Understanding Mechanical Emulsification (Nanofat) Versus Enzymatic Isolation of Tissue Stromal Vascular Fraction (tSVF) Cells from Adipose Tissue: Potential Uses in Biocellular Regenerative Medicine

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ABSTRACT

Background: *With rapid growth of experiences and techniques in the field of Biocellular Regenerative Medicine, clinical scientists and biotechnical advancements constantly seek to understand and optimize uses of the multipotent heterogeneous cellular populations found in adipose tissue complex. The value of including the living, native bioscaffolding within the adipose tissues has likewise gained importance associated with the biologically trophic effects and cellular attachment capabilities believed to positively influence undifferentiated stromal cells in the native sites and biocellular grafts placed. These bonds are felt necessary for cellular activation, proliferation, and contributing to an auto-amplification system within the processes of homeostasis, regeneration, and repair in a "site specific" manner. Appreciation of uses of biologicals (such as platelet-rich and bone marrow concentrates) has grown, and felt to provide a trophic influence on a variety of sites and applications.*

Study: *This study reports on the differences in use of microcannula lipoaspirates undergoing mechanical emulsification in order to provide adult mature adipocyte lysis, preserving tissue stromal vascular fraction (tSVF) in small particle form which can be injected through small bore needles (25-30 gauge). Comparison of the compressed (centrifuged) lipoaspirates with emulsified adipose specimens is made for differences in viability, cell numbers, and total nuclear counts. Simultaneous harvesting of both specimen groups via closed syringe microcannula system was carried out. Following centrifugation at 800 g-force for 5 minutes, comparative 10 cc compressed specimens were submitted for incubation, agitation, and cell separation using a CentriCyte 1000 closed semi-automated system and Vitacyte Clyzme AS at a 1::1 ratio per manufacturer's instructions. Cellular testing was carried out using flow cytometry for viability, counts and total nucleated counts (following RBC lysis) and compared. Lastly, each emulsified specimen was tested with 1 cc luer syringes through 25, 27, and 30 gauge needles. NOTE: Compressed adipose-derived tissue stromal vascular fraction (AD-tSVF) specimen harvested through 2.11 mm OD microcannulas <u>will not</u> inject through such small bore needles without plugging, and typically require 18-20 gauge needles to easily pass.*

Conclusions: *Comparative testing of 20 specimens each (n=20) revealed no statistically significant differences in mean cell viabilities, numbers or total nucleated cell counts between the <u>non-emulsified</u> AD-tSVF versus the <u>emulsified</u> AD-tSVF specimens. Examination of the emulsified AD-tSVF using fluorescent microscopy and live-dead staining did reveal many small fragments (extracellular matrix or microvascular remnants) with viable stromal cells remaining attached. This suggests that the mechanical emulsification process was effective in reducing the particle size permitting the small needle injection capability while preserving or promoting potentially important cellular attachments, while maintaining a comparable stromal cell viability (without large size mature adipocytes having been mechanically lysed).*

This emulsified AD-tSVF has been referred to as "microfat" or "nanofat" in current literature. Uses of such microfat or nanofat injections have important potentials in anti-aging, hair regeneration, radiation/sun damage skin, chronic wounds, abnormal scarring, and many ultrasound guided placements in musculoskeletal applications currently using a compressed AD-tSVF + high density Platelet concentrates. Reduction of needle diameter requirement permits significantly less patient discomfort during injections, and permits intradermal placement and small joint placements, currently challenging therapeutic sites.

Each mechanically emulsified AD-tSVF specimen successfully achieved injection ability using 1 cc luer syringes and down to 30 gauge needles. This ability changes many applications which involve intradermal patterned injections, scars, radiation damaged skin and chronic wound areas. Patient comfort is significantly improved with use of smaller bore injection needles, whether using sharp or blunt types.

NOTE: It is important to clearly understand that the mechanical emulsification alone does not create a true cellular stromal vascular fraction (cSVF), and should not be thought of as a substitute for true cellular isolation and concentration (often reported in laboratory, pre-clinical research, and clinical studies and papers). Due to the regulatory environment in the

United States, many seek to provide cellular isolates without use of digestive enzymes in the clinical setting which currently would need an IRB trial and tracking to pursue in the human patient at this time. Testing of the infranatant from both tissue samples revealed a relatively small number of viable stromal cells (typically ranging from 50,000 to 300,000) mixed within cellular debris and RBC/WBC). The infranatant debris and cellular elements are best discarded, as they are very limited in value, and not recommended for routine administration. The vast majority of AD-tSVF available in the compressed graft bears the most important stromal elements, and highest stem/stromal cell numbers.

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KEYWORDS: Adipose-Derived Tissue Stromal Vascular Fraction (AD-tSVF); Adipose-Derived Cellular Stromal Vascular Fraction (AD-cSVF); Nanofat; Emulsification; Stem Cell; Stromal Cell; Biocellular; Fat Grafting; Platelet-Rich Plasma (PRP); Cell Isolation; Regenerative Medicine.

Introduction

While clinical applications for adipose-derived stem/stromal cell products are rapidly evolving, terminology is progressively more confusing to the clinical provider. This article attempts to clarify some often used and confusing terminology, particularly use of the term "Stromal Vascular Fraction" (SVF), by defning the fundamental differences betwe the terms tissue SVF (tSVF) and cellular (cSVF). Recognizing the defnitive differences is imperativ clinical providers to interpret peer-reviewed sci and applied clinical Journal publications. This paper examines creation of a small particle AD-tSVF using a simple closed, mechanical system intending to physically destroy the majority of the larger, mature adipocytes, while preserving the stem/stromal cellular elements including reduction in the size of remaining bioactive matrix. Adipose-derived stem/stromal cells have rapidly gained popularity as a safe and easily accessible source of a large, heterogeneous, multipotent cellular population and bioactive matrix found within the largest microvascular organ in the body.[1] This is clearly reported in rapidly growing peer-reviewed literature and clinical reports exploring the potential important therapeutic advantages using the autologous adipose tissue complex (ATC). By defnition, AD- ... ipos stromal cell population AND the bioactive scaffolding (known as extracellular matrix, native scaffolding, and all components in the microvascular environment of adipose tissue). This product represents lipoharvested adipose extracted via *en bloc* excision or aspiration protocols. In the context of this paper, it also refers to the "nanofat" product produced via use of disruption procedures, since it is composed of both cellular and native structural fragments, created without use of enzymatic digestion.[2-5]

Laboratory applications seeking to completely isolate stem/stromal cell elements typically involve the chemical digestion of the stroma to accomplish separation of the extensive cell-to-cell and cell-to-matrix connections. To date, there is no mechanical means capable of producing a pure cSVF, but instead advance the fragmentation of the AD-tSVF effectively accomplishing reduction in the large mature adipocytes (rupture) and creation of a small particle size cells retaining some native, bioactive adipose scaffolding. This size reduction permits the injection of a combination of emulsifed tS (PRP) concentrates through very small bore needles into dermal and subdermal targets, but only if preserving the viability of its components.

