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ORIGINAL ARTICLE

Biochemical profiling of adipose-derived mesenchymal stem cell conditioned media: Reproducible protein quantification and growth factor analysis

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ABSTRACT

Mesenchymal stem cell-conditioned media (MSC-CM) represents a promising cell-free therapeutic strategy in regenerative medicine, leveraging the paracrine effects of stem cells. Standardized biochemical characterization of MSC-CM is crucial for ensuring its reproducibility and therapeutic potential. However, statistical validation of batch-to-batch reproducibility has been rarely reported. Therefore, this study aimed to establish a reproducible protocol for AD-MSC-CM production and quantitatively demonstrate batch-to-batch reproducibility of total protein and key growth factors. Adipose-derived MSCs (AD-MSCs) were isolated from rat adipose tissue, expanded, and used to produce conditioned media (MSC-CM). The MSC-CM was biochemically profiled using the Bradford assay for total protein concentration and specific enzyme-linked immunosorbent assays (ELISAs) for quantifying Transforming Growth Factor-Beta (TGF-β) and Vascular Endothelial Growth Factor (VEGF). Analyses were performed on three independent biological replicates. The MSC-CM showed a significant and consistent increase in total protein concentration compared to control serum-free medium. Quantitative analysis revealed reproducible and stable secretion of key growth factors. The concentrations of both TGF-β and VEGF showed no significant variation across the biological replicates, confirming the high reproducibility of the MSC-CM preparation protocol. This study established a robust method for generating reproducible AD-MSC-CM, characterized by a stable and enriched content of total protein and specific growth factors (TGF-β and VEGF). The findings confirm the reliability of the production process and highlight the hypothesized potential of this well-characterized MSC-CM as a consistent therapeutic agent for applications requiring immunomodulation and angiogenesis, pending direct functional validation.

Keywords: Mesenchymal stem cell-conditioned media (MSC-CM), Adipose-derived Mesenchymal stem cell, Secretome, Cell-free therapy, Regenerative medicine.

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Introduction

Mesenchymal stem cells (MSCs) have emerged as a basic cell source in regenerative medicine due to their multipotent differentiation capabilities, immunomodulatory properties, and potent paracrine activity (1) While the therapeutic potential of MSCs themselves has been extensively studied, recent research has increasingly shifted focus towards the bioactive molecules they secrete (2). The secretome of MSCs, comprising a complex mixture of proteins, growth factors, cytokines, and extracellular vesicles, is now recognized as a primary mediator of their therapeutic effects, including tissue repair, angiogenesis modulation, and anti-inflammatory responses (3).

The collection and characterization of conditioned media (MSC-CM) from MSC cultures, termed mesenchymal stem cell-conditioned media (MSC-CM), has become a critical area of investigation. This cell-free approach offers advantages over whole-cell therapies, including reduced risks of immune rejection, tumorigenicity, and embolization, while allowing for precise dosing and standardization (4). MSC-CM has demonstrated efficacy in diverse experimental models, including cholestatic liver fibrosis(5, 6), type 2 diabetes (7), cerebral ischemia-reperfusion injury and sepsis-induced lung damage(8, 9).

A critical challenge, however, lies in standardization and reproducible characterization, as the protein composition and growth factor content can be influenced by MSC source, culture conditions, passage number, and preconditioning stimuli (10).

Despite this potential, comprehensive biochemical analyses of MSC-CM from adipose-derived MSCs (AD-MSCs), particularly from rat models, are still needed to ensure reproducibility and functional efficacy. Despite the growing body of evidence supporting MSC-CM therapeutic potential, a major translational hurdle remains: batch-to-batch variability. Most published studies report data from a single batch of MSC-CM or pool multiple batches without statistically verifying consistency across independent biological replicates. Without demonstrated reproducibility, attributing observed therapeutic effects to specific MSC-CM components and reliably reproducing those effects across different production runs becomes problematic. Therefore, a critical gap in the literature is the lack of formal, statistically validated protocols for assessing and ensuring MSC-CM consistency.

Here, we addressed this critical gap by performing a quantitative, statistically validated characterization of MSC-CM from rat AD-MSCs, with specific emphasis on demonstrating batch-to-batch reproducibility across three independent biological replicates

Methods

Study design

This experimental laboratory study was designed to isolate adipose-derived mesenchymal stem cells (AD-MSCs) from adult Wistar rats, prepare conditioned media under standardized serum-free culture conditions, and perform biochemical characterization of the collected conditioned media through total protein quantification and growth factor analysis. In the present study, the inclusion criteria were healthy adult male Wistar rats weighing 200–250 g, and the exclusion criteria was signs of illness, infection, abnormal behavior, weight loss, or visible abnormalities in adipose tissue. Three independent biological replicates were performed, each using one rat. All experimental procedures were carried out in triplicate at the technical level, with two technical replicates per biological replicate for ELISA measurements.

