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Original Article

Secretome from hypoxic mesenchymal stem cells as a potential therapy for ischemic stroke: Investigations on *VEGF* and *GFAP* expression

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Abstract

Ischemic stroke is a sudden onset of neurological deficit resulting from a blockage in cerebral blood vessels, which can lead to brain tissue damage, chronic disability, and increased risk of mortality. Secretome from hypoxic mesenchymal stem cells (SH-MSC) is a potential therapy to improve neurological deficit by increasing the expression of vascular endothelial growth factor (VEGF) and reducing glial fibrillary acidic protein (GFAP). These effects can reduce the infarction area of ischemic stroke. Therefore, the aim of this study was to analyze the effect of 150 µL and 300 µL SH-MSC injection on *VEGF* and *GFAP* expression as well as the improvement of infarction area in ischemic stroke animal model. A post-test-only experimental design with consecutive sampling was used, with *Rattus norvegicus* as subjects. Stromal mesenchymal stem cells (S-MSCs) were isolated from the umbilical cords of rats at 21 days of gestation. Secretome production by the S-MSCs was induced under a hypoxic condition, and subsequently isolated. The resultant secretome was administered to rats subjected to middle cerebral artery occlusion (MCAO) at doses of 150 µL (P1 group) and 300 µL (P2 group). The results showed that the infarction area was reduced in P1 (p<0.001) and P2 groups (p<0.001). SH-MSC at a dose of 300 µL increased the expression of *VEGF* (p=0.028) and reduced the expression of *GFAP* (p=0.001). In conclusion, secretome from hypoxic S-MSC could potentially improve ischemic stroke by upregulating *VEGF* expression and downregulating *GFAP* expression.

Keywords: *GFAP*, ischemic stroke, secretome, stem cell, *VEGF*

Introduction

Stroke is a focal or global neurological deficit of sudden onset, with symptoms often lasting more than 24 hours, due to vascular injury (infarction or hemorrhage) of the central nervous system. The lack of oxygen to the brain is caused by the blockage of blood vessels that leads to neuronal cell death [1]. An epidemiological study conducted from 1990 to 2016 identified stroke as the second leading cause of both death and disability worldwide [2]. Approximately 13.7 million people are affected, and 5.5 million die each year due to stroke. The incidence of ischemic stroke was estimated at 87% of the total stroke cases, and the prevalence increased significantly between 1990 and 2016 [1]. According to Indonesian Basic Health Research data from 2018, the stroke

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prevalence in Indonesia among those aged >15 years is 10.9 per mile. The highest rate is in East Kalimantan and the lowest is in Papua [3].

Proper management has been advanced in all phases of ischemic stroke. Recent advancements in the acute phase include neuroimaging, tenecteplase use, and endovascular thrombectomy [4]. To maximize the benefits of rehabilitation therapy, medical professionals and rehabilitation therapists focus on developing a wide range of therapeutic approaches, such as physical therapy, robotic assistance, and artificial intelligence [5]. A study on exergame-based rehabilitation therapy has also been conducted [6]. Intravenous thrombolysis and endovascular recanalization are the standard treatments for ischemic stroke in the acute phase (<4.5 hours). These treatments open the blockage of blood vessels and save the penumbra area. However, the recanalization treatment is difficult, and only 3,2–5,2% of ischemic stroke patients qualify for intravenous thrombolysis [7]. Endovascular interventional therapy is limited to patients, as it is only applicable to significant arterial occlusions. For patients who are not qualified to receive thrombolytic treatment or thrombectomy, the rates of impairment are significantly higher.

Rehabilitation therapy has a limited curative impact even though it helps patients regain neurological function [8]. Recently, therapy based on stem cells has become a new approach to treating various diseases [9] including ischemic stroke. Clinical trials using stem cells have been carried out, and the results showed the improvement of neuroprotective qualities through increased trophic factor release, a more potent anti-inflammatory impact, and enhanced neuro-/angiogenesis [10]. Furthermore, stem cell therapy has double effects on animal models, such as prolonging the penumbra period (acute phase), suppressing the pro-inflammatory effect (subacute stage) and initiating neuro/angiogenesis (chronic phase). These three phases prove the effectiveness of mesenchymal stem cells (MSCs) as a treatment [10].