Further, this paper examines method, and reports the eff AD-tSVF ("nanofat") in examining the cellular impact based on fow cytometric tes stromal cell concentrations and total nuclear counts (TNC) after cell membrane lysis. It well known that removal of the large, native, mature adipocytes (which provide approximately 95% by volume) in subcutaneous deposits, while contributing <10-12% of the nucleated cells within the adipose tissue complex (ATC). (*See Figure 1.*)

Figure 1. SEM: Adipose Tissue Complex: Adipocytes providing >85% by volume; 12-15% by cell numbers in AD-cSVF.

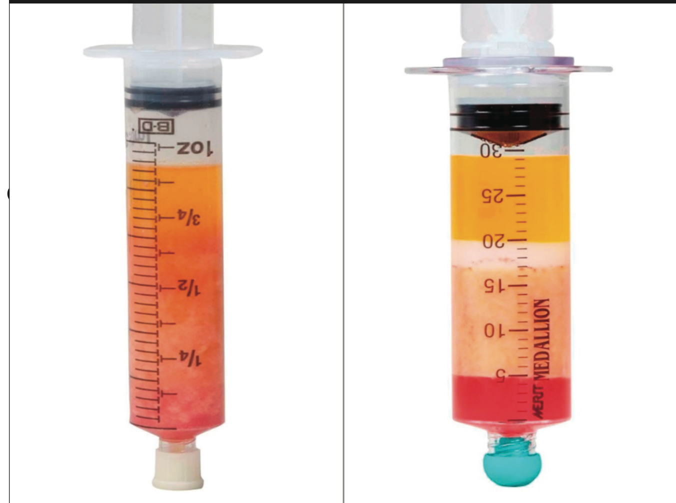
Adipocytes are not considered the most important cellular group, as it is now known that, due to anoxic conditions, these mature adipocytes are known to be lost in lipoaspiration. It is the precursor and complex group of stromal cells replacing them during such loss in traditional adipose structural grafting procedures.[5,6] The long-term assumption that adipocyte numbers were a constant since no traditional "mitotic" activity had been identifed, has now been heost means of cellular maint proce of asymmetrical cell division within the precursor populations of the ATC, involved in proliferation of multipotent adult cells in the microvascular tissues. It is now accepted that the human turnover rate of mature adipocytes is approximately 10-20% per year.[7]

We examine cellular viability changes and numbers between enzymatic digestion (used as baseline) versus mechanical emulsifcation for potential clinical transplantatio We report fndings made resulting from emulsifying AD-tSVF (Healeon ACM System Newport Beach, CA, USA by Tulip Medical) as compared to same patient, split samples, using enzymatic digestion (*See Materials and Methods.*) to create AD-cSVF recorded (CentriCyte 1000, Healeon Medical, Newport Beach, CA, USA). Same patient comparisons of viability and numbers within compressed, <u>non-emulsifed</u> AD-tSVF to compressed <u>emulsifed</u> AD-tSVF are reported.

The emulsifcation product produced in emulsifed AD-tSVF clearly should <u>NOT</u> be considered a pure isolated, concentrated *cellular* product which can be a means to avoid use of enzymatic digestion, or culture-expansion processing, as some suggest. Examination of the infranatant "pellet", created when harvested adipose is compressed to create a density gradient, has been suggested to represent an "SVF" alternative to use of enzymes by various biotechnical companies including Healeon ACM (Healeon Medical, Newport, CA, USA); Stromacell (Microaire Aesthetics, Charlottesville, VA, USA); LIPOGEMS (Lipogems Int'l, Milan, IT); REVOLVE Lifecell, Bridgewater, NJ, USA).

Centrifugation has long been an accepted means used to create quality density gradients (layer separation), and has shown as an effective means to safely reduce extracellular fuid, dilute local, free lipids, and permit quality compression and separation. (*See Figure 2.*)

Figure 2. Gradient Density Separation: Diagrammatic Decantation Versus Centrifugation: Left: Standard Decantation; Right: Post-Centrifugation (1000 g-force for 4 minutes) using lipid separation disk and infranatant layer.



The so-called "pelletization" found at the bottom of the centrifuged specimen created in the process does reveal the presence of some viable stem/stromal cells, but in relatively low numbers compared to stem/stromal cells of the full ATC remaining in the harvested graft.[8] In addition, besides relatively low viable stem/stromal cell numbers (compared to the full lipoaspirated graft), that "pellet" is also contaminated with cellular debris, RBCs, WBCs, Miscellaneous Stromal Cells, and non-viable matrix populations.[9] This <u>should not</u> be confused with production of a pure stem/stromal cellular product produced by enzymatic digestion.

The major advantage of creating a mechanical AD-tSVF is to offer the ability to provide a bioactive cellular/matrix product which can be injected via very small needles.[10] This includes the potential value realized in addition of PRP concentrates to create a true biocellular therapeutic modality. Uses of such offers several important potential contributions in clinical applications including anti-aging skin treatments, hair regeneration, sun and radiation damaged skin[11], superfcial fller[12], and facilitate a variety of uses in treatment of musculoskeletal disorders (particularly small joint and improved comfort of guided injections). An important value of using is that it permits inclusion of important native stroma (including residual stromal attachments) to be placed into the target tissues, including the hair bearing scalp and dermal targets. It has become clear that development, or maintenance, of cellular attachments are optimal to

favor activation and proliferative changes within native tissues and transplanted biocellular matrix. This paper examines potential changes in cellular viability and concentration when lipoaspirated fat is submitted for mechanical emulsifcatio instructions in the ACM System. (*See Figure 3.*)

Figure 3. Component Assembly Healeon ACM Device (NanoFat mfg. by Tulip Medical for Healeon). Top: Male Input Luer; O-Ring to prevent leakage; Emulsification Screen (Non-Aligned 600 u/400 u screen basket) for final emulsification.

