RESEARCH ARTICLE

Acute effects of cigarette smoke on Endothelial Nitric Oxide synthase, vascular cell adhesion molecule 1 and aortic intima media thickness [version 1; peer review: 1 approved with reservations]

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Abstract

Background. Cigarette smoking could induce endothelial dysfunction and the increase of circulating markers of inflammation by activation of monocytes. This can lead to increased intima media thickness (IMT) of entire blood vessels and result in acceleration of the atherosclerosis process. However, to our knowledge, little is known about the role of cigarette smoking in this atherosclerotic inflammatory process.

The aim of this study is to explore the link between cigarette smoking and its effect on endothelial nitric oxide synthase (e-NOS) and vascular cell adhesion molecule 1 (VCAM-1).

Methods. An experimental study with a post-test only controlled group design was used. We used 18 Wistar rats (*Rattus norvegicus*) randomly subdivided into two groups: group K (-) were not exposed to tobacco smoke, whereas group K (+) were exposed to smoke equivalent of more than 40 cigarettes for 28 days daily. After 28 days, samples were analyzed for e-NOS, VCAM-1 and aortic IMT.

Results. Our results indicate that tobacco smoke can enhance the expression of VCAM-1 on rat cardiac vascular endothelial cells, resulting in a decreased expression of e-NOS level and increase of aortic IMT. Linear regression model found that eNOS level negatively correlated with aortic IMT ($r^2 = 0.584$, $\beta = -0.764$, $p < 0.001$), whereas VCAM-1 expression did not correlate with aortic IMT ($r^2 = 0.197$, $p = 0.065$).

Conclusion. Low e-NOS level and high VCAM-1 level observed after cigarette smoke exposure which may increase aortic IMT.

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Keywords

aortic tissue, atherosclerosis, cigarette smoking, endothelial-NOS, intima media thickness, VCAM-1

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Abbreviations

ANOVA: analysis of variance
 ELISA: enzyme-linked immunosorbent assay
 e-NOS: endothelial Nitric Oxide Synthase
 H_2O_2 : Hydrogen peroxide
 IACUC: Institutional Animal Care and Use Committee
 IHC: Immunohistochemistry
 IMT: Intima-media thickness
 IRS: Immunoreactivity Scoring System
 LSAB: Labeled Streptavidin Avidin Biotin
 NIH: National Institutes of Health
 PCR: Polymerase Chain Reaction
 SA-HRP: Streptavidin-Horseradish Peroxidase
 SD: standard deviation
 SEM: standard error of the mean
 SPSS: Statistical Package for the Social Sciences
 VCAM-1: Vascular Cell Adhesion Molecule-1

Introduction

Cigarette smoking is the most important modifiable risk factor for developing atherosclerosis including cerebrovascular accident, peripheral arterial disease, and coronary heart disease.¹ In a meta-analysis from 55 eligible studies (43 cross-sectional, ten cohort and two case-control studies), the odds ratio (ORs) of peripheral arterial disease (PAD) associated with cigarette exposed was 2.71 (95% CI: 2.28-3.21; $p < 0.001$).² In a meta-analysis from 75 cohorts (2.4 million participants) that adjusted for cardiovascular risk factors other than coronary heart disease, multiple-adjusted pooled ORs of smoking versus non-smoking was 1.25 (95% CI: 1.12–1.39, $p < 0.0001$).³

Even though epidemiologic studies clearly stated the negative effect of cigarette smoke for cardiovascular diseases, the underlying mechanisms have yet to be confirmed. The pathogenesis and pathophysiologic mechanisms by which exposure to cigarette smoke could accelerate atherosclerosis cardiovascular disease are complex and challenging, due to more than 5,000 different mixtures of chemicals inside the cigarette smoke itself.⁴ Several potential contributing factors to atherogenesis inside cigarette smoke are (1) polycyclic aromatic hydrocarbons, (2) oxidizing agents, (3) particulate matter, and (4) nicotine.⁵

One of the most important factor contributing for pro-atherogenic is nicotine, which has commonly been studied using cigarette smoke condensates.⁶ In addition to its role as the habituating agent in tobacco, nicotine also accelerates atherosclerosis cardiovascular disease. There are several potential mechanisms of the pro-atherogenic effects of nicotine: (1) inducing endothelial dysfunction, (2) modifying lipid profile, (3) increasing inflammatory response, (4) inducing the release of catecholamines, which may increase heart rate and blood pressure, (5) increases platelet aggregability, (6) direct actions on the cellular elements participating in plaque formation, and (7) induces the proliferation and migration of vascular smooth muscle cells into the intima, mediated in part by TGF β .⁷ These pathomechanisms of nicotine could lead to the increase of intima media thickness of the entire blood vessel, leading to the greater risk of developing atherosclerosis.⁸