Isolation and culture of rat adipose-derived mesenchymal stem cells

All animal procedures were conducted in strict accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The experimental protocol was reviewed and approved by the Research Ethics Committees of Laboratory Animals-Academic Center for Education, Culture and Research (Ethical Approval Code: IR.ACECR.AEC.1404.066). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Adipose tissue was aseptically harvested from three adult male Wistar rats weighing between 200-250 grams. The animals were housed under standard laboratory conditions (12-hour light/dark cycle, 22 ± 2°C, 50–60% humidity) with ad libitum access to food and water. The rats were acclimatized for at least one week prior to any surgical procedure.

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For tissue harvest, rats were humanely euthanized via CO_2 asphyxiation following approved protocols. Subcutaneous adipose tissue deposits from the inguinal or epididymal region were immediately excised under sterile conditions. The harvested tissue was rinsed several times in sterile phosphate-buffered saline (PBS) containing 1% antibiotic-antimycotic solution to remove contaminating blood cells. Subsequently, the tissue was mechanically minced into 1 mm pieces and then digested with a 0.1% collagenase type I (Gibco, USA) solution at 37°C for 60-90 minutes with gentle shaking. The digest was neutralized with complete culture medium, consisting of Dulbecco's Modified Eagle Medium (DMEM:Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS:Gibco, USA) and 1% Penicillin-Streptomycin (Gibco, USA). The cell suspension was centrifuged, and the resulting pellet, containing the stromal vascular fraction (SVF), was resuspended and passed through a 70-micrometer cell strainer to remove debris. The isolated cells were plated in T-25 culture flasks (Falcon, USA) and maintained in a humidified incubator at 37°C with 5% CO_2 . After 48 hours, non-adherent cells were removed by a PBS wash, and the culture medium was replaced every three days thereafter.

Characterization and passage of adipose-derived mesenchymal stem cells

Upon reaching 70-90% confluence, the adherent cells were detached using a 0.25% trypsin-EDTA (Gibco, USA) solution and sub-cultured at an appropriate seeding density. Cells from passage 3 (P3) were used for all experiments. The mesenchymal phenotype of the isolated cells was confirmed through morphological and phenotypical characteristics and surface marker analysis using flow cytometry. The cells were positive for typical MSC markers (CD90, CD44) and negative for hematopoietic markers (CD34, CD45) (Figure 1).

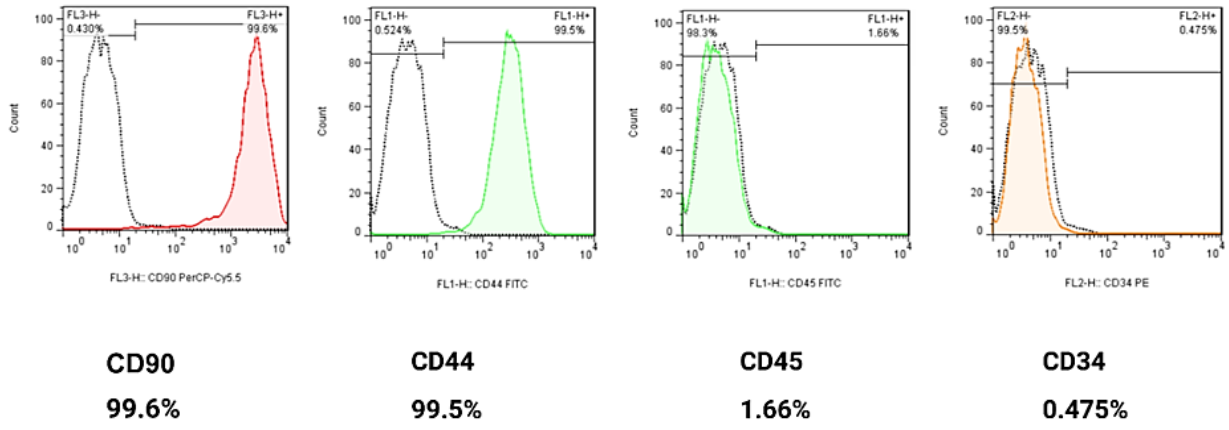


Figure 1. Immunophenotypic Characterization of Rat Adipose-Derived Mesenchymal Stem Cells (AD-MSCs) by flow cytometry.

