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PHENOTYPIC EXPRESSION OF MESENCHYMAL STEM CELLS: COMPARING SELECTIVE TISSUE-ENGINEERED PHOTOSTIMULATION TECHNIQUE AND CONVENTIONAL LIPOSUCTION TECHNIQUE.

KEY WORDS: Adipose tissue-derived stem cells, adipose tissue, conventional liposuction, Selective Tissue Engineering Photo-stimulation.

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ABSTRACT

Introduction: Adipose-derived stem cells (ASCs) show some membrane markers that enable cell identification by flow cytometry. One of them which is essential during the angiogenesis process is CD105 (Endoglin). This is very important for various pathologies where it is required angiogenesis to regenerate tissues or organs affected by various acquired or congenital noxae. **Objective:** To determine the phenotypic expression, CD105 angiogenesis factor, of ASCs obtained by Selective Tissue Engineering Photostimulation (STEP ™) with infrared light of 1210 nm compared to those obtained by Gold Standard Suction assisted lipectomy/conventional liposuction technique (SAL). **Results:** ASCs obtained by STEP™ technique with 1210 nm laser were found to be highly viable (> 97%) and showed increased CD105 expression (90%) and only <5% with the SAL. **Conclusion:** ASC had been obtained after application of the STEP™ technique is highly viable and show higher expression of specific marker CD105 than the ASC obtained by SAL.

INTRODUCTION

A stem cell is characterized by its ability to renew itself and to differentiate into multiple cell lines. There are diverse kinds of stem cells available: embryonic stem cells (ESCs) and adult stem cells. Although the therapeutic potential of ESCs seems great due to their capacity to renew and their pluripotentiality, they pose many practical and ethical limitations. Adult stem cells can be obtained from different tissues and do not have the ethical limitations of ESCs. Mesenchymal stromal cells (MSC) are a type of adult stem cells that can be obtained from adipose tissue, bone marrow, umbilical cord blood and other fetal tissues. MSCs were originally called fibroblast colony-forming units and display adhesion capabilities in culture medium, have great capacity to proliferate and generate connective tissue lineage progenies (bone, cartilage, tendon, fat)[1].

The multilineage differentiation of cells obtained from enzymatic digestion of human adipose tissue was described as early as 2001[1]. Various research studies that assessed such findings observed multilineage differentiation from one single cell [2,3,4,5]. Adipose tissue-derived stem cells (ASC) are different because they have a high proliferation capacity and multilineage differentiation, express CD 105/ endoglin, CD 44, CD 90/ Thy 1 and SH2 as well, and are CD45 and CD31 negative [6,7]. Phenotypically they are very similar to MSCs obtained from bone marrow.

Adipose-derived stem cells (ASCs) have been reported to be less demanding than bone marrow MSCs in their nutrition requirements for *in vitro* growth [1,2]. MSCs are known to represent the 1:50 000 to 1:1 000 000 of the bone marrow total cellularity in adults, while the amount of ASCs varies between 1:1000 to 1:30 in adult adipose tissue [8,9]. The relative frequency of clonogenic cells is an important reason for using adipose tissue as a source of stem cells. Another reason is the relative easiness and low mortality rate when obtained from adipose tissue. A total volume of up to five liters of tissue can be immediately available after a simple process that poses minimum risk for the patient[10].

Major advances in technology applied to aesthetic surgery have allowed us to offer benefits to patients like less trauma and better natural aesthetics results [11]. Liposuction, the Suction assisted lipectomy (SAL) a technique born in the 70's [12,13] has gone through several modifications that have optimized its application thereby casting no doubt on its effectiveness [14]. Laser (with affinity for water) applied to the treatment of lipodystrophy has revolutionized the liposuction technique, offering less surgical trauma for patients which translates into less pain, less blood loss, no hematomas, minimum ecchymosis, less edema and insignificant skin retraction, with a short recovery period and reduced hospital cost [15,16,17]. Unfortunately, the adipocytes and the stroma were destroyed during the laser emission making the aspirated tissue an inadequate material for fat grafting[18].

