**TITLE:**

Mechanical Micronization of Lipoaspirates for Regenerative Therapy

**AUTHORS AND AFFILIATIONS:**

Huidong Zhu1, Jinbo Ge1, Junrong Cai1 \*, Feng Lu1 \*

1Department of Plastic and Cosmetic Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, P. R. China

\*These authors contributed equally to this manuscript.

**Corresponding Authors:**

Junrong Cai (Drjunrongcai@outlook.com)

Feng Lu (doctorlufeng@hotmail.com)

**E-mail Addresses of the Co-authors:**

Huidong Zhu (824970453@qq.com)

Jinbo Ge (37768973@qq.com)

**KEYWORDS:**

Adipose-derived stem cell, mechanical process, lipoaspirates, fat grafting, stem cell therapy, stromal vascular fraction gel

**SUMMARY:**

Here, we present a protocol to obtain stromal vascular fraction from adipose tissue through a series of mechanical processes, which include emulsification and multiple centrifugations.

**ABSTRACT:**

Stromal vascular fraction (SVF) has become a regenerative tool for various diseases; however, legislation strictly regulates the clinical application of cell products using collagenase. Here, we present a protocol to generate an injectable mixture of SVF cells and native extracellular matrix from adipose tissue by a purely mechanical process. Lipoaspirates are put into a centrifuge and spun at 1200 x *g* for 3 min. The middle layer is collected and separated into two layers (high-density fat at the bottom and low-density fat on the top). The upper layer is directly emulsified by intersyringe shifting, at a rate of 20 mL/s for 6x to 8x. The emulsified fat is centrifuged at 2000 x *g* for 3 min, and the sticky substance under the oil layer is collected and defined as the extracellular matrix (ECM)/SVF-gel. The oil on the top layer is collected. Approximately 5 mL of oil is added to 15 mL of high-density fat and emulsified by intersyringe shifting, at a rate of 20 mL/s for 6x to 8x. The emulsified fat is centrifuged at 2,000 x *g* for 3 min, and the sticky substance is also ECM/SVF-gel. After the transplantation of the ECM/SVF-gel into nude mice, the graft is harvested and assessed by histologic examination. The result shows that this product has the potential to regenerate into normal adipose tissue. This procedure is a simple, effective mechanical dissociation procedure to condense the SVF cells embedded in their natural supportive ECM for regenerative purposes.

**INTRODUCTION:**

Stem cell therapies provide a paradigm shift for tissue repair and regeneration so that they may offer an alternative therapeutic regimen for various diseases1. Stem cells (*e.g.*,induced pluripotent stem cells and embryonic stem cells) have great therapeutic potential but are limited due to cell regulation and ethical considerations. Adipose-derived mesenchymal stromal/stem cells (ASCs) are easy to obtain from lipoaspirates and not subject to the same restrictions; thus, it has become an ideal cell type for practical regenerative medicine2. In addition, they are nonimmunogenic and have abundant resources from autologous fat3.

Currently, ASCs are obtained mainly by collagenase-mediated digestion of the adipose tissue. The stromal vascular fraction (SVF) of adipose tissue contains ASCs, endothelial progenitor cell, pericytes, and immune cells. Although obtaining a high density of SVF/ASCs enzymatically was shown to have beneficial effects, the legislation in several countries strictly regulates the clinical application of cell-based products using collagenase4. Digesting the adipose tissue with collagenase for 30 min to 1 h to obtain SVF cells increases the risk of both exogenous material in the preparation and biological contamination. The adherent culture and the purification of ASCs, which takes days to weeks, require specific laboratory equipment. Moreover, in most studies, SVF cells and ASCs are used in suspension. Without the protection of extracellular matrix (ECM) or another carrier, free cells are vulnerable, cause a poor cell retention after injection, and compromise the therapeutic result5. All of these reasons limit the further application of stem cell therapy.

To obtain ASCs from adipose tissue without collagenase-mediated digestion, several mechanical processing procedures, including centrifugation, mechanical chopping, shredding, pureeing, and mincing, have been developed6-9. These methods are thought to condense tissue and ASCs by mechanically disrupting mature adipocytes and their oil-containing vesicles. Moreover, these preparations, containing high concentrations of ASCs, showed considerable therapeutic potential as regenerative medicine in animal models8-10.

