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Mechanical Micronization of Lipoaspirates for Regenerative Therapy

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TITLE:

Mechanical Micronization of Lipoaspirates for Regenerative Therapy

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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 diseases[1]. 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 medicine[2]. In addition, they are nonimmunogenic and have abundant resources from autologous fat[3].

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 collagenase[4]. 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 result[5]. 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 developed[6-9]. 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 models[8-10].

In 2013, Tonnard *et al.* introduced the nanofat grafting technique, which involves producing emulsified lipoaspirates by intersyringe processing[11]. 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-gel[12]. 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.

2. 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.

3. 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.

4. 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.

5. 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% H_2O_2 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 tissues[15]. However, there are limitations to expanding its clinical application, since it requires complicated procedures to isolate cells and collagenase for processing[6]. 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-gel[12]. 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 10^5 cells/mL[12].

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 fiber[16,17]. 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 regeneration[14]. 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 healing[12]. Moreover, it helped to improve the survival rate of the free flap in a mouse model by accelerating angiogenesis[10].

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.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

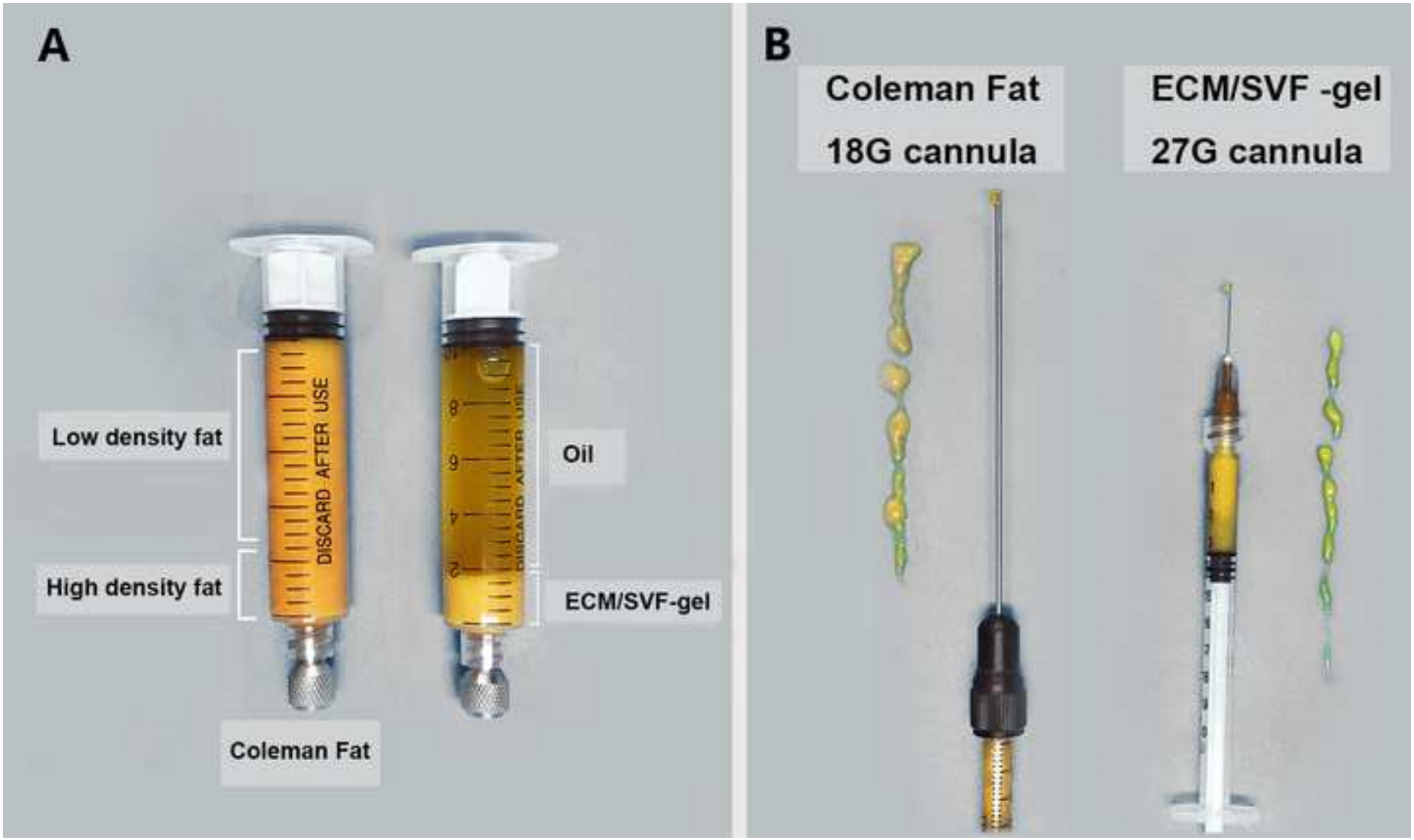
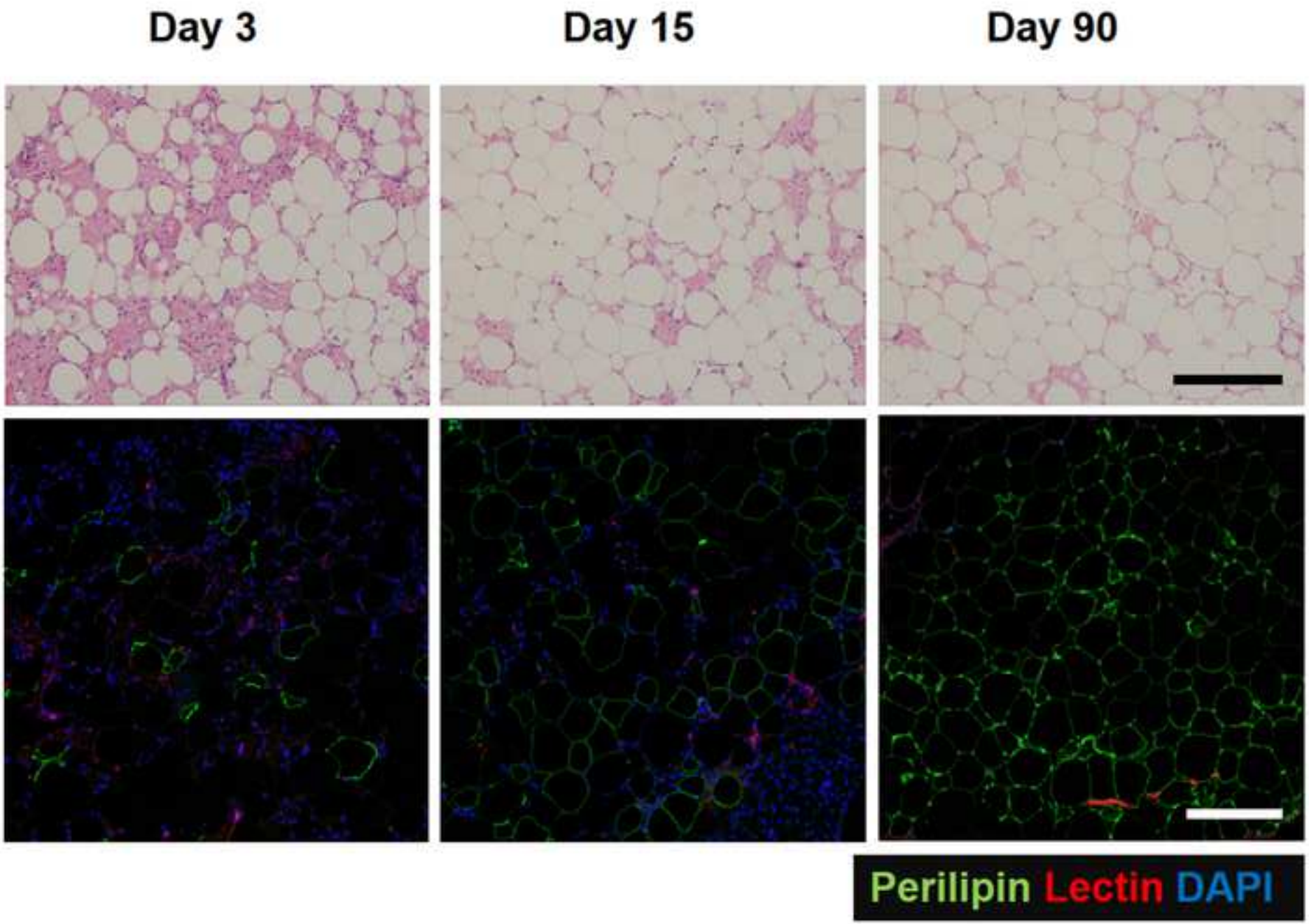


Figure2



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Signature: Junwei Cai Date: 2018-8-19

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Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
Author's response: We have corrected the reference style.

3. Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials (e.g., 3-mm multiport cannula, sterile bag, 50 mL tubes, wide tip pipette, Centrifuge, 20-mL syringes, Luer-Lok connector, nude mice, ethanol, the device used to cut sections, Eosin Y dye, xylene I and II and III, microscope, etc.).
Author's response: We have revised the table of the essential supplies, reagents, and equipment.

4. Please also address specific comments marked in the attached manuscript.
Author's response: We have addressed all the specific comments marked in the attached manuscript.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author's response: We have proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Author's response: Though we present similar results in this article, the figures that we used here is different from the previous publication.

3. Please revise the title to be less wordy and avoid punctuations.

Author's response: We have revised out title as you suggested.

4. Please provide an email address for each author in the manuscript.

Author's response: We have provided an email address for each author in the manuscript.

5. Keywords: Please provide at least 6 keywords or phrases.

Author's response: We have provided at least 6 keywords or phrases.

6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Author's response: We have used SI abbreviations.

7. Please use centrifugal force (x g) for centrifuge speeds.

Author's response: We have used centrifugal force (x g) for centrifuge speeds.

8. Long Abstract: Please include an overview of the method and a summary of its advantages, limitations, and applications.

Author's response: We have included an overview of the method and a summary of its advantages, limitations, and applications.

9. Please remove the square brackets enclosing the reference numbers.

Author's response: We have remove the square brackets enclosing the reference numbers.

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Author's response: We have not included any commercial language in this manuscript.

11. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
Author's response: We have adjusted the numbering as you suggested.

12. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
13. 1.1: Perform liposuction on what?
Author's response: We perform the liposuction on human.

14. 2.1: What is the container used in this step? Can a sterile bag stand?
Author's response: The sterile bag (blood bag-like) is the container. The sterile bag can be hung up or lean on something to make it stand still. Or you can transfer the lipoaspirates into four 50 mL tubes.

15. 2.2: What is used to collect the fat layer? Is it transferred to a centrifugation tube?
Author's response: The fat layer is collected in a 50 mL tube.

16. 2.3: How many tubes are used?
Author's response: It depends on how much fat you can obtain. In most cases, after discarding the liquid portion, 100mL fat tissue can be obtained and two 50mL tubes are needed.

17. 2.4: Technically, the upper layer should be collected before discarding the bottom portion, correct? What is used to collect the upper layer?
Author's response: A wide tip pipette is used to collect the upper layer. After collecting the fat, the liquid portion at the bottom was discarded.

18. 2.5, 2.6: What is used to transfer the upper 2/3 or lower 1/3 of Coleman fat?
Author's response: A wide tip pipette is used to transfer the upper 2/3 or lower 1/3 of Coleman fat.

19. 3.4: What is used to collect the oil portion? How is it stored? Technically, the fluid at the bottom layer can be only discarded after the middle layers is removed, correct?
Author's response: The oil portion was collected in a 10 ml tube by using a wide tip pipette at room temperature.

20. 3.5: What is used to collect the middle layer?
Author's response: A wide tip pipette is used to collect the middle layer.

21. 4.1: Is the oil used in this step from step 3.5 (i.e., the sticky substance in the middle layer)? Please specify. How to mix oil with the high density fat?
Author's response: The oil was collected from the step 1.3.4 and mixing the oil and high density fat can be achieved by inter-syringe shifting in the next step.

22. 3.5 and 4.5: The products from both steps are referred to as ECM/SVF-gel. Should they be distinguished as one is from low density fat and the other is from high density fat? Are the products from both steps mixed?
Author's response: The products from both steps are mixed

23. Line 144: Please specify the age, gender and type of mice. Please mention how proper anesthetization is confirmed. Do you apply eye ointment? Do you perform the injection in a sterile environment?
Author's response: The nude mice (8-week old, female) were anesthetized with isoflurane (1-3%) inhalation anesthesia in an animal operation room. Eye ointment is not applied.

24. Line 145: Please specify ECM/SVF-gel from which step is used here. Anything visible by naked eye after injection or after the days when the sacrifice is performed? What is the control injection?
Author's response: ECM/SVF-gel was transferred into a 1 mL syringe before injection. The transferred ECM/SVF-gel can be visualized by naked eyes on their back beneath the skin.

25. Line 152: Please describe how this is done. Please specify all surgical instruments used.
Author's response: Mice dorsal skin was cut open alone the midline with surgical scissors. SVF/ECM gel grafts' were dissected and harvested beneath the mice skin.

26. Line 153: What container is used?
Author's response: The anesthesia induction was conducted by putting single mice in a 1L transparent drop jar for 1 minute.

