DOI: http://dx.doi.org/10.22122/arya.v17i0.2150 Published by Vesnu Publications

The effect of subchronic cigarette smoke exposure on oxidative stress parameters and endothelial nitric oxide synthase in a rat aorta

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Original Article

Abstract

BACKGROUND: The compounds in cigarette smoke are believed to cause oxidative stress, leading to endothelial dysfunction. Understanding the mechanism of endothelial dysfunction due to cigarette smoke is useful for the development of early and preventive therapy for cardiovascular diseases (CVDs) with smoking risk factors.

METHODS: In this experimental study, a posttest-only control group design was used. 20 Wistar rats were divided into two groups: a smoking group (exposed to 40 cigarettes per day for 4 weeks) and a control group. After the exposure, the animals were sacrificed and aortas were removed for measurement of malondialdehyde (MDA), superoxide dismutase (SOD), endothelial nitric oxide synthase (eNOS), intima-media thickness (IMT), and for histological analysis.

RESULTS: Exposure to cigarette smoke caused a significant decrease in SOD activity (24.28 ± 4.90; P = 0.027) and eNOS levels (50.81 ± 4.18; P = 0.014), but no significant effect on the level of MDA (17.08 ± 5.78; P = 0.551). Histological analysis showed an increase in IMT (13.27 ± 2.40; P = 0.000) and disorganization and vacuolation of smooth muscle cells in tunica media after exposure to cigarette smoke. The regression analysis showed a significant negative relationship between the eNOS level and IMT (β = –1.012, P = 0.009).

CONCLUSION: Subchronic exposure to cigarette smoke caused a decrease in SOD activity and eNOS levels, but no significant change in MDA levels. This study also indicated that smoking causes IMT thickening and pathological structural changes in the aorta. Another finding indicated that a decrease in eNOS levels could cause an increase in the IMT of the aorta.

Keywords: Cigarette Smoke; Vascular Endothelium; Oxidative Stress; Nitric Oxide Synthase

Date of submission: 02 Apr. 2020, ***Date of acceptance:*** 13 July 2020

Introduction

Smoking is an important risk factor for cardiovascular diseases (CVDs). It is one of the largest causes of CVDs in the world, so that it contributed to 7.2 million deaths in 2015.[1] The World Health Organization (WHO) has reported that smoking causes 10% of CVD cases.[2] The American Heart Association stated that smoking is the most preventable risk factor of CVDs.[3] Most countries already have smoking prevention programs and policies, but the prevalence of smoking remains high.[4]

The contents of cigarette smoke each have a role in the pathophysiology of CVDs.[2] The components of cigarette smoke can cause free radical formation in the body by triggering different processes, such as uncoupling of endothelial nitric oxide synthase (eNOS), xanthine oxidase activation, and the mitochondrial electron transport chain (METC).[5,6] High concentrations of free radicals in the body can cause an imbalance between pro-oxidants and antioxidants, causing a condition called oxidative stress.[7]

How to cite this article: Ardiana M, Susetyo Pikir B, Santoso A, Oky Hermawan H, Jibril Al-Farabi M. **The effect of subchronic cigarette smoke exposure on oxidative stress parameters and endothelial nitric oxide synthase in a rat aorta.** ARYA Atheroscler 2021; 17: 2150.

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Oxidative stress plays an essential role in the pathogenesis of endothelial dysfunction.[8] Endothelial dysfunction is the initial phase of most CVDs, and, therefore, it can be used to predict the progression of CVDS.[9-11] Oxidative stress in the endothelium is marked by the formation of lipid peroxidation products, such as malondialdehyde (MDA), and decreased the activity of antioxidant enzymes, such as superoxide dismutase (SOD).[10] Lipid peroxidation is described as a process in which free radicals attack lipids that cause cell damage, producing MDA. MDA is the final product in the decomposition of polyunsaturated fatty acids, which are the main constituents of the cell membrane.[12] The activity of SOD can inhibit lipid peroxidation. SOD is an enzyme that can catalyze the conversion of the free radical O_2^- to H_2O_2, which is then converted to H_2O by glutathione peroxidase or catalase.[13]

