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Effect of Astaxanthin on High Mobility Group Box-1 (HMGB-1) Expression, Histopathological Differences, and Arterial Oxygen Partial Pressure in Lung Contusion Model

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ABSTRACT:

Background: Lung contusion is a frequent outcome of chest trauma and contributes substantially to morbidity and mortality, primarily through the development of acute respiratory distress syndrome (ARDS) and related inflammatory processes. High Mobility Group Box-1 (HMGB-1) is recognized as an essential mediator involved in the late phase of inflammation during lung injury. Astaxanthin, a potent antioxidant and anti-inflammatory carotenoid, has shown therapeutic potential in various inflammatory conditions. This study aimed to investigate the effects of astaxanthin administration on HMGB-1 expression, histopathological changes, and arterial partial pressure of oxygen (PaO₂) in an experimental rat model of lung contusion.

Methods: A controlled experimental study was conducted on adult female Sprague-Dawley rats. Lung contusion was induced using a standardized weight-drop method. The rats were randomly divided into groups: a normal group (no contusion, no treatment), a control group (contusion without treatment), and treatment groups receiving fixed doses of astaxanthin after contusion. HMGB-1 mRNA expression in lung tissue was measured using real-time PCR. Lung tissues were examined histopathologically to assess alveolar

edema and leukocyte infiltration using a semi-quantitative scoring system. Arterial blood gas analysis was performed to evaluate PaO₂ levels at specific time points after injury.

Results: Astaxanthin treatment significantly reduced HMGB-1 mRNA expression in lung tissue compared to the untreated contusion group ($p < 0.05$). Histopathological evaluation showed a notable decrease in alveolar edema and leukocyte infiltration in groups receiving astaxanthin ($p < 0.05$). In addition, PaO₂ levels improved significantly with astaxanthin treatment, indicating enhanced oxygenation ($p < 0.05$). A dose-dependent response was observed, with higher doses providing greater protective effects in several parameters.

Conclusion: Astaxanthin administration demonstrates significant therapeutic benefits in an experimental model of lung contusion by downregulating HMGB-1 expression, mitigating lung histopathological damage and improving arterial oxygenation. These findings suggest that astaxanthin holds promise as a potential adjunctive therapy for lung contusion, warranting further investigation in clinical settings.

1. INTRODUCTION

Lung contusion, a direct injury to the lung parenchyma without laceration, represents a frequent and potentially life-threatening complication of blunt thoracic trauma.^{1,2} This condition is marked by alveolar hemorrhage and edema, which compromise gas exchange, decrease lung compliance, and elevate the likelihood of severe respiratory complications, including pneumonia and acute respiratory distress syndrome (ARDS).^{3,4} In cases of blunt thoracic trauma, lung contusion occurs in approximately 25% to 75% of patients and is strongly linked to considerable morbidity and mortality, especially when the injury is bilateral or widespread.^{5,6} Despite advances in trauma care, the management of lung contusion remains largely supportive, highlighting the need for novel therapeutic strategies targeting the underlying pathophysiological mechanisms.

The pathophysiology of lung contusion involves a complex cascade of events initiated by the initial mechanical injury. Among the array of inflammatory mediators, High Mobility Group Box-1 (HMGB-1) has emerged as a crucial late-phase cytokine that perpetuates and amplifies the inflammatory cascade.⁹ HMGB-1 is a widely distributed nuclear protein that, once released from necrotic or activated immune cells into the extracellular environment, serves as a strong damage-associated molecular pattern (DAMP).¹⁰ Increased concentrations of HMGB-1 are implicated in the development of several inflammatory lung disorders, such as ARDS and ventilator-induced lung injury, with its expression closely linked to the extent of lung injury.^{13,14}

As a xanthophyll carotenoid mainly derived from marine species, astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4'-dione) has been widely recognized for its remarkable antioxidant and anti-inflammatory effects.¹⁵ Beyond its antioxidative role, astaxanthin mitigates inflammation through inhibition of NF- κ B activation, reduction of pro-inflammatory cytokine levels, and suppression of adhesion molecule expression.^{17,18} Evidence from several preclinical models highlights the organ-protective role of astaxanthin in cardiac, renal, and neurological injury, an effect mainly linked to its anti-inflammatory mechanisms and antioxidative.^{19,20} The present investigation was designed to explore the therapeutic role of astaxanthin in lung contusion, focusing on HMGB-1 expression, key histological changes including alveolar edema and leukocyte infiltration, and arterial oxygenation (PaO₂) using a standardized rat model.

