

Hypoxic Mesenchymal Stem Cell Secretome Restores Hematopoiesis and Modulates Immune Responses in A Murine Model of Aplastic Anemia

Citra Primavita Mayangsari^{1,*}, Harsono Salimo¹, Yulia Lanti Retno Dewi¹,
Agung Putra^{2,3}, Betty Suryawati¹ and Annang Giri Moelyo⁴

¹Doctoral Program of Medical Science, Faculty of Medicine, Universitas Sebelas Maret, Surakarta 57126, Indonesia

²Stem Cell and Cancer Research (SCCR) Laboratory, Semarang 50112, Indonesia

³Department of Doctoral Biomedical Science, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang 50112, Indonesia

⁴Department of Child Health, Faculty of Medicine, Universitas Sebelas Maret/Dr.Moewardi Hospital, Surakarta 50112, Indonesia

(*Corresponding author's e-mail: citraprimavita@gmail.com)

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Abstract

Aplastic anemia (AA) is a severe bone marrow failure syndrome characterized by pancytopenia and hypocellular marrow, driven by disrupted hematopoietic stem/progenitor cell (HSPC) pathways and elevated inflammatory cytokines, particularly IFN- γ . Current therapies have limitations, underscoring the need for innovative regenerative approaches. The mesenchymal stem cell (MSC) secretome, especially from hypoxic preconditioning (SH-MSCs), exhibits enhanced paracrine factor secretion, survival, and immunomodulatory efficacy by mimicking the bone marrow niche. This study evaluated the therapeutic potential of SH-MSC secretome in a busulfan-induced AA mouse model. Thirty Balb/c mice were divided into 5 groups: Healthy, AA (control negative), AA + standard medication (control positive), AA + 200 μ L SH-MSCs, and AA + 200 μ L SH-MSCs + standard medication. AA was induced by busulfan injection. SH-MSCs were isolated from human umbilical cords and cultured under hypoxic conditions (2% O₂). Hematological parameters (RBC, platelets, leukocytes, hemoglobin), gene expression (IL-3, IFN- γ), EPO levels, and bone marrow cellularity were assessed. The AA model exhibited significant reductions in RBC, platelet, and leukocyte counts compared to healthy controls ($p < 0.05$). SH-MSCs (200 μ L) significantly restored RBC counts, while SH-MSCs alone and combination therapy significantly increased platelet, leukocyte, and hemoglobin levels compared with AA controls ($p < 0.05$). IFN- γ expression was significantly reduced, and bone marrow cellularity was markedly enhanced by both treatments ($p < 0.05$). No significant differences were observed between SH-MSCs alone and combination therapy for RBCs, hemoglobin, or other parameters ($p > 0.05$). These findings demonstrate that hypoxic preconditioned MSC secretome effectively promotes hematopoiesis, suppresses inflammation, and restores bone marrow function in AA, offering comparable efficacy to combination therapy and strong potential as a novel standalone or adjunctive treatment.

Keywords: Aplastic anemia, Mesenchymal stem cell secretome, Hypoxia, Hematopoiesis, Immunomodulation, Busulfan, Murine model

Introduction

Aplastic anemia (AA) is a life-threatening bone marrow failure syndrome marked by severe peripheral pancytopenia and hypocellular marrow, resulting from the depletion and dysfunction of hematopoietic

stem/progenitor cells (HSPCs) [1]. AA pathogenesis is driven by disrupted apoptotic and differentiation pathways in CD34+ cells. Transcriptomic analyses reveal upregulation of pro-apoptotic genes, including

Bax and caspase-3, along with downregulation of survival signals, such as Bcl-2 and PI3K/AKT [2]. This imbalance is further aggravated by the actions of inflammatory cytokines, particularly interferon-gamma (IFN- γ), which plays a central role in disease severity [3]. IFN- γ disrupts the generation and differentiation of common myeloid progenitors (CMPs), granulocyte/monocyte progenitors (GMPs), and megakaryocyte/erythrocyte progenitors (MEPs), leading to a marked reduction in the production of red blood cells (RBCs), leukocytes, and platelets [4]. This cytokine-mediated inhibition is intrinsic to HSPCs and results in profound marrow hypocellularity and peripheral pancytopenia, hallmarks of AA [5].

Interleukin-3 (IL-3) is another critical hematopoietic growth factor whose deficiency impairs the proliferation and differentiation of hematopoietic progenitors, exacerbating cytopenias [6]. Additionally, erythropoietin (EPO) levels are often elevated in AA patients as a compensatory response to anemia. Yet, this increase is insufficient to overcome the block in erythropoiesis caused by stem cell depletion and cytokine-mediated suppression [7,8]. The reduction in RBCs leads to decreased hemoglobin concentrations, while thrombocytopenia and leukopenia further increase the risk of bleeding and infection, respectively [9]. The severity of AA is thus closely linked to the degree of suppression in these parameters, red blood cells (RBC), hemoglobin, leukocyte, and platelet levels, making their analysis essential for assessing disease progression and therapeutic efficacy [9,10].

The bone marrow environment in AA is typically hypocellular, reflecting the loss of hematopoietic elements and their replacement by fat. However, in some cases, areas of hypercellularity can be observed, particularly during early or recovery phases, or in response to certain therapies, indicating attempts at regeneration or dysregulated hematopoiesis [11]. The global incidence of AA varies widely, with estimates ranging from 0.6 to 6.1 cases per million population annually [3,5]. The burden is notably higher in Asia, where incidence rates are 2 to 3 times greater than in Europe or Israel. In Indonesia, the prevalence is estimated at 2 - 5 cases per million population, according to the Ministry of Health data. Mortality remains high: without treatment, most patients die within the first year of diagnosis, and the one-year

mortality rate can reach up to 70% [12,13]. These sobering statistics highlight the urgent need for innovative therapies that can restore marrow function and improve patient survival, particularly in high-incidence regions such as South and Southeast Asia.

Current therapies, including immunosuppressive regimens (e.g., antithymocyte globulin and cyclosporine A) and bone marrow transplantation, are limited by donor availability, risk of graft-versus-host disease, and incomplete restoration of the hematopoietic niche [14]. Mesenchymal stem cells (MSCs) and their secretome have emerged as promising therapeutic candidates due to their immunomodulatory and regenerative properties. The MSC secretome, especially when derived under hypoxic conditions, is enriched in growth factors and cytokines including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase-1 (IDO-1), interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1Ra), and prostaglandin E2 (PGE2) that support hematopoiesis, modulate immune responses, and promote tissue repair [15].

Importantly, hypoxic preconditioning of MSCs has been demonstrated in various in vivo disease models, exhibiting significant immunomodulatory and regenerative effects. Studies demonstrate that hypoxia-conditioned MSCs' secretome enhances proliferation, migration, angiogenesis, and anti-inflammatory functions more effectively than that of normoxic MSCs [16,17]. A previous study in rat models of osteochondral defects and acute kidney injury showed that hypoxia preconditioning improves cartilage regeneration and tissue repair by enriching extracellular vesicles and elevating trophic factors, such as VEGF and basic fibroblast growth factor (bFGF) [17-19]. Furthermore, hypoxic MSC secretome modulates signaling pathways including phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), hypoxia-inducible factor 1-alpha (HIF-1 α) stabilization, and transforming growth factor-beta (TGF- β)/SMAD2, which are critical for anti-apoptotic and immunoregulatory activities [18,20]. Although direct studies in aplastic anemia models are lacking, these findings suggest that hypoxic MSC secretomes hold therapeutic potential in immune-mediated and degenerative disorders.

While previous studies have demonstrated the benefits of MSC transplantation in AA, there is a lack of systematic evaluation of hypoxia-optimized MSC secretome in robust animal models that recapitulate the molecular and cellular features of human AA [4,21]. The busulfan-induced AA rat model, which mimics the oxidative stress and stem cell depletion observed in human diseases, provides an ideal platform to investigate whether hypoxic MSC secretome can restore hematopoietic parameters (RBC, leukocyte, platelet, hemoglobin) and rebalance cytokine profiles (IL-3, IFN- γ , EPO) [22]. By targeting these molecular and cellular pathways, this approach offers a novel and potentially safer strategy for treating AA, with particular relevance for regions with high disease burden and limited access to advanced therapies.

Materials and methods

Experimental animals and ethical considerations

This study used BALB/c mice (*Mus musculus*) as subjects. Mice aged 8 to 10 weeks and weighing between 20 and 35 g were selected. Before their inclusion in the study, the mice were deemed suitable by a veterinarian from the Animal Model Research Center at SCCR Indonesia in Semarang. Ethical approval for this study was obtained from the Ethics Committee of the Faculty of Medicine at Universitas Islam Sultan Agung Semarang, under reference number 43/I/2025/Komisi Bioetik.

