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ORIGINAL ARTICLE

Optimization of a Culture Protocol for Murine Bone Marrow-Derived Mesenchymal Stromal Cells

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Abstract

Background: Mesenchymal stem cells (MSCs) have attracted extensive research due to their high proliferative capacity, multipotent differentiation, and essential role in tissue regeneration. Despite their application in clinical research, there are limitations in the culture and maintenance of these cells. This study aimed to optimize a cost-effective and efficient protocol for the culture and expansion of murine MSCs (M-MSCs).

Methods: MSCs were isolated from BALB/c mouse femoral bone marrow (6–8 weeks old) and cultured in DMEM-Low Glucose with 20%-5% fetal bovine serum (FBS), penicillin/streptomycin (1%), and gentamicin (0.5%). At passage 3, cells exhibited fibroblastic morphology and were immunophenotyped for MSCs (CD73-PE, CD105-PerCP-Cy5.5) and hematopoietic markers (CD34-PE, CD45-FITC). The growth rate, post-freezing cell viability, and population doubling time were evaluated.

Results: Fibroblastic morphology was observed at 48 hours post-primary culture, with cells forming a scaffold and reaching 80% confluency by day 5. Under 5% FBS conditions, the time to reach 90% confluency decreased to <5 days by passages 2 and 3. Flow cytometry confirmed a typical MSCs profile: high CD73 (99.9%) and CD105 (98.5%) and low CD34 (5.99%) and CD45 (2.86%) expression. Results show a positive correlation between increasing passage number and enhanced MSCs growth rate, viability and functional characteristics ($p \leq 0.05$).

Conclusion: Optimization of M-MSCs culture through a FBS reduction (20% to 5%) is shown to preserve key cellular properties: fibroblastic morphology, proliferation rate, and immunophenotypic markers. Our protocol reduces microbial contamination associated with animal serum and provides an economical, and viable strategy for large-scale M-MSCs production in regenerative medicine.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of self-renewal and differentiation into mesodermal lineages, including osteoblasts, chondrocytes, and adipocytes, a property designated as trilineage differentiation potential (1). This defining characteristic underlies their widespread application in regenerative medicine and tissue engineering (2). Although MSCs can be isolated from multiple tissues such as adipose tissue, umbilical cord, and dental pulp (3), bone marrow represents the most extensively charac-

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terized source in both preclinical and clinical investigations (4). MSCs serve as essential experimental models for elucidating fundamental mechanisms of cellular differentiation, tissue development, and repair (5). Furthermore, their immunomodulatory properties position them as promising candidates for cell-based therapies targeting inflammatory (6), autoimmune, cancer(7, 8) and, degenerative conditions (9).

Moreover, murine and human MSCs exhibit conserved biological properties, including comparable immunophenotypic profiles ($CD73^+/CD90^+/CD105^+/CD34^-/CD45^-$)(10), trilineage differentiation capacity, and immunomodulatory mechanisms, making murine models highly relevant for protocol development. Optimization of culture conditions in murine MSCs provides a framework that can inform and accelerate the refinement of human MSCs expansion protocols, thereby enhancing reproducibility and stability for clinical applications while reducing reliance on high serum concentrations (4).

Despite their therapeutic potential (11), technical challenges limit the isolation and expansion of bone marrow-derived MSCs in murine models, specifically BALB/c mice, are constrained by several technical challenges (5). The inherently low concentration of bone marrow cellular content, stromal microenvironment, limits isolation efficiency and low initial cell yield. This limitations, coupled with reduced proliferative capacity and phenotypic changes induced during serial passaging. Conventional protocols typically require high fetal bovine serum (FBS) supplementation ($\geq$10%). One of the aim of the present study is to reduce FBS use for MSCs culture and passaging, making the process both economical and less prone to animal-derived contamination. Alternatively, cells can be maintained without additional growth factors, relying on the secretory factors of MSCs themselves.

2. Materials and Methods

2.1. Animal

Female BALB/c mice were purchased from the Pasteur Institute of Iran. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Bu-Ali Sina University (Ethics Code: IR.BASU.REC.1404.012) and conducted. Female BALB/c mice (6–8 weeks old, 20–30 g) were housed under standardized conditions: 12-h light/dark cycle, temperature of 22 ± 1°C, 50–60% relative humidity.