In 2013, Tonnard *et al.* introduced the nanofat grafting technique, which involves producing emulsified lipoaspirates by intersyringe processing11. The shearing force created by intersyringe shifting can selectively break mature adipocytes. Based on their findings, we developed a purely mechanical processing method that removes most of the lipid and fluid in the lipoaspirates, leaving only SVF cells and fractionated ECM, which is ECM/SVF-gel12. Herein, we describe the details of the mechanical process of human-derived adipose tissue to produce the ECM/SVF-gel.

**PROTOCOL:**

This research was approved by the Ethical Review Board in Nanfang Hospital, Guangzhou, China. Adipose tissue was collected from healthy donors who gave written informed consent to take part in the study. All animal experiments were approved by the Nanfang Hospital Institutional Animal Care and Use Committee and performed according to the guidelines of the National Health and Medical Research Council (China).

1. **ECM/SVF-gel Preparation**

1.1 Harvest fat.

1.1.1 Perform liposuction on a human with a 3-mm multiport cannula, which contains several sharp side holes of 1 mm in diameter, at -0.75 atm of suction pressure.

1.1.2 Collect 200 mL of lipoaspirates in a sterile bag.

1.2 Prepare Coleman fat.

1.2.1 Transfer the lipoaspirates into four 50 mL tubes and allow the harvested fat to stand still for 10 min.

1.2.2 Collect the fat on the top layer into two 50 mL tubes by using a wide-tip pipette to transfer, and discard the liquid portion at the bottom layer.

1.2.3 Using 50 mL tubes, centrifugate the fat layer at 1,200 x *g* at room temperature (RT) for 3 min.

1.2.4 Define the upper layer (approximately 80 mL) as Coleman fat.

1.2.5 Transfer the upper 2/3 of the Coleman fat to a 20 mL tube by using a wide-tip pipette, and define this portion as low-density fat.

1.2.6 Transfer the lower 1/3 of the Coleman fat to another 20 mL tube by using a wide-tip pipette, and define this portion as high-density fat.

1.3 Produce ECM/SVF-gel from low-density fat.

1.3.1 Using two 20 mL syringes connected by a female-to-female Luer-Lock connector (with an internal diameter of 2.4 mm) to intershift 20 mL of low-density fat.

1.3.2 Keep the shifting speed stable (at 20 mL/s) and repeat for 6x to 8x.

1.3.3 Centrifugate the mixture at 2,000 x *g* at RT for 3 min.

1.3.4 Collect the oil portion on the top in a 10 mL tube by using a wide-tip pipette at RT for further use.

1.3.5 Collect the sticky substance in the middle layer, which is ECM/SVF-gel (**Figure 1A**), by using a wide-tip pipette, and discard the fluid at the bottom layer.

1.4 Produce ECM/SVF-gel from high-density fat.

1.4.1 Add 5 mL of oil (collected from step 1.3.4) to 15 mL of high-density fat.

1.4.2 Intershift the mixed fat between syringes 6x to 8x until a flocculate is observed within the emulsion.

1.4.3 Centrifugate the mixture at 2,000 x *g* at RT for 3 min.

1.4.4 Discard the oil portion on the top.

1.4.5 Collect the sticky substance in the middle layer (ECM/SVF-gel) by using a wide-tip pipette and discard the fluid at the bottom layer.

1.4.6 Mix the ECM/SVF-gel from steps 1.3.5 and 1.4.5.

1. **Nude Mouse ECM/SVF-gel Graft Model**

2.1 Anesthetize the nude mice (8 weeks old, female) with isoflurane (1% - 3%) inhalation anesthesia in an animal operation room.

2.2 Transfer the ECM/SVF-gel to a 1 mL syringe.

2.3 Connect the 1 mL syringe with a blunt infiltration cannula.

2.4 Insert the cannula subcutaneously into each flank of the mouse.

2.5 Inject 0.3 mL of the ECM/SVF-gel.

1. **Tissue Harvesting on 3, 15, and 90 Days after ECM/SVF-gel Injection**

3.1 Anesthetize the mice with isoflurane (1% - 3%) inhalation anesthesia.

3.2 Sacrifice the mice by the cervical dislocation method.

3.3 Make an incision at the midline of the mouse’s dorsal skin with surgical scissors.

3.4 Dissect and harvest the fat grafts on both sides of the mouse. Embed the fat grafts in the 4% paraformaldehyde at RT overnight.

