Influence of Negative Pressure on the Viability of Adipocytes and Mesenchymal Stem Cell, Considering the Device Method Used to Harvest Fat Tissue

Luiz Charles-de-Sá, MD; Natale F. Gontijo de Amorim, MD; Danielle Dantas, MD; Joh Victor Han, MD; Paola Amable, PhD; Marcus Vinicius Telles Teixeira, PhD; Pedro Luiz de Araújo, PhD; Walter Link, PhD; Radovan Borojevich, PhD; and Gino Rigotti, MD

Abstract

Background: Transplanted adipose tissue has many applications in regenerative medicine. However, fat grafting yields unpredictable results because the fat that is transferred can suffer variable degrees of fat reabsorption. It is necessary to identify methods and maneuvers to minimize reabsorption rates and provide predictable long-term results.

Objectives: Our study aimed to identify the optimal method of harvesting, as well as the optimal pressure regime for fat aspiration. The primary objective was to assess the degree of adipocyte and mesenchymal stem cell death that occurred with the various devices and pressure levels used to harvest fat.

Methods: This study was a prospective, randomized, comparative study in 15 healthy male and female subjects aged 25 to 60 who were undergoing abdominal cosmetic surgery. Various apparatuses and pressure regimens were used to harvest 8 samples of fat tissue. These samples (R1 = R8) underwent histological analysis in order to verify the integrity and functionality of the adipocytes and mesenchymal stem cells that had been harvested.

Results: A total of 14 females and 1 male underwent abdominal cosmetic surgery. Quantitative analysis revealed that the adipocytes in all 8 samples had homogeneous quantitative profiles. The adipose mesenchymal stem cell (AMSC) analysis, according to Friedman ANOVA, revealed no significant variation in the percentage of mesenchymal stem cells ($P = .045$) between the various samples.

Conclusions: The type of device, nozzle diameter tip, and pressure regimen used in this study for harvesting fat tissue did not significantly affect the number of the adipocytes or viable AMSC harvested.

Level of Evidence: 3

Accepted for publication August 7, 2014.
The use of adipose tissue in regenerative medicine is attractive because tissue from the patient is used to correct contours without concern of allergic or immune system reactions. Adipose tissue is readily available, abundant, inexpensive, and biocompatible, and it can be harvested easily and repeatedly. Unfortunately, fat grafting can be unpredictable because transferred fat can suffer variable degrees of reabsorption. Consequently, it is necessary to identify methods and techniques that minimize reabsorption and provide more predictable and stable long-term results.

Data have shown that the rate of absorption following fat grafting ranges from 40% to 60%. Histological studies after biopsy of grafted fat have shown fat reabsorption and replacement by fibrous tissue. The most acceptable explanation for this absorption is based on Peer’s theory of cell survival, which states that the number of viable adipocytes at transplantation may correlate with graft survival in a volume ratio of 1:1. In 1994, Coleman first described the technique of using a syringe with a 2 to 3 mm cannula along with a centrifuge for fat grafting. His technique has been popularized as the lipostructured Coleman technique.

Adipose tissue has become one of the most interesting materials in bioengineered tissues of the human body because of its enormous potential to produce special regenerative cells and its capacity to provide material for autologous volume replacement. The techniques used to harvest, process, and transfer adipose tissue significantly impact the survival of adipocytes and adipose mesenchymal stem cells (AMSC) and the longevity of the fat grafts. Long-term success of fat transplantation is dependent on several elements, including harvesting, processing methods, bioactivation, transplantation methods, and management of the recipient site.

The primary objective of this study was to identify the ideal method of harvesting by analyzing the effects of various factors, including the pressure exerted on the adipocyte and mesenchymal stem cell at the time of harvest; the type of device used (syringes and apparatus); the pressure regime used in the maintenance of the adipocytes; mesenchymal cell morphologies; and cellular viability. A secondary objective was to determine the optimal pressure regime for fat aspiration by comparing the diameter of the nozzle tip of the syringe and its interference with the integrity of adipocytes and AMSCs at the time of harvesting.