2.2. Isolation and Primary Culture of Bone Marrow-Derived MSCs

BALB/c mice were euthanized under deep anesthesia (ketamine/xylazine, 90/10 mg/kg, i.p.). Femurs were aseptically harvested, cleared of adherent soft tissues, and epiphyses were excised. Bone marrow was flushed using 5 mL of DMEM(Dulbecco's minimal essential medium)-Low Glucose (DMEM-LG) supplemented with 1% penicillin/streptomycin (Pen/Strep). The cell suspension was collected in 15-mL tubes and centrifuged at 300 × g for 5 min. The supernatant was discarded, and the cell pellet was washed three times with PBS containing 5% Pen/Strep. The final pellet was resuspended in complete growth medium (DMEM-LG supplemented with 20% FBS, 1% Pen/Strep and 0.5% gentamicin) and seeded into T12.5 culture flasks. Cultures were maintained at 37°C in a humidified incubator with 5% CO2. Twenty-four hours post-seeding (passage 0), 90% of the medium was replaced to remove non-adherent hematopoietic cells, using DMEM-LG containing 10% FBS and 1% Pen/Strep and 0.5% gentamicin. Subsequent medium exchanges occurred on days 3 and 5. The culture media was gradually replaced with percentages of 80, 60, and 50 percent from passage 1 and FBS concentration was progressively decreased from 20% → 15% → 10% → 5% across three sequential passages, while maintaining 1% Pen/Strep and 0.5% gentamicin supplementation throughout.

2.3. Cell Passaging

Cells were passaged upon reaching 80% confluency (typically day 5 for P0, and <5 days for subsequent passages under 5% FBS). The culture medium was aspirated, and adherent cells were washed twice with PBS containing 5% Pen/Strep. Cells were detached using 0.25% trypsin-EDTA (3 min, 37°C), neutralized with complete medium, and transferred to 15-mL tubes. After centrifugation (300 × g, 5 min), the pellet was washed three times with PBS containing 5% Pen/Strep, resuspended in DMEM-LG containing 10% FBS and 1% Pen/Strep and 0.5% gentamicin.

2.4. Freezing and Storing

One of the major challenges in long-term storage of mesenchymal stem cells is their low resistance to freezing and damage by liquid nitrogen. For this purpose, third-passage cells were trypsinized, suspended in cryotubes containing 90% FBS and 10% dimethyl sulfoxide (DMSO) and quickly placed in liquid nitrogen.

2.5. Immunophenotypic Characterization

At passage 3, cells were harvested, washed twice with PBS, and counted using trypan blue exclusion (1×10^6 cells/sample). Cells were then incubated for 30 min at 4°C with fluorochrome-conjugated monoclonal antibodies: CD34-PE and CD45-FITC (negative markers), CD73-PE and CD105-PerCP-Cy5.5 (positive markers; all from BD Biosciences). Isotype-matched controls were included for gating validation. After two

washes with FACS buffer (PBS+2% FBS), samples were analyzed on a BD FACSCalibur flow cytometer (v10.8)(12).

2.6. Morphological Assessment and Data Analysis

Cell morphology was documented using a digital camera mounted on an inverted phase-contrast microscope (Olympus). The proliferation ratio were assessed by recording the time required to reach 80–90% confluency at each passage. Flow cytometry data were acquired on a BD FACSCalibur instrument and analyzed using FlowJo software (version 10.8). The data was statistically analyzed using GraphPad prism 8. One and Two-way ANOVA following post-hoc Tukey's test were performed ($P\text{-}value \leq 0.05$).

2.7. Growth Rate

Cell growth rate was determined by seeding cells at a specific density and monitoring cell numbers over a defined period. Cells were harvested at regular intervals, and viable cell counts were performed using a hemocytometer. The growth curve was plotted with cell number on the y-axis against time on the x-axis.

2.8. Post-Thaw Viability

Post-thaw viability was assessed by thawing cryopreserved cells according to standard procedures. Immediately after thawing, cells were stained with trypan blue, and the percentage of viable (unstained) cells was determined using a hemocytometer. Viability was calculated as: Viability (%) = (Number of viable cells / Total number of cells) × 100.

2.9. Population Doubling Time

The population doubling time (PDT) was determined from the exponential growth phase. Cells were seeded at time T1 with an initial cell number (N1), and at time T2, the cell number reached N2.

