ORIGINAL ARTICLE

Injectable adipose tissue combined with stem cells for soft-tissue augmentation: A pilot study for dental applications

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Abstract *Background/purpose:* Bone resorption and soft-tissue defects are the typical physiologic responses after tooth extraction. Various dental ridge augmentation techniques have been applied and lack of the soft tissue is the major factor causing the failure. We propose that the adipose-derived stem cell can be useful in soft-tissue augmentation in dental applications. The objective of this study was to optimize the operation procedures for the isolation of adipose stem cells and tissues. Accelerated clinical protocols for effective transplantation of adipose tissue with high amount of adipose stem cells shall be developed.
Materials and methods: Operation parameters were designed and optimized for the extraction of adipose tissue-derived stromal vascular cells. The optimized accelerated procedure was washing the lipoaspirate samples one time. Collagenase was then added and samples were incubated in a water bath for 30 minutes at $37^{\circ}C$ and centrifuged at $1200g$ for 3 minutes. A mouse animal model was applied to evaluate the soft-tissue-filling effects using the optimized procedure.
Results: The animal model tests demonstrated the filling and regeneration of the soft tissues with significant angiogenesis.

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Conclusion: This pilot study demonstrated the feasibility of soft-tissue augmentation applications.

Introduction

Alveolar bone resorption is the typical physiologic response following tooth extraction.[1] Soft-tissue defects in conjunction with severe bone defects are commonly observed clinically. In addition, ridge defects present important challenges for aesthetic dentistry. Several ridge augmentation techniques have been developed to replace tissue loss, including soft tissue or/and alveolar bone.[2-5] The critical factor for successful ridge augmentation is the coverage of soft tissues. It is indicated that mucosal dehiscence and premature exposure of the bone graft are the most common failure mechanisms.[6] Therefore, prior to performing grafting procedures, plastic periodontal procedures to augment soft tissue may be necessary. The traditional procedures include the use of free gingival grafts, subepithelial connective grafts, and various types of roll and pedicle flaps.[7-10] However, these advanced surgical procedures are highly technique sensitive and usually result in postoperative complications. The development of a rapid protocol for soft-tissue augmentation is therefore needed.

Injectable adipose tissue obtained from patients by liposuction can be used as autologous filling materials to improve multiple defect healing.[11] This also presents a nonsurgical alternative for augmenting soft tissues. So far, injection of adipose tissue has safely been applied in cosmetic medicine. Adipose tissue is not only suitable as a soft-tissue filling material, but also serves as a plentiful source of adipose-derived stem cells (ADSCs). Clinical studies of ADSCs for the regeneration of periodontal tissues have shown that the ADSCs with platelet-rich plasma can regenerate alveolar bone, cementum, and periodontal ligament.[12,13] ADSCs in combination with functional biomaterials could be used to accelerate bone healing in peri-implant defects caused during dental implant placement.[14]

ADSCs are contained in adipose tissue-derived stromal vascular fraction (SVF) cells, which are freshly isolated from adipose tissue by enzymatic digestion. ADSCs can further be selected and expanded by culturing extracted SVF cells in plastic,[15] and if the SVF cells are harvested, a sufficient number of these cells can be used clinically without the need for expansion of ADSCs. SVF cells supplemented fat tissues can improve calcification, fibrosis, and reduce the transplant absorption rate as these cells secrete vascular endothelium growth factor, fibroblast growth factor, and tissue growth factor-beta.[16-20] Therefore, injectable adipose tissue supplemented with SVF cells has the potential for augmenting soft tissues in clinical applications. It is suggested that SVF cells contain ADSC-assisted autologous adipose tissue that may assist in augmentation of ridge defects healing.

SVF cell-assisted autologous adipose tissue augmentation is a process that relies on the isolation of SVF cells. However, most methods of isolation of SVF cells are tedious and time consuming. Similar methods reported in the literature for isolating the adipose cells and their principles are shown in Figure 1. At present, a typical isolation process takes ~3 hours. During this period, patients, doctors, and healthcare personnel are forced to wait until the completion of stem cell extraction through a surgical procedure. As a result, such isolation process may increase the cost and affect the quality of the care. In addition, each step of the isolation process may affect the cell viability. With the increasing number of experimental procedures, the viability of the extracted cells starts to decrease.

The objective of this study was to develop an accelerated procedure for adipose tissue augmentation. A series of experiments were designed to optimize the parameters for extraction of SVF cells. Animal tests were carried out to evaluate soft-tissue augmentation with adipose tissue and SVF cells extracted using the optimized procedure presented herein.

Materials and methods

Harvest of human adipose tissue

Human adipose tissue was harvested from individual female donors during liposuction surgery using a 16-gauge suction cannula under a pressure of 760 mmHg. All procedures were approved by the Institutional Review Board of Tri-Service General Hospital, Taipei, Taiwan with the informed consent of the donors (TSH-IRB-100-05-143). Adipose tissue specimens were preserved at 4°C within 3 days after the surgery for subsequent use.