Representative flow cytometry histograms demonstrate the characteristic surface marker profile of rat AD-MSCs at passage 3. The cells showed high expression (>95%) of the positive mesenchymal markers CD90 and CD44. In accordance with the minimal defining criteria for MSCs, the cells were negative (<2%) for the hematopoietic lineage markers CD45 (pan-leukocyte) and CD34 (hematopoietic stem/progenitor cell). This profile confirms the isolated population's identity as mesenchymal stem cells.

Preparation of mesenchymal stem cell-conditioned medium

For MSC-CM collection, AD-MSCs (P3) at 80-90% confluence was thoroughly washed with PBS to remove residual FBS. The culture medium was then replaced with serum-free medium. The cells were incubated for 48 hours, after which the supernatant was collected. The collected medium was centrifuged at 300 × g (equivalent to 1,500 rpm) for 5 minutes at 4°C to remove cellular debris, and the supernatant was subsequently filtered through a 0.2 µm sterile filter. The MSC-CM was aliquoted and stored at -80°C for a maximum of 2 months. No freeze-thaw cycles were

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performed before analysis. The resulting MSC-CM was aliquoted and stored at -80°C until further analysis. Control medium, consisting of serum-free medium that was not exposed to cells, was processed identically.

Biochemical characterization of mesenchymal stem cell-conditioned medium

Total Protein quantification by Bradford assay

The total protein concentration in the MSC-CM was determined using the Bradford protein assay. Briefly, a standard curve was prepared using known concentrations of Bovine Serum Albumin (BSA: Sigma, USA). Triplicate biological replicates of each MSC-CM sample and control medium were mixed with Bradford reagent, and the absorbance was measured at 595 nm using a microplate reader. The total protein concentration (mg/ml) for each sample was interpolated from the standard curve (Figure 2).

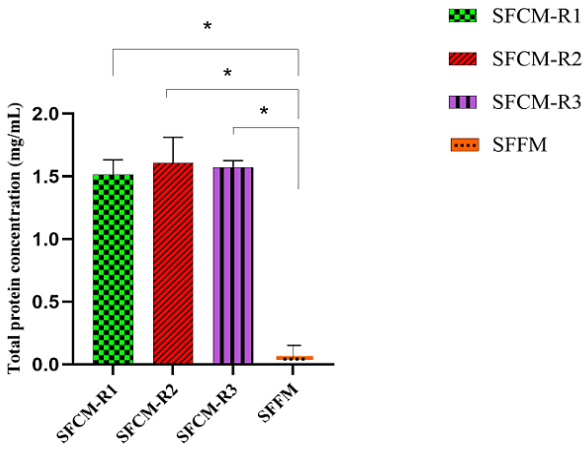


Figure 2. Total protein concentration (mg/ml) in different biological replicates of mesenchymal stem cell-conditioned media (MSC-CM) compared to serum-free control medium.

Quantification of total protein content in MSC-CM versus control. The bar graph shows the mean total protein concentration (± SD) measured by the Bradford assay across three independent biological replicates of MSC-CM (SFMSC-CM-R1, 2, 3) and a control group (SFFM: fresh serum-free medium). Each biological replicate of MSC-CM was assessed with three technical replicates (n=3 biological replicates/group). One-way ANOVA with post-hoc analysis revealed no significant difference in protein concentration among the three MSC-CM biological replicates ($p > 0.05$), indicating high reproducibility. However, all MSC-CM replicates showed a statistically significant increase in total protein concentration compared to the control group (*$p < 0.001$ compared to the control group (SFFM)). This demonstrates consistent and enriched protein secretion by adipose-derived mesenchymal stem cells into the conditioned medium. SFMSC-CM: serum-free conditioned media; SFFM: serum-free fresh media; R: replicate

Growth factors quantification by ELISA

The concentrations of specific growth factors, TGF-β (Cat No: DB100C, R&D systems Co., USA) and VEGF (Cat No: RRV00, R&D systems co., USA) secreted into the MSC-CM were measured using commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits, following the manufacturer's instructions (Figures 3, 4). Standards and samples (in triplicate biological replicates) were added to the antibody-coated wells. After incubation and washing, a detection antibody was added, followed by an enzyme-conjugated secondary antibody and a colorimetric substrate. The reaction was stopped, and the absorbance was read. The concentration of each growth factor (pg/ml) was determined by comparing the absorbance (optical density at 450 nm at 450 nm with a correction wavelength of 620 nm) to the standard curve. All samples were measured in duplicate technical replicates. The minimum detectable concentrations were 15.4 pg/ml for TGF-β and 5.0 pg/ml for VEGF according to the manufacturer's datasheets.