Aplastic anemia model induction

Aplastic anemia was induced in mice by administering Busulfan (Sigma-Aldrich, Catalog No. MFCD00007562, St. Louis, MO, USA) through intraperitoneal injection at a dose of 10 mg/kg of body

weight [23]. The Busulfan was given on days 1, 3, 8, 11, 15, 18, 21, and 23 to achieve a toxic dose. Busulfan was dissolved in acetone at a concentration of 5 - 10 mg/mL [24]. Immediately before administration, deionized water was added to the Busulfan-acetone solution in a 3 - 10:1 water-to-acetone ratio. This solution was then administered by intraperitoneal injection at a volume of 0.1 - 0.2 mL per mouse, up to a maximum of 10 mL/kg body weight.

Treatment of experimental animals

In this study, 30 mice were divided into 5 groups. The groups are Healthy Group, which consists of healthy mice without any conditions. Control Negative Group (C-): Mice with Aplastic Anemia who received no treatment. Control Positive Group (C+), Mice in this group have Aplastic Anemia and received standard medication, specifically methylprednisolone (Sigma-Aldrich, Catalog No. M0639, St. Louis, MO, USA) and cyclosporine (Sigma-Aldrich, Catalog No. 30024, St. Louis, MO, USA). SH-MSCs Group, mice with Aplastic Anemia that received 200 μ L of hypoxic MSC secretome (SCCR Indonesia, batch 11078925; total protein concentration 1.625 mg/mL, corresponding to 325 μ g total protein per mouse per injection) along with standard medication. Combination Group, mice with Aplastic Anemia that received a combination of 200 μ L of hypoxic MSC secretome (1.625 mg/mL; 325 μ g protein/mouse) and standard medication. The standard therapy involved administering a methylprednisolone intraperitoneal injection at a dosage of 1 mg/kg/day to the mice for 14 days. Additionally, oral cyclosporine was administered at a dosage of 5 mg/kg/day on days 1, 7, and 14. Both the methylprednisolone injections and the oral cyclosporine were started simultaneously on the first day of treatment.

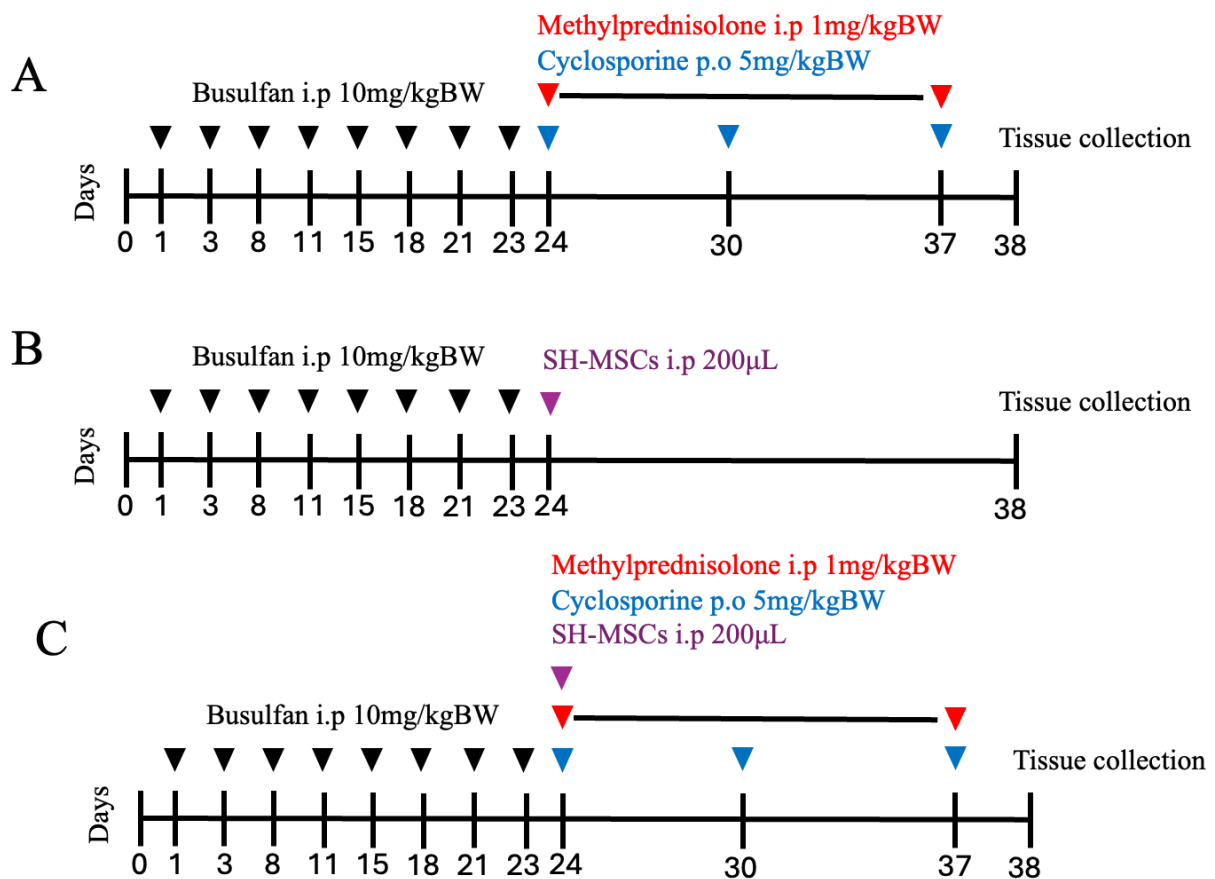


Figure 1 Experimental design for (A) standard therapy, (B) SH-MSCs, and (C) a combination of standard therapy and SH-MSCs.

Isolation of MSCs from human umbilical cord

All procedures were conducted under aseptic conditions in a Class II biosafety cabinet using sterile instruments and high-sterility techniques. Human umbilical cords were obtained from healthy full-term deliveries (gestational age, 38 - 40 weeks) after written informed consent was obtained from the mothers. Only cords from cesarean section deliveries were included to minimize the risk of contamination. The collection and use of human umbilical cords were approved by the Institutional Ethics Committee of the Faculty of Medicine at Universitas Islam Sultan Agung Semarang, under reference number 43/I/2025/Komisi Bioetik, in accordance with the Declaration of Helsinki. Immediately after delivery, cords were transferred to sterile containers containing 0.9% NaCl (Sigma-Aldrich, S5886, St. Louis, MO, USA) and transported to the laboratory within 4 h. Each umbilical cord sample was processed individually. In total, umbilical cords from 3 donors were used for MSCs isolation. For the preparation of the secretome, equal cell numbers

(7.5×10^6 cells in Flask T75) and culture conditions were maintained for MSCs derived from each donor. The collected conditioned media were then pooled to minimize donor variability. In the laboratory, a section of the cord was transferred to a sterile petri dish and thoroughly washed with phosphate-buffered saline (PBS) (Gibco, 10010023, Waltham, MA, USA) to remove blood residues. The washed cords were finely minced into small fragments (approximately 2 - 3 mm³) and evenly spread in a 25 cm² tissue culture flask (Thermo Fisher Scientific, 145004, Waltham, MA, USA). After allowing 3 min for tissue adherence, complete culture medium comprising Dulbecco's Modified Eagle Medium (DMEM, Gibco, 11965092), amphotericin B 0.5% (Gibco, 15290018), penicillin-streptomycin 1% (Gibco, 15140122), and fetal bovine serum 10% (Gibco, 16000044) was gently added to cover the explants. Cultures were maintained in a humidified incubator at 37 °C with 5% CO₂. MSCs began to emerge from the tissue approximately 14 days after culture initiation. The medium was refreshed every

3 days by replacing half with fresh complete medium. Subculture and maintenance continued until the cells reached 80% confluence, at which point they were harvested for subsequent expansion and secretome collection.

Characterization and validation of human umbilical cord-derived MSCs

The identity and multipotency of the isolated umbilical cord-derived MSCs were confirmed by surface marker expression and differentiation assays. Flow cytometry analysis was performed to assess MSC-specific markers following the criteria established by the International Society for Cellular Therapy (ISCT). For flow cytometry, cells at passage 3 - 4 were detached using TrypLE Express (Gibco, 12604013) and washed twice with phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA). Approximately 1×10^6 cells were incubated with fluorochrome-conjugated monoclonal antibodies for 30 min at 4 °C in the dark. The antibodies used were anti-CD90-FITC (BioLegend, 328110, San Diego, CA, USA), anti-CD29-PE (BD Biosciences, 555443, Franklin Lakes, NJ, USA), anti-CD45-PerCP (BioLegend, 304024), and anti-CD31-APC (BioLegend, 303116). Unstained cells served as negative controls. After incubation, cells were washed twice with PBS-BSA and analyzed using a BD Accuri C6 flow cytometer (BD Biosciences, USA). MSCs were identified by positive expression of CD90 and CD29 and negative expression of hematopoietic and endothelial markers, including CD45 and CD31. Data analysis was performed with FlowJo™ software version 10.8 (Tree Star Inc., Ashland, OR, USA) [25].