METHODS

This was a prospective, randomized, comparative study in 15 healthy male and female subjects between 25 and 60 years of age who were undergoing a cosmetic abdominal surgery (complete abdominoplasty, mini-abdominoplasty, or liposuction). All patients were invited verbally to participate in this study. Patients were excluded if they had unstable clinical pathologies such as type II diabetes and others, used tobacco, and/or had previous surgery of the abdomen (abdominoplasty and liposuction).

We aimed to assess the percentage of adipocyte and AMSC death that occurred with various devices used to harvest fat at a variety of pressure levels. Initially, we measured the varying degrees of pressure generated in each type of syringe (10 mL, 20 mL, and 60 mL) commonly used for liposuction, as well as different pressures (350 mmHg and 700 mmHg) on apparatuses used for harvesting fat. All devices were analyzed at the Center for Technology, Department of Mechanical Engineering, Laboratory of Metrology of the Rio Grande do Norte Federal University in order to measure system pressure and nozzle diameter of each syringe (Figure 1A-D). Negative pressures were measured by pulling the plunger of each syringe at 5 position intervals (Pressure Calibrator PC-500; Presys Instrumentos, São Paulo, Brazil) (Figure 2A, B and Table 1).

We used these devices (syringes and apparatus) under a particular pressure regimen, previously established on an increasing pressure scale, to harvest samples of fat tissue. These samples were submitted for histological analysis to the Excellion Laboratory in Rio de Janeiro (Brazil) and the cytopathology department of the Rio de Janeiro Federal University in order to verify the integrity of the adipocytes and AMSCs that had been subjected to the previously assessed pressure values. The preoperative work-up included laboratory studies, blood tests, and cardiac examination.

Patients were clearly informed of benefits, risks, operative complications, and postoperative care. All patients were operated on under spinal anesthesia (epidural) on an inpatient basis in hospitals and signed an informed consent form that conformed to the Brazilian investigation ethical committee board (protocol no. 60243) and IRB approved (REBEC-UTN: U1111-1155-3912) documents.

Fat Harvesting

Under epidural anesthesia, adipose tissue was collected from the patient’s lower abdomen using the super wet technique. A solution containing normal saline with 1:500,000 units of epinephrine was injected through a 22-gauge spinal needle before aspiration. The solution was infiltrated into the area of liposuction at a ratio of 1 mL of solution per mL of aspirated tissue. Eight areas were marked on the patient’s lower abdomen where adipose tissue was to be harvested. All 8 harvested samples were collected in specific areas using 2-hole blunt canulas measuring 3 mm in diameter. Each canula hole measured 1.5 mm in diameter and was 15 cm in length (tip model, Richter, Sao Paulo, Brazil). The canula was attached to a 10 mL, 20 mL, or 60 mL Luer Lock syringe or a 60 mL catheter tip syringe (Becton Dickinson, Franklin Lakes, NJ) and 2 conventional liposuction apparatuses, according to each negative pressure as previously assessed (Table 2). The main author harvested all samples.
For each syringe volume, we collected 2 samples of adipose tissue submitted to 2 different pressure regimes. As a technical matter, Areas R7 and R8 were aspirated through a blunt cannula of 3 mm in diameter and 15 cm in length, each hole measuring 1.5 mm in diameter, attached to a hose coupled latex and a liposuction apparatus of either 350 mmHg (Area R7) or 700 mmHg (Area R8) in a conventional suction-assisted liposuction model. The tissue was harvested and stored in a sterile flask of 250 mL. Liposuction was performed in the patient’s lower abdomen on the marked area as shown in Figures 3A and B. During the harvesting process, the pressure inside the syringe was kept stable by pulling back the plunger in order to conserve the vacuum regime. Each sample was harvested using a device specific to each unique area.