To learn more about the pathomechanisms of the diseased endothelium, we need to study all the oxidizing, inflammatory, and thrombotic molecules which are not in an equilibrium state. In the model of atherosclerosis cardiovascular diseases, a pathological imbalance between prothrombotic and antithrombotic state, prooxidant and antioxidant state, and pro-inflammatory and anti-inflammatory state are observed.⁹ Considerable evidence supports the importance of inflammation and hypercoagulability to promote atherogenic state.¹⁰ There is abundant literature concerning the role of biomarkers of pathological imbalance in atherosclerosis.

Cell adhesion molecules are the essential pro-inflammatory and pro-atherogenic proteins that represent a hallmark of endothelial dysfunction and atherosclerosis. P-selectin, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and PECAM-1 were demonstrated to be involved in the formation of atherosclerosis plaque.¹¹ Beyond the other cell adhesion molecules, VCAM-1 plays as an important factor in neointima proliferation following nicotine-induced arterial injury, an area of research important for atherosclerosis cardiovascular diseases.¹² In the nicotine-induced arterial injury model, VCAM-1 expression is highly induced in the proliferation and migration of neointimal smooth muscle cells.¹³

Previous studies showed that upregulation of endothelial nitric oxide synthase (e-NOS) expression and activity has its important role in the protection of endothelium.^{14–16} e-NOS could stimulate endothelium-dependent relaxation and protect against the development of VCAM-1-induced endothelial dysfunction.¹⁷ However, to our knowledge, little is known about the role of cigarette smoke in this atherosclerotic inflammatory process. This study aims to explore the link between cigarette smoke on e-NOS and VCAM-1, which results in the development of aortic intima media thickness (IMT) of the experimental animals.

Clinical significance

Increasing evidence suggests that cigarette smoke exposure could induce VCAM-1 (enhance pro-atherogenic property), and a decrease of e-NOS level (anti-atherogenic depletion). Thus, cigarette smoke may represent a significant risk factor for atherosclerosis by increasing aortic IMT. This evidence is discussed herein.

Methods

Ethics approval

This article was reported in line with the ARRIVE guidelines. Animal experimental study were conducted under the approval of the Institutional Animal Care and Use Committee of Universitas Airlangga (UNAIR), Surabaya, Indonesia (animal approval no: 2.KE.184.10.2019) under the name of Meity Ardiana as the Principal Investigator. All efforts were made to ameliorate any suffering of animals through using anaesthetic to euthanize the rats at the end of the experimental procedure.

Animals

The present study used 18 male Wistar rats (*Rattus norvegicus*), eight weeks of age (average body weight 150-200 grams). The rats were adapted to their environment for seven days before the experiment start. They were nurtured at the Animal House at Faculty of Veterinary Medicine, Universitas Airlangga in polycarbonate cage, which measured 480 mm × 265 mm × 210 mm. Each cage had wood shavings on the floor, and contained three or four animals, which were marked for each subgroup. The rats were housed in microisolator cages and maintained in a constant room temperature ranging from 22°C to 25°C, with a 12-h light/12-h dark cycle, under artificially controlled ventilation, with a relative humidity ranging from 50% to 60%. The rats were fed a standard balanced rodent diet and water was provided *ad libitum*. The rats were chosen for each group by simple random sampling. Study was carried out in strict accordance to internationally-accepted standards of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and in line with the 'Animal Research: Reporting in vivo Experiments' (ARRIVE) Guidelines. All efforts were made to ameliorate any suffering of animals and details of how this was achieved should be provided.

Experimental design and groups

The present study design was a randomized post-test only controlled group design using quantitative method. We extracted 18 male Wistar rats, randomized and then allocated them into two groups. Group 1 were not exposed to tobacco smoke, whilst group 2 were given 40 or more cigarette smokes daily for 28 days as seen in Figure 1. Each cigarette smoke dose contains 39 mg of tar and 2.3 mg of nicotine. The enrolled subjects were analyzed for vascular cell adhesion

Figure 1. Illustration of how to exposed rats in the K(+) group to the cigarette smokes. Exposure to tobacco smokes were done using sidestream technique from peristaltic pump, smoke producer chamber, and inhalation chamber, connected by modified silicon tube.

molecule 1 (VCAM-1), endothelial nitric oxide synthase (e-NOS), and aortic intima media thickness (IMT) after 28 days of consecutive experiments.

