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β-Hydroxybutyrate Attenuates Cigarette Smoke-Induced Aortic Injury and Improves Endothelial Function in Wistar Rats

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Abstract

Background: Cigarette smoking is a major risk factor for cardiovascular disease. Oxidative stress plays a central role in endothelial dysfunction and atherosclerosis. β-hydroxybutyrate (β-OHB), a ketone body, has been proposed to exert antioxidant and vascular protective effects. This study aimed to evaluate the effects of β-OHB on aortic histopathology and von Willebrand Factor (vWF) levels in Wistar rats exposed to cigarette smoke.

Methods: This experimental randomized controlled animal study included 25 male Wistar rats divided into five groups: negative control (K−), positive control (K+; cigarette smoke exposure), and three treatment groups receiving β-hydroxybutyrate-(R)-1,3-butanediol monoester (DeltaG®, TDeltaS Global Inc., Orlando, FL, USA) at doses of 1.5 g/kg/day (P1), 3 g/kg/day (P2), and 6 g/kg/day (P3). Rats were exposed to 40 cigarettes/day for 28 days. Aortic tissues were collected after euthanasia on day 28 for histopathological analysis and vWF measurement using ELISA. Data were analyzed using ANOVA with Fisher's LSD post-hoc test.

Results: β-OHB significantly reduced aortic histopathological alterations compared to the smoke-exposed control group (degeneration $p = 0.001$, hyperemia $p = 0.002$, hemorrhage $p = 0.001$, necrosis $p = 0.000$). vWF levels differed significantly among groups ($p = 0.034$), indicating improved endothelial function.

Conclusions: β-OHB attenuates cigarette smoke-induced aortic injury and improves endothelial function. These findings support its potential role as a cardiometabolic therapeutic strategy.

Keywords: β-Hydroxybutyrate, cardiovascular disease, aorta, endothelial dysfunction, cigarette smoke

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INTRODUCTION

Atherosclerosis is the primary underlying cause of cardiovascular disease (CVD)[1]. CVD remains the leading cause of death worldwide, accounting for more than 17.3 million deaths annually, of which approximately 7.3 million are due to coronary heart disease (CHD). It is projected that the global burden of CVD-related mortality will increase to 23.6 million deaths per year by 2030[2,3].

Atherosclerosis, the primary cause of CVD, is a degenerative inflammatory condition that predominantly affects the intima of large- and medium-sized arteries[4,5]. This disease leads to thickening and stiffening of the arterial walls, resulting in reduced oxygen and blood flow to tissues[6]. The atherosclerotic process is initiated when endothelial cells are damaged by reactive oxygen species (ROS). While low concentrations of ROS are involved in normal vascular signaling, excessive ROS levels induce oxidative stress, causing cellular damage[7]. High ROS concentrations contribute to endothelial dysfunction, migration and proliferation of vascular smooth muscle cells, monocyte adhesion and migration with foam cell formation, and reflecting a pro-inflammatory and pro-atherogenic state characterized by increased monocyte-to-HDL ratio (MHR)[8,9].

Von Willebrand factor (vWF) is a glycoprotein synthesized by endothelial cells that mediates platelet adhesion and aggregation at sites of vascular injury[10,11]. Platelets form the glycoprotein (GP) IIb-IIIa complex by binding to ligands such as fibrinogen, vWF, fibronectin, and vitronectin. This interaction triggers intracellular calcium influx, leading to the release of α-granules and dense granules containing molecules such as 5-hydroxytryptamine, adenosine diphosphate (ADP), adenosine triphosphate (ATP), thromboxane A2, leukotriene B4 (LTB4), and other pro-inflammatory mediators. These mediators affect vascular smooth muscle cells, endothelial cells, monocytes, and macrophages[12]. Cigarette smoking exacerbates endothelial dysfunction by increasing vWF and tissue factor (TF) levels, partly through the reduction of nitric oxide (NO) bioavailability[13].

Free radicals play a critical role in tissue injury through lipid peroxidation, which predominantly targets polyunsaturated fatty acids (PUFAs) in cellular membranes[14]. This process disrupts membrane integrity and function, impairs molecular exchange between intracellular and extracellular environments, and may ultimately lead to reversible or irreversible cellular damage[15].

