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Research Article

A Comparative Analysis of Cell Yield and Viability of Stromal Vascular Fraction from Lipoaspirates Harvested by Ultrasound and Suction-assisted Liposuction

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ABSTRACT

This study aims to compare two methodologies routinely used for lipoaspiration- a standard Suction Assisted Liposuction (SAL) and Ultra-sound Assisted Lipoaspiration (UAL) on yield, viability, surface markers and trilineage differentiation potential of isolated SVF from both these samples. Subcutaneous fat tissue was collected by UAL and SAL from the same patient (n=8). Stromal Vascular fraction was isolated by enzymatic digestion and the cell yield and viability were compared. Further, the surface markers from both UAL and SAL isolated SVF was assessed. The isolated SVF was used to isolate adipose-derived stem cells (ADSC's) and the surface markers and trilineage differentiation potential were compared. Statistical analysis: All statistical analysis and graph generation were performed using GraphPad Prism version 9.1.1. Results: The results indicate no significant difference in cell, viability and surface markers of SVF isolated from UAL and SAL. Further, we demonstrate that ADSC's isolated from the SVF of both UAL and SAL are capable of trilineage differentiation. There is no statistically significant difference in the yield and viability of SVF isolated from both UAL and SAL techniques. Since UAL can be used for larger volumes of lipoaspiration, we suggest that UAL would be a suitable method for large volume aspirations that do not affect cell yield and viability. Further expansion of these cells demonstrates that they are capable of trilineage differentiation, indicating their possible use in regenerative therapies.

Introduction

Regenerative medicine is a rapidly emerging field that focuses on restoring injured tissue instead of symptomatic treatment. The domain of regenerative medicine encompasses numerous strategies ranging from bio-scaffolds and growth factors to live cells.[1] Several such strategies are commercially available while several are under trial.[1]

Adult stem cells are emerging as a promising cell type for cell-based therapies in regenerative medicine. Human adipose tissue has evolved as a new source of adult stem cells for obtaining multi-potent stem cells. Emerging literature has identified these Adipose Derived Stem Cells (ADSCs) as an ideal application for regenerative therapies. The main advantage of these ADSCs over Mesenchymal Stem Cells (MSCs) derived from other sources like bone marrow is the ease of harvesting, using less invasive techniques. ADSCs have been reported as multi-potent and have demonstrated their tri-lineage differentiation potential.[2] Upon isolation, these cells have the potential to proliferate for many passages in their undifferentiated form. Also, under the right stimulus, these cells can

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differentiate into all three lineages – mesoderm, ectoderm and endoderm.[3] Additionally, ADSCs are considered as immune-privileged cells since they do not express the major histocompatibility complex (MHC) II and co-stimulatory molecules, such as CD86, CD40 or CD80 and express a low level of MHC I,[4-6] thereby making them viable candidates for allogenic therapy.

Among adult MSC, ADSCs are most widely studied. Adipose tissue or fat is obtained abundantly from routine liposuction surgeries and is generated as a biological waste tissue post-surgery. First isolated by Zuk *et al*,[6] the AD-MSC can be isolated and propagated with routine cells culture methodologies. They share many biological properties with the bone marrow-derived MSC and are also effective in clinical applications.[7] ADSCs' therapeutic and regenerative potential in all three lineages has been extensively studied.[8]

Liposuction or lipoaspiration is a widely used, effective, and safe method for obtaining adipose tissue from the body.[9,10] In addition to the traditional suction-based aspiration, a new approach of ultrasound-assisted liposuction, known as VASER (Vibration Amplification of Sound Energy at Resonance), is now widely used. While the traditional method uses suction to break up the fat cells from tissues, the VASER technique uses pulsed ultrasound waves to break up the fat tissue.[10] The VASER technique has improved skin retraction and reduced blood flow compared to the traditional suction-based aspiration.[11]

The quality of ADSCs obtained from the adipose tissue can be affected by the tissues harvesting method. For instance, the laser-assisted lipoaspiration has demonstrated a lower cellular yield and viability of ADSCs compared to the traditional method. However, cells obtained by both methods showed differentiation potential,[12] whereas ultrasound-assisted lipoaspiration has shown comparable cell numbers, viability and differentiation potential to traditional suction-based aspiration[13].

