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*Original Research Article*

# Optimization of Enzymatic Digestion Conditions for Isolation of Stable and Functional Mesenchymal Stem Cells from the Infrapatellar Fat Pad

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***Abstract***: ***Background***: The infrapatellar fat pad (IFP) is an emerging and clinically accessible source of mesenchymal stem cells (MSCs) with promising applications in cartilage repair and regenerative medicine. However, variability in enzymatic digestion protocols during cell isolation may significantly influence MSC yield, viability, and functional stability. This study aimed to evaluate the effect of different collagenase digestion conditions on the biological characteristics of IFP-derived MSCs (IFP-MSCs). ***Methods***: IFP tissue was obtained from patients undergoing knee arthroplasty following ethical approval and informed consent. Tissue samples were enzymatically digested using 0.1% collagenase type I under three conditions: (G1) 37 °C for 1 hour with agitation, (G2) 37 °C for 3 hours with agitation, and (G3) 37 °C for 1 hour without agitation. Isolated cells were cultured up to passage 3. Cell yield, viability, morphology, proliferation rate, population doubling time (PDT), colony-forming unit–fibroblast (CFU-F) efficiency, and immunophenotype were assessed. ***Results***: Group G2 demonstrated a significantly higher initial cell yield compared to G1 and G3. Cell viability across passages remained stable in G2, whereas significant variations were observed in G1 and G3. All groups exhibited typical spindle-shaped MSC morphology and adherence to plastic. Proliferation rate was significantly higher in G2 during later culture periods, while PDT remained comparable among groups. Flow cytometry confirmed expression of MSC markers (CD29, CD73, CD90) and absence of hematopoietic markers (CD45) across all conditions. ***Conclusion***: Enzymatic digestion at 37 °C for 3 hours with agitation optimizes isolation efficiency while preserving viability, proliferative capacity, and phenotypic stability of IFP-MSCs. These findings support protocol standardization to enhance the translational potential of IFP-derived MSCs for cartilage repair and regenerative therapies.

**Keyword**: Infrapatellar fat pad, Mesenchymal stem cells, Enzymatic digestion.

 

## INTRODUCTION

The infrapatellar fat pad (IFP), also known as Hoffa's fat pad, is a yellowish adipose tissue structure located posterior to the patellar tendon, between the femoral condyle and tibial plateau(1). Adipose tissue is a rich source of mesenchymal stem cells (MSCs), which are fibroblast-like, plastic-adherent cells with multilineage differentiation potential. MSCs can be isolated from various sources, including bone marrow, Wharton's jelly, umbilical cord blood, and adipose tissues such as the infrapatellar fat pad(2). Among these, the IFP has gained considerable attention due to its easy accessibility, minimal donor site morbidity, and high yield of viable MSCs.

Mesenchymal stem cells are characterized by their ability to adhere to plastic surfaces, differentiate into adipogenic, osteogenic, and chondrogenic lineages, and express specific surface markers such as CD29, CD44, CD73, CD90, CD105, and CD166, while lacking expression of hematopoietic markers including CD14, CD34, and CD45(3). Compared with bone marrow–derived MSCs, harvesting IFP tissue is associated with reduced procedural pain and lower risk of complications such as infection or sepsis, while providing a substantially higher initial cell yield(4).

The infrapatellar fat pad has emerged as a promising cell source for cartilage repair and regenerative therapies. Several studies have demonstrated its suitability for cell-based and tissue-engineering applications(5). IFP-derived adipogenic stem cells seeded on three-dimensional printed chitosan scaffolds have shown successful chondrogenic differentiation under the influence of transforming growth factor-β3 (TGF-β3) and bone morphogenetic protein-6 (BMP-6). Additionally, IFP-MSCs have been shown to retain their proliferative capacity, epitope profile, and osteogenic potential even with advancing age(6). Other investigations have reported maintained trilineage differentiation potential at later passages and successful application of stromal vascular fraction derived from IFP in the treatment of focal cartilage defects. These findings collectively support the therapeutic relevance of IFP-MSCs(7,8).
Enzymatic digestion, most commonly employing collagenase-based protocols, facilitates efficient dissociation of the extracellular matrix (ECM) of the infrapatellar fat pad, enabling the isolation of a high-yield stromal vascular fraction enriched with viable MSCs. This approach ensures minimal mechanical stress compared to purely mechanical methods, thereby reducing cellular damage, apoptosis, and phenotypic drift. Preservation of membrane integrity and intracellular signaling pathways through controlled enzymatic digestion is critical for maintaining MSC stability during early isolation and expansion phases.