For functional validation, MSCs were induced to differentiate into adipogenic and osteogenic lineages using standard differentiation media. Adipogenic differentiation was induced by culturing cells in adipogenic induction medium consisting of DMEM supplemented with 10% FBS, 1 µM dexamethasone (Sigma-Aldrich, D4902), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, I5879), 10 µg/mL insulin (Sigma-Aldrich, I9278), and 0.2 mM indomethacin (Sigma-Aldrich, I7378) for 14 days with medium changes every 3 days. Lipid accumulation was visualized by staining with Oil Red O (Sigma-Aldrich, O0625) for 20 min at room temperature, followed by washing with distilled water. [26] Osteogenic

differentiation was induced using osteogenic medium composed of DMEM supplemented with 10% FBS, 0.1 µM dexamethasone (Sigma-Aldrich, D4902), 10 mM β-glycerophosphate (Sigma-Aldrich, G9422), and 50 µg/mL ascorbic acid (Sigma-Aldrich, A5960) for 21 days, with medium refreshed every 3 days. Mineralized calcium deposits were confirmed by staining with Alizarin Red S (Sigma-Aldrich, A5533) for 10 min, followed by rinsing with distilled water. The formation of lipid droplets and calcium nodules confirmed the adipogenic and osteogenic differentiation potential of the UC-MSCs, respectively [27,28].

Preparation and isolation of Hypoxia-induced MSC-Derived secretome (SH-MSCs)

Human umbilical cord-derived MSCs at passages 3 - 4 were used to collect secretomes. Cells were cultured in 75 cm² flasks at 80% confluence in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco, 11965092) supplemented with 10% fetal bovine serum (FBS; Gibco, 16000044), 1% penicillin-streptomycin (Gibco, 15140122), and 0.5% amphotericin B (Gibco, 15290018). Prior to secretome collection, the cultures were washed twice with phosphate-buffered saline (PBS; Gibco, 10010023) to remove residual serum and replaced with a defined, serum-free DMEM (10 mL per flask) to prevent contamination by exogenous proteins.

The flasks were placed inside an airtight hypoxia chamber (Billups-Rothenberg MIC-101, USA). Nitrogen gas was introduced through the inlet valve until the oxygen level reached 5%, as verified with an oxygen meter at the chamber port. The chamber was sealed and incubated at 37 °C for 24 h to induce hypoxic conditions. After incubation, the conditioned medium containing the hypoxia-induced MSC secretome was carefully collected and centrifuged at 300×g for 10 min to remove detached cells. This was followed by a second centrifugation at 2,000×g for 15 min to remove cell debris. The resulting supernatant was concentrated and purified using tangential flow filtration (TFF) with a 100-2 kDa molecular weight cut-off filter cassette (Pellicon, Merck Millipore, Darmstadt, Germany). The filtration process was performed under aseptic conditions in a closed-loop system until the desired concentration was achieved, approximately 20-fold concentration. The concentrated secretome solution was

then sterilized by passing through a 0.22 μm PES syringe filter (Millipore, SLGP033RS). The final hypoxia-induced MSC secretome (SH-MSC) preparation was aliquoted into sterile cryovials and immediately stored at -80°C until use.

Termination and bone marrow tissue collection

The aspiration site for bone marrow was clearly marked. An anesthetic solution (2% lidocaine) was prepared in a syringe. A separate 20 mL syringe was filled with a small amount of EDTA (Sigma-Aldrich, Catalog No. E7889, St. Louis, MO, USA) for cytology examination or heparin (Sigma-Aldrich, Catalog No. H3393, St. Louis, MO, USA) for cytogenetics. Aseptic and antiseptic procedures were performed at the aspiration site using sterile gauze moistened with 10% povidone-iodine (Betadine, Purdue Pharma) or chlorhexidine, starting from the center and moving outward in a circular motion until approximately 8 - 9 cm from the site. Sterile drapes were then placed around the area. After the pre-procedural preparations, the anesthetic was administered. A sufficient amount of aspirate was collected and dropped onto a glass slide (Thermo Fisher Scientific, Catalog No. 12-550-123, Waltham, MA, USA), where it was spread evenly to ensure the presence of bone marrow particles. If the specimen was deemed appropriate, the remaining aspirate was transferred to a collection bottle (BD Vacutainer, Catalog No. 366430, Franklin Lakes, NJ, USA) and sent to the laboratory for analysis. Thin smears were made on additional glass slides to facilitate better evaluation of the bone marrow particles and to prevent the accumulation of fat or bone debris. This technique was similar to that used for preparing peripheral blood smears. The prepared specimens were immediately stained using the May-Grünwald-Giemsa method (Sigma-Aldrich, Catalog No. 48900, St. Louis, MO, USA).

Mouse orbital blood sample collection

Mice were carefully removed from their cage and held securely by the head with the right hand. An eye anesthetic ointment was applied to each mouse. The skin around the mouse's eye was gently pulled taut using the thumb and forefinger. Blood was collected from the mouse's tail by inserting a capillary tube into the medial canthus of the eye at a 30-degree angle towards the

mouse's nose. After filling, the capillary tube was lifted and wiped with sterile cotton. Blood was collected into an Eppendorf tube until a sufficient volume was obtained. A micropipette was then used to aspirate 1 mL of blood, and the pipette was cleaned with cotton. The pipette, containing the blood sample, was placed into a tube until its tip touched the bottom. The tip was then pressed to ensure the entire liquid was mixed. Gentle pressure was applied to the area around the mouse's eye to stop any bleeding. Finally, the mouse was returned to its cage and re-examined 30 min later to check for any post-procedure lesions.

RNA extraction and cDNA synthesis

A 100 mg sample was taken from RNALater (Thermo Fisher Scientific, Catalog No. AM7020, Waltham, MA, USA), cut into small pieces, and placed into a tube containing 50 μL of RNAiso Plus (Takara Bio, Catalog No. 9108, Kusatsu, Shiga, Japan). The sample pieces were crushed using a micropestle, and another 50 μL of RNAiso Plus was added. The mixture was stored at room temperature for 5 min. After that, 20 μL of chloroform (Merck, Catalog No. 102445, Darmstadt, Germany) was added, and the solution was vortexed until it turned milky white. The sample was incubated at room temperature for 2 - 3 min, then centrifuged at 15,000 rpm for 15 min at 4°C , yielding 3 distinct layers: the top layer contained RNA (liquid phase), the second layer contained DNA (semisolid phase), and the bottom layer contained cell debris. The uppermost RNA layer was transferred to a new centrifuge tube, and its volume was measured. Isopropanol (Merck, Catalog No. 100983, Darmstadt, Germany) was then added in the same volume as the RNA taken from the uppermost layer. The Eppendorf tube was shaken until white threads appeared, then centrifuged again at 15,000 rpm for 10 min at 4°C . The supernatant was discarded until a visible white pellet formed at the bottom of the tube. After drying, 100 μL of 70% ethanol in Diethyl pyrocarbonate (DEPC)-treated water (Thermo Fisher Scientific, Catalog No. AM9915, Waltham, MA, USA) was added, and the tube was inverted repeatedly before being centrifuged again at 15,000 rpm for 5 min at 4°C . The supernatant was discarded, and an additional 30 - 50 μL of DEPC water was added. The mixture was incubated at 55°C for 10 min, yielding the total RNA solution, which was stored

at -80°C . The RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Model ND-1000, Catalog No. ND-1000), and the quantification results were adjusted to 3,000 ng. cDNA synthesis was performed by preparing mixture A, which combined the quantified RNA sample with 1 μL of Oligo(dT) primer (Thermo Fisher Scientific, Catalog No. 18418020) and PCR-grade water to achieve a final volume of 10 μL . This mixture was incubated for 5 min at 70°C . Mixture A was then combined with mixture B, consisting of 4 μL of 5X buffer (Toyobo, Catalog No. FSQ-201, Osaka, Japan), 5 μL of DEPC-treated water, and 1 μL of ReverTra Ace reverse transcriptase (Toyobo, Catalog No. TRT-101, Osaka, Japan). The combined mixture was incubated sequentially at 25°C for 5 min, 42°C for 50 min, and 85°C for 5 min.

IL-3 and IFN- γ gene expression examination using real time-polymerase chain reaction (RT-PCR)

Primers for IL-3 and IFN- γ genes were designed using the NCBI Primer-BLAST tool (National Center for Biotechnology Information, Bethesda, MD, USA; <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The sequences of the primers used in this study are listed in **Table 1**. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for normalization. Total RNA was extracted from bone marrow tissue samples using RNAiso Plus reagent (Takara Bio, Catalog No. 9108, Kusatsu, Shiga, Japan),

following the manufacturer's protocol. RNA quality and quantity were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, ND-1000), ensuring an A260/A280 ratio between 1.8 and 2.0. RNA samples were then adjusted to a concentration of 3,000 ng/ μL for downstream application. Complementary DNA (cDNA) synthesis was performed using the ReverTra Ace reverse transcriptase kit (Toyobo, Catalog No. TRT-101, Osaka, Japan) according to the manufacturer's instructions. Briefly, 1 μL of Oligo(dT) primer (Thermo Fisher Scientific, Catalog No. 18418020) and total RNA were incubated at 70°C for 5 min, followed by reverse transcription at 42°C for 50 min, and enzyme inactivation at 85°C for 5 min. qRT-PCR amplifications were conducted on an Eco Real-Time PCR System (Illumina, Catalog No. 20018781, San Diego, CA, USA) using SensiFAST SYBR No-ROX Mix (Bioline, Cat. No. BIO-98020, London, UK). Each 20 μL reaction mixture consisted of 10 μL of 2 $\times$ SYBR Green Master Mix, 1.6 μL of primer mix (forward and reverse, each at 0.8 μL), 1 μL of cDNA, and nuclease-free water up to 20 μL . The thermal cycling program included an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 56°C for 20 s. Relative gene expression levels were quantified using the $2^{-\Delta\Delta\text{Ct}}$ method, normalizing target gene expression to GAPDH. Data analysis was performed using EcoStudy software (Illumina, San Diego, CA, USA).

