Comparison of Mechanical Isolation Procedures of Stromal Vascular Fraction through Five Different Commercial Device

Abstract

Background: Usage of adipose-derived MSCs (ASCs) and stromal vascular fraction (SVF) are a promising treatment modality in regenerative medicine and orthopedics, particularly for osteoarthritis (OA). The SVF is comprised of a diverse population of stem and stromal cells that are derived from adipose tissue. Mechanical isolation methods rather than enzymatic digestion to obtain SVF from fat are advantageous for human derived cell-based therapies because of fewer regulatory restrictions, ease-of-use and cost efficiency. The purpose of this study is to compare five different, novel commercial systems for obtaining SVF from adipose tissue using mechanical concentrating and non-concentrating approaches.

Materials and Methods: Five unique mechanical isolation methods, including Lipogems, Lipocell, Mystem, Lipocube Nano and Lipocube™ SVF (Lipocube™ SVF Hybrid) were compared. After processing the lipoaspirate from 3 patients using these five kits in accordance with the manufacturer's instructions, the cell yield and the cellular activity for each sample was measured by analyzing Cluster Differentiation (CD) markers and the differentiation capacity was examined in adipogenic, chondrogenic, and osteogenic pathways. As the device with the best in vitro results, was used for our preliminary clinical study to demonstrate clinical efficacy of SVF treatment on OA patients of various grades.

Results: When compared to the only cell-concentrating device Lipocube™ SVF Hybrid, the non-concentrating Lipogems, Lipocell, Lipocube Nano and Mystem devices yielded significantly lower cell yield and resulted in a low percentage of cell activity. When comparing non-concentrating devices, we observed that the Lipocube Nano has more active cells and cell numbers.

Conclusions: Following the in-vitro optimization study, the device with the best results was selected for a preliminary clinical study. Hybrid-SVF treatment with the Lipocube™ SVF Hybrid was used and evaluated on OA patients of Grades II-IV to demonstrate clinical efficacy. Although substantial improvement in Grade II and Grade III cases was demonstrated, more research is to be required to support and improve our findings.

Key Words

Stromal Vascular Fraction, Adipose Derived Stem Cell, Osteoarthritis, Stromal, Vascular Matrix, Mechanical Isolation, Enzymatic Isolation

Introduction

Osteoarthritis (OA) is a very common form of arthritis disease over the world [1]. The detailed initiation and progression of the molecular mechanism for osteoarthritis are not fully understood yet. It has been seen mostly in elderly and athletes due to the fact that their cartilage tissues have limited regenerative capacities to renew themselves [2]. The risk factors of OA include age, obesity, hereditary and joint injury (occupation/ sports activities) [3]. Even though OA is a very common illness, it is hard to identify the exact signs since it may be confused with other arthritic conditions, thus making it difficult to diagnose the illness. Joint pain and stiffness are the most common symptoms of OA. Patients might also experience balance issues and muscle weakness [1].

The anatomic and physiologic basis of the illness is chronic inflammation and degeneration of the connective tissues of joints. Long-term damage of chondrocytes, extracellular matrix (ECM), and chondroplasty ([4]), caused by oxidative stress, inflammatory factors, and mitochondrial dysfunction leads to DNA damage, resulting in the degeneration of cartilage, damage to the underlying bone, and morphological changes to the joint. It has been a major health concern and burden because it affects the quality of life especially for the rapidly aging world population [5]. Cellular therapy is one of the breakthroughs in the field of regenerative medicine. There have been some developments to treat osteoarthritis like the method of cultured chondrocyte transplantation; however, these methods have been limited by the lack of cultured chondrocyte sources. Moreover, not all of the cultured chondrocytes are viable after long-term culture because of the variability in cell maturation [6]. Therefore, there are no approved medical treatments currently, that control or reverse the morphological changes [7] nor a precise blood test that has been recognized for OA diagnosis [8,9].

