Exosomes derived from human umbilical cord mesenchymal stem cells attenuate kidney inflammation in a 5/6 subtotal nephrectomy rat model

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ABSTRACT

Introduction: Chronic kidney disease (CKD) is a non-communicable disease that contributes to the rise of global mortality rate. The condition is marked by reduced kidney function persisting for three months or longer. The main cause of CKD is kidney fibrosis, resulting from chronic inflammation. Current CKD therapies, such as hemodialysis and kidney transplants, are limited in effectiveness. Stem cell-derived therapy, particularly mesenchymal stem cells (MSC), provide great potential for reducing inflammation and fibrosis. Notably, exosomes secreted by MSC offer a safer and more effective alternative by carrying bioactive molecules that can repair kidney function through modulating inflammatory processes.

Materials and Methods: Twenty-five male 3-month-old Wistar rat were divided into five groups: Sham operation (SO, n=5), 5/6 subtotal nephrectomy (SN, n=5), SN with exosome treatment at total protein concentration of 48.30 µg (SNE1, n=5), 96.61 µg (SNE2, n=5), and 193.21 µg (SNE3, n=5). The rat was euthanized, and the kidneys were harvested for analysis. The mRNA expression levels of NF-κB and MCP-1 were measured using RT-PCR. Macrophage infiltration was assessed using immunohistochemistry (IHC) staining with anti-CD68 antibodies.

Results: The mRNA expression of NF-κB was significantly higher in the SN group compared to the SO group. In the exosome groups (SNE1, SNE2, and SNE3), NF-κB expression was significantly lower than in the SN group (p = 0.011, 0.029, 0.026, respectively). The mRNA expression of MCP-1 in the exosome groups was not significantly different from the SN group. IHC staining showed the SN group had a more dominant macrophage infiltration compared to the SO group. The exosome group exhibited a less dominant macrophage infiltration compared to the SN group.

Conclusion: Exosomes may attenuate kidney inflammation by inhibiting inflammatory gene expression and macrophage infiltration in a 5/6 subtotal nephrectomy rat model.

KEYWORDS:
Exosome, HUC-MSC, NF-κB, MCP-1, macrophage

INTRODUCTION

Chronic kidney disease (CKD) is a non-communicable disease that contributes to the rise of global mortality rate. The condition is characterized by reduced kidney function persisting for three months or longer. CKD affects 1 in 10 people worldwide.[1,2] In Indonesia, the prevalence of CKD increased from 0.2% in 2013 to 0.38% in 2018.[3] The primary cause of CKD is kidney fibrosis, where healthy kidney tissues are replaced by extracellular matrices components, leading to diminished kidney function. The fibrosis is primarily induced by chronic inflammation.[4,5]

Inflammation is a key factor that drives the development of kidney fibrosis, making its reduction a crucial therapeutic intervention.[4] Inflammation is mediated by damage-associated molecular patterns (DAMPs), which activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) through toll-like receptor 4 (TLR4).[6] NF-κB acts as a transcription factor that regulates the production of cytokines and chemokines, including monocyte chemoattractant protein-1 (MCP-1).[7,8] MCP-1 is a chemokine that attracts macrophages to the injury site, where they release pro-inflammatory and pro-fibrotic cytokines. Persistent inflammation ultimately causes progressive damage to the glomeruli and tubules of the kidneys.[8,9]

Current treatments for CKD are limited to hemodialysis and kidney transplants.[10,11] Stem cell-based therapies provide a

This article was accepted: 25 September 2025
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promising alternative due to their potential to mitigate inflammation and fibrosis.[12] However stem cell therapies also face challenges, some types of stem cells, such as induced pluripotent stem cells (iPSCs), carry a risk of tumorigenesis.[13] Previous studies have reported that mesenchymal stem cells derived from human umbilical cord (HUC-MSCs) may reduce the risk of tumorigenesis.[14] Recently, attention has shifted toward MSC-derived bioactive compounds, particularly exosomes, which are considered a safer and more stable therapeutic option with a lower risk of tumorigenesis.[15,16]