Isolation of human adipose tissue-derived SVF cells

The collected fat tissue was divided into 15 mL samples for experiments. The adipose tissue was washed using Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO, USA) at $1200g$ for 3 minutes. The tissue was then digested with an equal amount of 0.2% collagenase I (Sigma-Aldrich) in DPBS at 37°C for various amounts of time (30 minutes, 45 minutes, or 60 minutes). The collagenase was inactivated using Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) with 10% (v/v) fetal bovine serum (FBS; HyClone); the digested tissue was then centrifuged at different forces ($600g$, $1200g$, or $2800g$) for various periods (1 minute, 3 minutes, or 5 minutes; UNIVERSAL 320 R-1406-01; Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The supernatant was removed and
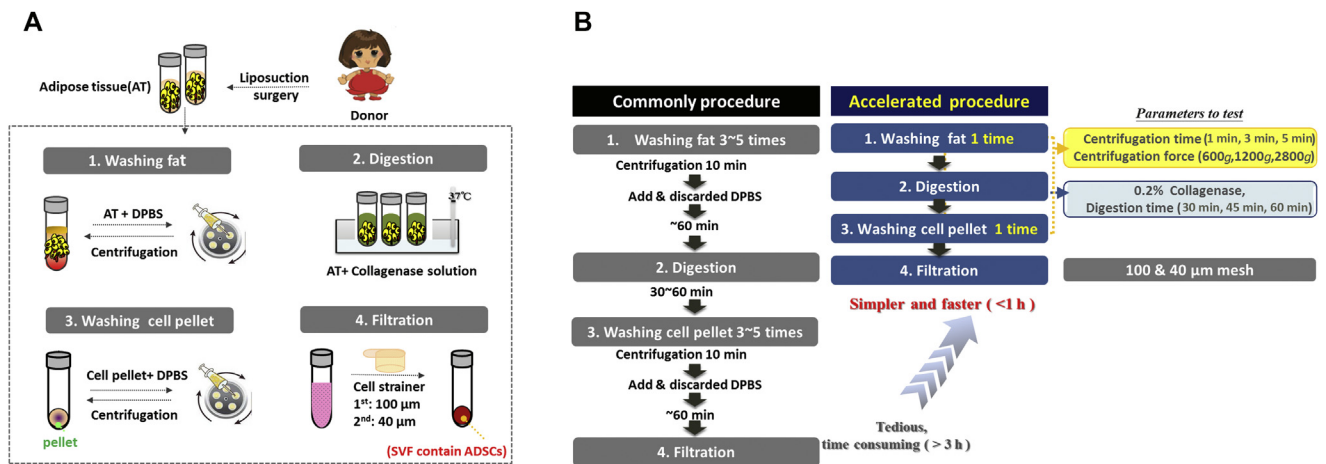
Figure 1 Development of the accelerated procedure by optimizing the key parameters from common methods for isolation of stromal vascular fraction (SVF) cells. (A) Commonly used procedure for isolation of adipose-derived stem cells (ADSCs); (B) key parameters for isolation of SVF cells. DPBS = Dulbecco's phosphate-buffered saline.

the remaining pellets were filtered through 100- and 40-μm mesh cell strainers (BD Biosciences, San Jose, CA, USA).

Cell counting of SVF cells

The isolated SVF cells and the accompanying medium were transferred to a test tube, where the cells were dyed using Trypan blue (ScienCell Research Laboratories, Carlsbad, CA, USA). The number of blue cells and the number of white cells were observed under an inverted microscope (Ti-DH; Nikon Inc., Melville, NY, USA) and counted.

Flow cytometry analysis

The ADSCs were counted using an FACS Calibur flow cytometer (BD Biosciences). Flow cytometry was used to conduct immunophenotype characterization of the ADSCs isolated using the accelerated procedure. The SVF cells were centrifuged and incubated at 4°C for 30 minutes with phycoerythrin-, fluorescein isothiocyanate-, peridinin–-chlorophyll–protein-, or Alexa Fluor-conjugated mouse antihuman monoclonal antibodies for CD34, CD90, CD105, CD146, CD31, and CD45 antigens (BD Biosciences). The flow cytometer was used to determine the number of cells with different immunophenotypes. The isolated ADSCs were cultured in DMEM to produce four generations of cells. Immunophenotyping was performed on the cell cultures. The expressions of surface markers (CD34, CD45, CD90, CD146, CD105, and CD31) and surface marker combinations (CD34$^-$/CD90$^+$/CD105$^-$/CD31$^-$, CD34$^+$/CD45$^-$/CD105$^-$/CD31$^-$, and CD34$^+$/CD146$^-$/CD105$^-$/CD31$^-$) were analyzed in each of the four generations.

Colony-forming unit assay

A colony-forming unit (CFU) assay was conducted to measure the number of progenitors in the sample. ADSCs were cultured at a concentration of 8 cells/cm^2 in DMEM. The medium was changed every 2 days or 3 days for 12 days, and then the cells were washed with DPBS for 15 minutes. The CFU-fibroblasts (CFU-F) were stained with crystal violet stain (Sigma-Aldrich) for 30 minutes and colonies that were clusters of 50 cells or more were counted under an optical microscope.