3.5 Dehydrate the tissue in increasing concentrations of ethanol: 70% ethanol, two changes, 1 h each; 80% ethanol, one change, 1 h; 95% ethanol, one change, 1 h; 100% ethanol, three changes, 1.5 h each; xylene, three changes, 1.5 h each.

3.6 Infiltrate the tissue with paraffin wax (58 ‐ 60 °C), two changes, 2 h each.

3.7 Embed the tissue into paraffin blocks. Cut sections at a thickness of about 4 µm and put them on slides.

1. **Hematoxylin and Eosin Staining**

4.1 Deparaffinize the paraffin block slides by soaking them in xylene I, II, and III (10 min each).

4.2 Rehydrate the tissue sections by passing them through decreasing concentrations (100%, 100%, 95%, 80%, 70%) of ethanol baths for 3 min each.

4.3 Rinse the tissue sections in distilled water (5 min).

4.4 Stain the tissue sections in hematoxylin for 5 min.

4.5 Rinse the tissue sections in running tap water for 20 min. Decolorize in 1% acid alcohol (1% HCl in 70% alcohol) for 5 s. Rinse in running tap water until the sections are blue again.

4.6 Add two to three drops of Eosin Y dye directly onto the slides by pipette, and let the dye set for 10 min.

4.7 Wash the slides in tap water for 1 - 5 min.

4.8 Dehydrate the slides in increasing concentrations (70%, 80%, 95%, 100%, 100%) of ethanol for 3 min each.

4.9 Clear the slides in xylene I and II for 5 min each.

4.10 Mount the slides in mounting media.

1. **Immunofluorescent Staining**

5.1 Deparaffinize the tissue sections in xylene I, II, and III (5 min each).

5.2 Rehydrate the tissue sections by passing them through different concentrations (100%, 100%, 95%, 95%, 70%) of alcohol baths for 3 min each.

5.3 Incubate the sections in a 3% H2O2 solution in methanol at RT for 10 min.

5.4 Rinse the slides 2x with distilled water, 5 min each.

5.5 Drop the slides in a slide basket. Add 300 mL of 10 mM citrate buffer (pH 6.0) and incubate the slides at 95 - 100 °C for 10 min.

5.6 Cool the slides in RT for 20 min.

5.7 Rinse the slides 2x with phosphate-buffered saline (PBS), 5 min each.

5.8 Add 100 µL of 10% fetal bovine serum onto the slides and incubate in a humidified chamber at RT for 1 h.

5.9 Incubate the sections with primary antibody solution (guinea pig anti-mouse Perilipin, 1:400) at 4 °C overnight.

5.10 Rinse the slides with PBS 3x, 5 min each.

5.11 Incubate the sections with secondary antibody solution (goat anti-guinea pig-488 IgG) for 2 h at RT.

5.12 Rinse the slides with PBS 3x, 5 min each.

5.13 Wipe off the water around the section with clean tissue paper.

5.14 Drop 4′,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488-conjugated isolectin into the circle to cover the section on the slide.

5.15 Mount the coverslips and let them dry in the dark.

5.16 Observe the slides with a fluorescent microscope.

**REPRESENTATIVE RESULTS:**

After processing the Coleman fat to ECM/SVF-gel, the volume of discarded oil takes up 80% of the final volume, and only 20% of adipose tissue preserved under the oil layer is regarded as ECM/SVF-gel (**Figure 1A**). ECM/SVF-gel has a smooth liquid-like texture that enables it to go through a 27 G fine needle; however, Coleman fat is comprised of an integral adipose structure with large fibers and can only go through an 18 G cannula (**Figure 1B**).

On day 3 after the transplantation, large numbers of small-sized preadipocytes with multiple intracellular lipid droplets, extensive well-vascularized connective tissue, and infiltrated inflammatory cells appeared. Beginning on day 15, the number of inflammatory cells started to get reduced gradually, and adipocytes began to mature. By day 90, most of the vascularized connective tissue in the grafts had been replaced by mature adipocytes (**Figure 2**, upper panel).

ECM/SVF-gel grafts contained a few perilipin-positive adipocytes, 3 days after the transplantation. Small-sized preadipocytes with multiple intracellular lipid droplets began to appear on day 15. Each field of the ECM/SVF-gel graft sections on day 90 showed numerous perilipin-positive adipocytes and newly formed blood vessels (**Figure 2**, lower panel).

