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RESEARCH ARTICLE

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Hypoxic Umbilical Cord-Derived Mesenchymal Stem Cells Decrease TNF-α and IL-1β Levels in an Acute Ischemic Stroke Rat Model

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Background: Tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) contribute to secondary neuronal injury after ischemic stroke by promoting blood brain barrier disruption and neuronal apoptosis. Mesenchymal stem cells (MSCs) have anti-inflammatory and neuroprotective effects, but their efficacy may be limited under ischemic conditions due to poor survival and inadequate paracrine activity. Hypoxic preconditioning may enhance MSC potency, yet in vivo evidence on cytokine modulation remains limited. This study evaluated the effects of hypoxic-preconditioned umbilical cord MSCs (UC-MSCs) on TNF-α and IL-1β levels in an acute ischemic stroke model.

Materials and methods: Twenty-eight male Wistar rats were randomized into four groups: a healthy control group, an untreated stroke group, and two treatment groups receiving UC-MSCs at doses of 1.5×10^6 and 3.0×10^6 cells, respectively. Stroke was induced using middle cerebral artery occlusion (MCAO). UC-MSCs were administered intravenously on day 3 post-occlusion. Brain tissue TNF-α and IL-1β were measured by ELISA and analyzed using one-way ANOVA with post hoc testing.

Results: Compared with the untreated stroke group, both treatment groups receiving UC-MSCs (1.5×10^6 and 3.0×10^6 cells) showed significantly lower TNF-α and IL-1β levels ($p<0.001$). The reduction was greater in the 3.0×10^6-cell group, indicating a dose-dependent anti-inflammatory effect, with the lowest cytokine levels observed in this group.

Conclusion: Hypoxic UC-MSCs decrease the levels of TNF-α and IL-1β in acute ischemic stroke, supporting their immunomodulatory and neuroprotective potential. Further studies should assess long-term outcomes and histopathological correlations.

Keywords: *ischemic stroke, UC-MSCs, hypoxic preconditioning, TNF-α, IL-1*β

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Introduction

Ischemic stroke is a major cause of mortality and disability worldwide, characterized by acute cerebral ischemia followed by secondary inflammatory injury.[1–4] It occurs when blood flow to the brain is obstructed, leading to oxygen deprivation and neuronal injury that trigger oxidative stress and excessive inflammatory responses.[5,6] During the acute phase of ischemia, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) play key roles in blood–brain barrier disruption and neuronal apoptosis.[7,8] Although conventional treatments such as recombinant tissue plasminogen activator (rtPA) and mechanical thrombectomy can restore cerebral perfusion, their effectiveness is limited to a narrow time window and carries a high risk of hemorrhage.[9,10] Consequently, new therapeutic approaches that address both inflammation and tissue repair are urgently needed.

Mesenchymal stem cells (MSCs), particularly those derived from the umbilical cord (UC-MSCs), demonstrate strong anti-inflammatory and neuroprotective effects due to their low immunogenicity, high proliferation rate, and secretion of trophic and immunomodulatory factors.[11–13] Hypoxic preconditioning further enhances these properties by increasing MSC viability and the release of bioactive molecules such as microRNA and exosomes with potent anti-inflammatory activity.[14,15]

However, growing evidence supporting the therapeutic potential of hypoxic-preconditioned UC-MSCs, there remains limited in vivo data specifically elucidating their effects on key pro-inflammatory cytokines, particularly TNF-α and IL-1β, during the acute phase of ischemic stroke.[16–18] The lack of direct experimental evidence addressing cytokine modulation in acute ischemic injury represents an important knowledge gap in the current literature. This in vivo experimental study aims to determine the effect of UC-MSCs on TNF-α and IL-1β levels in Wistar rats with acute ischemic stroke. This research addresses a critical gap by providing evidence on the cytokine-decresing mechanisms of UC-MSCs, contributing to the development of more effective and targeted stem cell-based therapies for ischemic stroke.