In this study, we have compared the cell yield, viability, differentiation potential and surface marker expression of ADSCs obtained by Ultrasound Assisted Liposuction (UAL) to those obtained by traditional Suction Assisted Liposuction (SAL).

Materials and Methods

The study was conducted under the Institutional review board of Wockhardt Hospitals, Mumbai, India (study number WHIRB/04-WH/WCRM/Auotologous/ATSVF – CALA /2011), and all written consents were obtained before the procedure. Human Lipoaspirates were collected from 8 healthy adult female patients, undergoing elective cosmetic surgery. Each patient underwent liposuction using UAL and SAL techniques from two incisions at an identical site. Lipoaspirates were collected, the volume of each paired lipoaspirate was 100 ml. The collected lipoaspirate was transferred to sterile 500 mL self-locking, leak proof collection bottles (Genetix – Cat. No. GX11500B) and the bottles are labeled appropriately as "SAL" or "UAL" with a unique identification for every patient. The sample bottles were transported to the lab for further processing.

Isolation of Stromal Vascular Fraction (SVF) and Immunophenotyping Flow Cytometry:

Stromal Vascular Fraction was isolated using the method described.[14,15] SVF was isolated from samples of 8 patients between the ages 32 and 46 years, had no medical comorbidities and were undergoing elective liposuction of the abdomen.

Briefly, 100 mL of the UAL and SAL sample from a single donor were digested using Type 1 Collagenase (GIBCO – Cat. No. 17100017) for 2 hours, with vigorous shaking. The lipoaspirate was transferred to 50 mL sterile conical tubes and centrifuged to obtain the SVF pellet. The pellet was further processed to obtain a uniform cell suspension. The pellet was subjected to Red blood cell (RBC) lysis and the cells were washed using 1X sterile Phosphate Buffered Saline (PBS) by spinning at 300xg for 10 minutes. The pellet was resuspended in 1X PBS to form a single cell suspension and strained through 100µm (BD Cat. No. 352360), 70µm (BD Cat. No. 353250) and 40µm (BD Cat. No. 353240) cell strainers. Cell viability was performed using 7AAD. The cell count was taken using an automated cell counter – Biorad TC-20. Yield of SVF, per ml of lipoaspirate from UAL and SAL fat samples, was compared.

SVF cells were characterized using surface markers CD31, CD34, CD45, CD73, CD90, CD105, CD146, CD271, HLA-ABC, HLA-DR. Viability was performed using 7AAD as per the manufacturer's instruction using a BD FACS Calibur.

Cell Culture

The freshly isolated SVF was plated on a 24 well plate, at a seeding density of 40,000 cells / cm^2, in DMEM-LG (GIBCO – Cat No. 11885084) supplemented with 10% FBS (GIBCO – Cat. No. 10270-106) and 1% Antibiotic / Antimycotic solution (GIBCO – Cat No.15240062). The SVF cells were cultured at 37°C in the presence of 5% CO_2 and 95% humidity. Media change was done every 4 days till attached cells were confluent. For each sample, there were two 24 well plates seeded, one plate seeded with freshly isolated SVF obtained from UAL and the second plate seeded with freshly isolated SVF obtained from SAL method.

9 wells of each plate were used for differentiation (3 wells used as control – undifferentiated, 2 wells used for adipogenic differentiation, 2 for chondrogenic differentiation, 2 for osteogenic differentiation). The remainder 15 wells were used for immunophenotyping as passage 0.

In vitro Adipogenic Differentiation

For adipogenic differentiation the freshly isolated SVF obtained from the patients (n=8) was seeded in duplicate as described earlier.[16] At 80% confluency the cells were exposed to adipogenic differentiation media (GIBCO - A1007001). Oil Red O staining was performed after 21 days.