Aortic Intima Media Thickness (IMT)

Thoracic aortas were prepared as distal aortic arch by cutting from the left ventricle of each rat. The post mortem samples of descending thoracic aortas obtained by dissection were fixed in 10% formaldehyde, embedded in paraffin, and sectioned at a thickness of 6 μm . The mounted tissues were stained using hematoxylin and eosin. Aortic intima media thickness was measured via Leica DMD 108 (Leica Microsystems GmbH, Wetzlar, Germany). Each sample was measured as “micrometer (μm)” from six different locations of the vessel wall. Arithmetic averages of these six measurements are presented in the results section.

Vascular cell adhesion molecule 1 (VCAM-1)

We used streptavidin-biotin method which uses a biotin conjugated secondary antibody to link the primary antibody to a streptavidin-peroxidase complex for Immunohistochemistry (IHC) staining. The labeled streptavidin-biotin (LSAB) method were utilized to measure expression of VCAM-1 in the aortic tissue of the rats. Firstly, the aortic tissue were prepared and preserved through deparaffinize models following fixation. Secondly, the aortic tissue was rehydrated by immersing the slides through the xylene (three washes, five minutes each), 100% ethanol (two washes, 10 minutes each), 95% ethanol (two washes, 10 minutes each), 70% ethanol (two washes, 10 minutes each), 50% ethanol (two washes, 10 minutes each), and deionized water (two washes, five minutes each). Thirdly, the aortic tissue was washed using Phosphat Buffer Sollution and then dipped into 3% of H_2O_2 solution withing 20 minutes. Fourthly, we added 1% of Bovine Serum Albumin to the Phosphat Buffer Sollution and then incubated them within 30 minutes in the room temperature. Fifthly, primary antibody anti-VCAM-1 (Santacruz biotech SC-13160) was added and incubated within 30 minutes, then washed again using Phosphat Buffer Sollution. Secondary antibody (Anti-Rat IgG Biotin Labelled) was added and incubated within 30 minutes in the room temperature, then washed using Phosphat Buffer Sollution. Sixthly, SA-HRP (Streptavidin-Hoseradish Peroxidase) complex was added and incubated within 10 minutes in the room temperature and then washed using Phosphat Buffer Sollution. Seventhly, Chromogen DAB (3,3-diaminobenzidine tetrahydrochloride) was added and incubated within 10 minutes in the room temperature, and then washed using Phosphat Buffer Sollution and sterile water. Finally, counterstain Hematoxylin-Eosin was added into the object glasses and expressions of VCAM-1 were measured and analyzed by a biological microscope (400 $\times$ magnification) from tunica intima and tunica media of the aortic tissue. Semiquantitative measurements of VCAM-1 were done by immunoreactivity scoring system (Table 1).

Endothelial Nitric Oxide Synthase (e-NOS)

All samples were assessed by direct-sandwich enzyme-linked immunosorbent assay (ELISA) under the manufacture's system (R&D System Europe Ltd, Abingdon, UK) according to the National Institute for Biological Standards and Controls (Blanche Lane, South Mimms, Potters Bars, Hertfordshire, UK) protocol. We used eNOS kit from the elabscience (catalogue number: E-EL-R0367). Briefly, samples from the aortic tissue were collected and stored at -70°C (-94°F) at the Institute of Tropical Diseases Universitas Airlangga (UNAIR). Samples were homogenized into solution. Then, 100 μL of the solution was mixed with the well-coated primary antibody for e-NOS. Overnight incubation was done in a temperature of 4°C with a shaking machine for minimum 24 hours. Wash Buffer (20 $\times$) were diluted to 1 $\times$ working solution with D.I. water prior to ELISA wash procedures. After that, 50 μL of the stop solution was added into each sample. A minimum value of 0.01 pg/mL were assigned for below the limit of detection.

Statistical analysis

All measurements were performed and replicated at least three times. Results were presented as (1) means $\pm$ standard deviations (SD) for normally distributed data; (2) medians with lower and upper value for abnormally distributed data. The assumption of the normality for the complete data was assessed by Shapiro-Wilk test. Test of homogeneity of variances was assessed by Levene Statistics. Statistical significance were examined by Independent T-test, Mann-Whitney U test, and logistic regression using SPSS version 17.0 for Microsoft (IBM corp, Chicago, USA).

