Indexed by : DOAJ Google Crossref Dimension sinta GARUDA

J.Biomed.Transl.Res
ISSN: 2503-2178

JOURNAL OF BIOMEDICINE AND TRANSLATIONAL RESEARCH

Available online at JBTR website: https://jbtr.fk.undip.ac.id

Original Research Article

Cigarette Smoke Exposure, but Not High Fat Diet, is able to Induce Atherosclerosis in Wild-Type Rats

Qorry Amanda[1], Nyoman Suci Widyastiti[2], Yan Wisnu Prajoko[3], Nani Maharani[4], Udin Bahrudin[5]*

[1]*Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia*
[2]*Departement of Clinical Pathology, Faculty of Medicine, Universitas Diponegoro, Indonesia*
[3]*Departement of Oncology Surgery, Faculty of Medicine, Universitas Diponegoro, Indonesia*
[4]*Departement of Pharmacology, Faculty of Medicine, Universitas Diponegoro, Indonesia*
[5]*Departement of Cardiology and Vascular Medicine, Faculty of Medicine, Universitas Diponegoro, Indonesia*

Article Info
History
Received: 01 Sept 2022
Accepted: 18 Oct 2022
Available: 30 Dec 2022

Abstract

Background: High-fat diet (HFD) and cigarette smoke exposure (CSE) have been used to induce atherosclerosis in wild-type (WT) rats however, their effectivity remains unclear.

Objective: To confirm and compare the effectivity of HFD and CSE on both the histopathology of aorta and the level of malondialdehyde (MDA) in WT rats.

Methods: Thirty-six WT Wistar rats were divided into four main groups (K0, K1, K2, and K3) and four subgroups (K3A, K3B, K3C, and K3D). The negative control group (K0) was fed with regular diet. Group K1 was treated with an intravenous adrenaline followed by high-fat diet (HFD), K2 was fed with regular diet and given CSE, while K3 was treated with a combination of CSE and HFD. The serum and cardiac MDA levels were measured using ELISA. Hematoxylin eosin and oil red O staining of aorta were done for measuring of the intima-media thickness (IMT) ratio and for counting of foam cells, respectively.

Results: Both serum and cardiac tissue MDA levels in either K1, K2, or K3 were significant higher ($p<0.01$) than that of in K0. IMT ratio in K3 was significant higher compared to other groups ($p<0.01$). Foam cell numbers were significant higher in K2 and K3 groups than that of in either K0 or K1 ($p<0.01$).

Conclusion: While the HFD fails to induce atherosclerosis in WT rats for 28 days, either CSE or combination of CSE and HFD is able to induce it, and the combination is better than alone.

Keywords: *Atherosclerosis, Intima-Media Thickness Ratio, Foam Cells, Malondialdehyde, Cigarette Smoke Exposure, High Fat Diet*
Permalink/ DOI: https://doi.org/10.14710/jbtr.v8i3.15744

INTRODUCTION

Atherosclerosis is the main leading cause of cardiovascular mortality both in developed and developing countries.[1] Thus, research efforts have been continuously made into understanding the complexity of atherosclerosis in order to achieve the best treatment for it.[2] The small animal including rat remains favourable choice of model for studying atherosclerosis.[3,4] While genetically-modified rats are not widely available and less affordable in the regular animal laboratory, the wild type (WT) rats are resistant to atherosclerosis due to their lipoprotein metabolism pathway.[5] Several methods of inducing atherosclerosis have been known, including the diet containing extremely high-fat, high-salt, high-purine, giving propylthiouracil drug, inducing of hypercortisolemia condition, or giving smoke exposure [6-10]. Anazawa et al. found an increase in the intimal-media thickness (IMT) ratio of WT rats exposed to cigarette smoke.[10] Cigarette smoking is known for its capability in inducing endothelial dysfunction, an early change for atherosclerosis development and remains the most preven risk factor of cardiovascular diseases.[11]

* Corresponding author:
E-mail: *bahrudin00@lecturer.undip.ac.id*
(Udin Bahrudin)

