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Modulation of inflammatory pathways in ischemic stroke rats by hypoxia-primed umbilical cord mesenchymal stem cells (UC-MSCs): Implications for IFN-γ and IL-10 signaling

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ABSTRACT

Ischemic stroke is a major global cause of disability and mortality. Inflammation plays a central role in its pathogenesis, characterized by elevated pro-inflammatory cytokines such as IFN-γ and reduced anti-inflammatory cytokines like IL-10. Mesenchymal stem cells (MSCs), especially those derived from the umbilical cord (UC-MSCs), exhibit enhanced immunomodulatory potential when preconditioned under hypoxia. This study aims to evaluate the effect of hypoxic-preconditioned UC-MSCs administration on IFN-γ and IL-10 levels in an ischemic stroke rat model. This *in vivo* experimental study employed a randomized posttest-only control group design with four groups of male Wistar rats (n=6 each), ranging from healthy controls, untreated group to stroke-induced groups treated with hypoxic UC-MSCs at different doses ($1.5x10^6$ and $3x10^6$). IFN-γ and IL-10 levels in brain tissue of each group were measured via ELISA. Significant reduction in IFN-γ and elevation in IL-10 were observed in UC-MSC-treated groups, particularly at the $3×10^6$ cell dose compared to the untreated ischemic group ($p<0.05$). Hypoxic UC-MSCs reduce post-stroke inflammation by lowering IFN-γ and enhancing IL-10, indicating a promising immunomodulatory potential.

ABSTRAK

Stroke iskemik merupakan salah satu penyebab utama kecacatan dan kematian di seluruh dunia. Inflamasi berperan sentral dalam patogenesisnya, yang ditandai dengan peningkatan sitokin proinflamasi seperti IFN-γ dan penurunan sitokin antiinflamasi seperti IL-10. Sel punca mesenkimal (*Mesenchymal Stem Cells*, MSCs), khususnya yang berasal dari tali pusat (*Umbilical Cord*-MSCs/UC-MSCs), menunjukkan potensi imunomodulator yang lebih tinggi ketika diberi prapengondisian hipoksia. Penelitian ini bertujuan untuk mengevaluasi efek pemberian UC-MSCs yang diprapengondisikan dalam kondisi hipoksia terhadap kadar IFN-γ dan IL-10 pada model tikus stroke iskemik. Penelitian eksperimental *in vivo* ini menggunakan rancangan *randomized posttest-only control group design* dengan empat kelompok tikus Wistar jantan (n=6 per kelompok), terdiri atas kelompok sehat (kontrol), kelompok tanpa terapi, serta kelompok stroke yang mendapat terapi MSC hipoksia dengan dosis berbeda ($1,5×10^6$ dan $3×10^6$ sel). Kadar IFN-γ dan IL-10 pada jaringan otak setiap kelompok diukur menggunakan metode ELISA. Hasil menunjukkan penurunan signifikan kadar IFN-γ dan peningkatan kadar IL-10 pada kelompok yang mendapat terapi UC-MSC, terutama pada dosis $3×10^6$ sel dibandingkan dengan kelompok stroke tanpa perlakuan ($p<0,05$). UC-MSC hipoksia menurunkan inflamasi pascastroke melalui penurunan IFN-γ dan peningkatan IL-10, sehingga menunjukkan potensi imunomodulator yang menjanjikan.

Keywords:
ischemic stroke;
UC-MSCs;
hypoxia;
IFN-γ;
IL-10 i

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INTRODUCTION

Ischemic stroke remains a major global health burden, accounting for approximately 87% of all stroke cases and causing over 2.7 million deaths annually worldwide.[1] It occurs due to the obstruction of cerebral blood flow, leading to a cascade of neurovascular events such as neuronal ischemia, mitochondrial dysfunction, and blood–brain barrier disruption.[2] These processes initiate a robust immune response characterized by the infiltration of immune cells and the release of pro-inflammatory cytokines. One of the key mediators in this process is interferon-gamma (IFN-γ), a cytokine that promotes macrophage activation, increases adhesion molecule expression, and exacerbates neuronal injury.[3] In contrast, interleukin-10 (IL-10) functions as a critical anti-inflammatory mediator that mitigates immune-mediated damage by suppressing pro-inflammatory signalling and promoting tissue repair.[4] The imbalance between IFN-γ and IL-10 has been strongly correlated with larger infarct volumes and worse neurological outcomes in both clinical and experimental studies.[3-5]