Figure 1. (A) Measure of the nozzle diameter of 10 mL syringe (Luer Lock tip): Nozzle diameter = 1.77 mm. Radius = 0.889 mm. Perimeter: 5.585 (form error: 0.020 mm; misunderstanding: 0.040 mm). (B) Measure of the nozzle diameter of 20 mL syringe (Luer Lock tip): Nozzle diameter = 1.87 mm. Radius = 0.93 mm. Perimeter: 5.90 mm (form error: 0.020 mm; misunderstanding: 0.040 mm). (C) Measure of the nozzle diameter of 60 mL syringe (Luer Lock tip): Nozzle diameter = 1.96 mm. Radius = 1.03 mm. Perimeter: 6.47 mm (form error: 0.020 mm; misunderstanding: 0.040 mm). (D) Measure of the nozzle diameter of 60 mL syringe (catheter tip): Nozzle diameter = 3.60 mm. Radius = 1.87 mm. Perimeter: 11.37 mm (form error: 0.020 mm; misunderstanding: 0.040 mm).
Management of Harvested Fat

All aspirated fat was transferred to plastic containers through a 50 mL tube to be submitted for cytological and histochemical evaluation (Figure 3C). All samples were kept at 4°C for up to 16 hours.

Laboratory Analysis

The 8 fat samples collected for each patient (samples R1 through R8) were sent for cytological and histochemical analysis in an polystyrene insulator following conservation standards dictated by laboratory policies. All
samples were refrigerated for 12 hours before being analyzed.

**Cytological and Histochemical Analysis of Adipocytes and Mesenchymal Cells**

Fat tissue samples were transferred to 50 mL tubes and weighed. After decantation, each sample was divided into 3 layers: a superior oil layer, a fat tissue layer in the middle, and a pellet of blood cells. The oily fraction was collected and the blood cells were discarded, leaving only the fat tissue layer. Adipocytes in the oily fraction were counted in a Neubauer chamber using an inverted microscope (Eclipse E800, Nikon®, Japan) and photographed using a camera (MicroPublisher 5.0 & 3.3 RTV®, QImaging, Surrey, BC, Canada) (Figure 4A).

Phosphate buffered saline (PBS) was used to wash the middle fat tissue layer to remove any remaining blood cells. Fat tissue was digested using 1.76 mg collagenase I per gram of tissue (Sigma, #C9891) over 30 minutes at 37°C under agitation. Alpha-MEM (LGC Biotechnology) supplemented with 10% FBS (LGC Biotechnology) was added (1:2) in order to inhibit enzyme activity. After centrifugation at 700 g for 10 minutes, the sample was divided into three layers: a superior oily fraction containing adipocytes, a pellet of small cells (endothelial cells, pre-adipocytes, mesenchymal stem cells), and a middle fraction containing...
nondigested extracellular matrix. The upper layer containing adipocytes was collected for analysis (adipocyte counting and photography).

**Flow Cytometry Analysis of the Adipose-Derived Stem Cells**

A pellet of mesenchymal cells was obtained after enzymatic digestion of fat tissue and centrifugation. Cells in the pellet were counted in a Neubauer chamber, and expression of surface and intracellular molecules was evaluated by flow cytometry. The following monoclonal antibodies were used: CD31-FITC, CD146-PE, CD34-PECy5, CD45-FITC, CD90-PE, CD73-FITC, CD13-PE, and CD49d-PECy5 (BD Biosciences San Diego, CA). The cells were incubated with the antibodies for 30 minutes, then washed and fixed with BD FACSTM Lysing Solution (BD Biosciences). Flow cytometry analyses were performed using a FASCalibur™ (Becton Dickinson and Co., San Jose, CA). At least 70,000 total events were acquired, and data analysis was performed with CELLQuest™ software (Becton Dickinson and Co.). Apoptosis and viability were also determined using the Annexin V-FITC and Propidium Iodide (PI) tests (Apoptosis Detection Kit I; BD Pharmingen #556547), following the manufacturer’s instructions. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes, including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometry analysis of cells undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes, such as DNA fragmentation. FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to allow the investigator to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes are