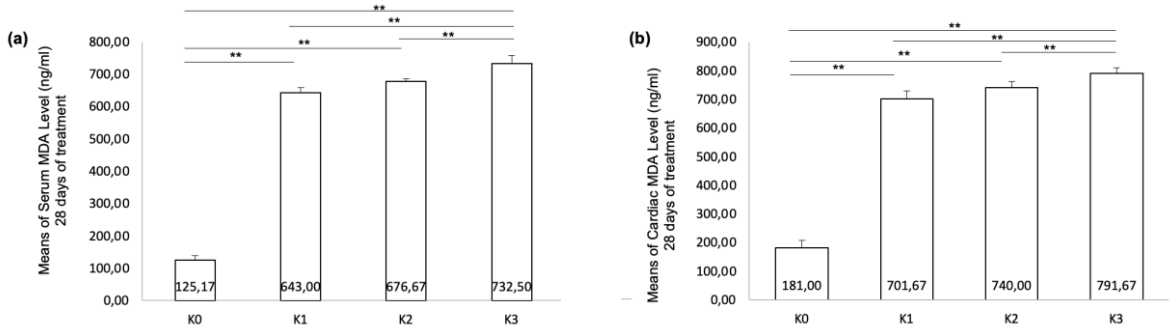

Figure 1. Comparison of effect of the atherosclerosis induction methods on the levels of MDA in either serum (a) or cardiac tissue (b). K0, the negative control group; K1, rats treated with an intravenous adrenaline followed by high-fat diet (HFD); K2, rats with regular diet and given cigarette smoke exposure (CSE); K3, rats treated with a combination of CSE and HFD. ** $p<0.01$.