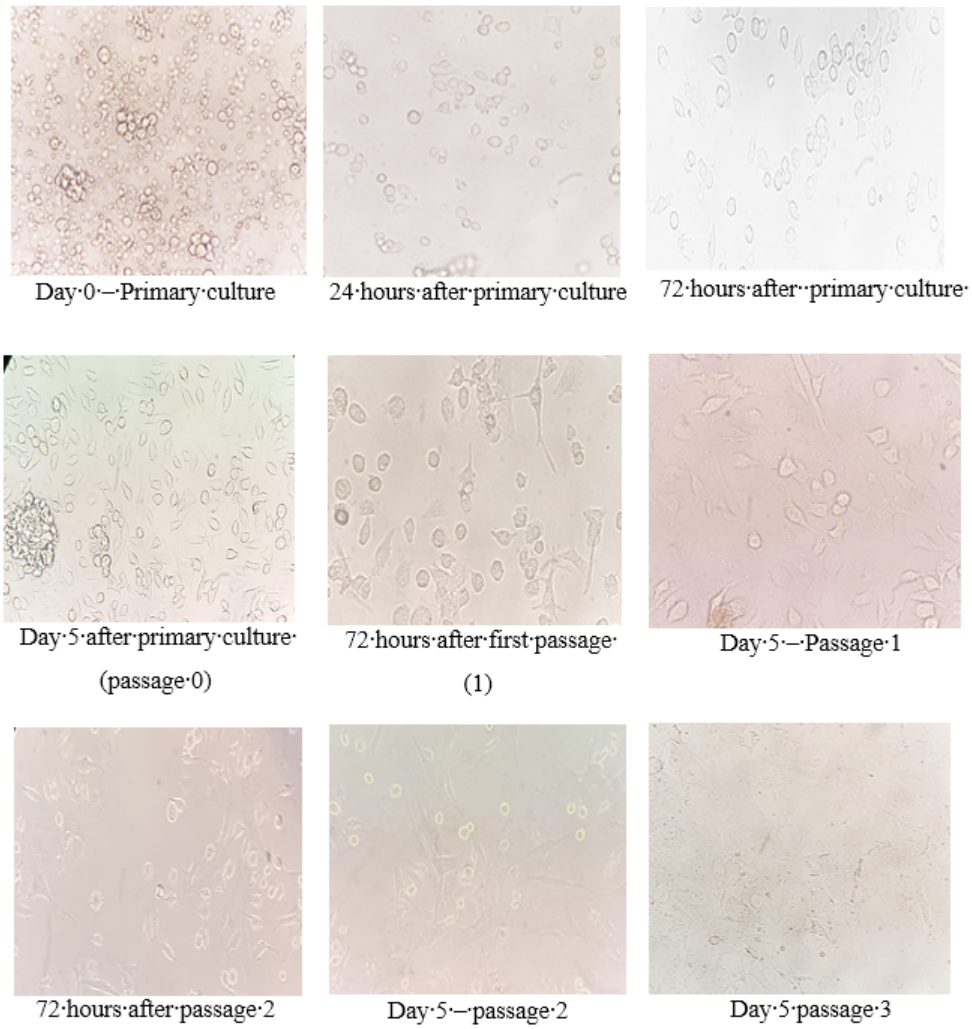

Fig. 1. Morphological changes of murine bone marrow-derived mesenchymal stem cells: from isolation to subsequent passages.

