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Adipose-derived Stromal Vascular Matrix (SVM): a new paradigm in regenerative medicine

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Abstract

Objective: **The conventional method of harvesting the Stromal Vascular Fraction (SVF) from adipose tissue is enzymatic digestion of extracellular matrix (ECM) from the lipoaspirate. This process necessarily affects the viability and potency of the cells, eliminates the majority of pericytes embedded in the fibrous matrix and structural/functional support of ECM.**

Patients and Methods: **Thirteen healthy subjects underwent abdominal liposuction and the aspirates were split into enzyme and mechanical digestion of stromal vascular fraction (E-SVF and M-SVF, respectively). The E-SVF and M-SVF were mixed with the ECM concentrate (buffy coat) to form the "Stromal Vascular Matrix" (SVM). The SVM was then compared to E-SVF and M-SVF.**

Results: **The preparation of SVM using the Lipocube™ mechanical digestion technology results in a source of autologous and minimally manipulated adipose-derived stem cells for use in cosmetic and regenerative medicine procedures. Cell counts, viability assessments, CD antigen expression and gene expression analysis showed that the SVM is an optimal product for therapy.**

Conclusions: **The SVM has a higher regenerative cell potency and provides a greater ECM support compared to common enzymatic digestion methods. In clinical applications, SVM seems to be suitable for suboptimal recipient conditions and skin regeneration purposes.**

Introduction

The use of stromal vascular fraction (SVF) cells in both basic research and clinical trial has significantly increased during the last decade due to a variety of newly suggested therapeutic indications, including treatment of facial aging, chronic wound healing, improvement of radiation damage and hypertrophic scars, breast augmentation/reconstruction, as well as treatment for inflammatory and degenerative orthopaedic conditions[1-6]. The SVF is the population of cells which results from the mechanical or enzymatic digestion of lipoaspirate without culture or expansion. The SVF is a heterogeneous mixture of vascular endothelial progenitors and adipose-derived stem cells, various blood cells, preadipocytes, fibroblasts, and smooth muscle cells[7-9]. The most common method for digestion of the extracellular matrix (ECM) for SVF isolation is by the use of tissue dissociation enzymes, particularly collagenase, to break down adipose tissue niches and thus to isolate regenerative cells. Recent mechanical digestion methods have been shown to give comparable cell counts and yields[10-12]. Mechanical processing technology has the advantage of being a closed, bedside, processing system re-

sulting in fewer regulatory implications. The overall cost of the procedure is therefore considerably less than enzymatic methods[13].

The ECM is a complex network which holds all cells, tissues and organs together[14,15]. The adipose tissue-derived ECM has been proposed to be a valuable starting point for future clinical applications[16]. The most abundant component of the ECM is collagen, which acts as the "backbone" of the extracellular matrix. Collagen and elastin are not only structural proteins with high tensile strength, but also have facilitative roles influencing cell adhesion, differentiation, migration, signaling and attachment[17,18]. Other proteins such as fibronectin or laminins function by forming bridges between the cell surface receptors such as integrins and other proteoglycans, and also mediate various functions such as adhesion[19], differentiation[20], migration[21] and survival[22].

Enzymatic digestion of adipose tissue effectively disrupts the functional ECM and the SVF, resulting in a heterogeneous mixture of "naked" adipose-derived stem cells, blood cells, pericytes, macrophages, fibroblasts, and vascular endothelial progenitors[23]. The literature also shows that the discarded ECM has a substantial amount of adipose-derived stromal cells as well as endothelial cells, which supports its use in regenerative medicine[24]. Moreover, enzymatic digestion of adipose tissue has been deemed by the FDA and other regulatory authorities as a more than minimal manipulation of tissue, which makes it clinically more complicated to use than the minimally manipulative mechanical methods of isolation[25].

In order to optimize SVF cell counts in the yield, viability and functional quality (and therefore clinical results), in the present study we re-mixed the mechanically isolated SVF with the ECM concentrate before use, making it into the "Stromal Vascular Matrix" (SVM), where the extracellular "backbone" is not totally discarded (Figure 1). Our study compares the cellular contents and properties of enzymatically and mechanically isolated SVF and SVM.