Examination of any residual perivascular/extracellular matrix remaining may, in fact, offer an advantage of placement for many stem/stromal cells. It is widely understood that existing, available stroma may actually potentiate early cellular activation, proliferation, and response to site specifc sho retained in the emulsifed AD-tSVF. Addition of plat rich plasma (PRP) to emulsifed AD-tSVF as describe in this paper permits a simple means to incorporate concentrates of platelets, which are believed to promote many of the site healing and repair processes by provision of important growth factors and signal proteins for the entire healing cascade.[13-16]

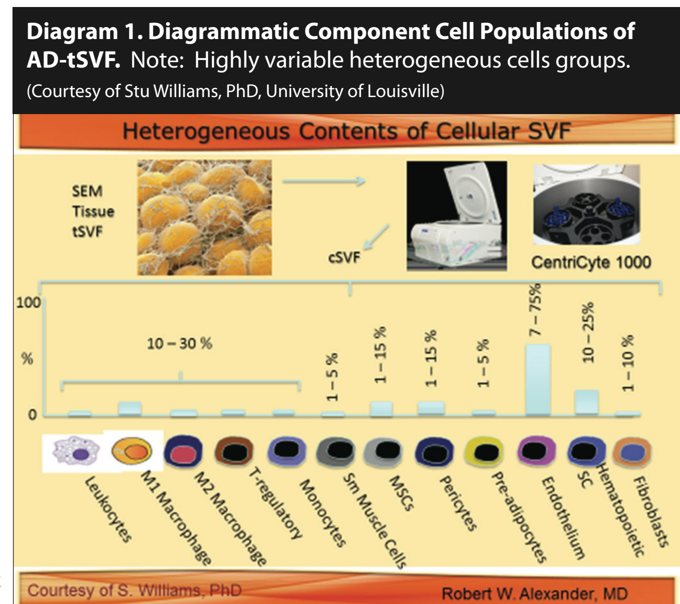
Background

Clinical translation of uses of autologous adipose-derived stem/stromal cells has been rapidly expanding with favorable safety and effcac reconstructive and regenerative applications have increasingly been reported, unfortunately with an array of terms and descriptions and confusion with commercial manufacturers' differences between the two "SVF" entities.

DEFINED: ADIPOSE-DERIVED TISSUE STROMAL VASCULAR FRACTION (AD-TSVF)

As example, in small volume clinical uses (<100 cc), AD-tSVF is acquired by sterile, closed system, microcannula syringe lipoharvesting of adipose tissue complex (ATC). ATC includes all components of adipose such as stem/stromal cellul structural elements (perivascular & extracellular matrix). This tissue is comprised of a highly heterogeneous, highly variable stem/stromal cell group, and its contents include a highly variable donor site population. (*See Diagram 1.*)

Diagram 1. Diagrammatic Component Cell Populations of AD-tSVF. Note: Highly variable heterogeneous cells groups. (Courtesy of Stu Williams, PhD, University of Louisville)



Attempts at identifcation of or singularly critical activated growth factors and signal proteins remains elusive at best. AD-tSVF can be easily harvested from subdermal fat deposits using disposable, microcannula harvest techniques using cannulas ranging from 1.65 mm – 2.4 mm diameters. Centrifugation to compress the graft is recommended to reduce the unwanted extracellular fui infusion of carrier fuids i isolating extracellular lipids and debris is well accepted.[17] Since it is known that the actual mature adipocytes are

resorbed after placement, structural volumes attained in grafting should be credited to the stromal matrix and stem/stromal cell population in the AD-tSVF, and their replacement functions to return to a homeostatic state dictated by the site.

The latest advances in the use of micronized AD-tSVF has progressed from relatively crude beginnings in open emulsifcation process (“nanofat”) as describe Tonnard et al (2013), to the availability of mechanical systems such as low frequency ultrasound[18], Revolve, Stromacell, LipoGems, and ACM devices. The desired outcome of each is to effectively reduce the AD-tSVF to a near non-adipocyte cellular SVF product. Although the size of the native adipose matrix (including extracellular matrix (ECM) and peri-adventitial structures is most modifed in the LipoGem and ACM devices, neither achieve a pure cellular SVF (AD-cSVF) product.

Researchers and clinical scientists are providing increasing evidence of adventitial and peri-adventitial cell groups serve to provide a much greater importance than initially thought, and are now appreciated as a probable source of true pluripotent or multipotent stromal cells (mesenchymal, pericyte/endothelial cell group).[19] Peault, Caplan, and others believe that the pericyte/endothelial (EC) groups, may in fact, represent the true “stem” cell group responsible for maintaining the mesenchymal cell group, and, on that basis, considered potentially very important to contribute to mesenchymal cell elements. It has become increasingly clear, that mesenchymal and peri-adventitial cells offer extensive overlapping and important capabilities *in vitro*, regardless of the tissue of origin in the microvascular system. These cells are preserved and included i ... tes in this study.[20]

Due to the relative rarity of mesenchymal cells in the bone marrow, adipose has effectively become the tissue of choice when striving to acquire higher numbers of viable stem/stromal cells for transplantation, avoiding complex and expensive culture/expansion to achieve a minimal therapeutic number of such cells.[21] One of the core questions to be examined in this pilot study was whether microcannula lipoaspirat ... emu and still maintain similar viabilities and stem/stromal cell numbers found in compressed AD-tSVF. We report comparative testing of the centrifuged AD-tSVF followed by enzymatic cell isolation[22-24] tested for viabilities and numbers compared to testing of same patient volume following mechanical emulsi the same testing protocols. (*See Materials & Methods.*) Tonnard reports a high safe these emulsifed AD-tSVF, bu regarding the cellular viabilities and numbers that are achievable.[25] Advances in techniques and testing analyses began in 2013, resulting in reasonably inexpensive, effective means of providing small particle AD-tSVF for injection into the dermis, tissues.

Of concern is the misconce is a pure cellular product OR capable of parenteral introduction (IV, IA, IP, or IT). Suggestions that mechanical means to completely separate high numbers of stem/stromal cells and create a cSVF “pellet” is not true in our experience. Understanding the complexity of vast connections (cell-to-cell and cell-to-matrix), such intricate contacts not being simple, single, or few in numbers, mitigates against creation of such a fully separated cellular product. (*See Figures 4-6.*)

Figure 4. Pericytes attachments along microcapillary wall.