2. METHODS

Study Design

The study was carried out using a post-test only control group experimental design with Sprague-Dawley rats as the animal model. A total of 27 rats were randomly assigned into three experimental groups ($n = 9$ per group); (1) **Normal group (N)**: Rats without any intervention, serving as the physiological control, (2) **Contusion group (K-)**: Rats subjected to lung contusion induction without astaxanthin administration, (3) **Treatment group (K+)**: Rats subjected to lung contusion induction followed by astaxanthin administration (100 mg/kg body weight, administered starting immediately after contusion and continued once daily for 48 hours).

Ethical Approval

All animal experiments were performed in full compliance with established ethical standards for animal research and were approved in advance by the Institutional Animal Ethics Committee of the Faculty of Medicine, Hasanuddin University (Makassar, Indonesia).

Experimental Animals

Female Sprague-Dawley rats were used in this study. The inclusion criteria were: 4–6 months of age, weighing 180–265 g, and in a healthy condition. The exclusion criteria included: damaged biopsy samples, presence of wound infection, or death of the animal before the completion of the study. All animals were obtained from a certified local breeder and housed in standard polypropylene cages under controlled environmental conditions (temperature $22 \pm 2^{\circ}\text{C}$, relative humidity $55 \pm 5\%$, and a 12-hour light/dark cycle). Rats were provided with standard pellet chow and water ad libitum and allowed to acclimatize to the laboratory environment for at least one week prior to the initiation of the experimental procedures.

Experimental Protocol

Pulmonary contusion in this study refers to an injury model in rats induced by high-impact energy using an experimental device, in which a 200 g metal cylinder was dropped from a height of 100 cm onto the thoracic area of the rat, delivering a force of 1.96 J. Prior to this procedure, the rats were anesthetized intraperitoneally with ketamine HCl 50 mg/kgBW and xylazine 10 mg/kgBW. During anesthesia, the rats remained asleep, allowing spontaneous respiration to continue.

Outcome Measurement

At 48 hours post-contusion, animals were euthanized with an overdose of anesthetic, and blood samples were obtained via cardiac puncture for arterial blood gas analysis. Arterial blood was obtained in heparinized syringes and directly examined for oxygen tension (PaO_2) employing a calibrated pHox Ultra blood gas analyzer (Nova Biomedical). After euthanasia, the lungs were excised; the right lung was preserved in 10% neutral buffered formalin for 24 hours, processed with graded alcohols and xylene, embedded in paraffin, and cut into 5 μm sections for histopathological evaluation using Hematoxylin and Eosin staining. Histopathological changes, including alveolar edema, hemorrhage, inflammatory cell infiltration (particularly neutrophils and macrophages), and alveolar septal thickening, were evaluated using a semi-quantitative scoring system by a pathologist blinded to group allocation. In addition, a portion of lung tissue was snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis of HMGB-1 mRNA expression. Total RNA was extracted from the frozen lung tissue samples using a commercial RNA extraction kit (Quick-RNATM MiniPrep Plus, Zymo Research) according to the manufacturer's instructions. RNA concentration and purity were assessed with a spectrophotometer, and first-strand complementary DNA (cDNA) was generated from total RNA using a reverse transcription kit (ReverTra Ace® qPCR RT Master Mix, Toyobo). RT-qPCR was performed to quantify the mRNA expression levels of HMGB-1. The specific primer sequences for rat HMGB-1 and a reference housekeeping gene (β -actin) were designed or obtained from published literature. RT-qPCR reactions were carried out using a SYBR Green-based detection method on a real-time PCR system (e.g., Applied Biosystems 7500 Real-Time PCR System). Relative HMGB-1 mRNA expression was calculated with the housekeeping gene used for normalization. Results were expressed as fold change relative to the normal group.