In addition to the changes in oxidative stress parameters, endothelial dysfunction is marked by a decrease in the bioavailability of nitric oxide (NO) in endothelial cells. A reduction in NO causes an imbalance in the production of mediators that regulate tone, platelet aggregation, and coagulation in blood vessels.[14-16] eNOS is an enzyme involved in the production of NO in endothelial cells.[6,15] Free radicals can also interrupt the work of eNOS cofactors, causing a decrease in eNOS production. The failure of eNOS biosynthesis amplifies the oxidative stress process as it can cause the formation of other free radicals. As a result, the oxidative stress process continues, forming a vicious circle.[6,17,18]

All the biochemical changes that occur during the endothelial dysfunction could cause structural changes and an increase in the intima-media thickness (IMT) of blood vessels. Both of these changes are signs of early atherosclerosis, which appears soon after endothelial dysfunction occurs.[19] However, the processes that cause an increase in the IMT are still unclear.

Many studies have evaluated the effects of cigarette smoke exposure on the emergence of CVDs, but only a few have investigated its initial process called endothelial dysfunction. Understanding the mechanism of endothelial dysfunction is essential because it is useful for the development of early and preventive treatment for CVDs. In this study, we aimed to assess the effects of subchronic cigarette smoke exposure on endothelial dysfunction by measuring MDA levels, SOD activity, eNOS levels, IMT, and aortic structural changes. We hypothesized that subchronic cigarette smoke exposure could increase MDA levels, decrease SOD activity, decrease eNOS levels, increase IMT, and repair structural damages of the aorta.

Materials and Methods

Study Design: This was an experimental study with a posttest-only control group design. The study was conducted in the animal laboratory of the Faculty of Veterinary Medicine, Airlangga University, Indonesia, from January 2020 to February 2020. The experimental protocol was approved by the Ethics Committee, Airlangga University.

Experiment Animals: 20 male Wistar rats were used in this study. The rats aged approximately 8 weeks, with body weights ranging from 150 to 200 g. The animals were kept in groups of 5 per cage in a standard animal laboratory with proper ventilation, a temperature of 23 °C, and a 12 hour light/dark cycle. All rats were fed a standard diet and water ad libitum. The rats were divided randomly into two groups [control (C)] and smoking (S)], with each group containing 10 rats, and they were adapted in the animal laboratory for one week before the intervention. The smoking group was exposed to cigarette smoke with certain protocols, while the control group did not receive any treatments. After the intervention, the animals in both groups were anesthetized with ketamine and killed with cervical dislocation and their aortas were removed through medial thoracotomy. After the aortas were excised, the adventitial fat was removed. The specimens were placed in individual containers with 10% buffered formalin solution for histopathological preparations, and fresh tissue without fixation was used for MDA, SOD, and eNOS measurements.

Cigarette smoke exposure: For cigarette smoke exposure, we followed the methods described by Ali et al.[20] and Jaldin et al.,[21] with some modifications. The rats were put in a transparent chamber with a volume of 95 × 80 × 65 cm^3, which was connected to the specially designed smoking machine. Smoke from the cigarettes was extracted by the machine, flowed into the chambers, and then incubated for 30 minutes in the chambers. 10 rats in the smoking groups were exposed to cigarette smoke at a rate of 8 cigarettes at a time, 5 times a day (a total of 40 cigarettes per day), every day (a total of 280 cigarettes per week). The exposure was performed for four weeks (subchronic). The cigarettes used in this study were the best-selling non-filter cigarette brands in Indonesia. The cigarette contained 39 mg of tar and 2.3 mg nicotine, according to the manufacturer (HM Sampoerna, Indonesia).