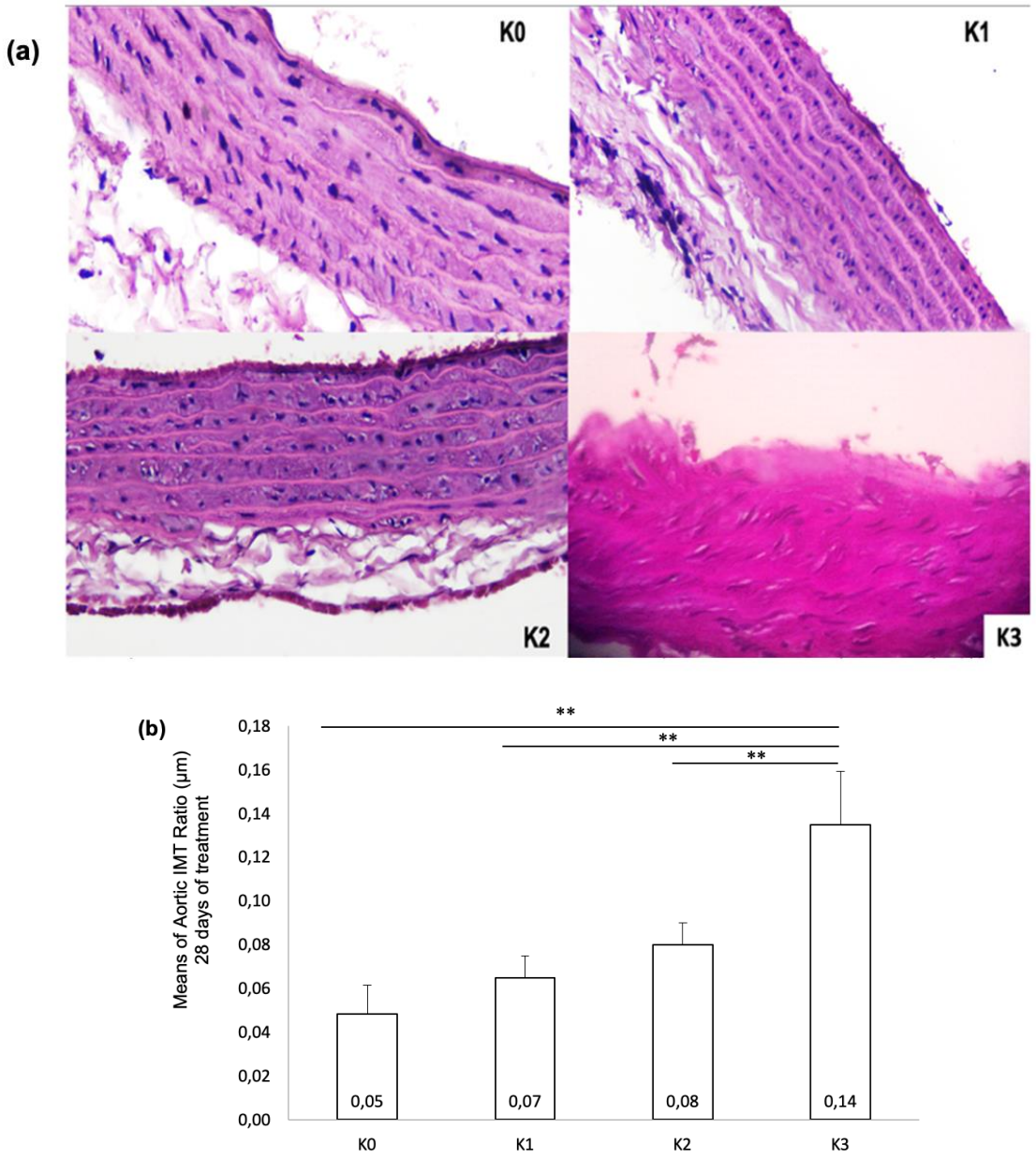

Figure 2. Comparison of effect of the atherosclerosis induction methods on the thickness of intimal layer of aortic wall. (a) Representative transverse sections of the abdominal aorta were stained with hematoxylin-eosin and the microscopic magnification of 400x. (b) Bar graph shows the mean comparison of aortic IMT ratios. ** $p< 0.01$. See the figure legend 1 for description of K0, K1, K2, and K3.

Table 1. Data of maximum, minimum, and mean values of IMT ratio and Foam Cell Counts among four main groups treated for 28 days.

Groups	IMT Ratio			Foam Cell Counts		
	Maximum Value	Minimum Value	Mean	Maximum Value	Minimum Value	Mean
K0	0.07	0.04	0.05	3	0	2
K1	0.09	0.05	0.07	9	3	6.67
K2	0.1	0.05	0.08	25	12	18.67
K3	0.16	0.1	0.14	26	14	21.17