27. Line 157-159: Please describe how these steps are actually done. For instance, how to embed tissues into paraffin blocks, what tool is used, and what is used to cut sections?
Author's response: Tissue dehydration were conducted with Leika Biosystem Tissue Processor automatically by soaking in the following liquids: 70% ethanol, two changes, 1 hour each; 80% ethanol, one change, 1 hour; 95% ethanol, one change, 1 hour; 100% ethanol, three changes, 1.5 hour each; xylene, three changes, 1.5 hour each; Paraffin wax (58- 60℃), two changes, 2 hours each.

28. Lines 163 and 179: What is deparaffinized and how?
Author's response: Paraffin was removed by deparaffinazation process: the paraffin slides were soaking in xylene solution for 10 min, and for three changes (Xylene I and II and III).

Line 174: This step is unclear. Please clarify.
Author's response: 2-3 drops of Eosin Y dye were added onto the slides directly by pipette. Let the dye set for 10 minutes.

30. Line 175: What is mounted and what is the composition of the mounting media?
Author's response: We mount the coverslips to the slides with a xylene based mounting media Mount-quick (Newcomer supply, WI, US)

Line 183: What slides? They have never been mentioned in the previous steps. When and how and what did you make a slide?
Author's response: Same paraffin slides were prepared in the tissue harvest part under the subtitle " Tissue Harvesting on 3, 15 and 90 Days after ECM/SVF-gel Injection. "

32. Line 184: What container? How many slides are placed in one container?
Author's response: The slides were loaded onto a specialized slide basket, which contains 30 slides and soaked into the compatible soaking boxes sequentially.

33. Line 186: What does it mean by arranging the container?
Author's response: Manuscript was edited as following: Let the container cool in room temperature for 20 min.

34. Line 196: What is used to wipe off the water?
Author's response: The excess water was wipe off with clean tissue paper.

35. Line 198: Is the slide observed using a microscope?
Author's response: Manuscript was edited as following.
Line 201: Mount the coverslips and let dry in dark. Slides were then observed with a fluorescent microscope.

36. Lines 142-198: Please continue numbering (therefore it should be 5. Nude Mouse…).
Author's response: We have make the numbering system more understandable.

37. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Author's response: We have made according changes.

38. Please include single-line spaces between all paragraphs, headings, steps, etc.
Author's response: We have made according changes.

39. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Author's response: We have highlighted the essential steps of the protocol with yellow highlight color.

40. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
Author's response: We have highlighted the essential steps as you requested.

41. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Author's response: We have highlighted the essential steps as you requested.

42. Discussion: As we are a methods journal, please also discuss any modifications and troubleshooting of the technique, and any limitations of the technique.
Author's response: We have added modifications, limitations and troubleshooting of the technique in the discussion part.

43. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
Author's response: We have revised the reference style as you suggested.

44. References: Please do not abbreviate journal titles.

Author’s response: We have revised journal title as you suggested.

45. Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
Author’s response: We have made the revision as you suggested.

Reviewers' comments:

Reviewer #3:
Manuscript Summary:
The authors describe their method of producing ECM/SVF-gel in detail and show that it results in high graft retention with histologically normal-appearing adipose tissue.

Major Concerns:
None

Minor Concerns:
The authors should make it clear that the experimental component of this study is not new. The authors have previously described these findings in more details in previous publications. However, this detailed description is of value because it will help other scientists to replicate these methods and perform additional experiments with ECM/SVF-gel, especially if the authors include a video showing the production steps.

Author’s response: This article mainly focuses on the introduction of the method to produce the ECM/SVF-gel. A video showing the production steps will be produced with the journal’s help.

Reviewer #4:
Manuscript Summary:
The manuscript describes the major steps in an appropriate manner. Some concerns need to be answered and revised.

Major Concerns:
-line 60: ASCs = adipose-derived mesenchymal stromal/stem cells. PLEASE REVISE
Author’s response: We have made revisions as you suggested.

-Introduction: You should describe the cells within the SVF...there are not only ASCs/preadipocytes.....
Author’s response: As you implied, stromal vascular fraction (SVF) of adipose tissue contains ASCs, endothelial progenitor cell, pericytes, and immune cells. We have added this background into the Introduction.

-Fig. 2: Please provide better figures of the histology (higher resolution). Please mark preadipocytes by arrows !!!
Author's response: We have made revisions as you suggested.

Minor Concerns:
-line 58: delete comma after e.g
-line 63: ...abundant resources. Please clarify and describe in an appropriate manner
Author's response: We have made revisions as you suggested.