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SECRETOME OF HYPOXIA-PRECONDITIONED MESENCHYMAL STEM CELLS PROMOTES RECOVERY IN A RAT MODEL OF ACUTE ISCHEMIC STROKE

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ABSTRACT

Stroke is the second leading cause of disability in the world, for which alternative and effective therapies are urgently needed. Mesenchymal stem cells (MSCs) show promise for the treatment of stroke after ischemic injury and subsequent studies indicate that hypoxia MSCs-derived secretome (S-MSCs) can replace MSCs advantageous effects. The impact of S-MSCs for treating acute ischemic stroke in rats was assessed in this study. Rats underwent a permanent common carotid artery (CCA) ligation to replicate ischemic cerebral stroke. Six hours following stroke, S-MSCs (300 µL; n = 8) were injected into tail vein and compared to control (ischemic stroke + 300 µL NaCl; n = 8) and healthy rats. Modified neurological severity score (mNSS) was used to assess behaviour. Brain injury was assessed by histopathological examination. Immunohistochemical analyses on the brain was used to evaluate CD31 and CD105. The level of interleukin-10 (IL-10) in serum was analysed using enzyme-linked immunosorbent assay (ELISA). We found that S-MSCs can significantly improve mNSS seven days following S-MSCs treatment and optimum at day 21. S-MSCs can significantly enhance CD31, CD105 and IL-10 expression at day 21 compared with control. S-MSCs also significantly improve neuron cells recovery of brain ischemia. Overall, our results demonstrate S-MSCs therapeutic benefits for acute ischemic stroke in rats.

KEYWORD: Acute Ischemic Stroke, Permanent Middle Cerebral Artery Occlusion, Secretome, Umbilical Cord-Mesenchymal Stem Cell.

1. INTRODUCTION

Stroke is one of the primary contributors to mortality and incapacity in adults.[1] In 2019, there are more than 10 million new cases of cerebral stroke worldwide, with over 6 million fatalities. Almost 90% of strokes are ischemic strokes.[1] On average, stroke survivors live for 6 to 7 years, with over 85% of patients surviving beyond the first year, often experiencing long-term disability and neuropsychiatric issues.[2,3] When reaching the chronic phase of stroke, the patient's well-being is impacted, and there are noticeable burdens on both the family and society. Currently, there are no successful medications or treatments for chronic stroke; primary therapy consists of rehabilitation or supportive measures to avoid significant tissue damage and ongoing decline.[4] Hence, it is crucial to discover a novel strategy for managing acute ischemic stroke.

To date, mesenchymal stem cells (MSCs) are considered one of the leading options for restorative therapy.[5] According to prior studies, umbilical cord derived MSCs (UC-MSCs) transplantation to the striatum, spinal cord, cerebral cortex, hippocampus, and cerebellum has been shown to be a successful treatment for Parkinson's disease, spinal cord injury, acute stroke, epilepsy, and cerebellar atrophy.[6–11] A recent study has also found that MSCs can effectively reduce strong proinflammatory cytokines and repair significant tissue damage when exposed to low oxygen levels, by releasing various anti-inflammatory cytokines and growth factors.[12] Soluble molecules released by H-MSCs may serve as an alternative approach to promote recovery on ischemic stroke. Treating MSCs with hypoxia precondition can increase the secretion of their active soluble molecules, referred to as secretome of hypoxia preconditioned-MSCs (S-MSCs), such as interleukin-10 (IL-10), transforming growth factor-ß (TGF-ß), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF). These molecules are beneficial in reducing inflammation, angiogenesis, and enhancing tissue repair.[13,14]

In previous findings, middle cerebral artery ligation was performed in rats for 90 minutes followed by reperfusion to monitor cerebral cortex changes. There was severe edema in the cerebral cortex from day 1 to day 2 after the stroke. Moreover, 24 hours following stroke, or during acute phase, MSCs transplantation into the rat's damaged area of the brain's cerebral cortex notably reduced the amount of tissue loss and enhanced its movement ability. However, some of the therapeutic effects of transplanted MSCs were due to the paracrine signaling factors secreted by the implanted MSCs, including anti-inflammatory cytokines and growth factors.[11] In this study, permanent common carotid artery (CCA) ligation was employed, and we investigated the impact of S-MSCs injection for treating acute ischemic stroke in rats.