Ketone bodies, particularly β-hydroxybutyrate (β-OHB), have demonstrated therapeutic potential in cardiovascular disease. As a stress-response molecule, β-OHB modulates antioxidant defense mechanisms to maintain redox homeostasis[16]. Advances in medical technology that enhance targeted therapeutic delivery may further improve its efficacy[17]. Moreover, interventions that increase circulating ketone levels have been shown to ameliorate endothelial dysfunction. As an initial step toward the development of ketone-based therapies, this study investigates the effects of β-OHB on cigarette smoke-induced endothelial dysfunction in male Wistar rats, with a particular focus on aortic histopathology and von Willebrand factor (vWF) levels.

MATERIALS AND METHODS:

Animals

Twenty-five male Wistar rats (*Rattus norvegicus*), aged eight weeks and weighing 150–200 grams, were randomly assigned into five groups (n=5 each): K–, K+, P1, P2, and P3. Their health was evaluated by the Ministry of Agriculture's Farma Veterinary Center, Directorate General of Livestock and Animal Health Services, Surabaya, East Java, Indonesia. The rats were housed in ventilated cages at room temperature (22–30°C) with a 12-hour light/dark cycle, and had ad libitum access to water and standard rodent feed. All procedures were approved by the Institutional Animal Care and Use Committee of Universitas Airlangga, Surabaya, Indonesia (Approval Certificate No. 2.KE.113.08-2022) and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The rats were randomly assigned to five groups (n = 5 per group): K- (negative control, no intervention), K+ (positive control, daily cigarette smoke exposure), and three treatment groups (P1, P2, P3) receiving oral β-hydroxybutyrate-(R)-1,3-butanediol monoester (DeltaG; KE) at 1.5, 3, and 6 g/kg/day, respectively, along with daily cigarette smoke exposure. After 28 days, aortic tissues were collected to analyze histopathological evaluation of the aorta and measurement of von Willebrand factor (vWF). Data were analyzed using one-way ANOVA.

Study Design
This was an experimental, randomized controlled animal study conducted to evaluate the protective effects of β-hydroxybutyrate against cigarette smoke-induced vascular injury.

Ethics Approval
All procedures were approved by the Institutional Animal Care and Use Committee of Universitas Airlangga (Approval No. 2.KE.113.08-2022) and conducted in accordance with international guidelines for laboratory animal care.

Cigarette Smoke Exposure
Side-stream cigarette smoke was delivered using a peristaltic pump into an acrylic exposure chamber (95 × 80 × 65 cm) housing five rats at a time. Rats were exposed to smoke from 40 cigarettes/day for 30 minutes per session over 28 days. Indonesian unfiltered cigarettes (Dji Sam Soe®, HM Sampoerna) containing approximately 39 mg tar and 2.3 mg nicotine per cigarette were used.

β-hydroxybutyrate
β-hydroxybutyrate (β-OHB) (DeltaG Performance, TDeltaS Global, Inc., Orlando, Florida) was administered orally once daily at doses of 1.5, 3, and 6 g/kg/day.

Tissue Collection
All animals were euthanized under anesthesia on day 28 of the experiment, and aortic tissues were subsequently collected for histopathological analysis and quantification of von Willebrand factor (vWF) levels.

vWF Measurenment
Aortic von Willebrand factor (vWF) levels were quantified using a sandwich ELISA kit (Rat VWF ELISA Kit, Elabscience, E-EL-R1079) according to the manufacturer's instructions. In brief, microplate wells pre-coated with anti-Rat vWF antibody were incubated with standards or samples, followed by a biotinylated detection antibody specific for Rat vWF and an avidin–horseradish peroxidase (HRP) conjugate. Unbound components were removed by washing, and substrate solution was added. The enzyme-substrate reaction produced a blue color that turned yellow after adding stop solution. Optical density (OD) was measured at 450 ± 2 nm, and vWF concentrations were calculated from a standard curve.