The use of secretome is interesting because it is capable of crossing the blood-brain-barrier (BBB), has no first-pass metabolism effect, lowers microvascular thrombosis risk and produces convenient extracellular vesicles/secretome on a large scale [11]. According to several studies, secretome-mesenchymal stem cell (S-MSC) is a potential therapy to regenerate brain cell injury [11,12]. S-MSC increases *VEGF* expression which provides effects of neurogenesis, angiogenesis and neuroprotectors after focal cerebral ischemia [13]. It also induces microglia changes from a neurotoxic phenotype that releases a pro-inflammatory molecule to a neuroprotective phenotype. This occurs by producing an anti-inflammatory molecule through CX3-CL1 release and decreasing the expression of microglia activating markers (ED1 and Iba) as well as the proliferation of astrocytes that causes the reduction of *GFAP*, often found in neuronal apoptosis in the brain injury process [14]. Based on the available evidence, S-MSC given to animal models of ischemic stroke can stimulate sensorimotor symptoms improvement and infarction area reduction [15,16]. A few methods can be used to create S-MSC, including the hypoxia-conditioned method. Secretome under the hypoxic condition (5% oxygen) induces increased cell migration, proliferation, viability and in vitro angiogenesis. Growth factors such as thrombocyte-derived growth factors, hepatocyte growth factors, placenta growth factors and vascular endothelial derivative growth factors are regulated under hypoxia condition (SH-MSC) [17]. However, the specific role of SH-MSC in ischemic stroke has not been reported. Therefore, the aim of this study was to analyze the effect of 150 µl and 300 µl of secretome from SH-MSC injection on *VEGF* and *GFAP* expression as well as the improvement of infarction area in Wistar rats (*Rattus norvegicus*) with ischemic stroke.

Methods

Study design and setting

A post-test-only experimental study was conducted at the Stem Cell and Cancer Research Laboratory (SCCR) and the Animal House Integrated Biomedical Laboratory Facility, Medical Faculty of Universitas Islam Sultan Agung, Semarang, Indonesia, for 12 months spanning from June 2023 to June 2024. A total of 24 Wistar rats (*Rattus norvegicus*) were selected by consecutive sampling with inclusion criteria. The sample size was calculated using the Federer formula for experimental study design with minimum error degrees of freedom being 15. The

surgery was performed on rats by clamping the common carotid artery (CCA) to induce middle cerebral artery occlusion (MCAO).

Animal selection and randomization

This study used the Wistar rats (*R. norvegicus*) from Animal House Integrated Biomedical Laboratory Facility Medical Faculty of Universitas Islam Sultan Agung, Semarang, Indonesia. The sample size consisted of 24 adults male *R. norvegicus,* weighed 200–250 g, aged 12–16 weeks, healthy and physically active. The exclusion criteria were aggressive rats that attacked the others, could not survive until the end of this study and those declared diseased. The rats were acclimatized for seven days in the 37 × 40 × 18 cm cages, each containing two to three rats, at a room temperature of 25°C. Standard food (AIN-76A) was given with water ad libitum, as well as 12 hours of light intensity. Furthermore, the rats were divided into four groups, including the sham group (healthy rats), control group (MCAO), P1 group (MCAOs + 150 µl SH-MSC) and P2 group (MCAO + 300 µL SH-MSC). Randomization into control and treatment groups was carried out using a manual randomization method. Each rat was given a unique identification number and randomized through a paper lottery.

Mesenchymal stem cell (MSC) isolation and secretome analysis

In this study, 21-day-old pregnant rats were anesthetized using lethal dosage anesthesia with 10 mL of cocktails containing ketamine 50 mg/kg BW, xylazine 10 mg/kg BW and acepromazine 2 mg/kg BW injected intramuscularly. Umbilical cords (UC) were collected and stored in a petri dish containing 0.9% NaCl using a tweezer. Washing was performed with PBS (Gibco BRL, Grand Island, NY, USA), and then the UC was cut into smaller pieces (1 mm), and placed on a T75 flask (Corning, Life Sciences, USA). Complete media consisting of Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, New York, USA), Fungizone®, Penicillin-Streptomycin, and 10% Fetal Bovine Serum (FBS) (Givco BLL, Grand Island, New York, USA) were added to cover tissues of about 3 mL. Subsequently, incubation was performed in incubators at 37°C and 5% CO2. Cell maintenance was carried out until the cells reached 80% confluency.