Measurement of MDA, SOD, and eNOS synthase in the aorta: The MDA levels, SOD activity, and eNOS levels were measured in the aorta to ensure that changes in these parameters could represent local processes in the aorta. Total MDA levels (nmol/mg prot) were determined by measuring the levels of thiobarbituric reactive species using the colorimetric method following the manufacturer's instructions for the kit (E-BC-K025, Elabscience, USA). The SOD activity (U/mg prot) was determined using a colorimetric water-soluble tetrazolium salt (WTS-1) method. The procedures followed the kit's instructions (E-BC-K025, Elabscience, USA). Measurement of the eNOS levels (ng/mL) was performed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (EL-R0367, Elabscience, USA). All measurements of the MDA levels, SOD activity, and eNOS levels used a kit intended for experimental animals.

Histology of the Aorta: Histopathological preparations were made using the paraffin method and the fixation was performed by immersing the aortic tissue in 10% formalin for 24 hours. Then, the tissue was processed using a tissue processor and mounted in paraffin blocks. In the next step, the paraffin blocks were cut to a thickness of 5 µm using a microtome and the sections were placed on glass slides and stained with Hematoxylin and eosin stain (H&E). The slides were examined using a light microscope with 400× magnification to determine structural changes in all layers of the aorta and to calculate the IMT. Structural changes were identified as the presence of a loss of endothelial cells in the tunica intima and/or disorganization and vacuolization of smooth muscle cells in the tunica media.[20,21] Each sample was blindly analyzed by independent pathologists.

Statistical Analysis: Data analysis was performed using Statistical Package for the Social Sciences (SPSS) software (version 25, IBM Corporation, Armonk, NY, USA). The data were expressed as mean and standard deviation (SD) and the Shapiro-Wilk test was employed to examine the normal distribution of the data. Accordingly, the independent t-test was used if the data were normally distributed and the Mann-Whitney U test if the data were not normally distributed. The mean difference was considered to be significant if the *p* value was less than 0.050. The association between the independent and dependent variables was analyzed using regression analysis.

Results

Effect on MDA Levels: The median MDA level in the smoking group (15.25) was higher than that in the control group (14.42), but the difference was not significant (p = 0.551) (Table 1). Figure 1 represents the graphs of the MDA levels.

Table 1. Measurement results of malondialdehyde (MDA), superoxide dismutase (SOD), endothelial nitric oxide synthase (eNOS), and intima-media thickness (IMT)

Variables	Control (n = 10)	Smoking (n = 10)	P
MDA (nmol/mg prot)			
Mean ± SD	14.42 ± 0.83	17.08 ± 5.78	0.551**
Median	14.42	15.25	
Min-Max	13.58-15.25	11.92-31.92	
95% CI	12.34-16.49	12.95-21.22	
SOD (U/mg prot)			
Mean ± SD	43.06 ± 8.44	24.28 ± 4.90	0.027*
Median	39.90	21.20	
Min-Max	36.66-52.62	10.22-43.39	
95% CI	22.10-64.01	15.02-33.54	
eNOS (pg/ml)			
Mean ± SD	100.72 ± 5.35	50.81 ± 4.18	0.014*
Median	101.99	48.63	
Min-Max	94.85–105.33	47.19–57.67	
95% CI	87.42–114.02	47.31–54.30	
IMT (µm)			
Mean ± SD	3.74 ± 0.55	13.27 ± 2.40	< 0.001*
Median	3.69	13.49	
Min-Max	3.22-4.31	8.72-16.87	
95% CI	2.38-5.09	11.55-14.99	

SD: Standard deviation; 95% CI: 95% confidence interval; MDA: Malondialdehyde; SOD: Superoxide dismutase; eNOS: Endothelial nitric oxide synthase; IMT: intima-media thickness
*Significant at P < 0.050 (independent t-test), **Significant at P < 0.050 (Mann-Whitney U test)

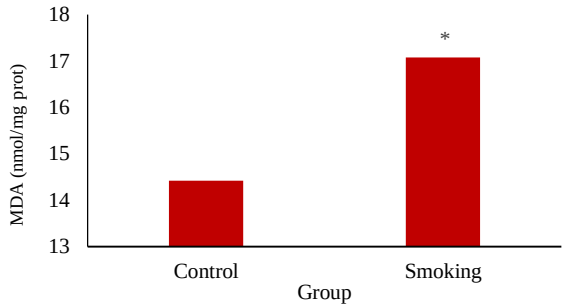

Figure 1. There was no significant difference in the mean of malondialdehyde (MDA) levels in the smoking group compared to the control group (*P > 0.050).