While previous studies have broadly utilized MSC transplantation or raw conditioned medium, this study introduces a novel approach by employing a tangential flow filtration (TFF)-based isolation strategy to obtain a molecularly defined S-MSCs preparation from hypoxia-preconditioned UC-MSCs.[11] Unlike prior single-endpoint studies, we specifically characterized the cytokine profile of this preparation, notably VEGF, IL-10, and IFN-γ, and also linked these molecular constituents to *in vivo* therapeutic outcomes in a permanent common carotid artery (CCA) ligation model.[11] Furthermore, to provide a comprehensive understanding of its efficacy, we conducted a multi-outcome assessment within a single experimental cohort. This encompassed evaluations of behavioral recovery using the modified neurological severity score (mNSS), anti-inflammatory responses via serum IL-10 levels, vascular remodeling through CD31 and CD105 expression, and neuronal survival via histological examination.[11] Ultimately, this study aims to fill the existing gap in cell-free regenerative stroke therapies by establishing a clear link between a highly defined S-MSCs secretome and multifaceted neurorestorative mechanisms.

2. MATERIALS AND METHODS

2.1. Animals

Male adult Wistar rats (*Rattus norvegicus*) were utilized for the *in vivo* ischemic stroke model, while female pregnant rats were used exclusively as the source for umbilical cord-derived mesenchymal stem cells (UC-MSCs) isolation. All procedures were conducted following approval from the Ethics Review Board and Committees, Faculty of Medicine, Universitas Diponegoro (No. 31/EC-H/KEPK/FK-UNDIP/IV/2023). The Investigators conducting functional evaluation, as well as molecular and histological studies, were kept unaware of the treatment groups. The rats were accommodated in the Animal Model Research Center, Stem Cell and Cancer Research (SCCR) Indonesia under a 12-hour light-dark cycle had free access to food and water.

2.2. Cerebral ischemia animal model

The permanent CCA-ligation was employed for the induction of ischemic stroke in male adult Wistar rats. Rats weighing 250–300 g were anesthetized. with ketamine hydrochloride (100 mg/kg) and xylazine (5 mg/kg). Heating pads were used to keep

the rectal temperature at 37°C throughout the surgical procedure. Permanent CCA-ligation was performed following previous study.[15] Briefly, a 2 cm midline incision was made to expose the left CCA by separating the overlying tissue and the vagus nerve. Next, a 4-0 surgical nylon suture was placed into the left CCA. The thread remained in position until the rats were euthanized.

2.3. Preparation of MSCs from umbilical cord

UC-MSCs isolation was performed as described previously.[12] The umbilical cord (UC) was obtained from 19-day pregnant rat. The UC was mechanically dissected and cultured in Dulbecco's Modified eagle medium (DMEM) low glucose (Gibco, NY, USA) containing 10% Fetal bovine serum (FBS) (Gibco), 1.5% penicillin/streptomycin (Gibco), and 0.25% amphotericin B (Gibco) and incubated at 37°C and 5% CO_2. The medium was changed every three days. UC-MSCs at the 5th passage and under 80% confluence were employed for subsequent studies. The cells were characterized by cluster of differentiation (CD) surface markers using flow cytometry (BD Accuri C6 Plus Flow cytometer, BD Biosciences, San Jose, USA). The cells were stained with rat monoclonal antibodies against CD90-FITC, CD29-PE, CD31-perCP, and CD45-APC (BD Bioscience, CA, USA) by incubating them for 20 minutes in the dark at room temperature to label the cell surface markers.

2.4. Hypoxia preconditioning in MSCs

To create a hypoxic environment, UC-MSCs that reached 80% confluence were placed in a hypoxic chamber (Stem Cell Technologies). A 5% oxygen concentration was employed to the chamber and monitored by oxygen controller (BioSpherix, Lacona, NY, USA). The cells were kept in the chamber for 24 hours at 37°C and 5% CO_2. Following the incubation, the conditioned medium (CM) was collected.

2.5. S-MSCs preparation and profiling

The CM-MSCs was centrifuged at 13,000 g for 10 min and 4°C. To separate S-MSCs, the tangential flow filtration (TFF) approach was used, as previously described in previous study.[14] The CM were filtered using filter cassettes with pore sizes of 10-30 kDa (50%) and 30-50 kDa (50%) to isolate defined population of secreted protein and cytokines in CM-MSCs, the end results is secretome-MSCs (S-MSCs) that has desired cytokines and protein such as VEGF, IL-10 and interferon-gamma (IFN-γ). The VEGF, IL-10 and IFN-γ level were confirmed and measured using in enzyme-linked immunosorbent assay (ELISA) (Elabscience, TX, USA) according to the manufacturer's instructions. The data were analysed using a microplate reader (Bio-Rad, CA, USA) with a wavelength of 450.