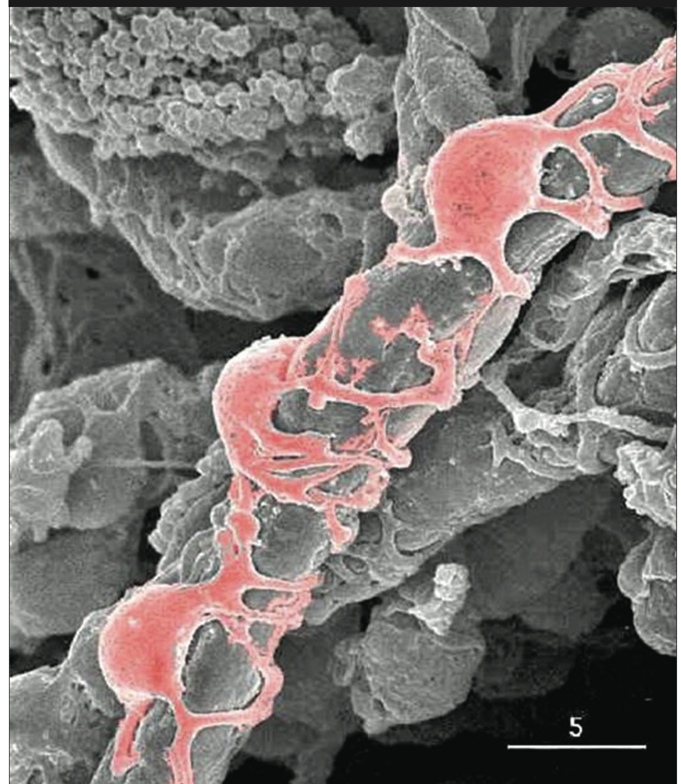


Figure 5. Mesenchymal Stromal Cells: In Vivo Extensive cell-to-cell and cell-to-matrix connections (Calcein AM; Hoechst Nuclear Stain).

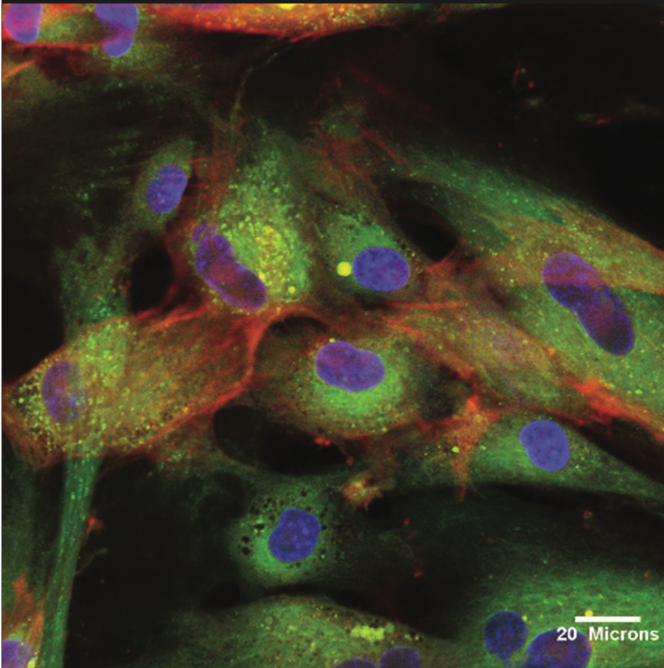
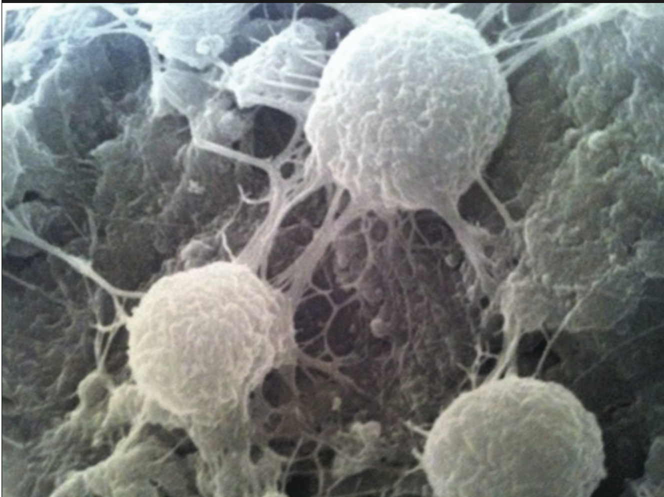


Figure 6. SEM Image Mesenchymal Stromal Cells demonstrating extensive connections within microenvironment of AD-tSVF.



Following harvest of ATC via disposable microcannulas and subsequent centrifugation (at an optimal range of 800 – 1200 g-force (rpm values cannot be used as the sole information of g-force delivery) for 3-4 minutes is effective in layer separation.[26,27] The described so-called "SVF Pellet" found at the bottom of the conical tube or syringe centrifuged spun immediately after AD-tSVF harvest does have some loose nucleated and stem/stromal cell elements, but in relative *much fewer* in number. In our examinations, true stem/stromal cells are found to range in number (typically between 50-300,000 nucleated cells, miniscule compared to the numbers found in the main adipose tissue complex (ATC) graft of the lipoaspirate.[28]
Signifcant amounts of cell
and heavier materials are mixed within the residual infranatant "pellet". It is the author's opinion that this should **not** be considered a substitute for utilizing the much higher number of stem/stromal cells and the native bioactive matrix found in the compressed adipose graft. With the ability to utilize the entire harvest specimen in the mechanical processing, it seems more logical and prudent to use the higher concentration product without the debris.

DEFINED: ADIPOSE-DERIVED CELLULAR **STROMAL** VASCULAR FRACTION (AD-CSVF)

In research publications, papers referencing "SVF" typically describe true cell isolates achieved by digestion/incubation techniques.[29] Rather than use of a generic term such as "SVF", it should be more correctly
identifed as "AD-cSFV) as t
This should be technically
and should be identifed tha
of enzymatic digestion and incubation/agitation were utilized to accomplish.[30] Many papers now exist outlining such preparations, characterizations, and culture/expansion components, beginning with Zuk (2001, 2002)
and revisited in 2013, have
variable, heterogeneous populations comprising the actual AD-cSVF. In current regulatory terms, current guidelines suggest these may fall into the "more than manipulated"
category and, thereby, requi
to examine and report the l
of such products in human clinical uses. Many clinical
trials are, or recently hav
outcomes in a very wide variety of targeted and systemic applications. (www.clinicaltrials.gov).