Although acute management of ischemic stroke has advanced through the implementation of intravenous thrombolysis and mechanical thrombectomy, these interventions are only applicable within a narrow time window and fail to address post-ischemic inflammation, which continues to contribute to secondary brain injury.[6] Furthermore, no currently approved pharmacological treatments are capable of modulating the immune microenvironment effectively during the subacute and chronic phases of stroke.[7] This highlights a critical therapeutic gap: the absence of immunomodulatory strategies capable of attenuating neuroinflammation and promoting endogenous repair mechanisms.[7,8] Therefore, there is an urgent need to explore novel biological approaches that can rebalance inflammatory responses, particularly by reducing IFN-γ and enhancing IL-10—to limit long-term neurological deficits.

Over the past decade, mesenchymal stem cells (MSCs) have emerged as a promising regenerative and immunomodulatory therapy in the context of stroke. MSCs derived from umbilical cord tissue (UC-MSCs) offer distinct advantages over bone marrow or adipose-derived MSCs, including non-invasive harvest, higher proliferation rates, and lower immunogenicity.[9,10] Preclinical studies have shown that UC-MSCs can migrate to ischemic sites, promote angiogenesis, reduce apoptosis, and exert anti-inflammatory effects.[10-12] Notably, hypoxic preconditioning, a technique involving the culture of MSCs under low oxygen (1–5%) (UC-MSCs), has been shown to enhance the expression of hypoxia-inducible factors (e.g., HIF-1α) and increase the secretion of paracrine factors such as VEGF, IL-10, and TGF-β.[10,11] These changes improve cell survival, homing capacity, and therapeutic efficacy in ischemic environments. Despite this, few studies have directly assessed how hypoxia-conditioned UC-MSCs influence the balance between IFN-γ and IL-10, particularly in *in vivo* models of ischemic stroke.[12]

To address this gap, the present study aims to evaluate the immunomodulatory effects of hypoxic preconditioned UC-MSCs on IFN-γ and IL-10 levels in a rat

model of ischemic stroke. By investigating these specific cytokines, this research seeks to elucidate the potential of UC-MSCs as a targeted therapy to modulate post-stroke inflammation, offering insights into their mechanisms of action and therapeutic value in neuroinflammatory conditions.

MATERIAL AND METHODS

Animals and study design

This *in vivo* experimental study employed a randomized post-test-only control group design using male Wistar rats (*Rattus norvegicus*) aged 6–8 wk and weighing 180–200 g. Twenty-four rats were randomly assigned to four groups (n = 6 per group): N (healthy control), C (stroke model without treatment), T1 (stroke model treated with UC-MSCs at 1.5×10^6 cells/rat), and T2 (stroke model treated with UC-MSCs at 3×10^6 cells/rat). Animals were housed in the animal facility adjacent to the SCCR Indonesia research center. Animals received a commercial rodent pellet diet *ad libitum* and had continuous access to filtered drinking water via drinking bottles. The environment was maintained at 22 ± 2 °C, relative humidity of 45–60%, and a 12:12-hr light–dark cycle (lights on 07:00–19:00). Animals were group-housed (2–3 animals per cage) in polypropylene cages with appropriate bedding and environmental enrichment. Animals were allowed at least 7 d of acclimatization before experimentation. The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia (No. 417/ VIII/2025/Komisi Bioetik).

Induction of ischemic stroke rats

Induction of ischemic stroke was conducted using the middle cerebral artery occlusion (MCAO) technique. Rats were anesthetized with ketamine (30 mg/kg BW, i.p.), and a midline scalp incision was made to expose the skull. The middle cerebral artery was carefully ligated using sterile surgical suture for 90 min, after which the occlusion was released to allow reperfusion. Post-operative care included administration of enrofloxacin (2.5 µg/g BW, i.p.) as antibiotic prophylaxis and paracetamol (18.9 mg, i.p.) twice daily for analgesia, continued for 7 d. Rats were monitored for signs of distress or neurological deficit, and successful induction of stroke was validated based on behavioral and anatomical criteria.

UC-MSC culture

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) were isolated from pregnant rats under sterile conditions. Umbilical cords were collected in sterile containers with 0.9% NaCl and washed thoroughly with phosphate-buffered saline (PBS). Wharton's jelly was dissected, minced, and plated in T25 flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and fungizone. Cultures were maintained at 37°C in 5% CO_2, with medium changes every three days. Cells exhibiting fibroblast-like morphology appeared after 10–14 d and were subcultured until reaching 80% confluency.