2.6. In vivo experimental protocol

A total of 24 rats were randomly divided into three groups: healthy, control (ischemic stroke + 300 µL NaCl), and treatment (ischemic stroke + 300 µL S-MSCs) groups (n = 8 per group). Treatments were injected intravenously six hours following ischemic stroke. All animals survived after NaCl or S-MSCs treatment. Blood serum was taken three- and 21-days following treatments to analyse IL-10 level. Rats were sacrificed three weeks following treatments to prepare ischemic brain tissue samples for immunohistochemistry and histological analysis.

2.7. Modified neurological severity score (mNSS)

The modified neurological severity score was used to determine neurological function in rats on day 1, 7 and 21 following S-MSCs treatment. The mNSS includes a mix of motor and sensory assessments such as walking and circling behaviours, wire grip, resistance to lateral push, forelimb flexion, thorax twisting when tail-suspended, grasping reflex, and spontaneous activity. Scores range from 0 to 7, with points awarded for tasks animals cannot complete (normal score of 0, maximum deficit score of 7). Behavioural assessments were conducted by a blind independent observer.

2.8. Histological analysis

Brain tissue samples were fixed in 4% neutral-buffered formalin at 4°C and processed for paraffin embedding. The sections were then deparaffinized and rehydrated using xylol and alcohol before staining. Sections were subsequently treated using acetone for haematoxylin-eosin (H&E) staining following standard protocols.

2.9. Immunohistochemical examinations of CD31 and CD105

Brain tissue samples embedded in paraffin underwent deparaffinization with xylene and alcohol. The slides were rehydrated before being treated with rat primary monoclonal antibody for CD31 and CD105 (1:100, Abcam, Cambridge, MA, United States followed by a biotinylated secondary antibody. Streptavidin peroxidase was utilized to assess for detection, while ImageJ was employed for semi-quantification of marker expression. CD31 and CD105 were selected as key markers of endothelial cells and angiogenesis, to evaluate vascular remodelling in response to ischemic injury and S-MSCs therapy.[16,17] Although CD105 is also

expressed by mesenchymal stem cells, its co-expression with the endothelial-specific marker CD31 allowed for the identification of endothelial cells and MSCs, facilitating accurate assessment of tissue-level vascular regeneration.

2.10. Serum IL-10 level

Blood serum from rats on day 3 and 21 were collected. All samples were centrifugated 1000 xg for 20 min at 4-8°C to remove debris. ELISA was employed to measure IL-10 level following guidelines provided by the manufacturer (Elabscience, TX, USA). The data was processed utilizing a microplate reader at a wavelength of 450 (Bio-Rad, CA, USA).

2.11. Statistical analysis

Data is shown as either the Standard Deviation (SD) from the average or as the average ± Standard Error Mean (SEM). Statistical analysis was conducted using ANOVA, followed by post hoc Tukey test. Immunohistochemistry, histological and MSCs figures was also analysed using ImageJ software. A p-value less than 0.05 is regarded as statistically significant.

3. RESULTS

3.1 Isolation and characterization of UC-MSCs

Cultured UC-MSCs isolated from umbilical cord of female 19 days pregnant rats appeared as fibroblast-like spindle shape characteristic in tissue culture flask (Fig. 1A). To determine the membrane marker expression of UC-MSCs, we employed flow cytometry analysis at the 5th passage. The immunophenotyping profile found thar UC-MSCs were positive for CD90.1 and CD29, and negative for CD45 and CD31 (Fig. 1B).