Statistical Analysis

Quantitative variables are presented as mean $\pm$ standard error of the mean (SEM) or as median with interquartile range, depending on data distribution. Statistical analyses were carried out using GraphPad Prism software (version 10, GraphPad Software, San Diego, CA, USA). Data normality was evaluated with the Shapiro–Wilk or Kolmogorov–Smirnov test. For normally distributed datasets, comparisons among groups were made using one-way ANOVA, followed by Tukey's post hoc test. For non-normally distributed data, the Kruskal–Wallis test with Dunn's post hoc analysis was applied. A p -value < 0.05 was considered statistically significant.

3. RESULTS

Effect of Astaxanthin on HMGB-1 mRNA Expression in Lung Tissue

Lung contusion induction in the K group resulted in a significant upregulation of HMGB-1 mRNA expression in lung tissue compared to the N group ($p < 0.001$) (Figure 1). Administration of astaxanthin (K group+ASTA) significantly attenuated this

increase in HMGB-1 mRNA expression. Specifically, the K +ASTA group showed a more pronounced reduction in HMGB-1 levels ($p < 0.01$ vs K), bringing the expression closer to that of the N group, although still significantly higher ($p < 0.05$ vs N).

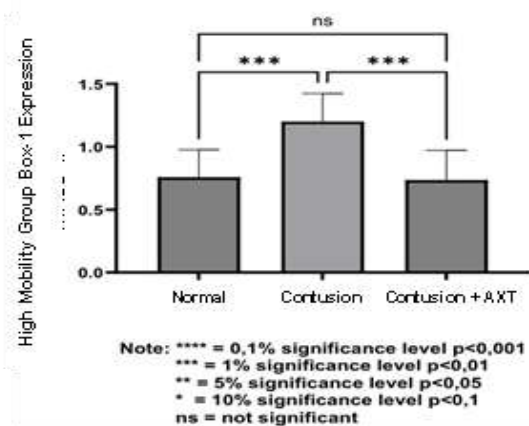


Figure 1. Post Hoc Result Tukey's Ekspresi High Mobility Group Box-1

Effect of Astaxanthin on Lung Histopathology

Histopathological examination of lung tissue from the K group revealed significant pathological changes characteristic of lung contusion, including widespread alveolar edema, hemorrhage, and marked infiltration of inflammatory cells, primarily neutrophils, within the alveolar spaces and interstitium. The mean histopathological scores for alveolar edema and leukocyte infiltration were significantly higher in the K group compared to the N group ($p < 0.001$ for both parameters). Treatment with astaxanthin ameliorated these histopathological changes in a dose-dependent manner. K+ASTA groups exhibited significantly reduced scores for alveolar edema ($p < 0.05$) and leukocyte infiltration ($p < 0.05$).

Effect of Astaxanthin on Arterial Partial Pressure of Oxygen (PaO₂)

Lung contusion in the K group led to a significant decrease in arterial PaO₂ levels at [Time point, e.g., 24 hours] post-injury, indicative of impaired gas exchange ($p < 0.001$ vs N group). Astaxanthin treatment significantly improved PaO₂ levels. Compared with the K group, PaO₂ levels were markedly higher in the K+ASTA group ($p < 0.01$), and these levels did not differ significantly from those observed in the N group ($p > 0.05$). The K+ASTA group also demonstrated a significant improvement in PaO₂ compared to the K group ($p < 0.05$), though the effect was less pronounced than that observed with the high dose.