From a biological standpoint, enzymatic digestion maintains the native characteristics of IFP-MSCs by conserving key surface markers (CD73, CD90, CD105) and suppressing hematopoietic markers (CD34, CD45), consistent with the International Society for Cell & Gene Therapy (ISCT) criteria. Importantly, optimized enzymatic protocols help preserve the cells 'differentiation potential toward chondrogenic, osteogenic, and adipogenic lineages, which is essential for translational and therapeutic applications.

Despite growing interest, variations in enzymatic digestion conditions during isolation may influence cell yield, viability, and functional characteristics. Optimizing isolation parameters is therefore critical for enhancing the efficiency and clinical applicability of IFP-derived MSCs

## MATERIALS AND METHODS

### Procurement of Infrapatellar Fat Pad Tissue

Infrapatellar fat pad tissue was obtained from patients undergoing knee arthroplasty procedures following institutional ethical approval and written informed consent. The collected tissue samples were processed under sterile conditions for MSC isolation.

### Isolation and Culture of IFP-Derived Mesenchymal Stem Cells

The harvested IFP tissue was washed three times with Dulbecco's phosphate-buffered saline (DPBS) to remove blood and debris. The tissue was then finely minced into small fragments using sterile surgical instruments. The samples were divided into three experimental groups (Group I, Group II, and Group III) and subjected to enzymatic digestion using 0.1% collagenase type I under different incubation conditions:

- **Group I:** Incubation at 37 °C for 1 hour with constant agitation

 

- **Group II:** Incubation at 37 °C for 3 hours with constant agitation
- **Group III:** Incubation at 37 °C for 1 hour without agitation

$$\mathrm{PDT} = \frac{t \times \log 2}{\log N_t - \log N_0}$$

Following digestion, the cell suspension was filtered through a 100-µm cell strainer and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, and the resulting cell pellet was washed with DPBS and resuspended in complete culture medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 20% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$. Culture medium was replaced twice weekly until cells reached 80–90% confluence. Adherent cells were detached using 0.25% trypsin–EDTA and subcultured up to passage 3 for further analyses.(9,10)

**Cell Yield Assessment**
Cell yield was defined as the total number of viable cells obtained immediately following enzymatic digestion(11). Initial cell yield is a critical determinant for clinical and tissue-engineering applications, as prolonged in vitro expansion may reduce proliferative capacity, multipotency, and genomic stability. Therefore, early-passage cells were considered optimal for downstream therapeutic use(11)

**Morphological Assessment**
Morphological characteristics of IFP-MSCs were evaluated at different time intervals and passages using a phase-contrast inverted microscope. Cellular features such as adherence, shape, and confluency were documented.

**Cell Viability Assay**
Cell viability was determined using the trypan blue exclusion method. Cells were stained with 0.4% trypan blue and counted using a hemocytometer. Cells excluding the dye were considered viable, whereas stained cells were considered nonviable.

$$\text{Cell viability} = \frac{\text{Total cells} - \text{Dead cells}}{\text{Total cells}}$$

Cell viability was calculated using the formula (12)

**Proliferation Rate and Population Doubling Time**
For proliferation analysis, IFP-MSCs were seeded at a density of $2 \times 10^3$ cells per well in 12-well plates and cultured for 12 days. Media were replaced every three days. Cells were harvested and counted on days 3, 6, 9, and 12 using a hemocytometer. The average cell number from triplicate wells was used to calculate the proliferation rate.

Population doubling time (PDT) was assessed at passage 2 using a seeding density of 10,000 cells per well. Cell counts were recorded every three days over a 12-day period. PDT was calculated using the following formula(13).

where $t$ represents time in days, $N_0$ is the initial cell number, and $N_t$ is the final cell number.