Patients and Methods

Adipose Tissue Processing

60 mL of lipoaspirate was harvested through abdominal liposuction from 13 healthy subjects and it was divided into three 20 mL aliquots and submitted to enzymatic or mechanical digestion for SVF isolation. In the enzymatic SVF group (E-SVF), lipoaspirate was enzymatically digested using GMP grade collagenase NB6 (Serva Electrophoresis, Heidelberg, Germany) at a concentration of 0.1 U/mL and a ratio of 1:1 (v/v), washed and centrifuged twice at 300 g for 5 minutes, and the pellet was re-suspended. The other two groups were submitted to mechanical SVF isolation using Lipocube™ SVF, where ordinary pistons of 20 mL Luer-lock syringes were replaced with disarmable pistons with concave, cell-adhesive gaskets from the kit. The lipoaspirate was transferred into syringes, connected to a closed unit, harnessing 3 different sets of blade grids on three Luer-lock ports on a rotating canal. The lipoaspirate was placed in the first port, passed back-and-forth 10 times through the first blade grid containing multiple 1200 μm-holes. The direction of the rotating canal was changed to the second port and the lipoaspirate was passed through the second blade grid containing 750 μm-holes and through the 500 μm-holes blade grid for full dissociation. Dulbecco's phosphate buffered saline with calcium and magnesium (Merck Life Science UK Limited, Dorset, England) was added to the lipoaspirate in the syringes at a ratio of 1:3, incubated and shaken for 10 minutes at room temperature to wash the erythrocytes and cell debris. When isolating SVF cells with tissue dissociating enzyme (TDE) mixtures, it is important to select a buffer which is both suitable for optimal enzymatic activity as well as preserving the viability of the cell population. Commonly used buffers include Lactate Ringer's (LR) solution, Phosphate buffered Saline (PBS) and balanced salt solutions such as Hanks' Balanced Salt Solution (HBSS) with calcium. The pistons of the syringes were detached and the detached syringes containing the dissociated lipoaspirate were centrifuged at continuous centrifuge speeds (2000 g for 1 minute, 800 g for 5 minutes, 1500 g for 2 minutes) with the Luer-lock tips directed inward so that the SVF could be collected in the concave gaskets. Finally, the pistons were reattached. In the mechanical SVF group (M-SVF) the whole supernatant was discarded, and the isolated pellet re-suspended in phosphate buffer solution. In order to optimize the SVM preparation, the M-SVF from mechanical digestion and adipose buffy coat with high ECM content - which is the lowest part of the adipose fraction after centrifugation - were sequentially resuspended and mixed together to form the SVM. This means that the extracellular "backbone" in SVM preparations was not totally discarded (Figure 1).

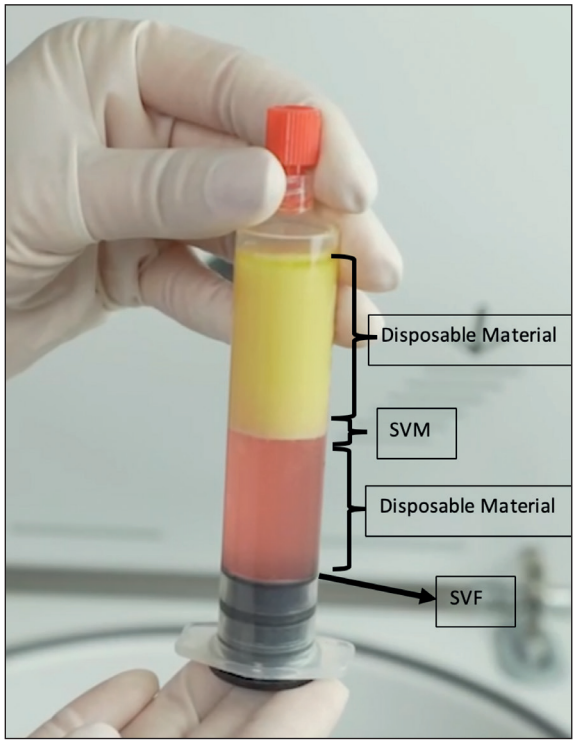


Figure 1. After centrifugation, the top oil and fat layers were disposed. The adipose buffy coat, the very bottom ECM-rich portion of the adipose column was harvested, then the serum/buffer solution was disposed of and the SVF over the top of the cell adhesive gasket was harvested and re-mixed with the ECM concentrate, making it into the "Stromal Vascular Matrix" (SVM). *Abbreviations:* ECM, Extracellular matrix; SVF, Stromal vascular fraction; SVM, Stromal vascular matrix.