Successfully cultivated cells from the UC were validated and characterized according to S-MSC using flow cytometry techniques with CD44, CD73, CD 90 and CD 105 markers. To validate the flow cytometry method, 1 × 10^7 cells/ml were released from the flask using BDTM AccutaseTM Cell Detachment Solution. The cells were then washed with phosphate-buffered saline (PBS) cells and placed in a 5 mL Falcon tube. Finally, flow cytometric was performed using 1–5 tubes as a control to set up the cytometer.

The T75 flask contains a fourth passage S-MSC with a 95% confluence placed in the hypoxia chamber. The hypoxia chamber was discharged with CO2 gas and the O2 content was measured using DO meters to reach 5%. The S-MSC in the hypoxia chamber was supplied in the incubator at a temperature of 37°C for 24 hours. The gas content was normalized after 24 hours of incubation. The hypoxia medium was collected and inserted into a 50 mL conical tube for filtration. S-MSC was then stored at -80°C until further analysis. The levels of cytokines and growth factors in the secretome under hypoxia conditions were assessed using an enzyme-linked immunosorbent test (ELISA) following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). The VEGF, PDGF, FGF, TGF-β, IL-10 and IL-6 assays were conducted at room temperature. The outcomes were evaluated using a microplate reader (Bio-Rad, California, USA) at a wavelength of 450 nm.

Isolation of RNA and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Rats were euthanized on the fifteenth day, and the brain tissue was examined using hematoxylin and eosin (H&E) staining. Subsequently, *VEGF* and *GFAP* expression were evaluated, followed by data processing and analysis. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the expression of mRNA in VEGF and GFAP from the brain. Total RNA was extracted from the cells using the TRIzol (Invitrogen, Thermo Fisher Scientific, Canada, USA) reagent according to the factory protocol. Approximately 25 µl of reaction volume consisted of 12.5 µl PCR buffer 2× for KOD FX (PCR amplification enzyme), 5 µl 2 mM dNTPs, 2 µl primer, 0.1 µl KOD, 2.4 µl water and 1 µl DNA. The standard conditions for PCR included 95°C for 2

minutes, followed by 40 cycles at 95°C for 30 seconds, 62°C for 1 minute, and the final extension at 72°C for 5 minutes. The 2–ΔΔCt method was used to determine the relationship of the target gene expression between the experimental and control groups. The primer sequences of *VEGF* and *GFAP* were as follows: VEGF: forward 5'-GTACCTCCACCATGCCAAGT-3' and reverse 5'-AATAGCTGCGCTGGTAGACG-3'; GFAP: forward 5'-GCGAAGAAAACCGCATCACC-3' and reverse 5'-TCTGGTGAGCCTGTATTGGGA-3'.

Infarct volume analysis

The post-ischemic tissue was examined after processing paraffin-embedded samples for H&E staining. All preparations were visualized and evaluated under a light microscope, followed by a histopathological evaluation of all samples. Triphenyltetrazolium chloride (TTC) staining examination of a coronal slice from the rats' brains showed that the white parts indicated the infarction area and the red part represented the normal area. The tissue was photographed using the Z1 slide scanner axio scan camera system (Carl ZEISS, Oberkochen, Germany) with a 20×/0.8 objective lens, processed and exported using ZenBlue software (Version 3.5, Carl ZEISS, Oberkochen, Germany). The file was transferred to J image software. The infarct lesion was measured in pixels and would be converted into percentages using J images software [18,19].

Statistical analysis

Data obtained from this study were processed, edited, tabulated and tested for normality distribution using the Shapiro-Wilk test. When the data were distributed normally, a one-way ANOVA test was carried out (95% significant level), followed by the post-hoc LSD test. However, when data were not distributed normally, transformation was first carried out, and if data remained non-normal, the analysis was continued with the non-parametric Kruskal-Wallis and Mann-Whitney tests to observe the differences between groups. Data analysis and processing were performed using SPSS version 26.0 (IBM, New York, USA).

Results

MSC characterization and secretome

The S-MSC specimen with 80% confluence after the fifth passage expansion showed cells attached to the base of the flask with spindle-like cell morphology under microscopic examination. The specimen incubated in a hypoxia chamber with 5% O2 for 24 hours showed a greater homogeneous spindle-like cell morphology (**Figure 1**). MSC flow cytometry analysis showed that the cells expressed specific markers of S-MSC, including positive expression of CD90 (97.6%), CD29 (97.7%), as well as negative expression of CD45 (1.5%) and CD31 (3.2%) (**Figure 1**). This was in line with the 2006 International Society of Cellular Therapy standards [20,21]. The differentiation test showed that S-MSC differentiated to osteogenic cells characterized by the deposits of red-colored calcium on the Alizarin Red staining, as well as adipogenic cells marked by the image of oil dots on the Oil red O staining (**Figure 1**).

