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The Effect of Vitamin D_3 (Cholecalciferol) on Histopathological Pulmonary Emphysema of The White Rats (*Rattus norvegicus*) Lungs

Riedho Aulia Anggara[1], Elfina Dinyyatika Arifin[2], Anwar Ma'ruf[3], Jola Rahmahani[3], Wiwik Misaco Yuniarti[3], Ratna Damayanti[3], Hani Plumeriastuti[3], Boedi Setiawan[3], Nusdianto Triakoso[3], Kuncoro Puguh Santoso[3], Rury Mega Wahyuni[3]*, Lita Rakhma Yustinasari[3], Rahmi Sugihartuti[3], Abdullahi Baso[4]

[1]Vaccinology and Immunotherapeutics, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia; [2]Reproductive Biology, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia; [3]Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia; [4]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Bayero University Kano, Nigeria.

Abstract | Prevalence of smoking population in Indonesia was raising year by year; it's a bad habit of Indonesian society whose become the main contributors to chronic obstructive pulmonary disease (COPD) and emphysema. The content of various dangerous chemical compounds in cigarette smoke can trigger oxidative stress, inflammation, and lung tissue damage. Vitamin D_3 has been studied to have potential as an antioxidant and anti-inflammatory, which may provide a protective effect against lung damage caused by cigarette smoke. This study, using white rats as model animals, aims to determine the impact of vitamin D_3 on histopathological pulmonary emphysema in white rats (*Rattus norvegicus*) exposed to cigarette smoke. This study used 25 white rats divided into 5 groups: the negative control (K-) in the form of healthy rats and positive control (K+) in the form of rats exposed to cigarette smoke without giving vitamin D_3, 3 treatment groups (P1, P2, and P3) each of which received vitamin D_3 at a dose of 0.00125 mg/50 IU, 0.0025 mg/100 IU, and 0.005 mg/200 IU orally after being exposed to cigarette smoke. Pulmonary emphysemas were analysed histopathological to assess the protective effects of Vitamin D_3 against cigarette smoke. Taken together, these findings demonstrate that vitamin D3 effectively attenuates cigarette-smoke–induced pulmonary emphysema and preserves lung health. This study also highlights the suitability of the white rat as a reliable animal model for investigating respiratory disorders, providing valuable insights for advancing animal health research and improving animal welfare within the context of animal health and production.

Keywords | Antioxidant, Cigarette smoke, Emphysema, Health, Inflammation, Vitamin D_3

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***Correspondence** | Rury Mega Wahyuni, Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia; **Email:** rury.mega@fkh.unair.ac.id
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INTRODUCTION

Smoking is a poor lifestyle that causes social and health problems, which is its prevalence continues to increase in Indonesia. A survey conducted by the Ministry of Health (Kemenkes, 2023) disclosed several significant findings, including the fact that 22.46% of individuals aged ≥10 years in Indonesia smoke daily, while 4.56% smoke

occasionally. Approximately 27.22% (75 million) of the total population in Indonesia are estimated to be smokers (Kemenkes, 2023). Additionally, research has demonstrated that exposure to cigarette smoke can lead to elevated serum cotinine levels in domestic dogs (Gropetti *et al*., 2023). Also study conducted by Demirtas *et al*. demonstrated that domestic cats exposed to cigarette smoke experienced an increase in Total Oxidant Status (TOS) and Oxidative Stress Index (OSI), followed by a substantial increase in proinflammatory cytokines (INF-γ, Il-1β, IL-2, and IL-6), as well as a plummet in Total Antioxidant Status (TAS) (Demirtas *et al*., 2023). More than 7,000 chemicals, including oxidants such as hydrogen peroxide (H_2O_2) and free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), are present in the smoke produced by the combustion of tobacco products or cigarettes (NTP, 2016). These substances have the potential to induce oxidative stress and initiate the production of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8. Emphysema is the hallmark of Chronic Obstructive Pulmonary Disease (COPD), the third most prevalent cause of mortality worldwide. Exposure to cigarette smoke contributes to 90% of instances of emphysema in humans (Zachary, 2022).

Smoking will demonstrate a mismatch of ventilation and perfusion by measuring blood flow in the pulmonary capillaries. Low perfusion also occurs in tobacco smokers by assessing lung perfusion problems from 8.6% to 9.1% (Simanjuntak *et al*., 2023). An excess of free radicals relative to antioxidants can induce oxidative stress in cells, leading to lipid peroxidation. Cigarette smoke leads to the imbalance between oxidants and antioxidants through exogenous reactive oxygen species (ROS). Moreover, inflammation and mitochondrial dysfunction induced by free radicals may potentially facilitate the progression of COPD (Suryadinata, 2018).