Exosomes derived from MSC offer a novel therapeutic approach with a Reno-protective effect and enhanced stability.[17] These exosomes carry bioactive molecules that are capable of repairing kidney function more effectively than MSC alone.[18,19] Previous studies have demonstrated that MSC exosomes reduce inflammation in various doses, presenting a safer and better alternative.[20,21] Specifically, exosomes containing micro RNAs, miR-26a and miR-146a-5p have been shown to downregulate inflammation by modulating NF-κB.[22-24] However, research on the effect of human umbilical cord mesenchymal stem cells (HUC-MSC) exosomes in CKD remains limited. Therefore, further investigation is needed to elucidate their impact on inflammation and kidney function in animal models. The 5/6 subtotal nephrectomy model serves as a representative CKD model due to its similarity to human kidney injury.[25]

MATERIALS AND METHODS

Animal subjects

The subjects were 25 male 3-month-old Wistar rats weighing around 150-300 grams. The rats were acclimated for 7 days before treatment. They were divided into five groups of five rats: sham operation group + exosome solvent (SO), 5/6 subtotal nephrectomy group + exosome solvent (SN), 5/6 subtotal nephrectomy group + exosome with 48.30 µg total protein (SNE1), 5/6 subtotal nephrectomy group + exosome with 96.61 µg total protein (SNE2), and 5/6 subtotal nephrectomy group + exosome with 193.21 µg total protein (SNE3).

During the experiment, the rats were kept at room temperature, 60% humidity, and 12 hours of dark-light cycle. They were given ad libitum food and drink. All procedures were conducted following the ethical clearance from The Medical and Health Research Ethics Committee (MHREC) of FK-KMK UGM number KE/FK/1964/EC/2023. The study was carried out from October 2023 to January 2024 in the Anatomy Laboratory of FK-KMK UGM, the Anatomical Pathology Laboratory of FK-KMK UGM, and the Integrated Research Laboratory of FK-KMK UGM.

Preparation of 5/6 Subtotal Nephrectomy Model

The 5/6 subtotal nephrectomy model involved two main procedures: unilateral nephrectomy and subtotal nephrectomy. The unilateral nephrectomy was performed on the right kidney, followed by the removal of the superior and inferior poles of the left kidney after seven days. rats were anesthetized with ketamine (100 mg/kg body weight, intraperitoneal) and laid on their stomach. Their lumbar hairs were shaved. An incision of 1.5 cm width was performed on the right lumbar region, followed by ligation on the renal pedicle using a 3/0 silk suture. Afterward, the right kidney was cut, and the peritoneum and skin were sutured. After 7 days, the left kidney was taken by incising the left lumbar region, followed by making cuts on the inferior and superior poles. Bleeding was stopped with a microcauter, and the peritoneum and skin were sutured.

Administration of Exosome

Exosomes were derived from human umbilical cord mesenchymal stem cells (HUC-MSC) and provided by PT. Kalbe Farma Ltd®. Exosome was dissolved with an exosome solvent and given intravenously with three variations on total protein: 48.30 µg, 96.61 µg, and 193.21 µg. Exosomes were injected twice a week for 28 days (in week 2nd, 3rd, 4th, and 5th) after 5/6 subtotal nephrectomy procedure. There were no exosome injections on week 6th in all intervention groups.

Termination and measurement of urine/serum creatinine ratio

Prior to termination, rats were placed in metabolic cages for 24-hour urine collection to measure creatinine levels. Urine samples were collected at three time points: prior to the 5/6 subtotal nephrectomy procedure, at week 2 as a mid-treatment assessment, and prior to termination. Serum samples were collected before the 5/6 nephrectomy and again prior to termination to enable calculation of the urine/serum creatinine ratio.

The rats were anesthetized with 100 mg/kg body weight of ketamine intraperitoneally. Afterward, an incision was made from the abdominal region to the thorax. NaCl 0.9% perfusion was carried out in the left ventricle while cutting the right auricular to observe perfusion flow. The kidney was taken and divided into two parts: one part was stored in a formalin fixative solution for making paraffin blocks, while the other part was stored in an RNA stabilization solution at -20°C for RNA extraction. After taking the kidneys, the carcasses were incinerated in an incinerator. Histopathological examination was performed using periodic acid–schiff (PAS) staining to visualize glomerular and tubular structural changes, thereby confirming the successful establishment of the CKD model. Representative histological images were selected from each group based on consistent and characteristic morphological features observed across all subjects.

