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Experimental Research

Effect of erythropoietin administration on expression of mRNA brain-derived Neurotrophic factor, levels of stromal cell-derived Factor-1, and neuron specific enolase in brain injury model *Sprague Dawley*

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ABSTRACT

Background: Traumatic brain injury (TBI) is a complicated condition that is the primary cause of death and disability in children and young adults in developed countries. Various kinds of therapy have been carried out in the management of brain injury, one of which is the administration of erythropoietin (EPO). There are not many studies in Indonesia have proven that EPO administration is effective on parameters such as stromal cell-derived factor 1 (SDF-1), brain-derived neurotrophic factor (BDNF mRNA), and neuron-specific enolase (NSE) in brain injury patients. The purpose of this study was to see how EPO affected BDNF mRNA expression, SDF-1 serum levels, and NSE levels in experimental rats with TBI.

Methods: This study was conducted using a rat head injury model. Fifteen rats were randomly assigned to one of three groups: A, B, or C. EPO was administered subcutis with a dose of 30.000 U/kg. Blood samples were taken after brain injury (H0), 12 h (H12), and 24 h (H24) after brain injury. Serum level of SDF-1 and NSE were measured using mRNA BDNF gene expression was measured with Real-Time-PCR, and ELISA.

Results: This study found EPO increase BDNF mRNA expression in group C at H-12 ($7,92 \pm 0,51$ vs $6,45 \pm 0,33$) compared to group B, and at H-24 ($9,20 \pm 0,56$ vs $7,22 \pm 0,19$); increase SDF-1 levels in group C at H-12 ($7,56 \pm 0,54$) vs $4,62 \pm 0,58$) compared to group B, and at H-24 ($11,32 \pm 4,55$ vs $2,55 \pm 0,70$); decrease serum NSE levels in group C at H-12 ($17,25 \pm 2,02$ vs $29,65 \pm 2,33$) compare to group B and at H-24 ($12,14 \pm 2,61$ vs $37,31 \pm 2,76$); the values are significantly different with $p < 0,05$.

Conclusion: EPO may have neuroprotective and anti-inflammatory properties in TBI by increasing mRNA BDNF expression and serum SDF-1 levels, and decrease serum NSE levels.

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1. Introduction

Traumatic brain injury (TBI) is a complicated condition that is the primary cause of death and disability in children and young adults in

developed countries [1–3]. In 2019, De Leon et al. conducted a systematic review and the results found that 69 million (95% CI 64–74 million) people worldwide will experience brain injury each year. The highest percentages of brain injuries resulting from road traffic accidents are

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from Southeast Asia and Africa (both 56%), and the lowest outcomes are in North America (25%). North America (1299 cases, 95% confidence interval 650–1947) and Europe (1011 cases, 95% confidence interval 911–1113) had the highest overall TBI incidence per 100,000 people, while Africa (801 cases, 95% confidence interval 732–871) and the Eastern Mediterranean had the lowest (897 cases, 95% CI 771–1023) [1].

Tissue injury and interruption of cerebral blood flow (CBF) characterize the early stages of brain injury following a TBI. This induces anaerobic glycolysis, which results in lactic acid buildup, increased membrane permeability, and edema. Anaerobic metabolism fails to maintain cellular energy levels, causing ATP stores to decrease and the energy-dependent membrane ion pump to fail [4,5].

The end of membrane depolarization, as well as excessive release of excitatory neurotransmitters (e.g., glutamate, aspartate), activation of *N*-methyl-*D*-aspartate, -amino-3-hydroxy-5-methyl-4-isoxazolpropionate, and Ca^{2+} and Na^{+} voltages, characterize the second stage, or secondary damage. The continuous currents of Ca^{2+} and Na^{+} cause intracellular catabolic (self-digesting) processes. Ca^{2+} activates lipid peroxides, phospholipases, and proteases, resulting in a rise in intracellular free fatty acid and free radical concentrations [6]. Caspases (ICE-like proteins), translocases, and endonucleases are also activated, causing structural alterations in cellular membranes and nucleosome DNA (DNA fragmentation and inhibition of DNA repair).

