Drake-Hicks Protocol For *In Situ* Release of Mesenchymal Stem Cells and Cytokines Following Abdominal Lipolysis

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Abstract: The Authors propose the hypothesis that the lipolysis of abdominal adipose tissue and associated cellular matrix will release mesenchymal stem cells, endothelial cells, vascular endothelial growth factor, as well as a host of other cells and cytokines into the peripheral blood. This could lead to various systemic therapeutic events. This *in situ* abdominal lipolysis could be achieved through any of several lipolytic procedures, with some being more effective than others. The procedure is proposed as a possible substitute methodology for the current practice of isolating of stem cells from a mini-liposuction followed by *ex vivo* processing, prior to re-injection into the same patient.

Key Words: Adipolysis, Lipolysis, Adipose-derived stromal/stem cells, Adipose tissue, *In situ* cell therapy, Mesenchymal stem cells, Regenerative medicine; Stem cells, Stromal vascular fraction, Adipose tissue growth factors, Adipose tissue cytokines, Release of stem cells from adipose tissue *in situ,* Intra-abdominal subcutaneous injection

1. Introduction

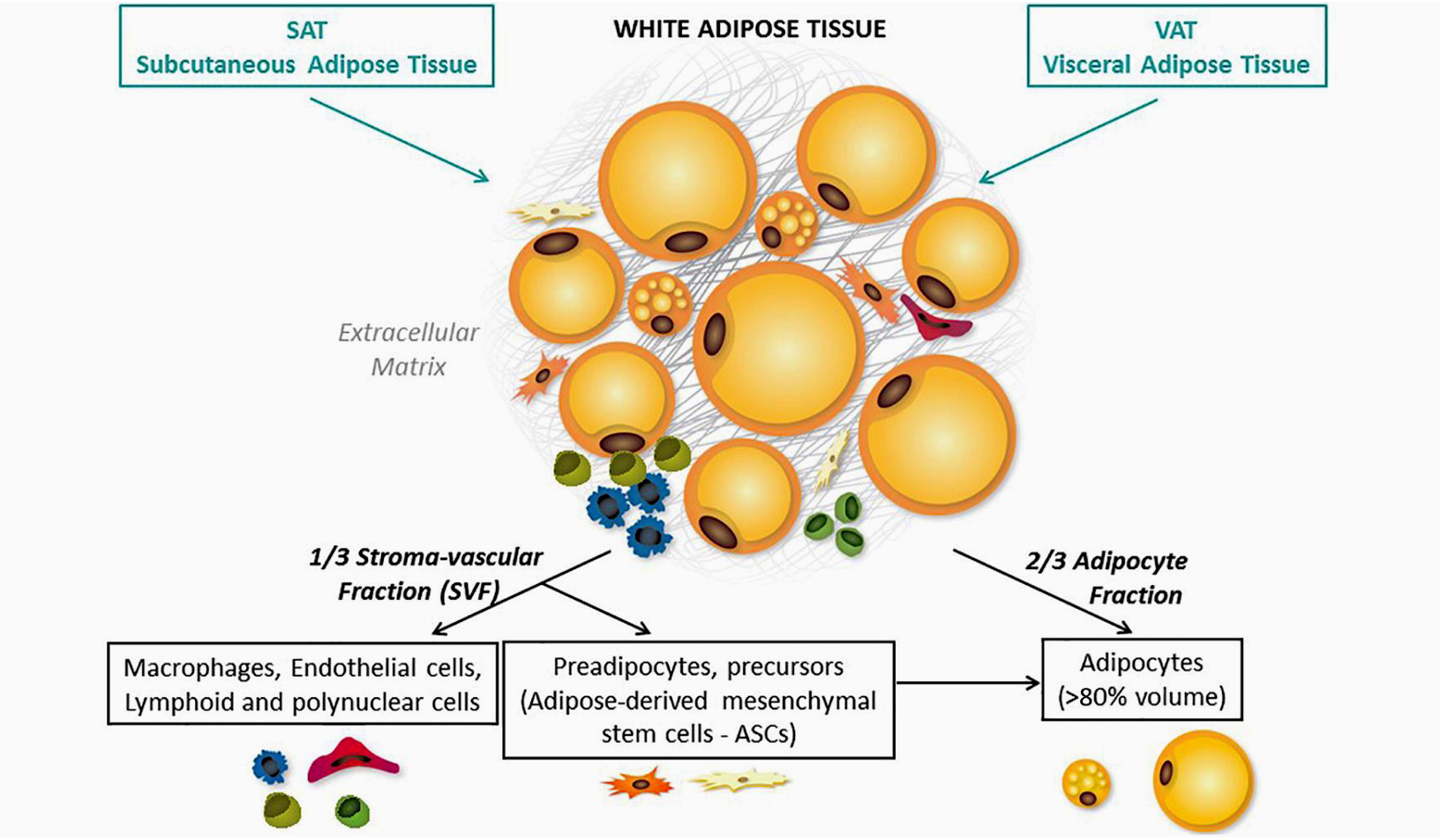
The Stromal Vascular Fraction (SVF), obtained from abdominal liposuction, is among the most widely used biologics in stem cell therapy for the treatment of many conditions and diseases. Although some international centers base their therapies on cord blood and other birth tissues, isolating autologous mesenchymal stem cells and various growth factors and cytokines from abdominal adipose tissue for re-injection into the patient has been the mainstay of stem cell therapy for many years.