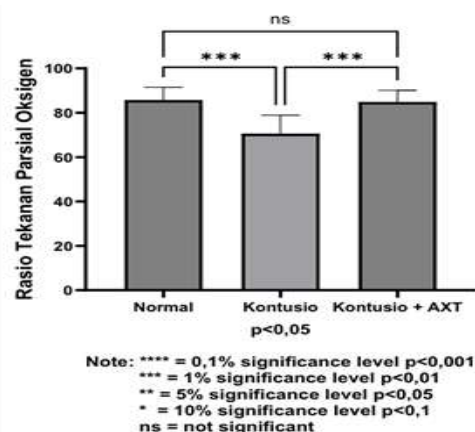


Figure 2. Post hoc Result Tukey's Partial Pressure of Oxygen

4. DISCUSSION

The present study demonstrates the significant therapeutic potential of astaxanthin (AXT) in mitigating inflammatory responses and tissue damage in pulmonary contusion models through multiple molecular pathways. Our findings reveal that AXT administration effectively reduces High Mobility Group Box-1 (HMGB-1) expression and alveolar edema, two critical pathological manifestations of pulmonary contusion. Our results demonstrate that AXT administration significantly affects HMGB-1 expression in pulmonary contusion models. The AXT-treated group exhibited significant differences compared to the contusion-only group, as evidenced by Tukey's test results. Pulmonary contusion itself induces elevated HMGB-1 expression, indicating heightened inflammatory response and cellular damage. This finding aligns with previous research⁵, which reported increased HMGB-1 expression in rats with acute lung injury, peaking at 24 hours post-injury. While HMGB-1 expression typically begins to decline after 24 hours post-injury and persists up to 72 hours, remaining above control levels, our study found that AXT administration resulted in decreased HMGB-1 expression at 48 hours post-injury, with values approximating those of the control group. This suggests AXT's potential to alleviate inflammation and repair lung tissue damage caused by contusion injury, consistent with its anti-inflammatory properties that reduce oxidative stress and inflammation while accelerating tissue healing processes. This therapeutic approach aligns with other studies,⁸ aimed at inhibiting pro-inflammatory responses, although the HMGB-1 pathway represents just one of several pro-inflammatory pathways.

Previous research found that HMGB-1 levels increase significantly in plasma and lungs in rat models with injury, highlighting HMGB-1's role in lung injury mechanisms.¹⁶ This research supports HMGB-1's involvement in inflammation and tissue injury processes, which AXT can effectively inhibit. Our findings are further supported by multiple molecular mechanisms through which AXT modulates HMGB-1 expression. The influence of AXT on HMGB-1 is largely regulated through the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway.⁶ In their dry eye disease model, AXT increased phospho-Akt (p-Akt) expression while significantly reducing HMGB-1 expression. When PI3K inhibitor (LY294002) was administered before AXT treatment, it eliminated AXT's effect in reducing HMGB-1 expression, indicating the PI3K/Akt pathway's crucial role in AXT's mechanism for reducing HMGB-1 expression.

The PI3K/Akt pathway, when stimulated by AXT, can downregulate HMGB-1 expression via several regulatory mechanisms: (1) Akt activation can inhibit NF- κ B activation, a transcription factor regulating HMGB-1 expression; (2) Akt can phosphorylate and inactivate forkhead box O (FOXO) transcription factors involved in HMGB-1 expression regulation; and (3) Akt activation can increase anti-apoptotic protein expression such as Bcl-2 and Bcl-xL, reducing HMGB-1 release from stressed cells. Inflammatory processes are strongly influenced by the HMGB-1/TLR4/NF- κ B signaling pathway, which serves as a major regulatory mechanism. After release into extracellular space, HMGB-1 interacts with TLR4, activating NF- κ B and leading to pro-inflammatory cytokine production. Our research aligns with previous findings showing that AXT suppresses the HMGB-1/TLR4/NF- κ B signaling pathway in rat spinal cord injury models.¹ In their study, intrathecal AXT administration after spinal cord injury significantly reduced HMGB-1, TLR4, and NF- κ B expression.¹ This pathway suppression was associated with decreased spinal cord edema, reduced astrocyte activation, and improved motor function in AXT-treated rats. AXT can suppress the HMGB-1/TLR4/NF- κ B pathway through multiple mechanisms: (1) reducing HMGB-1 release from damaged or activated cells; (2) inhibiting HMGB-1 interaction with TLR4, possibly through conformational modification of HMGB-1 or TLR4; and (3) inhibiting TLR4-induced NF- κ B activation, possibly through inhibition of NF- κ B inhibitor (I κ B) phosphorylation and degradation.

Pulmonary contusion effects include oxidative stress and inflammatory reactions occurring both locally and systemically. This oxidative stress plays a crucial role in HMGB-1 release and activation. Reactive oxygen species (ROS) can induce HMGB-1 translocation from the nucleus to cytoplasm and subsequently to extracellular space. Additionally, ROS can post-translationally modify HMGB-1, altering its biological activity. AXT is a potent antioxidant capable of scavenging various ROS types, including superoxide radicals, singlet oxygen and hydroxyl radicals. Our research is strengthened by previous findings demonstrating that AXT modulates neuroinflammation by reducing oxidative stress, partly through activation of nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor, which regulates antioxidant enzyme expression.⁷ By reducing oxidative stress, AXT can inhibit HMGB-1 release and activation through several mechanisms: (1) decreased ROS can reduce HMGB-1 translocation from nucleus to cytoplasm; (2) decreased ROS can reduce HMGB-1 oxidative modifications required for its pro-inflammatory activity; and (3) Nrf2 activation by AXT can induce heme oxygenase-1 (HO-1) expression, which has been shown to inhibit HMGB-1 release.