**Colony-Forming Unit–Fibroblast (CFU-F) Assay**
The clonogenic potential of IFP-MSCs was evaluated using a CFU-F assay. Cells were seeded at a density of $0.1 \times 10^3$ cells per well in 6-well plates and cultured for 14 days, with media changes every three days. Cells were fixed with 4% formaldehyde and stained with 0.4% crystal violet. Colonies containing more than 25 cells were counted and recorded as CFUs.

**Immunophenotypic Characterization**
Cell surface marker expression was analyzed using flow cytometry. IFP-MSCs were assessed for the expression of mesenchymal markers CD44, CD29, CD73, and CD90, and the absence of hematopoietic markers CD34 and CD45.

Cells at 80–90% confluence were washed with PBS and fixed in 3.7% paraformaldehyde. Primary antibodies were incubated for 2 hours at 37 °C, followed by incubation with FITC-conjugated secondary antibodies for 1 hour at room temperature. Isotype-matched antibodies were used as negative controls. A total of 10,000 events were acquired and analyzed using flow cytometry software. Experiments were performed in duplicate, and mean values were recorded.

**STATISTICAL ANALYSIS**
All experiments were performed in triplicate unless otherwise stated. Data were expressed as mean ± standard deviation. Statistical significance between groups was determined using appropriate statistical tests, with $p < 0.05$ considered statistically significant.

## RESULTS

**Cell Yield of IFP-MSCs Prior to Primary Culture**
The initial cell yield of infrapatellar fat pad–derived mesenchymal stem cells (IFP-MSCs) obtained before primary culture initiation was compared among the three experimental groups (G1, G2, and G3). A statistically significant

 

difference in cell yield was observed among the groups (Fig **1A**). Among them, Group 2 (G2) demonstrated a significantly higher cell yield compared to Groups 1 (G1) and 3 (G3), indicating superior cellular recovery efficiency prior to culture establishment.

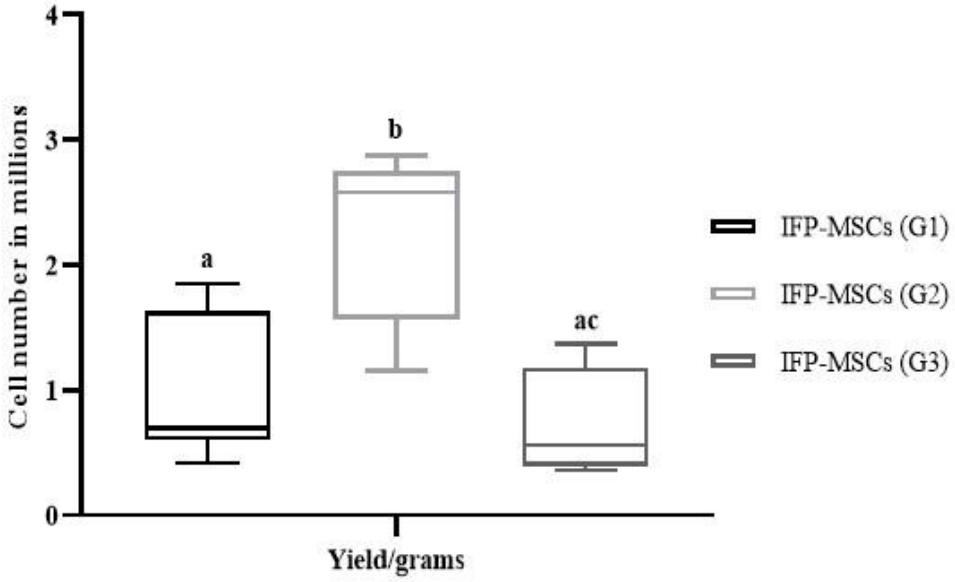


Figure 1 A: Cell yield of IFP-MSCs between three groups (G1,G2 G3) were recorded prior to primary culture . A significant difference in cell yield of IFP-MSCs were observed in three groups (G1,G2,G3), Whereas IFP-MSC (G2) group showed comparatively higher cell yield among other IFP-MSCs groups (G1,G2).