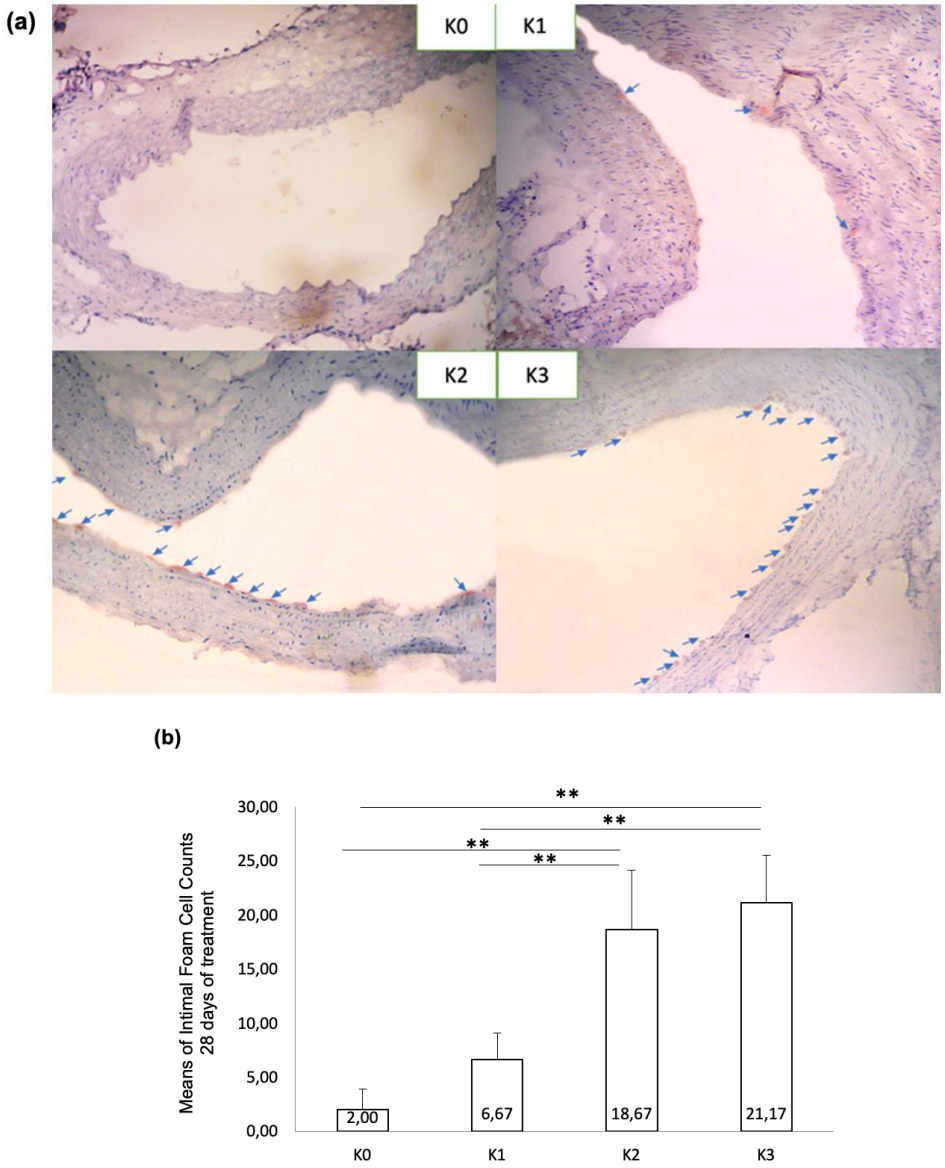

Figure 3. Comparison of effect of the atherosclerosis induction methods on the number of foam cells in the intimal layer of aortic wall. (a) Representative transverse sections of thoracic aorta stained with oil red O, with microscopic magnification of 100x. (b) Bar graph shows the mean comparison of intimal foam cell counts$^{**}p<0.01$. Arrows indicate the intimal foam cells-stained red. See the figure legend 1 for description of K0, K1, K2, and K3.

An early atherosclerosis lesion was also occurred in WT rats given initial adrenaline injection followed by an egg-yolk diet for 21 days.[12] Blood level of adrenaline (epinephrine) is increased in chronic stress condition, activating sympathetic nervous system and thus causing hypertension. Hypertension causes endothelial dysfunction which initiates vascular remodelling and atherosclerosis development.[13,14] Initial adrenaline injection was conducted in order to transiently elevate animals' blood pressure and thus causing endothelial barrier disruption and dysfunction. Egg-yolk acts as a form of high-fat diet which was also proven in inducing

atherosclerosis in rats through its effect of impaired lipid metabolism.[15] Other studies also tried to induce atherosclerosis in WT rats however, data in the published paper remain unclear.

This study aimed to confirm and compare the effectivity of induction methods of atherosclerosis in the WT rats, i.e., the initial adrenaline injection followed by a high-fat diet (HFD), a cigarette smoke exposure (CSE), and combination of them for 28 days, on both the histopathology of aorta and the level of a lipid peroxidation marker malondialdehyde (MDA). MDA is a toxic product from lipid peroxidation process and a biomarker for oxidative stress as well. A previous study reveals that level of MDA correlates with intima-media thickness and thus showing a potential of it being one of atherosclerosis biomarker.[16]

MATERIALS AND METHODS

Study Design

This study was an experimental study with a post-test-only control-group design, with the total of sample was 36 Wistar rats (*Rattus norvegicus*). The animal study was conducted at the Central Laboratory of Food and Nutrition Universitas Gajah Mada, Yogyakarta, while laboratory works were done at the Pathology Laboratory of Central General Hospital of dr. Sardjito, Yogyakarta and Physiology Laboratory of Medical Faculty of Universitas Brawijaya, Malang, East Java from August 2021 to September 2021.