Table 1 Primer sequences for qRT-PCR.

Gene	Primer sequence
IL-3	Forward: CCTGCCTACATCTGCGAATGAC Reverse: GAGGTTAGCACTGTCTCCAGATC
IFN- γ	Forward: CAGCAACAGCAAGGCGAAAAAGG Reverse: TTTCCGCTTCCTGAGGCTGGAT
GAPDH	Forward: CATCACTGCCACCCAGAAGACTG Reverse: ATGCCAGTGAGCTTCCCGTTCAG

EPO level examination

Bone marrow response to erythropoietin (EPO) was assessed by measuring Fe-heme synthesis in bone marrow suspensions cultured for 3 days with and without erythropoietin addition. Bone marrow cells were isolated and cultured in RPMI 1,640 medium (Gibco, Thermo Fisher Scientific, Catalog No.

11875093, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Catalog No. 16000044), 1% penicillin-streptomycin (Gibco, Catalog No. 15140122), and 2 U/mL of recombinant human erythropoietin (rhEPO) (PeproTech, Catalog No. 100-64, Rocky Hill, NJ, USA) for the treatment group. Cultures were maintained at 37°C , 5% CO_2 for

72 h. Following incubation, Fe-heme synthesis was quantified by measuring incorporation of radioactive iron (Fe-59) (PerkinElmer, Catalog No. NEZ033, Waltham, MA, USA) or using a colorimetric heme assay kit (Sigma-Aldrich, Catalog No. MAK316, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, bone marrow suspensions were incubated with Fe-59 for 4 h at 37 °C. After centrifugation, cell pellets were washed and solubilized, and radioactivity was measured using a gamma counter (PerkinElmer, Model Cobra II Auto-Gamma, Waltham, MA, USA). For colorimetric assays, absorbance was measured at 400 nm using a microplate reader (BioTek, Model Synergy H1, Winooski, VT, USA). The EPO effect was evaluated by comparing Fe-heme synthesis in treated versus untreated cell cultures.

Red blood cell count examination

Blood was slowly extracted from the mouse's tail by applying pressure to the base of the tail and massaging it toward the tip. Blood was then aspirated into a Thoma pipette (Hawksley, Catalog No. HA/13140, Sussex, UK) up to either the 0.5 or 1.0 mark. Hayem's solution (ENG Scientific, Catalog No. ES5601, Dunstable, UK) was added to the Thoma pipette until it reached the 101 mark, resulting in a dilution of either 1/200 or 1/100. Both ends of the pipette were sealed with the thumb and middle finger, and the contents were mixed by inverting the pipette. After inverting, 1 - 2 drops of the fluid from the Thoma pipette were expelled and discarded. The tip of a micropipette was then placed on one side of a counting chamber (Hausser Scientific, Catalog No. 7150, Horsham, PA, USA), which had a coverslip and tissue paper on the opposite side. The fluid from the Thoma pipette was allowed to flow into the counting chamber, which was subsequently placed under a microscope (Olympus, Model BX51, Tokyo, Japan). Erythrocytes in the counting chamber were counted in a zigzag pattern starting from the left side. To ensure accurate counting, erythrocytes located on the border lines of the left and top sides of each small chamber were considered as being within that chamber. The actual number of erythrocytes was calculated using the following formula: Number of erythrocytes per $\text{mm}^3 = N \times p / V$ (S), where N = number of erythrocytes counted from 5 counting fields, p = dilution factor (either 100 or 200),

and V = volume of 5 counting fields (calculated as 0.2 mm side length of 1 R-cell multiplied by 0.1 mm depth of the counting chamber).

Platelet count examination

Blood was slowly collected from the mouse's tail by pressing at the base and gently massaging toward the tip. The blood was drawn into a Thoma pipette until reaching the 0.5 or 1.0 mark. A dilution of 1/200 or 1/100 was then prepared. Both ends of the pipette were sealed with the thumb and middle finger, and mixing was accomplished by inverting the pipette. One to 2 drops of fluid were expelled from the Thoma pipette and discarded. Next, the tip of a micropipette was placed against one side of a counting chamber, while a coverslip and tissue paper were positioned on the opposite side. The fluid from the Thoma pipette flowed into the counting chamber, which was then placed under a microscope. The platelet count was conducted immediately using a hematology analyzer as a control. Venous blood samples were examined after resting at room temperature. The platelet count was re-evaluated using an Abacus 3CT hematology analyzer.

Hemoglobin level examination

Hemoglobin levels were measured using a spectrophotometer at a wavelength of 540 nm. First, 5 mL of Drabkin solution was added to a reaction tube. Next, 20 μL of blood was carefully drawn using a micropipette and added to the tube containing the Drabkin solution (Sigma-Aldrich (Catalog No. D5941). The sample in the reaction tube was then transferred to a cuvette. The spectrophotometer was set to a wavelength of 540 nm. A standard Drabkin solution was used as a blank, and the instrument was calibrated to full scale with an absorbance reading of 0. After calibrating, the sample was inserted into the spectrophotometer, and the absorbance was recorded from the screen. The hemoglobin level was then calculated using the formula: Hemoglobin level (g/dl) = Absorbance $\times$ 36.8.

Leukocyte level examination

Blood samples were diluted with Turk's solution (Sigma-Aldrich, Catalog No. 109277, St. Louis, MO, USA), which contains glacial acetic acid (the weak acid responsible for hemolyzing erythrocytes) and gentian violet dye to stain leukocytes for counting. The dilution

was typically performed using a pipette or leukocyte tube, resulting in either a 10x or 20x dilution. The diluted sample was then loaded into a counting chamber (Hausser Scientific, Catalog No. 7150, Horsham, PA, USA). Under a microscope (Olympus, Model BX51, Tokyo, Japan) at low magnification, leukocytes were counted in 4 large squares of the chamber. The number of leukocytes per milliliter of blood was calculated using the formula $(N/0.4) \times 20$ or $N \times 50$, where N is the number of leukocytes counted, 0.4 represents the volume of the counting chamber, and 20 is the dilution factor.

Histological analysis of bone marrow

Femurs were collected from all experimental animals at the end of the treatment period to evaluate bone marrow recovery and cellularity. The bones were carefully dissected, cleaned of adherent tissue, and immediately fixed in 10% neutral-buffered formalin (Sigma-Aldrich, HT501128) for 24 h at room temperature. After fixation, femurs were decalcified in 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4; Sigma-Aldrich, E5134) for 14 days, with the decalcification solution refreshed every 2 days. After complete decalcification, tissues were washed in phosphate-buffered saline (PBS), dehydrated through a graded ethanol series, cleared with xylene, and embedded in paraffin. Longitudinal bone sections of 5 μ m thickness were prepared using a rotary microtome (Leica RM2235, Germany) and mounted on glass slides. The slides were dried overnight at 37 °C and then stained with hematoxylin-eosin (HE). For staining, sections were deparaffinized in xylene, rehydrated through a series of decreasing ethanol concentrations, and rinsed in distilled water. The sections were stained with Mayer's hematoxylin (Sigma-Aldrich, MHS16) for 5 min, washed under running tap water, and counterstained with eosin Y (Sigma-Aldrich, HT110132) for 1 min. After staining, slides were dehydrated, cleared in xylene, and mounted using DPX mounting medium (Sigma-Aldrich, 06522). Stained sections were examined under a light microscope (Olympus BX53, Tokyo, Japan) at 400x magnification. Bone marrow cellularity was evaluated semi-quantitatively based on the proportion of hematopoietic cells occupying the marrow cavity relative to adipose tissue components. Five randomly selected high-power

fields (HPFs) per section were evaluated for each animal, and images were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Data analysis

After acquiring the data, we performed processing, editing, tabulation, and cleaning. We then conducted descriptive statistics on the dataset and followed up with a Shapiro-Wilk test to assess the data's normality. If the data were normally distributed, we tested for homogeneity using Levene's test. For homogeneous data, we analyzed the differences among treatment groups using parametric One-Way ANOVA ($p < 0.05$), which was followed by Post Hoc LSD testing. The data analysis was conducted using SPSS 29.0.

Results and discussion

Mesenchymal stem cells (MSCs) exhibit remarkable self-renewal and multipotency, making them attractive candidates for regenerative therapy. In this study, we isolated and characterized MSCs derived from neonatal umbilical cord tissue and evaluated the therapeutic potential of their hypoxia-conditioned secretome (SH-MSCs) in a busulfan-induced aplastic anemia (AA) model. The following results detail the phenotypic characterization of MSCs, validation of the AA animal model, and the hematopoietic and immunomodulatory effects of SH-MSCs in restoring bone marrow function.