For hypoxic preconditioning, confluent MSC cultures were placed in a

hypoxia chamber flushed with nitrogen gas until oxygen levels reached 5%, as verified by an oxygen meter. The cultures were incubated under these conditions at 37°C for 24 hr.[13] This condition was selected based on our previous work, which confirmed that exposure to 5% O_2 for 24 h induces a robust hypoxic response characterized by upregulation of the HIF-1α protein and increased expression of VEGF mRNA in UC-MSCs.[14] This method was used to enhance the paracrine activity and survival potential of the MSCs. Validation of MSC identity was conducted using flow cytometry. Cells were stained with fluorochrome-conjugated antibodies and analysed for expression of surface markers. Rat UC-MSCs were confirmed to be positive for CD73, CD90, and CD105, and negative for CD34, CD45, CD31, and HLA-DR, in accordance with the criteria set by the International Society for Cellular Therapy (ISCT).

Six hours following stroke induction, rats in the treatment groups (T1 and T2) received a single intravenous injection of hypoxia MSCs via the tail vein. The control group (C-) received 300 µL of normal saline. On day 3 post-treatment, rats were anesthetized using a high dose of ketamine (>200 mg/kg BW, i.p.) and euthanized. Brain tissues were collected and homogenized for cytokine analysis by ELISA.

ELISA

The IFN-γ and IL-10 levels were quantified from homogenates of the ipsilateral (ischemic) hemisphere, not from the entire brain. This region was selected because it directly represents the inflammatory microenvironment after middle cerebral artery occlusion (MCAO). Levels of IFN-γ and IL-10 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, TX, USA), following the manufacturer's instructions. The assay included serial dilution of standards, sample incubation with capture and detection antibodies, and colorimetric detection at 450 nm using an ELISA microplate reader.

Data analysis

All data were analysed using SPSS version 22.0 for Windows. The Shapiro–Wilk test was used to assess normality, and the Levene's test was used to determine homogeneity of variance. If data met the assumptions of normality and homogeneity, one-way analysis of variance (Anova) was used, followed by the least significant difference (LSD) or Tamhane's post-hoc test where appropriate. For non-normally distributed data, the Kruskal–Wallis test was applied, followed by the Mann–Whitney U test for post-hoc comparisons. A p-value of <0.05 was considered statistically significant.

RESULTS

Characterization and validation of cultured MSCs

Prior to their use in subsequent experiments, it was essential to confirm that the cultured cells exhibited the defining characteristics of MSCs. Validation ensures the purity of the cell population, prevents contamination by hematopoietic or endothelial cells, and verifies the multipotent differentiation

potential that is the hallmark of MSCs. This process followed the minimal criteria set by the International Society for Cellular Therapy (ISCT), which include specific surface marker expression and the ability to differentiate into multiple mesodermal lineages.[14] Therefore, MSC validation serves not only as a quality control step but also as a safeguard to ensure the consistency and reliability of experimental outcomes dependent on MSC biological activity.

Flow cytometric analysis demonstrated that the cultured cells expressed high levels of the mesenchymal marker CD90.1 (98.6%), while showing minimal expression of the hematopoietic marker CD45 (0.41%) and the endothelial marker CD31 (5.12%), confirming their mesenchymal phenotype (FIGURE 1A). The slightly higher CD31 expression compared with the ISCT threshold (<2%) may reflect species-specific differences in antigen expression or the presence of a small endothelial-like subpopulation commonly observed in early-passage rat umbilical cord MSCs, rather than true endothelial contamination.[15-16] Functional differentiation assays further validated MSC identity, as the cells successfully underwent adipogenic differentiation, evidenced by the accumulation of Oil Red O–positive lipid droplets (FIGURE 1B), and osteogenic differentiation, indicated by Alizarin Red S staining of calcium deposits (FIGURE 1C). Collectively, these findings confirm that the isolated cells fulfill the minimal defining criteria for MSCs as recommended by the ISCT, while acknowledging minor interspecies phenotypic variability. Although CD31 is typically used as an exclusion marker for endothelial cells in human MSC standards, previous studies have shown that rodent MSCs, particularly from umbilical cord sources, can exhibit low-level CD31 expression without displaying endothelial functionality.[16,17] This is often attributed to transient endothelial-like characteristics or antibody cross-reactivity, rather than true lineage contamination.

