Research

In-Vitro Comparative Examination of the Effect of Stromal Vascular Fraction Isolated by Mechanical and Enzymatic Methods on Wound Healing

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Abstract

Background: Enzymatic digestion has been the gold standard for stromal vascular fraction (SVF) isolation but remains expensive and raises practical and legal concerns. Mechanical SVF isolation methods have been known to produce lower cell yields, but may potentially produce a more robust product by preserving the extracellular matrix niche.

Objectives: The aim of this study was to compare mechanically dissociated SVF (M-SVF) and enzymatically digested SVF (E-SVF) in terms of wound-healing efficacy.

Methods: Lipoaspirate was partitioned into 2 equal groups and processed by either mechanical or enzymatic isolation methods. After SVF isolation, cell counts and viabilities were determined by flow cytometry and cell proliferation rates were measured by the WST-1 test. A wound-healing scratch assay test, which is commonly used to model in-vitro wound healing, was performed with both cell cocktails. Collagen type 1 (*Col1A*) gene expression level, which is known for its role in wound healing, was also measured for both groups.

Results: As predicted, E-SVF isolated more cells (mean [standard deviation], 1.74 [3.63] × 10^6/mL, n = 10, $P = 0.015$) than M-SVF (0.94 [1.69] × 10^6/mL, n = 10, $P = 0.015$), but no significant difference was observed in cell viability. However, M-SVF expressed over 2-fold higher levels of stem cell surface markers and a 10% higher proliferation rate compared with E-SVF. In addition, the migration rate and level of *Col1A* gene expression of M-SVF were found to be significantly higher than those of E-SVF.

Conclusions: Although the cell yield of M-SVF was less than that of E-SVF, M-SVF appears to have superior wound-healing properties.

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Wound healing is an orderly and methodical physiologic process that has evolved to repair and maintain anatomic and functional integrity.[1] Chronic wounds are those that have failed to undergo such a process and are often a co-morbidity of an underlying condition, such as diabetes or obesity.[1] In the United States alone, 6.5 million patients are affected by chronic skin ulcers caused by pressure, venous stasis, or diabetes mellitus.[2] Meanwhile, the treatment cost of venous leg ulcers represents 1% of the entire annual healthcare budget, with healthcare costs continuing to

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rise.[3,4] Although wound healing has been intensively researched, current treatments remain unsatisfactory due to their high costs, low efficiency, and possible side effects.[3]

Stem cells derived from various sources, including adipose tissue, bone marrow, peripheral blood, cord blood, and amnion, are known to enhance wound repair of damaged tissues, and therefore provide a useful resource for chronic wound treatment. In fact, recent studies have demonstrated that adipose-derived stem cells (ADSCs) from in-vivo animal tests showed human dermal fibroblast proliferation by both direct cell-to-cell contact and secretory-induced paracrine activation, thereby significantly reducing wound size, accelerating re-epithelialization, and thus shortening the time required for wound closure.[5]

The use of a heterogeneous cell population of adipose-derived stromal vascular fraction (SVF) is known to be one of the most promising therapeutic strategies for wound healing based on certain physiologic characteristics such as epithelialization, angiogenesis, and immunomodulation.

Although adipose tissue consists mostly of adipocytes, it is an important mesenchymal stem cell reservoir. SVF is a heterogeneous cell cocktail that is obtained by the destruction of adipose tissue through surgery means. SVF contains stem cells, mesenchymal cells, preadipocytes, endothelial cells, hematopoietic cells, fibroblasts, and pericytes. The use of SVF is a recent development with important clinical implications in plastic surgery and other surgical branches.[6,7]

The traditional isolation method for obtaining SVF is through enzymatic digestion of human lipoaspirate, which yields E-SVF. Approximately 100,000 to 1,300,000 nucleated cells per gram of lipoaspirate can be obtained by enzymatic digestion with more than 80% cell viability.[8] Enzymatic digestion effectively disrupts the functional extracellular matrix, leaving SVF as a heterogeneous mixture of “naked” ADSCs, pericytes, macrophages, fibroblasts, vascular endothelial progenitors, and blood cells. However, enzymatic digestion of adipose tissue is considered to involve more than minimal manipulation of tissues and has therefore not been approved by the Food and Drug Administration and other regulatory authorities. Furthermore, this isolation method remains expensive, requires “good manufacturing practice” facilities, has long isolation times (90-120 minutes), and raises legal and administrative concerns.[7] In spite of these disadvantages, automated devices used in the isolation of SVF for clinical and surgical applications have spread worldwide. The main advantages of automated devices include a closed environment, minimal risk of contamination, standardization in clinical practice, and the ability to perform the application in operating rooms or health clinics. However, the widespread use of these devices is limited due to their high costs and the requirement to be trained to operate them.