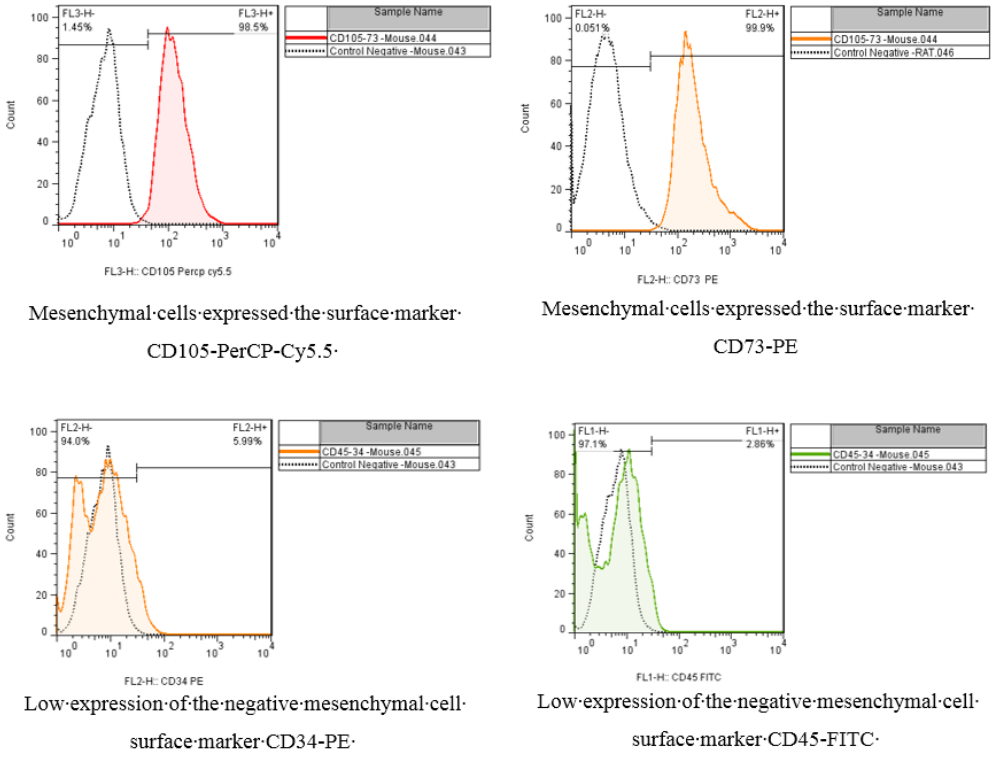

Fig. 2. Immunophenotyping of bone marrow-derived mesenchymal stem cells.

3. Result

3.1. Cell Culture and Passage

Adherent spindle-shaped fibroblastic cells were observed within 24 hours following primary culture initiation. By day 5, the monolayer reached 80% confluency and was subjected to enzymatic passaging using 0.25% trypsin-EDTA. Post-passage assessment revealed preservation of characteristic morphology without cytoskeletal disruption. Notably, cellular attachment and formation of interconnected stromal networks accelerated progressively across sequential passages under the optimized serum-reduced protocol. By gradually replacing the cell culture medium with varying percentages of serum-reduced medium, the growth factors present in the culture supernatant supported the cells. Despite the reduction in FBS, the growth of non-specific cells decreased, while the growth and proliferation rate of mesenchymal cells increased. The use of the antibiotic gentamicin was also effective in controlling contamination of the culture medium (Fig. 1).

4. Immunophenotypic Characterization

Flow cytometric analysis of surface markers confirmed high purity of the bone marrow-derived mesenchymal stem cell population, with minimal contamination by hematopoietic lineages. The cells exhibited very low expression of hematopoietic markers CD34 (5.99%) and CD45 (2.86%). Concurrently, characteristic MSCs markers were highly expressed: CD73 (99.9%) and CD105 (98.5%). This immunophenotypic profile, $CD73^{+}/CD105^{+}/CD34^{-}/CD45^{-}$, validates the efficacy of the optimized serum-reduction culture protocol in maintaining phenotype and purity throughout expansion(Fig. 2).

4.1. Cell Growth Rate

Analysis of cell growth rates across three passages revealed a consistent increase in growth rate with time. Passages 2 and 3 exhibited a higher initial growth velocity compared to passage 1, reaching maximum cell density between days 20 and 28 post-seeding. Notably, the growth rate accelerated with subsequent passages. Passage 3 cells demonstrated a 100% growth rate by day 10, while passage 2 reached this threshold by day 20. In contrast, passage 1 cells did not reach a 100% growth rate within the observed experimental period ($P \leq 0.05$) (Fig. 3).

4.2. Post-Thaw Viability

Cryopreservation and subsequent thawing of (MSCs) commonly present significant viability challenges, potentially leading to a decrease in cell survival of up to 50%. In this study, we observed a significant increase in post-thaw viability that correlated with increased passage number. Earlier passages, particularly passage 1, demonstrated diminished recovery. In contrast,

MSCs at passage 3 exhibited a significantly more functional post-thaw phenotype, characterized by substantially higher viability percentages, reflecting their more mature characteristics at this stage (P≤0.05) (Fig. 4).