Procedure for Making Histopathology Preparations
Necropsied organs were fixed in 10% neutral-buffered formalin (NBF), dehydrated in acetone (I, II; 1 hour each at room temperature), cleared in xylene (I, II; 0.5–1 hour each), and infiltrated with xylene-paraffin (I, II; 30 minutes each at 54–56˚C). Tissues were embedded in paraffin, sectioned at 3 μm, mounted on glass slides, and covered with Canadian balm and a coverslip. Slides were incubated at 37˚C overnight before routine hematoxylin and eosin (H&E) staining.

Histopathological Parameters
Histopathological parameters were evaluated in aortic tissue sections, including hyperemia, hemorrhage, degeneration, and necrosis. Observations were performed in 10 fields per slide at 400× magnification. Lesions were graded semi-quantitatively as mild (+; 1–10 affected cells), moderate (++; 11–20 affected cells), or severe (+++; >20 affected cells)[18].

Histopathological Analysis
Aortic tissues were fixed, processed, and stained with hematoxylin–eosin (H&E) for histopathological evaluation. Degeneration was characterized by cellular swelling, cytoplasmic vacuolization, and loss of structural integrity, whereas hyperemia was defined as vascular congestion with dilated vessels containing erythrocytes. Hemorrhage was identified by the presence of extravascular erythrocytes within the tissue, and necrosis was determined based on nuclear changes, including pyknosis, karyorrhexis, and karyolysis. All histopathological alterations were evaluated using a semi-quantitative grading system.

Statistical Analysis
Data were expressed as mean ± standard deviation (SD) or median (range), depending on distribution. Normality was assessed using the Shapiro-Wilk test. For normally distributed data, comparisons were made using independent t-tests or one-way ANOVA. Non-normally distributed data were analyzed using Mann-Whitney or Kruskal-Wallis tests. Post hoc analysis was performed with Fisher's LSD test. Statistical analyses were conducted using SPSS version 25.0 (IBM Corp., Chicago, IL, USA).

RESULTS AND DISCUSSION

Aortic Histopathology

β-hydroxybutyrate (β-OHB) significantly reduced degeneration, hyperemia, hemorrhage, and necrosis in aortic tissue compared to the positive control group. Each treatment group consisted of five tissue samples corresponding to the number of animals per group. Histopathological evaluation was performed by examining 10 fields of view per slide at 400× magnification, and all observations were conducted by an experienced anatomical pathologist. The results of the analysis are presented in the table below.

Tabel 1. Histopathological features of the aortic tissue degeneration

Group	n	Degeneration		*p*-value
		x ± SD	Min-Max	
K(-)	5	0.14± 0.313[a]	0.00-0.70	0.001*
K(+)	5	1.42 ± 0.558[b]	0.80- 2.20	
P1	5	1.18 ± 0.785[b]	0.40-2.50	
P2	5	0.22± 0.228[a]	0.00-0.50	
P3	5	0.40 ± 0.693[a]	0.00-0.08	

* significant at α=0.05 (*One-way* ANOVA)
[ab] different superscript shows significant differences between groups (post-hoc analysis: LSD test for degeneration)

Tabel 2. Histopathological features of the aortic tissue hyperemia

Group	n	Hyperemia		*p*-value
		x ± SD	Min-Max	
K(-)	5	0.50± 0.608[a]	0.00-1.30	0.002*
K(+)	5	1.56 ± 0.477[b]	0.80- 2.10	
P1	5	1.30 ± 0.674[a]	0.90-2.50	
P2	5	0.22± 0.228[a]	0.00-0.50	
P3	5	0.62 ± 0.438[b]	0.20-3.00	

* significant at α=0.05 (*One-way* ANOVA)
[ab] different superscript shows significant differences between groups (post-hoc analysis: LSD test for hyperemia)

Tabel 3. Histopathological features of the aortic tissue hemorrhage

Group	n	Hemorrhage		*p*-value
		x ± SD	Min-Max	
K(-)	5	0.14± 0.313[a]	0.00-0.70	0.001*
K(+)	5	1.42 ± 0.558[b]	0.80- 2.20	
P1	5	1.18 ± 0.785[b]	0.40-2.50	
P2	5	0.22± 0.228[a]	0.00-0.50	
P3	5	0.40 ± 0.693[a]	0.00-0.08	