Mesenchymal stem cell (MSCs) isolation and validation

Mesenchymal stem cells (MSCs) were successfully isolated from neonatal umbilical cord tissue using enzymatic digestion and subsequently cultured in a specialized MSC medium. By passage 4, the cells exhibited the hallmark characteristics of MSCs. These included strong adherence to the surface of culture flasks. Morphologically, the cells displayed spindle-like shapes with thin, elongated cytoplasm and centrally located, elliptical nuclei. They either grew sparsely or formed small colonies, showing a fibroblastic pattern characteristic of healthy, proliferating MSCs (**Figure 2**). These features confirmed the successful isolation of viable MSCs that remained undifferentiated.

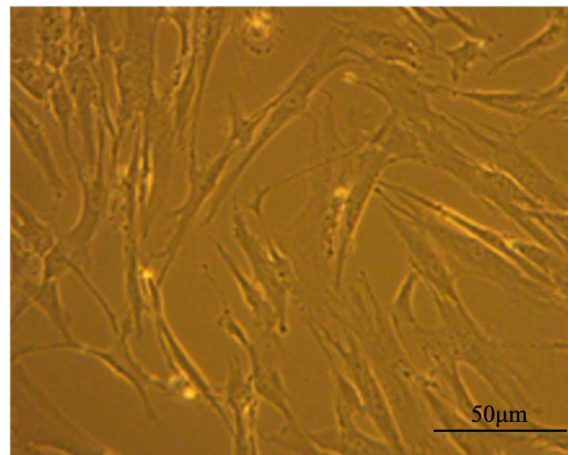


Figure 2 Morphological characterization of isolated MSCs showing a spindle-like morphology of MSCs isolated from neonatal umbilical cord tissue after 4 passages, as observed under brightfield microscopy at 100x magnification. Scale bar = 50 μ m.

Flow cytometry validation

The identity of the MSCs was further confirmed using flow cytometry. This analysis showed that the isolated cells exhibited high expression levels of the surface markers CD90 (99.80%) and CD29 (94.20%). In contrast, there was minimal expression of hematopoietic

markers, with CD45 at 1.60% and CD31 at 6.60% (**Figure 3**). These results strongly validate the isolated cells as mesenchymal stem cells, which is crucial for their potential therapeutic applications, particularly in stem cell-based therapies for hematopoietic regeneration.

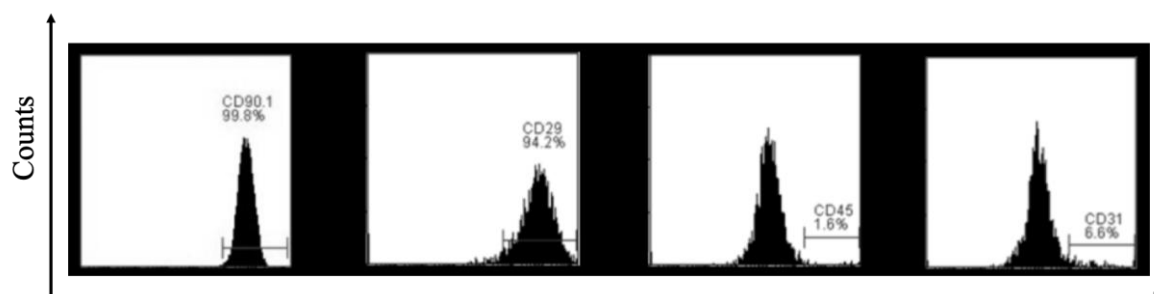


Figure 3 presents a flow cytometry analysis of mesenchymal stem cell (MSC) surface markers. The analysis demonstrates the expression of CD90 and CD29, while confirming the absence of hematopoietic markers CD45 and CD31. These findings establish the mesenchymal identity of the isolated cells. The results are based on 3 independent isolations, with the percentage of positive cells calculated from a minimum of 10,000 events.

Differentiation assays

The differentiation capacity of MSCs was assessed through adipogenic and osteogenic differentiation assays. Adipogenic differentiation was confirmed by Oil Red O staining, which revealed lipid droplets, an indication of successful adipocyte differentiation (**Figure 4(A)**). Similarly, osteogenic differentiation was

demonstrated through Alizarin Red staining, which showed calcium deposition, a key marker of osteogenesis (**Figure 4(B)**). This ability of MSCs to differentiate into multiple cell types highlights their versatility and supports their potential application in regenerative medicine, particularly in promoting hematopoietic recovery.

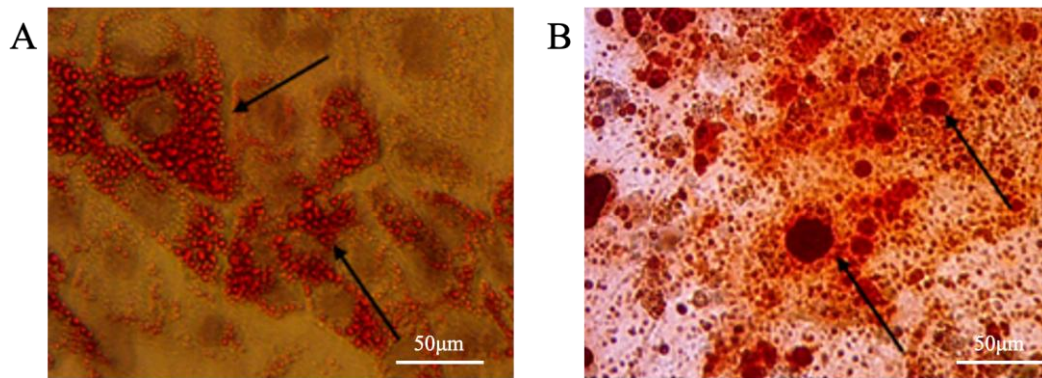


Figure 4 Differentiation Potential of MSCs. (A) Adipogenic differentiation of MSCs was confirmed by Oil Red O staining, which shows the formation of lipid droplets in the cytoplasm. (B) Osteogenic differentiation of MSCs was confirmed by Alizarin Red staining, demonstrating calcium deposition in the extracellular matrix. Images are shown at 40x magnification. Scale bar: 50 μm.

MSC secretome isolation and characterization

To investigate the paracrine mechanisms of mesenchymal stem cells (MSCs), we isolated their secretome from MSCs cultured under hypoxic conditions (5% oxygen) for 24 h. Hypoxia is known to enhance the secretion of bioactive factors that are critical for tissue regeneration. After incubation, we concentrated the culture medium containing the MSC secretome using tangential flow filtration (TFF) to enrich for soluble factors secreted by the cells. We

analyzed the cytokine profile of the secretome using an enzyme-linked immunosorbent assay (ELISA), which revealed the presence of key cytokines: IL-10 (an anti-inflammatory cytokine), TNF- α , and IL-6 (pro-inflammatory cytokines), VEGF (which promotes angiogenesis), and TGF- β (which regulates growth and differentiation) (**Figure 5**). These findings highlight the therapeutic potential of SH-MSCs to modulate the immune response and promote tissue repair, particularly in inflammatory conditions such as aplastic anemia.

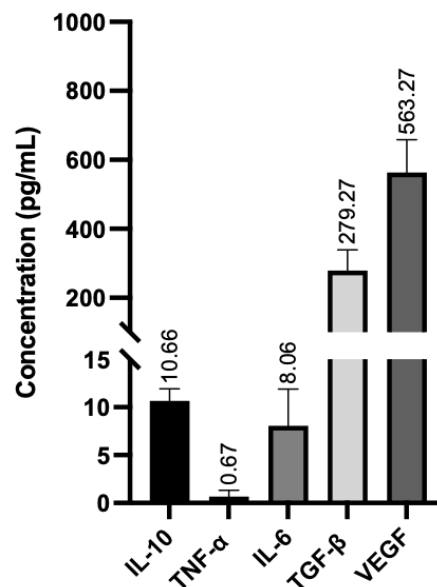


Figure 5 Cytokine and Growth Factor Profile of SH-MSCs. The levels of cytokines and growth factors in the secretome of SH-MSCs, analyzed using ELISA. The secretome was obtained from MSCs cultured under hypoxic conditions (5% O₂) for 24 h. Data is based on 3 independent isolations and is presented as mean $\pm$ standard deviation (SD).