Effect of hypoxia UC-MSCs on IFN-γ and IL-10 levels

Measurement of IFN-γ levels in brain homogenates revealed a significant elevation in the stroke model without treatment (C), indicating a strong pro-inflammatory response post-ischemia. In contrast, rats treated with both doses of hypoxia MSCs (T1–T2) showed reduced IFN-γ levels compared to the untreated stroke group. Notably, the administration of hypoxic UC-MSCs at a dose of 3×10^6 cells (T2) resulted in the greatest suppression of IFN-γ expression, with levels approaching those of the healthy control group (N). The reduction in IFN-γ was dose-dependent and more pronounced in the hypoxia-conditioned groups than in the normoxic MSC group, suggesting an enhanced anti-inflammatory potential of hypoxic preconditioning. Statistical analysis using one-way Anova confirmed that the differences in IFN-γ levels among the groups were statistically significant ($p< 0.05$). Post-hoc comparisons demonstrated that the T2 group differed significantly from both the untreated stroke group (C) and the low-dose of hypoxic MSC group (T1) in both cytokines. These findings confirm the dose-dependent and conditioning-dependent potency of UC-MSCs in modulating the post-stroke immune environment.

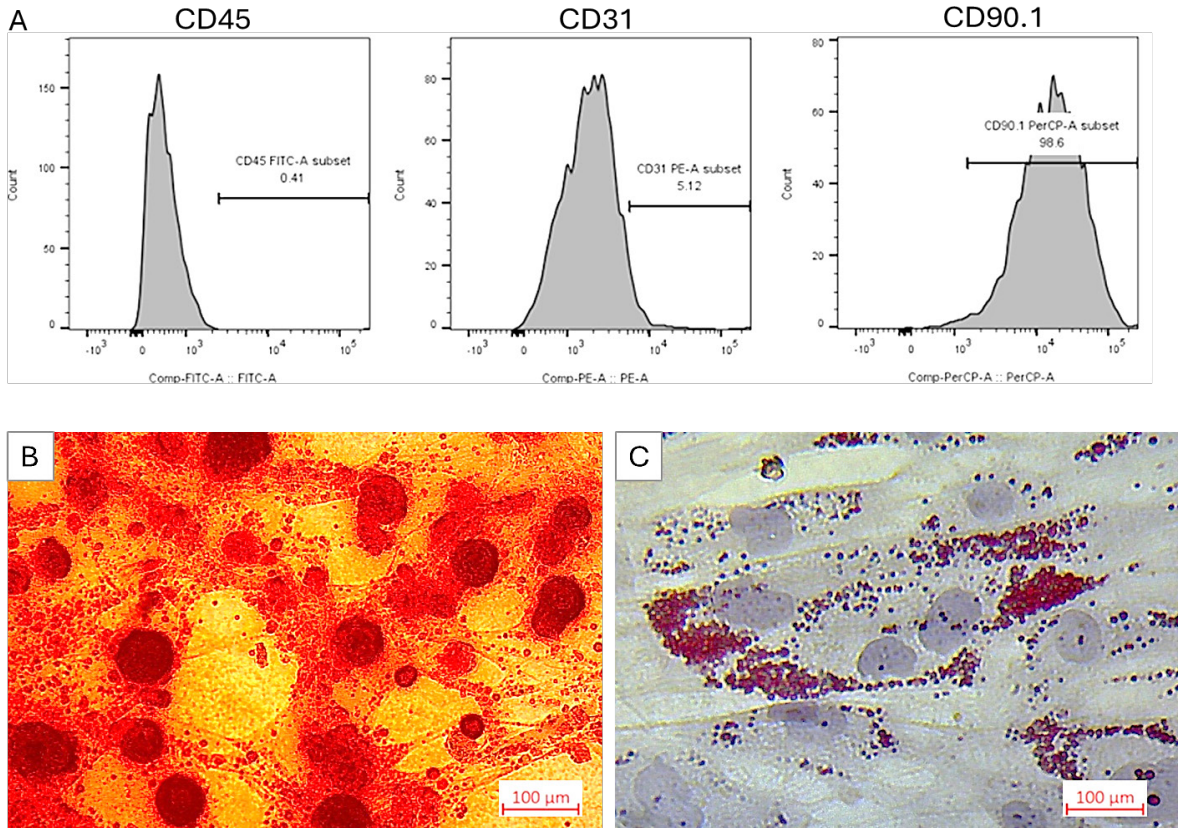

FIGURE 1. Characterization of MSCs by flow cytometry and differentiation assays. (A) Flow cytometric analysis showing low expression of hematopoietic marker CD45 (0.41%) and endothelial marker CD31 (5.12%), and high expression of MSC surface marker CD90.1 (98.6%). (B) Adipogenic differentiation potential of MSCs, evidenced by Oil Red O staining of intracellular lipid droplets (red) after induction. Scale bar = 100 µm. (C) Osteogenic differentiation potential of MSCs, confirmed by Alizarin Red S staining indicating calcium mineral deposition (red) after induction. Scale bar = 100 µm.