Table 2. Devices and Negative Pressures for Each Sample (R1 Through R8)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Devices</th>
<th>Negative Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1 (R1)</td>
<td>Syringe 10 mL: 2 mL</td>
<td>−77.5 mmHg</td>
</tr>
<tr>
<td>Sample 2 (R2)</td>
<td>Syringe 10 mL: 4 mL</td>
<td>−142.0 mmHg</td>
</tr>
<tr>
<td>Sample 3 (R3)</td>
<td>Syringe 20 mL: 5 mL</td>
<td>−159.0 mmHg</td>
</tr>
<tr>
<td>Sample 4 (R4)</td>
<td>Syringe 20 mL: 10 mL</td>
<td>−265.0 mmHg</td>
</tr>
<tr>
<td>Sample 5 (R5)</td>
<td>Syringe 60 mL: 10 mL</td>
<td>−260.5 mmHg</td>
</tr>
<tr>
<td>Sample 6 (R6)</td>
<td>Syringe 60 mL: 30 mL</td>
<td>−466.0 mmHg</td>
</tr>
<tr>
<td>Sample 7 (R7)</td>
<td>Liposuction AP: 350 mmHg</td>
<td>−350 mmHg</td>
</tr>
<tr>
<td>Sample 8 (R8)</td>
<td>Liposuction AP: 700 mmHg</td>
<td>−700 mmHg</td>
</tr>
</tbody>
</table>

10 mL, 20 mL, and 60 mL syringes were submitted to vacuum analyses in specific mL level to determined negative pressure. Each sample was harvested using a device specific to each unique area. Eight adipose tissue samples were harvested using a specific device with negative pressure previously determined.

Figure 4. (A) Adipocytes were counted in a Neubauer chamber using an inverted microscope (Nikon T100) and photographed using a camera (MicroPublisher 5.0 & 3.3 RTV). (B) The number of adipocytes per mL was analyzed for each of the 8 negative pressure regimes used to harvested adipose tissue. The adipocytes quantitatively analyzed in the samples revealed a homogeneous quantitative profile between samples R1–R8 (381.691/mL to 430.909 adipocytes/mL), and mean total adipocytes were 406.939 adipocytes/mL.
impermeable to PI (and therefore exclude it), whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive.

**Statistical Analysis**

Descriptive statistics present observed data and were expressed as mean, standard deviation, median, and minimum (Figures 4-6). The inferential analysis was completed with Friedman ANOVA\textsuperscript{21} to check for significant variation in cell count adipocytes and the percentage of cells phenotyping from 8 samples (R1 to R8). The test for multiple comparisons was applied to identify the samples that differed significantly, and followed methods by Nemenyi.\textsuperscript{22}

Nonparametric tests were used because variables did not show Gaussian distribution due to the wide dispersion, small sample size, and rejection of the hypothesis of normality according to the Kolmogorov-Smirnov test. Statistical significance was set at $P < .5$. Statistical analysis was performed with SAS 6.11 software (SAS Institute, Inc., Cary, NC).

**Figure 5.** (A) Viable cells: functional analysis of AMSCs considering the 8 negative pressure regimes adopted to harvest adipose tissue. There were no significant changes in viable cells ($P = .97$) between samples R1–R8. (B) Dead cells: Functional analysis of AMSCs revealed significant variation in the percentage of dead cells ($P = .039$) between samples. In samples R1, R2, and R3, the percentage of dead cells was significantly higher than that in samples R7 and R8. There was no significant difference between other pairs of samples. (C) Recent apoptosis: functional analysis of AMSCs showed no significant changes in recent apoptosis cells ($P = .18$) between samples. (D) Late apoptosis: functional analysis of AMSCs showed no significant changes in late apoptosis ($P = .37$) between samples.
RESULTS

A total of 15 patients were included in this study: 14 females who underwent abdominoplasty and 1 male who underwent liposuction. They ranged in age from 25 to 60 years old (mean, 40 years), and body mass index (BMI) ranged from 24.6 to 33.2 (mean, 29.2).