Secretome content analysis

After 24 hours, the culture media was collected and filtered using tangential flow filtration (TFF) to obtain SH-MSC. The biomolecular content profiles are presented in **Table 1**. SH-MSC consisted of VEGF, PDGF, FGF, IL-10, TGF-β and IL-6. The results showed that VEGF was present in higher amounts compared to the other soluble molecules in the secretome.

Table 1. The value of soluble molecules of secretome from a hypoxic-mesenchymal stem cell (SH-MSC)

Molecules	Mean±SD (ng/mL)
Vascular endothelial growth factor	1,228.86±27.71
Platelet-derived growth factor	1,043.06±24.49
Fibroblast growth factor	1,085.34±28.92
Interleukin-10	415.02±12.14
Transforming growth factor-β	282.83±9.28
Interleukin-6	323.99±10.04

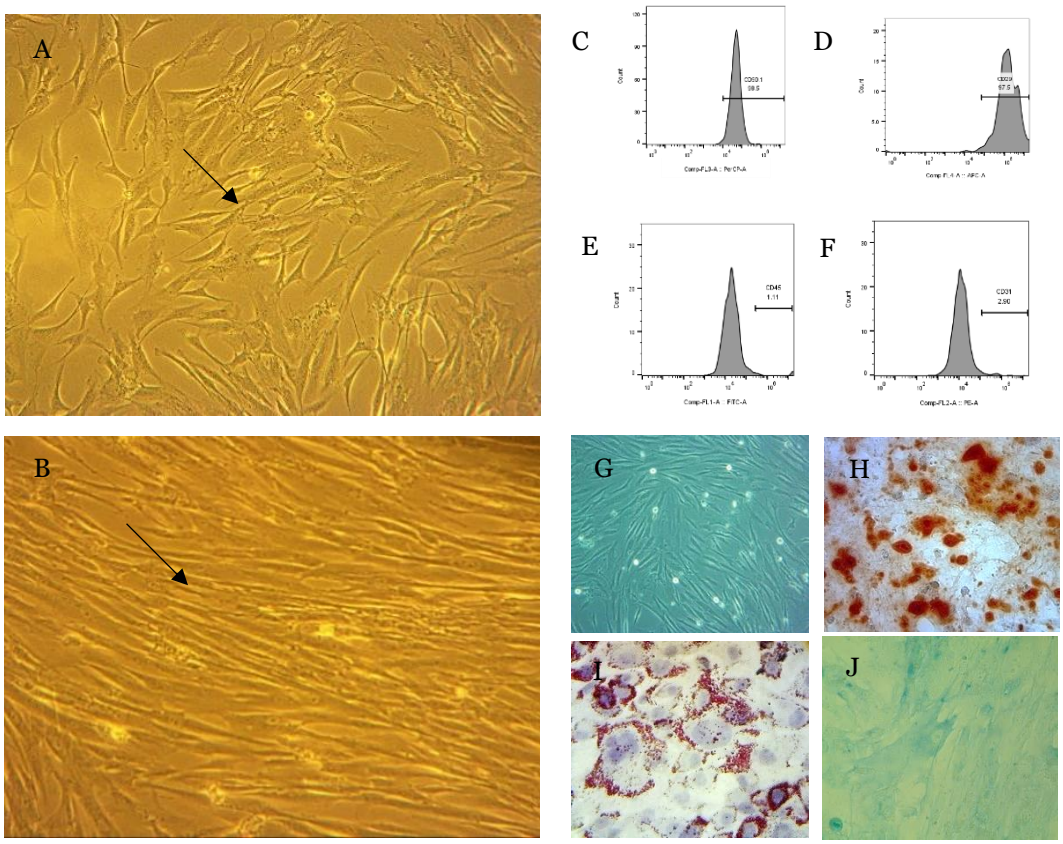

Figure 1. Secretome mesenchymal stem cell (S-MSC) isolation of 80% confluence with 100× magnification shows a spindle-like shape indicated by the arrow (A) and greater homogeneous spindle-like morphology in hypoxia condition with 200× magnification by the arrow (B). Flow cytometry histograms showing secretome from hypoxic MSC (SH-MSC) expression of CD90.1 (C) and CD29 (D), and lack of CD45 (E) and CD31 (F). Spindle-like morphology with 10× magnification (G). The color red denotes osteogenic differentiation, which is reflected by calcium deposition on the SH-MSC culture (H). The color red denotes adipogenic differentiation, which is reflected by lipid deposition in the SH-MSC culture (I). The color blue denotes chondrogenic differentiation in the SH-MSC culture (J).