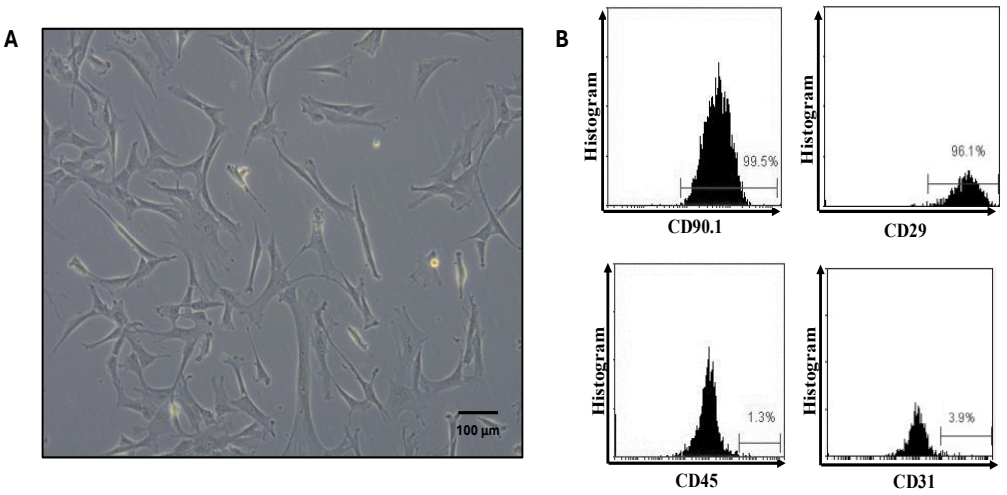

Figure 1. Characterization of UC-MSCs. A. At passage 5, the cells showed a fibroblast-like spindle-shaped morphology (100× magnification). B. UC-MSCs exhibited positive expression for CD90.1 (99.5%) and CD29 (96.1%), and negative expression for CD45 (2.0%) and CD31 (2.1%).

3.2 Molecules contained in S-MSCs

The CM was collected after MSCs incubation in a 5% O_2 environment for 24 hours. To obtain purified S-MSCs, we separated the molecules from CM of hypoxia MSCs using a TFF strategy based on Molecular Weight Cut-Off (MWCO) categories as described in a previous study.[13] We used filters with a 10-50 kDa range (50%) and a 50-100 kDa range (50%) to isolate the molecules. After filtration, ELISA was employed to measure the VEGF, IL-10, and IFN-γ contained in S-MSCs (Table 1). ELISA found that the level of VEGF, IL-10 and IFN-γ contained in S-MSCs, respectively were 1965.22 ± 123.31pg/mL, 53.86 ± 6.27pg/mL, and 9.49 ± 2.84

Table 1. Molecules contained in S-MSCs. Analysis of VEGF, IL-10, and IFN-γ molecules contained in S-MSCs was employed using ELISA.

Molecules	S-MSCs Value ± SE (pg/mL)
VEGF	1965.22 ± 123.31
IL-10	53.86 ± 6.27
IFN-γ	9.49 ± 2.84

3.3 S-MSCs ameliorates functional deficit in rat ischemic stroke

Systemic administration of MSCs provides functional benefits in several stroke animal models. Our hypothesis suggested that S-MSCs significantly contribute to stroke animal model providing similar advantages as MSCs through signalling molecules

secreted from hypoxia microenvironment. To examine our hypothesis, we induced an ischemic stroke model in rats and analysed the therapeutic effect of S-MSCs. A single injection of S-MSCs (300 µL) was administered 6 h after ischemic stroke induction via tail vein. Behavioural performance of rats was assessed using mNSS score 1, 7 and 21 days after acute ischemic stroke. Rats from the control and treatment groups displayed severe functional impairments on day 1 after the surgery, while the healthy rats did not show any signs of functional deficits. We observed that the administration of S-MSCs significantly improved behavioural outcome 7 days following treatment compared to control. The optimum improvement was shown 21 days following S-MSCs administration (Fig. 2). These results suggest that S-MSCs facilitate therapeutic effect to improve neurological outcome in acute ischemic rats.

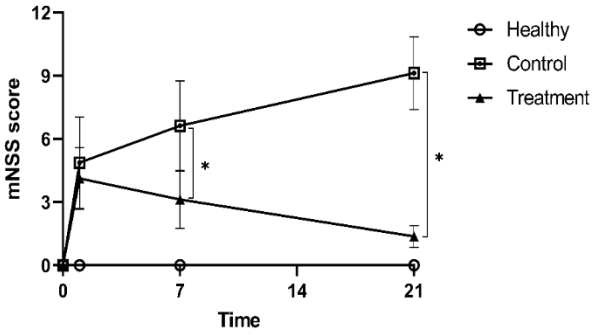

Figure 2. mNSS analysis shows significant functional improvement in S-MSCs treatment at day 7, with optimal enhancement by day 21, compared to control. *P<0.05; n=8 per group; Day 0: surgery and S-MSCs injection (6 h post-surgery); Day 1, 7, 21: post-injection days.

