PCR. MI+CS showed a significant increase in the expression of *Spp1*, the bone sialoprotein gene, and *Alpl*, the alkaline phosphatase by 3- and 2.2-fold, respectively, compared to the MI control group ($p < .05$; Figure 4c). No significant difference for *Spp1* was found in sham mice in the absence or presence of CS (Figure S1).

4 | DISCUSSION

Numerous studies reported a strong association between smoking and cardiovascular disease (CVD) events. Smoking is a worldwide

public health burden and is associated with detrimental clinical outcomes. In this study, female mice subjected to MI were exposed to 14 days of CS before MI induction and for 7 days after MI to investigate the effect of smoking on female mice aorta after acute cardiac injury. The importance of the findings revealed in this study is threefold: (a) CS did not exacerbate cardiac remodeling after MI as both MI and MI+CS females exerted the same level of injury and cardiac dysfunction for at least 3 weeks of CS exposure; (b) No difference in systolic BP after CS exposure and before MI induction was seen between the groups, and 3-aortic injury was more pronounced in the MI group in the presence of CS when compared to the relative non-CS MI group. These findings suggest that the impact of CS on the aorta at early stages after MI might be due to direct effects rather than complication of worsened cardiac remodeling or altered vascular dynamics.

Several studies have implicated the effect of smoking in the development of CV remodeling by driving changes in endothelial and vascular smooth muscle cell physiology. For instance, the increase in the expression of adhesion molecules (ICAM-1 and VCAM-1; Giebe et al., 2017), pro-inflammatory cytokines (interleukin [IL]-6 and IL-1 β), and chemoattractant molecules after CS exposure alters endothelial function, empowering therefore atherogenicity. Moreover, nicotine exposure increases the incidence of aortic aneurysms in parallel with MMP-2 and MMP-9 expression and activity in a mouse model of aneurysm and hypertension (Colombo et al., 2013; Wagenhauser et al., 2018). In addition, it is well established that the

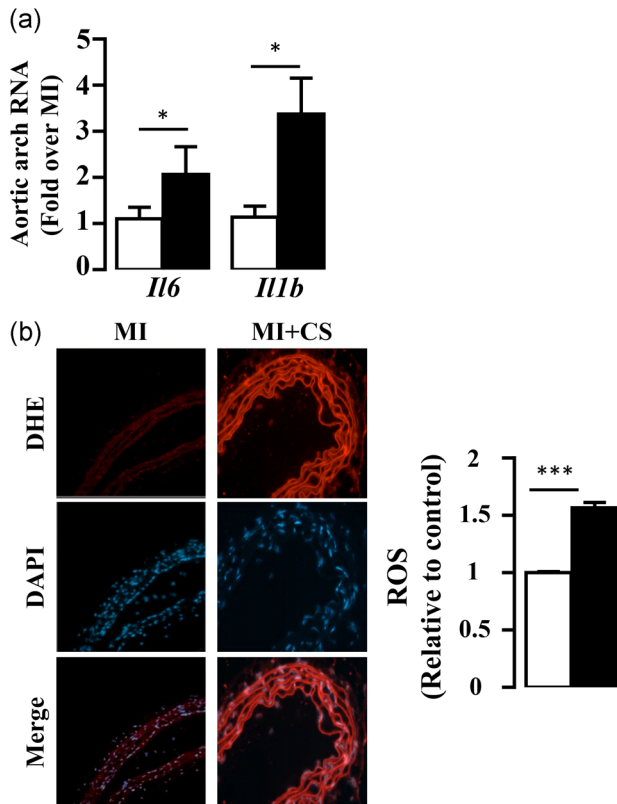


FIGURE 3 CS exposure reduces gene expression of pro-inflammatory cytokines and induces reactive oxygen species (ROS) generation in aortic arch of mice subjected to MI. (a) Gene expression of *Il6* and *Il1b* ($n = 5-8$); (b) Representative sections of mice aorta ($n = 8$) stained with DAPI for nuclei and DHE for ROS. Intensity of ROS staining was determined from ZEN software and normalized to DAPI level relative to MI alone. Quantification is described in the method section. Data correspond to mean \pm SEM; * $p < .05$, *** $p < .001$ for CS+MI compared with MI. Mann-Whitney test. DAPI, 4',6-diamidino-2-phenylindole; DHE, dihydroethidium; MI, myocardial infarction; SEM, standard error of the mean

loss of smooth muscle 22α of VSMCs and the expression of bone-associated proteins like tissue nonspecific alkaline phosphatase (*Alpl*) and osteocalcin decrease elasticity and increase stiffness and the differentiation of the VSMCs into osteochondrogenic cells (Durham, Speer, Scatena, Giachelli, & Shanahan, 2018). In this study, we show that CS exposure exaggerated inflammation, ROS production, and vascular calcification in the context of MI injury in female mice.

Although premenopausal women show a higher level of protection after a CV injury than men, their clinical risk of CVD increases with cigarette smoking and with menopause. Results of several experimental, clinical, and epidemiological studies attributed this protection to estrogen, mainly due to its antiatherogenic effects on the CV system (Morselli et al., 2017; Nakamura et al., 2004). Although both $ER\alpha$ and $ER\beta$ have been reported in the VSMCs of the human female aorta (Nakamura et al., 2004; Register & Adams, 1998), $ER\alpha$ atheroprotective effects seem to predominate (Nakamura et al., 2004). Nakamura et al. (2004) were among the first to report

the protective antiproliferative effects of $ER\alpha$ on aortic VSMCs in premenopausal women. In a recent study, McRobb et al. (2017) showed that $ER\alpha$ or $ER\beta$ antagonism promotes aortic calcification and VSMCs differentiation to osteoblast-like cells.