* significant at α=0.05 (*One-way* ANOVA)
[ab] different superscript shows significant differences between groups (post-hoc analysis: LSD test for Hemorrhage)

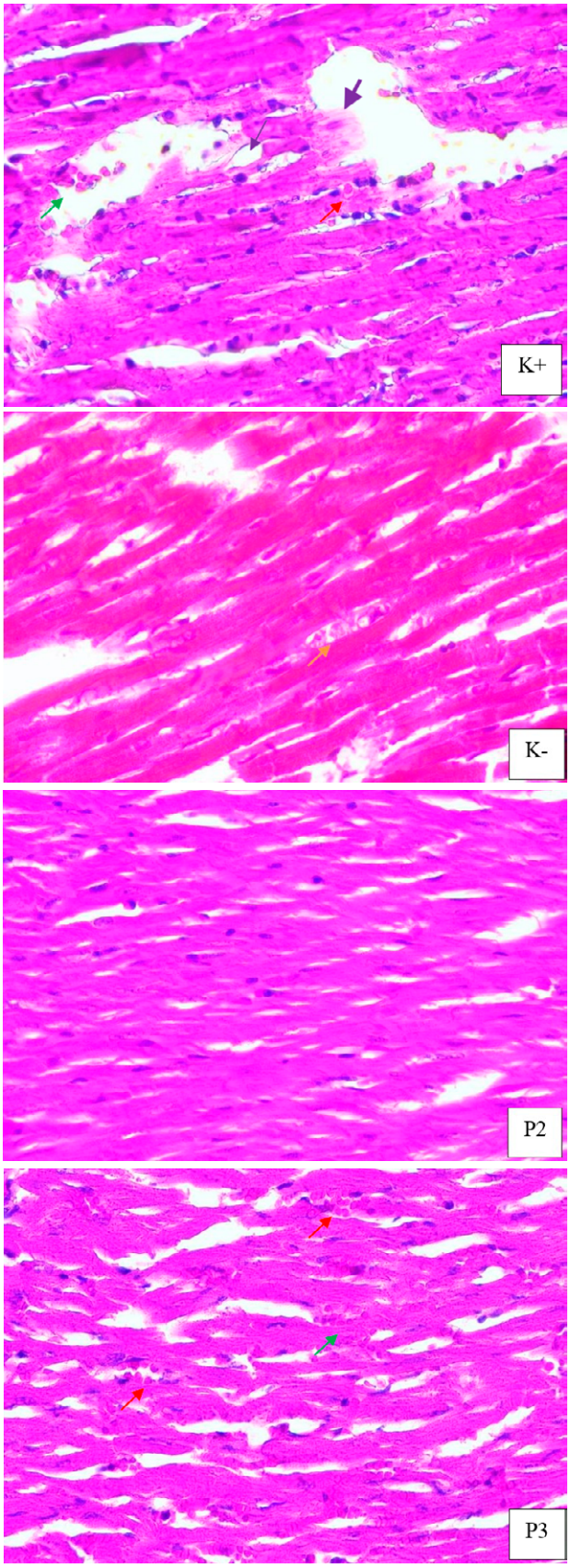
Tabel 4. Histopathological features of the aortic tissue necrosis

Group	n	Necrosis		*p*-value
		x ± SD	Min-Max	
K(-)	5	0.06± 0.089[a]	0.00-0.20	0.000*
K(+)	5	1.96 ± 0.856[b]	0.90- 3.00	
P1	5	0.6 ± 0.367[a]	0.00-1.10	
P2	5	0.22± 0.134[a]	0.00-0.50	
P3	5	0.58 ± 0.831[a]	0.00-3.00	

* significant at α=0.05 (*One-way* ANOVA)
[ab] different superscript shows significant differences between groups (post-hoc analysis: Tamhane test for necrosis)

The results of the ANOVA analysis demonstrated significant differences between the positive control group and the treatment groups across all assessed histopathological parameters. Specifically, the p-values for degeneration, hyperemia, hemorrhage, and necrosis were 0.001, 0.002, 0.001, and <0.001, respectively, indicating statistically significant differences ($p < 0.05$). These findings suggest that β-hydroxybutyrate treatment significantly reduced the severity of degeneration, hyperemia, hemorrhage, and necrosis in aortic tissue compared to the positive control group.