Experiment Animals

Determining of sample size was conducted using the Federer's formula and the result for minimal sample size for animal experiment was 6 animals per group. The 36 rats were male weighing 180-200 grams, actively moving and not having any anatomical abnormality. They were divided into four main groups and four subgroups. The negative control group (K0) had 9 rats and was fed with regular standard diet. K1, K2, and K3 groups were induced atherosclerosis. K1 group (6 rats) was treated with an intravenous (i.v.) 0.006 mg/200 grams body weight adrenaline on day one and followed by a high fat diet using 10 grams of egg yolk (HFD) given via feeding tube intermittently (every 2 days), to avoid toxicity,(12) for 28 days. K2 group (6 rats) was fed with a regular standard diet and given a "sidestream" cigarette smoke exposure (CSE) as much as four clove cigarettes, five times a day, five times per week for 28 days[17] K3 group (15 rats) was induced by combining of methods for K1 and K2, CSE and HFD. The K3 was then divided into 4 subgroups (3 rats), i.e., K3A (3 rats), K3B (3 rats), K3C (3 rats), K3D (6 rats) in whom was treated for 7, 14, 21, and 28 days, respectively. On the end of day 7, 3 animals from K0 and 3 animals from K3 were sacrificed as well to see whether the combination of induction had already given significant result in 7 days of treatment compared with negative control group or not. After 28 days of treatment, 6 animals in all four main groups (K0, K1, K2, and K3) were anesthetized using diethyl ether and killed with cervical dislocations.

Malondialdehyde and Histopathological Analysis

Both serum and cardiac malondialdehyde (MDA) levels were measured using the ELISA method. The heart was separated for MDA examination, the aortas were excised while the adventitial fat was removed. The heart specimens were cut into smaller pieces and centrifugated with speed of 1000xg for 20 minutes. The serum was separated from blood using separator tube before being centrifugated 1000xg for 20 minutes as well. The supernatants were collected for MDA examination using ELISA methods for Rat-Kit.

The aortas were divided into two parts for histopathology examination, the thoracic part was for foam cell counts using oil red O preparation while the abdominal part was for intima-media thickness (IMT) ratio using hematoxylin-eosin (HE) one. IMT was measured by dividing the thickness of intima layer interface with the thickness of media layer.[18] The thoracic aortic tissue was paraffin embedded and stained hematoxylin-eosin (HE) before IMT ratio was measured microscopically using calibrated Image-J software.[19] The abdominal aortic tissue was immediately prepared for fresh frozen section before stained by oil red O. The number of foam cells was calculated microscopically from average 8 field of views from these preparations.

Data Analysis and Ethical Clearance

Statistical analysis was done using One-Way ANOVA test and continued with the post hoc test. Significant result is obtained when $p < 0.05$. All animals in this study were treated in compliance with the latest version of Declaration of Helsinki by World Medical Association. The study was ethically approved by the Institutional Review Board of Faculty of Medicine, Universitas Diponegoro (number 81/EC/H/FK-UNDIP/VIII/2021).

RESULTS

To know the effect of HFD, CSE and combination of them on the oxidate stress both in systemically and locally in the heart, we analyzed the levels of MDA both in serum and cardiac tissue. Figure 1 shows that levels of serum MDA in K3 were significantly higher ($p<0.01$) than that in either K0, K1, and K2 (Figure 1(a)). While the levels were not different between K1 and K2, it was significant higher ($p<0.01$) in the K3 than that of other groups. Similar results were found in cardiac tissue as well (Figure 1(b)). These results indicated that either HFD, CSE, or combining of CSE and HFD for 28 days induce oxidative stress in both the blood and the heart however, the later method provides the highest degree of the stress.