Innovative research in regenerative medical field continues to make major advances. One of the possible treatments of OA relies on local application of stromal vascular fraction (SVF) which is derived from adipose tissue. It has been proven that SVF contains many different regenerative cell types including mesenchymal stem cells (MSCs), fibroblasts, endothelial cells, macrophages, etc. and has shown to be efficient in cartilage repair [10,11]. Adipose tissue can be easily obtained through small volume liposuction and case-control clinical studies have documented improved quality of life and reduced pain levels in OA patients [5]. In addition to

the safety and efficacy of these treatments in pain relief and cartilage defect repair, studies have shown actual improvement in cartilage function [12].

Although the potential use of SVF in regenerative medicine has made its place, and become quite popular as a therapeutic modality, there are some challenges to overcome such as the optimization of SVF isolation in order to obtain the maximal number of cells and enhance treatment outcomes. A number of systems has been developed to process or isolate adipose tissue elements, while overcoming donor variability, infection risk, and final product unpredictability. Cell isolation based on enzymatic digestion has been the most effective method for obtaining SVF, however, the clinical applications of enzymatically harvested SVF remain limited because enzymatic digestion of adipose tissue has been deemed by the FDA and other regulatory bodies, as a ''more than minimal manipulation'' of tissues, which implies that the final product is considered effectively as a ''drug''. [13, 14, 15] Therefore, many methods of mechanical isolation of SVF have surfaced, some non-concentrating methods like shaking, vibrating, or centrifuging only, and some concentrative approaches harnessing mechanical digestion, incubation, and centrifugation in a more comprehensive fashion. [16, 17] Some of these recent mechanical digestion methods have been shown to give comparable cell counts and yields with fewer regulatory implications than enzymatic methods [18].

New concentrating isolation methods are emerging to improve not only the quantity, but also the quality of mechanically digested SVF, in which the extracellular matrix is not completely discarded, but rather used as Stromal Vascular Matrix (SVM). According to Tiryaki et al. [19], SVM resulted in 75% cell yields compared to enzyme-based digestion. To improve the cellular yield, viability, and functional quality of our cell suspension (and thus clinical outcomes), Tiryaki et al. combined mechanically isolated SVF with Stromal Vascular Matrix to create a 'Hybrid SVF' which produces approximately the same cell yield and comparatively higher regenerative capacity to enzymatic digested SVF [20]. Non-concentrating mechanical methods involve reducing the volume of fat tissue by removing the liquid in the adipose tissue via filtration or dialysis, utilizing the phase gradient difference. The concentrating mechanical method is to concentrate the regenerative cell population in high volume adipose tissue by centrifuging them after mechanical digestion into a highly concentrated mix of regenerative cells. The fact that the non-concentrative approaches do not provide any digestion step, thus they lack the same release of cells from perivascular spaces, add to the significant cell yield difference in favor of the concentrative approach. (Figure 1)

In this perspective, we observe two different approaches for mechanical SVF isolation. The first approach includes a concentration step for digested material by centrifugation, while the second approach is based on adipose tissue washing and limited volume reduction but no-concentration. In this study we evaluated first the in-vitro efficiency and quantity of the regenerative cell populations resulting from the addressed methods by examining the SVF extracted from subcutaneous abdominal fat from three consecutive patients using five different commercial mechanical isolation devices. Our focus was on cell viability, cell activity by flow cytometry and expression in differentiation clusters (CD), and the ability to differentiate in adipogenic, chondrogenic, and osteogenic lineages. The SVF isolation technology resulted with the highest efficiency and quantity in compared groups has been applied to OA patients who suffered from Grade II, Grade III and Grade IV.

Material and Methods

Research Study

Three consecutive patients (two women and one man) of ages ranging from 45 to 65 years (mean 54.3 years) underwent liposuction procedures in accordance with the policies approved by the Institutional Review Boards for in vitro characterization of SVF material. We evaluated four different non-concentrating mechanical devices; Lipocube™ Nano (Lipocube Biotech, London), Lipogems processing kit (Lipogems International Spa, Italy, Lipocell processing kit (Tiss'You, Domagnano, Republic of San Marino) and Mystem Processing Kit (MyStem LLC, Wilmington, Del.) and one cell-concentration device; Lipocube™ SVF (Lipocube Biotech, London). As the device with the best in vitro results, Lipocube™ SVF Hybrid was used for our preliminary clinical study to demonstrate clinical efficacy of SVF treatment on OA patients of various grades. Between February and June 2018, 42 knee and 7 hip joints of 28 patients with an average age of 42.5 ± 5.4 years were treated.

