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RESEARCH ARTICLE

The Effects of Ketone Body β-hydroxybutyrate on eNOS Levels and VCAM-1 Expression in Wistar Rats Exposed to Cigarette Smoke

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ABSTRACT:

Background and Aim: Cigarette smoking is strongly associated with coronary artery disease and atherosclerosis, both of which are influenced by endothelial dysfunction. Antioxidant therapy has the potential to inhibit the pathogenesis of atherosclerosis. purpose of the study was to assess the antioxidant potential of β-hydroxybutyrate by examining its effects on eNOS levels and VCAM-1 expression in male Wistar rats exposed to cigarette smoke. Material and methods:. There were 25 Wistar rats involved in this study under a laboratory experiment, which were distributed into five experimental groups. Two control groups were included, with one group receiving no intervention (K-) and the other group exposed to daily exposure to 40 cigarette smoke (K+). The remaining three groups received daily doses of β-hydroxybutyrate-(R)-1,3-butanediol monoester supplement (DeltaG; KE) at 1.5 g/kg/day (P1), 3 g/kg/day (P2), and 6 g/kg/day (P3), respectively, in addition to daily exposure to 40 cigarette smoke. After a 28-day exposure period, eNOS levels and VCAM-1 expression in the aortic tissue were measured. The data were analyzed using the ANOVA test, followed by Fisher's LSD post hoc test. Results: The administration of β-hydroxybutyrate led to a significant increase in eNOS levels in the Wistar rat aorta ($p = 0.036$; $p < 0.05$). However, there were no huge contrasts seen in VCAM-1 expression ($p = 0.426$; $p > 0.05$). Conclusion: This study demonstrated that while Wistar rats exposed to cigarette smoke for 28 days experienced an increase in eNOS levels, there was no decrease in VCAM-1 expression.These findings suggest the potential of β-hydroxybutyrate as a vasodilator in mitigating the effects of cigarette-induced endothelial dysfunction.

KEYWORDS: Ketone body, β-hydroxybutyrate, cigarette smoke, eNOS, VCAM-1.

INTRODUCTION:

Smoking is the most well-known risk factor, and inflammatory changes are a clear indication of pathological changes in cardiovascular disease[1]. Smoking not only causes physiological harm to the respiratory lot but also adds to the advancement of cardiovascular disease[2].

Smoking is a significant factor in causing endothelial dysfunction, and understanding the mechanisms linking smoking to atherosclerosis can be challenging. The numerous chemicals presents in cigarettes, exceeding 5,000 in number, disrupt the balance between prooxidant and antioxidant levels, promotes inflammation over anti-inflammation, as well as disturb prothrombin and antithrombin, leading to endothelial dysfunction[3-6]. Caffeine in cigarettes can increase breathing rate, diaphragm contractility, and sensitivity to carbon dioxide[7].

The biggest cause of death worldwide is cardiovascular

disease, which typically starts with endothelial dysfunction[8]It is a critical indicator of the seriousness and mortality of atherosclerosis confusion as it adds to the improvement of atherosclerosis[9]. Since atherosclerosis is a main source of coronary vein infection (computer-aided design), endothelial brokenness becomes a critical indicator for CAD[10-11]. The vast majority of deaths in developed countries are caused by cardiovascular disease, most of which are related to some form of irregular blood flow in the arteries[12]. In problems related to the heart, the arteries are affected by hardening due to the accumulation of fatty substances in the lumen or plaque formation[13]. The pathogenesis of endothelial dysfunction is largely influenced by the role of endothelial nitric oxide synthase (eNOS), an enzyme present in endothelial cells responsible for nitric oxide (NO) production[14,15]. Early indicators of atherosclerosis include reduced nitric oxide (NO) activity, increased production of reactive oxygen species, and augmented endothelial expression of the redox-sensitive vascular cell adhesion molecule 1 (VCAM-1) gene in the arterial wall[16]. Adenosine triphosphate (ATP) concentration as a functionally high energy carrier occurs in all cells and in the extracellular space[17]. Reactive oxygen species (ROS) have been implicated in the initiation and progression of atherosclerosis[18]. The fluid dynamics of blood through arterial segments play an important role in cardiovascular disease and can lead to strokes, heart attacks, and various cardiovascular diseases[19].