3.4 S-MSCs enhance IL-10 Level and mitigate acute inflammation in ischemic stroke rats

To assess the ability of S-MSCs to inhibit inflammation, we conducted ELISA analysis of IL-10 levels in rat serum on days 3 and 21. IL-10 was selected as a key marker due to its well-established role in suppressing pro-inflammatory responses following stroke ischemic injury. On day 3, IL-10 level in the control group were significantly higher than the healthy group, indicating an acute inflammatory response triggered by ischemia (Fig. 3A). Notably, the treatment group exhibited significantly elevated IL-10 levels compared to the control group, suggesting that S-MSCs enhanced anti-inflammatory environment early post-ischemia. However, by day 21, no significant differences in IL-10 level were observed among groups, indicating that the S-MSCs primarily exerted their effects during the acute phase of inflammation (Fig. 3B). These findings highlight the potential of S-MSCs to modulate early inflammatory responses after ischemic stroke by increasing IL-10 production.

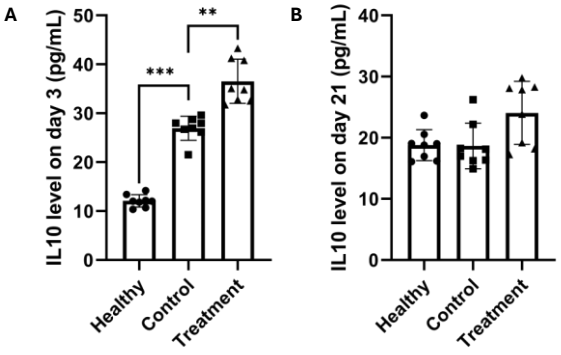

Figure 3. Anti-inflammatory effect of S-MSCs in ischemic stroke rats. ELISA was used to analyze IL-10 level on day 3 (A) and 21 (B). IL-10 level was significantly higher in control compared to healthy and reached optimum increase following S-MSCs treatment compared to control. Values are indicated as means ± SD. **P<0.01, ***P<0.001, n = 8 per group.

3.5 S-MSCs enhances vascularization in ischemic brain via CD31 and CD105 modulation of ischemic stroke Rats

Vascularization, a critical component of tissue repair following ischemic stroke, involves the formation of new blood vessels that restore blood supply to damaged areas. CD31 and CD105 are key markers of endothelial cells and angiogenesis, serving as important indicators of vascular regeneration. In this study, we analysed the expression of CD31 and CD105 in ischemic brain tissue on day 21 using immunohistochemistry. There was no significant difference in CD31 expression between the healthy and control groups, indicating that ischemia did not induce substantial vascularization. However, CD31 expression was notably higher in the treatment group compared to the control group, highlighting that S-MSCs enhanced endothelial cell proliferation and blood vessel formation in ischemic tissue (Fig. 4A and B). In contrast, CD105 expression was significantly lower in the control group than in the healthy group, suggesting impaired vascularization in the untreated ischemic rats. In contrast, CD105 expression in the treatment group was significantly increased compared to control group, highlighting the role of S-MSCs in stimulating endothelial cell activity and promoting angiogenesis (Fig. 4C and D). These results demonstrate that S-MSCs enhance vascularization in ischemic brain tissue by upregulating CD31 and restoring CD105 expression, facilitating tissue repair.