Mechanical Digestion of Adipose Tissue

For in vitro characterization of SVF, we collected 100 mL of lipoaspirate from each patient, which was processed by five different kits, 20 ml each for *in vitro* studies, considering the Lipogems, Lipocell, Mystem, Lipocube Nano and Lipocube™ SVF user manuals.

Surgical procedure was assisted by 14G (gauge) needle for incision entry. Local anesthesia was used with xylocaine (10mg/ml), adrenaline (0,005mg/ml) and 200 ml SF. Lipoaspirate was

harvested from each patient's abdominal area using 200 mm long, 3.0mm thick, 2.0mm pore size multi-hole cannula.

Lipogems: 20 ml of harvested adipose tissue was processed in the Lipogems processing kit (Figure 2a), a disposable system that gradually decreases adipose tissue clusters while removing fatty substances and blood residues. The lipoaspirate was first subjected to a cluster reduction in the Lipogems system, which was accomplished by moving the aspirated fat from the syringe into the device through the large filter (blue end) and allowing the corresponding amount of saline to exit into the wasting bag. The device's stainless-steel beads are needed to create a temporary emulsion between fat, blood, and saline that can be washed away against density by gravity-driven saline current. The saline flux was stopped, and the system reversed after this washing step, resulting in the second adipose cluster reduction. Pushing the floating adipose clusters through the second cutting hexagonal filter while pushing fluid from below with a 10-ml syringe achieved the reduction. Mechanically processed adipose tissue was then subjected to the enzymatic digestion method for the assessment. [21]

Lipocell: 20 ml of harvested adipose tissue processed in the Lipocell processing kit (Figure 2b). Lipocell has a semipermeable membrane that separates adipose tissue from waste elements, with the aid of continuous irrigation. By extracting blood and fat residues, tissue dialysis decreases tension and damage to cell and extracellular matrix structure. Lipocell was used to process the lipoaspirates according to the manufacturer's instructions. The lipoaspirate was located in the unit and dialyzed with a filter before being washed with 300 ml of washing solution (phosphate buffered saline (PBS)). To make washing easier, the outside of the bag was gently cleaned. The lipoaspirate appeared clear from blood at the end, and the flowing washing fluid was transparent; the filtered lipoaspirate is collected from the output link with a 10 ml syringe. Mechanically processed adipose tissue was then subjected to the enzymatic digestion method. [22]

Mystem: MyStem Package is a single-use kit for preparing autologous stromal tissue grafts (Figure 2c). Mystem Kit was used to process the lipoaspirates according to the manufacturer's instructions. Washing, filtration, and size-based separation of tissue fragments are all possible with this unit. The blood/saline fraction was extracted with a syringe through the specified connector after 20 ml of adipose tissue was harvested. The tissue fraction was washed by introducing 10 to 20 ml of sterile PBS solution into the system to minimize sample retention in

the device's "dead amount." After lipoaspirate fluid separation, the device's residual tissue fraction was obtained. Mechanically processed adipose tissue was then subjected to the enzymatic digestion method. [23]

Lipocube™ Nano: 20 ml of harvested adipose tissue was processed in the Lipocube™ Nano Device (Figure 2e). The Lipocube™ Nano is a single-use mechanical device for the processing of lipoaspirate, the autologous fat tissue. The fat graft was first passed through Port 1 once, resulting in 1mm parcel sizes. The fat was then passed back and forth between Ports 2 and 3 ten times, to smoothen and homogenize the fat tissue. Finally, the fat was passed through a 500-micron single filter once from Port 3 to Port 4 to create the end product. Mechanically processed adipose tissue was then subjected to the enzymatic digestion method. [24]