RT-PCR and Electrophoresis

RNA was extracted using RNA isoplus (GENEzol®, Cat. No. GZR100) and quantified using nanodrop. Complementary DNA was made by mixing 3000 ng with nuclease-free water to reach a volume of 12 µL, then added to 8 µL of PCR mixture (5x RT buffer 4 µL, random primer 1 µL, dNTP 2 µL, Reverse transcriptase enzyme 1 µL). The mixture was incubated in the PCR machine (30°C for 10 minutes, 42°C for 60 minutes, and 99°C for 5 minutes). Three microliters of cDNA were inserted into a 0.2 mL microtube with 22 µL PCR mixture (0.6 µL primer forward, 0.6 µL primer reverse, 12.5 µL master mix, 8.3 µL nuclease-free water). Reverse transcriptase PCR was done for assessing the expression of following genes: NF-κB (206 bp; forward GCCTGACACCAGCATTTGA, reverse

Table I: Ratio of urine/serum creatinine in each group

Sample	*Urine/serum creatinine ratio (mean ± SD)
SO	205,823 ± 72,003
SN	9,496 ± 2,222
SNE1	34,987 ± 26,691
SNE2	43,146 ± 29,423
SNE3	42,015 ± 37,175

*Urine specimens were collected prior to termination.

Table II: Ratio of NF-κB gene expression normalized to GAPDH in each group

Group	NF-κB/GAPDH (Mean ± SD)
SO	0.574 ± 0.097
SN	0.736 ± 0.061*
SNE1	0.582 ± 0.049#
SNE2	0.614 ± 0.068#
SNE3	0.612 ± 0.088#

*P < 0.05 vs SO group; #P < 0.05 vs SN group.

Table III: Ratio of MCP-1 gene expression normalized to GAPDH in each group

Group	MCP-1/GAPDH (Mean ± SD)
SO	1.18 ± 0.122
SN	1.69 ± 0.132*
SNE1	1.377 ± 0.197
SNE2	1.481 ± 0.302
SNE3	1.469 ± 0.236

*P < 0.05 vs SO group.

CAAACCAAACAGCCTCACG); MCP-1 (150 bp; forward CAGGTCTCTGTCACGCTTCT, reverse GTAGTTCTCCAGCCGACTCA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (163 bp; forward GTTACCAGGGCTGCCTTCTC, reverse TCCCGTTGATGACCAGCTTC). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control because its expression is relatively constant across different conditions. PCR was conducted with the following conditions: denaturation at 95oC for 10 seconds, annealing at 55°C for GAPDH, 60°C for MCP-1, 64°C for NF-κB for 1 minute, and extension at 72°C for 1 minute. This process is repeated for 30 cycles for GAPDH, 39 cycles for MCP-1, and 33 cycles for NF-κB. Electrophoresis was subsequently performed following RT-PCR to visualize the amplified DNA fragments, using a DNA ladder as a molecular size marker to verify fragment sizes.

Immunohistochemistry

Kidney tissue slides were deparaffinized, rehydrated, and rinsed under running water. Then, antigen retrieval was performed by heating in a citrate buffer with pH 6 for 15 minutes. After rinsing with phosphate-buffered saline (PBS), endogenous peroxidase (H2O2 3%) was inhibited for 20 minutes, then rinsed with PBS 3 times for 5 minutes. The slides were dried and background blocking was performed with immunoblock (Finetest Cat. No. IHC0009) for 30 minutes and rinsed with PBS. Afterward, the slides were dried and an anti-CD68 antibody (ABclonal A22239) was added and stored overnight at 4°C. The next day, the slides were rinsed with PBS and mouse/rabbit probe horseradish peroxidase (HRP) (Finetest Cat. No. IHC0009) was added and waited for 10 seconds to be rinsed. Then, the slides were stained with hematoxylin, dehydrated, cleared, and mounted. Immunohistochemical staining of CD68 was performed on all kidney samples, and representative images were selected to reflect consistent macrophage infiltration patterns observed across the samples. Slides were observed under a light microscope at 400x magnification and viewed in 10 fields of view.

Statistical Analysis

Data were analyzed with the Shapiro-Wilk test for normality and the Levene test for homogeneity. Normalized data were then tested with One Way ANOVA and LSD post hoc, while non-normalized data were tested with Kruskal Wallis and Mann Whitney U post hoc test. Significance was determined with $p < 0.05$.