In vitro Chondrogenic Differentiation

For chondrogenic differentiation, the freshly isolated SVF obtained from the patients (n=8) was seeded in duplicate as described earlier.[17] At 80% confluency the cells were exposed to chondrogenic differentiation media (GIBCO A1007101). Alcian Blue staining was performed after 21 days.

In vitro Osteogenic Differentiation

Freshly isolated SVF from all patient samples (n=8) were seeded in a 24 well plate in duplicates as described earlier.[18] On achieving 80% confluency, the cells were exposed to an osteogenic differentiation medium (GIBCO A1007201). Alizarin red staining was performed and quantified after 21 days to assay extracellular mineralization, as previously described.

Staining of all Samples was Performed in Duplicate

DNA Ploidy Analysis

To ensure that the isolated cells during culture have a stable genome , DNA ploidy of the freshly harvested attached cells from the 24 well plate was performed as per the manufacturers' instructions (Becton Dickinson, BD Cycletest^{TM} Plus DNA reagent kit – 340242).

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism. One way ANOVA was performed for Counts, Viability, and Cell Surface markers from UAL and SAL samples.

Results

SVF isolated from UAL and SAL human lipoaspirates were assessed for cell yields and cell viability. SVF from SAL lipoaspirates exhibited moderately higher cell yield (total cell counts) as compared to SVF from UAL lipoaspirates (Fig. 1A) with a p-value of 0.44, which was statistically insignificant. Next, we examined whether differences in cell yields impacted cell viability. Interestingly, SVF from SAL lipoaspirate had increased cell viability without statistical significance compared to SVF from UAL lipoaspirate (Fig. 1B), with a p-value of 0.33. Therefore, a comparison of Counts and Viability of SVF from UAL and SAL lipoaspirates showed that UAL does not affect the overall quality of SVF.

Further, we explored whether differences in cell yield and viability alter the expression of stem cell lineage markers as outlined by ISCT. Immuno-phenotyping was performed on ADSCs for the following surface markers using flow cytometry. CD73, CD105, CD90 and other stem cell markers of different origins like CD146, HLA-ABC, CD271, and negative markers like CD34, CD45, CD31 and HLA-DR in ex-vivo.

In *ex-vivo* analysis, statistically non-significant increases in ADSCs lineage markers CD73 (p=0.83), CD90 (p=0.66), CD105 (p=0.69), CD146 (p=0.31), HLA-ABC (p=0.78) were observed, while CD271 was moderately decreased (p=0.78) in SVF from SAL samples as compared to SVF from UAL samples. Similarly, ADSC negative markers like CD31 (p=0.87), HLA-DR (p= 0.54) and CD34 (p=0.38) was non-significantly increased in SVF from SAL as compared to SVF from UAL (Table 1). Though hematopoietic lineage markers differed, interestingly CD45 did not show any considerable differences between SVF from UAL and SVF from SAL samples (p=0.52) (Fig. 2).

Marker expression of ADSCs in cell cultures suggested non-significant reductions in CD45 (p=0.20), moderately increased CD105 (p=0.58), and unaltered CD73 (p=0.99) and CD90 (p=0.83) expression in ADSCs isolated from SAL samples as compared to ADSCs isolated from UAL samples (Table 2, Fig. 3).

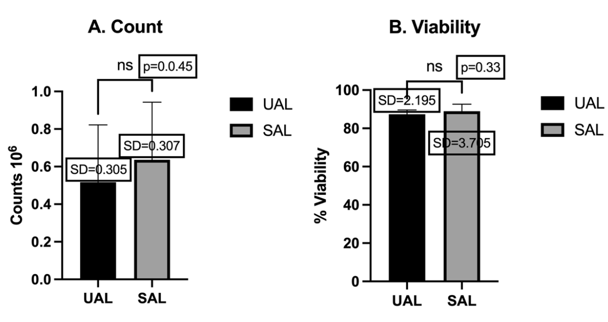

Fig. 1: A. Lipoaspirates obtained from UAL and SAL yield similar cell counts of SVF. counts compared from UAL and SAL are similar upon statistical analysis of one-way ANOVA with p-value= 0.45, which is non-significant.
B. Comparison of viability by flowcytometry using 7 AAD. The viability of SVF obtained from UAL and SAL was found to be similar with p=0.33 using one-way ANOVA, which is not significant.