To know effect of HFD, CSE and combination of them on the proliferation and/or migration of smooth muscle cells into intimal layer of aortic wall, we checked it indirectly by comparing the intima-media thickness (IMT) between the groups. Figure 2 shows that K1, K2, and K3, had higher ratio of intima-media thickness (IMT) than K0, but only K3 had a significant higher IMT compared with all other groups ($p<0.01$). These results showed that only combination of CSE and HFD for 28 days induces thickening of intimal media layer of aorta. Data of maximum, minimum, and mean values of IMT ratio and Foam Cell Counts among four main groups treated for 28 days are summarized in table 1.

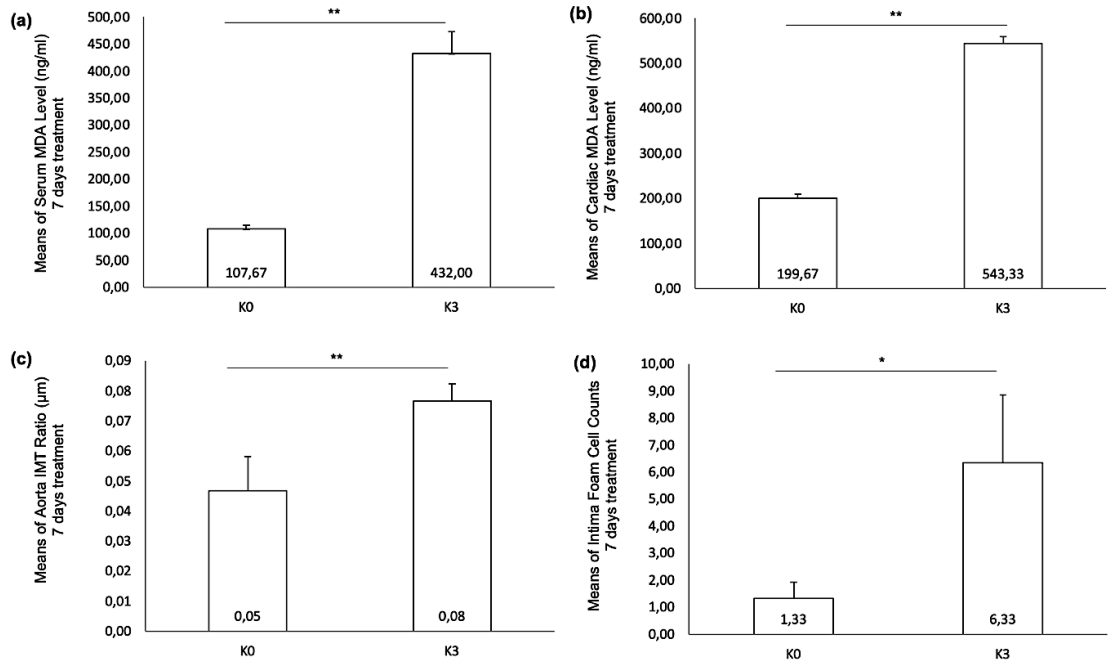

Figure 4 Comparison of effect of the atherosclerosis induction methods in K0 and K3 after 7 days of treatment on the levels of MDA in serum (a) and cardiac tissue (b), the Aorta IMT Ratio (c), and the Intimal foam cells (d). Combination of HFD and CSE gives significant difference ($p<0.05$). * $p< 0.05$, ** $p< 0.01$