Most of the cells from harvested adipose tissue were analyzed, discarding only blood cells. Adipocytes of the oily layer and middle layer were counted in a Neubauer chamber using an inverted microscope (Nikon T100) and photographed using a camera (MicroPublisher 5.0 & 3.3 RTV). The adipocytes quantitatively analyzed in the samples revealed a homogeneous quantitative profile between samples R1–R8 (381,691/mL to 430,909 adipocytes/mL), with mean total adipocytes of 406,939 adipocytes/mL (Figure 4B). Sample R4 (pressure, 265.5 mmHg; Luer Lock tip nozzle diameter, 1.9 mm) revealed 377,917 adipocytes/mL, and sample R5 was significantly higher than Sample R7. (D) Adipose mesenchymal stem cells (AMSC): Adipose mesenchymal subpopulation profile of cells in 8 samples revealed no significant variation in the percentage of mesenchymal cells (P = .045).

Figure 6. (A) Pericytes: Adipose mesenchymal sub-population profile of cells in 8 samples showed significant variation between them (P = .038). The percentage of pericytes in sample R7 was found to be significantly lower than those in samples R1, R2, R3, and R4. (B) Progenitor endothelial cells: Adipose mesenchymal subpopulation profile of cells in 8 samples showed no significant changes in percentage of endothelial progenitors cells (P = .43) between samples. (C) Pre-adipocyte cells: Adipose mesenchymal subpopulation profile of cells in 8 samples showed that the percentage of pre-adipocytes in sample R4 was significantly higher than that seen in samples R1, R3, R6, R7, and R8, and sample R5 was significantly higher than Sample R7. (D) Adipose mesenchymal stem cells (AMSC): Adipose mesenchymal subpopulation profile of cells in 8 samples revealed no significant variation in the percentage of mesenchymal cells (P = .045).
(pressure, 260.5 mmHg; catheter tip nozzle diameter, 3.60 mm) revealed 430,909 adipocytes/mL, respectively.

There were no significant changes in adipocyte cell count ($P = .81$), percentage of endothelial progenitors ($P = .43$), viable cells ($P = .97$), and rate of late ($P = .37$) or recent ($P = .18$) apoptosis between the samples as illustrated in Figures 4-6. According to Friedman ANOVA, significant variation between samples was found in the percentage of pericytes ($P = .038$). In the Nemenyi multiple comparison test, the percentage of pericytes in sample R7 was found to be significantly lower than those in samples R1, R2, R3, and R4, as shown in Figure 6A. There were no significant differences between other pairs of samples.

In the pre-adipocyte analysis, Friedman ANOVA showed significant variation in the percentage of pre-adipocytes ($P = .009$) between the samples. Nemenyi multiple comparison tests demonstrated significantly higher percentages of pre-adipocytes in sample R4 compared to samples R1, R3, R6, R7, and R8, and sample R5 was higher than sample R7, as shown in Figure 6C. There were no significant differences between other pairs of samples.

In the adipose mesenchymal stem cell (AMSC) analysis, Friedman ANOVA revealed no significant variation in the percentage of mesenchymal cells ($P = .045$) between samples. Nemenyi multiple comparison tests showed that the percentage of mesenchymal stem cells in sample R3 was significantly lower than that in samples R4, R5, R6, R7, and R8, as shown in Figure 6D. There was no significant difference between any other pairs of samples.

In the dead mesenchymal cell analysis, Friedman ANOVA revealed significant variation in the percentage of dead cells ($P = .039$) between samples. In the Nemenyi multiple comparison tests, samples R1, R2, and R3 had a significantly higher percentage of dead cells than samples R7 and R8, as shown in Figure 5B. There were no other significant differences between pairs.