**Cell Viability of IFP-MSCs at Different Passages**

The percentage of viable IFP-MSCs was assessed at passages P1, P2, and P3 in all three groups. A significant difference in cell viability was observed in Group 1 (G1) across passages P1, P2, and P3. Similarly, Group 3 (G3) showed significant variations in cell viability at passages P1 and P3. In contrast, Group 2 (G2) did not exhibit any statistically significant changes in cell viability across passages P1, P2, and P3, indicating stable cell survival during successive passaging (**Fig 1B**).

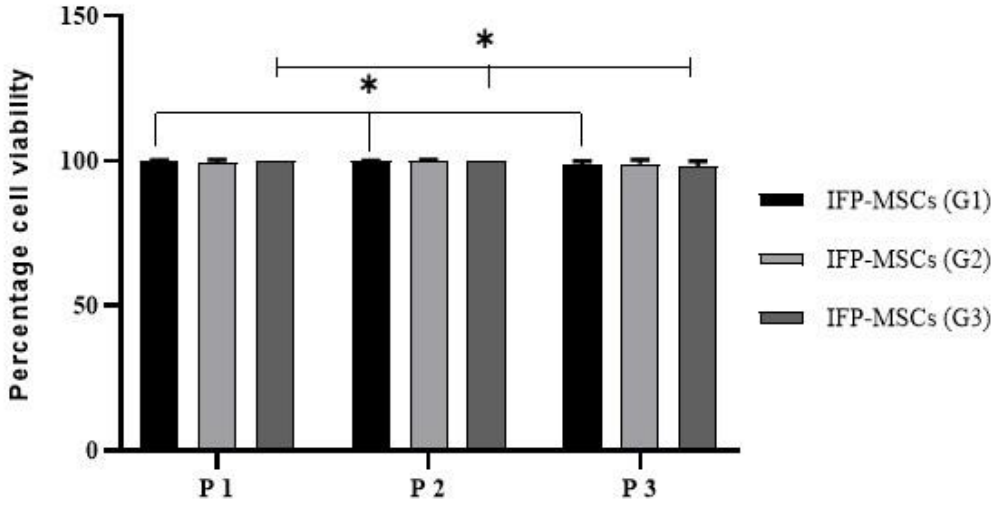


Figure 1 B: Percentage of cell viability IFP-MSCs between three groups at different passages was determined and recorded. A significant differences in percentage of viability was observed in IFP-MSCs (G1) at P1, P2, P3 passages respectively and also in IFP-MSCs (G3) at P1and P3, but there is no significant difference in cell viability of IFP-MSCs at P1,P2,P3 passages.

 

**Morphological Evaluation of IFP-MSCs**

Morphological assessment of IFP-MSCs revealed typical mesenchymal stem cell characteristics in all three groups. At passage P0, cells exhibited plastic adherence and spindle-shaped, fibroblast-like morphology (**Figure 2**). At passage P1, the cells maintained elongated morphology with uniform distribution and increased confluency. No marked morphological differences were observed among the groups, confirming phenotypic consistency during early passages.