Absorption/affinity curves in relation to (laser) light wavelength are currently known for its use in various targets, water, hemoglobin, including human fat. The Laser property depends on wavelength. This allows us the use of the most suitable laser equipment in protocols on fat tissue [18,19]. The One STEP liposculpture technique (STEP™) [20,21], using a novel 1210-nm wavelength infrared light, yields high quality adipose tissue (parenchyma & stroma) with less post procedure cell morbidity for laboratory processing, making it easier to verify whether the cellular material obtained is viable and possesses the proper phenotype. In previous studies applying the STEP technique, we confirm the presence of positive markers: CD44, CD73, CD90 and CD105, absence of (negative) differentiation markers such as CD11b, CD19, CD34, CD45 and HLA-DR [22].

Under this context, the purpose of this study is to determine the differences on phenotypic expression, of the CD105 angiogenesis factor, of adipose tissue-derived stem cells obtained by Selective Tissue Engineering Photostimulation (STEP™) compared to those obtained by SAL technique.

MATERIALSY METHODS

Thirty samples from six patients who underwent body contouring plastic surgery was analyzed. The extracted

adipose tissue was discarded, but the extracted adipose tissue was used with the patients' permission for analysis. Patients were divided into two groups of six each: the first group was the One STEP group and the second group was the SAL. All the fat collected was obtained from abdominal liposuction.

Technique:

One S.T.E.P.™ (Selective Tissue Engineering Photostimulation)

The One STEP, group followed the protocol established by the experience and research of the research team led by Dr. Centurión [20,21] was followed.

Adipose tissue samples were obtained from the abdominal area using the One STEP™ technique (Medilaser 1210 nm - DMC). This technique is based on the use of a 1210-nm wavelength infrared light, using a 600 μm optical fiber introduced through the subcutaneous layer inside a 2-mm diameter atraumatic cannula and subsequently collected by a 3.5-mm diameter aspiration cannula. The laser irradiation was performed after a cold solution at 4-degree C + adrenaline 1:500 injected between subcutaneous and muscular fascia (wet infiltration) in order to promote a vasoconstriction effect. The samples collected were stored in a sterile 1000 ml collector bag and transported immediately to the laboratory of the Institute for Cryopreservation and Cellular Therapy (ICCT-Criocord).

This infrared light has selective photochemical stimulation; this light stimulates the endogenous collagenase enzyme, allows the release of ASCs, present in the connective tissue into the fat tissue, without damaging them and stimulating the enzyme Cytochrome C oxidase of the ASC mitochondria [23,24].

Isolation of ASC with conventional liposuction or Suction-assisted lipectomy (SAL).

The SAL group was following the gold standard technique of conventional liposuction with tumescent infiltration technique and its mechanical disruption mechanism.

Stromal Vascular Fraction isolation for analysis

In order to isolate the stromal vascular fraction (SVF) in both groups, the aspirated tissue was washed with equal volumes of HBSS until the infranatant was clear and no blood clots were present. After that, using the collagenase enzyme the extracellular matrix digestion was performed. The enzyme with the aspirated tissue were incubated at 37 °C in slow and steady agitation for two hours until a uniform semi liquid appearance could be seen. The enzymatic activity was neutralized using Dulbecco's Modified Eagle medium with 10% FBS. The product was put into 50ml conical tubes and centrifuged at 400g at 20°C for 10 minutes. With a 50ml disposable sterile pipette all of the infranatant was discarded. The 50 ml conical tubes went through a rinsing process with HBSS and the material then filtered through a 100 μm nylon mesh. The red blood cell lysis was performed during the cytometry process using the aforementioned lysing solution.

All materials used to isolate the ASCs from the adipose tissue were from Becton Dickinson. The collagenase enzyme used was from Sigma-Aldrich. The Dulbecco's Modified Eagle medium, the fetal bovine serum (FBS) and the Hanks balanced salt solution (HBSS) were acquired from Gibco.

Analysis of fat derived ASCs.