For these reasons, research into mechanically dissociated stromal vascular fraction (M-SVF) was initiated by a number of groups within the regenerative medicine community. Various mechanical isolation methods based on shaking, vibration, and centrifugation were tested and evaluated against enzymatic protocols. Although low yields of SVF cells in terms of cell count and viability were initially reported,[9] subsequent studies have shown that phenotypic characterization of M-SVF is higher than that of E-SVF. The literature also demonstrated that M-SVF contains an existing amount of adipose-derived stromal cells and endothelial cells, which supports its use in regenerative medicine.[10] To further support these findings of higher phenotypic characterization in M-SVF, the aim of the present study was to investigate the effect of M-SVF and E-SVF by studying the effect of fat tissue on wound healing in vitro.[11-13]

METHODS

This study was started in September 2019 and concluded 5 weeks later in October 2019. It was not necessary to obtain approval from an institutional review board as the SVF isolation was performed in vitro and followed standard protocols. The Declaration of Helsinki was used as a guiding principle for this study. Ten consecutive, nonobese, female patients were preoperatively informed about all surgical procedures, anesthesia, intraoperative video recording, and photography, and gave written consent.

Cell Isolation Methods

A total of 40 mL of lipoaspirate per patient was obtained with a 2-mm-diameter multihole cannula. After decanting, the adipose tissue was partitioned into 2 equal parts for mechanical and enzymatic SVF isolation.

Enzymatic Isolation of SVF

A 20-mL portion of adipose tissue was shaken at a constant speed for 30 minutes in a 37 °C water bath with GMP-grade collagenase NB6 (Serva Electrophoresis, Heidelberg, Germany) at a concentration of 0.1 U/mL and a ratio of 1:1 (w/w). After shaking, the adipose tissue was washed with phosphate-buffered saline and centrifuged at 300 × *g* for 5 minutes. When centrifugation was completed, the supernatant, which contains fat cells and washing/enzyme liquid, was discarded. The pellet containing SVF cells was

then homogenized by adding phosphate-buffered saline in a 1:3 volume ratio. After the cells had been collected by homogenization of cell suspension, they were filtered with a 70-µm sterile filter (LSR00159, BD Biosciences, San Jose, CA, USA).

Mechanical Isolation of SVF

A 20-mL portion of adipose tissue was transferred to 20-mL Luer-lock jointed syringes with the help of a 3-way stopcock (Lipocube SVF Kit; Lipocube, London, UK). Mechanical isolation was achieved in a cube-shaped apparatus with 3 different blade grids at 3 separate Luer-lock ports with a rotating hub path. One syringe injector full of adipose tissue was placed into the first port, while an empty syringe injector was placed into the second port. Between these ports is a filter with 1000-µm holes that reduces the adipose tissue into smaller parcel sizes. This process is repeated with a 750-µm filter between the second and third ports, and a 500-µm filter located between the third and fourth ports, further mincing the adipose tissue into smaller parcels of fat. To remove contaminating erythrocytes, a 1:3 calcium-magnesium balanced buffer solution was added to the adipose product and incubated for 10 minutes at room temperature. Prior to centrifugation, ordinary pistons of 20-mL Luer-lock syringes were replaced with custom-made disarmable pistons with concave, cell-adhesive gaskets. The pistons were detached and the syringes containing the dissociated lipoaspirate were centrifuged at 2000 × *g* for 10 minutes with the Luer-lock tips directed inward so that the SVF could be collected in the concave gaskets. After centrifugation, the piston tops that had been removed were reinstalled and the fat tissue and buffer solution outside the cell pellet was transferred to an empty syringe with the aid of a 3-way stopcock. As a final step, the cell pellet was homogenized with a saline solution.

Cell Count and Viability

Total nucleated cell count and the viability of each cell cocktail isolated enzymatically and mechanically were measured by flow cytometry (Muse Cell Analyzer; Merck Millipore, Germany) following red blood cell lysis.

Flow Cytometry

Flow cytometry was used to quantify and compare adipose-derived stem phenotypes with using specific clustered of differentiation markers (CD surface markers) and SVF stem potential in all groups. Characterization of ADSCs (CD45–/CD90+/CD73+), endothelial cells (CD90+/CD44+), and pericytes (CD105+/CD73+) was performed. Staining was done with 5 mL of monoclonal antibodies (BD Biosciences, Le Pont de Claix, France). The binding efficiency of the surface markers CD73, CD90, CD105, and CD44 was also examined.