RESULTS

Exosomes Improve Kidney Function and Protect Tubules after 5/6 Subtotal Nephrectomy

The kidneys of rats subjected to 5/6 subtotal nephrectomy exhibited a significantly lower urine/serum creatinine ratio compared to the sham operation (SO) group, as shown in Table I. This reduced creatinine ratio indicates reduced kidney function, as healthy kidneys typically filter creatinine efficiently. However, after the administration of exosomes, there was a notable improvement in kidney function. Exosomes resulted in a higher urine/serum creatinine ratio, indicating a recovery in the kidney's filtration capacity (Figure 1A).

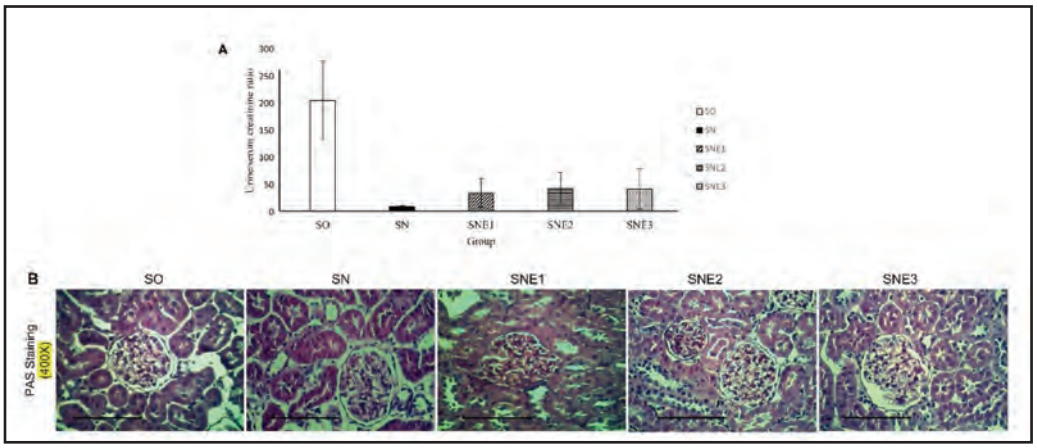

Fig. 1: Plot of urine/serum creatinine ratio in each group (A). Histological pictures (scale bar =100 µm) of kidney injury after SN model based on PAS staining (B).