**DISCUSSION**

The literature contains conflicting reports as to whether any one fat harvesting technique is superior to another.23 Five variables must be considered when evaluating the overall success of fat grafting: harvesting, processing, supplementation, transplanting, and management of the recipient site.24

This study evaluated the effects of fat harvesting methods on adipocytes and mesenchymal stem cells (morphological and functional correlation) that were subjected to a range of negative pressure applied during liposuction. The level of adipocytes and the AMSC damage in the harvested fat tissue were compared across samples. There are many harvesting techniques, and it can be confusing to surgeons as to which one is the best, because it is not fully understood how negative elements associated with the various collection methods and devices used to harvest fat tissue affect the structure and viability of adipose and mesenchymal stem cells.25-27

The Coleman lipostructural technique is a method whereby a light negative pressure container is created in the 10 mL syringe that is connected to a 3 mm cannula.8 However, one of the main concerns after grafting is the potentially high rate of absorption over time in the grafted site. The most acceptable explanation has been based on Peer’s cell survival theory, which compares the number of viable adipocytes at the time of lipotransfer with fat graft volume.7 In 2000, Sommer and Sattler28 studied the controversy surrounding the longevity of correction in autologous fat grafts and its relation to adipocyte survival. The authors found that the survival of aspirated fat cell grafts depends more on the anatomic site and less on the harvesting method used.3 Condé-Green and Gontijo de Amorim25 used a 3 mm cannula attached to a 10 mL Luer Lock syringe to harvest fat tissue. The lipoaspirate samples showed relatively intact, nucleated adipocytes and overall normal morphology with an elevated number of nucleated cells. The standard liposuction by machine (typically a high pressure is used for vacuum extraction) could potentially result in a high percentage of ruptured adipocytes and ADSMs according to other studies.29-32 However, this outcome was not supported by our study results. Smith et al33 found no significant difference in adipocyte viability between syringe and machine aspiration.

The adipocyte profile analyzed in lipoaspirate through conventional histologic methods require tissue fixation to avoid deterioration of the fragile tissue, and sectioning of the tissue leads to leakage of lipid contained in adipocytes, both of which deform the original structure of adipose tissue. In our study, we used a novel technique to investigate non-fixed adipose tissue without dissociating and sectioning adipocytes for analysis. This method enables two-dimensional visualization of living adipose tissue without coloring during light microscopy. The limitation of this method is that it cannot measure cells outside of tissue as ruptured adipocytes. For this reason, we carried out a quantitative analysis of the intact morphometric adipocytes by light microscopy in all samples. The results showed a homogenous pattern between them, a result that is underscored by other studies in the medical literature.32-34

The mesenchymal profile in the multicolor flow cytometry analysis revealed 4 subpopulations33 of mesenchymal cells in the 8 samples and showed a similar profile between samples, with a slight downward trend in the number of pericytes in the samples submitted to the highest suction pressure. However, the morphology of the AMSC may not reflect the same profile of the adipocyte in lipoaspirate tissue. The viability, cell death, and late and recent apoptosis tests revealed a profile with no significant difference between samples. Because the analysis was performed
approximately 12 hours after collection, the intervening time span may have interfered with the low level of viability. However, despite the low rate, there were no significant differences among all samples. In both the qualitative and quantitative analyses of adipocytes and mesenchymal cells, the nozzle tip diameter of syringes (Luer Lock and catheter tips) used for samples R4 and R5 made no difference to the integrity of the adipocytes and mesenchymal stem cells harvested.

These results demonstrate that the amount of negative pressure used for harvesting adipose tissue by syringes of 10 mL, 20 mL, and 60 mL, and by −350 mmHg and −700 mmHg pressure does not affect the integrity and viability of adipocytes and AMSCs. The various nozzles and the diameter tip of the syringes did not damage the adipose cells.

The two limitations of this study are the small sample size (n = 15) and the difficulty of having control with excised adipose tissue, due to difficulty around quantification of the cell sample excised using the handset in this study.

CONCLUSIONS

The type of device, nozzle diameter tip, and pressure regimen used in this study for harvesting fat tissue did not play a significant role in the number of the adipocytes and viable AMSCs extracted during the harvesting procedure. Considering the findings, choice of harvest device does not play a significant role in fat grafting outcomes. Consequently, no fat tissue harvesting procedure should be considered inferior or superior to another, and no harvesting procedure should be or dismissed or disqualified from normal surgeon practice.

Disclosures

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Funding

The authors received no financial support for the research, authorship, and publication of this article.

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