Flow Cytometry

The total nucleated cell number and viability of cells in E-SVF, M-SVF and SVM were determined by flow cytometry (Guava Muse Cell™ Analyzer, Luminex, Chicago, IL; USA) using the Muse Count and Viability Kit (Luminex) following erythrocyte lysis. The characterization of adipose tissue-derived stem cells (ADSCs; CD45-/CD90+, CD73+/CD90+), endothelial cells (CD45+/CD31+), macrophages and monocytes (CD45+/CD14+) was performed. The regenerative cell population in three groups was stained with 5 µl of monoclonal antibodies (BD Biosciences, Le Pont de Claix, France). The binding efficiency of the surface antigens CD13, CD73, CD90, CD146 and CD34 was also assessed. Cells were then seeded in T-75 tissue culture bottles (Thermo-Fischer, Waltham, MA, USA) in proliferation medium (NutriStem® MSC XF Medium, serum free, Sartorius, Goettingen, Germany) at 37°C and in 5% carbon dioxide. After 7 days, cell morphology was observed under phase contrast microscopy.

ADSC Differentiation

Adipogenesis differentiation medium was prepared by following the manufacturer instructions. StemPro Adipogenesis Differentiation Supplement (Gibco A1006501, Rockville, MD, USA) was thawed at 37°C in a water bath. 100 mL of differentiation medium was prepared by mixing the solutions with 90 mL of StemPro Adipocyte Differentiation Basal Medium, 10 mL of StemPro Adipocyte Supplement and 10 mg/mL of gentamicin solution. To induce adipogenic differentiation, $1x10^4$ cells/cm^2 were seeded into 12 well plate. Adipogenesis differentiation was carried out using the StemPro Adipogenesis Differentiation Kit. Media were replaced every three days for three weeks according to the manufacturers' protocol, and differentiation was evaluated using oil red staining and phase-contrast microscopy.

Gene Expression

The gene expression profile was examined by adipocyte-specific adiponectin and Peroxisome proliferator-activated receptors (*PPAR*) genes. Primers were designed using Primer-BLAST software from the National Center for Biotechnology (Bethesda, MD, USA). Total RNA isolation from differentiated cells of the three groups was performed using the Total RNA Purification Plus Kit (Norgen, Canada). The E-SVF group was used as negative control.

Statistical Analysis

Student's *t*-test was performed to compare cell count and viability parameters with a 95% confidence interval. A p-value ≤ 0.05 was considered to be statistically significant.