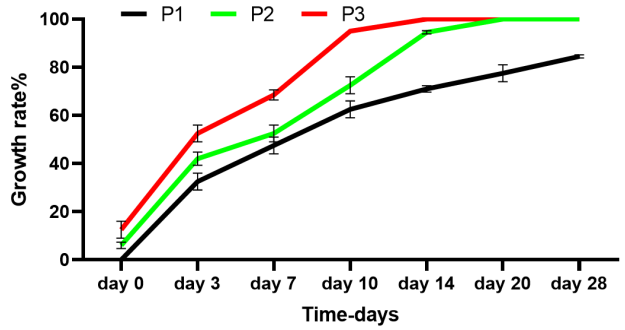

Fig. 3. Cell growth curve across different days for multiple cell passages.

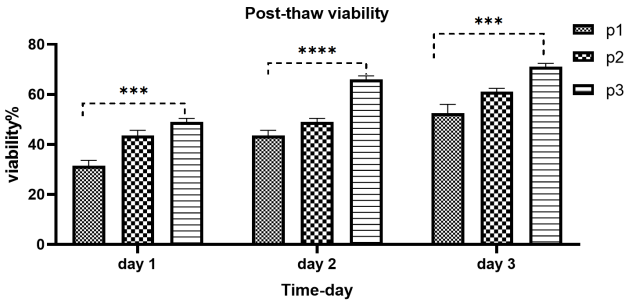

Fig. 4. Post-thaw cell viability during the initial three days.

4.3. Population Doubling Time

An increase in passage number was associated with a acceleration in cell proliferation. When cultured for an equivalent incubation period, a significant rise in total cell count was observed in higher passages. Cells at passage 3 exhibited approximately a three-fold increase in number compared with passage 1 and a two-fold increase compared with passage 2 (Fig. 5).

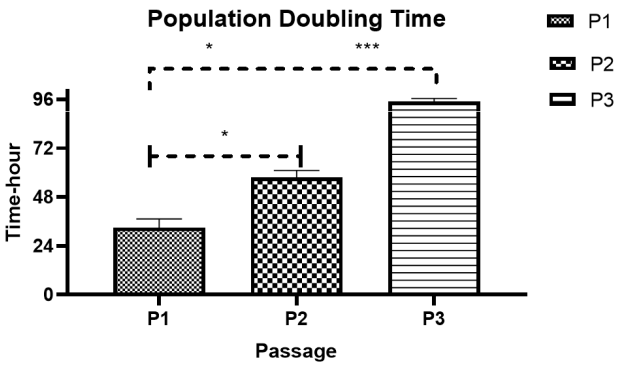

Fig. 5. Population doubling time of cells at different time points and across various passages.

4.4. Discussion

A principal limitation in the therapeutic application of MSCs is the difficulty to achieving optimal *in vitro* expansion while maintaining phenotypic stability and functional potency (13). The present study demonstrates that an optimized culture protocol, specifically adapted to the biological characteristics of bone marrow-derived MSCs from BALB/c mice, a strain known for low cellular yield, significantly improves both proliferation rates and phenotypic consistency. Key modifications in this study included: (i) gradual reduction of fetal bovine serum (FBS) concentration with medium adaptation while preventing microbial contamination; (ii) elimination of enzymatic digestion and filtration during primary isolation; (iii) accelerated growth following passaging; and (iv) efficient cryopreservation with high post-thaw viability. By passage 3 (P3), these adjustments produced a highly purified MSCs population without the use of exogenous growth factors, with a marked reduction in hematopoietic and non-mesenchymal contaminants and improved proliferative capacity and phenotypic stability. Phenotypic stability was maintained through p3, with cells reaching 80% confluence within 5–6 days after primary seeding. Flow cytometric analysis confirmed strong expression of CD105 and CD73 (>95% positive), and low expression of hematopoietic markers CD34 and CD45 (<6% positive) (Fig. 2). These immunophenotypic characteristics confirm the mesenchymal identity and high purity of the cultured cell population. The selection of an appropriate culture flask is critically important for successful monolayer expansion of MSCs (13). In this study, we utilized of T12.5 culture flasks, which have a smaller growth surface area compared with conventional T25 or T75 flasks. The reduced surface area in primary culture promoted higher effective cell density, thereby enhancing cell–cell interactions and supporting the formation of more scaffold-like cellular networks. Moreover, whereas standard protocols typically require 6–7 days to reach confluence for the first passage(5), our optimized approach achieved within 5–6 days. Accumulating evidence indicates that refined culture conditions profoundly influence the secretome of MSCs, upregulating trophic and immunomodulatory factors that are essential for self-renewal, differentiation potential, and *in vivo* functionality, thereby contributing to sustained phenotypic stability(14). Although batch-to-batch variability in culture medium components and reagents can also affect MSCs culture quality(15), this parameter warrants careful consideration in experimental design and protocol standardization. A defining characteristic of MSCs is their adherence to plastic culture surfaces(16), a property recognized as a fundamental criterion for MSCs identification and routinely used for their isolation and purification through plastic adherence-based selection(17).