Adipogenic differentiation

ADSCs were plated at 2 × 10^4 cells/cm^2 and cultured for 3 days; the cells were then induced in DMEM and 10% (v/v) FBS supplemented with 0.5mM isobutyl methylxanthine (Sigma-Aldrich), 1 μm dexamethasone (Sigma-Aldrich), 10 μm insulin (Sigma-Aldrich), 200 μm indomethacin (Sigma-Aldrich), and 1% (v/v) antibiotic/antimycotic (Sigma-Aldrich), with the medium being changed every 2 days.[21] After 28 days, the culture was fixed with 10% (v/v) formalin for 20 minutes at room temperature and stained with 0.6% (w/v) oil red O solution (60% isopropanol and 40% water; Sigma-Aldrich) for 1 hour at room temperature.

Osteogenic differentiation

ADSCs were plated at 2 × 10^4 cells/cm^2 and cultured for 3 days; the cells were then induced in DMEM and 10% (v/v) FBS supplemented with 0.1 μm dexamethasone, 50 μm ascorbate-2-phosphate (Sigma-Aldrich), 10mM β-glycerophosphate (Sigma-Aldrich), and 1% (v/v) antibiotic/antimycotic, with the medium being changed every 2 days. After 28 days, the culture was fixed with 10% (v/v) formalin for 20 minutes at room temperature and stained with 2% (v/v) alizarin red solution (Sigma-Aldrich) for 5 minutes at room temperature.[22]

Preparation of SVF cells-enriched fat tissues

The remaining untreated fat tissue was divided into either the experimental group or the control group. The experimental group was supplemented with SVF cells extracted from 0.5 cm^3 of aspirated fat tissue mixed with 0.5 cm^3 of untreated fat tissue (1:1 ratio); the control group received sterile PBS mixed with 0.5 cm^3 of untreated fat tissue. All

adipose tissue was from a single donor. To maximize biological function and to avoid any unexpected ADSCs effects, the untreated fat specimens and supplemental SVF cells were mixed by pushing and pulling at least ten times between two connected 1-cm^3 syringes. This ensured adherence of the supplemented SVF cells to adipocytes or connective tissue.

Animal study

All animal experimental procedures were approved by the National Defense Medical College Animal Care and Use Committee in Taiwan. In this work, 7-week-old BALB/c male nude mice were used (BioLASCO, Yi-Lan, Taiwan). This athymic nude mouse model was selected due to its limited ability to reject xenografts. Transplanted fat tissues were injected into the subcutaneous tissue of the bilateral flank region of the nude mice using an 18-gauge cannula under proper anesthesia. After 30 days, the animals were sacrificed and the specimens were dissected for further study. Specimens were weighed on an electronic scale.[20]

Histological evaluation

The specimens were fixed with paraformaldehyde and dehydrated with alcohol, and the alcohol was replaced with xylene gradient. Paraffin sections were then stained with hematoxylin and eosin (H&E) stain (Sigma-Aldrich). The sections were mounted with a mounting medium and examined for the integrity of the fat cells, angiogenesis, fibrosis, cyst formation, and infiltration of inflammatory cells. Specimens were examined and photographed using an inverted microscope under 100×, 200×, and 400× magnifications. Six selected 200× photographs of specimens from each study group and the control group were analyzed for angiogenesis, integrity of fat cells, infiltration of inflammatory cells, cyst formation, and fibrosis of necrotic tissues using Image-Pro 6.0 software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Results are expressed as mean ± standard derivation. Unpaired t tests were performed to evaluate differences between groups, and P values < 0.05 were considered statistically significant.

Results

Effect of centrifugation time on cell extraction

Figure 2 shows the effects of centrifugation time on the extraction of SVF cells and ADSCs from adipose tissue. It can be seen from Figure 2 that for Donor 1 when the centrifugation time was increased from 1 minute to 3 minutes, there was an increase in the numbers of extracted SVF cells and ADSCs. However, when we further increase the centrifugation time from 3 minutes to 5 minutes, the numbers of SVF and ADSCs decreased. For Donor 2, the numbers of extracted SVF cells are statistically

significantly elevated with the increase of the centrifugation time.

Effect of centrifugation force on cell extraction

As shown in Figure 3, the effects of centrifugation force on the extraction of SVF cells and ADSCs from adipose tissue are evaluated. For Donor 1, the numbers of isolated SVF cells and ADSCs are maximum at a force of 1200g and these decreased as the force increased to 2800g. There is a statistically significant difference between the numbers of extracted SVF cells and ADSCs at forces of 1200g and 600g. For Donor 2, the numbers of extracted SVF cells and ADSCs increased with the increasing force. There is a statistically significant difference between the numbers of isolated SVF cells and ADSCs at forces of 1200g and 600g.

Effect of digestion time on cell extraction

Figure 4 shows the effects of digestion time on the extraction of SVF cells and ADSCs. For Donor 1, there is no statistically significant difference between all three digestion times, for both SVF cells and ADSCs. However, the results of Donor 2 show a statistically significant difference in the numbers of SVF cells and ADSCs between 60 minutes and 30 minutes of digestion process.