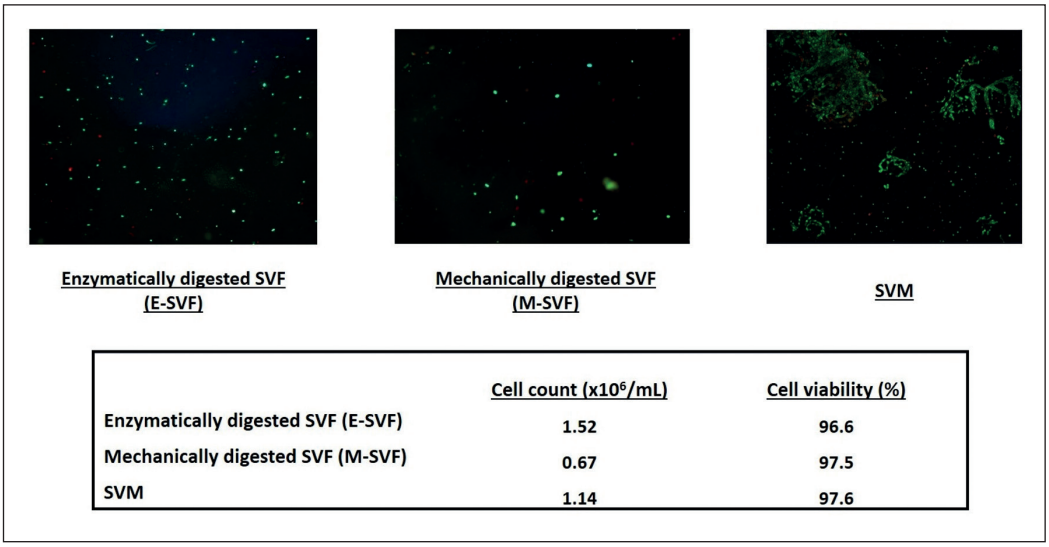
Ethics statement

All subjects provided written informed consent for inclusion before they participated in the study. The study was conducted in Turkey in accordance with the principles of the Declaration of Helsinki (1975). This study did not require IRB approval as fat-grafting is a long-established procedure and the microinjection device applied in this study received ISO 13485 certification and CE marking.

Results

Cell Count and Viability

The SVF cell yield obtained from the SVM was 25%, which is significantly lower ($1.14\pm1.33x10^6$/mL; n=13) than that obtained by E-SVF alone



	Cell count (x10⁶/mL)	Cell viability (%)
Enzymatically digested SVF (E-SVF)	1.52	96.6
Mechanically digested SVF (M-SVF)	0.67	97.5
SVM	1.14	97.6

Figure 2. Adipose tissue cell count and viability. The figure shows fluorescence microscopy images after SVF isolation. The SVF cell yield obtained from the SVM was 25% lower (1.14±1.33x10^6/mL; n=13) than that obtained by E-SVF alone (1.52±3.63x10^6/mL; n=13) and M-SVF alone (0.67 ±1.69 x 10^6/mL; n=13) [p-value=0.015; this p-value refers to the comparison between both groups]. The average cell viability was 97.6±4.58% for SVM, 96.6±10.68% for E-SVF and 97.5±5.74% for M-SVF. *Abbreviations:* E-SVF, Enzymatically Digested SVF; M-SVF, Mechanically Digested SVF; SVF, Stromal vascular fraction; SVM, Stromal vascular matrix.

(1.52±3.63x10^6/mL; n=13) and M-SVF alone (0.67 ±1.69 x 106/mL; n=13) [*p*-value=0.015; this *p*-value refers to the comparison between both groups]. The average cell viability was 97.6±4.58% for SVM, 96.6±10.68% for E-SVF and 97.5±5.74% for M-SVF (Figure 2).

CD Antigen Analysis by Flow Cytometry

The cluster of differentiation (CD) surface antigen expression of fresh ADSCs (CD73+/CD90+, CD45/CD90+) in SVM showed a 1.68 to 4.47-fold increase compared to M-SVF and a 4.1 to 7.5-fold increase in E-SVF (30%, 3.97%, 6.7%; 14.55%, 8.63%, 3.51%). The endothelial progenitor cell (EPC) content of SVM was 6.72%. The EPC content of M-SVF was 3.98%, whereas the EPC content of E-SVF was 1.47%. These results confirm that the SVM has a higher EPC content than either M-SVF or E-SVF. The macrophage and monocyte cell contents were approximately the same in the three groups (SVM: 3.34%; M-SVF: 3.41%; E-SVF: 4.11%) (Figure 3). SVM was shown to have a significantly higher expression of specific phenotypic markers. It was observed that all relevant CD markers, including CD13, CD73, CD90, CD146, CD34 and CD45 increased 1.2-fold (22.27%; 18.74%), 3.4-fold (46.19%; 13.65%), 1.3-fold (35.01%; 27.9%), 1.4-fold (20.6%; 14.4%), 4.3-fold (18.62%; 4.29%) and 1.2-fold (18.38%; 15.15%), respectively, when SVM was compared to M-SVF, and 2.1-fold (22.27%; 10.81%), 7.5-fold (46.19%; 6.5%), 2.5-fold (35.01%; 13.89%), 2.3-fold (20.6%; 14.4%), 2.8-fold (18.62%; 4.29%) and 1.7-fold (18.38%; 10.69%), respectively, when SVM was compared to E-SVF (*p*-value=0.05) (Figure 3).

Gene Expression Analysis

The mRNA expression levels of *PPAR2* and adiponectin genes were examined in ADSCs after the differentiation protocol and were 1.4- and 1.3-fold higher than the SVM group compared with E-SVF which were 2- and 3.6-fold higher than the SVM group compared with E-SVF (*p*-value=0.04). These findings strongly substantiated that mechanical SVF digestion increased the mRNA level of adipocyte complement-related protein (also known as adiponectin), which results in lipid droplet formation and increased adipogenic differentiation (Figure 4).