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Various kinds of therapy have been carried out in the management of brain injury, one of which is the administration of erythropoietin (EPO). Existing research has proven that EPO can provide neuroprotective effects after ischemia, hypoxia, metabolic, neurotoxic, and excitotoxic events in the nervous system [7]. EPO acts on several parts of the central nervous system (CNS), including limiting the production of reactive oxygen species and glutamate, modulating neurotransmission, reducing vasospasm, promoting angiogenesis, inhibiting apoptosis, reducing inflammatory cells and stem cells. Improvement of neurogenesis and angiogenesis function after EPO administration is the most essential for the healing process of traumatic brain injury patients. Based on this, EPO is used in human and animal studies [8].

Brain-derived neurotrophic factor (BDNF) is a type of protein that is part of neurotrophins and is part of the Nerve Growth Factor family. The central nervous system and the peripheral nervous system are both affected by BDNF. BDNF can help neurons survive and stimulate new growth and synapses [9]. Studies of BDNF in TBI patients are rare and the results are highly controversial, thus, its role in TBI remains unclear. The relationship between TBI patient outcomes and BDNF levels in serum or cerebrospinal fluid has not been determined [10,11].

Pro-BDNF selectively activates the high-affinity receptor, the p75 receptor, largely triggering the proapoptotic signaling pathway, via the BDNF receptor and signaling pathway. BDNF binds to the tropomyosin-associated type B receptor kinase (TrkB) and the low-affinity neurotrophin receptor p75 with great specificity, then acts by interacting with these two transmembrane receptors, either independently or together, to cause neuron death or survival [12].

Stromal cell derived factor-1 (SDF-1) is a member of the CXC [also known as CXC motif chemokine 12 (CXCL12)] family of chemokines, which is expressed by a variety of cell types. By RNA differential splicing, two SDF-1 isoforms (SDF-1a and SDF-1b) are produced from a single gene, differing only by four amino acids at the C-terminus. SDF-1 has the same basic structure in humans and mice, with only one amino

acid variation [13].

The study of Yoo et al. showed that increased levels of SDF-1 promote neuronal proliferation and enhance neurogenesis after stroke, suggesting a neuroprotective role of SDF-1 in brain injury [14]. SDF-1 therapy has also been shown to cause apoptosis in acute myeloid leukemia patients, while inhibiting apoptosis in endothelial progenitor cells and bone marrow mesenchymal cells. Previous results have shown that SDF-1 exerts pro-apoptotic or anti-apoptotic effects on different cell types [15].

Neuron-specific enolase (NSE) is a biomarker of acute brain damage seen in the CSF and blood, caused by the rupture of the neuron cell membrane, and is a sensitive sign of brain injury caused by hypoxia, ischemia, or CNS trauma. NSE with its longer half-life in blood compared to other biomarkers, can indicate the presence of brain inflammatory processes and neuronal cell death. High levels of NSE are associated with neuronal cell injury [16].

There are not many studies in Indonesia that have proven that EPO administration is effective on parameters such as stromal cell-derived factor 1 (SDF-1), brain-derived neurotrophic factor (BDNF mRNA), and neuron-specific enolase (NSE), especially in brain injury patients.

2. Material and method

This study examined the effect of subcutaneous (s.c) injection of EPO on serum gene expression of mRNA BDNF, level of SDF-1, and NSE in weight-drop brain injury model. The model was categorized into three groups: A, B, and C. Group A were control group, brain injury (–) and EPO (–), group B with brain injury (+) and EPO (–), and group C with brain injury (+) and EPO (+).

This study was conducted at Hasanuddin University's Molecular Biology and Immunology Laboratory. The Ethics Committee of Hasanuddin University's Faculty of Medicine accepted this study (Reference Number 48/UN4.6.5.31/PP36/2021). This work was also carried out in line with the ARRIVE guidelines for reporting animal research [17,18].

Fifteen healthy male Sprague-Dawley rats, weighing 150–250 g and older than 2 months, were assigned into groups A, B, and C at random and equally. With a 12-h day and night cycle, water and diet were fed ad libitum (Comfeed AD-2), the rats were acclimatized and cared for according to Ethical animal use and care principles. There were no controls for confounders in this study.