Lipocube™ SVF Hybrid: 20 ml of harvested adipose tissue was processed in the Lipocube™ SVF Device (Figure 2d). The Lipocube™ SVF in closed sterile system with proprietary complex structural geometry blades coupled with a flow pattern mechanically digests the adipose tissue. The lipoaspirate was transferred into syringes, connected to the Lipocube™ SVF, a closed unit, harnessing 3 different sets of blade grids on three luer-lock ports on a rotating canal. The lipoaspirate was placed in the first port, passed back-and-forth 10 times through the first blade grid containing multiple 1200-micron holes. The direction of the rotating canal was changed to the second port and the lipoaspirate was passed through the second blade grid containing 750-micron holes and through the 500-micron holes blade grid for full dissociation. In the system ordinary pistons of 20 ml of luer-lock syringes were replaced with disarmable pistons with concave, cell-adhesive gaskets from the kit. The pistons were detached and the syringes containing the dissociated lipoaspirate were centrifuged at 2000 g for 10 minutes with the luer-lock tips directed inward so that the M-SVF could be collected in concave gaskets, and SVM part with high ECM content is the collected in middle of the adipose fraction. The pistons were reattached, and supernatant was discarded until SVM part. SVM part was taken, and supernatant is discarded until M-SVF part. M-SVF part was taken into flacon and 1 ml of SVM part with high ECM content was digested with enzymatic digestion protocol. [19] After SVM part enzymatic digestion M-SVF and SVM part was resuspended for further in-vitro examinations.

For the in-vitro studies, lipoaspirate from all groups was enzymatically digested using GMP graded collagenase NB6 (Serva Electrophoresis, Heidelberg, Germany) at a concentration of

0.1U/ml and a ratio of 1:1 (v/v) for 30 minutes, washed and centrifuged twice at 300xg for 5 min, the pellet re-suspended and drained. [18]

In Vitro Characterization of SVF

Cell Count and Viability Assay

The total nucleated cell number and the viability of all groups were determined by flow cytometer (Muse Cell™ Analyzer) by using manufactures protocol after the lysis of red blood cell.

Flow Cytometry Analysis for Phenotypic Characterization

Cells, obtained by digestion of lipoaspirate by all groups after 4 different processes as mentioned above were subjected to flow cytometry analysis. The characterization of ADSC (CD45-/CD90+, CD73+/-CD90+), endothelial cells (CD45/CD31+), macrophages and monocytes (CD45+/CD14+) was performed. Staining was done with 5µl of monoclonal antibodies (BD Biosciences, Le Pont de Claix, France). Cells were analyzed with flow cytometer (FACSCalibur, BD Biosciences) by collecting 10,000 events, and the data were analyzed using the FACSCalibur Software (BD Biosciences).

Differentiation and Gene Expression Assay

A random group was selected from the repetitive SVF cell populations obtained by 5 different mechanical isolation methods. Isolated cell pellet from five group were re-suspended and seeded in culture flasks in NutriStem Proliferation medium; (MSC XF Medium/serum free-Biological Industries) supplemented with antibiotics (200 units/ml penicillin, 100 µg/ml streptomycin) at 37°C in a humidified atmosphere with 5% CO2. All cell suspensions were cultured in a T-75 flask (Corning, Milan, Italy). Medium was changed every 4 days, but the non-adherent fraction of three groups products were removed from the culture only after 2 weeks. At confluence, cells were detached by treatment with trypsin-EDTA and re-suspended for cell differentiation (Sigma-Aldrich).

To induce adipogenic differentiation, $1x10^4$ cells/ cm2 were seeded in 12 well plate. Adipogenesis differentiation was carried by StemPro Adipogenesis Differentiation kit Medium was replaced every three days for three weeks. According to the manufacturers' protocol, it was evaluated through oil red staining and investigated by phase contrast microscopy.

To induce osteogenic differentiation, $1x10^4$ cells/ cm2 were seeded in 12 well plate. Osteogenic differentiation was carried by StemPro Osteogenic Differentiation kit Medium was replaced every three days for three weeks according to the manufacturers' protocol. Differentiated cells were stained with alizarin red S staining and investigated by phase contrast microscopy.