Animal model validation for aplastic anemia

The busulfan-induced model of aplastic anemia was validated by significant reductions in key hematological parameters, including red blood cell (RBC), platelet, leukocyte, and reticulocyte counts. In the aplastic anemia group, the RBC count dropped significantly to $8.67 \pm 2.56 \times 10^{12}$ cells/L, whereas the healthy control group showed an RBC count of $11.72 \pm 1.75 \times 10^{12}$ cells/L, confirming the successful induction of anemia (**Figure 6(A)**). Additionally, the platelet count in the aplastic anemia group was significantly lower at 248.11 ± 86.10 compared to the healthy control group, which had a platelet count of 846.22 ± 196.74 . The decrease in platelet count further supports bone marrow suppression induced by busulfan (**Figure 6(B)**). A significant decrease in leukocyte count is also observed in the aplastic anemia group (2.85 ± 1.61)

compared to the healthy group (7.34 ± 3.72) (**Figure 6(C)**). This indicates that busulfan not only suppresses erythrocyte and platelet production but also leukocyte production, leading to pancytopenia in the animal model, consistent with the characteristics of aplastic anemia. The reticulocyte count in the aplastic anemia group (409.73 ± 297.42) was numerically higher than in the healthy group (209.94 ± 144.63), but the difference was not statistically significant (**Figure 6(D)**). This pattern may reflect compensatory reticulocyte release early after busulfan exposure or high inter-animal variability typical of murine models, despite confirming pancytopenia through significant reductions in RBCs, platelets, and leukocytes [29]. All validation parameters collectively confirm the presence of successful busulfan-induced aplastic anemia, characterized by bone marrow suppression.

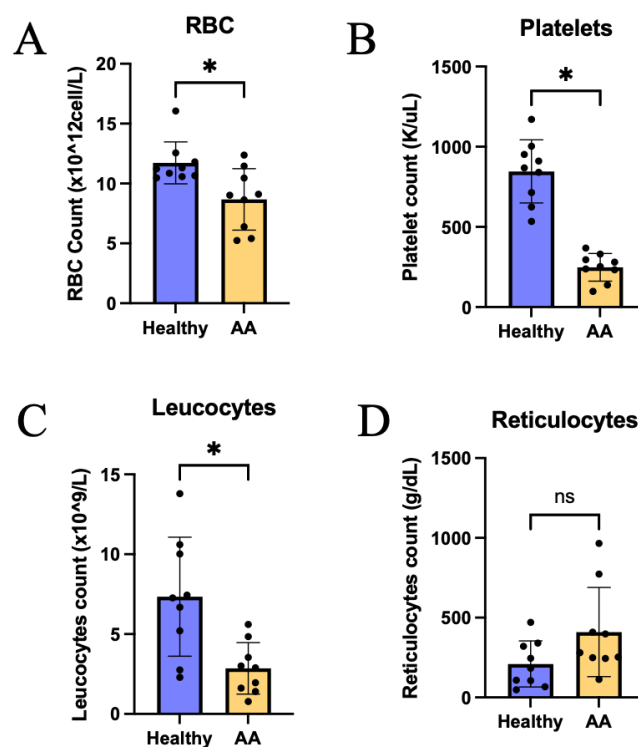


Figure 6 Hematological Parameters in a Busulfan-Induced Aplastic Anemia Model. The hematological parameters, including (A) red blood cell (RBC) counts, (B) platelet counts, (C) leukocyte counts, and (D) reticulocyte counts, were compared between the healthy group and the aplastic anemia (AA) group. This data validates the busulfan-induced model of aplastic anemia. There were 6 samples per group. Blood collection and analysis were performed using automated hematology analyzers. AA: Anemia aplastic. * $p < 0.05$, ns $p > 0.05$.

The effects of SH-MSCs on hematological parameters in an aplastic anemia model

Given the critical role of RBC production in anemia, the effects of SH-MSCs on RBC recovery were assessed. In the healthy control group, RBC counts were maintained at $9.79 \pm 0.66 \times 10^{12}$ cells/L, whereas the C- group showed a significant decrease in RBC count to $7.30 \pm 1.82 \times 10^{12}$ cells/L, confirming the development of aplastic anemia. Notably, administration of SH-MSCs at a dose of 200 μ L significantly restored RBC counts to $10.76 \pm 0.62 \times 10^{12}$ cells/L (**Figure 7(A)**). This demonstrates that SH-MSCs effectively stimulate erythropoiesis, offering a potential alternative therapy for the treatment of anemia aplastic induced by busulfan. In contrast, the combination therapy with C+

and SH-MSCs did not show significant improvements over SH-MSCs alone, suggesting that SH-MSCs alone are sufficient to enhance RBC recovery. Furthermore, platelet count is another critical component in assessing hematopoietic recovery, particularly in conditions like aplastic anemia. The C- group showed a dramatic decrease in platelet count to 206.67 ± 71.73 , compared to the healthy control group (658.67 ± 218.25), confirming the suppression of hematopoiesis induced by busulfan. Platelet recovery was observed in the positive control group (585.33 ± 319.65), SH-MSCs (535.00 ± 182.62), and the combination therapy group (555.50 ± 113.98), indicating the potential of these treatments to restore hematopoietic function (**Figure 7(B)**).

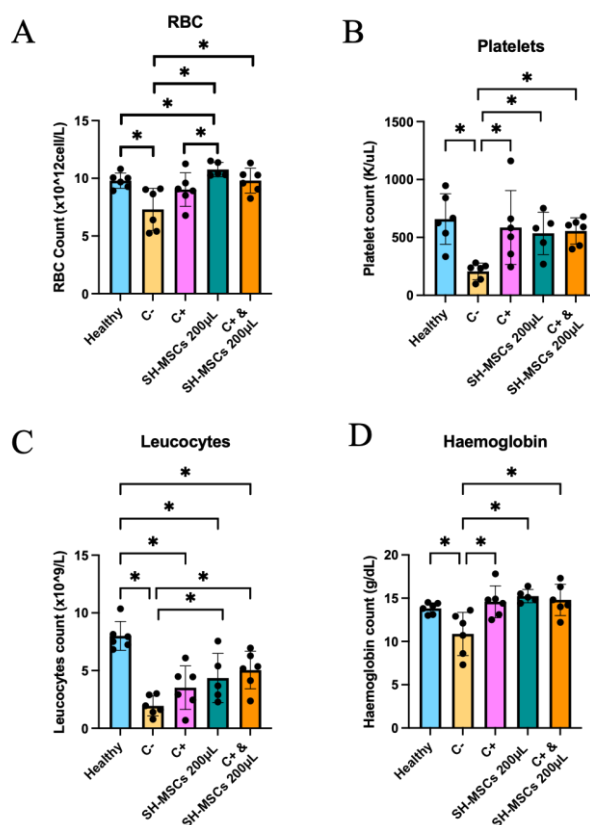


Figure 7 Effects of SH-MSCs on Hematological Parameters in Aplastic Anemia Model (A) RBC Count that demonstrates the effect of SH-MSCs on erythropoiesis recovery in the model of aplastic anemia. (B) Platelet Count shows the impact of SH-MSCs on platelet recovery in aplastic anemia. (C) Leukocyte Count, which indicates the effect of SH-MSCs on immune cell recovery in the aplastic anemia model. (D) Hemoglobin Levels highlight SH-MSCs' influence on hemoglobin recovery in aplastic anemia. $n = 6$ per group for each parameter. Statistical analysis used One-Way ANOVA, followed by post hoc LSD test for multiple comparisons. Data are presented as mean $\pm$ SD. * $p < 0.05$.

Leukocyte count, reflecting immune function, was significantly reduced in the C- group (1.94 ± 0.86)

compared to the healthy control group (8.00 ± 1.24). In response to SH-MSCs (200 μ L) and combination

therapy, leukocyte counts increased significantly to 4.36 ± 2.12 and 5.04 ± 1.63 , respectively (**Figure 7(C)**). The significant recovery of leukocytes in both the SH-MSC and combination therapy groups underscores SH-MSCs' ability to support the immune system in aplastic anemia. On the other hand Hemoglobin levels, an essential indicator of RBC function, were significantly lower in the K- group (10.87 ± 2.49) compared to the healthy group (13.82 ± 0.64). SH-MSCs at 200 μ L and the combination therapy showed significantly higher hemoglobin levels (15.24 ± 0.76 and 14.80 ± 1.80 , respectively), surpassing the values in the healthy control group (**Figure 7(D)**).

Effects of SH-MSCs on IL-3 gene expression in the aplastic anemia model

IL-3, a key cytokine involved in hematopoiesis, was significantly reduced in the C- group (0.63 ± 0.37) compared to the healthy control group (1.08 ± 0.44). Although SH-MSCs (200 μ L) and combination therapy slightly increased IL-3 expression (0.78 ± 0.34 and 0.91 ± 0.29), the differences were not statistically significant ($p > 0.05$) (**Figure 8**). These findings suggest that while SH-MSCs may have a mild effect on IL-3 expression, their primary therapeutic mechanism may lie in promoting hematopoiesis via other pathways.

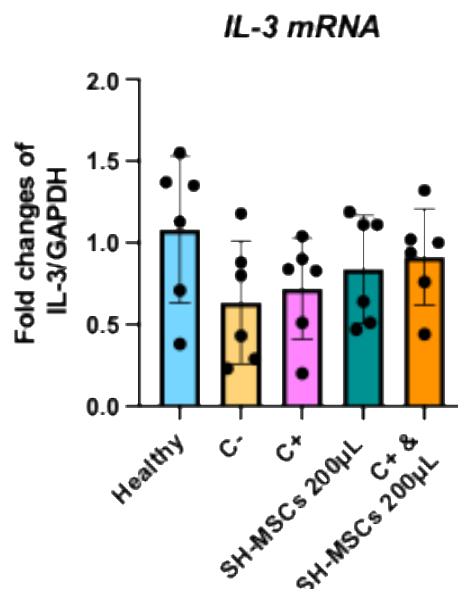


Figure 8 Effects of SH-MSCs on IL-3 gene expression in an aplastic anemia model under qRT-PCR analysis. n = 6 per group. Statistical analysis used One-Way ANOVA, followed by post hoc LSD test for multiple comparisons. Data are presented as mean $\pm$ SD. * $p < 0.05$.