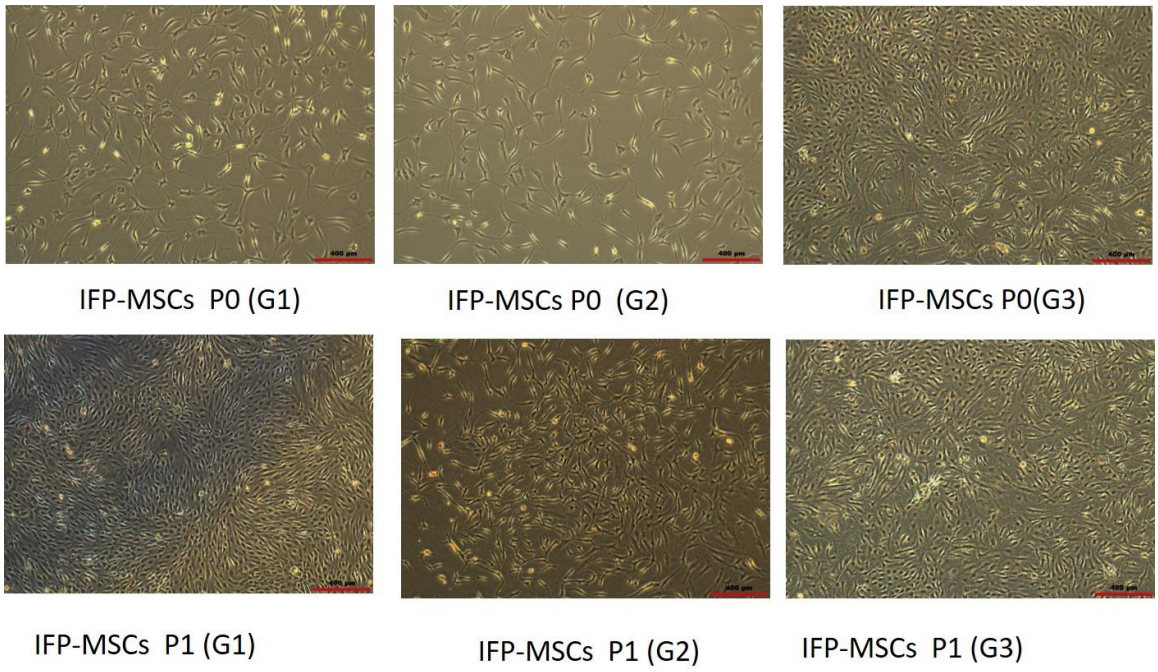


**Figure 2**. Morphology Images of human Infrapatellar fat pad derived MSCs of (Group 1, Group 2 Group3) at Passage 0 (P0) and Passage 1 (P1). Magnification of 4x

**Proliferation Rate of IFP-MSCs**

The proliferation rate of IFP-MSCs from Groups G1, G2, and G3 was evaluated at passage P2 over a 12-day culture period. From day 0 to day 3, proliferation was comparable and relatively slow across all groups. However, a marked increase in proliferation was observed from day 6 to day 12. During this phase, a statistically significant difference in proliferation rate was noted among the three groups (**Figure 3**), with Group 2 exhibiting a comparatively higher proliferative capacity than the other groups.

 

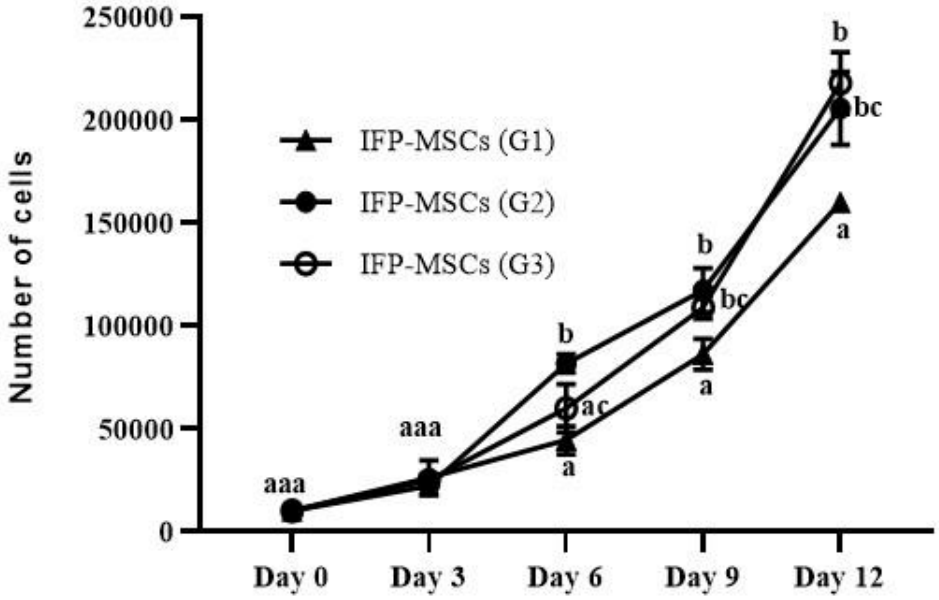


Figure 3 : Proliferation rate of IFP-MSCs from three groups at passage P2 was determined and changes in the proliferation rate between the groups were recorded. The proliferation of IFP-MSCs from day 0 to day 3 was comparatively similar and slower further increased spontaneously from day 6 to 12 but during this period A Significant difference was observed in proliferation rate of IFP-MSCs among the groups (G1,G2 G3).