To induce chondrogenic differentiation, 1x104 cells/ cm2 were seeded in 12 well plate. Chondrogenic differentiation was carried by StemPro Chondrogenic Differentiation kit Medium was replaced every three days for three weeks according to the manufacturers' protocol. Differentiated cells were stained with lcian blue (Sigma) for detecting glycoproteins in the extracellular matrix and investigated by phase contrast microscopy. [25]

Gene expression profiles were examined by adipocyte specific Adiponectin and Lipoprotein lipase (LPL) genes, chondrogenic specific Sox9 and collagen type II (COL2) genes and osteogenic specific Osteocalcin (OCN) and collagen type I (COL1) genes. Primers were designed using Primer-BLAST software from the National Center for Biotechnology (Bethesda, MD). Total RNA isolation from differentiated cells of two groups was performed according to the manufacturer's protocol (Total RNA Purification Plus Kit, Norgen, CAN). [25]

Clinical Evaluation

The concentrating method using Lipocube™ SVF Hybrid isolation technology, which resulted in the highest efficacy according to the in vitro evaluations, was used in 42 knee and 7 hip joints of 28 osteoarthritis patients who suffered from Grade II III and IV OA. The outcome was assessed on the basis of patient's physical examinations and standard questionnaires using Visual Analogue Scale (VAS) for the pain, the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pre-operatively, at 6 weeks, 3 months, 6 months and 1 year following the intervention. VAS and WOMAC scores were collected from the patients by the senior investigator, during the in person follow up visits. All patients were instructed about the potential adverse effects and asked to report or come back for an examination immediately.

Statistical Analysis

The statistical analysis of the data was performed by using a one-way analysis of variance and the t test, assuming a value of $p<0.05$ as the limit of significance.

Results

Cell Counts and Viability: SVF yield was calculated by dividing the number of viable nucleated cells in SVF per ml of end product. The quantity of fat harvested from the patients is same for all groups. According to the isolation methods, total nucleated cell number, cell viabilities are shown in the figure 3. The number of viable nucleated cells of the SVF material to be injected in the final product was 0.84 x 10^6 (± 0.06)/cc in the Lipogems group, 1.48x10^6 (± 0.08)/cc in the Mystem group, 1.04x10^6 (± 0.12)/cc in the Lipocell group, 1,2x10^6 (± 0.21)/cc in Lipocube Nano group and 10.6x10^6 (± 0.1)/cc in the Lipocube™ SVF Hybrid group. The average of the cell viability was 94,03 ± 0,9 by Lipogems Group, 96,36 % ±1 by Mystem Group, 43,5%±0,8 by Lipocell Group, 99,3±0,4 by Lipocube Nano and 99,2% ±0,6 by Lipocube™ SVF Hybrid group. No statistical difference was determined in the cell viability obtained from all methods used except Mystem Group. ($p<0.05$)

Cell Activity Results

Freshly obtained lipoaspirate product from non-concentrating Lipogems, Lipocell, Mystem, Lipocube Nano devices products and concentrating Lipocube™ SVF Hybrid device products were analyzed for the stem cell expression markers. Results from comparative flow cytometry analysis of selected stem cell markers in nonexpanded cellular components of all groups are reported in Figure 4. Interestingly, the Lipocube™ SVF Hybrid approach differed significantly from the four non concentrating groups. The expression pattern CD90+/CD73, CD90+/CD45−, identifying cells with Adipose derived stem cell identity, was higher in the Lipocube™ SVF Hybrid approaches group (85,31%±8/82,01%±10) than Lipogems (63,31%±7/65,7%±9,2), Mystem (72,3%±10/73,2%±11), Lipocube Nano (78,2%±6,5/72,1%±7,1) and Lipocell (57,8%±5/46,7%±7) groups. The endothelial progenitor cell (CD45-CD31+) of the Lipocube™ SVF Hybrid approaches product (35,4%±10) also shows a significantly higher proportion of CD45-/ CD31+ elements, compared to the Lipogems (20,04%±4), Mystem (29,5%±6), Lipocube Nano (32,4%±6,2) and Lipocell group (8,9%%±2,9). Compounding these differences among the five SVF isolation products, the percentage of Macrophages/Monocytes positive for CD45 was reduced in the Lipogems (5,61±3), Lipocube™ SVF Hybrid (8,1%±4), Lipocube Nano (7,9±3,4) and Lipocell product (7,6%±3) compared to the Mystem product (42,3%±9).