ADSCs surface marker expression

Figure 5 indicates that the cultures are positive for CD90 and CD105 and negative for CD45, CD146, and CD31 for all four generations, and these results are consistent with recent guidelines for ADSCs. The expression levels of CD34 decreased as the cells were grown in culture. A previous study has shown that the expression of CD34 decreases in *in vitro* cultures over time, which is consistent with the results of our study.[23]

Proliferation and multiplication assay

The results of the CFU assay showed that the ADSCs were capable of forming colonies of fibroblastic cells (CFU-F; Figure 6A). Adipogenic and osteogenic differentiation were achieved in the cultures established using the optimized isolation procedure. Cells cultured in the adipogenesis-inducing medium showed liquid droplet accumulation (Figure 6B). Cells cultured in the osteogenesis-inducing medium showed calcium deposition (Figure 6C). These observations demonstrate that the ADSCs isolated using the optimized cell isolation procedure are capable of both adipogenic and osteogenic differentiation potential.

Gross appearance of animal tests

Adipose tissue and SVF cells extracted by optimized accelerated procedure were applied to the nude mice animal model to evaluate soft-tissue augmentation. Photos of each step are shown in Figure 7. After 4 weeks of
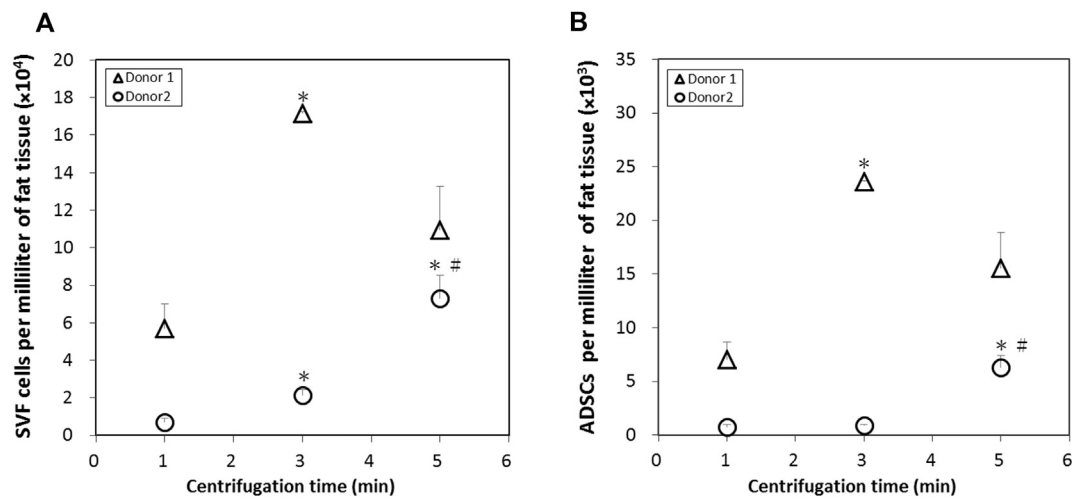
Figure 2 Numbers of stromal vascular fraction (SVF) cells and adipose-derived stem cells (ADSCs) obtained from two donor's lipoaspirate samples by centrifugation at 1200g for 1 minute, 3 minutes, and 5 minutes. (A) SVF cells yield; (B) ADSCs yield. Significant difference between the SVF cells or ADSCs numbers of centrifugation time of 1 and 3 or 5 minutes is indicated by an asterisk and between centrifugation time of 3 and 5 minutes is indicated by a hashtag (*$p < 0.05$, #$p < 0.05$). Data represent the mean $\pm$ standard deviation of three repeats.
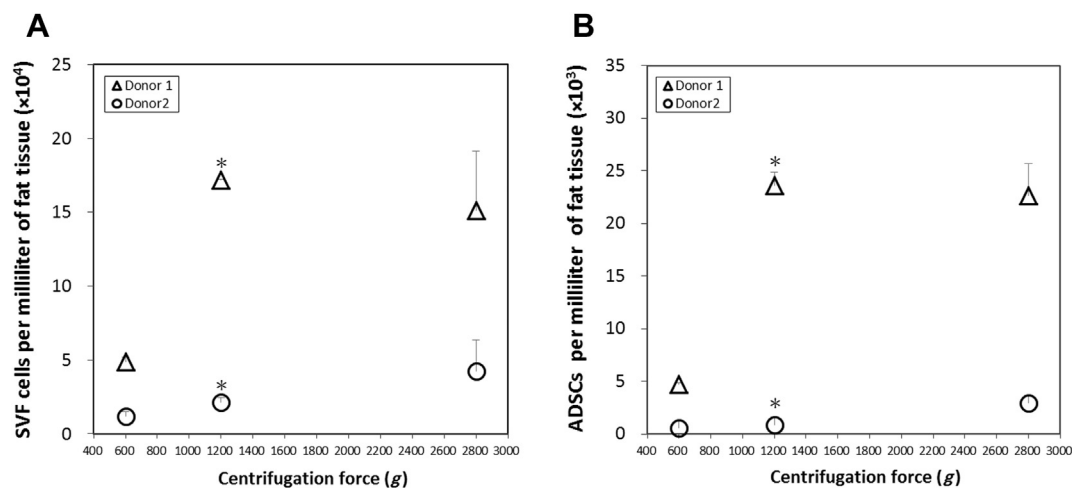