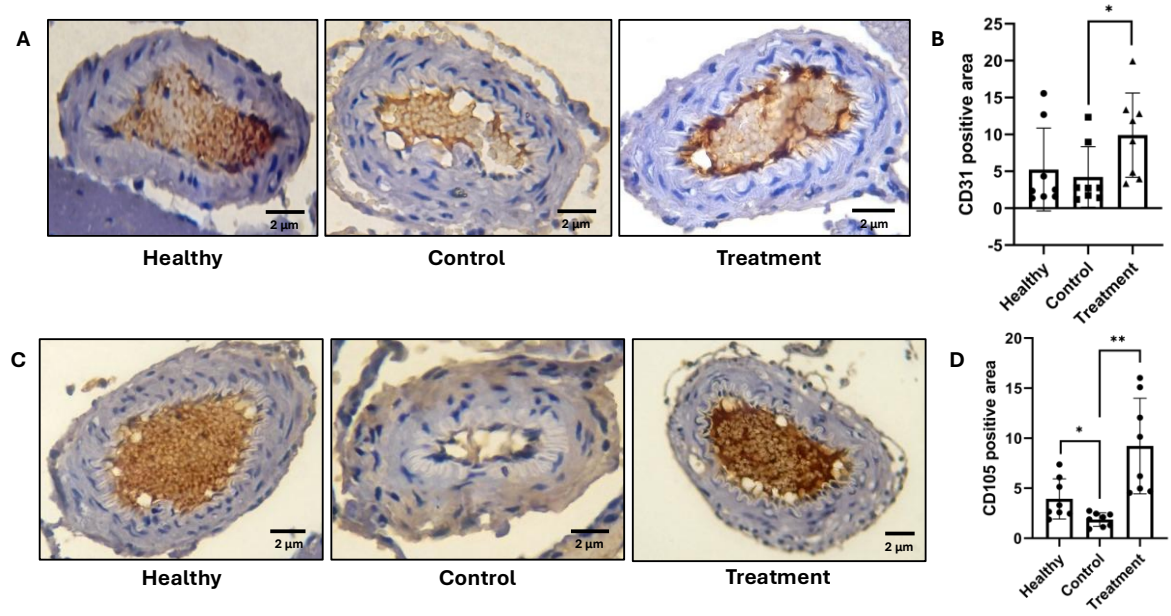

*Figure 4. S-MSCs promoted angiogenesis in rats with ischemic stroke. A. Immunohistochemistry shows a significant increase of CD31 in S-MSCs treatment on day 21 compared to control ($p<0.05$). B. Bar graph of CD31-positive area across all samples. C. Immunohistochemistry also shows a significant increase of CD105 in S-MSCs treatment on day 21 compared to control ($p<0.01$) D. Bar graph of CD105-positive area across all samples. CD31 and CD105 positive staining appears brown (400x magnification). Data are indicated as means ± SD. *P<0.05, **P < 0.01 compared with control, n = 8 per group.*

3.6 S-MSCs enhances neuronal survival in ischemic stroke rats

Neuronal loss is a hallmark of ischemic brain damage, with ischemic stroke often leading to widespread neuron death due to the lack of oxygen and nutrient supply. Neuron regeneration is critical for functional recovery, and therapies that enhance neuronal survival and promote neurogenesis hold promise for stroke treatment. In this study, we evaluated the effects of S-MSCs on neuron cell survival in the ischemic brain by counting living neurons in brain tissue on day 21 histologically using H&E staining. On day 21, the control group showed a significant decrease in the number of living neuron cells compared to the healthy group, reflecting the extensive neuronal damage caused by ischemia. However, the treatment group demonstrated a significant increase in the number of surviving neurons compared to the control group, indicating that S-MSCs promoted neuronal survival and mitigated cell death in the ischemic brain (Fig. 5). These findings suggest that S-MSCs supports neuron survival and may contribute to neuronal regeneration in ischemic stroke of rats.

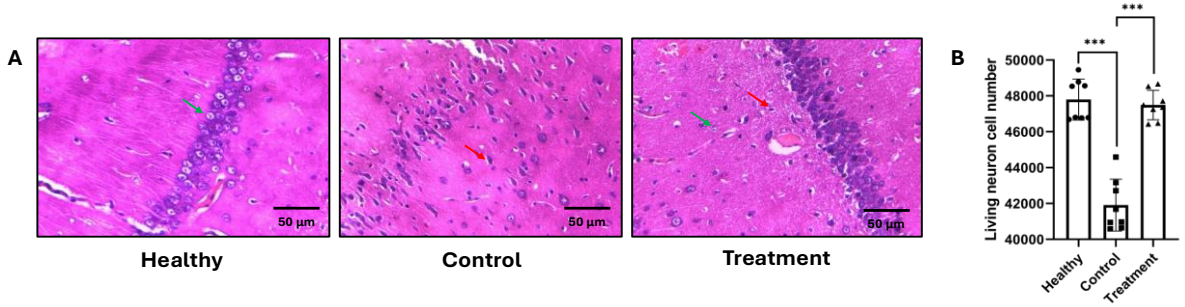

*Figure 5. S-MSCs increase living neuron cells in ischemic stroke rats. A. H&E analysis showed a significant increase of living neuron cell number in S-MSCs treatment on day 21 compared to control. B. Representative bar graph depicting living neuron cell number of all samples. Living neuron cells marked as green arrows and dead neuron cells marked as red arrow (40x magnification). Data are indicated as means ± SD. ***P<0.001, n = 8 per group.*

4. DISCUSSION

A number of studies demonstrated that the use of MSCs from several resources were beneficial for stroke and other neurodegenerative diseases.[18–21] Other studies also reported that therapeutic benefits were due to anti-inflammatory cytokines and growth factors paracrine factors released by the transplanted MSCs.[9,22] This study demonstrates the therapeutic potential of S-MSCs in promoting recovery from ischemic stroke in rats.