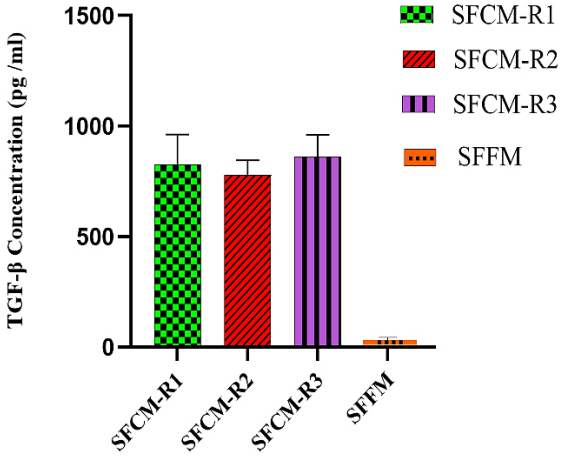


Figure 3. Consistent Secretion of Transforming Growth Factor-Beta (TGF-β) in different biological replicates of Mesenchymal Stem Cell-Conditioned Media.

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The concentration of TGF-β (pg/ml) was quantified using ELISA in three independent biological replicates of mesenchymal stem cell-conditioned media (MSC-CM). Each bar represents the mean TGF-β concentration (± SD) of one biological replicate, which was measured using three technical replicates. TGF-β was undetectable in the control medium (below the ELISA kit's lower limit of quantification). Statistical analysis by one-way ANOVA showed no significant difference ($p > 0.05$) in TGF-β concentration among the different biological replicates. This result demonstrates the reproducible secretion of this key growth factor by adipose-derived mesenchymal stem cells, confirming the reliability of the conditioned media preparation protocol. SFMSC-CM: serum-free conditioned media; SFFM: serum-free fresh media; R: replicate.

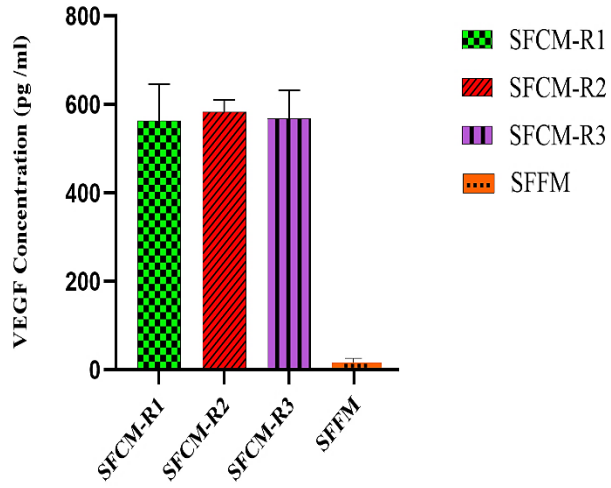


Figure 4. Reproducible Secretion of Vascular Endothelial Growth Factor (VEGF) in different biological replicates of Mesenchymal Stem Cell-Conditioned Media.

The graph represents the concentration of VEGF, a key angiogenic factor, measured by enzyme-linked immunosorbent assay (ELISA) in three independent biological replicates of mesenchymal stem cell-conditioned media (MSC-CM). Each bar represents the mean VEGF concentration (± SD) for one biological replicate. VEGF was undetectable in the control medium (below the ELISA kit's lower limit of quantification). Statistical analysis by one-way ANOVA confirmed no significant difference ($p > 0.05$) in VEGF levels among the biological replicates. This result highlights the consistent secretory profile of adipose-derived mesenchymal stem cells and reinforces the reliability of the conditioned media preparation protocol for yielding a stable composition of bioactive factors. SFMSC-CM: serum-free conditioned media; SFFM: serum-free fresh media; R: replicate