Many existing IRB trials are underway and beginning to report outcomes requiring a 3-5 year period of tracking outcomes and compiling data. In the interim, there have
been signifcant numbers of
cases and multiple case series within the United States and these should not be ignored. Published experiences
have reported excellent cli
reviewed settings. These publications, and trial reports, sets the stage for the much needed standardization and

Long-term outcome analyses offered in defned clini trials. Many plastic and cosmetic surgeons are beginning (2016) to submit to a common Registry-type data base which, may encompass use of cell-enrichment protocols proven effective in many international papers, but not within the traditional IRB pathways. This paper makes no effort to compare or characterize the specifc ce components between the AD-tSVF and true AD-cSVF.

Use of platelet concentrates with AD-tSVF is reported to be important contributor to enhanced outcomes in both aesthetic and musculoskeletal applications.[31-34] This treatment entity is known as a "Biocellular" therapy group, has shown very effective in provision of high concentration of growth factors and important signal proteins. This combination of AD-tSVF and HD PRP concentrates (defned as >4-6 times measured patien baselines) are more effective than ***either*** entity alone.[35] The addition of HD PRP to the AD-tSVF is well accepted by tissues, and appears to provide important trophic effects early in the healing processes, and has been referred to as contributing to an auto-amplifcati impact within the desired sites.[36]

The concept of provision of a high concentration of growth factors and important signal proteins and appear to offer a" bridge" to enhance the site-secreted or chemotactic agents as a result of interaction with biocellular elements augment recipient site activities.[37] As the clinical experiences using Biocellular AD-tSVF grows, improvement of the high safety and effcacy outcomes have become well documented. Gradually, follow up analytics in the laboratory are offering a much better understanding of the various chemical elements contributing has been reported.[37]

Recipient site importance has long been established, for example, adipose structural grafting consistently has been reported to do much better in areas where native structural fat deposits were located. Many examples of similarities within many site-specifc contribu are available in the peer-review publications outside of grafts of adipose to adipose. This has been reported in musculoskeletal applications, with tendons and ligament structures.

There are extensive clinical reports in aesthetic plastic surgery literature, reporting claims of high variability in "resorption" rates in doing structural fat transplantation. In most of these reports there was no accounting taken into consideration for the volume of fuids contained (30-40% by volume). These v been described as residual volume "load" placed within the location of injection into recipient sites, and does impact the actual volume of graft delivery. Centrifugation provides a more effective gravity density layered separation, permitting replacement of n (tumescent) with high-density platelet concentrates. This effectively reduces the fui so without obligating the p This reduction in unwanted advantage of using HD PRP in plastic and orthopedic uses, an important fact, si load potentially impact the perfusion of area capillaries and lymphatics, and may complicate the successful graft placements due to vascular impedance in recipient sites. Such overflling fuid loads i is likewise not desirable to maintain extremity perfusion.[38]

SUN AND RADIATION DAMAGED SKIN — PRP IN MESOTHERAPY AND PROLOTHERAPY

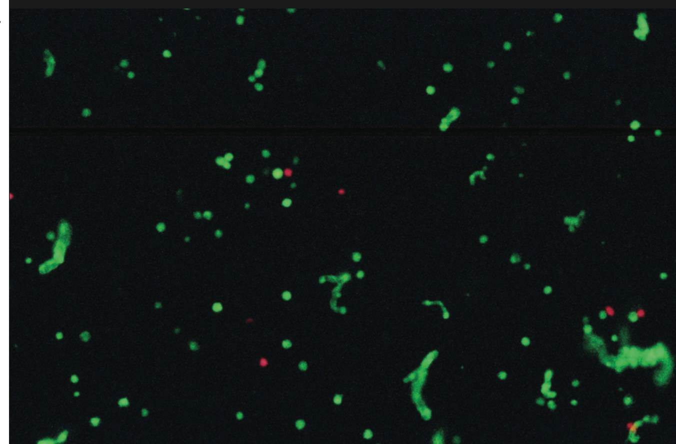
Over the past decade, many dermatologic and aesthetic-plastic surgeons have noted skin surface texture, vascularity, and change in the photo-damaged skin of the face associated with the emerging popularity of biocellular-enhanced structural fat grafting. It is important to note that initially, most observations were anecdotal, as the primary focus remained on volumetric retention. In the past 10 years, it has become relatively obvious that a signifcant a appearance and skin health changes have resulted from biocellular structural grafting techniques placed below the dermis, infuencing thes For many years, the desire to have the ability to do mid-reticular dermal placement of cellular and PRP products has increased. Until the ability to create a small particle graft of emulsifed adipose attempt injection through the very small needles. This led to examination of options to best create a derivative of grafts which could be reduced in the volume density. Since 2011, Yoshimura's group clearly demonstrated that the vast majority of mature adipocytes in AD-tSVF do not survive the anoxic conditions creating during and after lipoaspiration, it would suggest that long-term changes in volumes attained may more importantly relate to the precursor cell populations found within the tissue stromal vascular fraction.[39,40] With the advent of

is clear that the mature adipose contributes relatively little to any recipient sites, and suggests that placement in the dermal tissues might have great potential in improving vascularity of the skin and adnexa.[41] This paper focuses on the changes encounter sife of AD-tSVF and open the potential use of mechanically emulsifed samples to off abil numbers suggesting effective alternative in cases of severe radiation damage, aging changes, hair regenerative capabilities, and wide variety of uses in damaged-degenerative musculoskeletal and neurological issues.