Cell Proliferation Test

The SVF cell populations isolated from each protocol were then grown under the same cell culture conditions. For the cell proliferation analysis, cells obtained by the 2 different methods were plated in 96-well cell culture plates at a concentration of 3×10^3 cells/hole and treated with low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco Thermo Fisher Scientific, Loughborough, UK) and 1% penicillin-streptomycin-amphotericin mixture for 24, 48, and 72 hours. Cell proliferation was evaluated with the WST-1 test (BioVision, Milpitas, CA), as discussed in the literature.[13] The absorbance value of WST-1 was measured at 540 nm in a spectrometer. The experiment was completed by 3 biological replicates. ATCC adipose-derived mesenchymal stem cells (American Type Cell Culture, Manassas, VA) were used as a control group in order to compare stem phenotypic characterization in the SVF samples. Only 1 cell batch was used throughout the study (adipose-derived mesenchymal stem cells; normal, human, ATCC PCS-500-011).

Wound-Healing Scratch Assay

The two SVF cell populations were plated on 6-well cell culture plates (TPP, Trasadingen, Switzerland) at a concentration of 1×10^6 cell/hole.[13-15] Allantoin (50 µg/mL), a plant-derived commercialized drug, was used as a positive control to increase migration. ATCC adipose-derived mesenchymal stem cells served as a negative control group without any treatment, and 2% fetal bovine serum (FBS) was used to decrease the migration proliferation potential. Each group was fed with fresh medium containing 2% FBS. Once cells have been incubated and have achieved full surface coverage, a slit model was created in the middle of the container under sterile conditions with the aid of a 200-µL pipette tip. After 48 hours, the closure speed and spacing of this slit was measured by light microscopy with an inverted microscope (Nikon Eclipse TE200; Nikon, Tokyo, Japan) and wound closure analysis was performed with ImageJ software according to the formula: percentage wound closure = ((measurement at 0 h – measurement at 48 h)/measurement at 0 h) ×100.

Gene Expression Analysis with Real-Time Polymerase Chain Reaction

Collagen type I primer, which plays an active role in wound healing, was designed with Primary-BLAST software

from the National Biotechnology Center (Bethesda, MD). Total RNA isolation from SVF cell populations obtained by the 2 different methods was isolated with a Total RNA Purification Kit (Norgen, Ontario, Canada) according to the manufacturer's protocol. A QuantiTect Reverse Transcription kit (Qiagen, Les Ulis, France) was used to transform RNA into cDNA. The mRNA expression level of collagen type 1 gene (*Col1A*) was analyzed with a QuantiTect SYBR Green polymerase chain reaction (PCR) kit (Qiagen, Les Ulis, France). Reactions were read with an iCycler real-time (RT) PCR system (Bio-Rad, Hercules, CA) according to the manufacturer's protocols. In the analysis, *18S rRNA* was used as a reference gene. ATCC ADSCs served as a control group.

Table 1. Cell Count and Cell Viability Results Obtained After Mechanically Dissociated SVF (M-SVF) and Enzymatically Digested SVF (E-SVF) Isolation Protocols

Viable cell number/mL ($\times 10^6$)		Cell viability, %
M-SVF	0.94	97.55
E-SVF	1.74	96.67

Statistical Analysis

One-way analysis of variance and GraphPad Prism 5 (GraphPad, La Jolla, CA) software were used for statistical