Effect on SOD Activity: The mean ± SD of the SOD activity was significantly lower in the smoking group (24.28 ± 4.90) compared to the control group (43.06 ± 8.44), with P = 0.027 (Table 1). The graphs of the SOD activity are presented in figure 2.

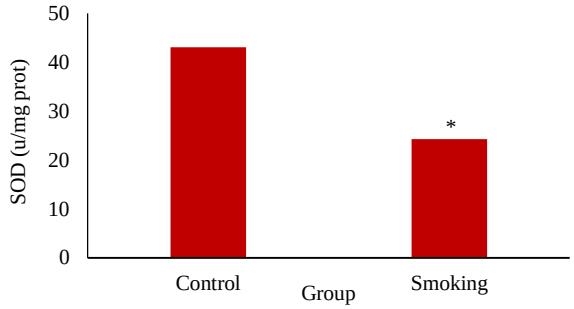

Figure 2. There was a significant difference in the mean of superoxide dismutase (SOD) activity in the smoking group compared to the control group (*P < 0.050).

Effect on eNOS Levels: The median eNOS level was significantly lower in the smoking group (48.63) compared to that in the control group (101.99), with P = 0.014 (Table 1). The graphs of the eNOS levels are presented in figure 3.

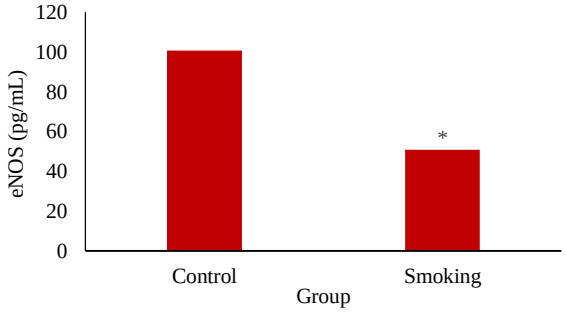

Figure 3. The mean of endothelial nitric oxide synthase (eNOS) levels was significantly lower in the smoking group compared to the control group (*P < 0.050).

Effect on the Histological Structure and IMT of the aorta: Cigarette smoke exposure for 4 weeks did not change the structure of the tunica intima. The endothelial lining was still intact and there were no atherosclerosis lesions in the subendothelial region. The alterations were found in the tunica media, which showed the degenerative process, characterized by disorganization and vacuolation of smooth muscle cells (Figure 4 A and B). There were no changes in the tunica adventitia layer. Beside the structural changes, there was also a significant increase in the mean ± SD of the IMT in the smoking group (13.27 ± 2.40) compared to that of the control group (3.74 ± 0.55) (P < 0.001) (Table 1). The graphs of the IMT are presented in figure 4C.