Alveolar edema represents a primary manifestation of severe lung injury. Based on Kruskal-Wallis and Dunn's Test analyses, AXT administration demonstrated significant influence in reducing alveolar edema resulting from pulmonary contusion. The AXT-treated group experienced significant alveolar edema reduction compared to the contusion-only group. This confirms AXT's ability to reduce capillary permeability and inhibit fluid accumulation in alveolar spaces, crucial for reducing lung tissue damage and accelerating pulmonary function recovery after injury. These results are supported by findings that lung injury often involves disruption of the oxidation and anti-oxidation balance, potentially causing uncontrolled inflammation and edema.¹⁷ Under these conditions, HMGB-1 exacerbates alveolar edema by stimulating pro-inflammatory cytokine secretion.

AXT functions as a potent antioxidant that can reduce inflammation and oxidative stress, primary factors in alveolar edema formation. The HMGB-1 expression reduction caused by AXT can decrease inflammatory cell and cytokine infiltration, reducing alveolar edema. Another study, mentioned that alveolar edema accompanied by lung tissue apoptosis facilitates sepsis and acute lung injury development.⁸ Our results are consistent with findings from studies using lipopolysaccharide (LPS)-induced acute lung injury models in rats.¹⁸ Administration of AXT led to a significant decrease in respiratory resistance (Re), inspiratory resistance (Ri), dynamic lung compliance (Cdyn), wet-to-dry lung weight ratio (W/D), bronchoalveolar-to-serum albumin ratio (BA/SA), and myeloperoxidase (MPO) activity relative to the LPS group. The numbers of total cells, neutrophils, and macrophages were notably decreased. Furthermore, histopathological analysis showed less alveolar interstitial edema, reduced hemorrhage, and decreased erythrocyte extravasation following AXT treatment. Compared to the LPS group, Th17 cell percentage and interleukin (IL)-17 and tumor necrosis factor- α (TNF- α) content in the ASTA pretreatment group decreased significantly, while transforming growth factor (TGF)- β content and Treg cell percentage increased significantly. Western blot results demonstrated that AXT increased the expression of suppressor of cytokine signaling-3 (SOCS3) while reducing the levels of phosphorylated Janus kinase 2 (p-JAK2) and phosphorylated STAT3 (p-STAT3) in lung tissue.

In alternative models of LPS-induced acute lung injury, AXT has been shown to attenuate lung injury by modulating oxidative stress, inflammatory pathways, the P2X7 receptor, NF- κ B, Bcl-2, and Caspase-3. Sepsis was verified by elevated serum levels of IL-1 β , IL-6, IL-10, and TNF- α . Lung injury was assessed through histopathological alterations. In septic lung tissue, expression of P2X7R, malondialdehyde (MDA), IL-1 β , TNF- α , NF- κ B, and Caspase-3 was increased, whereas Bcl-2 and glutathione (GSH) levels were reduced. Treatment with AXT improved MDA, GSH, IL-1 β , TNF- α , P2X7R, NF- κ B, Caspase-3, and Bcl-2 profiles while decreasing P2X7R immunoreactivity and ameliorating histological abnormalities in the lungs.¹⁹ A study using a lung injury model induced by lower extremity ischemia-reperfusion revealed marked congestion in the pulmonary veins and alveolar septa, along with partial accumulation of alveolar macrophages and erythrocytes.² In addition, edema was noted in certain bronchiolar and alveolar lumens in the second and fourth groups compared to the controls. The second group exhibited a significantly higher damage score (3.41) than the controls ($p < 0.001$), whereas the fourth group's score (0.92) was not different from the controls but showed a significant reduction compared with the I/R group ($p < 0.001$). As a result, AXT's protective effect was demonstrated through biochemical, histopathological, and immunohistochemical effects.