Materials and methods

Animal Models Preparation

Male Wistar rats were used as experimental subjects. The animals were randomly allocated into four groups: a normal control group without ischemia or treatment (G1), an untreated stroke group (G2), a stroke group treated with 1.5×10^6 hypoxic-preconditioned UC-MSCs (G3), and a stroke group treated with 3.0×10^6 hypoxic-preconditioned UC-MSCs (G4) (Figure 1).

Male Wistar rats (6–8 weeks old, 180–220 g) were obtained from the Animal House, SCCR Laboratory. All rats were healthy, infection-free, and acclimatized for seven days under controlled environmental conditions (temperature 22–24°C, 12-hour light/dark cycle, good ventilation). Standard pellet feed (PT. Charoen Pokphand, Indonesia) containing 20–25% protein, 45–55% carbohydrates, and 10–12% fat was provided ad libitum, along with clean drinking water. A total of 28 rats were randomly selected using simple random sampling and divided into four groups (n = 7 per group). The minimum sample size was calculated based on Federer's formula resulting in $n \geq 6$; one additional rat per group was added to compensate for possible dropout.

Hypoxic Preconditioning of UC-MSCs

The isolation of UC-MSCs was performed under aseptic conditions according to the method described in the previous study.[19] Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin-streptomycin (Sigma-Aldrich, USA), and 1% amphotericin B (Fungizone; Thermo Fisher Scientific, USA). Cultures were maintained at 37°C in 5% CO_2 for 72 hours. The medium was replaced every two days until the cells reached 80–90% confluence. MSC validation was performed according to the method described in the previous study.19 MSC identity was supported by plastic adherence, spindle-shaped morphology, and flow cytometric immunophenotyping with positive CD90.1 and CD29 expression and negative CD45 and CD31 expression. Because validated anti-rat antibodies for CD73 and CD105 were not available in our laboratory, and CD105 expression in rodent MSCs has been reported to vary across studies,[20,21] these markers were not included in the present analysis.

For hypoxic preconditioning, UC-MSCs were initially cultured under standard normoxic conditions (21% O_2 and 5% CO_2) at 37°C until they reached the desired level of confluence. Subsequently, the cells were transferred into a hypoxia chamber (Billups-Rothenberg, USA) and exposed to a controlled hypoxic environment consisting of 5% O_2, 5% CO_2, and 90% N_2 for 24 hours at 37°C. This preconditioning strategy was performed to mimic the low-

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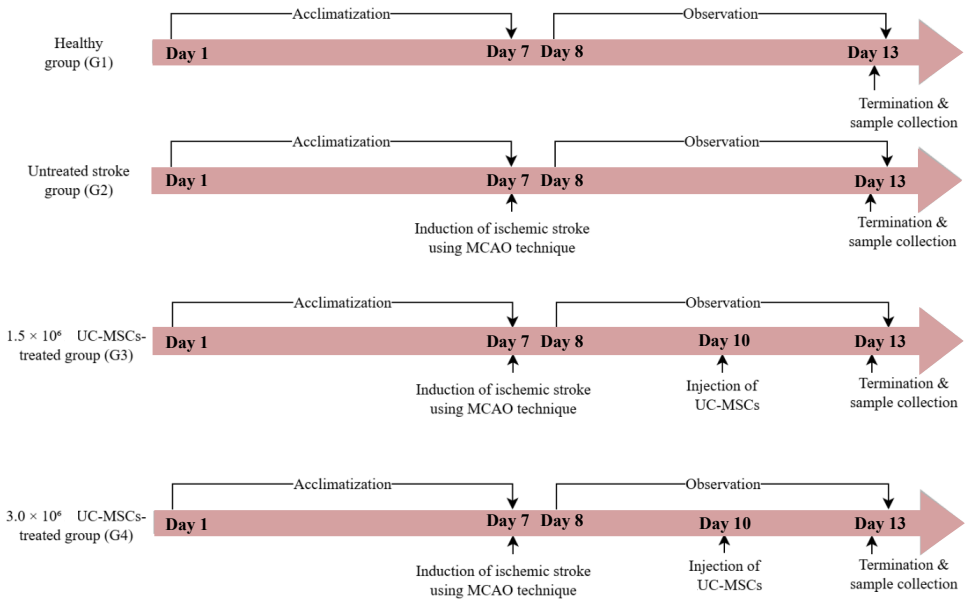


Figure 1. Schematic diagram of the study design and experimental timeline.

oxygen microenvironment encountered in injured tissues, thereby inducing adaptive cellular responses that improve cell survival, paracrine activity, and immunomodulatory capacity. Hypoxic exposure has been shown to enhance the secretion of anti-inflammatory and regenerative factors by MSCs, thereby increasing their therapeutic potential. Therefore, hypoxia-preconditioned UC-MSCs were used for subsequent transplantation experiments to maximize their anti-inflammatory efficacy.

