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EFFECTS OF COLLAGENASE INCUBATION TIME ON THE ISOLATION OF STROMAL VASCULAR FRACTION AND VIABILITY OF MESENCHYMAL STEM CELLS

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ABSTRACT

There are three groups of potential stem cells that are widely used in regenerative medicine in clinical practice: hematopoietic stem cells, mesenchymal stem cells, and epithelial stem cells. Among them, adipose-derived mesenchymal stem cells (ASCs) are very common in the human body and can be easily obtained without causing major damage to the body. The use of human adipose-derived stromal vascular fraction (SVF) therapy has increased in recent years. SVF is a rich source of mesenchymal stem cells (MSCs) used in regenerative medicine. This study evaluated the effect of collagenase incubation time on the efficiency of SVF isolation and MSC viability. The aim of the study was to optimize the SVF isolation time to obtain MSCs with a high survival rate in clinical applications while ensuring the stemness and differentiation ability of MSCs after isolation. Collagenase digestion at different time points (60, 120, and 180 minutes) was compared, measuring MSC yield and viability via flow cytometry and differentiation assays. Results indicated that 120 minutes of collagenase treatment provided optimal MSC yield and cell viability, demonstrating significant differentiation potential, making this time point ideal for clinical applications.

Keywords: Adipose-derived stem cells, collagenase, mesenchymal stem cells, regenerative medicine stromal vascular fraction

INTRODUCTION

Stromal vascular fraction (SVF) is a heterogeneous cell population isolated from adipose tissue, typically through enzymatic digestion using collagenase. SVF contains a variety of cell types, including mesenchymal stem cells (MSCs), endothelial progenitor

cells, T regulatory cells, macrophages, and preadipocytes (Senesi *et al*., 2019). Due to its diverse cellular composition, SVF has attracted attention in regenerative medicine, especially because of its rich content of MSCs, which holds promise for tissue engineering and therapeutic applications.

MSCs, specifically adipose-derived MSCs (ASCs), can differentiate into a variety of cell types such as osteoblasts, chondrocytes, myocytes, and adipocytes (Hahn *et al*., 2018). These stem cells are important due to their accessibility and abundance in adipose tissue, making them a better alternative to bone marrow-derived MSCs (Pittenger *et al*., 1999). White adipose tissue, in particular, serves as a major source of ASCs. ASCs are typically isolated through biochemical and mechanical methods. Mechanical isolation is often suboptimal in terms of cell quantity and quality. Instead, collagenase digestion is more efficient in separating cells. Collagenase disrupts the extracellular matrix, releasing a mixture of cells from adipose tissue. However, the efficiency of this process depends on the digestion conditions, especially the incubation time. Incubation time can significantly affect the yield and viability of cells after separation.

Although collagenase is widely used for SVF isolation, achieving an optimal digestion time that balances MSC yield and viability remains a challenge. While longer digestion times may result in greater cell dissociation, they also risk damaging the cell membrane and reducing viability (Zuk *et al*., 2002). On the other hand, insufficient digestion may not release enough MSCs from the tissue.

This study aimed to evaluate the impact of varying collagenase digestion time on SVF isolation and ASC viability. By optimizing the digestion protocol, this study sought to identify optimal conditions that maximize both MSC yield and viability for clinical and research applications in regenerative medicine.

MATERIALS AND METHODS

Sample preparation

Fat tissue fragments (negative for HBV, HCV, HIV and syphilis) from a healthy donor were approved by the Medical Ethics Council of Hanoi Obstetrics and Gynecology Hospital (No. 2206 CN/PS). Fat tissue fragments were collected from the abdomen of the mother who had undergone cesarean section. Fat tissue fragments were preserved in Phosphate Buffered Saline (PBS) supplemented with 1% Antibiotic Antimycotic (Sigma Aldrich, USA) at 4^{o}C and transported to the laboratory within one hour after birth (Kien *et al.*, 2024; Thanh *et al.*, 2024).