**Population Doubling Time (PDT)**

Population doubling time (PDT) analysis was performed to evaluate growth kinetics of IFP-MSCs across the three groups. Statistical comparison showed no significant difference in PDT among Groups G1, G2, and G3 , indicating that overall cell cycle duration remained comparable despite differences observed in proliferation rates.

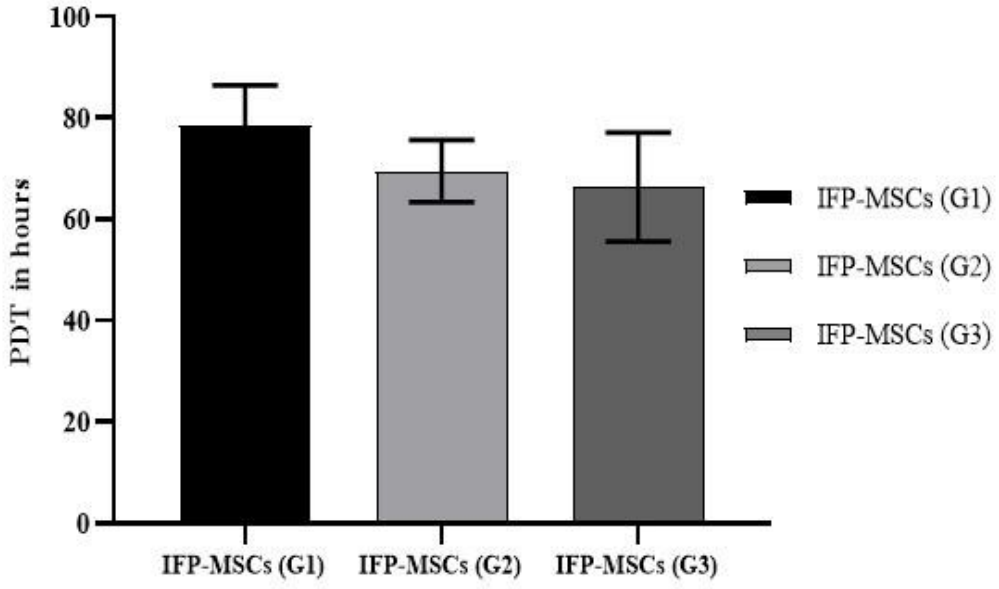


Figure 4: In case PDT of IFP-MSCs moreover, there is no significant difference in PDT was observed between three groups (G1,G2,G3).

**Colony forming unit-fibroblast (CFU-F) ability**

CFU-F ability of IFP-MSCs between three supplemented groups were determined at passage 3. Crystal violet stained colonies were recorded in 6-well culture plates with an initial seeding density of $0.1\times10^3$ cells/well. CFU-F assay was

 

performed on 14th day of culture, and a higher number of colonies (more than 25 cells) was visualized in all three groups. **(Fig. 5A and 5B)**