2.1. Surgical procedure

Rats were weighed and anesthetized with 2–10 mg/kg intramuscular ketamine. In an antiseptic and aseptic technique involving povidone iodine, a linear incision was made on the midline, 1.5 cm posterior to the coronal suture, and the periosteum was then lifted. To expose the dura mater, one burr hole with a diameter of 0.5–1 cm was drilled. The modified Marmarou model was used to conduct this brain damage experiment [19,20]. The exposed dura mater was placed on a platform with a tube connecting to it. A 20 g mass was dropped from a height of 20 cm, with the tube acting as a tract, allowing the impact to land directly on the exposed surface. To avoid secondary impact following recoil, a thread was attached to the end of the mass and readily pulled. The incision was sutured using 3–0 silk and antibacterial ointment after the brain injury. The rats in group A, who had no brain injury, did not lose any weight. Before being returned to their allocated group cages, all rats were examined in a rehabilitation room.

2.2. Erythropoietin administration

Erythropoietin was purchased as human recombinant erythropoietin (Renogen) (NCPC Genetech Biotechnology Co., Ltd. P.R of China), administered as s.c. Injection, 30 min after brain injury at a dose of 30.000 U/kg.

2.3. Sample collection and examination

10–15 min after brain damage (H0), 12 h (H12), and 24 h (H24) after brain injury, 0.3 ml of blood was taken from the lateral tail vein using a 0.5 ml syringe. A sample of serum was taken for ELISA testing. There were no samples that were excluded in the study. The levels of SDF-1 (cat. No. LS-F21608) and NSE (cat. No. LS-F5577) in the blood were determined using an ELISA kit from LSbio (USA), following the manufacturer's instructions [21]. Blood samples were split into two parts: one for quantitative RT-PCR of mRNA BDNF gene expression, which was combined with L6 buffer solution and processed into nucleic acid extract, and the other for serum extraction. Before RT-PCR and ELISA test [63], all samples were kept at -80°C .

The house-keeping gene GADPH was used to quantify mRNA BDNF gene expression (Oligo, Macrogen, catalog number: OG250521). Primers are: GADPH forward: AGGTCGGTGTGAACGGATTGTG; GADPH reverse: GGGGTCGTTGATGGCAACA; BDNF forward: GAGAA-GAGTGATGACCATCCT; BDNF reverse: TCACGTGCTCAAAGTGT-CAG'. A PCR-Bio-Rad BR004129USA machine was used to perform the PCR. 22.5 μl PCR Mastermix and SYBR green QRT were used in a combination. 2.5 μl of DNA extract were added to a 22.5 μl PCR mix combination. The first stage of amplification was carried out for 2 s at 94°C , followed by 60 s at 94°C , and 45 s at 57°C , for a total of 40 cycles. According to earlier study, mRNA expression was calculated.

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2.4. Statistical analysis

Data were presented as means \pm SD. The One-way ANOVA statistical test was used to process and evaluate the data, followed by the Repeated Measure ANOVA test. Statistic significant values were determined at $p < 0.001$.

3. Results

The average weight of the rats in groups A, B, and C was 204.8 g, 203.8 g, and 205.6 g, respectively. Between the three experimental groups, there were no significant differences ($p > 0.05$).

3.1. BDNF gene expression

A summary of the results of one-way ANOVA and Repeated Measure ANOVA tests to assess the effect of EPO on BDNF gene mRNA can be seen in Table 1 and Fig. 1.

Table and Fig. 1 show that BDNF mRNA was lower in the trauma group (B and C), the mean BDNF mRNA levels in both groups were significantly lower than in group A on H-0 (10.17 ± 0.25 vs 5.74 ± 0.47 vs 6.08 ± 0.52) and no significant difference was found between groups B and C. After 12 h, these values increased in both trauma groups but were higher in group C compared to group B (7.92 ± 0.51 vs 6.45 ± 0.33), this increased continued and at H-24, where group C remained significantly higher than group B (9.20 ± 0.56 vs 7.22 ± 0.19). This means that the administration of EPO can stimulate the production of BDNF by the BDNF gene to restore the decreased BDNF during brain

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Table 1
Effects of EPO on mRNA BDNF expression in Rats with Experimental Brain Injury.