Collagenase digestion

Adipose tissue was washed with PBS supplemented with antibiotics, then finely chopped and treated with collagenase solution (1:1) at 37ºC for various time frames (0, 60, 120, 180 minutes) in a shaker (Lee et al., 2020). At the 0-min time point, the mixture of adipose tissue and collagenase at a 1:1 ratio was collected immediately after being placed in the 37°C incubator. The mixture was centrifuged at 1500 rpm for five minutes to separate the SVF, removing the supernatant oil and fat cells. The SVF was washed twice with primary culture medium, filtered through a 100 µm filter to remove non-MSC cells, and then supplemented with primary culture

medium Dulbecco's modified Eagle's medium (DMEM, PAN-Biotech, Germany) supplemented with 1% fetal bovine serum (FBS, PAN-Biotech, Germany), as described in the N-BIOTEK patent (Korea, No. 10-1624514, 2017), and 1% Antibiotic Antimycotic Solution 100X (A5955, Sigma Aldrich, USA). The cells were cultured in T-flasks with optimized culture medium at 37ºC, 5% CO_2, and growth was monitored under an inverted microscope (Lasfargues *et al*., 1971).

Cell culture

Primary culture: After 24 hours of isolation, the old medium is removed, and the cells are washed with PBS solution warmed to 37ºC. If excess cells remain after the first wash, continue washing a second time to ensure complete cleanliness. Fresh medium DMEM supplemented with 10% FBS, 1% fibroblast growth factor (FGF; Sigma Aldrich, USA), and 1% Antibiotic Antimycotic is added and changed periodically every 2-3 days until the cell density reaches 80-90%, then proceed to subculture. Secondary culture: The cells are transferred to a flask, washed with PBS containing antibiotics, and treated with trypsin/EDTA for 5 minutes. When the cells are rounded, trypsin is inactivated with DMEM medium supplemented with 10% serum, the suspension is centrifuged, the cells are collected, and diluted in fresh medium. The cells are continued to be cultured in an incubator at 37ºC, 5% CO_2. After 5-7 days, the cells grow to 90% of the bottle bottom area, have a morphology similar to the primary stage and are selected according to MSC characteristics (Kien *et al.*, 2024; Thanh *et al.*, 2024).

Flow cytometry analysis

Flow cytometry was performed to characterize MSCs using surface markers. Cells were labeled with antibodies against CD73, CD90, CD105, and a negative marker (HLA). Cell populations were analyzed after collagenase digestion for 30, 60, 180, and 360 minutes. Cells from the third passage (P3) were fixed and antibody-labeled for flow cytometry analysis. In the fixation phase, cells were resuspended with trypsin, centrifuged at 1500 rpm for 5 minutes, then washed twice with PBS and fixed with 95% cold methanol for 15 minutes at 4ºC. After fixation, cells were washed again with PBS, centrifuged, and PBS was added to maintain them for the next step. Then, cells were divided into two samples, centrifuged, and the supernatant was removed. Each sample was added with 95 µl buffer and 5 µl isotype antibody and incubated in the dark at 2-8ºC for 15 minutes. The samples were then centrifuged, and washed twice with buffer. Flow cytometry was performed with a MACS Quant VYB system and software (Miltenyi Biotec, Germany).

Differentiation assays

MSCs were tested for their differentiation potential. Cells were cultured in StemMACS™ AdipoDiff Medium (Miltenyi Biotec, Germany) for up to 21 days. Adipogenesis was validated via the uptake of lipid droplets visualized by Oil Red-O staining (Ogando *et al.*, 2019).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from differentiated cells after 21 days at different time points (60, 120 and 180 minutes) using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Total RNA was converted into cDNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher

Scientific, USA). The PowerUp SYBR Green Master Mix (ThermoFisher Scientific, USA) was used to measure Relative gene expression. The primers used for qRT-PCR were synthesized by Phusa Genomics Co., Ltd. (Can Tho, Vietnam). The QuantStudio™ 6 Pro Real-Time PCR System with Design & Analysis Software v2.6.0 was used for qRT-PCR reactions and analyses. The relative expression levels of the *LPL* (Lipoprotein lipase) gene were calculated based on the 2-ΔΔCt method (Livak and Schmittgen, 2001). The *β-actin* gene was used as a housekeeping gene to normalize gene expression levels. Microsoft Excel was used to process the graphs and data, with the p-value determined by the t-test method. The primer sequences are LPL-F 5'-GAGATTTTCTCTGTATGGCACC-3', LPL-R 5'-CTGCAAATGAGACACTTTC TC-3'; beta -Actin-F (NM_001101) 5'-CACCATTGGCAATGAGCGGTTC-3'; beta-Actin-R 5'-AGGTCTTTGCGGATG TCCACGT-3'(Deeb and Peng, 1989).

Statistical analysis

The statistically significant differences among 60, 120, and 180 minutes were assessed by ANOVA and Student's t-test. The data are shown as the means ± standard deviations (SD) Statistical significance was considered by using P values < 0.05.

RESULTS AND DISCUSSION

SVF isolation and MSC culture

The SVF was successfully isolated from adipose tissue using collagenase digestion at different time points (0, 60, 120, and 180 minutes). At 0 minutes, the adipose was not digested. Visual observations showed that at 60 minutes, the adipose tissue was not fully digested. At 120 minutes, more than 90% of the tissue had been digested, with a visible layer of yellow liquid fat forming on top. By 180 minutes, complete digestion of the adipose tissue was observed, resulting in optimal release of the SVF. This indicates that, while complete collagen degradation in the sample may require up to 180 minutes of incubation, a 120-min timeframe is a viable option to optimize time and cost efficiency.

After isolation, SVF was cultured to proliferate adipose-derived mesenchymal stem cells (AD-MSCs). The results in Figure 1 showed that after four days, the adherent cells proliferated, reaching approximately 40–50% confluence. The images at the 0-min time point show unclear cell adhesion signs, with limited cell proliferation after that time. By this time, a significant number of non-stem cells had been eliminated, leaving a pure AD-MSC population. AD-MSCs then continued to proliferate, with cells becoming confluent after 4 days. The morphology of AD-MSCs, observed at different collagenase digestion times (60, 120 and 180 minutes), showed a typical fibroblast-like appearance. At 120 minutes, cell growth was faster, with cell density reaching 70–80% confluence by day 10. The results indicate that at the 180-min incubation time, a higher number of cells were obtained, and collagen was fully degraded. However, over the culture period, the 120-min incubation time showed a faster proliferation rate. According to the N-BIOTEK user manual for the Stempia kit, a good collagenase treatment time is about 60-120 minutes. However, under the conditions in Vietnam, with a source sample of different characteristics and a distinct extraction method, the time points need to be studied to ensure the optimal time frame.

This study showed that 120-min was the appropriate time to achieve the best balance between cell yield and viability. Longer digestion times may result in reduced cell viability, possibly due to excessive digestion of the extracellular matrix, while shorter times may not release enough ASCs from the tissue matrix. Compared with previous studies that typically used a collagenase treatment time of 30-min to isolate ASCs with relatively good results (Lasfargues *et al*., 1971), this study determined that a treatment time of 120-min was optimal, significantly increasing the quality and quantity of ASCs. The number of adherent cells per dish at the 120-min time point was 5.1×10^5, which was higher compared to 3.6×10^5 and 3.5×10^5 at the 180-min time point and the 60-min time point, respectively. Fewer than 1,000 cells were observed at the 0-min time point after 4 days of cultivation. The morphology of mesenchymal stem cells shows fibroblast-like homogeneity, and the proliferation rate of mesenchymal stem cells is consistent with previous studies (Kien *et al.*, 2024; Thanh *et al.*, 2024). The lack of consistency in collagenase treatment times in previous studies has made it difficult to compare results, emphasizing the importance of optimizing this procedure. In addition, using tissue fragments not treated with collagenase as controls is useful for assessing the effects of collagenase (Leslie *et al*., 2013). These results provide a scientific basis for standardizing collagenase treatment times to ensure the highest quality of ASC isolation.