Figure 3 Numbers of stromal vascular fraction (SVF) cells and adipose-derived stem cells (ADSCs) obtained from two donor's lipoaspirate samples by centrifugation at 600g, 1200g, and 2800g for 3 minutes. (A) SVF cells yield; (B) ADSCs yield. Significant difference between the SVF cells or ADSCs numbers of centrifugation at 600 and 1200g is indicated by an asterisk (*$p < 0.05$). Data represent the mean $\pm$ standard deviation of three repeats.

implantation, the animals were sacrificed and the specimens were dissected as shown in Figure 8. The gross appearance of the specimens from SVF cells-enriched fat tissue (Figure 9B) was brighter and shinier compared with those from the control group (Figure 9A), which were duller.

Histological assay

The harvested specimens were H&E stained and then observed under a light microscope at 200× magnification. Figure 10 shows the observed images from three donors at four different locations; two of the locations were SVF cells supplemented (A and C), whereas the other two acted as

controls (B and D). Specimens and grafts from the control group exhibited more fibrosis and cyst formation. The SVF cells-supplemented specimens exhibited very little fibrosis and cyst formation, and demonstrated neovascularization.

Discussion

During the general procedure of ADSCs isolation, multiple parameters are available for the washing process such as the number of repetitions and the duration of each wash, which are time consuming. Therefore, the washing process must be optimized to create an accelerated procedure. The number of washing was reduced to one in our accelerated procedure. In addition, various centrifugation times (1
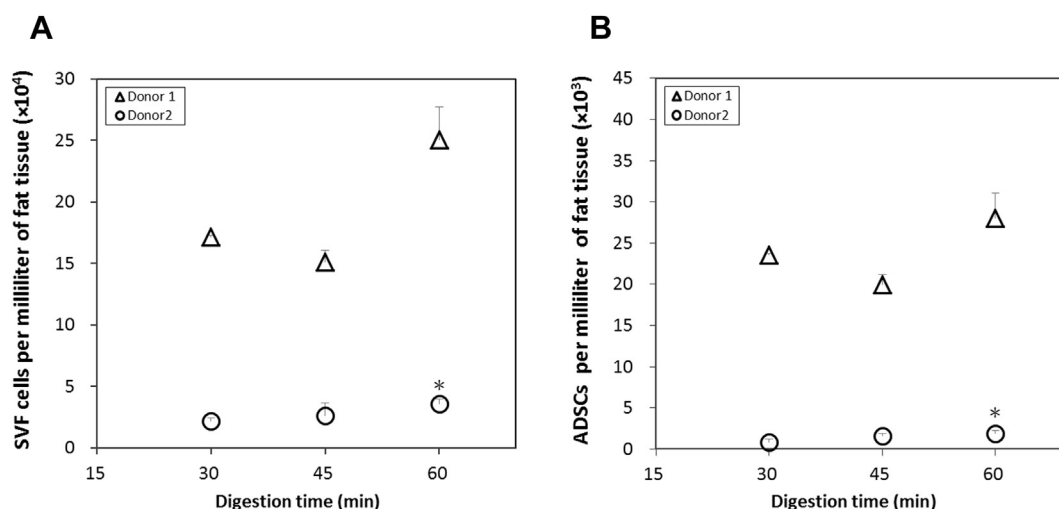
A

B

Figure 4 Numbers of stromal vascular fraction (SVF) cells and adipose-derived stem cells (ADSCs) obtained from two donor's lipoaspirate samples using 30-, 45-, and 60-minute digestion time with 0.2% collagenase concentration. (A) SVF cells yield; (B) ADSCs yield. Significant difference between the SVF cells or ADSCs numbers of digestion time of 30 and 60 minute is indicated by an asterisk (*$p < 0.05$). Data represent the mean ± standard deviation of three repeats.
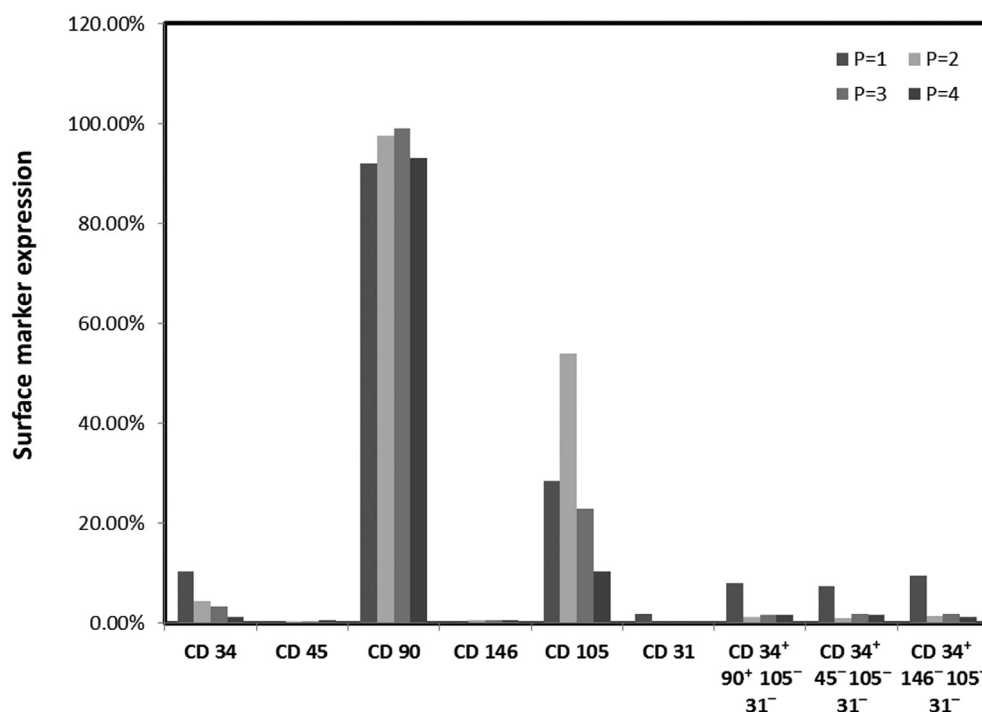