Group	mRNA BDNF Gene Expression (Mean \pm SD)			Repeated Measure ANOVA
	H-0	H-12	H-24	
(A)	10,17 (0,25) c	-	-	
(B)	5,74 (0,47) ^a	6,45 (0,22) b	7,22 (0,19) c	$p < 0,001$
(C)	6,08 (0,52) ^a	7,92 (0,51) c	9,20 (0,56) d	$p < 0,001$

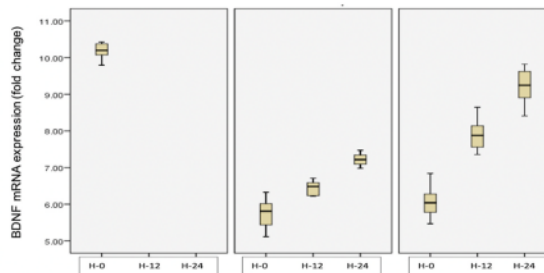


Fig. 1. Changes in the BDNF mRNA before and after EPO therapy.

injury.

3.2. Serum SDF-1 level

A summary of the results of the one-way ANOVA and Repeated Measure ANOVA tests to assess the effect of EPO on SDF-1 serum can be seen in Table 2 and Fig. 2.

Table and Fig. 2 show that the levels on H-0 serum levels of SDF-1 were lower in the trauma group (B and C), with the mean serum levels of SDF-1 in groups B and C significantly lower than in group A (12.42 ± 0.837 vs 7.15 ± 0.77 vs 6.32 ± 0.32) and no significant difference was found between group B and group C. After H-12 the value decreased in group B (from 7.15 ± 0.77 to 4.62 ± 0.58) and to (2.55 ± 0.70) on H-24, while in group C there was an increase in serum levels of SDF-1 (from 6.32 ± 0.32 to 7.56 ± 0.54), and became (11.32 ± 4.55) at H-24. This means that EPO administration increases SDF-1 serum levels several hours after brain injury to restore SDF-1 serum levels that decreased during brain injury.

3.3. Serum NSE level

A summary of the results of the One-Way ANOVA and Repeated Measure ANOVA tests to assess the effect of EPO on serum NSE can be seen in Table 3 and Fig. 3.

Table and Fig. 3 show that at H-0 the serum NSE levels in groups B and C were much higher than in group A (4.27 ± 2.17 vs 19.66 ± 2.99 vs 20.59 ± 2.45). After several hours after brain injury, group B still increased at H-12 (19.66 ± 2.99 to 29.65 ± 2.33) and became (37.31 ± 2.76) at H-24, whereas at group C actually decreased serum NSE levels from (20.59 ± 2.45) to (17.25 ± 2.02) on H-12 and to (12.14 ± 2.61) on H-24. This means that EPO administration can reduce serum NSE levels to restore serum NSE levels that increase during and several hours after brain injury.

Table 2
Levels of SDF-1 in rats with experimental brain injury.

Group	SDF-1 levels (Mean \pm SD)			Repeated Measure ANOVA
	H-0	H-12	H-24	
(A)	12,42 (0,83) d	-	-	
(B)	7,15 (0,77) ^c	4,62 (0,58) b	2,55 (0,70) ^a	$< 0,001$
(C)	6,32 (0,32) ^c	7,56 (0,54) c	11,32 (4,55) d	$< 0,001$

If there are different Superscripts in the same column, the values are significantly different ($p < 0.05$), as well as if the Superscripts are different in the same row. If the Superscript is the same, it means that the value is not significantly different ($p > 0.05$).

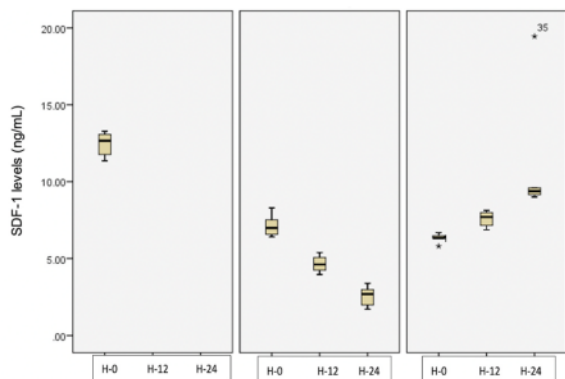


Fig. 2. Changes in the SDF-1 serum levels before and after EPO therapy.