The desire to improve surface textures and radiation damaged skin common to the aging processes led clinical researchers to explore a method to deliver a small needle capable adipose graft. This process was advanced with the patenting of a sterile, disposable microcannula system (Tulip Medical, San Diego, CA, USA) providing very small cannulas ranging from 1.6 mm-2.4 mm, thereby capable of creating a small particle lipoaspirate. Novel concepts of using mechanical reduction of the grafts lead to use of terms such as "microfat" and "nanofat" relating to particle size for transplantation. There are now signifcantly improved system devices to obtain CLOSED mechanical emulsifcation process compared to the relatively crude form which used OPEN processing using nylon mesh compression in an open setting. The early protocol was most utilized in the operating room setting, but presented concerns of safety when attempted in the small clinical settings without the optimal room pressurization and HEPA filtration capabilities. Th said, remarkably safe and effective outcomes have been experienced by a growing number of practitioners involved in aesthetic medicine.[42,43]

Clarifcation of the actual nature of mechanicall disrupted adipose product is important. A potentially dangerous misconception is that the emulsifed disru is able to create a cellular SVF and thereby avoiding of enzymatic digestion to create a true "cellular SVF". This is not considered reasonable, especially due to the relative inability to mechanically release the majority of stem/stromal cells from their multi-faceted connections between cell-to-cell and cell-to-matrix.[44-46] Such cells as pericyte/endothelial or mesenchymal groups may experience some smaller degree of mechanical separation, but the vast majority of such cells remain with the ATC, albeit in much smaller fragments. (*See Figure 7.*)

Figure 7. Live/Dead Stain in Flow Cytometer. Shows viable mononucleated cells (green); strings of emulsified matrix with viable cells attached (green linear); non-viable mononucleated cells (red).



Currently, confusing regulatory guidelines and conditions within the USA pushes interested practitioners to strongly attempt to avoid use of enzymatic digestion. Guidelines suggest chemical dissociation constitutes "more than minimally manipulation" as such, suggest it creates a "drug" which would require a complex and expensive IND pathway. Use of mechan biocellular applications are currently considered as falling within the clinical classif practice of medicine" exclusion.

REMINDER: The author warns that the stromal product achieved by mechan ***should NOT considered as safe for IV or other parenteral placement alternative, even using*** microfltration devices.

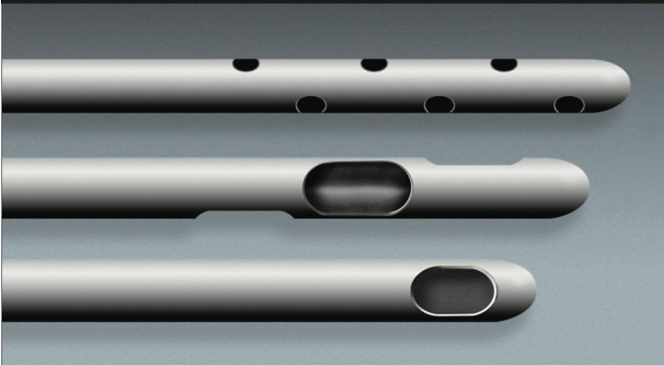
Materials & Methods

Previously reported by this author in the *Journal of Clinical, Cosmetic, Investigative Dermatology* (2013), use of a patented, sterile, disposab and harvesting system (Tulip Medical, San Diego, CA. USA), this project specifc standard protocol. (*See Figures 8a & 8b.*)

Standard coated, disposable super-luerlok™ microcannulas of 2.11 mm OD were utilized (20 cc of dilute xylocaine 0.05% with 1 x 10^{-6} epinephrine) and microcannula harvest using 2.11 mm OD offset Carraway Harvesting cannula. Samples were harvested

Figure 8a. Disposable Microcannula Setup for closed syringe Tulip Medical GEMS tm for AD-tSVF lipoharvesting. Top: Internal locks for plunger; Middle: 3 microcannula tips (see Figure X for close up); sterile, Bottom: Clear luer-to-luer transfer.

Figure 8b. Close Up Tulip GEM microcannulas: Top: 2.11 mm Multiport Infiltrator; Middle: 2.11 mm Off-Set Spiral (Carraway) Harvestor; Bottom: Single Port, Blunt Injector (variable sizes).



into standard 20 cc BD Syringes (Becton Dickenson, MD, USA) following the published protocol. Graft sizes were standardized to approximately a compressed volume (i.e. following specimen centrifugation, 5 min at 800g force) of lipoaspirate of 10 cc for each sample examination in a Healeon Medical CentriCyte 1000 centrifuge.

Each patient was cleared by routine history and physical examination, and provided a complimentary CBC taken at time of venipuncture for testing as actual platelet baselines. Each patient was individually consented for the harvested adipose tissues, and informed of the purpose of the removal to test for differences of AD-tSVF compared to emulsified AD-tSVF. No patients were treated using this sampling process or its products. Each sample was tested for the ability to inject through a 1 cc luer syringe mounted with a 5/8" 30 gauge needle.

Following harvest, each donor site was covered with small sterile absorbing pad, light triple antibiotic ointment to opening, and donor site closed using a closed cell medical grade foam over the areas of actual harvest, followed by light elastic compression (to greatly reduce post-harvest bruising). Male subjects were harvested from the lower abdomen or fank areas, and to primary distribution (fr lateral femoral sites).

Emulsifcation was carried ou Matrix Device (ACM) (Healeon Medical, Newport Beach, CA, USA) The protocol of Healeon Medical Inc., Newport Beach, CA, USA) was carried out according the standard steps to creat AD-tSVF. This involved three distinct steps:

1. Multiple (30) passes through a sterile standard 2.4 mm luer-to-luer transfer;
2. Multiple (30) passes through a sterile standard 1.2 mm luer-to-luer transfer;
3. Single Passage through sterile, non-aligned mesh screen chamber (600 micron low hematocrit PRP concentrates to displace all air from the screen device acquired through use of a Healeon PRP (Healeon Medical, Newport Beach, CA., USA) per manufacturer spin centrifugation and used the platelet poor plasma (PPP) during the neutralization phase of cellular testing protocol. (*See Figure 9a & 9b.*)

Figure 9a. Chamber Filling with High-Density Platelet-Rich Plasma to displace air, and create the biological (HD PRP) + cellular mix (AD-tSVF) known as Biocellular Therapeutic Modality.

Figure 9b. Final Emulsification of AD-tSVF through screen mesh chamber. Bottom: Lipoaspirate (AD-tSVF) attached to luer on INPUT side; Top: Receiving syringe containing HD PRP and Emulsified AD-tSVF.