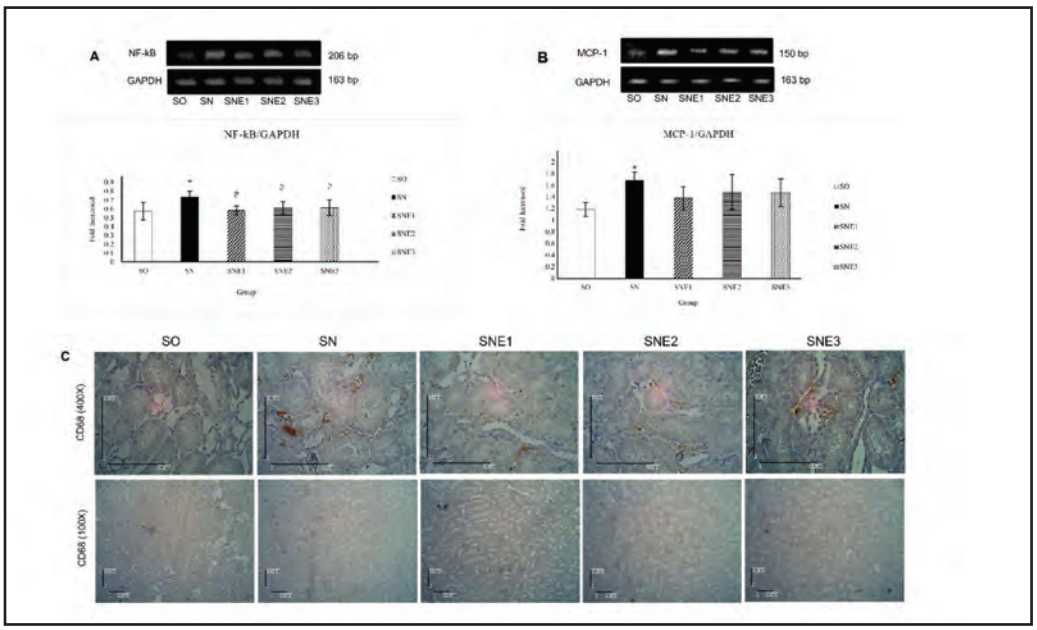

Fig. 2: Effect of Exosomes on mRNA Expression of Inflammation-Related Genes in Kidney Tissue after 5/6 Subtotal Nephrectomy. (A-B) Shows the upregulation of NF-κB and MCP-1 mRNA expression in the SN group compared to SO. NF-κB mRNA expression is significantly lower after exosome administration, while MCP-1 shows no significant change. (C) Representative image of CD-68 immunostaining shows positivity in interstitial tubules. *p<0.05 vs SO; # p<0.05 vs SN.

The 5/6 subtotal nephrectomy also caused severe tubular injury in the kidneys. This injury was characterized by several pathological changes, including interstitial inflammation, tubular dilation, and loss of the brush border, as identified through PAS staining. Figure 1B presents PAS-stained kidney sections as representative examples from each group, selected to reflect the consistent histological findings observed across all samples. The administration of exosomes showed protective effects by reducing inflammation, preventing further tubular dilation, and aiding in the recovery of the brush border.

Exosome Treatment Effects on Kidney Inflammation and Macrophage Infiltration

Gene expression related to inflammation, such as NF-κB and MCP-1, increases after CKD injury. NF-κB mRNA expression was significantly ($p < 0.05$) higher in the SN group compared to the SO group. Exosome treatment (SNE1, SNE2, SNE3) significantly reduced NF-κB mRNA expression compared to the SN group, with p-values of 0.011, 0.029, and 0.026, respectively (Table II and Figure 2A). The differences among the exosome-treated groups were not statistically significant. Figure 2B showed that MCP-1 mRNA expression was significantly higher in the SN group than in the SO group. Treatment with exosomes (SNE1, SNE2, SNE3) did not show a statistically significant difference in MCP-1 expression compared to the SN group, with p-values of 0.052, 0.178, and 0.156 (Table III).[26]

To further investigate whether exosomes can suppress inflammation, we conducted immunohistochemical staining using a CD68 antibody on kidney tissue. CD68 is a

membrane-bound glycoprotein expressed by macrophages, used to assess macrophage infiltration. Immunohistochemical staining with an anti-CD68 antibody revealed macrophage infiltration in the interstitial areas of tubules. The infiltration appeared less dominant in the exosome-treated group compared to the SN group. Exosome treatment consistently reduced macrophage infiltration across all treatment groups. As shown in Figure 2C, representative CD68-stained kidney sections from each group were selected to reflect the consistent macrophage infiltration patterns observed across samples.

DISCUSSION

This study highlights that exosome plays a vital role in inhibiting the progression of CKD by regulating inflammation in rat kidneys. Evidence supporting this includes analyses of NF-κB and MCP-1 mRNA expressions, which were involved in regulating inflammation and macrophage infiltration.

The results of this study revealed that the mRNA expressions of NF-κB and MCP-1 in the SN group were significantly higher than in the SO group. This finding aligns with a prior study that found an increased mRNA expression of NF-κB in 5/6 subtotal nephrectomy models.[27] In CKD, the expression of NF-κB increases, especially in the kidney cortex tubule cells.[7] In addition, an increase in the expression of MCP-1 was found in a 5/6 subtotal nephrectomy mouse model.28 This indicates that the model can induce kidney inflammation.

Increased mRNA expressions of NF-κB and MCP-1 as a response to injury showed that inflammation acts in the mechanism of tissue repair. Cellular injury prompts the production of signaling molecules that activate NF-κB. This activation happens through the canonical or non-canonical pathways, initiating the transcription of inflammatory genes including MCP-1, which facilitates macrophages recruitment to the site of injury for tissue repair.[29,30] However, in CKD, persistent inflammation can aggravate kidney damage.[31,32] Several studies have identified strategies to prevent kidney inflammation by targeting NF-κB. Inhibition of NF-κB can be achieved through reducing the expression of NF-κB component genes or by targeting its activating signaling pathway. For instance, exosomes derived from human umbilical cord mesenchymal stem cells (HUC-MSC) containing miR-22-3p suppress inflammation by downregulating NLRP3 expression, thereby reducing the activation and release of proinflammatory cytokines like IL-1β, which participates in the canonical pathway of NF-κB activation.33 Similarly, exosomes derived from adipose mesenchymal stem cells carrying miR-26 inhibit the NF-κB pathway by targeting TLR-4, thereby reducing the expression and phosphorylation of protein involved in NF-κB signaling.[22] Exosomes derived from HUC-MSC have also been shown to ameliorate kidney injury by inhibiting NF-κB phosphorylation.[34] Additionally, a study in a diabetic kidney disease (DKD) model reported that exosomes derived from bone marrow mesenchymal stem cells (BM-MSC) suppress NF-κB activation by reducing P65 expression.[35]