Briefly, a patient is subjected to liposuction of 50-60 ml of abdominal adipose tissue, which is then processed by enzymatic digestion or ultrasound to disrupt the cellular matrix, thereby yielding a “soup” composed primarily of mesenchymal stem cells (MSC’s), endothelial cells, as well as associated growth factors and cytokines.

By 2012, stem cell therapies using SVF were well underway worldwide, except for the United States, where this new medicine continues to be blocked by the medical and pharmaceutical industries. Robert W. Alexander presented an excellent review in which he described the various cell fractions and identified many growth factors and other cytokines in the SVF [1]. Many international stem cell centers and a few pioneering clinicians in the U.S. began treating patients with SVF. For example, Dr. Steven Victor of New York began treating patients by this method, even filing a U.S. patent application in 2011 “Ultrasonic Cavitation Derived Stromal or Mesenchymal Vascular Extracts and Cells Derived Therefrom Obtained From Adipose Tissue

and Use Thereof", Patent No. US 8,440,440 B2. In discussing SVF on his website, (RegenMedical) at that time, he noted: "SVF, also known as stromal vascular fraction (SVF) is the lipoaspirate obtained from small volume fat harvesting minus the fat cells (adipocytes). The SVF contains a wide number of cellular types including pre-adipocytes, endothelial cells, smooth muscle cells, pericytes, fibroblasts, and adult stem cells (ASCs). In addition, the SVF also contains blood cells from the capillaries supplying the fat cells. These include erythrocytes or red blood cells, B and T cells, macrophages, monocytes, mast cells, natural killer (NK) cells, hematopoietic stem cells and endothelial progenitor cells and more. Also the SVF includes adipocyte endocrine secretions, and importantly, contains growth factors such as transforming growth factor beta (TGF), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF), among others."

A "soup" of cells including cytokines and growth factors is used for therapy. We know that endothelial cells have been shown to work in harmony with stem cells to help grow them in large numbers. Endothelial cells are included. We know that cytokines and unknown growth factors help in therapy, which , in other treatment centers, is why a soup of fetal tissue is so effective in therapy. Cytokines and growth factors are included. Many other beneficial cells are also included. This image depicts adipose tissue cellular composition:



From: Christine Bourgeois et al., "Specific Biological Features of Adipose Tissue, and Their Impact on HIV Persistence" Front. Microbiol. (2019) 10: (Article 2837): 1-25. https://www.frontiersin.org/articles/10.3389/fmicb.2019.02837/full

SVF practitioners generally consider the SVF to contain at least the following, not all cytokines listed:

Vascular Fraction Cellular Composition
Adult autologous stem cells (Mesenchymal)
Endothelial Cells
Fibroblasts
Growth Factors
Pericytes Preadipocytes cells
Smooth muscle cells

Blood Cells from the capillaries supply including:
B&T cells
Erythrocytes Hematopoietic stem cells Endothelial progenitor cells Macrophages
Mast Cells
Monocytes
Natural killer (NK) cells

Between about 2010 and 2018 the field of stem cell therapy with SVF continued to blossom in the U.S. and particularly worldwide, as the methodology replaced the older methodology of harvesting autologous stem cells from peripheral blood. Adipose tissue was found to have 400 times more mesenchymal stem cells than any other source. In the U.S. the field hit its apex with the publication in 2017 of the treatment of 676 patients with SVF in a wide variety of conditions and diseases [2]. Other papers were being published by that time on the utility of SVF as a therapeutic modality [3,4].