Table 1: Flowcytometry surface markers of SVF isolated by UAL and SAL with their mean and standard deviation (SD)

	UAL			*SAL*		
CD Marker	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*
CD 31	26.731	10.023	8	27.968	18.547	8
CD 34	56.443	21.370	8	64.899	16.282	8
CD 45	34.409	13.826	8	29.733	14.987	8
CD 73	58.395	10.322	8	56.560	22.237	8
CD 90	65.000	23.053	8	69.750	19.345	8
CD 105	30.570	21.660	8	35.261	25.100	8
CD 146	41.139	22.334	8	52.503	21.299	8
CD 271	19.243	13.109	8	19.993	19.276	8
HLA ABC	73.500	22.956	8	76.246	16.714	8
HLA DR	45.376	18.297	8	51.534	21.282	8

The induction of differentiation of ADSCs isolated from SVF, at P0 showed differentiation into Adipocytes, Osteocytes and Chondrocytes (Fig. 4).

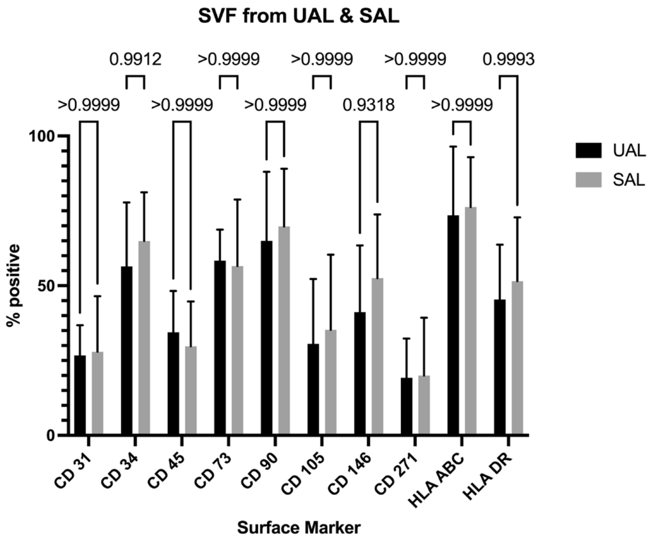

Fig. 2: Flow-cytometric analysis of surface markers of SVF isolated by UAL and SAL. analysis was performed as per ISCT guidelines. key markers for adipose derived stem cells are CD 73, CD 90, CD 105 and CD 146. on comparing these markers using one way ANOVA, the p values indicated no significant difference in SVF isolated by UAL and SAL.

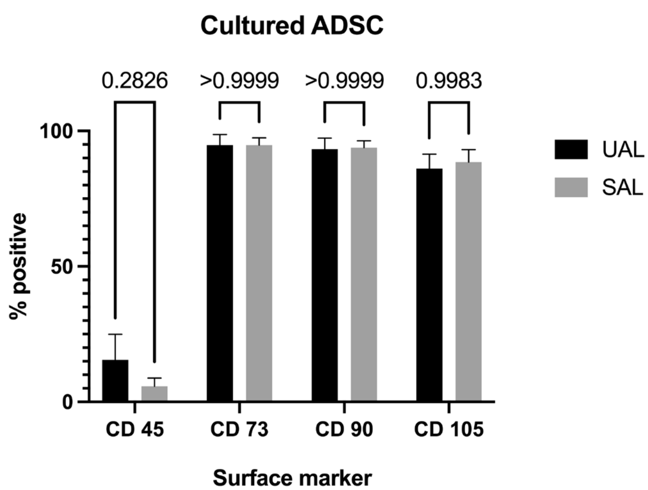

Fig. 3: Surface marker analysis of cultured ADSCs isolated by UAL and SAL. SVF obtained from both UAL and SAL were plated and adherent cells were analysed for flowcytometry markers. markers were selected as per ISCT guidelines.