Vitamin D is a secosteroid hormone which is well-known for its immunomodulatory and antioxidant properties. This was shown in a study that explained that the enzymes 1α-hydroxylase and VDR are found widely in the body, including in the lungs and immune system cells (Daryabor *et al*., 2023). Vitamin D_3 needs to be hydroxylated by 1α-Hydroxylase in the kidney to become its active form, calcitriol or 1,25-dihydroxyvitamin D_3. Calcitriol can also be produced locally in other organs, including the lungs.

Vitamin D has the power to enhance lung function through its ability to regulate inflammatory responses, stimulating the production of alpha 1-antitrypsin (AAT), maintaining the health of respiratory tract smooth muscle cells, and inducing antimicrobial peptides. Meanwhile, the function of vitamin D as an antioxidant in protecting the lungs is by preventing the formation of ROS and stimulating upregulation of glutathione (GSH) by modulate the expression levels and activity of the enzymes GSH Peroxidase (GPx1), Superoxide Dismutase (SOD), and glutathione reductase (GR) in GSH metabolism (Ansari *et al*., 2020). In COVID-19 study vitamin D levels in blood measured by blood 25(OH)D3 levels strongly correlated with decreasing chance of COVID-19 recurrent infection, cause vitamin D enhance the system immune including function of macrophages, neutrophils, and T lymphocytes (Nurjanah *et al*., 2024). Moreover, study in sepsis model mice has found that mice treated with vitamin D exhibited decreased necrosis, inflammation, and hemorrhagic lesions relative to untreated sepsis mice, showing the impact of vitamin D as antioxidant agent (Fajri *et al*., 2024). Serum 25(OH)D levels indicate the body's overall vitamin D production and can be used as the best biomarker to determine a person's vitamin D status (Risanti *et al*., 2024).

Indonesia is a tropical country, which is hassle-free to obtain the sources of Vitamin D and so easy to get sunlight throughout the season. The sun radiates UVB rays that are catalysts for endogenous vitamin D production, 7-dehydrocholesterol in the epidermis absorbs UVB radiation during exposure, it is converted to previtamin D_3 which in turn isomerizes into vitamin D_3 (Cholecalciferol). Marine fish commodities which are a source of cholecalciferol in Indonesia are also very abundant, considering that Indonesia is a maritime country, Vitamin D supplements in the form of vitamin D_3/cholecalciferol are widely available on the market store.

MATERIALS AND METHODS

This research is an experimental laboratory and the design used in this study is a Completely Randomized Design (CRD). The number of treatment groups is five groups, and each group contains five white rats. This research was conducted for 52 days. This research was conducted at animal testing laboratory of Faculty of Veterinary Medicine Universitas Airlangga, Surabaya. The experimental animal for this study was male white rats (*Rattus norvegicus*) aged 8–12 weeks and weighing around 150–200 grams. There were as many as 25 samples with five repetitions each.

There are 5 treatment groups, i.e. K-, K+, P1, P2, and P3 groups. K- is a group that was only given food and drink without being exposed to cigarette smoke, K(+) is a group that was only exposed to 5 cigarette smoke per day without being given vitamin D_3 supplementation, and in the treatment group that was given 5 cigarettes per day, after that it is continued with the provision of vitamin D_3 supplementation with a dose of P1 = 0.00125 mg (50 IU)/day, P2 = 0.0025 mg (100 IU)/day, and P3 = 0.005 mg (200 IU)/day diluted into 0.2 ml of solvent/day orally.

The determination of the vitamin D_3 dose in this study stuck to the research of Rimbun *et al.* (2015) as a reference basis for similar research, the dose of vitamin D_3 (cholecalciferol) can be given orally at a dose of 6.25 µg/kg BW, 12.5 µg/kg BW, and 25 µg/kg BW in white rats (*Rattus norvegicus*).

Research procedure

The implementation of the study began with adaptation for 1 week. After that, the animals were grouped according to the treatment group and on the same day continued with exposure to cigarette smoke as much as 5 cigarettes to the K+, P1, P2, and P3 groups. Potrait of the smoking box can be seen in the picture below.