Ketone body, including β-hydroxybutyrate, have shown potential benefits for patients with cardiovascular disease and exhibit potential for clinical applications[20]. β-hydroxybutyrate acts as a stress response molecule, regulating the antioxidant defense program to maintain redox homeostasis[21]. Development in the field of medicine with accelerated drug delivery systems to target organs can increase therapeutic efficacy[22]. In addition, therapies aimed at increasing the circulating ketones holds potential for improving endothelial dysfunction. In this study, the investigation was to determine the effects of β-hydroxybutyrate in animal models exposed to cigarette smoke as an initial step towards developing ketone body therapy. The essential goal of this study is to look at the effect of β-hydroxybutyrate on endothelial brokenness, zeroing in on eNOS levels and VCAM-1 articulation in male Wistar rodents presented to tobacco smoke.

MATERIALS AND METHODS:

Materials:

Animals:

In this study, 25 male Wistar rats (Rattus norvegicus) were eight weeks old and weighed between 150 and 200 grams on average. The Farma Veterinary Center, Directorate General of Livestock and Animal Health Services, Ministry of Agriculture in Surabaya, East Java, Indonesia, evaluated the rats' health after they were obtained from an experimental animal farm. The rats were housed in ventilated cages with a 12-hour light/dark cycle and the room temperature was maintained between 2 and 30 degrees Celsius. The rats were fed a standard rodent-balanced diet and provided with water *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of Universitas Airlangga, Surabaya, Indonesia with the Number of Approval Certificate 2.KE.113.08.2022. The study adhered in the guidelines outlined the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Design and Groups:

A total of 25 Wistar rats were randomly allocated to five experimental groups. The study comprised two control groups: one group (K-) received no intervention, while the other group (K+) was exposed to daily cigarette smoke. In addition, three treatment groups were included, receiving daily doses of β-hydroxybutyrate-(R)-1,3-butanediol monoester supplement (DeltaG; KE) at 1.5 g/kg/day (P1), 3 g/kg/day (P2), and 6 g/kg/day (P3), respectively. All treatment groups were also exposed to daily cigarette smoke. After a duration of 28 days of exposure to cigarette smoke, samples of the aortic tissue were collected and subsequently analyzed to determine the levels of eNOS and the expression of VCAM-1. The collected data were subjected to analysis using the ANOVA test.

Cigarette Smoke Exposure:

The rodents were presented to sidestream smoke utilizing a peristaltic siphon. They were set inside an acrylic box with aspects of 95 x 80 x 65 cm, which would oblige five rodents. To expose the rats to the cigarette smoke, the cigarettes were lit simultaneously and placed in the box for 30 minutes. The rats were exposed to the smoke from a daily dose of 40 cigarettes, divided into five cycles per day and eight cigarettes per cycle. This exposure regimen was maintained for a period of four weeks. The cigarettes utilized in the study were Indonesian unfiltered cigarettes from Dji Sam Soe® by HM Sampoerna according to the manufacturer's label, these cigarettes contain approximately 39 mg of tar and 2.3 mg of nicotine.

β-hydroxybutyrate:

We used β-hydroxybutyrate-(R)-1,3-butanediol monoester (DeltaG Performance) purchased from TDeltaS Global, Inc. (Orlando, Florida). The exogenous ketone was administered orally to the rats at a daily

dose.

Measurement of eNOS Levels:
ELISA (Elabscience E-EL-R0367) was used to measure eNOS levels in the aorta in accordance with the manufacturer's instructions. In rundown, the examples were added to a well plate with the essential enemy of eNOS neutralizer and brooded for the time being at 4 degrees Celsius. After washing, the secondary anti-eNOS antibody was added and incubated for two hours. Following another wash, substrate solution was added, and after a 30-minute incubation, the reaction was stopped using a stop solution. The absorbance of the examples was then estimated at 450 nm utilizing an ELISA peruser.

VCAM-1 Immunohistochemistry:
The aortic tissue was prepared, deparaffinized, and incubated with the anti-VCAM-1 primary antibody (Santacruz biotech SC-13160) for approximately 30 minutes. After washing, the tissue was incubated with the secondary antibody, followed by streptavidin-horseradish peroxidase (SA-HRP). Staining was performed using Chromogen DAB, and counterstaining was done with hematoxylin-eosin. The VCAM-1 expression in the tunica intima was observed under a light microscope at 400x magnification. A semi-quantitative measurement of VCAM-1 expression was conducted using the immunoreactivity scoring system (IRS) outlined in Table 1. IRS score by calculating the score of the proportion of positive cells multiplied by the intensity of staining.