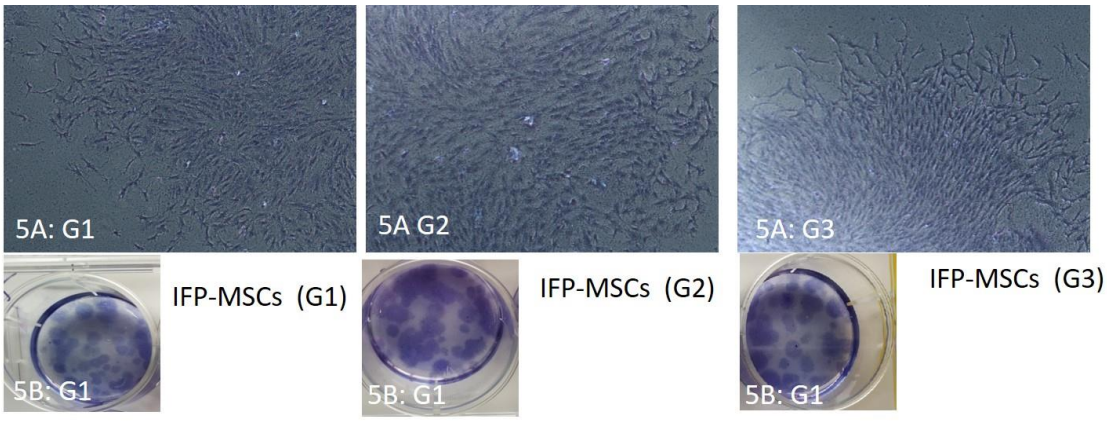


FIG.5A.Microscopic images of colony forming unit assay of Infrapatellar fat pad Group 1, Group 2, Group 3.

Figure.5B. Single well macroscopic images of colony forming unit assay staining of Infrapatellar fat pad A, B, C (Group 1, Group 2 Group 3) at P2 Passages after 14 days of culture

**Immunophenotypic Characterization of IFP-MSCs**

Flow cytometric analysis demonstrated that IFP-MSCs from all three groups were positive for mesenchymal stem cell markers CD29, CD73, and CD90, while negative for the hematopoietic marker CD45. Representative flow cytometry histograms are shown in **Figure 6.** This immunophenotypic profile confirms the mesenchymal identity and purity of IFP-MSCs across all experimental groups.

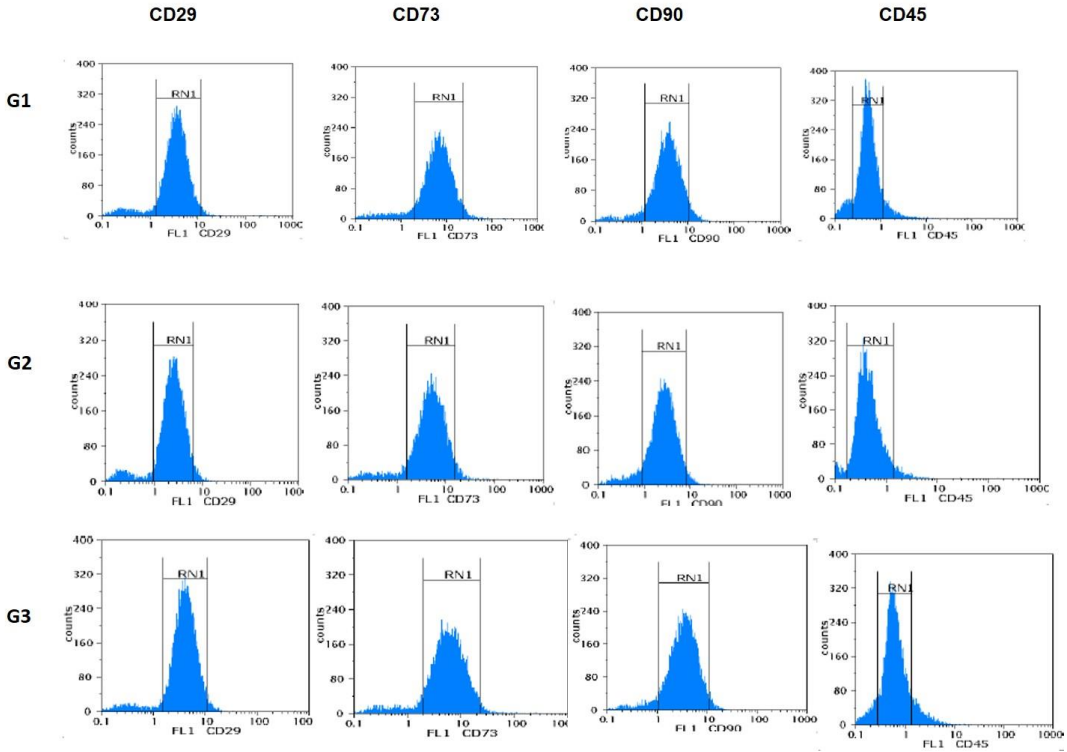


Fig 6 Representive histogram plot for cell surface epitope characterization of HIPFP derived cells following Flowcytometry analysis