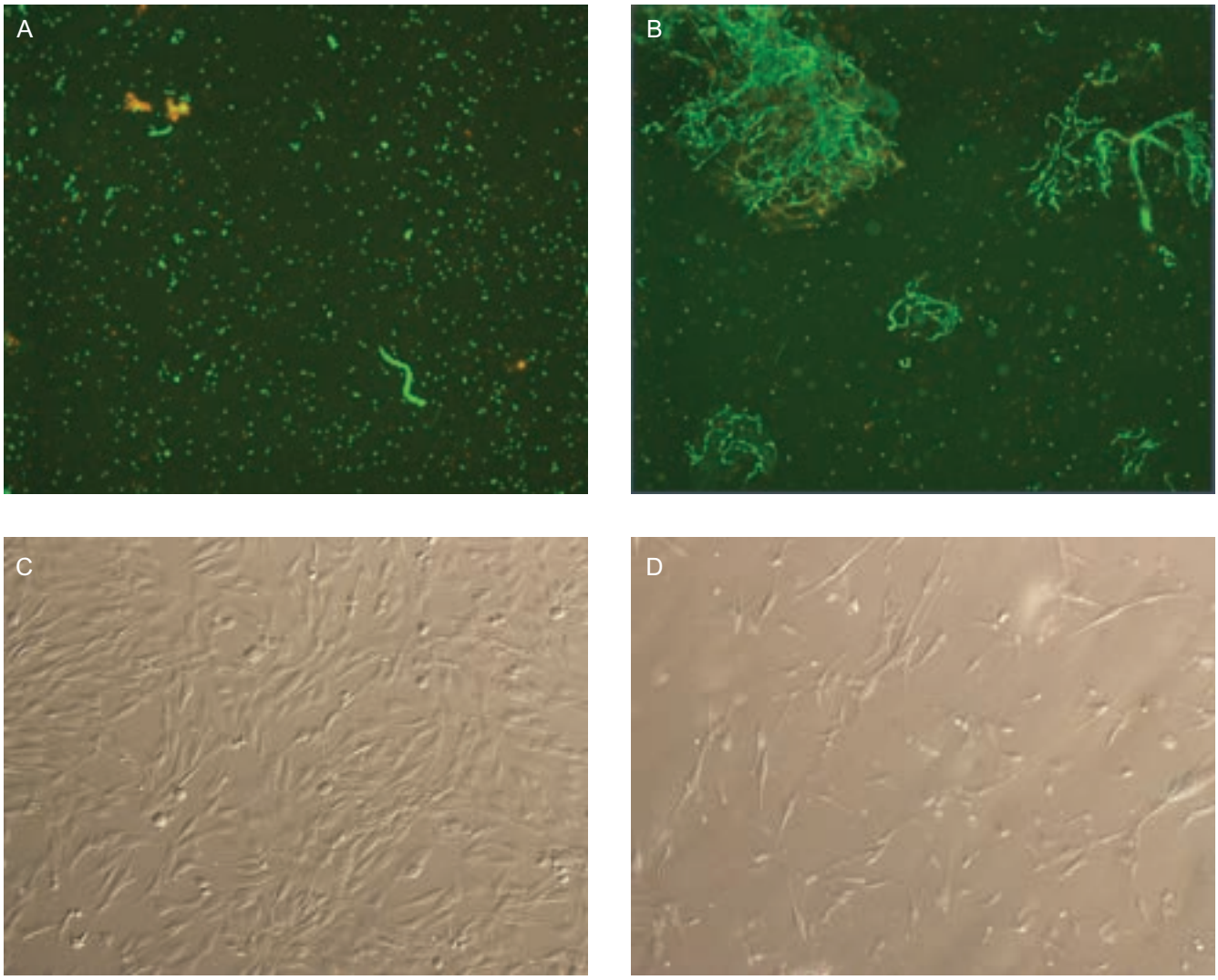

Figure 1. Microscopy images of cell-stained cocktails obtained after mechanical isolation and enzymatic isolation. Cell counts and cell viabilities were measured by laser-based fluorescence detection with a Muse flow cytometer (Merck Millipore, Germany). (A) Fluorescence microscopy of E-SVF. (B) Fluorescence microscopy of M-SVF. (C) Cell culture image of E-SVF by phase-contrast microscopy at ×40 magnification. (D) Cell culture image of M-SVF by phase-contrast microscopy at ×40 magnification.

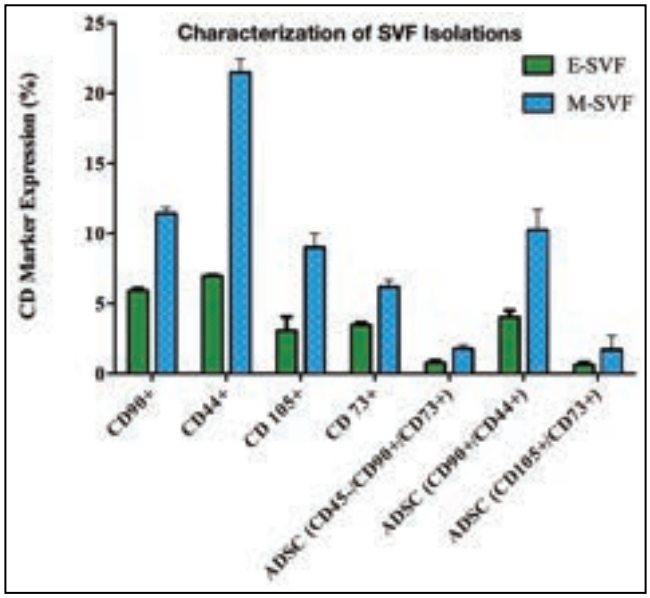

Figure 2. Characterization of adipose-derived stem cells (ADSCs) of enzymatically digested SVF (E-SVF) vs mechanically dissociated SVF (M-SVF). Individual CD markers (ie, CD90, CD44, CD105, CD73) were compared by flow cytometry along with combinations of commonly used adipose-derived stem phenotypic characterization markers (ie, CD45–/CD90+/CD73+, CD90+/CD44+, and CD105+/CD73+). These universal stem cell markers were approximately 2-fold higher in the M-SVF group than in the E-SVF group. When ADSC markers of phenotypic characterization were compared, a 1.93-fold increase in CD90 (11.39% vs 5.88%), a 3.0-fold increase in CD44 (21.45% vs 6.93%), a 2.9-fold increase in CD105 (9.0% vs 3.057%), and a 1.7-fold increase in CD73 (6.16% vs 3.44%) markers were observed.