Table 1: Immunoreactivity scoring system (IRS)

Score for percentage of cell 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	

Statistical Analysis:
The information examination included ascertaining mean and standard deviations (SD) for regularly dispersed information, and middle with higher and lower values for non-typically disseminated information. The Shapiro-Wilk test was used to determine whether the data were normal. For regularly circulated information, autonomous t-tests and one-way ANOVA tests were performed to look at mean qualities between factors. For non-ordinarily circulated information, the Mann-Whitney and Kruskal-Wallis tests were utilized all things being equal. Post hoc examination was directed utilizing Fisher's LSD test. SPSS adaptation 25.0 (IBM Corp., Chicago, USA) was utilized for the factual examination.

RESULT:

Impact of Cigarette Smoke Exposure and β-hydroxybutyrate Administration on eNOS Levels:
After 28 days of the experiment, we measured the eNOS levels in different groups. The results showed that the eNOS levels were 234.74 ± 225.50 in the K(-) group, 21.19 ± 12.50 in the K(+) group, 182.75 ± 111.07 in the P1 group, 263.39 ± 206.90 in the P2 group, and 21.40 ± 10.08 in the P3 group. Our analysis indicated significant differences between the groups (p = 0.036). Further examination using post-hoc testing revealed that the P2 group exhibited a significant decrease in aortic IMT compared to the K(-) group (p = 0.016). The summarized findings are presented in Table 2 and Figure 1 to provide a visual representation of the results.

Table 2: eNOS values (pg/ml) in each group

Group	n	*eNOS* (pg/ml)		*p*-value
		x ± SD	Min-Max	
K(-)	5	234.74± 225.50[a]	17.25-520.82	0.036*
K(+)	5	21.19 ± 12.50[b]	5.61- 39.39	
P1	5	182.75 ± 111.07[a]	74.04 - 357.25	
P2	5	263.39± 206.90[a]	53.32- 553.32	
P3	5	21.40 ± 10.08[b]	12.25- 34.54	

* Significant at p = 0.05 (one-way ANOVA)
ab Different superscripts shows significant differences between groups (post-hoc analysis: Fisher's LSD test for eNOS)

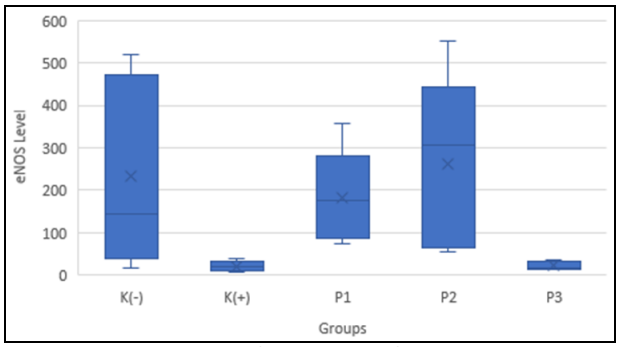

Figure 1: The mean value of eNOS levels after administration of β-hydroxybutyrate

Effects of Cigarette Smoke Exposure and β-hydroxybutyrate Administration on VCAM-1 Expression:
The administration of β-hydroxybutyrate and exposure to cigarette smoke did not result in any significant differences in aortic VCAM-1 expression (p = 0.426; p > 0.05). The measured VCAM-1 expression levels were as follows: 8.20 ± 2.48 in the K(-) group, 9.00 ± 2.12 in the K(+) group, 7.20 ± 3.03 in the P1 group, 6.60 ± 1.94 in the P2 group, and 6.40 ± 2.50 in the P3 group. The results are presented in the following Table 3 and Figure 2.