Differentiation and gene expression studies:

Lipocube Hyrid SVF technique demonstrated the best differentiation ability into adipogenic, osteogenic and chondrogenic linages (Figure 5a). Adiponectin and LPL levels were measured using RT-PCR to investigate adipogenic differentiation-related gene expression. Osteocalcin and Collagen type 1 was measure to check the osteogenic differentiation. Lastly, Sox9 and Collagen Type 2 was evaluated to check chondrogenic differentiation. Results demonstrated that there was a slight decrease in Adiponectin and LPL gene expression levels in Lipocube Hybrid when compared with the other groups but not a significant change. Lipocube hybrid had 2.67 -fold increase in SOX9 gene expression, 2.56 -fold increase in COL2 gene expression, levels when compared to Lipocube Nano group. Moreover, Lipocube hybrid had 2.29 -fold increase in SOX9 gene expression, 2.18 -fold increase in COL2 gene expression, levels when compared to Lipogems group. Lastly, Lipocube hybrid had 1.54 -fold increase in SOX9 gene expression, 1.33 fold increase in COL2 gene expression, levels when compared to Lipocell group. There is no significant change was detected in the gene expression levels of osteogenic differentiation markers. (Figure 5b)

Clinical Evaluation

Clinical evaluation of the study documented no negative conditions arising from any of the techniques of mechanically processing adipose tissue with concentration methods or the preparation of hybrid SVF approaches. It was observed that the mean number of purified SVF cells were $20{,}4 \times 10^6 \pm 1{,}2$ from 40 ml lipoaspirate for knee injection in 2ml end product and $10{,}2 \times 10^6 \pm 1{,}3$ from 20 ml lipoaspirate for hip injection in 1 ml end product, respectively. Mean hybrid SVF cell viability was reported as 94.01% ± 1,0% (Table 1). It was observed that the patients may feel some pain and swelling at both injection and fat harvesting areas however it was not long-lasting effect, ending in a few days and well controlled on the prescribed painkillers with the recommended dose. Furthermore, no other potential treatment-related adverse reactions such as decreased range of knee motion, fat embolism, deep venous thrombosis, septic arthritis, SVF-associated stiff knee, or superficial infection or intra-articular bleeding at injection sites in the knee, have been encountered during the study. Patient demographics are presented in the Table 1. 42 knee and 7 hip joints of 28 patients with an average age of 42.5 ± 5.4 years formed the study group. According to the Kellgren – Lawrence (KL) classification this study did not have any grade I patients.

In terms of the mean total WOMAC scores, a substantial improvement rate from the level of 59 ± 20.4 to the level of 31±21,9 for knee injections in Grade II patients was observed. Similarly, this improvement may be demonstrated from 59 ± 20.4 to 31±21,9 in *Grade II patients*, 62 ± 18,2 to 28± 18,7 in Grade III patient and 6767±21,1 to 52± 19,5 in Grade IV patients. On the other hand, our study revealed significant refinements in the matter of hip injection. Statistically and clinically meaningful difference in Grade III patients from the level of 63,22 ± 22,6 to 53±17,1 and also in Grade IV patients from the level of 56± 18,5 to 54± 16,7 was observed (Table 2). These points were measured both at the baseline and 12 months postoperatively. Preoperative VAS scores were recorded as 7±0.9, 7,9± 1,2, 8± 1,1 respectively (Tables 2).

Sustaining of this improvement was observed through a year. Moreover, it was reported that between six-week and the six-month follow-up visit, there is a dramatic improvement in the points of WOMAC and VAS.

Discussion

Autologous Stromal Vascular Fraction injection is a promising, non-surgical, minimally invasive option in the field of orthopedics disorders such as, osteoarthritis [26], which is one of the most common chronic diseases in middle-aged and elderly people causing a decline in quality of life over time. OA has been treated with a variety of stem cell procedures, including bone marrow isolated MSCs, adipose tissue-derived stem cells (ADSCs), SVFs, and peripheral blood-derived stem cells. These stem cells are used in various application mostly in plastic surgery and neurodegenerative disease because of their regenerative potential [27, 28]. Multiple paracrine factors generated by these cells inhibit pro-inflammatory cytokines and increase anti-inflammatory cytokines, resulting in decreased inflammation, immunomodulation, tissue repair, and increased proliferation and permeability of endothelial and epithelial cells. [29]