Comparative samples (10 cc compressed adipose graft) were placed into two separate 50 cc Healeon Specimen Containers, in a total volume of 10 cc compressed adipose graft each, and submitted for cell separation and isolation in the semi-automated, closed CentriCyte 1000 machine (Healeon Medical, Newport Beach, CA., USA) following device manufacturer's standard written protocol. Enzymatic digestion was completed using Vitacyte Clyzyme AS at a 1:1 ratio, and incubated/agitated at 37 degrees C for 30 minutes. Post-digestion, centrifugation for 5 minutes at 800 g- force, 2.5 cc of pellet and infranatant fuid was mixed with 2 cc platelet poor plasma and di to 50 cc. Following this neutralization and rinsing step, the specimen syringe was re-centrifuged for 3 minutes at 800 g-force and AD-cSVF pellet recovered per protocol. Testing of residual collagenase in the final pellet performed in each sample, and found to contain residual collagenase blend at levels beneath the measurable limits in all samples.

First testing was performed on the compressed AD-tSVF (non-emulsifed) usi manufacturer's protocol. The comparative sample of 10 cc compressed adipose graft device as described above. was then submitted for identical cell separation and isolation protocol as described for the compressed graft only sample. Each sample of AD-cSVF created was submitted for identical cellular testing using a MoxiFlow fow cytometer (ORFLO, Ketc their proprietary Viability Reagent (Cat # Mxa055). Recording of viabilities, cell concentrations, and followed by separate chamber TNC after use of ZAPoglobin II (Becton-Coulter, USA) for cellular membrane lysis and testing (excluding RBCs).

Twenty comparative samples (n=20) from each group were run according to the Healeon Medical CentriCyte 1000 protocol per manufacturer instructions outlined above. The mean sample sizes were 10.07 cc representing a post-centrifugation cycle of 800 g force for 5 minute per manufacturer's protocol and are described herein as "compressed adipose graft" or AD-tSVF.

Results

There were no discovered or reported donor complications from the sterile microcannula harvest, with each patient followed for a 30 day period following lipoaspiration procedure. Immediately after harvest, light compression of the donor sites was carried out for 24 hours, upon which removal was authorized, and replacement of dressing to simple type telfa pad covering until surface closure of an 18 gauge needle opening was noted. Patients were not treated with systemic antibiotic coverage. There were no medication reactions, surface irregularities, prolonged soreness, cellulitis, infections, hematomas, excessive hemorrhage, atypical scarring, or narcotic level pain encountered. Very minimal subdermal bruising was noted under the compressed closed cell, medical grade foam placed immediately post-operatively over the donor's harvest area. The harvesting was performed using a very dilute solution of 0.05% Xylocaine solution with 1:1,000,000 epinephrine as the carrier fuids in approxima solution to the volume extracted from each site. In the abdominal harvest sites, care was taken to accomplish the micro-aspiration beneath Scarpa's Fascia to avoid visible

surface irregularities. Post-harvesting medication recommended was acetaminophen 500 mg taken at 6-8 hour intervals if needed.

The frst half of the compressed fat grafts harvested (centrifugation only) was submitted for the standard closed protocol processing as described in Materials & Methods, providing enzymatic digestion and incubation cycles according the CentriCyte 1000 manufacturer's protocol. Neutralization and rinsing was completed using sterile 0.9% saline solution and 2 cc of autologous platelet poor plasma (PPP), rinsing the initial pelletized AD-cSVF from 2.5 cc to a volume of 50 cc per protocol. Final centrifugation protocol was then performed and 2.5 cc of pellet and infranatant removed for testing. These same numbers were used for comparison to the second sample which underwent emulsifcation using the ACM System device prior to enzymatic processing, and utilized to provide the baseline viabilities and counts which would potentially be altered by the mechanical disruption process. Prior to cellular separation, 0.2 cc of non-emulsifed AD-tSVF w attempted to be injected from a 1 cc luer syringe and standard 30 gauge needle, and found to consistently plug, preventing easy injection.

Table 1. Comparative outcomes of testing AD-tSVF versus emulsified AD-tSVF. (n=20 each sample set).

Sample Type n=20 (Each)	Volumes Tested Mean	cSVF Numbers /cc	Viability (Mean) %	TNC (RBC Lysis) Mean
Non-Emulsified AD-tSVF	10.08 cc Centrifuged (Compressed)	1.14×10^{-6}	98.2%	26.94×10^{-6}
Emulsified AD-tSVF	10.06 cc Centrifuged (Compressed)	1.36×10^{-6}	98.5%	28.38×10^{-6}

The second portion of compressed fat grafts was then submitted for the standard closed protocol recommended for use with the ACM (Nanofat) system device as described in Material & Methods above. At conclusion of this protocol, 0.2 cc of emulsifed AD-tSVF was loa in a 1 cc luer syringe and confrmed injection abi through a standard 5/6" 30 gauge needle. In each case, injection of emulsifed AD-tSVF was passed without diffculty. The balance of the emulsifed AD-tSVF wa submitted for the standard closed protocol processing exactly as the initial half of the compressed graft within 5 minutes of completion mechanical emulsifcation process. Neither specimen portion was thermally altered from time of harvest to introduction to the enzymatic digestion and shaker/incubation in the CentriCyte 1000 semi-automated machine. (*See Table 1.*)

The outcome analysis between the AD-tSVF and the emulsifed AD-tSVF specimens revealed no statistica signifcant difference in viability, numbers of cel cc, or total nucleated cells counts (TNC- measured with use of RBC lytic agent) between the compared sample groups. Paired t-test (< 0.202) and CI 1.96 (95%) was found between the paired samples tested, and reported as no statistical difference. It was observed that the cellular numbers, even with the loss of mature adipocytes created during the physica was compensated by slightly the collagenase blend compa AD-tSVF tested without enzymatic digestion. Author feels this may be a plausible explanation for the slightly higher, but comparable cellular concentrations in the tested emulsifed AD-tSVF an a result of more thorough enzymatic access to cellular attachments. (*See Table 1.*)

Importance of these observ signifcant in that the proc provided by the ACM (nanofat) device did not signifcantly lower stromal as measured with standard f no cell culture expansion, membrane characterizations, of isolation of component cells in the heterogeneous AD-tSVF populations typically encountered was examined. We are currently in the process of evaluation of these components, including *In Situ* processing and quantification of spec culture proliferative capabilities, and cryopreservation of cells derived from mechanic Testing of samples of emuls 1 cc luer syringes for injection ability through 25, 27, and 30 gauge needles. In every sample, this was demonstrated to be easily accomplished w resistance.