Effects on *VEGF* and *GFAP* expression

Expression of *VEGF* and *GFAP* following the SH-MSC treatment in the ischemic rat model is presented in **Figure 2**. The induction of MCAO resulted in a significant decrease in *VEGF* (p=0.004) and an increase in *GFAP* expression (p=0.011). Higher expression of *VEGF* was observed in P2 compared to control (p=0.028) and healthy groups (p=0.047). As for the *GFAP*, expression was reduced significantly in P2 group (p=0.001), similar to the healthy group (p=0.261).

Tetrazolium chloride (TTC) staining

TTC staining was observed through the software images on the 15th day to evaluate the infarction area in all groups (**Figure 3**). The examination showed that the white part in the coronal slices of rats' brains indicated the infarction area, while the red part represented the normal area. Infarction area was also found in the sham group due to the decapitation process that occurred until the measurement of the infarct lesion. This decapitation process caused hypoxic conditions that led to the occurrence of infarct lesions in the rats' brains without MCAO (**Figure 3**). The infarction area significantly increased following the MCAO induction (p=0.001) (**Figure 2**). Compared to the control group, the infarction area was significantly reduced in P1 and P2, with p-values of 0.001. There was no significant difference in the infarction area between P1 and P2 (p=0.587) (**Figure 2**).