However, a U.S. Federal Court ruled in 2019 that a patient's own stem cells were a drug subject to regulation by the Food and Drug Administration, and that was pretty much the end of stem cell therapy in the U.S. However in 2022, a California federal court ruled that the use of SVF in patient therapy was "… the practice of medicine, not the manufacture of pharmaceuticals", thereby possibly reopening the door to using this useful therapy in the U.S. [5]. While these court cases have had a chilling effect in the United States, stem cell therapy using SVF continues to be the methodology of choice in many international treatment centers, found particularly in Thailand, Mexico, and Dubai, many of which are operated by U.S. physicians.

The Authors reason that all the ingredients that make up the SVF and lead to therapeutic results are within each individual, although locked in the abdominal fatty tissue. The essence of the current SVF procedure is the extraction stem cells and growth factors from the patient's fatty tissue, and after some processing, the injection back into the patient. Currently, a surgical procedure, namely liposuction, is required to collect the SVF. Not only does this increase the cost of the procedure, a surgeon is required. The SVF procedure, while available, ***is still not widely available*** for this reason.

The Authors postulate, that if the adipose tissue could be dissociated ***in situ***, disrupting the cellular matrix, then it would be possible to release mesenchymal stem cells, and particularly bound up growth factors and cytokines, directly into the peripheral blood. The hypothesis being presented here is that by performing abdominal lipolysis, one would be able to skip the liposuction step altogether, and go directly to producing high levels of MSC's, growth factors, and cytokines within the patient. Many physicians and possibly even nurse practitioners or

physicians assistants in certain circumstances could perform the procedure. Moreover, being mostly non-invasive, repeat procedures could be done, thereby accelerating and increasing the number of MSC's and growth factors released into the periphery, which would hopefully yield a therapeutic effect.

This paper presents a proposed protocol for the *in situ* release of mesenchymal stem cells and cytokines into peripheral blood following abdominal lipolysis.

2. Methods

The proposed procedure consists of two parts: (A) Lipolysis; and (B) Testing for one or more cells and growth factors in the peripheral blood following the procedure.

A. Lipolysis:

Virtually any fat loss procedure would seem to be effective. All these procedures reduce abdominal fat, and hence would be expected to break up the adipose tissue extracellular matrix. Some lipolytic procedures are going to be found better than others in producing higher levels of MSC's and circulating cytokines. Methods which might be effective include:

Injection Lipolysis with lecithin
Injection Lypolysis with Lecithin plus Enzyme
Laser Lipolysis: involving photo thermal energy
Cryolipolysis: controlled cooling/freezing to destroy fat cells
Low Level Laser Therapy (LLLT), said to reduce fatty tissue without heat
Focused Ultrasound
Ultrasound Cavitation
Radio Frequency Lipolysis

B. Testing for MSC's.

Because Adipose tissue stem cells are comprised almost entirely of MSC's, testing peripheral blood for increased numbers of MSC's requires mixing/staining with a fluorescent antibody panel for MSC markers: CD45/CD73/CD90/CD105. A typical procedure is as shown by Mikrova et al., excerpted here with markers for MSC's substituted for HSC markers: "Peripheral blood mononuclear cells (PBMC) were isolated by the Ficoll-Hypaque method. Briefly, blood samples were diluted two-fold with PBS and layered onto Ficoll-Hypaque in 50-ml conical tubes. Each tube was centrifuged at 400 g for 30 min and the lymphocytes at the interface were collected. Cells were washed twice with RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, and subsequently resuspended in 100 µl (0.5 M cells per 100 µl) of buffer (PBS+0.5% BSA). Cells were stained with anti-CD45; anti-CD73; Anti-CD90; Anti-CD105. Specifically, 10 µl of antibody was added per 100 µl of resuspended cells and refrigerated in the dark for 15 min (4-8) °C. Cells were washed in 2 ml of PBS with 0.5% BSA and resuspended in 100 µl of buffer for analysis. Flow cytometry was performed with the total number of cells counted cells being 30,000 per sample" [6]. Counting cells with a hemacytometer and fluorescent microscope could be substituted for flow cytometry.