Statistical analysis

All experiments were performed with three independent biological replicates (n=3 per group). For each biological replicate, three technical replicates were measured and then averaged to obtain a single value per biological replicate. All statistical analyses were performed on the biological replicate means (n=3), not on individual technical replicates. Technical replicates were used solely to minimize measurement error. Normality of data distribution was assessed using the Shapiro-Wilk test ($p > 0.05$ considered normal). All datasets met the normality assumption. An unpaired Student's t-test (two-tailed) was used to compare the mean total protein concentration between the MSC-CM group and the control (serum-free fresh medium) group. To assess batch-to-batch reproducibility, a one-way between-subjects ANOVA (completely randomized design) was performed to compare total protein, TGF-β, and VEGF concentrations across the three independent biological replicates of MSC-CM. Following ANOVA, Tukey's Honest Significant Difference (HSD) test was used for pairwise comparisons between replicates when the overall ANOVA was significant. For non-significant ANOVA results ($p > 0.05$), post-hoc testing was not required. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

Results

Characterization of AD-MSCs

Isolated AD-MSCs were confirmed by flow cytometry analysis using MSCs–specific CD markers. At passage 3, most of the AD-MSCs exhibited very high levels of CD44 and CD90 expression (99.5% and 99.6%, respectively). In contrast, the cells were virtually negative for hematopoietic markers such as CD45 and CD34, with only 1.66% and 0.47% positivity, respectively. These findings show that the adherent cell population mainly consisted of AD-MSCs.

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While the ISCT minimal criteria for human MSCs recommend additional markers (CD73, CD105) and trilineage differentiation assays, the panel used here (CD90+/CD44+/CD34-/CD45-) combined with plastic adherence and characteristic morphology is widely accepted for immunophenotypic identification of rat AD-MSCs (11).In addition, while three biological replicates represent the minimum acceptable number for statistical comparison, the low variability observed (coefficient of variation = 5.3% for total protein) is encouraging and provides a foundation for future larger-scale validation studies.

Total protein content in different MSC-CM replicates versus control

The Bradford assay demonstrated a consistent total protein concentration across all biological replicates (Figure 2). The mean total protein concentration in MSC-CM was 6.24 ± 0.33 mg/ml (range: 5.99 to 7.05 mg/ml across technical replicates), while the control serum-free fresh medium showed a mean concentration of 0.15 ± 0.02 mg/ml. The low standard deviation (0.33 mg/ml) and non-significant variation between biological replicates (one-way ANOVA: $F(2,6) = 2.86$, $p = 0.13$) are consistent with the reproducibility of the conditioned media preparation protocol. The MSC-CM showed a statistically significant increase in total protein concentration compared to control medium (unpaired t-test: $t(4) = 32.45$, $p < 0.001$). This consistency reflects stable secretory activity by the adipose-derived mesenchymal stem cells (AD-MSCs).

Concentration of transforming growth factor-beta (TGF-β) in different MSC-CM replicates.

To quantitatively assess the presence of a key immunomodulatory and pro-regenerative factor in our prepared conditioned media, the concentration of TGF-β was determined via enzyme-linked immunosorbent assay (ELISA) (Figure 3). The mean concentration of TGF-β across three independent biological replicates of MSC-CM was [822.43 ± 42 pg/ml]. Statistical comparison confirmed no significant variation in TGF-β concentration among the different biological replicates (one-way ANOVA: $F(2,6) = 0.95$, $p = 0.42$), demonstrating the robustness of our MSC culture and conditioned media harvesting protocol. The reliable presence of this critical growth factor confirms that our adipose-derived mesenchymal stem cells maintain a functionally relevant secretory phenotype in vitro, contributing to the potential therapeutic value of the produced MSC-CM.

Concentration of vascular endothelial growth factor (VEGF) in different MSC-CM replicates

To further characterize the pro-angiogenic potential of the secreted factors, we quantified the concentration of VEGF, a pivotal cytokine for angiogenesis and endothelial cell function, in the conditioned media (Figure 4). The mean concentration of VEGF across three independent biological replicates of MSC-CM was [571.8 ± 10.2 pg/ml]. The absence of statistically significant variation in VEGF concentration among the replicates (one-way ANOVA: $F(2,6) = 1.12$, $p = 0.38$) underscores the stability of our MSC culture system and the reliability of the conditioned media production process.

Discussion

The present study successfully established a robust protocol for the generation and characterization of conditioned media from rat AD-MSCs. The primary novelty of this work is not the detection of TGF-β and VEGF, findings that align with established literature, but rather the quantitative demonstration of batch-to-batch reproducibility. Our findings demonstrate that the prepared MSC-CM is a rich and consistent source of total protein and specific growth factors, with statistically indistinguishable levels across three independent biological replicates ($p > 0.05$ for all parameters by one-way ANOVA). This level of statistical validation of batch-to-batch consistency has been rarely reported in the MSC-CM literature. This level of reproducibility, if confirmed in larger studies, could serve as a benchmark for the potential therapeutic application of MSC-CM, as it suggests the potential for a reliable and definable product, a prerequisite for regulatory approval and clinical translation.