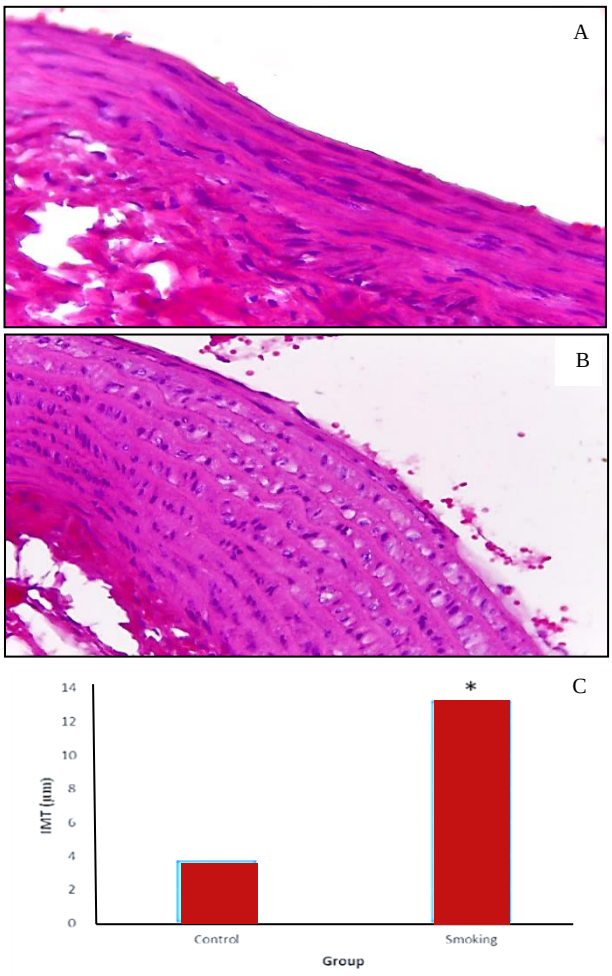

Figure 4. Photomicrographs of aorta cross-sections stained with hematoxylin eosin stain (H&E) with 400× magnification. (A) There were no structural changes in any of the layers of the control group. Note the regular arrangement of smooth muscle cells in the tunica media. (B) The aorta of the smoking group showed disorganization (white arrow) and vacuolation (black arrow) of smooth muscle cells in tunica media. (C) The mean intima-media thickness (IMT) was significantly higher in the smoking group compared to the control group (*P < 0.050).

Relationship between Variables: The regression analysis showed significant negative relationships between smoking and SOD activity (β = -0.659, P = 0.027) and also between smoking and eNOS levels (β = -0.881, P < 0.0001). There was no relationship between smoking and the MDA level (β = 0.290, P = 0.541). The relationships between SOD activity, MDA level, eNOS level, and the IMT were also measured. The regression analysis only showed a significant association between the eNOS level and the IMT (β = -1.012, P = 0.009).

Discussion

Cigarette smoke contains many free-radical-producing compounds.[5,22] Sidestream smoke is smoke that comes out of the tip of a cigarette and contains higher concentrations of free-radical-producing compounds compared to the smoke inhaled by smokers, called the mainstream smoke. The cigarette smoke contents can trigger the production of free radicals from endogenous sources of the cell.[5] The superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), reactive hydroxyl radical (HO), and NO are free radicals that are produced inside cells.[5,23] These free radicals can cause oxidative stress by triggering lipid peroxidation, DNA damage, and enzymatic antioxidant disorders in cells.[7,23] Many studies have shown that oxidative stress is an essential mechanism in the pathophysiology of endothelial dysfunction.[5,10,24] Oxidative stress parameters can be determined by measuring MDA levels and antioxidant enzyme activity levels, such as SOD.[25]

In this study, the MDA levels in the smoking group were not significantly different compared to those in the control group. This result is contrary to some studies that have shown a significant increase in MDA levels, but with a longer period of exposure of 8 weeks,[26] 12 weeks,[27,28] 24 weeks,[29] and 30 weeks,[30] while in this study we used a period of only 4 weeks. A study by Huang et al. showed that cigarette smoke exposure for 4 weeks did not increase the MDA levels and no damage occurred by oxidative stress due to cigarette smoke at 4 weeks of exposure.[29] Exposure to cigarette smoke for 4 weeks is a subchronic exposure, which still causes mild damage to the tissues in the body.[31]