C. Testing for various growth factors and other cytokines. One could select a number of growth factors and/or cytokines to test for. However, we believe it is not necessary to initially test for all or even many. If ***any*** growth factor or cytokine becomes elevated in the peripheral blood, it would demonstrate effective disruption of a portion of the adipose tissue extracellular matrix. One could determine by testing the patient before and after the procedure, the extent of elevation. Because the co-culture of stem cells with endothelial cells markedly increases the yield of stem cells in culture [7], and because endothelial cells as well as Vascular Endothelial Growth Factor are present in adipose tissue, we have settled on testing for VEGF levels in peripheral blood before and at intervals following lipolysis: -0.5 hr; 0.5 hr; 1 hr; 2 hr.

D. Injection Lipolysis With Laser Activation Sample Protocol: the materials and methods are as follows:

Injection Cocktail Possibilities:

Detergents. Lecithin or phosphatidylcholine (PC) or deoxycholic acid (DC) or any combination of these. These work by dissolving the fat cells, releasing their contents. It is stated that no other cells are affected. The results are stated to be "Immediate" [8]. With the adipose tissue somewhat disrupted, it is hoped that other components of the extracellular matrix become dissociated, thereby releasing stem cells, endothelial cells, and growth factors.

Deoxycholic acid (DC)

Kybella claims it contains 10mg/ml = 10mg/g = 0.01 = 1% of **deoxycholic acid** in water and buffer solution. Dosage Forms & Strengths, solution for injection 10mg/mL (supplied in 2mL vials). Dosage and Administration: A single treatment consists of up to a maximum of 50 injections, 0.2 mL (20 mg) each (up to a total of 10 mL), spaced 1 cm apart. 0.2 ml spaced 1 cm apart (total 4-6 ml) Kybella (deoxycholic acid) in the neck subcutis to chemically ablate adipose tissue. [9]

PC/DC Combo:

(A) 0.4 ml of combo prep seems to be the best: (20 mg phosphatidylcholine along with 10 mg deoxycholic acid) at a depth of 6-10 millimeters. Spacing per above [8].

(B) The volume of the solution (PC 25mg/mL, DC 21mg/mL) varied from 20 to 30cc per flank or saddle bag to 40 to 60cc per abdominal area in the two different practices. [10]

(C) 1 ml combo contained 50 mg PC, 42 mg DC, and 8mg benzyl alcohol. [11]

Enzymes: Enzymes work by digesting the extracellular matrix, and releasing all cells and cytokines, growth factors etc that are entwined in the adipose extracellular matrix. There is no better way to dissolve extra-cellular matrix than via enzymatic action which breaks down bonds holding adipose tissue together. In the standard SVF liposuction procedure, it has been reported that collagenase yields 1000-10,000 more cells and cytokines than mechanical dissociation, with incubation times varying widely between 20 minutes to 4 hours. Collegenase is currently in wide use at many cosmetic centers. "Proposed in vivo risks due to proteolytic enzymes include allergic reaction and unwanted tissue degradation. Preclinical and clinical studies have shown relatively low risk from small amounts proteolytic enzymes as a result of systemic exposure or localized injection [12].

Collagenase is approved by the FDA for cellulite. [collogenase clostridium histolyticum CCH]. 0.1 ml 2 cm apart, 1 aliquot perpendicular to the skin, 2 aliquots at 45 degree angle, indicated to be 0.84 mg CCH. [13]

Laser Activation: Low Level Laser Therapy (LLLT) has been used for fat reduction for over 10 years. Various lasers and wavelengths have been compared [14]. "Low-level laser therapy (LLLT), is also known as cold laser therapy. One device has 5 rotating diode laser heads that work at a wavelength of 635 nm. Treatment sessions last up to 30 minutes, and 6 to 8 sessions are required to obtain optimal results. Low-level laser therapy is a unique modality that is not based on thermal tissue damage, but rather on producing transient microscopic pores in adipocytes that allow lipids to leak out, leading to fat reduction. Because LLLT causes immediate emptying of targeted adipocytes, results are noticeable as soon as treatment is completed; however, there is no necrosis or apoptosis of adipocytes, so the recurrence of fat deposition is believed to be greater when compared to the other modalities. Because the results are temporary, long-term or permanent results should not be expected with LLLT" [15].