Induction of Ischemic Stroke Model

Ischemic stroke was induced using the middle cerebral artery occlusion (MCAO) technique.[22,23] The rats were anesthetized with 3% isoflurane (Baxter, USA) in oxygen and placed on a heated surgical table to maintain body temperature at 37±0.5°C. A laser Doppler flowmeter (Perimed, Sweden) was used to monitor cerebral blood flow (CBF).

A midline cervical incision was carefully performed under aseptic conditions to expose the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). The surrounding connective tissues and adjacent nerves were gently separated to provide clear visualization of the carotid vasculature while minimizing tissue damage. After isolation of the arteries, a silicone-coated monofilament nylon suture (Doccol Corp., USA) was introduced through the lumen of the CCA and carefully advanced into the ICA until the tip reached and occluded the origin of the middle cerebral artery (MCA). The occlusion was maintained for 60 minutes to induce transient focal cerebral ischemia. Successful establishment of the ischemic model was confirmed by continuous cerebral blood flow (CBF) monitoring, with ischemia defined as a reduction of at least 50% in CBF within the ipsilateral cerebral hemisphere compared with baseline values. Following the 60-minute occlusion period, the monofilament was gently withdrawn to restore blood flow, thereby initiating the reperfusion phase. The cervical incision was subsequently closed, and the animals were allowed to recover under appropriate postoperative monitoring before further experimental procedures.

Treatment Administration

Three days after MCAO, hypoxic-preconditioned UC-MSCs were administered intravenously via the tail vein in 300 µL of normal saline (NaCl 0.9%; Otsuka, Japan). The G3 group received 1.5×10^6 cells, while the G4 group received 3.0×10^6 cells. The control stroke group (G2) received only 300 µL of normal saline. All injections were performed using a 27G sterile syringe (Terumo, Japan).

ELISA-based Measurement of TNF-α and IL-1β

At day three post-treatment, rats were euthanized using chloroform inhalation. The brain tissue, particularly the ipsilateral cerebral cortex, was dissected and homogenized in cold PBS buffer. The concentrations of TNF-α (Cat. No. E-EL-R2856, Elabscience, USA) and IL-1β (Cat. No. E-EL-R0012, Elabscience, USA) were determined using enzyme-linked immunosorbent assay (ELISA)

kits (Elabscience, USA), following the manufacturer's protocols. Absorbance was read at 450 nm using a microplate reader (Bio-Rad, USA). Cytokine levels were expressed in picograms per milliliter (pg/mL).

TNF-α and IL-1β levels were quantitatively analyzed based on standard curves from serial dilutions of known cytokine concentrations. Each sample was tested in duplicate, and the mean value was recorded. A higher cytokine concentration indicated greater inflammatory response, while a lower concentration reflected anti-inflammatory effects of UC-MSCs.

Statistical Analysis

Data were analyzed using SPSS software version 26.0 (IBM Corp., USA). Descriptive statistics were used to summarize mean and standard deviation for each group. Normality of data distribution was tested using the Shapiro–Wilk test, and homogeneity of variance was assessed by Levene's test. If assumptions were met, comparisons between groups were performed using One-Way ANOVA, followed by Tukey's post-hoc test. If variances were unequal, Tamhane's T2 was applied. For non-parametric data, the Kruskal–Wallis test followed by Mann–Whitney U test was used. A p-value < 0.05 was considered statistically significant.