To our knowledge, this study provides the first formal statistical demonstration that independently produced batches of rat AD-MSC-CM contain consistent levels of total protein (6.24 ± 0.33 mg/ml), TGF-β, and VEGF. While qualitative or semi-quantitative detection of these factors has been reported, the absence of significant inter-batch

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variation has not been rigorously demonstrated using biological replicates and ANOVA-based statistics. This finding establishes a foundation for using MSC-CM as a reliable, off-the-shelf biologic agent in future preclinical studies.

Importantly, the primary objective of our study was not merely to report absolute values of total protein or growth factor concentrations in MSC-CM. Rather, our main goal was to demonstrate the absence of statistically significant differences across independent biological replicates, thereby establishing batch-to-batch reproducibility – a critical but frequently overlooked parameter for clinical translation. While many previous studies, including the work by Kusumawardani et al.(12), have reported protein concentrations from single batches or pooled samples without statistical validation of batch-to-batch consistency, we specifically focused on quantifying variability across multiple independently produced MSC-CM batches. A direct comparison of total protein concentrations between our study and the work by Kusumawardani et al. (2021) reveals notable differences that likely reflect variations in cell source, culture conditions, and analytical methods. Using a bicinchoninic acid (BCA) protein assay, Kusumawardani et al. reported a total protein concentration of 2.502 ± 0.06 µg/ml in gingival mesenchymal stem cell-conditioned medium (GMSC-CM) from human gingival tissue, with a concentrated preparation yielding 1.912 ± 0.08 µg/ml. In contrast, our study using adipose-derived MSCs from rat tissue with the Bradford assay demonstrated a substantially higher total protein concentration of 6.24 ± 0.33 mg/ml (equivalent to 6,240 ± 330 µg/ml) – approximately 2,500-fold higher than the GMSC-CM values. This striking difference can be attributed to several factors. First, the cell source differs considerably: we used rat adipose-derived MSCs, whereas Kusumawardani et al. employed human gingival MSCs, which may have inherently different secretory profiles. Second, the protein quantification methods differ: we used the Bradford assay, while they utilized the BCA assay, which can yield different absolute values for the same sample due to different detection chemistries.

Third, potential differences in protein concentration units or reporting (µg/ml vs. mg/ml) may contribute to the apparent discrepancy; our reported value of 6.24 mg/ml is approximately 2,500 times higher than 2.5 µg/ml. We suspect that the GMSC-CM value of 2.502 µg/ml reported by Kusumawardani et al. may represent a highly diluted or unconcentrated sample, as typical MSC-CM protein concentrations in the literature range from hundreds to thousands of µg/ml rather than single-digit µg/ml values. Despite these quantitative differences, both studies independently confirm that MSC-derived conditioned media contain measurable levels of total protein. However, our study uniquely contributes by providing formal statistical evidence (one-way ANOVA, $p > 0.05$) that three independently produced batches yielded indistinguishable total protein concentrations, thereby validating the reproducibility of our production protocol – a finding that has been rarely demonstrated in the existing literature.

For VEGF concentration, our findings are generally consistent with those of Jin et al. (2020) (13) regarding the detectable presence of VEGF in MSC-CM; a direct comparison of absolute concentrations reveals notable differences that likely reflect variations in cell source, culture conditions, and experimental design. Jin et al., using human gingival mesenchymal stem cells (hGMSCs) overexpressing FGF-2 via lentiviral transfection, reported a VEGF-A concentration of 39.19 ± 0.77 pg/ml in FGF-2-modified GMSC-CM, compared to 21.95 ± 0.77 pg/ml in non-modified (native) hGMSC-CM. Using human adipose-derived MSCs conditioned for 7 days in DMEM-LG, Sagaradze et al. reported VEGF concentrations of approximately 400–500 pg/ml in MSC-CM (14). In contrast, our study using rat adipose-derived MSCs (AD-MSCs) without any genetic modification demonstrated a substantially higher VEGF concentration of **[**571.8 ± 10.2 pg/ml**].** This discrepancy can be attributed to several factors. First, the cell source differs fundamentally: we used rat AD-MSCs, whereas Jin et al. employed human gingival MSCs; previous studies have shown that adipose-derived MSCs generally secrete higher levels of pro-angiogenic factors such as VEGF compared to other MSC sources. Second, species differences (rat vs. human) may influence secretory capacity. Third, our study used unmodified, native AD-MSCs, while Jin et al. reported data primarily from FGF-2-overexpressing GMSCs, which were designed for therapeutic optimization rather than baseline secretion characterization. Fourth, differences in culture conditions (e.g., cell density at collection, serum-free conditioning duration) can significantly affect VEGF