These findings are in line with our results, as evident by marked mineralization via increased calcium deposition, and a significant osteoblast-like marker expression including *Spp1* and *Alpl* as well as a marked decrease in α -SMA positive cells in the CS-MI group when compared to the non-CS MI control mice. Studies have also attributed the premenopausal anti-atherosclerotic effects of estrogen on the vascular system to significant anti-inflammatory and antioxidant effects mainly through their direct interaction with estrogen receptors expressed in the immune system, smooth muscle cells, and endothelial cells (Bowling et al., 2014; Meyer et al., 2014; Nofer, 2012). Inflammation and oxidation were markedly increased in our CS-exposed MI group when compared to the MI group only, as evident by the significant gene expression of IL-6 and IL-1 β and a pronounced production of ROS. *Col1a1* gene expression and collagen deposition were also elevated along with a significant increase in the extracellular matrix, *Mmp2* and *Mmp9* gene expression, suggesting a pronounced adverse aortic tissue remodeling in the CS-exposed MI group. Last but not the least, cigarette smoking is linked to estrogen alteration and a loss-of-function in multiple studies supporting our hypothesis and promoting the need for a thorough investigation in future

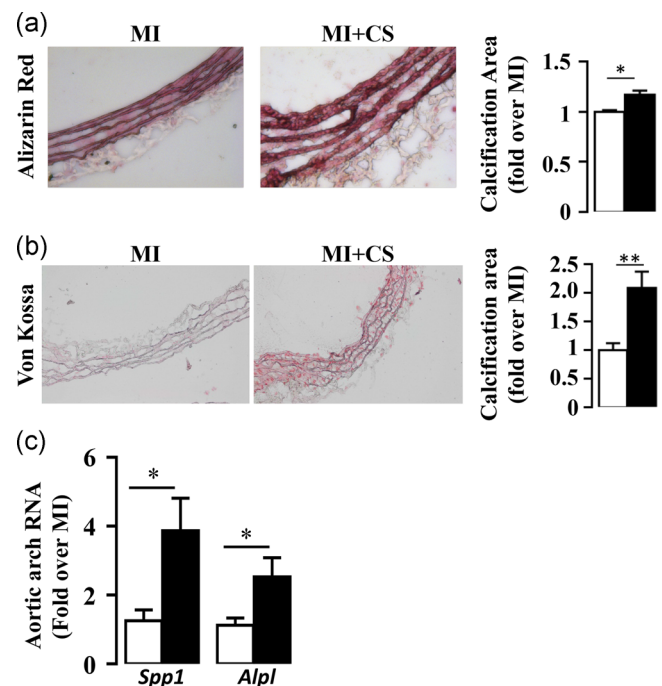


FIGURE 4 CS induces aortic calcification in mice subjected to MI. Representative image of aortic arch sections stained with Alizarin Red (a) or Von Kossa (b) stainings. Calcification areas were quantified using ImageJ and normalized to total area and expressed as fold of CS alone. Magnification: $\times 400$ for Alizarin Red and $\times 200$ for Von Kossa. Unpaired t test; ** $p < .01$ ($n = 3$). (c) Gene expression of calcification related genes, *Spp1* and *Alpl* ($n = 6-9$); * $p < .05$, Mann-Whitney test. MI, myocardial infarction

studies with experts in the estrogen field (Tanko & Christiansen, 2004). Our findings show a significant increase in estrogen receptors expression in the CS-exposed MI group, which highlights the importance of estrogen availability. Whether CS alters endogenous estrogen bioactivity or aortic estrogen receptor conformation and therefore alters estrogen protective effects, warrants investigation.

In summary, the modest findings of this study are of utmost value and with translational importance for multiple reasons. First, premenopausal CS-exposed women undergoing an MI might be at a higher risk of short and long-term worsened prognosis due to worsened aortic remodeling when compared to non-smokers MI ones. Second, although ER receptors are present in both the heart and vasculature, it seems, at least at the functional levels, that the female heart is more resistant to CS adverse effects after MI than the vasculature suggesting that worsened prognosis with female CS population undergoing an MI might be attributed initially to vasculature dysfunction. Future studies to unveil the mechanisms behind those findings are warranted.

ACKNOWLEDGMENTS

This study has been funded with support from the Ecole Doctorale Des Sciences et Technologie of the Lebanese University (to EH 28-1893), the Collaborative Research Stimulus 103556 and the AUBMC seed grant 100410 (To FAZ), and the Medical Practice Plan, the Faculty of Medicine of the American University of Beirut (to AHa, FK, AHu, and FAZ).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

E. H., A. Ha., F. K., and F. A. Z. gave the concept and designed the study. A. K., E. A., G. A. E., and A. A. K. acquired data. A. K. performed the MI surgery. S. Z., A. K., E. A., G. A. E., W. N., E. H., A. Ha., and F. A. Z. analyzed and interpreted the data. S. Z., E. H., A. Ha., and F. A. Z. did the statistical analysis. F. K. and A. Hu. critically read the manuscript and evaluated the C. S. system. S. Z., E. H., A. Ha., and F. A. Z. drafted the manuscript. E. H., A. Ha., and F. A. Z. supervised the study. E. H., A. Ha., F. K., and F. A. Z. provided financial support.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zalghout S, Kaplan A, Abidi E, et al. Tobacco cigarette smoking exacerbates aortic calcification in an early stage of myocardial infarction in a female mouse model. *J Cell Physiol*. 2020;235:1568–1575. <https://doi.org/10.1002/jcp.29075>