Results

Reduction of TNF-α Levels

The administration of UC-MSCs significantly decreased TNF-α levels compared with the untreated stroke model (Table 1, Figure 2A). The normal control group (G1) showed the lowest physiological TNF-α level, while the untreated stroke group (G2) had the highest concentration, reflecting strong inflammatory activation. The low-dose treatment group (G3) exhibited a notable reduction in TNF-α, and the high-dose treatment group (G4) showed the lowest TNF-α level among all groups, indicating a dose-dependent anti-inflammatory effect.

The data were normally distributed ($p>0.05$) but non-homogeneous; therefore, one-way ANOVA followed by Tamhane's post-hoc test was performed. Statistical analysis revealed a significant difference among groups ($p<0.05$), with post-hoc comparisons confirming that both treatment groups (G3 and G4) had significantly lower TNF-α levels than the untreated stroke group (G2), with the strongest decrese observed in the high-dose treatment group (G4) ($p<0.001$).

Reduction of IL-1β Levels

Similar to TNF-α, IL-1β concentrations decreased significantly following UC-MSC administration (Table 1, Figure 2B). The highest IL-1β level was observed in the untreated stroke group (G2), while the lowest was recorded in the high-dose treatment group (G4). The low-dose treatment group (G3) also demonstrated a clear reduction in IL-1β compared with untreated stroke group (G2).

Normality ($p>0.05$) and homogeneity ($p>0.05$) tests confirmed that the data met parametric assumptions; thus, one-way ANOVA followed by LSD post-hoc analysis was applied. The results showed a significant decrease in IL-1β levels among groups ($p<0.05$), and post-hoc testing indicated that both treatment groups differed significantly from the untreated stroke group (G2), with a stronger reduction in high-dose treatment group (G4) ($p<0.001$).

Discussion

The results demonstrated that UC-MSC administration, particularly at a dose of 3×10^6 cells on G4 group, significantly decreased both cytokines compared to the untreated stroke group (G2). These reductions were observed during the acute post-ischemic phase and support our hypothesis that UC-MSCs modulate early post-stroke inflammatory responses in a dose-dependent manner.[18] The more pronounced effect in G4 compared with the lower-dose group (G3) suggests that a threshold cell dose may be required to achieve consistent decreases of pro-inflammatory cytokines. Together, these findings indicate the potential of appropriately dosed UC-MSC therapy to attenuate acute neuroinflammation following ischemic stroke.

The reduction of TNF-α and IL-1β levels following UC-MSC treatment reflects the potent anti-inflammatory and immunomodulatory properties of these cells. TNF-α and IL-1β are pivotal cytokines in the inflammatory cascade of ischemic stroke, contributing to blood–brain barrier (BBB) disruption, neuronal apoptosis, and oxidative injury.[7,24,25] Elevated TNF-α levels activate the NF-κB signaling pathway, promoting leukocyte infiltration and neuronal death.[26] Similarly, IL-1β increases vascular permeability and triggers caspase-1–mediated neuronal apoptosis via the NLRP3 inflammasome.[8,27] The significant decline in both cytokines observed in this study indicates that UC-MSCs effectively modulated these inflammatory mechanisms, thereby limiting secondary neural damage.

These outcomes are consistent with prior findings that MSC therapy mitigates post-stroke inflammation and

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Table 1. Mean expression and statistical tests of TNF-α and IL-1β levels across groups..

Expression/Statistical Test	Healthy	Untreated Stroke (G2)	1.5×10^6 UC-MSCs (G3)	3.0×10^6 UC-MSCs (G4)	
TNF-α(pg/mL)	50.66±8.06	114.69±15.10	88.50±2.39	52.24±5.53	
Shapiro wilk (p)	0.865 †	0.455 †	0.407 †	0.205 †	
Lavene test (p)					0.004
Oneway ANOVA (p)					<0.001*
Post hoc Tamhane's T2 (p)					
Healthy (G1)		<0.001*	<0.001*	0,69375	
Untreated stroke (G2)	<0.001*		0.045*	<0.001*	
1.5×10^6 EH-MSCs (G3)	<0.001*	0.045*		0,69375	
IL-1β (pg/mL)	12.97 ±5.88	60.79 ±4.34	24.53 ±7.44	18.56 ±1.33	
Shapiro wilk (p)	0.556 †	0.549 †	0.540 †	0.420 †	
Lavene test (p)					0.065 ‡
Oneway ANOVA (p)					<0.001*
Post hoc LSD (p)					
Healthy (G1)		<0.001*	0.001*	0.080	
Untreated stroke (G2)	<0.001*		<0.001*	<0.001*	
1.5×10^6 EH-MSCs (G3)	0.001*	<0.001*		0.063	