The rats were put into a smoking box and exposed to cigarette smoke connected by a hose; the mechanism of cigarette smoke exposure was done manually using a syringe. After the cigarette burned out, the rats were left in the smoking box for 5 minutes and when finished, the rats were removed from the smoking box. The smoking box used was 50x40x25 cm3 in size and had 10 vents. Cigarette smoke was exposed for 52 days, on the 53rd day the rats were euthanized using the cervical dislocation method. After being euthanized, the rats were dissected by making an incision on the thoracic wall, then the rat's lung organs were taken and put into an organ pot containing 10% formaldehyde solution for 6-24 hours to fix the tissue so that it remains preserved while increasing the affinity of protoplasm for the painting process. The lung organs will then be made into histopathology preparations for scoring pulmonary emphysema.

Sampling and analysis

Emphysema

The severity degree of pulmonary emphysema was measured by a semi-quantitative method with 4 scales, assessed through a lung specimen with five fields of view in each variable for one specimen. The pulmonary emphysema scoring scale was citing the emphysema scoring system by Mets *et al.* (2015).

Statistical analysis

The data result from the research were then analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test to determine the comparison of variance between treatment groups. The data were analyzed with JupyterLab software and Python programming language.

RESULTS AND DISCUSSION

The results of histopathology specimens that have been inspected under a microscope, also utilizing Optilab viewer 4.0, were then graded in different five fields of view in each variable. The mean score of emphysema demonstrates a very substantial variance (P<0.05) between K(+) and K(-) (Figure 1, Table 2). Comparison between the treatment groups provided vitamin D_3, P2 and P3 shows a significant variance (p<0.05), but between groups P1 and P2 there is no significant variance (p>0.05). This revealed that smoking has a major impact on the development of pulmonary emphysema. The mean pulmonary emphysema score in white rats (*Rattus norvegicus*) from the smallest to the largest in sequence is the K-, P3, P2, P1, and K+ groups. P1, P2, and P3 treatment groups got significant betterment in emphysema scores compared to the K+ group (Figure 2). Administration of vitamin D_3 may have an effect on preventing pulmonary emphysema.

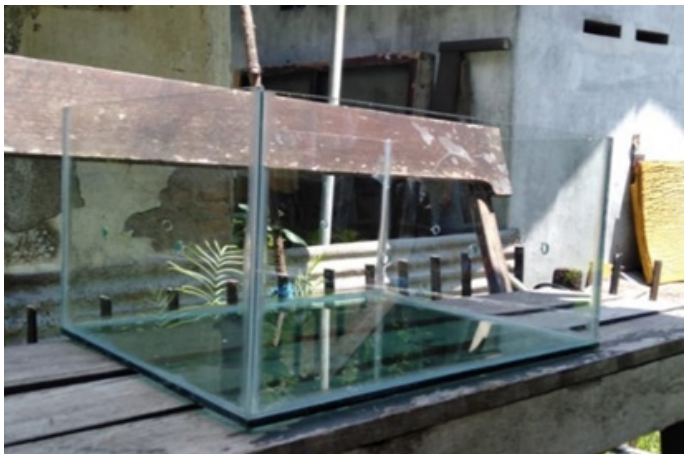

Figure 1: Smoking box design.

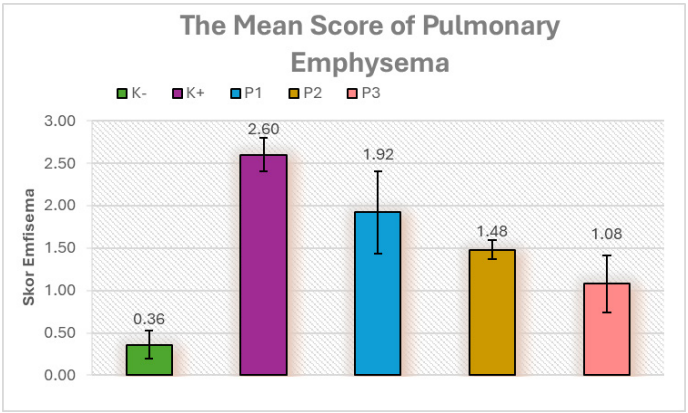

Figure 2: The mean score and standard deviation chart of pulmonary emphysema histopathology of white rats (*Rattus norvegicus*) exposed to cigarette smoke and administered vitamin D_3 in the control and treatment groups.

Table 1: Pulmonary Emphysema Scoring Index (Mets *et al.*, 2015).

Scoring	Observation
0	No emphysema
1	0-20% emphysema
2	20-50% emphysema
3	>50% emphysema

Table 2: The mean score and standard deviation of pulmonary emphysema histopathology of white rats (*Rattus norvegicus*) exposed to cigarette smoke and administered vitamin D_3 in the control and treatment groups.