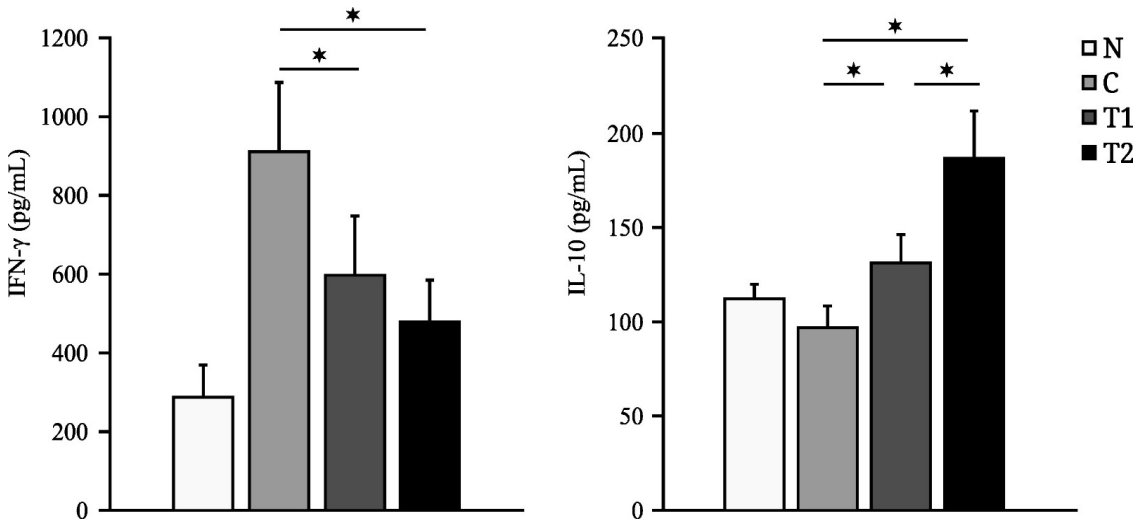

FIGURE 2. Effects of hypoxia-preconditioned umbilical cord mesenchymal stem cells (UC-MSCs) on IFN-γ levels in brain homogenates of ischemic stroke rats. Quantification of IFN-γ revealed a significant increase in the untreated stroke group (C) compared to healthy controls (N), indicating a strong post-ischemic pro-inflammatory response. Treatment with hypoxic UC-MSCs at both 1×10^6 cells (T1) and 3×10^6 cells (T2) reduced IFN-γ levels relative to C group, with the higher dose (T2) achieving suppression comparable to N. Data are presented as mean ± SD (n=6), $p < 0.05$, one-way Anova with post-hoc test.

Inversely correlated with IFN-γ, IL-10 levels were lowest in the untreated stroke group (C), reflecting a diminished anti-inflammatory response after ischemic injury. However, rats receiving hypoxic UC-MSCs (T1 and T2) demonstrated a significant elevation of IL-10, particularly at the higher dose of 3×10^6 cells (T2). The IL-10 levels in this group exceeded those of all other stroke model groups, indicating a robust immunoregulatory response. This finding supports the notion that hypoxic preconditioning enhances the capacity of UC-MSCs to promote anti-inflammatory cytokine expression in post-ischemic neural tissue. Statistical analysis using one-way Anova indicated that the variations in IL-10 levels among the experimental groups were statistically significant ($p < 0.05$). Further post-hoc analysis revealed that the T2 group exhibited significant differences in IL-10 when compared with the untreated stroke group (C) and the low-dose of hypoxic MSC group (T2). These results highlight the dose- and conditioning-dependent effectiveness of UC-MSCs in modulating immune responses following ischemic stroke.