† Normal (p>0.05); ‡ homogen (p>0.05); * significant difference (p<0.05)

promotes neuroprotection. The UC-MSCs have been shown to alleviate ischemic injury by regulating inflammatory and oxidative stress pathways.[12] Paracrine signaling from MSCs contributes to the downregulation of TNF-α and IL-1β while enhancing angiogenic and neurotrophic signaling.[11,28] Hypoxic preconditioning has been found to amplify these effects by increasing the secretion of anti-inflammatory cytokines such as IL-10, TGF-β, and PGE_2, along with growth factors including VEGF and IGF-1.[15,29,30] Hypoxic preconditioning stabilizes HIF-1α in MSCs, triggering a transcriptional program that increases secretion of angiogenic and anti-inflammatory mediators (e.g., VEGF, IGF-1, IL-10, TGF-β, PGE_2), upregulates homing receptors such as CXCR4, and activates prosurvival pathways (PI3K/Akt), thereby enhancing MSC survival, homing, and paracrine immunomodulation that suppresses NF-κB–driven TNF-α and IL-1β production.[31–33] These secreted molecules promote macrophage polarization from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype, thereby reducing inflammatory cytokine expression and supporting tissue repair.[34] Consistent evidence from inflammatory and degenerative disease models also supports the role of MSC-derived secretomes in shifting immune responses toward an anti-inflammatory profile.[18]

Although hypoxic preconditioning is reported to increase secretion of anti-inflammatory mediators (e.g., IL-10, TGF-β, PGE_2) and growth/chemotactic factors (e.g., VEGF, SDF-1/CXCL12) via HIF-1α signaling, the present study measured only TNF-α and IL-1β. Consequently, we could not directly confirm enrichment of these anti-inflammatory or pro-homing mediators in the UC-MSC secretome. The dose-dependent response observed in this research further underscores the therapeutic potential of UC-MSCs. An increased cell dose has been associated with greater decreases in TNF-α and IL-1β, reflecting enhanced paracrine activity and cytokine modulation.[16,17] Similar trends have been documented in experimental models where escalating MSC or exosome concentrations led to improved regulation of inflammatory mediators and better functional recovery.[35]

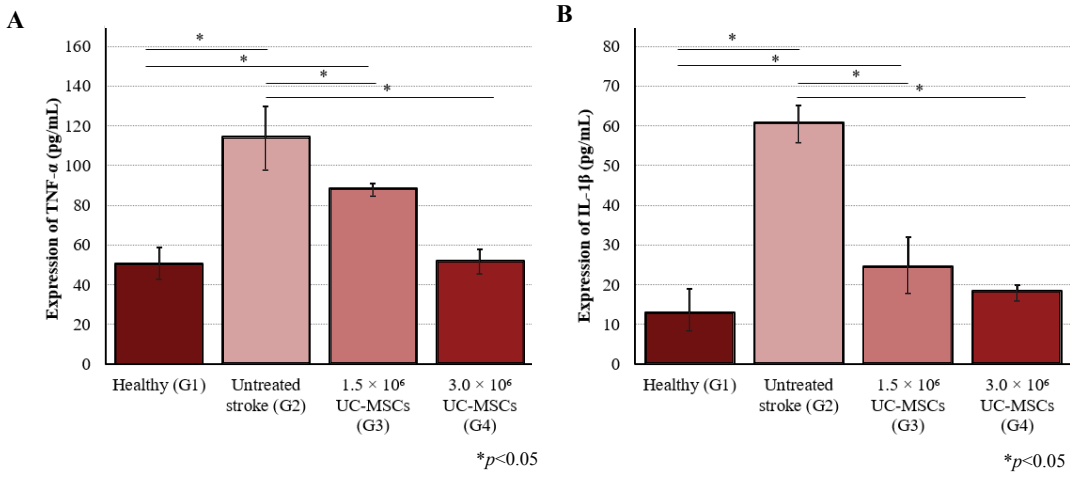


Figure 1. Expression of TNF-α and IL-1β in different treatment groups. A: TNF-α expression was significantly decreased in the high-dose treatment group (G4) compared to untreated stroke (G2) and low-dose treatment (G3) groups. B: IL-1β expression was also following the same manners. Bars represent mean ± SD; * indicates significant difference between groups (*p<0.05).