**FIGURE LEGENDS:**

**Figure 1: Images of the ECM/SVF-gel.** Centrifuged Coleman fat and ECM/SVF-gel after processing (**A**). ECM/SVF-gel can be easily injected through a 27 G needle; however, Coleman fat can only pass through an 18 G cannula (**B**).

**Figure 2: Histological change in the ECM/SVF-gel after transplantation.** ECM/SVF-gel showed extensive well-vascularized connective tissue and infiltrating inflammatory cells on day 3. Subsequently, most of the vascularized connective tissue was replaced by a structure containing mature adipocytes (upper panel). Immunofluorescent staining shows that most of the tissue is comprised of the perilipin-negative area on day 3. By day 15, a large portion of perilipin + adipocytes appeared. Numerous perilipin-positive adipocytes were found after 90 days (lower panel). The scale bars = 100 μm.

**DISCUSSION:**

Stem cell-based regenerative therapy has shown a great potential benefit in different diseases. ASCs are outstanding therapeutic candidates because they are easy to obtain and have the capacity for tissue repair and the regeneration of novel tissues15. However, there are limitations to expanding its clinical application, since it requires complicated procedures to isolate cells and collagenase for processing6. Thus, it is essential to develop a simple technique to obtain stem cells without the use of collagenase.

In this study, we presented a purely mechanical process of adipose tissue to obtain SVF cells, which are protected by the native adipose ECM. Furthermore, this process is collagenase-free. A previous study compared different intersyringe processing times and showed that the intersyringe processing of standard Coleman fat for 1 min at a flow rate of 10 mL/s is the optimal protocol for producing ECM/SVF-gel12. By using intersyringe shifting, most adipocytes in the lipoaspirates are destroyed, with most of the SVF cells and ECM preserved. Thus, intersyringe shifting is the key step of the whole process. The intersyringe shifting speed and the time spent on conducting the shifting determine the destruction level of the adipose tissue because the sheering force created by the intersyringe process is associated with the shifting speed. We suggest that the shifting speed should be stable at 20 mL/s. Insufficient destruction leads to remaining unwanted adipocytes, while overdestruction results in damaging the SVF cells. The product of this protocol can be defined as ECM/SVF-gel only if it reached the following criteria: 1) its final volume is ~20% of the initial volume; 2) it is easily injected through a 27 G needle. A previous study demonstrated that the ECM/SVF-gel contains high densities of both CD45-/CD31-/CD34+ ASCs and the SVF cell density is >4.0 x 105 cells/mL12.

During the process of creating ECM/SVF-gel, centrifugation was conducted 2x, before and after intersyringe shifting. Before the intersyringe shifting, we use centrifugation to create “graded densities” of fat. This is another key step. It has been proven that the high-density fat layer at the bottom, after centrifugation, is rich in condensed ECM but has less oil, while the low-density fat layer on the top has more oil but less ECM fiber16,17. Thus, these two layers should be processed in two different ways. Low-density fat can directly undergo the intersyringe processing to destroy the adipocytes. However, the high-density fat at the bottom layer requires additional oil to facilitate the destruction of adipocytes. The added oil can decrease the density of high-density fat, making the shifting much easier. Moreover, the oil can help extract more oil from the broken adipocytes; however, aqueous liquid cannot. Thus, we added extra oil to the high-density fat. The centrifugation after the intersyringe shifting is to separate the oil portion from the SVF cells and ECM. Oil should be avoided in the final product.

The transplantation of ECM/SVF-gel resulted in a great retention rate. As we mentioned above, the key step of the processing of ECM/SVF-gel is intersyringe shifting, which destroys most of the adipocytes. Thus, little adipocytes remained in the ECM/SVF-gel. As shown in **Figure 2**, a small perilipin-positive area appeared in the transplanted ECM/SVF-gel on day 3. However, after 15 days, lots of perilipin-positive adipocytes appeared and became mature after 90 days. A previous study has shown that the transplantation of ECM/SVF-gel induced host cell-mediated adipose tissue regeneration14. Using anti-human leukocyte antigen to identify the origin of the newly formed adipocytes, we discovered that, although most of the cells in the SVF were graft-derived, most of the newly formed adipose tissue was host-derived. ECM/SVF-gel has a great regenerative function, as we previously reported. This product had great therapeutic effects in wound healing12. Moreover, it helped to improve the survival rate of the free flap in a mouse model by accelerating angiogenesis10.