Discussion

Over the past decade, signi using compressed AD-tSVF and HD PRP combination

(Biocellular), has been extensively and effectively documented in clinical use. The Biocellular combination has confrmed a very high safety profile and has been reported in a large number of case reports, small and large case series, and from clinical trials using Adipose Tissue Complex derivatives.

Uses of both the non-emulsifed AD-tSVF and the Biocellular AD-tSVF have been published in peer-reviewed literature and well established in aesthetic-reconstructive surgery and variety of musculoskeletal indications. Uses of the emulsifed AD-tSVF product has been utilized for more than two years, and in initial reports have a very high safety and effcacy profles. Both forms of AD-tSVF continue to gain signifcant clinical uses and momentum, with providers utilizing the exclusion from guidelines claiming the "non- or less than minimally manipulated" category, using the Practice of Medicine exception in the USA (autologous, same day procedure, same surgeon, etc.). This study confrms the emulsifed AD-tSVF maintains very similar viabil and cellular numbers as the non-emulsifed AD-tSVF.

It is important to realize that the complex native cell groups and supportive tissues found in the ATC may, in fact, represent a more valuable and potent therapeutic entity, than trying to "guess" which cellular components or chemicals contained are the "key" to success. Many now believe, and have tested, advantages of leaving the component parts of the tSVF and reported that outcomes did best when the targeted site could choose from the variety of cells, growth factors, structural native scaffolding, and cytokines, rather than isolating cell types or structures. The "smorgasbord" approach seems to be more effective than selecting single cell types or chemical groups, all of known important in regenerative medicine. It is the author's experience that use of AD-tSVF combined with high density platelet-rich plasma (HD PRP) in aesthetic and orthopedic applications outperform either the tSVF <u>*OR*</u> the HD PRP alone. This combination is gaining acceptance as "Biocellular Therapy".

Current examination using clear, high-density platelet-rich platelets is currently underway, particularly for those applications seeking signifcantly higher concentra of growth factors, cytokine, and signal proteins with low hematocrit. In the author's experience, the use of AD-tSVF and HD PRP in which platelet concentrates containing > 1 x 10^6/ul tends to result in earlier and more ultrasound documentation of comprehensive improvement in most orthopedic applications. Uses for some facial and scalp skin surface changes (including hair regeneration) appear to respond to PRP concentrates of lower concentration (2.5 x measured baseline) levels when mixed with the emulsif injected in the dermal or close subdermal areas. This may be contributed to by the more extensive vascularity of the facial and scalp areas compared to distal extremities. Clinical trials are now underway to study the effects of lower and higher [illegible] measured patient baseline) products in these applications.

Clinical experiences for use of Biocellular treatment using emulsifed AD-tSVF and chronic wound healing suggest that, when combined with proper wound debridement, may offer additional trophic wound healing support to encourage marginal vascularity and healing effects within the surrounds epidermal and dermal elements.[47]

Great advantages for use of small needle injection ability is apparent from a clinical standpoint. Uses in aesthetic-plastic surgical and dermatological applications become feasible, permitting intradermal placement for improved vascularization, texture and appearance changes. In addition, uses for male and female hair loss can be addressed using a biocellular product, targeted at the hair bulge and follicular bulb area. The actual effects produced have been documented, but the mechanism of such changes remain a subject of current applied clinical research and controlled clinical trials. In Orthopedic Medicine, this facilitates use of much smaller diameter needles for patient comfort, while providing the ability to use in small joint or super

Conclusion

With the evolving applications in anti-aging, a variety of aesthetic and reconstructive uses, and small target guided injections in musculoskeletal applications, and the ability of using very small aperture needles are optimal for placement in dermal, chronic wounds, small joints, certain ligament-tendon targets. This is very attractive for the provider, and more easily tolerated by the patients.

This study provides evidence of the ability to achieve small particle AD-tSVF while preserving viable stem/stromal cells in high numbers can be attained using a sterile closed system with essentially minimal to no impact on stromal and cellular components. The ACM device provided a simple, closed mechanical processing in this comparison study, and was shown to effectively compared to use of AD-tSVF, in a safe and cost effective manner. Use of the StromaCell (Microaire Aesthetics, Charlottesville, VA, USA), Lipogems (Lipogems International S.p.A, Milan, Italy), REVOLVE systems do accomplish some degree of emulsifcation, but at much higher equipme consumable cost, and inconsistent inject ability via small needles. The use of the ACM system, as tested, was simple to use, markedly less expensive, required very little instrumentation, and provided an easy means to mix PRP to create a Biocellular therapeutic injectable. Reduction of consumable supplies costs required to create an emulsifed AD-tSVF + PRP concentrates can effectively reduce patient costs for such care, making it more available to patients in need.

At the time of this writing, the author is treating and tracking outcomes in each of the discussed areas of potential uses for Biocellular emulsifed AD-tSVF injections. The ability to provide a small particle stromal and Biocellular combination is clearly considered to offer a high potential for site-specific, cellular prolife reactive capabilities to targets. It is believed that the local microenvironment (niche) where the biocellular products are carefully placed, support the early acceptance and provide important immunomodulatory capabilities and advantages reported in use of components of the adipose-derived stromal complex.

We provide initial evidence to support the ability to effectively reduce the AD-tSVF particle size for small bore needle injections, while not destroying the cellular and stromal interactions. We are currently participating in a multi-centric, controlled Clinical Trials for use of emulsifed AD-tSVF with PRP and cell enrichment AD-tSVF with PRP concentrates in a hair regenerative applications. Many more controlled trials and studies are needed to further confirm the long-term safety effcacy of such processing and therapies, with eff to determine optimal cellular types and concentrations. Learning how mechanical emulsifcation impacts AD-tSVF, is an important first step in determining potential impact on the heterogeneous cell population.

Appreciation of the complex, heterogeneous elements comprising emulsifed AD-tSV knowledge of threshold viability, types, and numbers of cells and matrix needed for optimal clinical effects. This will be advanced with additional studies examining cell characterization, and examine their important proliferative capabilities and energy. We are currently examining the component processes and analyzing the important growth factors, exosomes, signal proteins, and various micro-environme to understanding how we can optimize the uses of biocellular regenerative medicine applications.

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There are no conficts of in

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