Effects of SH-MSCs on IFN- γ gene expression in the aplastic anemia model

IFN- γ , a pro-inflammatory cytokine, was significantly elevated in the C- group (1.92 ± 0.67) compared to the healthy control group (1.04 ± 0.33), reflecting an inflammatory state. SH-MSCs (200 μ L)

and combination therapy significantly reduced IFN- γ expression to 1.14 ± 0.37 and 1.13 ± 0.13 , respectively (**Figure 9**). This anti-inflammatory effect supports the potential of SH-MSCs to restore a healthy bone marrow microenvironment, promoting hematopoiesis in the process.

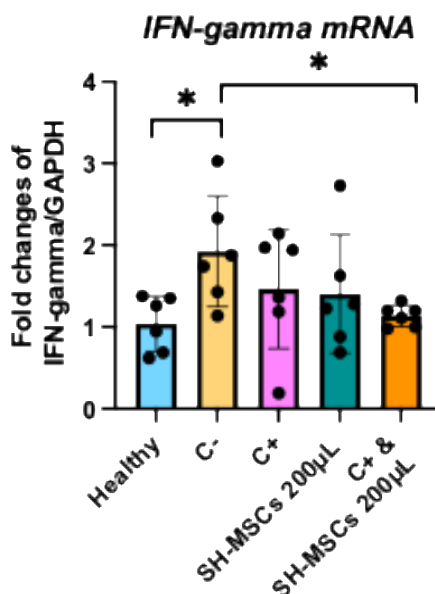


Figure 9 Effects of SH-MSCs on IFN- γ gene expression in an aplastic anemia model under qRT-PCR analysis. $n = 6$ per group. Statistical analysis used One-Way ANOVA, followed by post hoc LSD test for multiple comparisons. Data are presented as mean $\pm$ SD. * $p < 0.05$.

Effects of SH-MSCs on erythropoietin (EPO) levels in the aplastic anemia model

EPO, an essential hormone for RBC production, showed no significant differences between treatment

groups and the C- group (**Figure 10**). A numerical trend toward higher EPO levels was observed in the combination therapy group (59.20 ± 11.08 pg/mL), though this did not reach statistical significance.

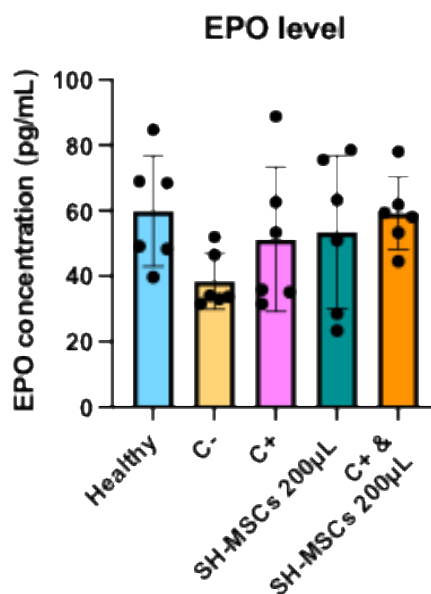


Figure 10 Effects of SH-MSCs on EPO levels in an aplastic anemia model. $n = 6$ per group. Statistical analysis used One-Way ANOVA.

Effects of SH-MSCs on bone marrow hypercellularity in the aplastic anemia model

Bone marrow hypercellularity, a marker of hematopoietic regeneration, was significantly reduced

in the C- group (24.53 ± 5.16) compared to the healthy group (50.14 ± 3.07). Both SH-MSCs (200 μ L) and combination therapy significantly increased bone marrow hypercellularity (to 31.07 ± 3.22 and $38.06 \pm$

8.38, respectively; **Figure 11**). These results indicate that SH-MSCs and combination therapy effectively support bone marrow regeneration, demonstrating their

potential in treating aplastic anemia and other hematopoietic disorders.

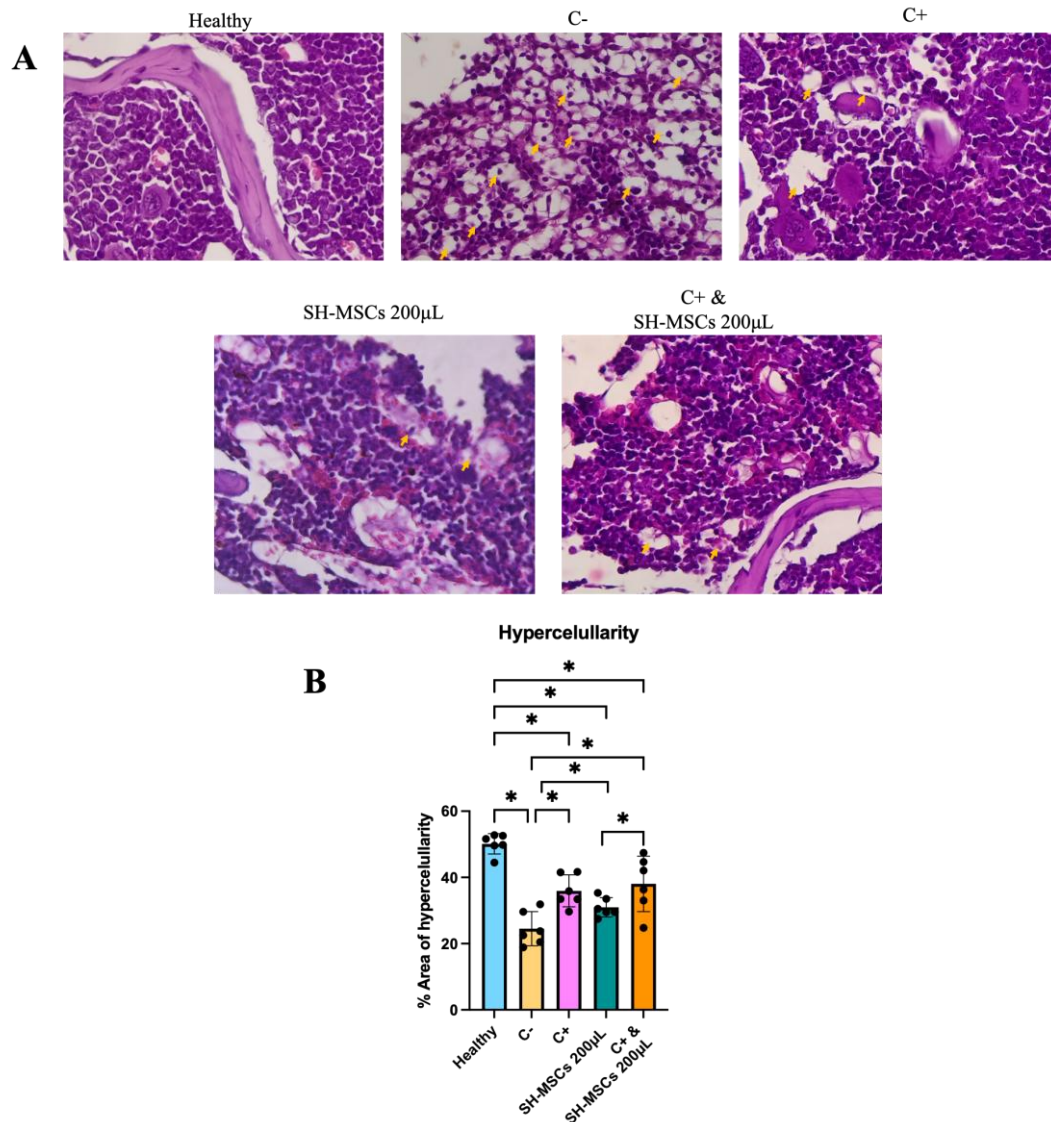


Figure 11 (A) HE staining of Bone marrow hypercellularity in all groups, indicating hematopoietic regeneration. (B) Quantification of % area of hypercellularity per total area. $n = 6$ per group. Statistical analysis used One-Way ANOVA, followed by post hoc LSD test for multiple comparisons. Data are presented as mean $\pm$ SD. $*p < 0.05$.