Table 3
Levels of NSE in rats with experimental brain injury.

Group	NSE levels (Mean \pm SD)			Repeated Measure ANOVA
	H-0	H-12	H-24	
(A)	4,27 (2,17) ^a	-	-	
(B)	19,88 (2,99) ^c	29,65(2,33) ^d	37,31(2,76) ^e	< 0,001
(C)	20,59 (2,45) ^c	17,25(2,02) ^b	12,14(2,61) ^a	< 0,001

If there are different Superscripts in the same column, the values are significantly different ($p < 0.05$), as well as if the Superscripts are different in the same row. If the Superscript is the same, it means that the value is not significantly different ($p > 0.05$).

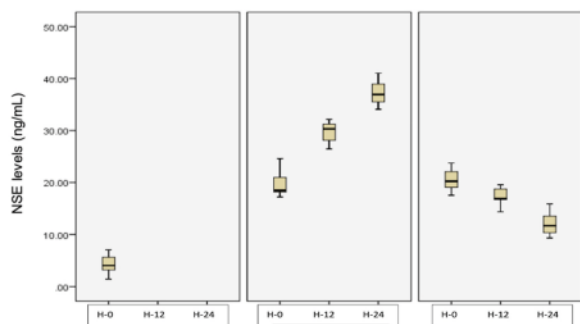


Fig. 3. Changes in the NSE serum levels before and after EPO therapy.

4. Discussion

This study assessed the effect and relationship between EPO treatment with level of SDF-1, NSE, and serum gene expression of mRNA BDNF in brain injury model. The brain injury model has been thoroughly described as one modality to study the neuroinflammation process.

The high amount of BDNF mRNA expression in group A is consistent with earlier research showing that BDNF is highly expressed in healthy brains and plays a function in excitatory and inhibitory synaptic transmission as well as activity-dependent plasticity [22,23].

BDNF mRNA expression will decrease after a stress or trauma event occurs. BDNF mRNA expression increased 1–6 h after brain damage, according to three earlier studies [24–26]. In addition, other studies reported decreased BDNF mRNA expression 20 h after brain injury and in the placebo group [27].²¹ Comparison of the treated groups illustrates

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the effect of EPO on increasing BDNF mRNA expression. The increase in BDNF mRNA expression in groups B and C were both increased at H-12 and 24 h, but there was a more significant increase in group C receiving EPO.

EPO-induced BDNF production is correlated with increased neurogenesis in response to stroke and brain injury [28]. Activation of Ca²⁺-channel voltage and recruitment of Ca²⁺-sensitive transcriptional factors promote BDNF mRNA production in neuronal cells. EPO consistently elevated intracellular Ca²⁺ and protein phosphorylation cAMP response element binding (CREB) within 5 min and 30 min, respectively [29]. These data suggest that EPO activates the CREB transcriptional pathway and increases the expression and production of BDNF mRNA, which contributes to neuroprotection [16,30].

The research data showed SDF-1 levels on H-0 in the control group (A) were higher than the brain injury group (B and C). This means that there is a decrease in SDF-1 levels after brain injury. This is in line with the study that explained that there was a decrease in SDF-1 levels in mice with TBI which showed that SDF-1 was involved in TBI pathology [31].

On H-12 and H-24, there was an increase in SDF-1 levels in group C, while there was a decrease in SDF-1 levels in group B. These results indicate that the administration of EPO after brain injury has a positive effect on increasing SDF-1 levels. This is consistent with the previous theory that EPO enhances neuroprotective and angiogenic effects in its role in hematopoiesis [32].

To the best of our knowledge, no studies have directly examined the direct relationship between EPO administration and serum level of SDF-1. TBI causes hypoxia in brain lesions, which affects the expression of angiogenic growth factors such as SDF-1, according to previous studies [33].

Pretreatment with EPO prevents high hyperglycemia-induced neuronal tubular cell death by inhibiting excessive ROS production, reversing the effect of high glucose on Bcl2, Bax, and caspase-3 expression, and reversing the effect of high glucose on Bcl2, Bax, and caspase-3 expression. Through anti-oxidative stress, EPO can block the pro-apoptotic effect of high hyperglycemia, according to this study [34].