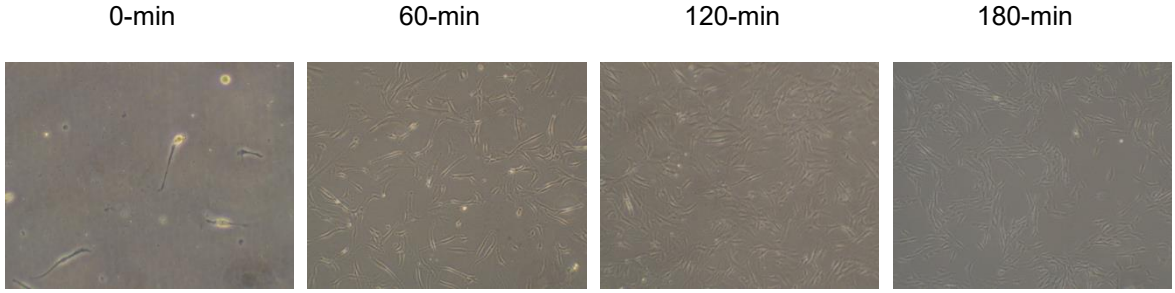

Figure 1. Morphology of mesenchymal stem cells from adipose tissue after 4 days of culture using a 10 X objective lens. Mesenchymal stem cells were incubated with collagenase for 0, 60, 120, or 180 minutes, then cultured in DMEM medium for 4 days. The results shown are representative of three independent experiments.

Flow cytometry analysis

Flow cytometry was performed on ASCs at P3 after being isolated with collagenase for 60, 120, and 180 minutes. The results (Figure 2) showed that ASCs expressed the positive markers CD73, CD90, and CD105, consistent with the ISCT criteria. In contrast, the expression of the negative marker HLA-DR was negligible in any of the time groups. In particular, the 120-min collagenase treatment group achieved the expression rates of CD73, CD90, and CD105 (97.83%, 97.57%, and 95.68%, respectively) similar to the 60-min and 180-min treatment groups of 96.32%, 96.75%, and 95.68%, respectively, and 97.27%, 97.32%, and 95.76%, respectively. The positive and negative expression of characteristic surface markers of mesenchymal stem cells, as shown in the research results consistent with Taghizadeh's research (Taghizadeh *et al.*,

2018), meets international requirements for mesenchymal stem cell characteristics and demonstrates that the cell population isolated in this study is homogeneous (Horwitz *et al.*, 2005). Overall, the results show no significant difference in the expression levels of characteristic positive markers of mesenchymal stem cells derived from adipose tissue.