This study showed that SOD activity levels were significantly lower in the smoking group compared to those in the control group. There was also a significant negative relationship between the cigarette smoke exposure and SOD activity. Studies by Ozkol et al.,[32] Ramesh et al.,[28] and Selim et al.[30] reported the same results as this study. The decrease in the SOD activity is thought to be due to the excessive production of H_2O_2 by free radicals, especially those originating from the tar phase, such as quinone/semiquinone.[28,33] The excessive amount of free radicals also makes other antioxidant enzymes, such as glutathione peroxidase and catalase, inactive so that H_2O_2 is not degraded, eventually inactivating SOD.[33,34]

The decrease in NO bioavailability is a central mechanism in the pathophysiology of endothelial dysfunction. eNOS is an enzyme that produces NO in endothelial cells; therefore, the level of eNOS can represent the NO bioavailability in endothelial cells. This study suggested that smoking decreases the eNOS levels. These two variables have a strong negative relationship. This result is consistent with the findings by Marwick et al., who showed reduced eNOS levels in animals exposed to cigarette smoke.[35] Free radicals might cause a decrease in eNOS. The free radical O_2^- can react with NO to form peroxynitrite ($ONOO^-$), which is very reactive and has pro-oxidant properties. This process makes NO no longer available as an active form. Additionally, peroxynitrite and other free radicals can inactivate an essential cofactor in eNOS production, namely tetrahydrobiopterin (BH_4). Inactivation of BH_4 causes the uncoupling of eNOS and the uncoupled eNOS is an inactive form of eNOS, making it unable to produce NO.[6,18,36] The decrease in NO leads to endothelial dysfunction marked by vascular tone disruption and increasing the expression of adhesion molecules that trigger coagulation and inflammation.[14]

Cigarette smoke exposure also affected the histological structure of the aorta. It was found in the current study that the disorganization and vacuolation of the smooth muscle cell in tunica media was the mostly marked structural change in the aorta. There was no change in the tunica intima region. Ali et al. also found the same results in their study[20], while Jaldin et al. found only the disorganization of smooth cells.[21] Vacuolation is one of the effects of the cytotoxic process in the cell and an early sign of cell death. Chemical components in drugs and pollutants have been known to cause irreversible vacuolation in the cell. Oxidative stress plays a role in this process. Vacuolation causes smooth muscle cells in the tunica media have different shapes and sizes, thus making the cells disorganized.[37]

The IMT was also found to increase in the smoking group, which was also found in the study

by Ali et al.[20] The increase in the IMT is an adaptive response to increased vascular wall tension. The regulation of vascular tone is regulated by NO, whose production depends on eNOS levels in endothelial cells. This theory could explain the result of this study, which showed a strong relationship between an increase in the IMT and a decrease in eNOS levels. Besides, smoking also caused several cells in the tunica intima and the tunica media to die, but the remaining cells proliferated excessively. This condition could also cause an increase in the IMT.[38,39]

The limitation of this study was that we did not examine all biochemical parameters comprehensively, which might have effects due to free radicals from cigarette smoke, such as catalase, glutathione peroxidase, and the BH_4 cofactor. Further research is needed to determine these parameters. Moreover, this study only used a limited number of intervention groups. A study with a larger number of treatment groups is needed to observe the effects of cigarette smoke exposure for periods longer than 4 weeks.

Conclusion

This study found that subchronic exposure to cigarette smoke causes changes to oxidative stress parameters, which were indicated by a decrease in SOD activity levels; however, no significant difference in MDA levels in the aorta was found. Subchronic cigarette smoke exposure can also decrease eNOS levels, IMT, and pathological structural changes in the aorta. Another finding indicated that a decrease in eNOS levels can cause an increase in the IMT of the aorta.

Acknowledgments

W The head of the animal laboratory of the Faculty of Veterinary Airlangga University is appreciated.

Conflict of Interests

Authors have no conflict of interests.

Authors' Contribution

MA, AS, and BSP: Study conception and design; MA and HOH: Acquisition of data; HOH and MJA: Analysis and interpretation of data; MA, HOH, and MJA: Drafting of manuscript; AS and BSP: Critical revision.

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