Table 2: Flowcytometry surface markers of cultured ADSCs isolated by UAL and SAL with their mean and standard deviation (SD)

	UAL			*SAL*		
CD Marker	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*
CD 45	15.577	9.390	3	5.763	2.987	3
CD 73	94.770	3.919	3	94.757	2.702	3
CD 90	93.257	4.103	3	93.867	2.535	3
CD 105	86.113	5.345	3	88.527	4.575	3

The collective data suggest that UAL lipoaspirates have lesser cell yield with reduced cell viability that affects the ADSC marker expression albeit without statistical significance.

DNA ploidy analysis was performed to assess DNA Stability and to ensure that the culture conditions did not alter the ploidy status of these cells. 95% of population of cells was distributed in two peaks of G1 and G2/M phase, indicating that culture conditions did not alter the DNA status of the cells (Fig. 5).

The adherent cells demonstrated high positivity of CD73, CD90 and CD105, indicative of mesenchymal stem cell fraction, which are present in adherent cultures in

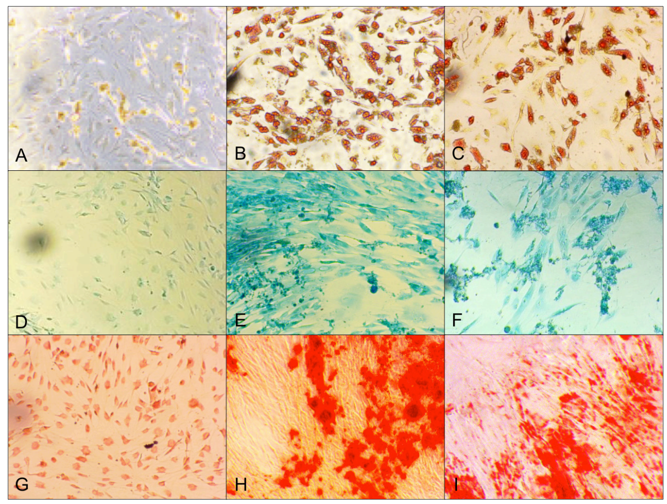

Fig. 4: UAL and SAL differentiation at P0. A - adipogenic control (10x), B – adipogenic differentiation – UAL (10x), C - adipogenic differentiation – SAL (10x), D - chondrogenic control (10x), E - chondrogenic differentiation – UAL (10x), F - chondrogenic differentiation – SAL (10x), G – osteogenic control (10x), H - osteogenic differentiation – UAL (10x), I - osteogenic differentiation – SAL (10x). all images taken at microscopic magnification of 10x.

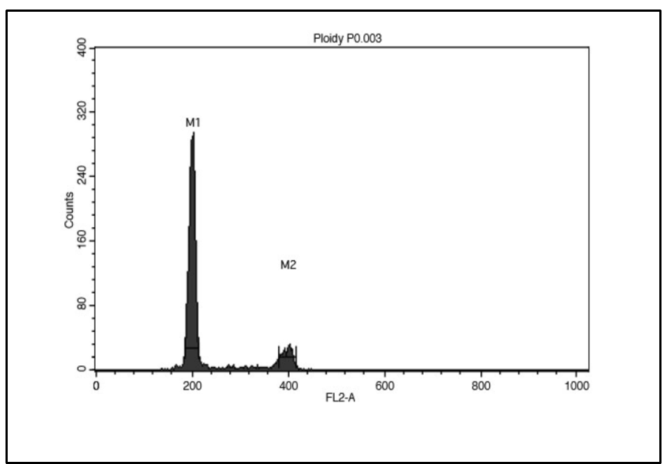

Fig. 5: DNA ploidy done on ADSCs isolated from UAL and SAL. DNA ploidy analysis was performed to assess DNA Stability and to ensure that the culture conditions did not alter the ploidy status of these cells. 95% of population of cells distributed in two peaks of G1 and G2/M phase, indicating that culture conditions did not alter the DNA status of the cells.

large numbers. low positivity of CD45 markers indicate that adherent cells are of mesenchymal origin. on performing one way ANOVA between UAL and SAL for each marker no significant difference was found.