DISCUSSION

Our findings indicate that hypoxia-preconditioned UC-MSCs effectively modulate the post-stroke immune response by downregulating pro-inflammatory IFN-γ and upregulating anti-inflammatory IL-10, thereby reinforcing their role as potent immunomodulatory agents. This cytokine shift aligns with the well-established immunoregulatory functions of MSCs, which act primarily through paracrine mechanisms rather than direct cell replacement. The MSCs have been shown to suppress the activity of pro-inflammatory macrophages, promote polarization toward the anti-inflammatory M2 phenotype, and inhibit the activation of NK cells and dendritic cells, thus creating a more favourable environment for tissue repair and resolution of inflammation.[15-16]

At the molecular level, previous studies have shown that hypoxic preconditioning enhances these therapeutic properties by stabilizing HIF-1α, a key transcription factor that regulates cellular adaptation to low-oxygen environments.[17,18] The HIF-1α activation leads to upregulation of critical genes involved in angiogenesis, cell survival, and metabolism, including VEGF and brain-derived neurotrophic factor (BDNF**)**. This molecular cascade improves MSC viability, enhances their homing to ischemic tissue, and amplifies their neuroprotective and regenerative potential.[19] Furthermore, preclinical studies have consistently demonstrated that hypoxia-preconditioned MSCs exhibit greater immunomodulatory potential compared to their normoxic counterparts, particularly through increased secretion of trophic and immunomodulatory factors that promote functional recovery after stroke.[20-22] In the present study, although we did not directly measure HIF-1α or VEGF expression, our findings are consistent with these previously described mechanisms.

Moreover, HIF-1α–overexpressing MSCs have been shown to modulate immune responses by promoting the expression of anti-inflammatory factors such as IL-6 and Galectin-1, as well as chemokines like CCL2/MCP-1 that foster recruitment of immunosuppressive monocytes and M2-like macrophages.[10,21] Notably, these cells demonstrated resistance to NK cell–mediated lysis by downregulating activating ligands like ULBP1 and B7H6, supporting their enhanced engraftment and persistence.[23] Supporting the relevance of IL10 enhancement, studies where MSCs were genetically modified to overexpress IL-10 revealed pronounced neuroprotective effects in stroke models—significantly

reducing infarct volume, inhibiting microglial activation, and improving motor function. Preconditioning with hypoxia—or combining hypoxia with inflammatory priming (e.g., IFN-γ)—further accentuates the anti-inflammatory and regenerative potential of MSCs through expanded secretion of immunoregulatory and angiogenic factors.[24]

Taken together, our data reinforce that the potential immunomodulatory potential of UC-MSCs in ischemic stroke is enhanced through hypoxic preconditioning via molecular pathways involving HIF-1α stabilization, augmented secretion of VEGF and IL10, improved cell survival, and strengthened immunomodulation.

Despite the promising findings, this study has several limitations. First, the analysis was limited to short-term cytokine expression (IFN-γ and IL-10) at a single time point (day 3 post-treatment), which may not fully capture the dynamic changes in immune responses or long-term effects of UC-MSC therapy. Second, functional neurological outcomes, infarct size, and histopathological changes were not assessed, limiting interpretation of the therapeutic relevance beyond molecular parameters. Additionally, the study was conducted in an animal model, and results may not fully translate to human physiology due to interspecies differences. Future research should incorporate extended observation periods, functional recovery assessments, and molecular pathway exploration to better understand the mechanisms and durability of the observed immunomodulatory effects. Future studies should extend beyond cytokine profiling to evaluate functional outcomes such as neurological scores, infarct volume reduction, and long-term behavioral recovery, and examine the downstream signaling pathways, such as IDO, PGE_2, and TGFβ, that mediate this response.

CONCLUSION

In conclusion, hypoxia-preconditioned UC-MSCs significantly shift the inflammatory balance in ischemic stroke rats by suppressing IFN-gamma and elevating IL-10, demonstrating their capacity for molecular immunomodulation. While these results are promising, future studies incorporating neurological scores and infarct volume assessment are necessary to establish the full therapeutic relevance of this approach in stroke management.

Declaration of AI

The authors only used generative AI tools to improve the language and grammar of their manuscripts.

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The authors declare that they have no conflict of interest

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