Treatment groups	N	Emphysema score (Mean ± SD)
K-	5	0.36^{a} ±0.167
K+	5	2.60^{d} ± 0.200
P1	5	1.92^{c} ± 0.482
P2	5	1.48^{c} ± 0.110
P3	5	1.08^{b} ± 0.334

Detail: different superscript$^{(abcd)}$ in emphysema score column present significant variance ($p<0.05$).

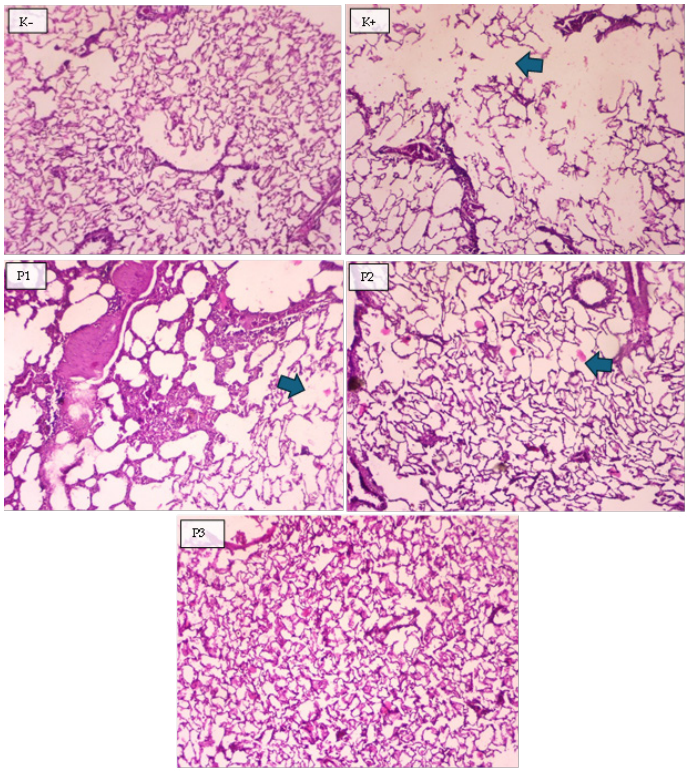

Figure 3: Histopathology of pulmonary emphysema in white rats (*Rattus norvegicus*) lung figures with 100x magnification with HE staining in each group of research samples. K-: emphysema score 0, K+: emphysema score 3, P1: emphysema score 3, P2: emphysema score 2, P3: emphysema score 0. Blue arrows indicate emphysema lesion.

The three main mechanisms that cause airway deterioration in the pathophysiology of COPD are oxidative stress, which is brought on by exposure to detrimental particles like cigarettes, chronic airway inflammation, and protease-antiprotease imbalance. Neutrophil Elastase (NE), a serine protease housed in azurophilic granules of neutrophils, actively contributes in airway remodeling and microbiocidal action. It hydrolyzes elastin, collagen, and other essential Extracellular Matrix Proteins (EMP) in the respiratory tissue. Furthermore, EMP degradation is accelerated by neutrophil elastase's activation of other key proteinases, including matrix metalloprotease (MMP)-2, MMP-9, Cathepsin B, Meprin alpha protease, and Calpain. Neutrophil elastase also activates macrophages, the major leukocyte responsible for lung parenchymal inflammation in COPD. However, neutrophil elastase level is strongly associated with the degree of airway inflammation and disease severity (Saputra *et al.*, 2023).

Cigarette smoke contains ROS and RNS which have a negative impact on antioxidant capacity, increasing oxidative stress, and also increasing inflammation, the lungs are very sensitive to damage caused by ROS. The pathogenesis process of emphysema involves oxidative stress, which is not only caused by an increase in the total of oxidants, but also by a decrease in antioxidant capacity.

Elevated endogenous synthesis of reactive oxygen species (ROS) can be induced by pro-inflammatory cytokines including leukotriene B4, interleukin (IL), and tumor necrosis factor alpha (TNF-α) (Lin and Thomas, 2010). Pro-inflammatory mediators such as IL-8 and leukotriene B4 in large amounts can trigger the recruitment and activation of neutrophils and alveolar macrophages to the lungs. Activation of neutrophils and alveolar macrophages produces free radicals, proinflammatory cytokines, and proteases such as matrix metalloproteinases (MMPs), and neutrophil elastase that can cause mucus hypersecretion, decreased lung elasticity, these enzymes also the main cause of extracellular matrix remodeling and emphysema (Atkinson *et al.*, 2011).