Upregulation of hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) in the bone marrow due to changes in the bone marrow microenvironment [35]. The mechanism by which ischemia induces increased ROS and hypoxic expansion in bone marrow is not fully understood. As a result, Nox2-derived ROS cause hypoxia expansion and HIF-1 expression in the bone marrow microenvironment, while ROS from ischemic tissue promote stem and progenitor cell mobilization [36].

Another study found that hypoxia-induced SDF-1 expression was important for selective cell repair and migration of CXCR4 progenitor cells to ischemic tissue, and that inducing SDF-1 expression via HIF-1 might direct regenerative progenitor cells to damage sites [37].

EPO binds to its receptor in hematopoiesis and activates the JAK/STAT5 signaling pathway [38]. EPO is a crucial growth factor that stimulates BMSC recruitment and further induces bone formation and angiogenesis. Since it was shown to be a regulator of erythropoiesis. In particular, SDF-1 has been reported to play a role in BMSC migration [39]. Kaigler et al. stated that BMSC will secrete sufficient amount of VEGF to promote growth and differentiation of endothelial cells in an *in vitro* model of angiogenesis [40]. SDF-1 expression of infarcted tissue is stimulated by overexpression of VEGF in human BMSCs, resulting in enormous mobilization and placement of BMSCs and cardiac stem cells, which is advantageous for infarct size reduction [41].

Other studies have found that EPO mobilizes BMSCs to the lesion site after TBI and increases their anti-apoptotic impact by modulating the SDF-1/CXCR4 axis expression [42].

NSE is not secreted normally, but when axon damage occurs, there will be an increase in NSE for homeostatic processes.³⁷ Therefore, NSE is the only direct marker of nerve cell damage [43]. Increased levels of NSE are also hazardous, as they can induce death in nerve cells by stimulating the release of pro-inflammatory cytokines (TNF- α , IL-6, and

IL-1 β). On monocytes, 46 macrophages, microglia, and astrocytes, surface endothelial cells can produce nitric oxide (NO), reactive oxygen species (ROS), and inflammatory cytokines (IL-1 β , TGF- β , and TNF- α) and chemokines (MIP-1 α and MCP-1) [44]. 14

This study showed that NSE levels were found to be considerably higher in the brain injury group (B and C) compared to the control group (A). Significant improvement continued in group B compared to group C. In most TBI, especially severe cases, NSE will remain high or tend to increase as a result of secondary brain injury. In severe cases, NSE levels will continue to increase. Therefore, NSE not only describes the level of nerve damage, but also assesses the progression of nerve damage due to TBI, so that NSE is a potential biomarker to assess prognosis and therapeutic indicators in neurology intensive care [43,45].

The administration of EPO that reduced NSE levels in the brain injury group (group C) showed that there was an improvement in nerve cell damage after brain injury. When compared with group B, this is in accordance with previous studies that there will be a persistent increase in NSE levels after brain injury.

A limitation of this study 61 was that the study time of mRNA BDNF expression, protein levels of SDF-1, and protein levels of NSE were only 24 h, so the long-term effects of EPO could not be evaluated. The administration of EPO was only given 1 dose so there was no comparison when given in several doses.

5. Conclusion

EPO may have neuroprotective and anti-inflammatory properties in TBI by increasing mRNA BDNF expression and serum SDF-1 levels, and decrease serum NSE levels. 32

Provenance and peer review

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amsu.2021.102877>. 25

Please state any conflicts of interest

The authors declare that they have no conflict of interests. 49

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Ethical approval

All procedure for Animal experiment has been approved by Ethics Commission Faculty of Medicine, Hasanuddin University Number: 408/UN4.6.5.31/PP36/2021.

Consent

This manuscript does not involve human participants, human data, or human tissue. 7

Author contribution

MFS, AAI, PR, MNM, and MH initiated and designed the study. IP performed the statistical analysis. NS, WA, HC, and RAN contributed in the data processing. All authors have read and approved the final manuscript.

Registration of research studies

This study was not involving human participants. Registration was not applicable.

Guarantor

Muhammad Fadli Said, Andi Asadul Islam, Muhammad Nasrum Massi, and Mochammad Hatta.

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