According to the literature, many methods have been used to isolate SVF cell from adipose tissue such as enzymatic or mechanical isolation methods. The gold-standard of SVF isolation was initially enzymatic digestion of extracellular matrix (ECM) from the lipoaspirate by using a chemical enzyme to disrupt the ECM, despite its restrictions. However, it is an expensive method with the need for special environment, equipment and personnel, but most significantly with serious regulatory obstacles. [30, 31]. Thus, a growing number of systems have been developed to process or isolate adipose tissue elements in accordance with the EU and US minimal manipulation rules. Non-enzymatic isolation methods use different methods to separate cells or clusters of cells from adipose tissue, such as centrifugation, sonication, and

filtering. According to the literature [17, ,31, 32], these non-enzymatic methods yielded lower cell counts than enzymatic methods, but substantially less time to perform than enzymatic methods [15]. To improve cell number and cell activity Tiryaki et al. (2021) described Stromal Vascular Matrix and Hybrid SVF hypothesis. [19, 20] Stromal Vascular Matrix refers to the destruction of the large mature adipocytes while preserving the stem/stromal cellular elements and ECM (identified in the microvascular setting of adipose tissue as extracellular matrix, native scaffolding, and all components) [33, 34] Adipose-derived stromal cells and endothelial cells are abundant in ECM in adipose tissue.

In this study, we examined SVF extracted from subcutaneous abdominal fat using five different commercial mechanical isolation devices, where cell yield and cell activity (flow cytometry and cell differentiation capacity) of SVF cells isolated mechanically by non-concentrative volume reduction methods and cell concentration methods were compared in vitro. [34] The first approach used by Lipogems, Lipocell, Mystem, and Lipocube Nano, is reducing the volume of the fat tissue by removing the liquid in the adipose tissue through filtration, or dialysis, taking advantage of the phase gradient difference. The alternative approach used by concentrating methods Lipocube™ SVF kit on the other hand is to concentrate the regenerative cell population in the high-volume adipose tissue by precipitating them by centrifugation after mechanical digestion into a very concentrated mix of regenerative cells. When we compared the non-concentration methods within its category we can observe that mystem has the highest cell number however, it has lowest cell viability. When the number of viable cells per ml is calculated, it can be said that Lipocube Nano device has the highest cell number.

When the flow cytometry results were examined, the ADSC cluster differentiation capacities of regenerative cells in the Lipocube™ SVF Hybrid group were found to be higher than the other groups as a result of flow cytometry. In the publications written by Banyard et al. [35] and Tiryaki et al. [18, 19], it has been shown that mechanical stress increases the activity of regenerative cells. In the comparative study conducted in the observation of this information, it can be said that the Lipocube™ SVF Hybrid group is mostly subject to mechanical stress. Because of their paracrine effect and multi-lineage differentiation capacity, stromal cells, which include pericytes, ASCs, and supra-adventitial cells, are the most important cell types in regenerative therapies [36, 37]. CD90+/CD73, CD90+/CD45, identifying cells with adipose derived stem cell identity and endothelial progenitor cell (CD45-CD31+) to detect the pericyte-like population in the SVF and isolated the highest percentage of regenerative cells using the

Lipocube™ SVF Hybrid procedure compared to other non-concentrating isolation procedures. When the monophage / macrophage contents are examined, it can be said that the Lipocube™ SVF Hybrid and Mystem group have the highest blood content. While comparing the non-concentrating device within its category, the best result of adipose derived stem cell marker and endothelial cell markers were observed in Lipocube Nano group.

On another level, fat of the ECM plays an important role in the effectiveness of some indications, such as chronic and acute wounds promoting healing by differentiation of multipotent cells into fibroblasts [38, 39]. The ability of adherent cells obtained in the SVF to differentiate toward adipocytes, chondrocytes, and osteocytes was used to assess the multi-lineage differentiation potential of the SVF obtained with five different mechanical isolation method. Lipocube Hyrid technique demonstrated the best differentiation ability and gene expression level to adipogenic, chondrogenic and ostoegenic at the appropriate time. In this study, we could not obtain differentiation from Mystem mechanical device.