Table 1. Immunoreactivity Scoring System (IRS).

Score for percentage of cells staining	Score for intensity of staining
0 = no stained cells	0 = no reaction
1 = <10% cells are stained	1 = mild intensity of staining
2 = 10-50% cells are stained	2 = moderate intensity of staining
3 = 51-80% cells are stained	3 = heavy intensity of staining
4 = >80% cells are stained	

Results

Comparison of IMT level between smoke and non-smoke groups

After 28 days following experiments, there was a significance difference in IMT level between both groups ($p < 0.001$). The mean of the aortic IMT in all subjects were $73.68 \pm 17.86 \mu\text{m}$. The mean of the aortic IMT in the cigarette smoke group was $88.39 \pm 2.51 \mu\text{m}$. The mean of the aortic IMT in the control group was $58.98 \pm 13.61 \mu\text{m}$. **Table 2** presents the impact of the exposure of smoke from 40 cigarettes daily on the aortic IMT profile of the experimental animals. The comparative analysis of IMT parameters demonstrated that there were statistically significant differences between the groups ($p < 0.001$; Mann-Whitney's test) (**Figure 2**).

Comparison of e-NOS level between smoke and non-smoke groups

After 28 days following experiments, there was a significance difference of e-NOS level between both groups ($p < 0.001$). The mean of the e-NOS in all subjects was $78.02 \pm 25.84 \text{ pg/ml}$. The mean of the e-NOS level in the cigarette smoke group was $101.22 \pm 11.8 \text{ pg/ml}$. The mean of the e-NOS level in the control group was $54.83 \pm 8.3 \text{ pg/ml}$. **Table 3** presents the impact of the exposure of daily smoke from the equivalent of 40 cigarettes on the e-NOS profile of the experimental animals. The comparative analysis of e-NOS parameters demonstrated that there were statistically significant differences between the groups ($p < 0.001$; Mann-Whitney's test) (**Figure 3**).

Comparison of VCAM-1 expression between smoke and non-smoke

After 28 days following experiments, the mean of the VCAM-1 expression in all subjects was 9.00 ± 3.51 . The mean of the VCAM-1 level in the cigarette smoke group was 10.33 ± 2.9 . The mean of the VCAM-1 level in the control group was 7.67 ± 3.7 . **Table 4** presents the impact of the exposure of daily 40 or more cigarette smokes on the VCAM-1

Table 2. Statistic table IMT between K(+) group which is exposed to the daily 40 cigarette smokes and K(-) group as the control group.

Group statistics						Sig (Independent T)	Sig (Mann-Whitney)
	Group	N	Mean	Std. Deviation	Std. Error Mean		
IMT	K(-)	9	58,9800	13,61075	4,53692	<0.001	<0.001
	K(+)	9	88,3911	2,51766	,83922	<0.001	<0.001

Figure 2. Median with lower and upper value of IMT between K(+) group which is exposed to the daily 40 cigarette smokes and K(-) group as the control group.

Table 3. Statistic table e-NOS between K(+) group which is exposed to the cigarette smokes and K(-) group as the control group.

Group statistics							
	Group	N	Mean	Std. Deviation	Std. Error Mean	Sig (Independent T)	Sig (Mann-Whitney)
eNOS_	K(-)	9	101,2233	11,80266	3,93422	<0.001	<0.001
	K(+)	9	54,8267	8,30862	2,76954	<0.001	<0.001

Figure 3. Median with lower and upper value of e-NOS between K(+) group which is exposed to the cigarette smokes and K(-) group as the control group.**Table 4.** Statistic table of VCAM-1 between K(+) group which is exposed to the cigarette smokes and K(-) group as the control group.

Group statistics							
	Group	N	Mean	Std. Deviation	Std. Error Mean	Sig (Independent T)	Sig (Mann-Whitney)
VCAM1	K(-)	9	7,67	2,915	,972	0.111	0.138
	K(+)	9	10,33	3,742	1,247	0.112	0.161

expression of the experimental animals. The comparative analysis of VCAM-1 expression demonstrated that there were no statistically significant differences between the groups ($p = 0.112$; independent t test) (Figure 4).