Aplastic anemia (AA) is a hematopoietic disorder characterized by bone marrow failure, resulting in pancytopenia [30]. The pathogenesis of AA is closely linked to damage to hematopoietic stem cells (HSCs), which can be triggered by abnormal immune responses, oxidative stress, and the loss of supportive hematopoietic microenvironmental factors [10,12]. Inflammatory processes play a central role, particularly through the activation of autoreactive CD8⁺ T cells that secrete pro-inflammatory cytokines such as interferon

gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α). IFN- γ has been shown to induce apoptosis in HSCs via the Fas/FasL pathway and to suppress the expression of key growth factors, such as stem cell factor (SCF) and interleukin-3 (IL-3), both of which are crucial for HSC proliferation and differentiation [31,32]. Immune dysregulation in AA also manifests as an imbalance in T cell subpopulations, with increased Th1 cells (producing IFN- γ) and Th17 cells (secreting IL-17) that promote HSC destruction. In contrast, regulatory T cells

(Tregs), which normally suppress autoreactive responses, are reduced in number and function [33]. The Th1 transcription factor T-bet drives IFN- γ expression, and the Th17 transcription factor ROR- γ t promotes IL-17 production, both of which contribute to bone marrow inflammation. The hypoxic MSC secretome can potentially restore this balance by inhibiting T-bet and ROR- γ t expression while enhancing Foxp3⁺ Treg differentiation via secreted factors such as TGF- β and IL-10 [14]. Hypoxic MSCs secrete a diverse array of bioactive factors, including cytokines, growth factors, and extracellular vesicles that contain microRNAs and proteins [34]. Under hypoxic conditions, MSCs upregulate hypoxia-inducible factor-1 α (HIF-1 α), which enhances the secretion of pro-angiogenic and anti-apoptotic factors [35]. These secreted factors act synergistically to promote HSC survival, proliferation, and differentiation while modulating the bone marrow inflammatory microenvironment.

Conventional immunosuppressive therapies such as methylprednisolone and cyclosporine have shown variable efficacy and are frequently associated with adverse effects, including hypertension, nephrotoxicity, electrolyte imbalances, hepatic inflammation, and gingival swelling [36]. This underscores the need for alternative therapies with improved efficacy and safety profiles. Emerging evidence suggests that secretome MSCs contain exosomes and microRNAs (miRNAs) derived from MSCs, which play crucial roles in intercellular communication and tissue regeneration. Secretome can transfer functional miRNAs that regulate gene expression in recipient HSCs, potentially modulating apoptotic pathways, immune responses, and hematopoietic recovery. Specifically, certain miRNAs have been shown to suppress IFN- γ production and restore Treg function, making them relevant therapeutic candidates for immune-mediated AA.

Our findings demonstrate that the combination therapy significantly improved red blood cell, platelet, and leukocyte counts, hemoglobin levels, IL-3, and erythropoietin (EPO) concentrations, as well as bone marrow cellularity, while reducing IFN- γ expression. Notably, administration of 200 μ L of SH-MSCs alone resulted in a greater increase in RBC count and hemoglobin than the combination therapy. The beneficial effects of SH-MSCs can be attributed to their rich content of cytokines and growth factors, such as

HGF, VEGF, SCF, and IL-6, which are known to stimulate the proliferation of hematopoietic progenitors [37,38]. Previous studies have also reported that MSC secretome enhances hematopoietic gene expression and promotes bone marrow regeneration [39], and that MSCs and their derivatives can ameliorate anemia through paracrine mechanisms that boost erythropoiesis and suppress pathological immune responses [40].

Recent studies increasingly support that hypoxia preconditioning enhances the therapeutic properties of the MSC secretome compared to normoxic conditions [34]. Hypoxic cultivation upregulates the secretion of pro-regenerative and immunomodulatory factors, such as VEGF, HGF, SDF-1, and TGF- β , thereby potentiating angiogenesis, anti-inflammatory activity, and the secretome's resilience to oxidative stress [41]. Research demonstrates that hypoxia-induced MSC secretome exhibits superior hematopoietic support and more pronounced inhibitory effects on pro-inflammatory cytokine production in experimental models of bone marrow failure and graft-versus-host disease [42]. In clinical settings, mesenchymal stem cell (MSC) infusions have resulted in improved hematopoietic reconstitution and reduced graft failure or severe graft-versus-host disease (GvHD) among patients undergoing transplantation for aplastic anemia. In addition, the enhanced expression of regulatory molecules, including interleukin-10 (IL-10), prostaglandin E2 (PGE2), and indoleamine 2,3-dioxygenase (IDO), in the hypoxia-conditioned secretome contributes to the effective suppression of aberrant T cell activation and the restoration of marrow homeostasis [43]. Previous studies have demonstrated that hypoxic MSC-conditioned medium can increase bone marrow cellularity by 40% - 60% and enhance HSC proliferation, as indicated by increased Ki-67 expression [44]. Hypoxia preconditioning thus not only increases the yield of bioactive factors but also achieves a secretome profile more closely matched to the requirements for immune modulation and tissue repair in AA and related hematopoietic disorders.

Importantly, the combination of standard therapy and SH-MSCs effectively suppressed IFN- γ gene expression, highlighting the immunoregulatory potential of this approach. The downregulation of IFN- γ not only supports hematopoietic activity but also indicates that SH-MSCs exert both hematopoietic and

immunomodulatory effects. This is consistent with prior findings that MSCs can inhibit T cell activation and IFN- γ production, thereby fostering a bone marrow microenvironment conducive to regeneration [45,46]. In the context of AA, the bone marrow microenvironment is often dysfunctional, marked by the loss of extracellular matrix components [47]. SH-MSCs can modulate the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), preserving tissue architecture [12]. Furthermore, TGF- β within SH-MSCs promotes angiogenesis and vascular integrity in the marrow Xu *et al.*, [48] Crane & Cao, [49], while the secretome can inhibit Th1 differentiation by suppressing T-bet and upregulating GATA-3 and FOXP3, thereby restoring immune balance [50,51].

At the molecular level, the combination of SH-MSCs, methylprednisolone, and cyclosporine reduces IFN- γ gene expression through several mechanisms: cyclosporine inhibits T cell activation via the calcineurin/NFAT pathway, methylprednisolone suppresses NF- κ B and AP-1 transcription factors, and SH-MSCs provide anti-inflammatory cytokines such as TGF- β and IL-10, which suppress Th1 differentiation and promote Treg expansion [52]. Additionally, SH-MSC components, such as IDO and PGE2, further inhibit T cell proliferation and activation. In the busulfan-induced AA model, these mechanisms collectively mitigate pro-inflammatory immune responses and facilitate marrow recovery [53].

Our study also observed increased IL-3 gene expression and EPO levels in groups treated with SH-MSCs, supporting the hypothesis that SH-MSCs strongly influence hematopoietic cytokine pathways. IL-3 accelerates multipotent progenitor proliferation, while EPO specifically induces erythroid differentiation [54]. Growth factors in SH-MSCs, such as HGF, VEGF, and IGF, activate the JAK/STAT, PI3K/AKT, and MAPK/ERK signaling pathways, promoting HSC proliferation and differentiation [55]. ERK1/2 activation is particularly important for erythroid progenitor proliferation and granulopoiesis in AA patients. Prior studies have also shown that MSC-conditioned medium can upregulate EPO receptor expression in the bone marrow of anemic mice [7,56].

Histological analysis in this study revealed increased bone marrow cellularity in the group receiving

combined SH-MSCs and standard therapy, suggesting enhanced marrow proliferative activity. While SH-MSCs alone and combination therapy both effectively restored hematological parameters and bone marrow cellularity, no significant differences were observed between them ($p > 0.05$), suggesting that the hypoxic MSC secretome provides robust benefits independent of or additive to standard medication. Overall, our results suggest that SH-MSCs hold considerable promise for enhancing AA through both hematopoietic and immunomodulatory mechanisms. This opens new avenues for the development of safer and more effective MSC secretome-based therapies for AA.

This study has several limitations, including the fact that the preclinical murine model of aplastic anemia provides valuable mechanistic insights but may not fully recapitulate the complexity of human disease. Furthermore, although key cytokines were quantified by ELISA (**Figure 5**), the precise molecular composition of the secretome, including detailed proteomics, exosomal content, and miRNA profiles, was not characterized. Future studies using targeted proteomic, exosomal, or miRomics analyses will be needed to confirm the specific bioactive factors, including potential miRNAs/exosomes, driving the observed therapeutic effects in this AA model. In this study, only a single dosing regimen and route of administration were evaluated; the optimal dose and delivery strategy for maximal hematopoietic recovery remain undetermined.

Conclusions

In summary, the hypoxia-conditioned MSC secretome significantly promotes hematopoietic and immune recovery in an aplastic anemia model by enhancing erythropoiesis, restoring bone marrow cellularity, and suppressing inflammatory cytokine expression. These findings support SH-MSCs as a potent, cell-free therapeutic strategy for bone marrow failure syndromes.

Declaration of Generative AI in Scientific Writing

The author utilized artificial intelligence tools solely for grammar and language editing purposes to enhance readability, as the author is not a native English speaker. The AI was not used for data analysis, interpretation, or content generation. The author takes

full responsibility for the accuracy, integrity, and originality of all content presented in the manuscript.

CRedit Author Statement

Citra Primavita Mayangsari, Harsono Salimo, Yulia Lnati Retno Dewi: Conceptualization; Methodology; Software. **Agung Putra:** Data curation; Writing - Original draft preparation. **Betty Suryawati:** Visualization; Investigation. **Agung Putra, Annag Giri Moelyo:** Supervision. **Citra Primavita Mayangsari:** Software; Validation. **Citra Primavita Mayangsari, Agung Putra:** Writing - Reviewing and Editing.

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