Discussion

Autologous fat transplantation is an ideal treatment for facial rejuvenation and soft tissue augmentations providing ‘‘like for like’’ tissue material. However, the success of traditional fat grafting has been unpredictable and often unsatisfactory, particularly in

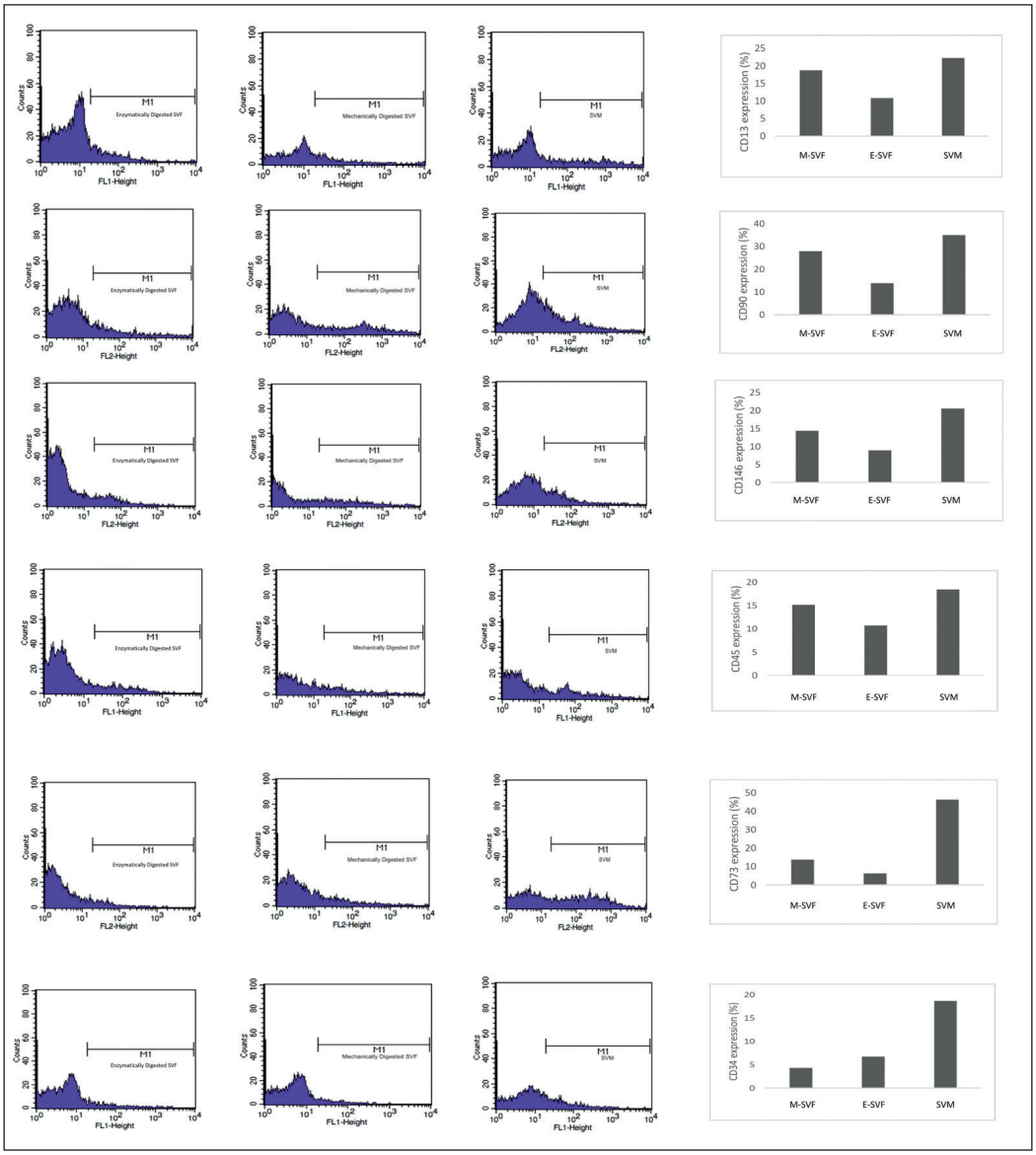


Figure 3. CD antigen analysis using flow cytometry. SVM demonstrated significantly higher expression of specific phenotypic markers. Considering the ADSC markers of cell activity, it is observed that all CD markers included in this part such as CD13, CD73, CD90, CD146, CD34 and CD45 increased 1.2-fold (22.27%; 18.74%), 3.4-fold (46.19%; 13.65%), 1.3-fold (35.01%; 27.9%), 1.4-fold (20.6%; 14.4%), 4.3-fold (18.62%; 4.29%) and 1.2-fold (18.38%; 15.15%), respectively, when SVM was compared to M-SVF, and 2.1-fold (22.27%; 10.81%), 7.5-fold (46.19%; 6.5%), 2.5-fold (35.01%; 13.89%), 2.3-fold (20.6%; 14.4%), 2.8-fold (18.62%; 4.29%) and 1.7-fold (18.38%; 10.69%), respectively, when SVM was compared to E-SVF (p-value=0.05). *Abbreviations:* ADSC, adipose tissue-derived stem cell; E-SVF, Enzymatically Digested SVF; M-SVF, Mechanically Digested SVF; SVF, Stromal vascular fraction; SVM, Stromal vascular matrix.

a hostile recipient bed. Many papers have been published, showing the added value of autologous SVF enrichment in terms of improved graft uptake as well as recipient tissue bed quality improvement[26,27].

The conventional method of processing SVF is enzymatic digestion of ECM from the lipoaspirate. However, enzyme-based SVF isolation techniques have many drawbacks. These techniques are expensive, need specialized personnel/environment and, last but not least, there are major regulatory obstacles related to the use of enzymes in the processing. In the United States, the main regulatory issue associated with the isolation of SVF cells from adipose tissue is minimal manipulation. In December 2014, the FDA released a series of draft guidance for the industry dealing with the minimal manipulation and homologous use of HCT/Ps (Human Cells, Tissues, and Cellular and