ASCs were analyzed by flow cytometry, which was performed immediately after obtaining the filtered material. To assess cell viability, CD34, CD45, CD90 and CD105 as well as 7AAD were used as markers for MSCs and ASCs. Along with cell identification, absolute cell counting was performed using flow counting fluorospheres.

Flow Cytometry was performed using a FC500 Beckman Coulter cytometer. The acquisition and analysis software used was CXP. The lysing solution was bought from Beckman Coulter. The absolute count was made with Flow Count Fluorospheres (Beckman Coulter).

Ethical considerations.

The patients followed the protocol established by the clinic's plastic surgery department. The patients were asked in writing, prior to surgery, for their authorization to use the adipose tissue for subsequent analysis. The purpose of the study was explained to each patient and informed consent was obtained, following international standards for ethical research in developing countries. Finally, during the implementation of the study, the ethical principles outlined in the Declaration of Helsinki were respected.

RESULTS

Flow cytometry analysis of material obtained from adipose tissue ASCs using STEP™ and SAL. It found cell viability greater than 97% in both groups (Figure 1).

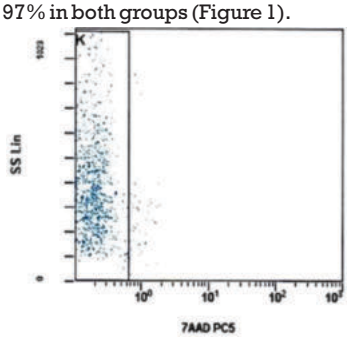

Fig 1. Viable cells can be seen (7AAD negative) at >97% in ASCs obtained via STEP™

It was found that in both groups all ASCs were CD45 negative, CD34 negative and CD90 positive. But there was a difference in the expression of cell surface markers in ASCs obtained by One STEP™ compared to SAL, this was the expression of CD105. In the SAL group it was decreased (<5%) or negative (Figure 2a) while in the One STEP group it was positive, with high expression (>90%) (Figure 2b).

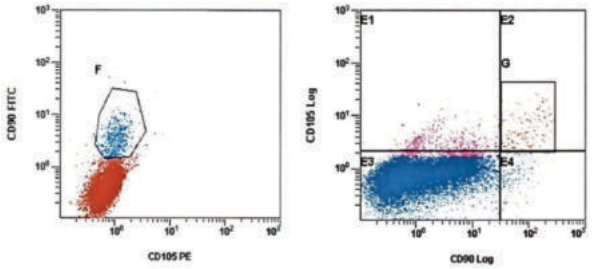

Fig 2. A) ASCs obtained via SAL exhibit CD90 positive and CD105 negative.
B) ASCs obtained via STEP™ exhibit CD90 positive and CD105 positive.

DISCUSSION

MSCs are mesodermal in origin and are considered to be multipotent with limited self-renewal capabilities and with capacity to develop tissues within the same embryonic layer. Recent publications, however, suggest they may differentiate from other embryonic origin tissues [25].

The function of the presence of stem cells in diverse tissues is to maintain and regenerate tissues. They can be found in connective tissues (stroma), mainly in mesodermal origin organs. This is why adipose tissue is recognized as an ideal source, because it allows to harvest a great number of cells safely and without causing adverse events during the liposuction procedure[28]. In 2000, the International Society for Cellular Therapy (ISCT) changed the term stem cell to "Mesenchymal Multipotent Stromal Cell", though the acronym MSC can still be used. The ISCT has established

criteria to define MSCs: First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, 95% of the MSC population must express CD105, CD73 and CD90 markers, as measured by flow cytometry and lack expression (<2%) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Third, the cells must be able to differentiate in osteoblasts, adipocytes and chondroblasts under standard culture conditions [26]. We demonstrate that the STEP™ samples showed all the criteria to be defined as MSC[22].