Table 3: VCAM-1 expression values in each group

Group	n	VCAM-1 Expression (IRS)		p
		x ± SD	Min-Max	
K(-)	5	8.20± 2.48	6.00-12.00	0.426*
K(+)	5	9.00± 2.12	6.00- 12.00	
P1	5	7.20± 3.03	4.00- 12.00	
P2	5	6.60± 1.94	4.00- 9.00	
P3	5	6.40± 2.50	4.00- 9.00	

* Significant at p = 0.05 (one-way ANOVA)

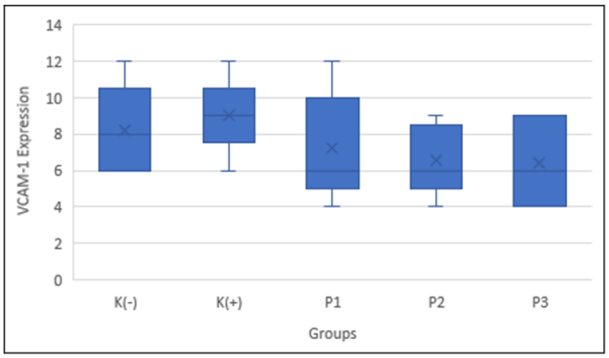

Figure 2: The mean value of VCAM-1 expression after administration of β-hydroxybutyrate

We examined the VCAM-1 expression through immunohistochemical analysis by counting the endothelial cells in the aortic tissue. Using a light microscope at 400x magnification, we observed ten different spots within the tunica intima of the aortic tissue. The findings indicated that after exposure to cigarette smoke, the K(-) group exhibited weak VCAM-1 expression in the intima (Figure 3A), while the K(+) group demonstrated strong expression (Figure 3B). Similarly, the P1, P2, and P3 groups, which received different doses of β-hydroxybutyrate along with exposure to cigarette smoke, displayed weak VCAM-1 expression in the intima. Notably, the P3 group showed the weakest VCAM-1 expression among these groups (Figure 3C, 3D, and 3E).

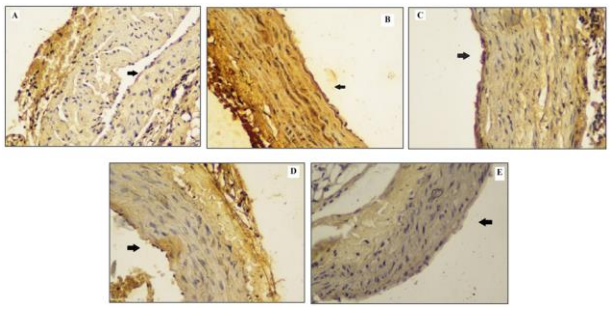

Figure 3. Observation results in the tunica intima of aortic tissue on the histopathological preparate with a light microscope at 400x magnification. (a) Control groups without intervention (K-). (b) Control groups with daily exposure to 40 cigarette smoke (K+). (c) Treatment groups with daily exposure to 40 cigarette smoke and daily doses of β-hydroxybutyrate 1.5 g/kg/day (P1). (d) Treatment groups with daily exposure to 40 cigarette smoke and daily doses of β-hydroxybutyrate 3 g/kg/day (P2). (e) Treatment groups with daily exposure to 40 cigarette smoke and daily doses of β-hydroxybutyrate 6 g/kg/day (P3).

DISCUSSION:

This study explored the impact of cigarette smoke exposure and the administration of β-hydroxybutyrate on eNOS levels and VCAM-1 expression, key factors in endothelial dysfunction, in the aorta. Our findings revealed that openness to cigarette smoke resulted in reduced eNOS levels, a ley player implicated in endothelial dysfunction[23]. Endothelial nitric oxide synthase (eNOS) is a fundamental compound associated with the development of nitric oxide (NO) inside endothelial cells.NO plays a crucial role in maintaining vascular homeostasis by regulating various physiological processes, including vasodilation, platelet aggregation, and inflammation. As eNOS is responsible for synthesizing NO, its levels can serve as an indicator of NO bioavailability within endothelial cells, reflecting their ability to maintain proper vascular function[24-25]. This lines up with past examinations showing diminished eNOS levels in endothelial cell societies presented to tobacco smoke, prompting cell brokenness and disabled multiplication, grip, and migration[26].