The dose-dependent response observed in this research further underscores the therapeutic potential of UC-MSCs. An increased cell dose has been associated with greater decreases in TNF-α and IL-1β, reflecting enhanced paracrine activity and cytokine modulation.[16,17] Similar trends have been documented in experimental models where escalating MSC or exosome concentrations led to improved regulation of inflammatory mediators and better functional recovery.[35]

Despite its promising findings, this study has several limitations. The short observation period of three days post-treatment restricts conclusions about long-term effects on cytokine dynamics and neurological recovery. The chloroform inhalation was used to euthanize animals in this study and that inhalant agents can induce vascular congestion or other post-mortem changes that might affect biochemical measurements of brain tissue.[36] In addition, only TNF-α and IL-1β were measured, and other hypoxia- and immunomodulation-related mediators such as IL-10, TGF-β, PGE_2, VEGF, and HIF-1α–associated signaling pathways were not assessed, limiting direct mechanistic interpretation of hypoxic preconditioning effects. Additionally, the absence of histopathological and immunohistochemical assessments precludes direct correlation between cytokine reduction and tissue preservation. Intravenous delivery ensured systemic administration, but intrathecal routes may offer greater local efficacy and should be considered in future studies.[16]

Overall, this study reinforces the potent anti-inflammatory effects of UC-MSCs, particularly through the downregulation of TNF-α and IL-1β, cytokines known to mediate acute ischemic injury. This mechanism likely involves HIF-1α activation, macrophage phenotypic switching, and enhanced secretion of regenerative paracrine factors. We did not directly measure HIF-1α stabilization in the present study; therefore, future work should quantify HIF-1α protein levels in UC-MSCs immediately after hypoxic preconditioning (e.g., by nuclear-enriched Western blot or ELISA and/or immunocytochemistry) and assess downstream targets (e.g., VEGF, CXCL12/SDF-1, IDO) and tissue HIF-1α by IHC or Western blot to confirm the proposed mechanism. These findings complement and extend existing evidence on MSC-based immunomodulation, highlighting hypoxic preconditioning as a promising strategy to improve therapeutic outcomes. Supporting data from other inflammatory conditions, further illustrate the immunoregulatory and cytokine-modulating properties of MSC therapies.[37–39] Further investigations should assess long-term outcomes, including neuronal regeneration and behavioral recovery, while incorporating histopathological and protein-level analyses to clarify underlying mechanisms. Comparative studies using different administration routes or exosome-derived therapies are also warranted to optimize therapeutic efficacy.

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Conclusion

The UC-MSCs significantly downregulate TNF-α and IL-1β in an acute ischemic stroke model, confirming their potent anti-inflammatory roles, with a greater reduction at 3×10^6 cells. The dose-dependent decrease in these cytokines highlights the enhanced paracrine activity induced by hypoxic conditioning, suggesting a mechanism that mitigates secondary neuronal injury following ischemia.

Authors' Contributions

IHD, AP, and ES were involved in concepting and planning the research; IHD performed the data acquisition/collection, calculated the experimental data, performed the analysis, and drafted the manuscript and designed the figures; AP, and ES interpreted the results. All authors took parts in giving critical revision of the manuscript.

Ethical Statement

The study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Islam Sultan Agung, Semarang (Approval No.: 603/XI/2025/Komisi Bioetik) and was conducted according to the Guidelines for the Care and Use of Laboratory Animals by the National Research Council. All experimental procedures were conducted at the Semarang Cancer Center and Research (SCCR) Laboratory, Semarang, Indonesia.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

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