Stem cells from adipose tissue are defined as "Adipose tissue-derived stromal cells" (ADSC), though currently called ASCs [26], they have become a sub-type of MSCs. The markers more widely used for identification of ASCs by cytometry are CD105, CD90, CD34 and CD45. CD105 (Endoglin) is a glycoprotein located in the cellular membrane, and acts as a receptor for the transforming growth factor-beta. It can be found in diverse cell types, such as activated macrophages, fibroblasts, endothelial cells and smooth muscle cells. Research studies in mice lacking the Endoglin gene (CD105 negative) showed mice die due to defective angiogenesis that evidenced impairment in the vascular smooth muscle sheath of embryonic blood vessels [27]. Hence, the CD105 (high) expression gives to ASCs a relevant characteristic for angiogenesis processes present in the perivascular ASC, which are the most important cells compared to the paravascular ASC with less angiogenesis property. Our study revealed that the STEP technique preserves and harvest the perivascular niche (ASCs) for adequate regenerative response, where the angiogenesis is a main goal for regenerative purposes. Zocchi (2019) used an Adipose Tissue sonication, an ultrasonic-assisted lipoplasty, in order to emulsified the fat to isolate the stroma and ASCs maintaining the integrity and vitality of the tissue, and after flow cytofluorometry he found only 3,8% CD 105 positive cells[28].

Several authors have recommended not to use laser energy to obtain ASCs by stating that it diminishes cell viability significantly [18]. In our experience, the use of STEP™ laser 1210-nm with its Selective Tissue Engineering Photo-stimulation property does not affect viability of ASCs, and it is an optimal technique to obtain adipose tissue[20,21,29]. Moreover, upon isolation, ASCs obtained via STEP™ exhibit a significant presence of the CD105 expression (90%) unlike those obtained via SAL (<5%) whose has a negative expression. We have concluded that the MSC harvest with STEP™ are perivascular with a higher regenerative and angiogenesis potential.

The use of laser in regenerative therapy has turned into a useful tool for the treatment of diverse pathologies (Markolf Niemz: Laser tissue interaction), and its use during liposuction allows us to isolate ASCs that have a special phenotype with pro angiogenic potential. The bio stimulating effects of laser have already been demonstrated in studies assessing the increase in the proliferation of fibroblasts and osteoblasts. It has been demonstrated that the migration and proliferation of endothelial cells with CD105 increase when stimulated under STEP™ [21,29].

The 1210 nm wavelength laser cause an intense absorption peak of the fat (CH_2 vibrational modes) [30], in contrast to other wavelength (940, 980, 1064, 1440 nm, 1470 nm, etc.) that exhibit lower absorption by fat, hence lower energies from the laser at 1210 nm are required in order to liberate both adipocytes and viable ASC from the collagen network, that prevent a thermal damage to the fat tissue takes place, as is commonly caused by other lasers. It is also hypothesized that photon-biostimulation (PBM) effects occur during the laser assisted harvesting step, however the exact mechanisms remain to be investigated.

It's important to point out that the used laser output power and linear energy density, LEED ((pull in and back by sweeping the fiber through tunnels within the subcutaneous fat layer) is not enough for preventing the damaging of the adipocytes nor the ASC liberated from fat or liberated from the connective tissue, is necessary to follow the STEP™ technique with the established parameters.

To our knowledge this is the first report worldwide about the high viability of ASCs obtained via STEP™ laser. Also, is the first report about ASC type in adipose tissues obtained through Gold standard technique (SAL) and compared with a new "fat friendly liposuction technique. The CD105 expression in ASCs obtained via STEP™ technique was determined and show an important difference when compared to the SAL, and it was found they were present at a high percentage. Further studies will focus on *in vitro* and pre-experimental ASCs obtained via STEP™ technique in order to confirm their pro-angiogenic potential.

CONCLUSION:

Our study demonstrates that the ASC obtained after application of the STEP ™ laser technique is highly viable and show a higher expression of the specific markers CD105 (an endoglin related to angiogenesis) than the ASC obtained by Conventional Liposuction/SAL.

The One STEP™ new Laser technique offer more ASC viability, with an optimal phenotype of MSC for Regenerative Medicine or Cryopreservation

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