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accumulation in MSC-CM. Despite these quantitative differences, both studies independently confirm that MSC-CM contains detectable levels of VEGF with pro-angiogenic potential. However, our study uniquely contributes by providing formal statistical evidence that three independently produced batches yielded indistinguishable VEGF concentrations, thereby validating the reproducibility of our production protocol, a finding that addresses the batch-to-batch variability challenge that has received limited attention in the existing literature, including the work by Jin et al. Moreover, regarding TGF-β concentration, a direct comparison of our findings with the landmark study by Salazar et al. (15) reveals both similarities and differences in approach and focus.

Using mouse bone marrow-derived and human umbilical cord blood-derived MSCs, Salazar et al. demonstrated by ELISA that MSC-CM contains significant amounts of TGF-β1, and importantly, they functionally validated that this TGF-β1 was biologically active, as it increased α1-procollagen gene expression in lung fibroblast target cells – an effect that was blocked by anti-TGF-β antibodies. In contrast, our study using rat adipose-derived MSCs detected TGF-β at **[**822.43 ± 42 pg/ml**]** in MSC-CM. While both studies independently confirm that TGF-β is a constitutive component of the MSC secretome across species (mouse, human, and rat) and tissue sources (bone marrow, umbilical cord blood, and adipose tissue), our study uniquely contributes by providing formal statistical evidence that three independently produced batches yielded indistinguishable TGF-β concentrations. This batch-to-batch reproducibility – a critical parameter for clinical translation that was not addressed by Salazar et al. – validates the consistency of our production protocol. It is worth noting that while Salazar et al. focused on functional validation of TGF-β biological activity (collagen gene expression), our study focused on biochemical reproducibility, and these two approaches are complementary. Future studies should combine both rigorous batch consistency testing (as we have done) with functional bioassays (as performed by Salazar et al.) to fully characterize MSC-CM for therapeutic applications.

The significant elevation in total protein concentration within the MSC-CM, as quantified by the Bradford assay, provides a foundational indicator of a complex secretome. This enrichment is consistent with the well-established paracrine hypothesis, which posits that the therapeutic benefits of MSCs are largely mediated by their secreted factors rather than by direct cell replacement (16). The lack of significant difference in total protein among the biological replicates underscores the stability of our AD-MSC isolation, expansion, and conditioning protocols, which is a prerequisite for any standardized cell-free therapeutic.

Further characterization of specific growth factors revealed that our AD-MSCs constitutively secrete significant and consistent levels of both TGF-β and VEGF. TGF-β is a master regulator of immune modulation and tissue repair, known to suppress T-cell proliferation, promote the differentiation of regulatory T-cells, and stimulate the production of extracellular matrix components such as collagen (17). The reliable presence of TGF-β in our MSC-CM batches suggests a potent and reproducible immunomodulatory and EMSC-CM remodeling capacity, which may warrant investigation for applications in wound healing. Concurrently, the stable secretion of VEGF, a potent mitogen for endothelial cells, is consistent with the hypothesized pro-angiogenic potential of our MSC-CM(18). Angiogenesis, the formation of new blood vessels from pre-existing ones, is a critical process in tissue regeneration and repair, ensuring the delivery of oxygen and nutrients to damaged sites (19). The consistent levels of VEGF across replicates indicate that our MSC-CM may have the capacity to promote vascularization, pending functional confirmation, a property highly valuable for treating ischemic conditions or in tissue engineering constructs.

The combination of a protein-rich secretome with stable concentrations of key regulatory factors like TGF-β and VEGF suggest that our AD-MSC-CM may serve as a multi-faceted cell-free therapeutic candidate, but this requires direct experimental validation**.** The synergistic action of these components could simultaneously orchestrate anti-inflammatory responses, promote matrix synthesis, and stimulate new blood vessel formation, thereby addressing multiple facets of the healing process (20). Future work should focus on functional in vitro and in vivo assays to validate these predicted bioactivities, such as lymphocyte proliferation tests for immunomodulation and tube formation assays or ischemic limb models for angiogenesis.