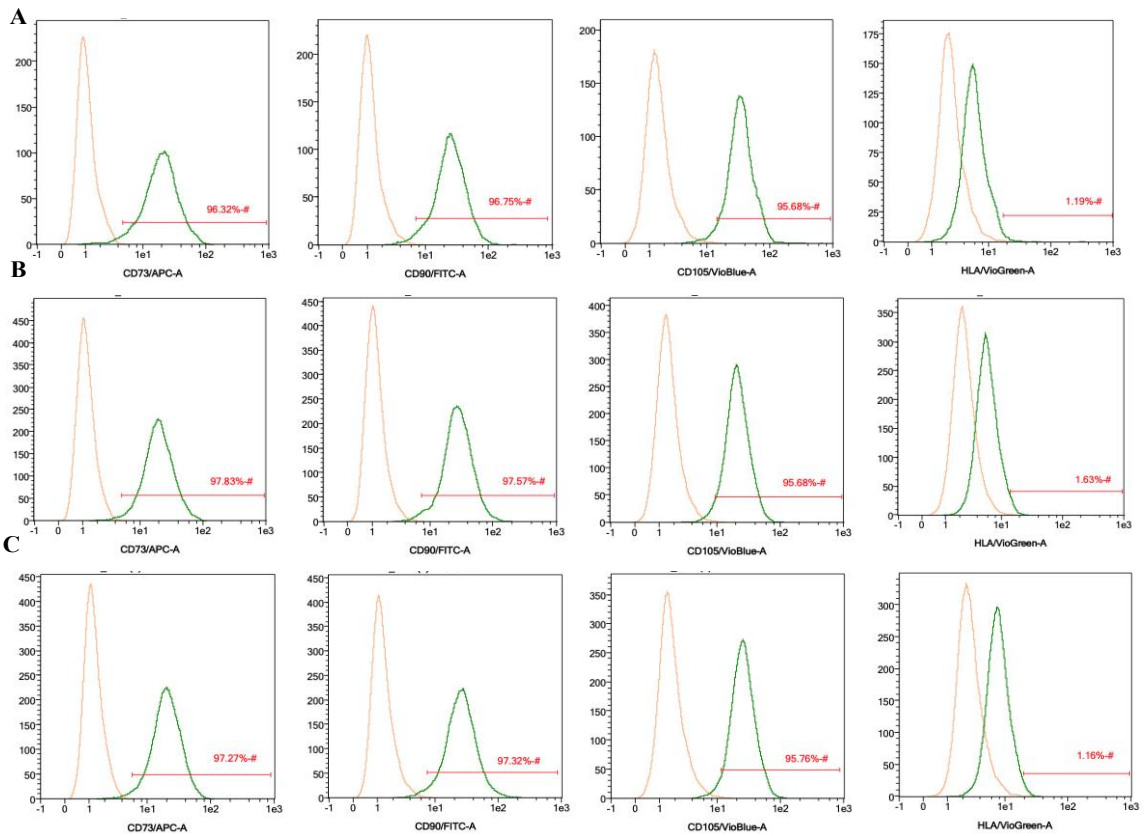

Figure 2. Positive expression of surface markers in adipose tissue-derived mesenchymal stem cells isolated from different collagenase-incubation time points: (A) 60-min, (B) 120-min, (C) 180-min. The results are representative of three independent experiments.

The differentiation of AD-MSCs into adipocytes and bone cells

The study evaluated the differentiation ability of ASCs through collagenase treatment time points (60, 120 and 180 minutes) with specific staining images using Oil Red-O under an inverted microscope. Meanwhile, undifferentiated cells did not take up the dye, indicating that they retained their original nature. The differentiation staining results shown in Figure 3 indicate that at the time points of 60 minutes, 120 minutes, and 180 minutes, mesenchymal stem cells up took Oil Red-O stain, demonstrating their ability to differentiate into adipocytes and meet the requirement of the International Society for Cellular Therapy (Horwitz *et al.*, 2005).

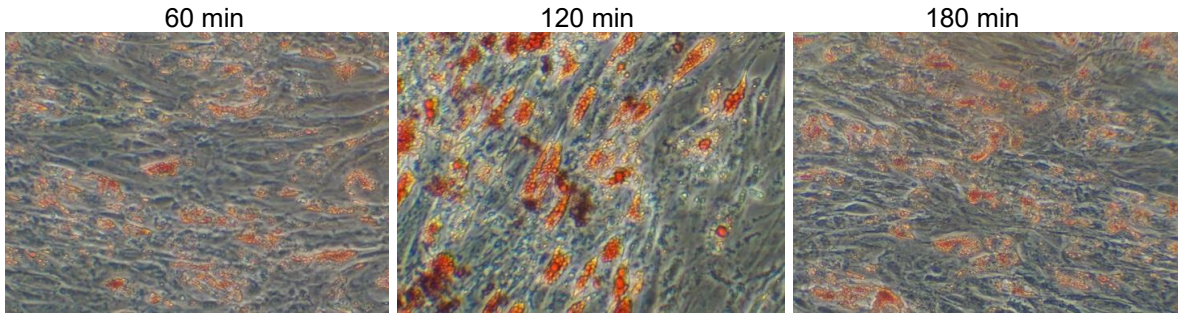

Figure 3. Oil Red-O staining images of adipose tissue-derived mesenchymal stem cells after differentiation into adipocytes. Cells were observed under a microscope using a 10 X objective lens.