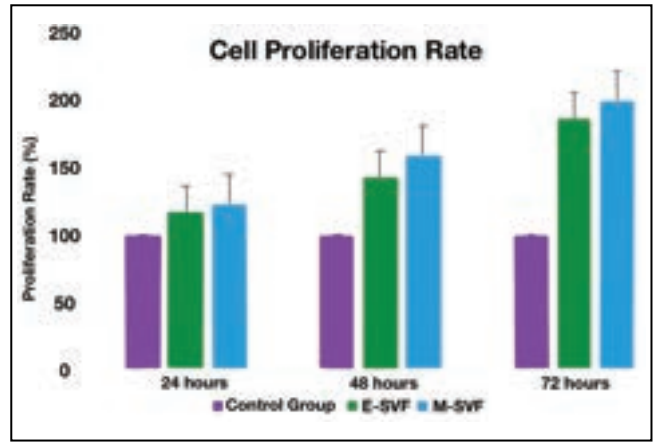

Figure 3. Cell proliferation was assessed by the WST-1 test. ATCC adipose-derived mesenchymal stem cells (American Type Culture Collection) were used as a control group. The analysis shows the measurements at 24, 48, and 72 hours of cell cocktails isolated by mechanical and enzymatic methods (M-SVF and E-SVF, respectively). Whereas in the E-SVF group the A_{490} value after 72 hours was 187 ± 0.3, in the M-SVF group it was 201 ± 0.1 ($P \leq 0.05$). Therefore, the cell proliferation rate of M-SVF cells was higher by 10% to 20% at the end of 72 hours compared with both the control group and the E-SVF group.

analysis with repeated samples. $P \leq 0.05$ values were considered as statistically significant. Because the analysis of variance was used to compare cell proliferation and gene expression between the 2 groups (E-SVF vs M-SVF), a post-hoc test was not required. Normal distribution was used. Nonparametric tests were not applied.

RESULTS

Patient Characteristics

After providing written consent, 10 consecutive, nonobese women, with an average age of 39 years (range, 26-52 years) and an average body mass index of 26 kg/m^2 (range, 17-35 kg/m^2) received liposuction to the lateral hip region.

Cell Count and Cell Viability Analysis

The total number of nucleated E-SVF cells isolated was 1.74 × 10^6 whereas the total number of nucleated M-SVF cells isolated was 0.94 × 10^6. Cell viability after isolation was 96.67% in the E-SVF group and 97.55% in the M-SVF group. There was no significant difference between the cell viabilities achieved with each SVF isolation method (Table 1, Figure 1).

Flow Cytometry Analysis

When ADSC markers of phenotypic characterization in M-SVF were compared to those in E-SVF, we observed a 1.93-fold increase in CD90 (11.39% vs 5.88%), a 3.0-fold increase in CD44 (21.45% vs 6.93%), a 2.9-fold increase in CD105 (9.0% vs 3.057%), and a 1.7-fold increase in CD73 (6.16% vs 3.44%), which are commonly used stem phenotypic characterization markers (Figure 2). When analyzing combinations of known ADSC CD markers, M-SVF showed 2.35-, 2.5-, and 2.8-fold greater expression than E-SVF.

Cell Proliferation Test

Whereas in the E-SVF group the A_{490} value after 72 hours was 187 ± 0.3, in the M-SVF group it was 201 ± 0.1 ($P \leq 0.05$). As shown in Figure 3, the proliferation rate of M-SVF cells