However, while this study provides a robust biochemical characterization of AD-MSC-CM, several

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limitations should be acknowledged. The most significant limitation is the absence of functional validation assays. Although our biochemical data demonstrate the presence of TGF-β and VEGF – factors known to mediate immunomodulation and angiogenesis – we did not directly assess the biological activity of the MSC-CM. Therefore, our statements regarding angiogenic and immunomodulatory potential remain speculative and hypothesis-generating rather than demonstrative. This limitation is particularly important because the presence of a growth factor does not guarantee its biological activity, as activity can be influenced by other components in the complex MSC-CM mixture (e.g., inhibitors, binding proteins, or degradation products). Our analysis was focused on total protein concentration and the quantification of two specific growth factors. The complete proteomic profile of the MSC-CM, including other crucial cytokines, chemokines, and the presence of extracellular vesicles (exosomes), remains uncharacterized. A more comprehensive analysis would provide a deeper understanding of the full spectrum of bioactive components responsible for the MSC-CM's therapeutic effects. Secondly, the functional bioactivity of the MSC-CM was not directly validated through in vitro or in vivo assays. While the presence of TGF-β and VEGF is strongly indicative of immunomodulatory and pro-angiogenic potential, respectively, functional experiments are necessary to confirm these biological activities. Furthermore, this study utilized MSCs from a single source (rat adipose tissue) and a standard culture condition. The influence of factors such as tissue source (e.g., bone marrow vs. adipose), donor variability, passage number, and specific preconditioning stimuli on the secretome composition was not investigated.

Another limitation of this study is the modest sample size of three independent biological replicates. While three replicates represent the minimum acceptable number for statistical comparison using ANOVA and are consistent with common practice in exploratory MSC-CM studies, we acknowledge that this sample size is insufficient to make definitive claims regarding long-term batch-to-batch consistency. The low variability observed among our replicates (e.g., coefficient of variation of 5.3% for total protein concentration) is encouraging and suggests that larger sample sizes would likely yield consistent results. However, future studies should include at least six to ten biological replicates to provide more robust statistical power for confirming batch-to-batch reproducibility. Until such studies are performed, our conclusions regarding batch consistency should be considered preliminary and hypothesis-generating rather than definitive.

Accordingly, based on the findings and limitations of this study, several promising avenues for future research are proposed. A primary next step would be to perform a comprehensive panel of functional assays, including pro-angiogenic assays, wound healing assays and immunomodulatory assays. Another requisite is proteomic analysis using techniques such as mass spectrometry to create a complete map of the MSC-CM's protein content. This would allow for the identification of novel factors and the discovery of potential synergistic interactions within the secretome. Subsequently, functional assays are crucial to link the MSC-CM's composition to its biological effects. For instance, in vitro lymphocyte proliferation assays or macrophage polarization studies could confirm the immunomodulatory activity suggested by the TGF-β data. Similarly, a tube formation assay using human umbilical vein endothelial cells (HUVECs) would directly demonstrate the pro-angiogenic capacity conferred by VEGF. Additionally, to enhance the therapeutic potency of the MSC-CM, future studies should explore preconditioning strategies. Exposing MSCs to hypoxic conditions or priming them with inflammatory cytokines (e.g., IFN-γ or TNF-α) can significantly alter their secretory profile, potentially boosting the concentration of desired factors(21, 22). Finally, the translation of these findings requires validation in relevant animal models. For example, the MSC-CM's efficacy in promoting wound healing could be tested in a diabetic mouse model, or its regenerative potential could be assessed in a model of myocardial infarction. Investigating the scaling-up of MSC-CM production under Good Manufacturing Practice (GMP) guidelines will also be an essential step toward clinical application.

This study established a robust, statistically validated protocol for generating mesenchymal stem cell-conditioned media (MSC-CM) from rat adipose tissue. The key contribution is the quantitative demonstration that three independently produced MSC-CM batches contained statistically indistinguishable levels of total protein, TGF-β, and VEGF ($p > 0.05$ for all parameters). This batch-to-

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batch reproducibility – rarely formally demonstrated in the literature – confirms the reliability of our production method and positions this well-characterized MSC-CM as a consistent therapeutic agent for applications requiring immunomodulation and angiogenesis.

Looking forward, the current in vitro biochemical analysis provides a necessary foundation but also outlines clear research directions. The primary limitations are the restricted scope of analysis and the need for functional validation. Future work should employ comprehensive proteomics to fully map the secretome and must progress to in vitro bioassays and in vivo preclinical models to conclusively demonstrate the MSC-CM's therapeutic efficacy. Investigating preconditioning strategies to enhance the secretome's potency will be a vital step toward developing this cell-free approach into a viable clinical therapy for regenerative medicine.

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