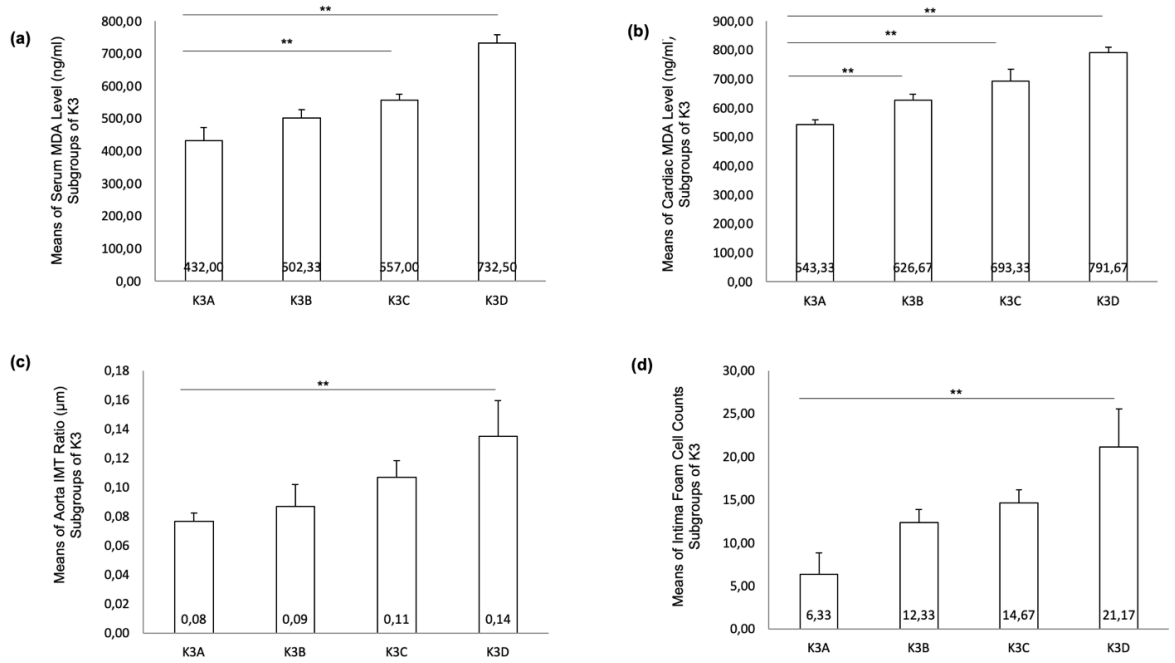

Figure 5 Comparison of effect of the atherosclerosis induction methods in subgroups of K3 after 7 days treatment (K3A), 14 days treatment (K3B), 21 days treatment (K3C), and 28 days treatment (K3D) on the levels of MDA in serum (a) and cardiac tissue (b), the Aorta IMT Ratio (c), and the Intimal foam cells (d). Combination of HFD and CSE in K3 were able to increase those variables overtime from day 7. * $p< 0.05$, ** $p< 0.01$.

To know effect of HFD, CSE and combination of them on the formation of foam cells in the intimal layer of aortic wall, we stained the aortic wall using oil red O. Figure 3 shows that the cell numbers were significantly higher in K2 and K3 groups, but not in the K1, than in the control. Both K2 and K3 groups have higher foam cell number than K1, but it was not significant different between K2 and K3 groups. These data indicated that either CSE or combination of CSE dan HFD for 28 days causes a significant formation of foam cell in aortic wall, but not by HFD alone.

To know whether the effect of combination of CSE and HFD has been occurred early period of induction, we analyzed the MDA level, IMT ratio, and foam cell formation on the day 7 of treatment. Figure 4 shows the comparison between K0 and K3 groups on the day 7 of treatment. Combination CSE and HFD in K3 group showed significant higher in serum MDA level ($p<0.01$),

cardiac MDA level ($p<0.01$), aorta IMT ratio ($p<0.01$), and intima foam cell counts ($p<0.05$) than that of the K0 control group. Furthermore, to know the progression of those parameters in K3 group, we analyzed the them in time dependent manner from the first to fourth week of induction. Figure 5 shows that serum MDA level, cardiac MDA level, aorta IMT ratio, and intimal foam cell counts were increased overtime from day 7 (K3A) today 28 (K3D). K3D had the highest levels of those all variables ($p<0.01$).

Taken together, either HFD, CSE, or combination CSE and HFD were able to induce oxidative stress systemically and locally in the heart, after 28 days of induction in rats. Although only the combination was able to increase IMT ratio of aorta, both the combination and CSE alone could increase number of foam cell in aortic wall. Thus, the combination of CSE and HFD was better than CSE alone in inducing atherosclerosis.