Figure 5 Immunophenotype characterization of adipose-derived stem cells isolated using the accelerated procedure.

minute, 3 minutes, and 5 minute) have been investigated and optimized.

A centrifugation time of 3 minutes was then chosen for the optimized procedure based on the experimental results. For Donor 1, the numbers of SVF cells and ADSCs reach a peak at 3 minutes and decrease when the centrifugation time was increased to 5 minutes. Previous studies have shown that when the centrifugation time increases, the centrifugation force remains the same, while extended duration may lead to more deaths of cells.[24,25]

The tissue was digested for the least tested amount of time, 30 minutes, and then various centrifugation forces were tested for 3 minutes, which was previously determined to be the optimized centrifugation time. The isolated SVF cells were then observed and counted. We further investigated the effects of centrifugation force on the cell viability. The forces of 600g, 1200g, and 2800g were tested for comparison in this study.

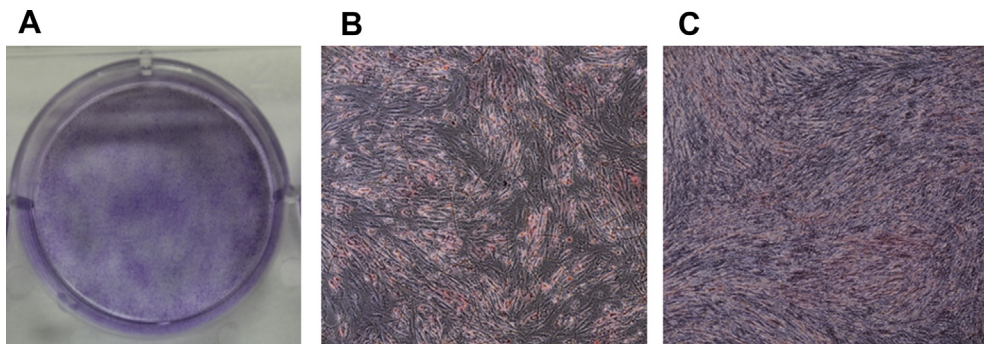Based on the aforementioned results, a centrifugation force of 1200g was then chosen for the optimized

Figure 6 Adipose-derived stem cells isolated using the accelerated procedure showing *in vitro* multipotentiality: (A) colony-forming units; (B) adipose tissue; and (C) bone.
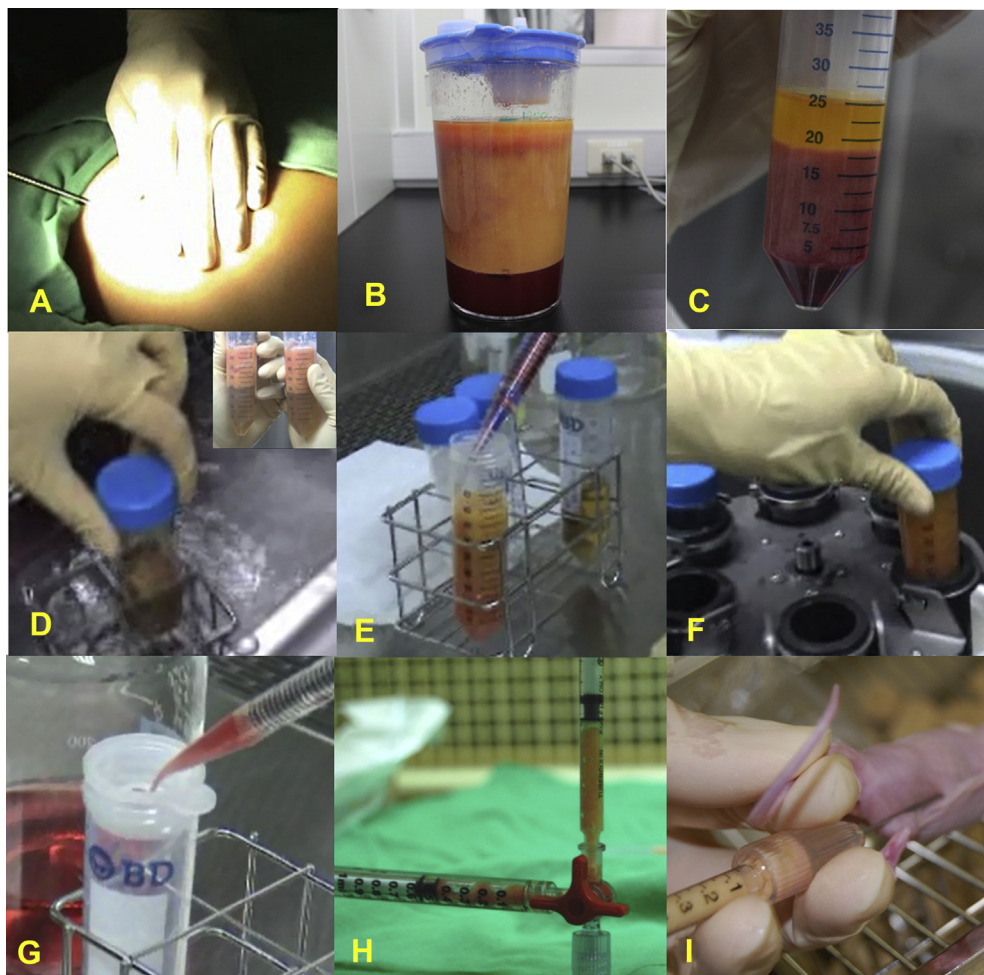