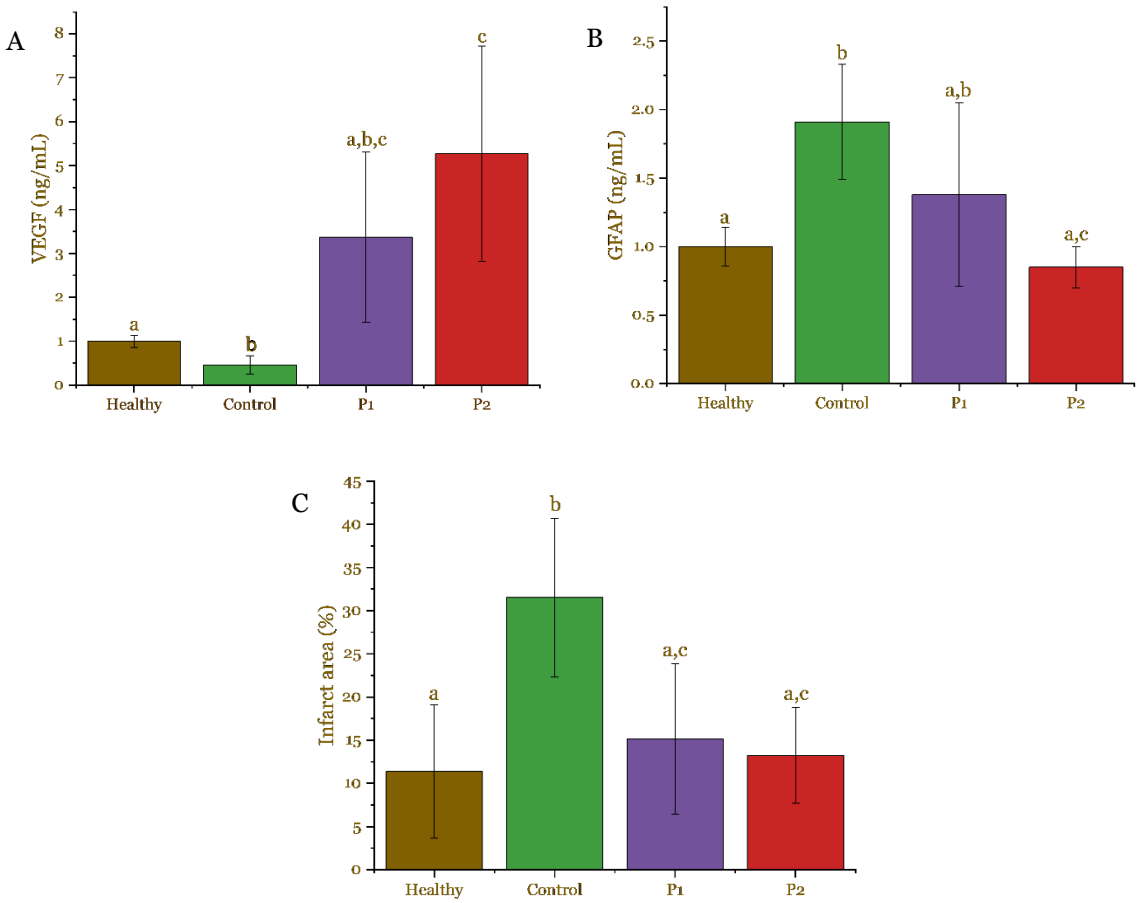

Figure 2. Effects of secretome from hypoxic mesenchymal stem cell (SH-MSC) on the expressions of *VEGF* (A) and *GFAP* (B) and infarction area (C) in rats with middle cerebral artery occlusion (MCAO). *Significantly different at $p<0.050$.

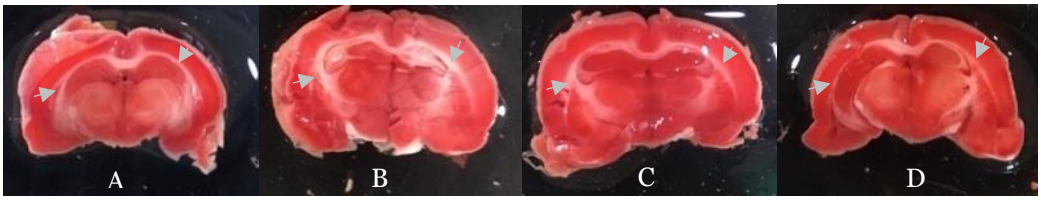

Figure 3. Triphenyltetrazolium chloride (TTC) staining in software images. Sham group with 11.36% infarction area (A), control group with 31.52% infarction area (B), P1 group with 15.15% infarction area (C), and P2 group with 13.26% infarction area (D). Gray arrows show infarction area of the brain appears as pale staining on the slice.

Discussion

The results suggested that *VEGF* expression significantly increased after SH-MSC treatment. VEGF is a specific mitogen of the endothelial cells and has the strongest specificity, in the role of the regulator of angiogenesis. SH-MSC has leukocyte characteristic that expresses the TLR 3 and CXCR3-R receptors capable of detecting the SDF-1, TNF-α and IFN-γ (released by cell injury and inflammation), leading to transmigration to the injury site, a process known as "homing." The paracrine mechanism secretes VEGF and the other growth hormones, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), TGF-β, nerve growth factor (NGF), IL-6 and IL-10 [22,23]. These are all important for vascularization, apoptosis inhibition, neurogenesis, synaptic function and angiogenesis stimulation of endothelial cells under hypoxic conditions. Based on the results, the model of MCAO injected with MSC intravenously 24 hours after onset, showed that neurotropic values of VEGF and Ang1/Tie2 expression increased on the 1st to 7th day after injection [24]. The treatment increases the capillary formation of endothelial cells in the brain, thereby triggering an angiogenesis response to accelerate tissue repair processing. Aside

from the secretion of pro-angiogenic factors such as IL-8, IGF-1 and VEGF, SH-MSC also activates angiogenic properties in endothelial cells. Angiocrine MSC in peripheral blood enhances the endothelium functional characteristics by triggering the *VEGF* signaling pathway through endothelin-1, platelet-derived growth factor-AA (PDGF-AA), and IGF-2. A previous study reported that *VEGF* expression significantly increased in the treatment group that was given MSC compared to the control (p<0.05) [25]. SH-MSC, through *VEGF*, increases the BrdU+ value in the microvessel to trigger angiogenesis in the peri-infarct cortex [25]. Furthermore, a previous study showed that SH-MSC was more potent in regenerating damaged tissues than MSC normoxic-conditioned, due to the ability to express higher Heat Shock Protein (HSP) molecules than normoxia [26]. The VEGF value (26,663.89 ng/L) in the SH-MSC was higher than in normoxia (1,577.88 ng/L) because the hypoxic condition induces the expression of VEGF mRNA. It is the target of the hypoxia-induced factor (HIF) transcription pathway through the VEGF receptor activation and helps recovery of tissue damage [26,27].