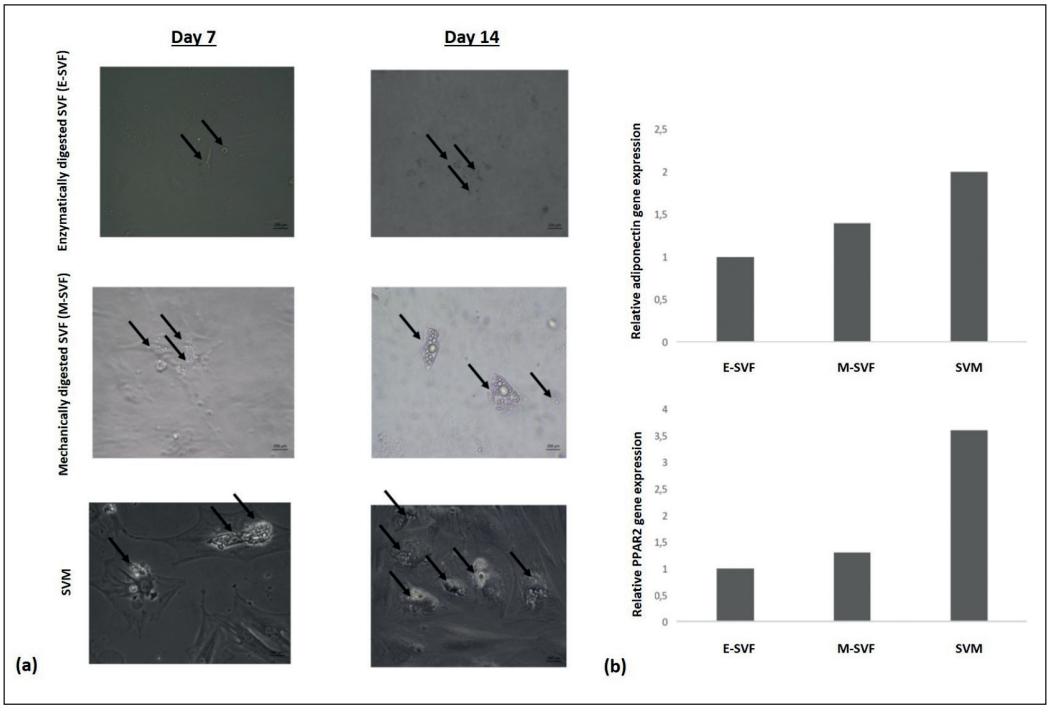


Figure 4. Phase-contrast microscopy images *(a)*; mRNA expression levels of *PPAR2* and adiponectin genes *(b).* mRNA expression levels of PPAR2 and adiponectin genes were examined in adipose tissue-derived stem cells after the differentiation protocol. *Abbreviations:* E-SVF, enzymatically digested SVF; M-SVF, mechanically digested SVF; PPAR2, Peroxisome proliferator-activated receptor 2; SVF, Stromal vascular fraction; SVM, Stromal vascular matrix.

Tissue-Based Products) from adipose tissue. In this draft guidance, the FDA very clearly states that usage of proteolytic enzymes is considered more than minimal manipulation since they are not water, crystalloids, or a sterilizing, preserving or storage agent. This has very serious clinical implications and restrictions for enzyme-based SVF therapies. Furthermore, the process of enzymatic digestion necessarily affects the viability and potency of the cells, hugely decreases the number of pericytes embedded in the fibrous matrix and eliminates structural and functional support of ECM.

In order to overcome these drawbacks of enzymatic SVF isolation, many methods of mechanical SVF isolation have been assessed from shaking, vibration, centrifugation and washing of the lipoaspirate manually and in automated closed devices[28]. The drawbacks of mechanical SVF isolation methods were the low cell yield (as they consist mostly in centrifuging or vortexing the lipoaspirate, without emulsification or digestion), the high number of peripheral blood cells and the low number of progenitor cells[29]. However, there are growing data showing higher cell yields from mechanical digestion compared to enzyme-based digestion, from new combined mechanical SVF isolation approaches. This increase in the cell count of the SVF does not eliminate all the negative effects of this process. Stripping the whole ECM from the SVF has been shown to have many disadvantages. The literature regarding the location of pericytes - the main "workhorse" of the SVF - suggests that preservation of the fibrotic ECM shall increase the number of regenerative mononuclear cells in the final product regardless of the chosen technique. Our results support the presumption that a less vigorous filtering of the fibrotic tissue generates higher cell numbers of regenerative potential in the cell yield.