Our data showed that all rats exhibited severe deficits on day 1 following ischemic stroke, except for the healthy group. S-MSCs injection significantly improved behavioural testing scores began within a week, with optimal recovery observed on day 21. The striking recovery in behaviour suggests that S-MSCs not only provides acute benefits but also highlight their therapeutic potential in enhancing neurorepair mechanisms over time. This improvement can be attributed to the S-MSCs ability to modulate inflammation and enhance neurovascular repair processes.

These data were further supported by the high levels of VEGF and IL-10 detected within S-MSCs. VEGF is known to play a crucial role in angiogenesis, promoting the formation of new blood vessels which is essential for restoring blood flow and repairing ischemic tissue damage in the brain.[23] The promotion of angiogenesis helps enhance oxygen and nutrient supply to the ischemic regions, which is critical for neuronal survival and regeneration following a stroke.[24] In conjunction, IL-10 acts as a potent anti-inflammatory cytokine, capable of inhibiting the release of proinflammatory mediators and reducing the extent of secondary damage due to inflammation.[25] This molecule helps counteract the detrimental proinflammatory milieu that is typically observed post ischemic stroke, thus reducing further injury to the affected tissues.[26] Moreover, we found that S-MSCs contained low level of IFN-γ, suggesting that S-MSCs did not elicit a significant pro-inflammatory response. The reduced presence of IFN-γ, a cytokine typically associated with activating inflammatory pathways, indicates that S-MSCs are less likely to trigger adverse immune reactions.[27]

Inflammatory regulation was further highlighted by IL-10 level measured in the study. On day 3, S-MSCs injection significantly increased IL-10 level in ischemic stroke rats, suggesting anti-inflammatory response enhancement during critical early phase post-ischemic stroke. Elevated IL-10 level is crucial for rapid inhibition of pro-inflammatory pathways, reducing neuronal injury and limiting secondary damage caused by the immune response. A previous study demonstrated that increased IL-10 expression following cell-based treatment significantly was in line with reduction of inflammatory markers and improved functional outcomes in a rat model of ischemic stroke.[28] These findings are consistent with our results, indicating that the early increase of IL-10 level induced by S-MSCs actively suppresses the acute inflammatory response, helping to prevent additional neuronal damage and promote neuroprotection. IL-10 levels were similar across all groups on day 21, implying that S-MSCs primarily modulate inflammation in the acute phase, preventing prolonged or chronic inflammation that could hinder recovery. This finding suggests that S-MSCs did not induce prolonged anti-inflammatory response, but instead, facilitate a transition to a steady-state inflammatory environment on day 21.

Vascularization is a critical factor in the recovery from ischemic stroke, as the formation of new blood vessels re-establishes the supply of oxygen and nutrients to the affected tissues.[29] Our data showed that S-MSCs contained high level of VEGF as a crucial factor in angiogenesis. This finding was correlated with the increased expression of CD31 on day 21 following S-MSCs injection in ischemic brain rats, indicating enhanced vascularization. The increase of CD31 specifically on day 21 suggests that the effect of S-MSCs on angiogenesis are not immediate but progressively develop over time, coinciding with the recovery phase of the ischemic tissue. CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), plays a vital role in endothelial cell junction integrity and angiogenesis.[17] A previous finding reported that CD31 expression was increased on later stages of recovery after MSC-based therapy, indicating the continued remodelling and maturation of new blood vessels.[30] These suggested that S-MSCs share similar roles and capacities to MSCs in promoting angiogenesis and vascular remodelling. Furthermore, our data showed that CD105 expression, which was significantly reduced in the control group, was restored in the treatment group, further suggesting potent proangiogenic effect of S-MSCs. CD105, a key marker of endothelial cell activation and proliferation, plays an essential role in the TGF-β signalling pathway, which governs angiogenesis and the differentiation of endothelial cells.[16] The co-expression of CD31 and CD105 highlights the dual role of S-MSCs in promoting both the formation and stabilization of new vasculature, reflecting a time-dependent process of vascular maturation that progresses from initial angiogenic sprouting to subsequent vessel remodelling and stabilization.