LPL is a central enzyme in lipid metabolism and fat biology. LPL is synthesized and secreted by adipocytes and muscle cells and is transported to the endothelial cells of capillaries (Thiemann *et al.*, 2022). RT-PCR analysis results show no expression (Ct value > 40) of the LPL gene at the 0-min time point, meanwhile the expression of LPL is detected at all other collagenase-incubation time points (Ct values were 25.43 ± 0.63; 25.14 ± 0.67; 26.084 ± 0.51 at 60-min; 120-min and 180-min, respectively). This suggests the incubation with collagenase potentially improves the differentiation capability via LPL expression. Interestingly, as we chose the 60-min time point as the control for the remaining 120-min and 180-min time points, statistical analysis showed no significant difference in LPL expression levels between 60 and 120 minutes (P = 0.788, t-test = 0.55), or between 60 and 180 minutes (P = 0.198, t-test = 0.16), as presented in Figure 4. ANOVA analysis among the three time points revealed no statistically significant differences (P = 0.133). These findings suggest that varying the collagenase incubation time (60, 120, and 180 minutes) does not significantly affect the differentiation capacity of the cells. Notably, at the 120-min time point, the ability to isolate a larger number of cells is observed, and the cells retain their basic stem characteristics.

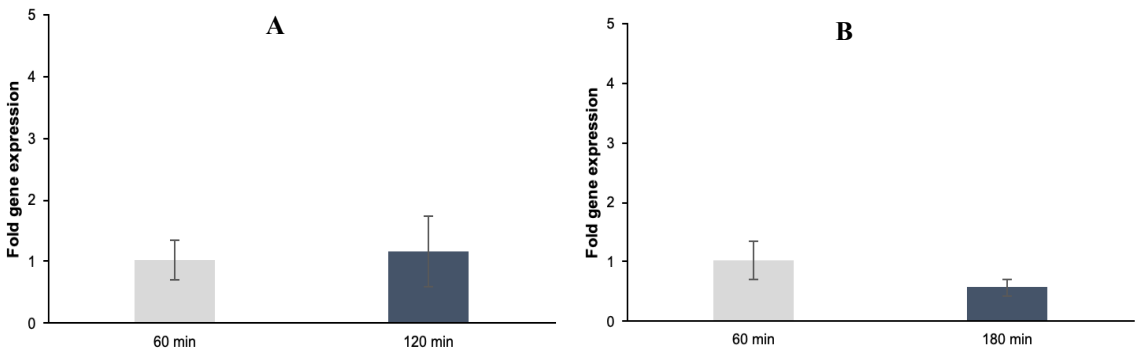

Figure 4. The fold gene expression of LPL genes at different time points. (A). Evaluation of LPL gene expression at the 60-min and 120-min time points; (B). Evaluation of LPL gene expression at the 60-min and 180-min time points. Pairwise comparisons between two groups were conducted using the Student's t-test. Data showed the mean ± SD from three independent experiments. * P < 0.05 compared with the 60-min time point.

CONCLUSION

Collagenase treatment time plays an important role in the isolation of living ASCs from adipose tissue. The characteristic features of adipose tissue-derived mesenchymal stem cells, including the cell expansion and the cell differentiation, show changes at different collagenase incubation time points. The time point that balances the separation efficiency as well as the characteristic features of mesenchymal stem cells from adipose tissue is indicated in this study as 120 minutes.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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