Although multiple isolation and expansion methodologies have been established for MSCs, including serial medium replacement, immunomagnetic selection (positive/negative), filtration(18), and enzymatic digestion(19), each technique presents limitations regarding yield, purity, stability, or cost-effectiveness. Thus, the development of simplified protocols that minimize technical complexity while maximizing cellular quality are necessary. Preservation of optimal culture parameters to ensure long-term phenotypic stability and replicative capacity is essential for both fundamental investigations and applications including the advancement of MSCs-based regenerative medicine(20). The proliferative capacity and post-cryopreservation resilience of (MSCs) are demonstrably influenced by their *in vitro* passage number, as evidenced by the presented data. A consistent trend of accelerated growth kinetics was observed with increasing passage numbers. Specifically, passages 2 and 3 exhibited a more vigorous initial growth rate than passage 1, achieving peak cell densities between days 20 and 28 post-seeding. This enhanced proliferative potential is further supported by the finding that passage 3 cells reached 100% growth by day 10 within the experimental duration. This observations suggests that serial passaging potentiates the intrinsic proliferative capacity of MSCs, likely through adaptive mechanisms acquired during *in vitro* expansion. Consistent with this, post-thaw viability assessments revealed a significant positive correlation between passage number and cell recovery following cryopreservation. Given that cryopreservation protocols can reduce cell survival by up to 50%, the enhanced resilience observed in higher-passage MSCs is of considerable relevance. Passage 3 cells exhibited a markedly more post-thaw phenotype and higher viability compared to earlier passages, particularly passage 1, which showed reduced recovery. This improved cryosurvival at later passages may reflect the acquisition of more stable cellular characteristics and enhanced membrane integrity with progressive *in vitro* maturation. Concurrently, population doubling time analysis indicated a significant increase in total cell yield for higher passages under equivalent incubation periods, with passage 3 cells producing approximately a three-fold increase relative to passage 1. These findings highlight a critical role of passage number that optimizes both proliferative and cryopreservation tolerance, providing valuable guidance for the strategic expansion and potential clinical application of MSCs.

Although the culture protocol described here was optimized for the isolation and expansion of MSCs from BALB/c mice, several limitations should be mentioned. Notably, all animals shared the same genetic background, sex, and age range. Biological variables, including sex hormones and age-related alterations in the bone marrow microenvironment are known to significantly influence MSCs yield, proliferative capacity, immunophenotype, and differentiation potential(21). Therefore the generalizability of these findings to other murine strains, sexes, or age groups requires further investigation. In addition, although surface marker profiling ($CD73^+/CD105^+/CD34^-/CD45^-$) confirmed phenotypic purity, functional validation of multipotency through tri-lineage differentiation assays (osteogenic, adipogenic, and chondrogenic) was not performed. Phenotypic characterization alone is insufficient to definitively confirm preservation of differentiation capacity. Accordingly, future studies applying this protocol should incorporate comprehensive trilineage differentiation assays complemented by molecular analyses of lineage-specific gene expression.

4.5. Conclusion

The development of optimized culture protocols that preserve MSCs identity while reducing dependence on animal-derived sera remains a critical priority. This study presents a step-by-step serum-reduction strategy for the isolation and expansion of bone marrow-derived MSCs from BALB/c mice. By progressively decreasing FBS concentration from 20% to 5% across sequential passages, combined with controlled medium replacement, the protocol yields high-density cultures that maintain characteristic spindle-shaped morphology, exhibit enhanced proliferative rate, and retain a stable immunophenotype ($CD73^+/CD105^+/CD34^-/CD45^-$). This approach significantly reduces culture-associated costs and biosafety concerns related to xenogeneic components, while providing a reproducible and stable methodology for preclinical MSCs production.

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Authors' contributions

Study concept and design: S. H.
Analysis and interpretation of data and drafting of the manuscript: S. H. and H. R
Statistical analysis: S. H
Experimental studies: M.H. P
All authors approved the manuscript.

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Conflict of Interest

We declare that there is no conflict of interest.

Data Availability

Data for this finding are available on request from the corresponding author.

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