The neuroprotective effects of S-MSCs were also evident in the analysis of neuron survival.[31] Rats with ischemic stroke exhibited a significant reduction of living neuron cells compared to healthy rats, reflecting the extensive neuronal loss typical of ischemic stroke. However, S-MSCs significantly increased the number of living neuron cells in ischemic stroke rats, suggesting that S-MSCs not only preserved existing neurons but may have also supported neurogenesis or reduced apoptosis in ischemic brain. A previous study demonstrated that H-MSCs enhance neuronal density and reduce apoptosis in an ischemic rat model, aligning with our findings and further supporting the notion that the S-MSCs is a key factor in promoting regeneration of neuronal cells.[32] This study suggests that the hypoxic environment primes MSCs to release a rich array of neuroprotective factors that contribute to neuronal survival and regeneration. By enhancing the local availability of neurotrophic factors and anti-apoptotic signals, S-MSCs likely plays a pivotal role in stimulating neurogenesis and facilitating the repair of damaged neural tissues.[33,34] Thus, beneficial effects observed in our study reinforce the idea that harnessing the S-MSCs, particularly those subjected to hypoxic conditions, could serve as an effective strategy for enhancing neuronal recovery following ischemic injury. Our results underscore the therapeutic potential of S-MSCs for stroke recovery, providing a promising avenue for future clinical applications in neuro-restoration.

Compared to other cell-free strategies, such as the isolation of exosomes or extracellular vesicles, our TFF-based S-MSCs preparation offers a distinct advantage. While exosome isolation typically requires time-consuming ultracentrifugation and yields narrowly defined vesicular fractions, the TFF approach efficiently captures a broader, yet highly defined, spectrum of soluble paracrine factors, including VEGF, IL-10, and IFN-γ. This collective molecular profile contributes synergistically to the observed neurorestorative effects, providing a scalable, efficient, and potentially more comprehensive therapeutic modality for acute ischemic stroke.

Although our study offers valuable insights into the therapeutic potential of S-MSCs in an ischemic stroke animal model, there are several limitations to consider. The study mainly concentrated on the acute and subacute recovery phases, with analyses performed up to 21 days post-injury. A longer follow-up period would be necessary to assess the durability of the observed effects and the long-term outcomes of neuroprotection and functional recovery. While we observed increases in key markers associated with angiogenesis and neuroprotection, the precise mechanisms through which S-MSCs exert these effects remain to be fully elucidated. Additional studies are required to investigate the specific signalling pathways and molecular interactions involved in these processes. The potential immunogenicity and long-term safety of administering S-MSCs in humans remain to be thoroughly evaluated. Preclinical studies should address these concerns before moving to clinical applications.

5. CONCLUSION

Overall, our study provides compelling evidence that S-MSCs offer significant improvement in an acute ischemic stroke animal model. Through a series of comprehensive analyses, we demonstrated that S-MSCs effectively enhance neuronal survival, promote angiogenesis, and modulate inflammation, all of which are critical factors for recovery following ischemic injury. Our findings advocate for the continued exploration of S-MSCs as a viable and effective therapeutic strategy for enhancing recovery in ischemic stroke patients, with the potential to improve clinical outcomes and advance regenerative medicine approaches. However, to bridge the gap between this preclinical animal model and potential clinical application, future studies must focus on optimizing the therapeutic dosage, determining the ideal administration window in larger mammalian models, and rigorously evaluating the long-term safety and immunogenicity of S-MSCs in human subjects.

AUTHORS' DECLARATION

- Conflicts of Interest: None.
- Figure and Table Ownership: We hereby confirm that all figures and tables included in the manuscript are our original work. Any figures or images that are not our own have been used with appropriate permissions for republication, and the necessary documentation is attached to the manuscript.
- Ethical Clearance: This project was approved by the local ethical committee from the Ethics Review Board and Committees, Faculty of Medicine, Universitas Diponegoro (No. 31/EC-H/KEPK/FK-UNDIP/IV/2023).

AUTHOR INFORMATION

R contributed to the conceptualization and methodology of the study, conducted formal analysis and investigations, and was involved in writing the original draft. She also supervised the research and participated in the review and editing of the manuscript. DTP was responsible for conceptualization, methodology, and supervision, and contributed to writing the review and editing of the manuscript. MIW performed data curation, formal analysis, and investigation, and wrote the original draft while also reviewing and editing the manuscript. EK contributed to the investigation, provided resources, and participated in writing the review and editing. ACB was involved in the methodology, investigation, and provided resources for the study. DP contributed to data curation and investigation. HS assisted with investigation and validation of the data. YA contributed to data curation, formal analysis, and methodology. TB was involved in the methodology, validation, and provision of resources. APW contributed to data curation, formal analysis, and writing the review and editing. MBR assisted with investigation and data validation. ABS participated in data curation and methodology. AC was involved in investigation and resource management. MKP contributed to data curation, investigation, and methodology, while PA played a key role in conceptualization, methodology, and resource management.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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