SVF-gel is an autologous injectable containing native ECM and functional cellular components. The product is generated from lipoaspirate by a simple mechanical process, which can be performed easily without any concerns regarding regulatory issues. However, the regenerative effect of ECM/SVF-gel remains unclear. In order to better characterize the beneficial effects of ECM/SVF-gel, further investigations to characterize the underlying molecular mechanisms of the regenerative effect of ECM/SVF-gel are on the way.

**ACKNOWLEDGMENTS:**

This work was supported by the National Nature Science Foundation of China (81471881, 81601702, 81671931), the Natural Science Foundation of the Guangdong Province of China (2014A030310155), and the Administrator Foundation of Nanfang Hospital (2014B009, 2015Z002, 2016Z010, 2016B001).

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1. Bateman, M. E. *et al.* Using Fat to Fight Disease: A Systematic Review of Non-Homologous Adipose-Derived Stromal/Stem Cell Therapies. *Stem Cells*. **36** (9), 1311-1328 (2018).

2. Baer, P. C., Geiger, H. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells International*. 812693 (2012).

3. Gimble, J. M., Katz, A. J., Bunnell, B. A. Adipose-derived stem cells for regenerative medicine. *Circulation Research*. **100** (9), 1249-1260 (2017).

4. Halme, D. G., Kessler, D. A. FDA regulation of stem-cell-based therapies. *New England Journal of Medicine*. **355** (16), 1730-1735 (2006)

5. Cheng, N. C., Wang, S., Young, T. H. The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. *Biomaterials*. **33** (6), 1748-1758 (2012).

6. van Dongen, J. A. *et al.* Comparison of intraoperative procedures for isolation of clinical grade stromal vascular fraction for regenerative purposes: a systematic review. *Journal of Tissue Engineering and Regenerative Medicine*. **12** (1), e261-e274 (2018).

7. van Dongen, J. A. *et al.* The fractionation of adipose tissue procedure to obtain stromal vascular fractions for regenerative purposes. *Wound Repair and Regeneration*. **24** (6), 994-1003 (2016).

8. Mashiko, T. *et al.* Mechanical Micronization of Lipoaspirates: Squeeze and Emulsification Techniques. *Plastic and Reconstructive Surgery*. **139** (1), 79-90 (2017).

9. Feng, J. *et al.* Micronized cellular adipose matrix as a therapeutic injectable for diabetic ulcer. *Regenerative Medicine*. **10** (6), 699-708 (2015).

10. Zhang, P. *et al.* Ischemic flap survival improvement by composition-selective fat grafting with novel adipose tissue derived product - stromal vascular fraction gel. *Biochemistry and Biophysics Research Communication*. **495** (3), 2249-2256 (2018).

11. Tonnard, P. *et al.* Nanofat grafting: basic research and clinical applications. *Plastic and Reconstructive Surgery*. **132** (4), 1017-1026 (2013).

12. Yao, Y. *et al.* Adipose Extracellular Matrix/Stromal Vascular Fraction Gel: A Novel Adipose Tissue-Derived Injectable for Stem Cell Therapy. *Plastic and Reconstructive Surgery*. **139** (4), 867-879 (2017).

13. Yao, Y. *et al.* Adipose Stromal Vascular Fraction Gel Grafting: A New Method for Tissue Volumization and Rejuvenation. *Dermatologic Surgery*. **44** (10), 1278-1286 (2018).

14. Zhang, Y. *et al.* Improved Long-Term Volume Retention of Stromal Vascular Fraction Gel Grafting with Enhanced Angiogenesis and Adipogenesis. *Plastic and Reconstructive Surgery*. **141** (5), 676e-686e (2018).

15. Sun, B. *et al.* Applications of stem cell-derived exosomes in tissue engineering and neurological diseases. *Reviews in the Neurosciences*. **29** (5), 531-546 (2018).

16. Allen, R. J. *et al.* Grading lipoaspirate: is there an optimal density for fat grafting? *Plastic and Reconstructive Surgery*. **131** (1), 38-45 (2013).

17. Qiu, L. *et al.* Identification of the Centrifuged Lipoaspirate Fractions Suitable for Postgrafting Survival. *Plastic and Reconstructive Surgery*. **137** (1), 67e-76e (2016).