In this study, the mRNA expressions of NF-κB in SNE1, SNE2, and SNE3 groups were significantly lower than the SN group. This finding was in line with a previous study demonstrating that exosomes from MSC, which were stimulated with melatonin containing miR-26a, effectively reduce NF-κB expression in the kidney.[21] These results underscore the role of exosomes in modulating inflammation by inhibiting NF-κB activation.

The expression of MCP-1 in the exosome-treated group did not significantly differ from that in the SN group. This result may be attributed to the activity of the NF-κB inflammatory pathway, which generates various other inflammatory mediators.[36] Previous research has demonstrated that exosomes inhibit NF-κB mRNA expression, subsequently leading to a reduction in IL-6 mRNA expression in renal ischemia-reperfusion models. IL-6 plays a crucial role in the differentiation of monocytes into macrophages by upregulating the expression of the macrophage colony-stimulating factor receptor (M-CSFR).37 Furthermore, HUC-MSC exosomes have been shown to reduce TGF-β expression in the kidney, which may potentially enhance M-CSF expression.[38] M-CSF, secreted by fibroblasts, binds to M-CSFR on monocytes to promote their differentiation into macrophages.[39]

Based on immunohistochemistry observation using anti-CD68 antibodies, rat treated with exosomes exhibited lower macrophage infiltration compared to untreated rat. This finding suggests that the inhibition of NF-κB synthesis correlates with decreased macrophage infiltration, demonstrating exosomes' potential to reduce macrophage infiltration, and thus, suppress excessive inflammation.[34]

Overall, the results of this study demonstrate the positive impact of exosome administration in reducing inflammation in the kidneys of a 5/6 subtotal nephrectomy rat model. These findings align with previous research indicating that exosomes may be more effective in reducing inflammation compared to MSCs.[40]

In this study, there were no significant differences in NF-κB and MCP-1 expression among the exosome-treated groups (SNE1, SNE2, and SNE3). This suggests that while exosomes can reduce inflammation, there is no difference in effectiveness among the three groups. These results are consistent with other studies showing that exosome administration with a total protein of 50 µg and 100 µg can reduce NF-κB expression to the same extent.[22] Therefore, exploring exosomes with varying total protein contents is important to maximize their ability to suppress inflammation. While this study focused on inflammatory response, it is also important to consider the potential long-term toxicity of exosome administration in future studies to ensure their safety for therapeutic use.

CONCLUSION

Exosomes attenuate the inflammatory response in 5/6 subtotal nephrectomy rat models by reducing mRNA expression of NF-κB and macrophage infiltration.

AUTHORS' CONTRIBUTIONS

ZNZ, NA, and DC contributed substantially to the conception and design of the study. ZNZ, EY, LRA, DA, and PBS performed the animal experiments, surgical procedures, post-operative care, and tissue processing. NF, HPW, KFM, and TMS isolated the exosomes, and HM, K and MAP performed their characterization. NF, YW, and VYS carried out the biochemical assays and assisted with data interpretation. DA and RY supervised the in vivo protocols and, together with NA, contributed to experimental troubleshooting and quality control. ZNZ, NA, and RY drafted the manuscript, which was critically reviewed and revised by DA, RY, and DC for important intellectual content. NA, as the corresponding author and principal investigator, oversaw the project, secured funding, and approved the final manuscript. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Tiara Kurnia Sari and Ando Gavintri for their assistance in animal maintenance. This study was funded by Rispro LPDP no. PRJ-9/LPDP/LPDP.4/2022 of the Indonesian Government 2023 – 2024. Part of the data in this study was used to fulfill the requirements of Zahara Nurfatihah Z's Master's program.

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