The mechanism by which vitamin D_3 addresses antioxidant insufficiency is through: (1) Enhance the activation of the transcription factor Nuclear factor E2-related factor 2 (Nrf2). (2) Prevent the production of advanced glycation end products (AGEs) (Kheirouri and Alizadeh, 2020). (3) Stimulate the upregulaton of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) enzyme expression, also suppress NADPH oxidase (NOX), and increase glutathione (GSH) synthetization. (4) Induce the expression and activity of gamma-glutamyl transpeptidase (GGT), which plays an important role in glutathione metabolism and serves the homeostatic function of lung epithelial cells (Moghaddam *et al.*, 2023). (5) Prevent the activation of NF-κB because VDR adheres with IκB kinase β (IKKβ) to hinder NF-κB activation since IKKβ is a NF-κB inhibitor (Chen *et al.*, 2013).

Vitamin D_3 in preventing cigarette smoke induced pulmonary emphysema through its ability to modulate the immune system's response to by: (1) Suppressing the production of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12 and promoting anti-inflammatory IL-10. (2) Down-regulating pro-inflammatory Th1/Th17 cells which have the effect of suppressing the production of

IFN-γ and IL-17. (3) Promoting Th2/Treg which plays a role in weakening excessive immunological responses by producing anti-inflammatory cytokines such as TGF-β1 and IL-10, also by expressing cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (Tang and Bluestone, 2008). (4) Induces modulation of tolerogenic dendritic cells (tolDCs) such that it can promote immunological tolerance dampen excessive inflammatory responses (Nikolic and Roep, 2013). Linear with this, the tolerogenic effect of vitamin D_3 on dendritic cells has been associated with its ability to inhibit the activation of the NF-κB pathway (Malaguarnera *et al*., 2017).

The action of vitamin D_3 in preventing emphysema more far is via limiting extracellular remodeling by MMP through increasing tissue inhibitor of metalloproteinases (TIMP), specifically TIMP-1, and TIMP-2 (Halder *et al*., 2013) as TIMP is an inhibitor of MMP. MMP expression can be decreased since its activation is suppressed by increasing TIMP concentrations (López-López *et al*., 2014). Increased production of the alpha-1-antitrypsin enzyme AAT will also occur along with increased vitamin D_3 administration. Alpha-1-antitrypsin itself is a serine protease inhibitor (serpin) which plays a major role in lowering neutrophil elastase activity, where AAT will provide lung protection from severe damage (Sapey, 2020). To overcome the protease-antiprotease imbalance through alternative pathway, vitamin D_3 actively reduces inflammation due to neutrophil and alveolar macrophage infiltration. Vitamin D_3 can lower neutrophil elastase activity by facilitating the binding of vitamin D-binding protein (VDB) to the actin on neutrophil plasma membrane (Kew, 2019).

CONCLUSION

Based on the results of the research that has been conducted, it can be concluded that vitamin D_3 (cholecalciferol) supplementation can prevent and/or reduce the severity of pulmonary emphysema in rats (*Rattus norvegicus*) exposed to cigarette smoke.

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NOVELTY STATEMENT

This study demonstrates a novel protective role of Vitamin D_3 in preventing cigarette smoke–induced pulmonary emphysema, evidenced through histopathological assessment in a controlled animal model. By evaluating graded doses of Vitamin D_3, the research provides new insight into its potential antioxidant and anti-inflammatory benefits against lung tissue damage. Additionally, the study reinforces the suitability of white rats (Rattus norvegicus) as a reliable model for investigating emphysema prevention strategies.

AUTHOR'S CONTRIBUTION

RAA: Original draft preparation, experiment and laboratory analysis execution, statistical analysis. A.M and JR: Supervised the research. WMY, RD, and HP: Methodology and validation. BS and NT: Observation the histopathological of lungs. KPS and RMW: Data interpretation. AB: Review and editing the manuscript. LRY: Validation, writing, review, and editing the manuscript.

Ethical statement

This research has accepted an ethical certificate from the Ethics Commission of the Faculty of Veterinary Medicine, Airlangga University (No: 1. KE.140.12.2021).

Generative AI and AI-assisted technology statement

Generative AI tools were used solely for language editing and grammar improvement. The authors reviewed, verified, and approved all content. No AI tools were used for data analysis, interpretation, or creation of scientific content.

Conflict of interest

The authors have declared no conflict of interest.

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