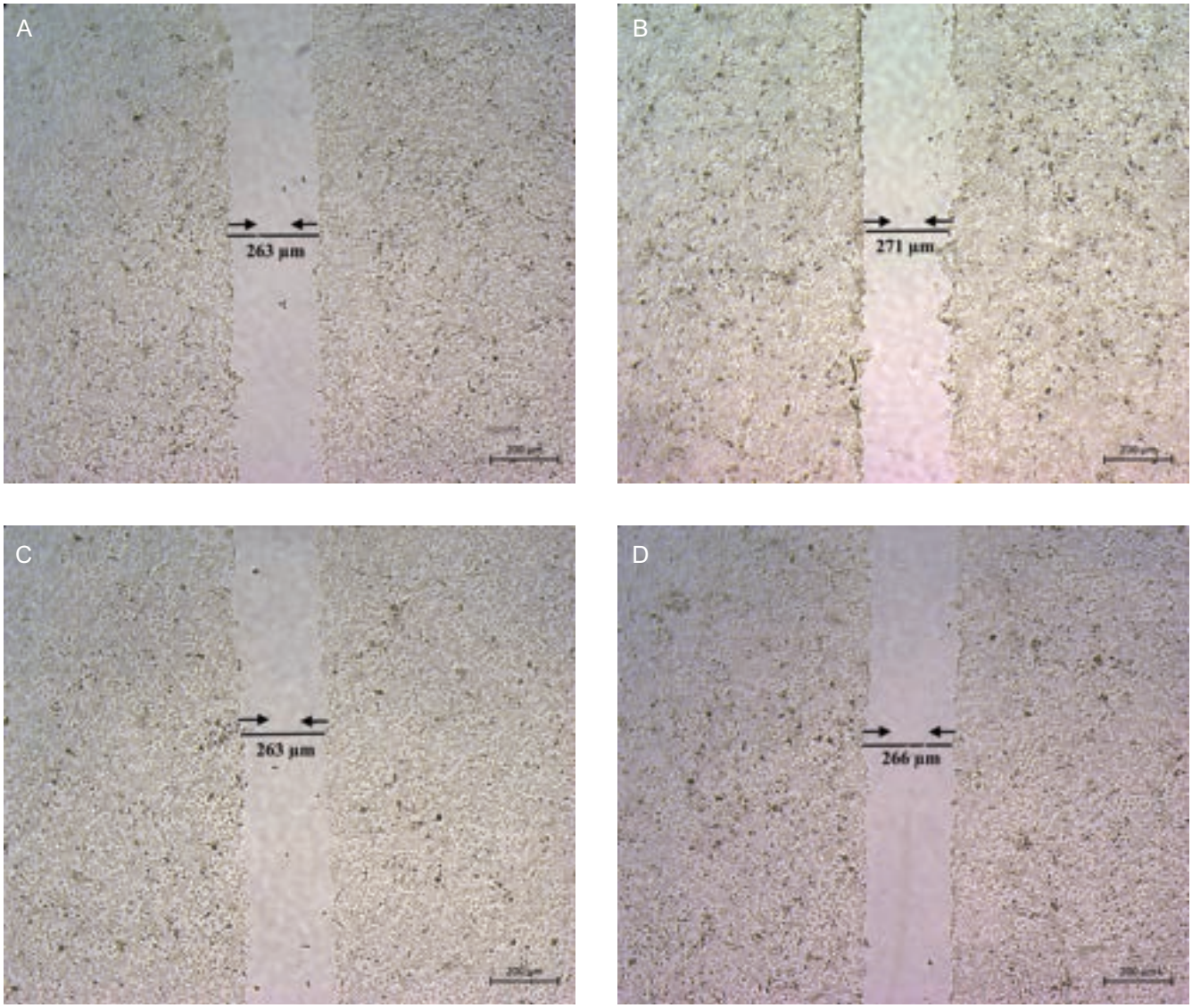

Figure 4. Scratch analysis and wound closure rates of cells obtained after mechanical and enzymatic isolation (M-SVF and E-SVF, respectively). Allantoin (50 µg/mL), a plant-derived commercialized drug, was used as a positive control to increase migration. ATCC adipose-derived mesenchymal stem cells (American Type Culture Collection) were used as a negative control group without any treatment, and 2% fetal bovine serum was used to decrease the migration proliferation potential. Microscope images with ×40 magnification taken between 0 and 24 hours for the (A) negative control, (B) positive control, (C) E-SVF, and (D) M-SVF, respectively. Microscope images with ×40 magnification taken between 24 and 48 hours for the (E) negative control, (F) positive control, (G) E-SVF, and (H) M-SVF, respectively. (I) Bar graph showing migration rates. No allantoin (negative control) showed a migration rate of 19% wound closure, moving from a 263 [2] µm gap at 0 to 24 hours to a 213 [3] µm gap at 0 to 72 hours. Allantoin (positive control) showed a migration rate of 98% wound closure, moving from a 271 [5.2] µm gap at 0 to 24 hours to a 5 [4.8] µm gap at 0 to 72 hours. The use of E-SVF showed a migration rate of 45% wound closure, moving from a 263 [4] µm gap at 0 to 24 hours to a 143 [1] µm gap at 0 to 72 hours. Conversely, M-SVF showed a migration rate of 89% wound closure, moving from a 266 [3] µm gap at 0 to 24 hours to a 29 [3] µm gap at 0 to 72 hours. The results indicate M-SVF may have a greater potential in wound healing.

was higher by 10% to 20% at the end of 72 hours compared with both the control group and the E-SVF group (Figure 3). ATCC adipose-derived mesenchymal stem cells were used as a control group.