Figure 7 Flowchart for isolation of stromal vascular fraction (SVF) cells using the accelerated procedure to transplant SVF cells-enriched fat to nude mice: (A and B) Harvest lipoaspirate samples by liposuction surgery from humans; (C) washing lipoaspirate samples (one time); (D) adding collagenase and incubation in water bath for 30 minutes at 37°C; (E) adding medium to stop collagenase reaction; (F) centrifugation at 1200*g* for 3 minutes; (G) filtration; (H) mix fat with SVF cells; (I) transplant SVF cells-enriched fat to nude mice.

procedure. For Donor 1, the maximum number of extracted SVF cells and ADSCs can be seen in Figure 3 with a force of 1200*g*. Further increase of centrifugation force may result in the death of cells, leading to the reduction of viable cells. Although it shows an increase in the number of isolated cells for Donor 2, there was no statistically significant difference between the numbers of isolated cells.[26]

Figure 8 Schematic of collection of transplanted fat after 4 weeks of implantation. (A) anesthetized the nude mice; (B) sacrificed the nude mice; (C) collected the transplanted fat with SVFs; (D) collected the transplanted fat without SVFs.
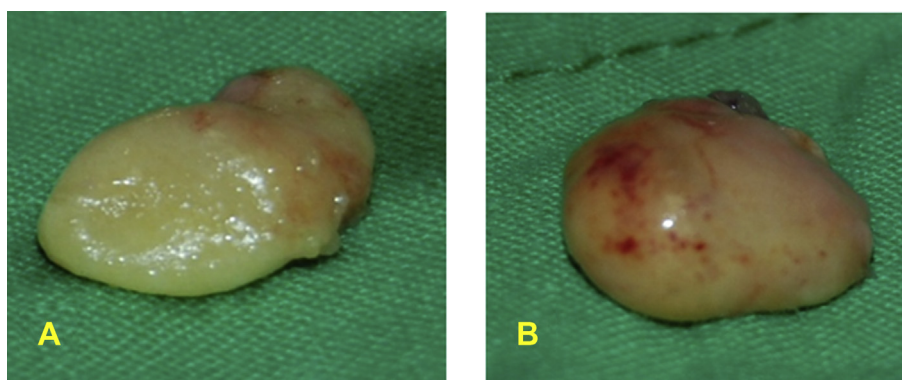

Figure 9 Gross appearance of transplanted fat with or without stromal vascular fraction (SVF) cells after 4 weeks of implantation: (A) transplanted fat without SVF cells; (B) transplanted fat with SVF cells.

Digestion time is a key parameter in the isolation process, as well as the critical step that can be accelerated. Various digestion times were tested in this study. Based on the optimized centrifugation time and force obtained earlier, the digested tissue was centrifuged at 1200g for 3 minutes and the isolated SVF cells were observed.

The data for Donor 1 did not show any statistically significant increase between the digestion time of 30 minutes and 60 minutes. Although the results for Donor 2 showed a statistically significant increase between 30 minutes and 60 minutes, only a slight increase of SVF cells and ADSCs was observed. To achieve the maximum generation rate of SVF cells, based on the experimental results, a 30-minute digestion time was chosen for the optimized procedure.

In addition, Bourin et al found that ADSCs were more than 90% viable if the expression of positive markers exceeded 80% and negative markers had less than 2% expression.[27] As shown in Figure 5, the expression of CD90 exceeded 80% and the expressions of CD45, CD146, and CD31 did not exceed 2%. Although the expression of CD105 did not reach 80%, previous research has shown that the expression of CD105 is often lower and less stable compared with the expression of other surface markers. The aforementioned results demonstrate that the cells obtained from the optimized isolation process are stem cells. Moreover, the CFU assay showed that the ADSCs are capable of forming colonies of fibroblastic cells (CFU-F; Figure 6A).