However, our study also demonstrated the potential of β-hydroxybutyrate, an active component of ketone body, to mitigate the effects of cigarette smoke on eNOS levels. β-hydroxybutyrate acts as a stress response molecule and regulates antioxidant defense mechanisms, thereby maintaining redox homeostasis[27]. By reducing free radicals through its radical scavenging activity, β-hydroxybutyrate may help preserve eNOS activity and prevent endothelial dysfunction induced by cigarette smoke[28]. The decrease in eNOS observed in our study can be attributed to the free radicals present in cigarette smoke, which can react with NO and form peroxynitrite, a highly reactive species with prooxidant properties, thereby reducing the availability of active NO[29]. Endothelial dysfunction, characterized by impaired vascular tone and increased expression of adhesion molecules such as VCAM-1, can lead to coagulation and inflammation when NO levels are diminished[30]. Notably, Soejima et al. demonstrated that ketone administration prevented a decrease in eNOS levels associated with vascular endothelial cell damage induced by low glucose exposure[31]. Additionally, Li et al. reported that medium doses of β-hydroxybutyrate increased the ratio of eNOS, resulting in a neuroprotective effect in ischemic stroke models[32].

In our study, we also evaluated the impact of β-hydroxybutyrate on VCAM-1 expression in response to cigarette smoke exposure. Although the differences were not statistically significant, the administration of β-hydroxybutyrate resulted in reduced VCAM-1 expression in the treatment groups. Ji et al. demonstrated that β-hydroxybutyrate can attenuate mitochondrial superoxide production and mitigate mitochondrial

oxidative stress in vascular endothelial cells by activating antioxidant pathways, thus potentially contributing to the observed decrease in VCAM-1 expression[33]. Although the difference did not reach statistical significance, there was an observed trend of dose-dependent reduction in VCAM-1 levels in our study.

Numerous previous studies have highlighted the pleiotropic effects of ketone body and their potential cardiovascular benefits[20]. Treatment with 1,3-butanediol, for instance, has been shown to enhance nitric oxide synthase activity in rat arteries[34]. Following transaortic constriction (TAC), the presence of beta-hydroxybutyrate dehydrogenase 1 (BDH1) in rats was associated with increased oxidation of cardiac ketone body, upregulation of antioxidant superoxide dismutase expression, and suppression of oxidative stress[35]. β-OHB, when present in mitochondria, serves as an alternative fuel and can mitigate the effects of oxidative stress[36]. Moreover, higher concentrations of ketone body (β-OHB) have been found to improve cardiac remodeling pathology and enhance cardiac function in small and large animal models[37].

Ketone body have also demonstrated immunoregulatory properties in animals and humans, attenuating pathological inflammation through various mechanisms[38]. β-hydroxybutyrate has been extensively studied and shown to decrease the production of reactive oxygen species (ROS), enhance mitochondrial respiration, and stimulate cellular endogenous antioxidant systems[39]. Oxidative stress is caused by an imbalance between free radicals and antioxidant defense mechanisms in the vascular system. It plays a crucial role in mediating the production of cytokines and linking reactive oxygen species (ROS) to endothelial dysfunction[40].. ROS-induced oxidative damage can exacerbate endothelial cell injury, triggering the activation of transcription factor Nrf2 in endothelial cells, which, in turn, protects against endothelial damage[41]. Huang et al. demonstrated that Nrf2 improves endothelial function by combating oxidative stress and mitigating mitochondrial damage, thus delaying atherosclerosis[42]. β-hydroxybutyrate, functioning as a stress response molecule, regulates antioxidant defense programs to keep redox homeostasis in response to environmental challenges, and its metabolism is key to its positive effects on stress response[20]. Ketone body induce oxidative stress, activating the transcription factor Nrf2, which leads to the transcription of target genes involved in cellular antioxidant defense systems[43]. The initiation of the Nrf2 pathway safeguards cells from cell demise incited by oxidative pressure through expanded articulation of cancer prevention agent proteins, for example, cytoplasmic superoxide dismutase (SOD1) and mitochondrial superoxide dismutase (SOD2)[44].Based on these findings, β-hydroxybutyrate emerges as a potent therapeutic agent with vasodilatory effects on the endothelium.

CONCLUSION:

In summary, our study provides evidence that exposure to cigarette smoke reduces eNOS levels, while the administration of β-hydroxybutyrate shows potential in mitigating the detrimental effects of cigarette smoke on eNOS levels. These findings suggest that β-hydroxybutyrate may have beneficial effects on endothelial function and oxidative stress management. Further exploration is justified to completely investigate the remedial capability of ketone body treatment as a vasodilator on the endothelium and its suggestions for cardiovascular health.

CONFLICT OF INTEREST:

There were no competing interests declared by the authors.

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