To optimize SVF cell yields (viability and functional quality), the mechanically isolated SVF was mixed with the ECM concentrate, or adipose buffy coat (the very bottom of the adipose column after centrifugation), making it into the so-called "Stromal Vascular Matrix" (SVM), where the extracellular "backbone" is not totally discarded. We hypothesized that we would increase the regenerative cell count in the SVF through adding the pericytes

attached to the ECM, as well as functional and mechanical durability to routine SVF cell yield.

In clinical applications, SVM seems to be suitable for suboptimal recipient conditions and skin regeneration purposes (Tiryaki, data unpublished). No infections, fat cysts, granulomas, or other unwanted side effects were observed. Needless to say, future studies should be performed, which include the use of a control group and a more accurate documentation of the volume of fat that is retained. To our knowledge, this is the first publication analyzing and describing a new concept of ECM-supported SVF usage.

Conclusions

Obtaining and resuspending the mechanically obtained SVF and the matrix rich lipoaspirate buffy coat together as SVM, delivers comparable cell yields to enzymatic digestion and has a higher regenerative cell potential. In clinical applications, SVM seems to be suitable for suboptimal recipient conditions and skin regeneration purposes. The SVM has a higher regenerative cell potency and a greater ECM support compared to those obtained with common enzymatic digestion methods. Further studies are being carried out to optimize the cell counts and to determine long term clinical outcomes.

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Author Contributions:
T. Tiryaki: conception and design of the study, analysis and interpretation of data, final approval of the version of the article to be published. S. Canikyan, P. Koçak, S. Cohen, A. Sterodimas, K. U. Schlaudraff, M. Scheflan: acquisition of data, analysis and interpretation of data. P. Hollands: drafting the article or making critical revisions related to relevant intellectual content of the manuscript; final approval of the version of the article to be published.

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Conflict of Interest:
The authors of this paper are all directly, or indirectly, associated with Lipocube Ltd.

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