Wound-Healing Scratch Assay

The closure speed and spacing of culture surface slits were measured by light microscopy at 0 and 48 hours. Positive and negative controls indicated the natural rate

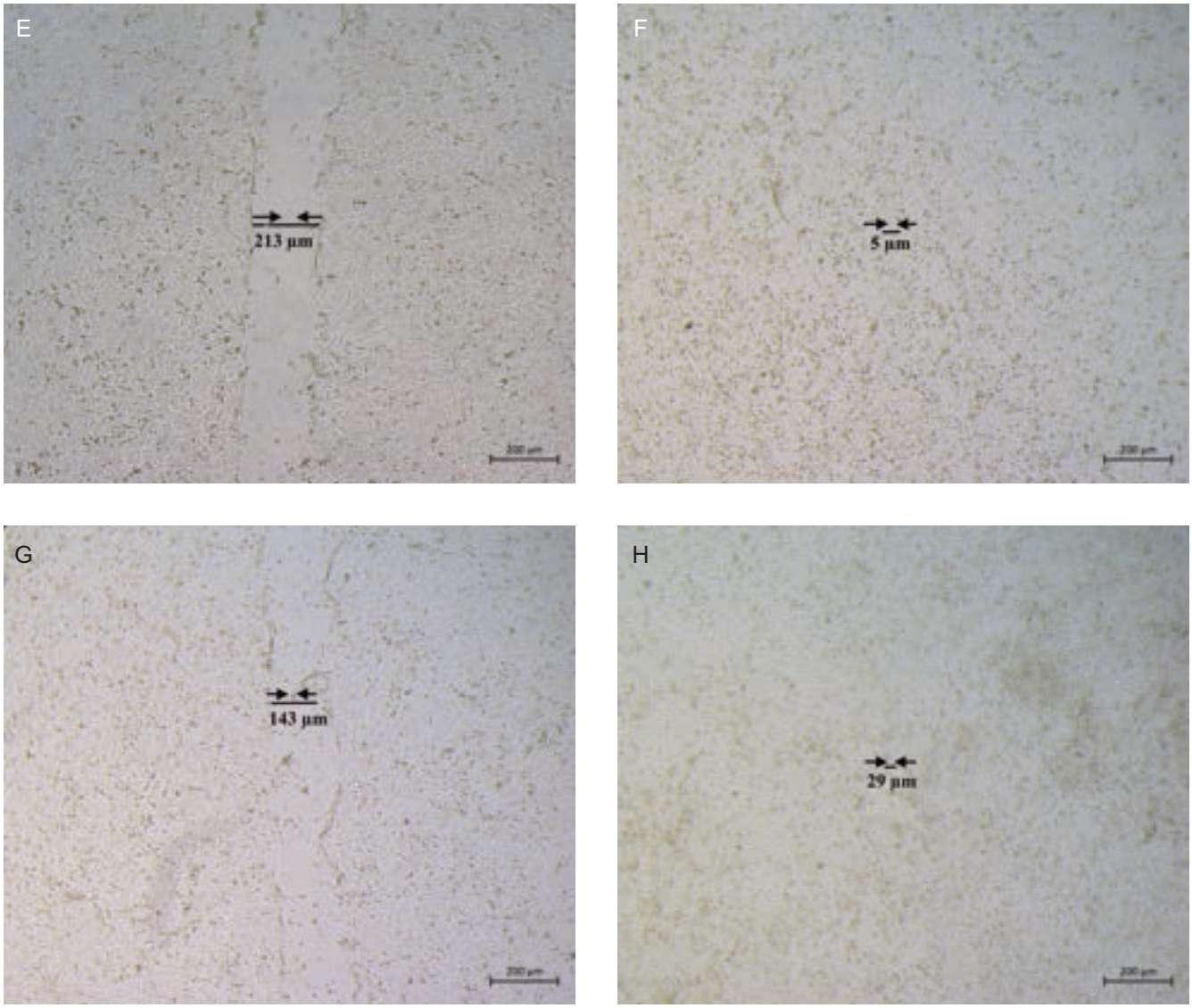

Figure 4. Continued.

of migration. Comparing results between 0 and 48 hours, allantoin (positive control) showed a migration rate of 98% wound closure. No allantoin (negative control) showed a migration rate of 19% wound closure. The use of E-SVF showed a migration rate of 45% wound closure, whereas M-SVF showed a migration rate of 89% wound closure. Hence, M-SVF presents a greater migration tendency, and therefore may provide greater wound-healing potential (Figure 4).

Gene Expression Analysis With RT-PCR

RT-PCR is a method to determine the number of genes that have been expressed.[16] A fluorescent luminescent dye (SYBR) binds to DNA, and when a sufficient product is formed, it emits light from which gene expression can be determined.[17] Supporting the results of the aforementioned cell viability and wound closure analyses, the expression level of the collagen type 1 gene, *Col1A*, known to be involved in wound healing, was 1.5 higher in M-SVF cell populations than in E-SVF cell populations (control, 2.5 [1.3] × 10^6; E-SVF, 7.5 [2.4] × 10^6; M-SVF, 12.5 [1.2] × 10^6; $P \leq 0.05$) (Figure 5).