Correlation of e-NOS level and aortic IMT

To determine if the level of e-NOS is correlated with atherosclerosis, we measured e-NOS as a parameter of endothelial cell function in aortic tissue of Wistar rats. Linear regression model found that e-NOS was negatively correlated with aortic IMT in our experimental study ($r^2 = 0.584$, $\beta = -0.764$, $p < 0.001$) (Figure 5).

Figure 4. Median with lower and upper value of VCAM-1 between K(+) group which is exposed to the cigarette smokes and K(-) group as the control group.

Figure 5. Relation between e-NOS level and aortic IMT in experimental rats. A negative linear relationship was found between e-NOS level and aortic IMT.

Correlation of VCAM-1 expression and aortic IMT

To determine if expression of VCAM-1 precedes atherosclerosis, we measured expression of this adhesion molecule in aortic tissue of Wistar rats. Linear regression model found that VCAM-1 expression did not correlate with aortic IMT ($r^2 = 0.197$, $p = 0.065$) (Figure 6).

Figure 6. Relation between aortic VCAM-1 expression and aortic IMT in experimental rats. A positive but non-significant linear relationship was found between aortic VCAM-1 expression and aortic IMT.

Discussion

Oxidative stress-mediated cigarette smokes precedes atherosclerosis

Smoking cigarettes is one of the well-established modifiable risk factors for developing atherosclerosis, which mechanisms remain closely linked to the increased oxidative stress. The total amount of cigarettes smoked per day plays an essential role in increasing the level of oxidative stress and depletion of the antioxidant system. Cigarette smoke contains high concentrations of reactive oxygen species and tiny particles that are easily inhaled in the human body.¹⁸ It is believed that smoking causes increased oxidative stress because of several mechanisms, including direct damage by radical species and the inflammatory response caused by cigarette smoking. The production of oxidative stress and reactive oxygen species due to the cigarette smoke is expected to increase VCAM-1 expression and decrease of e-NOS level. According to the previous research by Yang *et al* (2014), an increase of VCAM-1 expression in rat arteries after being exposed to cigarette smoke had been observed for seven days.¹⁹ Translational research completed by Teasdale *et al* (2014) and Pott *et al* (2017) also supported the findings that increased oxidative stress, reactive oxygen species, and VCAM-1 expression in endothelial cell cultures followed exposure to cigarette smoke.²⁰ Previously, researchers had been studying the influence of smoking on the levels of several biomarkers of oxidative stress, antioxidant status and redox status, including plasma hydroperoxides, e-NOS and VCAM-1. Using different assays to our study, they confirmed that smokers have elevated concentrations of VCAM-1 and compromised e-NOS status.²¹

Cigarette smoke extract induces expression of cell adhesion molecules

VCAM-1 is expressed in vascular endothelial cells, and expression of VCAM-1 may promote the adhesion of leukocytes to the endothelial cells. VCAM-1 accelerates the migration of adherent leukocytes along the endothelial surface, and promotes the proliferation of vascular smooth muscle cells; thus, VCAM-1 may play an essential role as a pro-atherogenic molecules.²² Exposure to cigarette smoke in this study can increase VCAM-1 expression in the aorta although the increase is not statistically significant between the two groups. An insignificant increase in VCAM-1 expression was also found in the previous human research held by Noguchi (1999). In his previous research, soluble VCAM-1 levels were increased in smokers' serum but not significantly when compared to non-smokers' serum.²³

Increase of VCAM-1 expression is a multifactorial process, smoking could not increase VCAM-1 independently without other risk factors such as dyslipidemia. Mu *et al* (2015) had proven this hypothesis by examining VCAM-1 expression in aortic tissue of dyslipidemia patients. As a result, VCAM-1 expression was positively correlated with triglyceride, total cholesterol and LDL levels while VCAM-1 and HDL had a negative correlation²⁴ because the expression of VCAM-1 in endothelial cells requires a trigger that is high lipid levels, especially LDL. An increase in oxidized LDL in the endothelium will be phagocytosed by macrophages. Recruitment of these macrophages requires the role of

VCAM-1.²⁵ In our study, other factors contributing to the development of atherosclerosis such as dyslipidemia weren't included. Our study did not use experimental animals with high-fat diets and serial lipid profile measurement. Therefore, results of our study didn't show any statistical significance of VCAM-1 expression between K (-) and K (+) groups.