Histologically, vascular tissue is tubular and shows red blood cell expression. The fat graft supplemented with SVF cells displayed the red coloring associated with red blood cells. In addition, the SVF cells with transplanted fat was full and rounded, and exhibited neovascularization. The region without SVF cells with transplanted fat had less transplanted adipose tissues and angiogenesis, but was infiltrated by inflammatory cells.

The accelerated procedure developed herein confirmed that SVF cells contain ADSCs. Rehman et al reported ADSCs
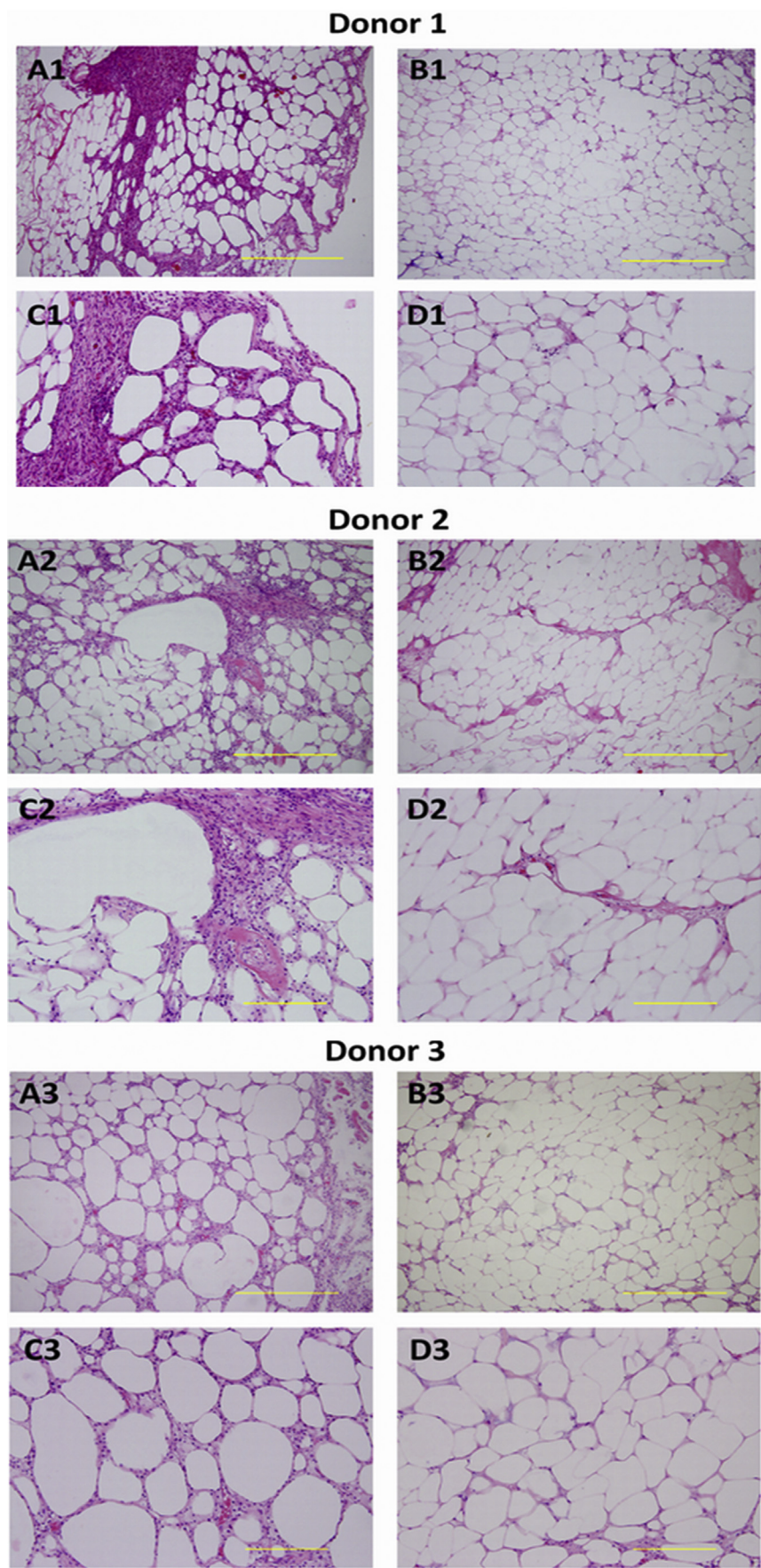
Figure 10 Hematoxylin and eosin staining results of transplanted fat with or without stromal vascular fraction cells after 4 weeks of implantation.

painted manufacture and release of vascular endothelial growth factor and other growth factors to promote angiogenesis in the hypoxic environment,[16] the organization of this study H&E staining of sections with rich support for the SVF cells can be transplanted fat graft area to promote angiogenesis.[28–30]

In conclusion, an optimized and accelerated procedure for isolation of adipose SVF cells has been developed. The yield of cell isolation and the results of animal tests have demonstrated the efficacy of this procedure for soft-tissue augmentation. Results of this pilot study suggest that the protocol may be further applied in dental applications, especially for augmentation of ridge defects healing.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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