Research has shown that AXT can prevent lung injury due to hypoxia and inflammation.³ In LPS-induced acute lung injury models, AXT showed protective effects against disease progression with the ability to inhibit oxidative/nitrative stress and reduce pulmonary edema, inflammatory cell infiltration, and ferroptosis. AXT demonstrates protective effects against alveolar edema and lung injury through several molecular signaling pathways. One primary mechanism is through SOCS3/JAK2/STAT3 signaling pathway activation, which promotes Treg cell differentiation and reduces inflammatory reactions and Th17 cell differentiation. This pathway plays an important role in immune response and inflammation regulation in the lungs.¹⁸ Additionally, AXT also regulates P2X7 receptor (P2X7R), NF- κ B, Bcl-2, and Caspase-3 expression in lung tissue. P2X7R is a purinergic receptor whose activation is known as a strong response in inflammation modulation. Adenosine 3'-triphosphate (ATP) enters extracellular fluid when cell integrity is disrupted, causing ion channel activation through purinergic receptor (P2X) signaling. The purinergic signaling cascade may be one important component for pathogen response. One of the most important oxidant producers during sepsis is phagocyte reactions in inflamed lung tissue that reduce respiratory capacity, where ATP leaking into extracellular space exacerbates reactive oxygen species (ROS) generation and mitochondrial damage.¹⁹

AXT also affects PI3K/AKT, Nrf2, NF- κ B, ERK1/2, JNK, p38 MAPK, and JAK-2/STAT-3 signaling pathways. These pathways are involved in inflammatory response and oxidative stress regulation in various tissues, including lungs. In acute lung injury models, AXT has been proven to inhibit NF- κ B pathway activation, a major inflammatory response regulator.⁴ AXT

also increases heme oxygenase-1 (HO-1) expression through Nrf2 transcription factor activation, a major cellular antioxidant response regulator. HO-1 has strong anti-inflammatory and antioxidant properties and has been proven to protect against various lung injuries, including LPS-induced acute lung injury.⁴ Furthermore, AXT also inhibits ferroptosis, an iron-dependent cell death form characterized by lipid peroxide accumulation. Ferroptosis has been proven to play a role in the pathogenesis of various lung diseases, including acute lung injury. By inhibiting ferroptosis, AXT can reduce cell damage and inflammation in the lungs, which in turn can reduce alveolar edema.¹⁸ From a microbiological perspective, AXT has significant effects on cellular immune responses involved in alveolar edema pathogenesis in pulmonary contusion. AXT has been proven to influence differentiation and function of various immune cells, including T cells, macrophages, and neutrophils, which play important roles in pulmonary inflammatory response.⁴

5. CONCLUSION

This study demonstrates the therapeutic potential of astaxanthin (AXT) in mitigating pulmonary contusion-induced inflammatory responses and improving respiratory function. The research findings reveal that AXT effectively normalizes HMGB-1 expression in contused lung tissue to levels comparable with normal conditions, indicating its anti-inflammatory properties. While AXT administration showed moderate effects in reducing alveolar edema and leukocyte infiltration in histopathological assessments, its most notable impact was observed in the restoration of partial oxygen pressure ratios, effectively returning pulmonary oxygenation function to normal levels following contusion injury. These results collectively suggest that AXT exerts protective effects against pulmonary contusion through multiple mechanisms, particularly through modulation of inflammatory mediators and enhancement of respiratory function, establishing a foundation for further investigation of AXT as a potential therapeutic agent for pulmonary contusion and possibly other acute inflammatory lung conditions.

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AUTHOR CONTRIBUTIONS

JR: Conceptualization, Investigation.

JR, WS, ID, AHA, MA, PRI: Methodology, Laboratory Analysis.

JR, WS, ID, AHA, MA, PRI: Data Curation, Formal Analysis.

JR, WS, ID, AHA, MA, PRI, KPY, ST, UU, AA, MAP, HN, SA, HC, HD: Supervision, Writing—Review & Editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

All animal experiments were performed in full compliance with established ethical standards for animal research and were approved in advance by the Institutional Animal Ethics Committee of the Faculty of Medicine, Hasanuddin University (Makassar, Indonesia).

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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