Cigarette smoke extract counteracts atheroprotective effects of endothelial nitric oxide synthase

Decreased bioavailability of NO is a central mechanism in the pathophysiology of endothelial dysfunction. Endothelial nitric oxide synthetase (e-NOS) is an enzyme that responsible to produce NO in endothelial cells, so the level of eNOS can represent the availability of NO in endothelial cells.²⁶ Endothelial-cell dysfunction itself could be tested by acetylcholine response function and adenosine coronary flow reserve tests.²⁷ Celermajer *et al* (1992) published a study showing that smoking reduces flow-mediated dilatation (FMD) in systemic arteries in healthy young adults.²⁸

Our study showed that exposure to cigarette smoke can reduce levels of eNOS in the aorta. Our results are consistent with the findings of He *et al* (2017), which shows a significant decrease of eNOS level in endothelial cell cultures exposed to cigarette smoke. He *et al* (2017) showed that exposure to cigarette smoke in endothelial cell culture can reduce the expression of eNOS genes and proteins, resulting endothelial-cell dysfunction.²⁹ On the other hand, Su *et al* (1998) had already proven that administration of cigarette smoke extract can reduce the expression of genes and proteins eNOS. The effect of eNOS reduction depends on the duration of exposure to the cells. The longer the duration of cigarette smoke exposure, the more eNOS levels will be decreased.³⁰ In addition to decreasing eNOS at the gene level, Pini *et al* (2016) showed that exposure to secondhand smoke had also been shown to reduce eNOS at protein levels. eNOS levels decreased in the aorta of guinea pigs after exposure to cigarettes for eight weeks.³¹

It has been demonstrated that smoking cigarettes triggers demethylation, leading to a consecutive reactivation of epigenetically silenced genes *in vitro* and *in vivo* of eNOS and NO production.³² Peroxynitrites, a very reactive oxygene species and pro-oxidant properties from cigarette extract, is believed to promote demethylation and inactivation of e-NOS.³³ In addition, peroxynitrite and other free radicals can deactivate BH4 which is an important cofactor in eNOS production. This was explained by the research of Abdelghany *et al* (2018) which showed that exposure to cigarette smoke has been shown to reduce the BH4 cofactor and correlated with the amount of superoxide and NO production in endothelial cell cultures.³⁴ A decrease of e-NOS and NO level will increase vascular tone, increase expression of adhesion molecules, and trigger coagulation cascade and inflammation.³⁵ In the final pathway, smoking cigarettes leads to increase of aortic intima-medial thickness as an earlier sign of atherosclerosis

Based on this literature and our own data, we suggest that the exposure to cigarette smoke for 28 days daily might be an independent risk factor for atherogenic process through several mechanisms. Aortic IMT in this study increased in group K (+) as was also found in studies conducted by Ali *et al* (2012).³⁶ Increased aortic and entire blood vessels' IMT are due to the pathological conditions such as apoptosis and excessive proliferation as a compensation mechanism.³⁷ In the previous study, the increase of IMT as a complication of endothelial dysfunction leads to the atherosclerosis process.³⁸ Cigarette smoke exposure underlies the endothelial dysfunction by the reduction of e-NOS level and increase of VCAM-1 expression.³⁹

Exposure to cigarette smoke also affects the histological structure of the aorta. In this study, we found not only an increase of IMT, but also structural changes marked by disorganization and vacuolization of smooth muscle cells in the tunica media of the aortic tissue. On the contrary, no changes were observed at the tunica intima level. Exposure to cigarette smoke for 28 days in the study of Ali *et al* (2012) also found the same results: no changes in the tunica intima were observed from the experimental rat.³⁶ Another experimental study from Jaldin *et al* (2013) found that exposure to cigarette smoke for eight weeks only resulted in a disorganization in the vascular smooth muscle cells in the tunica media.⁴⁰ Vacuolization is one of the complications from cytotoxic processes in the cells and an earlier marker of preclinical atherosclerosis. Chemical components from cigarette smoke can cause oxidative stress which is characterized by permanent vacuolization in cells. In the microscopic phenotyping, vacuolization makes the vascular smooth muscle cells have different shapes and sizes, thus resulting in the cells becoming disorganized and leading to atherosclerosis.⁴¹

Limitations

Every study has its limitations which emerge during the realization of the study, creating challenges and, thus, should be highlighted. Firstly, this study had limitations with regards to the small number of samples which can increase the likelihood of error and imprecision. Secondly, results from animal models often do not translate into replications in human models. Level of e-NOS and VCAM-1 expression in Wistar rats are typically transient, whereas in human they can persists for many years. Another crucial difference is IMT, which is usually much lower in the Wistar rats than in humans. These factors may have an impact on the interpretation of our results. Thus, the findings should be interpreted within the

context of this study and its limitations. The strengths of the study were its high statistical power and the homogeneity of each group to enable comparisons between groups and periods.