3. Discussion of Protocol Specifics

We have in this report referred to our procedure as **"in situ abdominal lipolysis"**. This is to emphasize that injections and external lipolysis applications such as laser lipolysis, ultra sound and an array of fat dissolving equipment are applied to the patient without the need for liposuction. Although injection lipolysis has also been referred to as "intra-abdominal subcutaneous injection", or "intra-adipose subcutaneous injection", these latter terms exclude laser lipolysis, lipolysis via ultrasound, radio frequency lipolysis, cryolipolysis, and other external modalities. Consequently, for our purposes, we believe "in situ abdominal lipolysis" to be the more applicable term for the procedure we are proposing.

We found the following additional reports instructive:

(A) Regarding injection lipolysis, **phosphatidylcholine** causes adipocyte-specific lipolysis and apoptosis in adipose and muscle tissues, as well as increased TNFα and IL-1β expression and release in 3T3-L1 adipocytes in a dose-dependent fashion [16];

(B) **PDC-DC injection** induces necrosis of adipose tissue and adipocyte apoptosis within 48 hours; and the lipoaspirate contains VEGF and other growth factors: basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF), necessary for engraftment of grafted tissue and secreted by ASCs but which could also act locally as an adequate regenerative stimulus [17];

(C) Regarding **enzymatic digestion**, "The extracellular matrix (ECM) is the vast and complex network that holds all cells, tissues and organs together. The most abundant component of the ECM is collagen. Collagens act as the backbone of the extracellular matrix" and "…clostridial collagenases…(cause) ECM degradation, cleavage of cell surface receptors, growth factor release from the ECM and more. Collagenases digest collagens in the ECM" [12].

We settled on the following procedure and Injection Cocktail: Lecithin gel (PDC) is obtained from a pharmaceutical supplier, or pharmacy via prescription. The patient is in the supine position. Grabbing a mound of abdominal flesh with one hand and lifting upwards, the lecithin is introduced using a 2" needle (25 gauge) at the center of the mound. The depth will depend on the patient, but the lecithin will be injected into the subcutaneous belly fat. The amount injected is estimated to be 5 ml, but would depend on the package insert guidance.

Following input of lecithin, **Collagenase** is also injected. A 1.84-mg vial should be reconstituted per the US prescribing information for buttock cellulite using the supplied diluent (8 ml; 0.23 mg/ml). Up to 0.84 mg is to be administered into subcutaneous fatty tissue.

Immediately following injection, the abdominal treatment area as well as the entire abdomen (4 quadrants) should be massaged for 3-5 minutes.

This can be followed by **LLLT (Low Level Light Laser Light Therapy)** to further break up the adipose tissue.

The release of Mesenchymal Stem Cells and cytokines from the extracellular matrix into the peripheral blood would be expected to be immediate.

Prior to and following Injection Lipolysis, blood testing for MSC's and VEGF proceeds as described above.

That both mesenchymal stem cells and cytokines from disrupted abdominal adipose tissue will be immediately detected in peripheral blood seems a certainty. Whether the increased levels will be sufficient for a generalized therapeutic effect is yet unknown, and further clinical studies are in the planning stages.

An important supporting observation was made by Jonathas Xavier Pereira et al in 2017 who reported that ALL adipolytic procedures used for fat reduction trigger local inflammation with the release of various cytokines and growth factors [18].

4. Final Comment and Future Directions

In this paper, the Authors have proposed that Injection Lipolysis of abdominal tissue with lecithin and collagenase, and/or with other external lipolytic techniques, such as laser or ultrasound, will disrupt the adipose extracellular matrix, thereby releasing mesenchymal stem cells, growth factors, and cytokines into the peripheral blood. Once elevated in a patient's peripheral blood, the biologics would be expected to exert the same therapeutic benefit as found in the standard SVF procedure requiring liposuction, external processing, and re-injection.

In a companion paper, it was postulated that serum factors and not exogenous stem cells are needed for a therapeutic effect, and that stem cells themselves, introduced ex vivo, may have a much more limited role than previously thought [19].

Consequently, elimination of liposuction from the current stem cell therapy protocol using SVF, through *in situ* adipolysis using one or more means, could thus open the door to affordable stem cell therapy for many patients. Each individual already has the body's curative materials, which only need to be stimulated or activated. We believe that *"in situ"* stimulation and activation of stem cells and supporting components are the way forward for stem cell therapy.

Clinical studies are underway to define the most appropriate of the lipolysis methods for yielding high levels of MSC's and cytokines in the peripheral blood.

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