This study also showed that *GFAP* expression between P2 and the control group significantly decreased (p=0.001) after SH-MSC treatment. GFAP is a monomeric filament of cytoskeletal protein that appears in astroglial cells in the white and gray matter of the brain. In brain injury conditions, GFAP is increased and secreted passively by damaged/dead astrocytes into the peripheral blood circulation through the leak of BBB. Various mechanisms of SH-MSC in reducing *GFAP* expression have been reported. It decreases the expression of microglia activating markers (ED1 and Iba) and the proliferation of astrocytes from a neurotoxic phenotype that releases a pro-inflammatory molecule to a neuroprotective phenotype by producing anti-inflammatory molecules through the releases of CX3-CL1, causing a decrease in *GFAP*. SH-MSC also induces the secretion of IL-6 and decreases the expression of TNF-α and IL-1 pro-inflammatory cytokines by inhibiting the activation of acid-sensing ion channels (ASICs) and reducing the inflammation response [16], while enhancing the signal on the Wnt/β-catenin pathway. Therefore, it decreases cell apoptosis and *GFAP* expression after ischemic stroke [28]. The decreased neuronal apoptosis causes the reduction of Bax and Kaspase-3 expression as well as an increase in Bcl-2 expression that improves cerebral ischemic condition [29,30]. Other studies have also shown a significant decrease in GFAP expression in rats with MCAO treated using MSC compared to the control group (p<0,001) [29,31]. In the previous study, histological staining of rats' brains with ischemic showed an increase in GFAP in the core of the lesion [32]. After the MSC injection, there was a reduction of *GFAP* expression in the ischemic area after the 7th day. This action can be attributed to stem cells that inhibit the astroglial pathway in neural progenitor cells and decrease astrogliosis of ischemic astrocytes due to the anti-inflammatory and immunomodulatory effects of MSC [32].

The results suggested that the infarction area after SH-MSC injection significantly decreased (p=0.001). Similar results were also shown by Zheng *et al*. where rats' brain infarction area on the 14th day with MCAO + S-MSC treatment significantly decreased compared to the treatment of MCAO + MSC treatment. Asgari Taei *et al*. showed that infarction area in rats' brains treated with human embryonic MSCs significantly decreased (p<0,001) [25]. The reduction in infarction area after MCAO and MSC exosome treatment also showed statistically significant (p<0.050) [33]. In this study, the reduction of infarction area was because SH-MSC increased VEGF expression, thereby affecting proliferation, migration and endothelial cell formation in blood vessels. VEGF triggers the inhibition of apoptosis, causes the reduction of GFAP, and also accommodates the migration of neuroblasts to the infarction zone, which affects the process of angiogenesis, neurogenesis, and neuronal survival in rats' brains with ischemic stroke.

The strength of this study is the application and observation of the SH-MSC treatment in an ischemic model to evaluate *VEGF* and *GFAP* expression through RT-PCR, histochemistry and infarction area measurement. This is the first study that examined the expression of *VEGF* and *GFAP* with 2 doses of SH-MSC at once to assess the neuronal survival through the infarction area reduction in rats' ischemic model. However, this study also had several limitations, including the absence of strain examination on the rats as well as the use of blinding procedures during data analysis and treatment. *VEGF* and *GFAP* were only assessed at the gene expression level, without measuring their concentrations. The levels of other inflammatory molecules that play important roles in angiogenesis and neurogenesis associated with neuronal survival in ischemic stroke were

not evaluated. This study was conducted over a period of only 15 days, making it impossible to monitor the long-term effects of SH-MSC administration. Moreover, repeated dosing was not implemented, which may have been necessary to achieve maximum reduction in the infarction area. Future studies should monitor the long-term effects of SH-MSC administration while addressing limitations, including randomization, blinding to reduce bias, as well as measurement of *VEGF* and *GFAP* concentrations.

Conclusion

The administration of 300 µL SH-MSC caused a reduction in the infarction area of a rat model, suggesting the potential as a treatment for ischemic stroke. This effect was likely mediated through the modulation of *VEGF* and *GFAP* gene expression. Further studies should administer SH-MSC treatment directly to ischemic stroke patients to evaluate the benefits in humans.

Ethics approval

This study was approved by the Ethical Committee of Universitas Sumatera Utara, Medan, Indonesia (No. 69/KEPK/USU/2024).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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