Conclusion

The present study indicates that smoking cigarettes adversely affects endothelial function and increases the risk of atherosclerosis. Smoking cigarettes as a risk factor for atherosclerosis is closely linked to the increased inflammatory process on the vascular endothelium. Low e-NOS level and high VCAM-1 level observed following smoke exposure may increase aortic IMT. Furthermore, smoking has also been found to influence the aortic IMT. Aortic IMT itself reflects the level of established CVD risk factors in apparently healthy men and women, adding to the evidence that cigarette smoking contributes to CVD through its inflammatory effects on the vascular endothelium.

Data availability

Underlying data

Figshare. Raw Data - Acute effects of cigarette smoke: <https://doi.org/10.6084/m9.figshare.14252639.v1>.⁴²

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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This study determines the effects of 1-month exposure to tar and nicotine on eNOS, VCAM-1, and aortic stiffness in a rats model.

- The authors should highlight the novelty of this study since there are a lot of studies that have been conducted in this area and for these parameters. The parameters also were limited (three parameters only). If the authors can add more parameters then it will be good.
- Out of 44 references, only 12 references are the latest (within 5 years).
- In the results section, the authors should show which statistical analysis has been conducted, rather than presenting both. For example, Table 2 presented both the t-test and Mann-Whitney, and actually one should be chosen based on the normality of the parameters. The mean value and SD in the table also should use a dot, instead of a comma.
- There were abbreviations used without the full names given, such as TGF and BH4.
- Please identify rats as animals and not subjects.
- The manuscript should be proofread as there were grammatical/spelling errors.
- A sample size of nine per group is more than enough and not the limitation of the study.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cardiovascular

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 07 Aug 2022

Ricardo Adrian Nugraha

This study determines the effects of 1-month exposure to tar and nicotine on eNOS, VCAM-1, and aortic stiffness in a rats model.

- The authors should highlight the novelty of this study since there are a lot of studies that have been conducted in this area and for these parameters. The parameters also were limited (three parameters only). If the authors can add more parameters then it will be good.

Author Response: The novelty of this study is the strong correlation between those three parameters in the setting of acute cigarette smoke exposure, which may change the paradigm about only chronic or sub-chronic cigarette smoke exposure can induce the atherogenic process. Increasing evidence suggests that cigarette smoke exposure, even in the short-term period could induce VCAM-1 (enhance pro-atherogenic properties), and a decrease in e-NOS level (anti-atherogenic depletion). Thus, acute cigarette smoke may represent a significant risk factor for atherosclerosis by increasing aortic IMT. This evidence is discussed herein.

- Out of 44 references, only 12 references are the latest (within 5 years).

Author Response: Thank you for your suggestion. We try to replace the reference with the latest one. However, several data and information are only available in the old references

- In the results section, the authors should show which statistical analysis has been conducted, rather than presenting both. For example, Table 2 presented both the t-

test and Mann-Whitney, and actually one should be chosen based on the normality of the parameters. The mean value and SD in the table also should use a dot, instead of a comma.

Author Response: Table 2 (IMT) and Table 3 (e-NOS) used Mann-Whitney u test. On the contrary, Table 4 (VCAM-1) used an independent t-test. This is based on the normality test by the Shapiro-Wilk test. Thank you for your constructive feedback to shape our manuscript better.

- There were abbreviations used without the full names given, such as TGF and BH4.

Author Response: Thank you for your suggestion, we have added TGF (transforming growth factor) and BH4 (Tetrahydrobiopterin) in the abbreviation lists

- Please identify rats as animals and not subjects.

Author Response: Thank you for your suggestion, we have replaced “subjects” with “animals”

- The manuscript should be proofread as there were grammatical/spelling errors.

Author Response: Thank you for your constructive feedback. We try to fix the grammatical/spelling errors in the second version of the manuscript.

- A sample size of nine per group is more than enough and not the limitation of the study.

Author Response: Thank you for your suggestion, we have removed the sample size in the limitation of this study

Competing Interests: Authors declare no competing interest

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