DISCUSSION

This study compared the difference effects of HFD, CSE, and combination of them for 28 days in wild-type rats, and we found that the atherosclerosis induction methods by the combining of CSE and HFD is better than CSE alone in inducing atherosclerosis. This is the first study comparing efficacy among atherosclerosis induction methods and combination of them in WT rats. The characteristic of lipoprotein metabolism pathway in those rats makes them resistance to atherosclerotic induction. The idea of combining of induction methods has been reported in a study using Apo E -/- rats treated with a mainstream cigarette smoking and high fat diet for 12 months.[20] In the current study, we use a side stream instead of mainstream cigarette smoke and an initial adrenalin injection prior to high fat diet is added.

The most important finding of this study is that the combination of CSE and HFD is more effective than CSE alone, while HFD alone is failed, in inducing atherosclerosis in WT rats for 28 days induction. A previous *in vitro* study demonstrated that the cells incubated with nicotine and oxLDL for 72 hours showed a higher cellular cholesterol content in cells and produced more foam cells than cells incubated with oxLDL alone. [21] The combination also increased the expression of CD36 and various pro-atherosclerotic inflammatory cytokines such as TNF-alpha, MCP-1, IL-1, IL-6, interferon-gamma, CXCL9, CXCL10, and CXCL11 compared to either OxLDL or nicotine-treatment only.[21]

In the dose and duration used in this study, the effect of CSE is more effective than HFD in inducing atherosclerosis. Smoking, both active and passive, is proven to be a major risk factor for atherosclerosis[22] Nicotine, a chemical in cigarettes, can increase both blood pressure and pulse.[23,24] They trigger shear stress in vascular lumen resulting in endothelial injury. (25) Nicotine also inhibits the activation of nitric oxide synthase and thus reduce the production and bioavailability of nitric oxide. Moreover, nicotine increases the expression of adhesion molecules on endothelial cells, such as adhesion molecular-1 and monocyte chemoattractant protein-1 which in turn increases the binding and migration of monocytes from the blood circulation to the blood vessel wall ,[25,26] and directly stimulates macrophages to produce more inflammatory cytokines, especially the TNF-alpha type. The later promotes a pro-inflammatory state in the subendothelial space.[27,28] The carbon monoxide found in CSE joins the bloodstream and is bound to the mitochondrial cytochrome oxidase system of cells, thus inhibiting the aerobic metabolism. The later interferes with mitochondrial function and the synthesis of ATP cells.[29,30] In endothelial cells, proteases released due to such mitochondrial disruption inhibit the protective mechanisms of blood vessels used against oxidative stress.[30] Those previous reports and data of this study support the idea that cigarette smoke induces oxidative stress and in turn promote inflammation in the subendothelial space.

An early atherosclerosis lesion was identified in rats given initial adrenaline injection followed by an egg-yolk diet.[12] However, in this study, a high fat diet following an adrenalin administration (HFD) was failed to promote it. The HFD increased oxidative stress systemically and locally in the heart but, was failed to promote IMT ratio as well as foam cell formation (Figure 1-3) for 28 days. A study confirmed the high fat diet given to rats could only give significant alteration in aorta on day 90.[31] Another study even confirmed the anti-inflammatory activity of organic duck egg yolks (300 mg/kg).[32] The levels of adiposity in animals given a high fat diet are affected by several factors including their age, body composition, phenotype, and metabolic profiles.[33] Thus, further study is warranted to confirm effect of a high fat diet on atherosclerosis event in rats.

This is by far the first animal study comparing the methods that were most often used as atherosclerosis induction in wild-type rats. This study confirms that despite its well-known resistance in developing atherosclerosis, wild-type rats are still capable of developing atherosclerosis if induced with the right method, that is, cigarette smoking exposure or combination of high fat diet with cigarette smoking exposure. This important finding can be the main consideration for any future researcher conducting atherosclerosis study in wild-type rats. Limitations of this study is not using liquid nitrogen for fresh frozen section procedure preparation used in *oil red O* staining after harvesting the aortas. Using liquid nitrogen to freeze the organs instantly after terminating the animals is highly recommended for the best staining result.

CONCLUSION

Finally, data of this study conclude that while the high fat diet fails to induce atherosclerosis in WT rats for 28 days, the atherosclerosis induction methods by either CSE or combination of CSE and HFD is able to induce it, and the combination is better than alone.

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