Histological observations of aortic tissue were performed using a light microscope at 400× magnification, and the findings are summarized in Figure 1. In the negative control group (K–), the aortic tissue showed minimal histopathological alterations. In the positive control group (K+), cigarette smoke exposure resulted in marked pathological changes, including necrosis and hyperemia. In the treatment group P1, aortic tissue demonstrated the presence of necrosis, hyperemia, and degeneration, although to a lesser extent than the positive control group. In group P2, no significant histopathological alterations, including degeneration, hyperemia, hemorrhage, or necrosis, were observed. In group P3, aortic tissue exhibited mild hyperemia and hemorrhage.



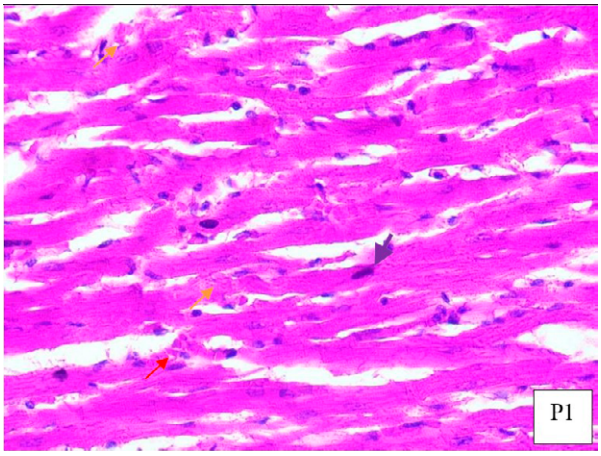


Figure 1. Comparison of aortic tissue in several treatment groups. Appears to show muscle degeneration (↗), hyperemia (↗), hemorrhage (↗) and necrosis (↗). (Hematoxylin-Eosin : 400x)

vWF Levels

After 28 days of the experiment, von Willebrand factor (vWF) levels were measured in all groups. The mean vWF levels were 244.75 ± 229.44 in the K– group, 25.30 ± 22.93 in the K+ group, 167.85 ± 113.97 in the P1 group, 276.17 ± 213.62 in the P2 group, and 20.19 ± 7.95 in the P3 group. Statistical analysis demonstrated significant differences among groups (p = 0.034), suggesting improved endothelial function in the treatment groups. The results are summarized in Table 5.

Table 5. Value of *vWF* (ng/mL) in groups

Group	n	vWF (ng/mL)		*p*-value
		x ± SD	Min-Max	
K(-)	5	244.75±229.44[a]	71.54-557.25	0.034*
K(+)	5	25.30±22.93[b]	4.04-62.96	
P1	5	167.85±113.97[a]	29.54-332.96	
P2	5	276.17±213.62[a]	46.89-546.18	
P3	5	20.19±7.95[b]	12.39-31.68	

* significant at a=0.05 (*One-way* ANOVA)
[ab] different superscript shows significant differences between groups (post-hoc analysis: Fisher LSD test for vWF)

Based on the assessment of necrotic cell counts in aortic tissue of Wistar rats, administration of β-hydroxybutyrate at doses of 1.5, 3, and 6 g/kg/day for 28 days resulted in distinct histopathological changes compared to the cigarette smoke-exposed control

group. β-hydroxybutyrate treatment reduced the number of cells exhibiting degeneration, hyperemia, hemorrhage, and necrosis in aortic tissue.