**DISCUSSION**

This study investigated the effect of different enzymatic digestion conditions on the yield, viability, and biological characteristics of infrapatellar fat pad–derived mesenchymal stem cells (IFP-MSCs). Optimization of isolation protocols is essential to

 

maximize cell recovery while preserving stem cell functionality for regenerative applications.

Enzymatic digestion of the infrapatellar fat pad contributes significantly to MSC stability by maintaining the paracrine and immunomodulatory properties of the isolated cells. IFP-MSCs obtained via optimized enzymatic methods exhibit sustained secretion of anti-inflammatory cytokines and growth factors, such as transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and interleukin-10 (IL-10). These factors are crucial for cartilage homeostasis, modulation of synovial inflammation, and tissue regeneration within the osteoarthritic joint environment.

Furthermore, enzymatic digestion enhances reproducibility and scalability, which are critical parameters for clinical-grade MSC manufacturing. High cell yield and consistent quality reduce the need for extensive in vitro expansion, thereby minimizing replicative senescence and genetic instability. This is particularly relevant for Good Manufacturing Practice (GMP) compliance, where cell stability and safety are paramount. Consequently, enzymatic digestion of the infrapatellar fat pad represents a cornerstone technique in ensuring the biological stability, therapeutic efficacy, and translational potential of IFP-derived MSCs.

Our results demonstrated that enzymatic digestion at 37 °C for 3 hours with agitation produced a significantly higher cell yield compared with shorter digestion or non-agitated conditions. This finding aligns with earlier reports showing that prolonged collagenase exposure enhances extracellular matrix breakdown, thereby improving MSC release from adipose tissues (14). Importantly, this digestion condition also maintained superior cell viability across passages, suggesting an optimal balance between enzymatic efficiency and cellular integrity (15).

Morphological assessment revealed typical fibroblast-like, spindle-shaped cells across all groups, consistent with the mesenchymal stem cell phenotype defined by the International Society for Cellular Therapy (Dominici et al., 2006). Differences in proliferation became evident during later culture periods, with cells isolated under optimized conditions exhibiting higher proliferative capacity. Similar observations have been reported for IFP-MSCs, which are known to possess high intrinsic proliferative potential influenced by isolation technique (16). Despite these differences, population doubling time remained comparable between groups, indicating stable growth kinetics following culture adaptation.

Clonogenic analysis further supported the enhanced stemness of cells isolated using prolonged digestion with agitation, as reflected by higher CFU-F efficiency. CFU-F formation is a key indicator of self-renewing stromal progenitors and correlates with therapeutic potential (Friedenstein et al., 1970). Flow cytometric analysis confirmed consistent expression of MSC markers (CD29, CD44, CD73, CD90) and absence of hematopoietic markers (CD34, CD45) across all groups, indicating preservation of MSC identity regardless of digestion conditions (17).

Overall, this study supports the infrapatellar fat pad as a reliable and efficient MSC source and demonstrates that enzymatic digestion at 37 °C for 3 hours with agitation is optimal for maximizing cell yield and functional quality. These findings contribute to protocol standardization and strengthen the translational potential of IFP-MSCs for cartilage repair and tissue-engineering applications (18).

This study was limited by a small donor sample size and evaluation restricted to early cell passages, which may not fully reflect long-term expansion behavior or functional differentiation potential. Additionally, lineage-specific differentiation and molecular analyses were not performed. Future studies should include larger cohorts, extended passaging, trilineage differentiation assays, and in vivo validation. Comparative studies with other MSC sources and development of GMP-compliant isolation protocols will further support the clinical translation of optimally isolated infrapatellar fat pad–derived mesenchymal stem cells.

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