Discussion

Regenerative medicine is an emerging and rapidly evolving field of research and therapeutics to restore, maintain and improve body functions and it aims at the 'repair, replacement or regeneration of cells, tissue or organs to restore impaired function.[19] It aids the body in forming new functional tissue to replace lost or defective tissue. Ultimately, this will help to provide therapeutic treatment for conditions where current therapies are inadequate.

Stem cells isolated from Bone marrow have been studied extensively, adipose stem cells have also emerged as an alternate source of stem cells. Adipose-derived stem cells have demonstrated efficacy in regenerative medicine.[20]

Adipose stem cells have not only demonstrated their comparable therapeutic effect as compared to bone marrow stem cells, and the adipose-derived stem cells have a significant advantage over bone marrow-derived stem cells in that they are more abundant in number and the ease of isolation makes adipose a suitable source for stem cells.[21]

Liposuction is routinely employed by plastic surgeons concerned with removing subcutaneous adipose deposits in various areas in the body to improve Fig. flaws and create a more balanced physique. Liposuction technologies have significantly advanced over time. The procedure has quickly become one of the most commonly performed cosmetic procedures worldwide. Numerous machines/ systems exist. No one system has definitively proven to be superior to the other. Liposuction is a safe and reliable method of removing subcutaneous fat to create a more harmonious silhouette in a disagreeable biological condition caused by excess fat deposition in relatively common distribution patterns.[10]

The emergence of lipoaspirates as an important source of stem cells has led to research exploring the effects of different liposuction techniques on the harvested tissue. Given the wide variety of techniques available for use in clinical practice, this research is critically important to determine the suitability of tissue and cells derived from lipoaspirate samples for regenerative medicine purposes.

The viability is an important indicator of the damage inflicted on adipose tissue during harvest. The multilineage differentiation capacity of adipose-derived stem cells dictates their value for tissue engineering.

Previous studies have demonstrated that SAL-derived adipose stem cells have significantly higher expression levels of Adipogenic differentiation markers.[12] There were no significant differences between UAL- and SAL-derived ASCs' Adipogenic gene expression profiles. Similarly, we could not detect any disadvantage for UAL-derived ASCs in a qualitative chondrogenic differentiation assay.

On analysis of counts and viability of SVF isolated from both UAL and SAL, there was no significant difference. To avoid any sample variability, UAL and SAL were collected from two different sites in the abdominal area during the same procedure. ADSCs isolated from both UAL and SAL at P0, differentiated into adipocytes, chondrocytes and osteocytes when induced with differentiation media. Hence, it can be concluded that even though the cell yield from SAL was more and viability along with cell surface markers did not have a significant difference, the safe aspiration of large quantities of UAL compensates for the lesser number of cells. It would be interesting to power this study with a larger number of patients and to analyze the effects of long-term cultures of ADSCs isolated from these two procedures. We have additionally analyzed DNA Ploidy on P0 ADSCs (Fig. 5) isolated from both these procedures and have found these cells to be normal diploid cells. DNA Ploidy status of these cells should be followed over a series of passages to ascertain the cells in culture retain their original ploidy characteristics.

These data suggest that UAL-harvested ADSCs retain their multipotency and have comparable efficacy to SAL-derived ADSCs for applications in tissue engineering. However, further in vivo studies are needed to confirm the full potential of UAL-harvested ADSCs in hard and soft tissue engineering.

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