Several factors may contribute to histopathological alterations in vascular tissue. In the positive control group exposed to cigarette smoke, marked aortic changes, including degeneration, hyperemia, hemorrhage, and necrosis, were observed. The accumulation of free radicals is associated with hypercholesterolemia and chronic low-grade inflammation, characterized by increased pro-inflammatory mediators, which further contribute to endothelial dysfunction[19,20]. Reactive oxygen species (ROS) may further disrupt lipid homeostasis by enhancing low-density lipoprotein (LDL) oxidation and impairing LDL receptor function, resulting in elevated circulating cholesterol levels. In this study, necrotic changes ranged from mild (focal) to moderate (multifocal) across treatment groups. Microscopically, necrosis is characterized by pyknosis, karyorrhexis, and karyolysis, and may trigger a localized inflammatory response in the surrounding tissue[21].

The reduction in endothelial abnormalities observed following β-hydroxybutyrate administration may be attributed to its antioxidant and anti-inflammatory properties, similar to other metabolic interventions shown to reduce systemic inflammatory biomarkers and improve vascular function[22,23]. Variability in histopathological findings among individual animals may reflect differences in metabolic response, immune status, and β-hydroxybutyrate dosage. Overall, β-hydroxybutyrate demonstrated protective effects by attenuating degeneration, hyperemia, hemorrhage, and necrosis in aortic tissue.

Von Willebrand factor (vWF) is a glycoprotein essential for primary hemostasis and the intrinsic coagulation cascade, mediating platelet adhesion to subendothelial collagen and stabilizing factor VIII. It is present in endothelial cells, platelets, the subendothelial matrix, and storage granules, and consists of repeating subunits that provide multiple binding sites for interacting proteins[24]. Hypercholesterolemia is associated with reduced antioxidant capacity, particularly in patients with vascular disease. Elevated vWF levels reflect endothelial dysfunction and are linked to oxidative damage and the progression of atherosclerosis[25].

Furthermore, ketone bodies exhibit immunoregulatory properties, reducing pathological inflammation through multiple mechanisms. β-hydroxybutyrate has been shown to decrease ROS production, enhance mitochondrial respiration, and activate endogenous defense systems[26]. An imbalance between antioxidant defenses and ROS leads to oxidative stress, which modulates cytokine production and contributes to endothelial dysfunction[27]. Activation of the transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) plays a critical role in protecting endothelial cells from oxidative injury[28]. Previous studies have demonstrated that Nrf2 activation delays atherosclerosis by reducing mitochondrial damage and oxidative stress, thereby improving endothelial function[29]. The beneficial effects of β-hydroxybutyrate are largely attributed to its role as a stress-response molecule that modulates antioxidant defense mechanisms to maintain redox homeostasis under physiological stress[30].

Collectively, these findings position β-hydroxybutyrate as a promising metabolic modulator capable of attenuating cigarette smoke-induced vascular injury. Through its antioxidant and endothelial-protective effects, β-hydroxybutyrate reduces histopathological damage and improves vWF levels, underscoring its role in mitigating oxidative stress and vascular dysfunction. Beyond its function as an energy substrate, β-hydroxybutyrate may act as a signaling molecule with therapeutic potential in cardiovascular disease. Further studies are warranted to validate these findings in long-term and clinical settings.

STUDY LIMITATIONS

This study has several limitations. The use of an animal model may limit the direct applicability of the findings to human clinical settings. In addition, the relatively short duration of the study (28 days) may not fully reflect the long-term effects of β-hydroxybutyrate. Furthermore, the absence of additional molecular biomarkers restricts a more comprehensive evaluation of the underlying mechanisms.

CONCLUSION

In summary, β-hydroxybutyrate attenuates cigarette smoke-induced aortic injury and improves endothelial function in Wistar rats. It reduces histopathological damage and modulates von Willebrand factor (vWF) levels, suggesting its potential to counteract oxidative stress and serve as a therapeutic strategy for cardiovascular disease.

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Ethics Statement and Conflict of Interest Disclosures

Financial support and sponsorship: All authors have declared that no financial support was received from any organization for the submitted work.

Conflict of interest: No known conflict of interest correlated with this publication.

Availability of data and materials: The data used and/or analyzed throughout this study are available from the corresponding authors upon reasonable request.

Competing interests: The authors declared that they have no competing interests.

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