DISCUSSION

Skin integrity is ensured by a series of intrinsic and extrinsic factors as well as stimuli from cytokines and growth factors

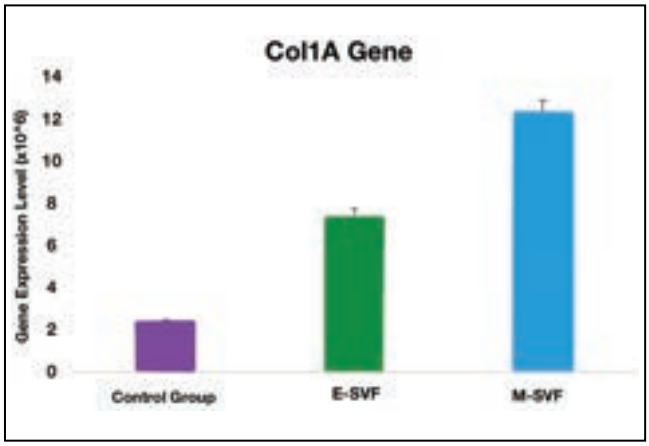

Figure 5. Real-time polymerase chain reaction determination of the expression of collagen type 1 (*Col1A*) level compared to the *18S RNA* reference gene of cells obtained after mechanical isolation and enzymatic isolation (M-SVF and E-SVF, respectively). *Col1A* expression was 1.5 higher in cell populations isolated by M-SVF than in cell populations obtained by E-SVF (control, 2.5 [1.3] × 10^6; E-SVF, 7.5 [2.4] × 10^6; M-SVF, 12.5 [1.2] × 10^6; $P \leq 0.05$).

that cooperate to maintain skin continuity in the event of any injury. Furthermore, extracellular matrix components such as collagen play an important role in wound healing. The wound-healing process requires replacing the fibrin clot with collagen matrix type I. The collagen produced by the surrounding fibroblasts controls both cell adhesion and cell migration during skin healing. The primary factors for wound regeneration rate, such as cell migration and proliferation, were imitated by using SVF cell cocktails isolated by 2 different methods. In our study, the cell count of M-SVF cells represented 30% to 60% of those found in the E-SVF cell cocktail. In terms of cell count alone, the E-SVF has an advantage. However, the present findings strongly indicate that despite M-SVF yielding lower cell counts, M-SVF cells have increased cellular activity, as exhibited by their increased rate of proliferation following cell culture seeding. Our findings indicated that M-SVF cells increase the level of *Col1A* gene expression, and therefore result in increased collagen production, increased proliferation of cells, and increased cell migration during wound healing.

Mechanical isolation is based on the dissociation of SVF cells by centrifugation as a result of the separation of adipocytes from the adipose matrix. Research has indicated that mechanical manipulation and mechanical forces can enhance cell functionality and effectiveness.[12] Increased phenotypic characterization results in increased gene expression levels of cells, which consequently leads to greater protein synthesis. The chemical used in enzymatic isolation is obtained from a pathogenic bacteria such as *Clostridium*, and the effects of this enzyme on human health are still unknown. In turn, many countries do not permit the use of the enzymatic processing of human tissue or human cells.[18] In order to overcome the disadvantages of the enzymatic method, various mechanical SVF isolation methods have been investigated. Mechanical isolation is not only easier and faster, and but is preferred over enzymatic isolation because it can be performed conveniently in the operating room environment. The disadvantage of mechanical isolation has been the lower cell counts, which were previously 10% those obtained by enzymatic methods.[8] Newly developed methods to produce M-SVF have, however, increased that number to 30% to 60%.[11,19] Furthermore, Banyard et al[12] have emphasized that the effect of cell count is not as important as previously assumed, but rather cell functionality and effectiveness are what matter. In light of this information, and supported by this study, M-SVF shows great promise in wound healing without the disadvantages of enzymatic digestion techniques. The results of this study should be reproduced in other laboratory settings and with larger sample sizes to negate any limitations of this study. In addition, controlled studies on wound healing should be performed clinically.

CONCLUSIONS

In this study, the wound-healing effect of SVF obtained by mechanical and enzymatic digestion methods was compared. Although the total cell yield of M-SVF was less than that of E-SVF, the phenotypic characterization and *Col1A* gene expression level of M-SVF was observed to be significantly higher by flow cytometry, gene expression, and cell proliferation studies compared with E-SVF, indicating better wound-healing properties. Our preliminary results suggest that M-SVF might be a better alternative for the treatment of skin injuries.

Disclosures

Dr Tiryaki is an investigator for Mentor, receives book royalties from Springer, and is on the advisory board and holds equity in the Mage Group and Lipocube Ltd. Dr Cohen has stock options and royalties with Millennium Medical Technologies (Carlsbad, CA); receives royalties from Tulip Medical; is a minor shareholder in Cytori, Inc; is a shareholder in the Mage Group and receives royalties; is an investigator for Allergan and Ampersand, Inc; and is an investigator with Thermigen. Ms Turkay is R&D officer in Lipocube Company, Dr Kocak, Ms Turkay, and Ms Hewett declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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