According to the literature, overexpression of adiponectin prevents high fat induced hepatic lipid accumulation obese rodents [40]. Therefore, when expression of adiponectin is decreased adipogenesis is also increased. Similarly, when isolation is performed with lipocube hybrid, expression of adiponectin is decreased. According to the research, fat cells release lipoprotein lipase to build fat storage, and this LPL activity is enhanced with weight gain [41]. In this study when Lipocube hybrid is compared with Lipocube Nano, Lipocell and Lipogems, it can be seen that LPL expression level is higher in Lipocube Hybrid. Besides, Sox9 and Col2 genes regulates chondrogenic differentiation, increase in these genes make chondrogenic differentiation enhance [42]. RT-PCR results indicate that cells which were isolated via Lipocube Hybrid has better potential for chondrogenic differentiation. Studies have shown that OCN (osteocalcin) is generated by mature osteoblasts and is mostly found in the extracellular matrix (ECM) of bones. OCN and Col1 are involved in bone mineralization and osteogenic differentiation [43]. It can be observed that OCN and Col 1 gene expression levels were decreased in other methods compare to Lipocube Hybrid. According to cell staining and gene expression results it can be clearly claimed that cells from Lipocube Hybrid method has better ability and potential for three different differentiation types compare to Lipocube Nano, Lipogems and Lipocell. While comparing the non-concentrating device within its category, we can say that Lipocell device has more Adiponectin and OCN expression compared with other non-concentrating devices and Lipocube Nano device has more Col1 expression compared with other non-concentrating devices.

Clinically, this study was performed on 20 knee injections of Grade II patients, 16 knee injections of Grade III OA patients and 6 knee injections of Grade IV patients: 2 hip injections of Grade II patients and 5 hip injections of Grade III patients. This study has used the result of Hybrid SVF application on the aforementioned patients accordingly. Following six weeks of Hybrid SVF injection to the specified areas of the patients, it was observed that all the scores of WOMAC and VAS significantly improved over baseline **(Figure 6).**

At 12 months postoperatively, clinical scores of WOMAC and VAS for Grade II and Grade III were significantly higher than for Grade IV in terms of knee injections, however there is small-scale improvement for hip injections. The WOMAC and VAS improvement rates among the KL classifications were not notably different (Table 2). Ultimately, it was observed that the improvement rates were reported as an average of 39% in Grade II, 50% in Grade III knee patients and also approximately 10% in hip patients. Moreover, regarding the VAS score, roughly 60% improvement was observed in Grade II and Grade III patients and 37% improvement was observed in Grade IV patients. Nevertheless, the recovery ratio is comparatively lower and observed as 35% for hip injection.

However, there are some limitations of this study. For example, there was no control group in this particular analysis, thus future trials are needed to investigate the relation between SVF cells and other intra-articular interventions. Secondly, clinical and imaging tests were not conducted during the study. Thirdly, no correlation was found between the dosage of intra-articular SVF cell injection and clinical/structural outcomes. Finally, SVF cells were given a single treatment in this study, whereas multiple injections might be needed for the best results.

Conclusion

In this study, we demonstrated that mechanical isolation devices allow the isolation of stromal vascular fraction in the form of free cells and connective tissue containing stromal cells and extracellular matrix. In terms of cell yield, viability, and SVF composition, five different mechanical isolation devices produced comparable results. It has been observed that non-concentrative approach used by Lipogems, Lipocell, Mystem and Lipocube Nano devices have lower cell efficiency and cellular activity compared to the cell-concentrative approach used by Lipocube™ SVF isolation kit. Based on these *in vitro* results, the Lipocube™ SVF Hybrid device approaches has been tested in OA patients with different grades. For knee and hip injection of OA patients, we conducted comprehensive clinical evaluations of intra articular autologous SVM cell injection. Both procedures were carried out in a secure manner. In Grade II and Grade III patients, the short-term clinical evaluation of intra-articular SVM cell injection on knee OA was very positive. We propose intra-articular SVF cell injection into the knee joint as a novel treatment option for knee OA patients.

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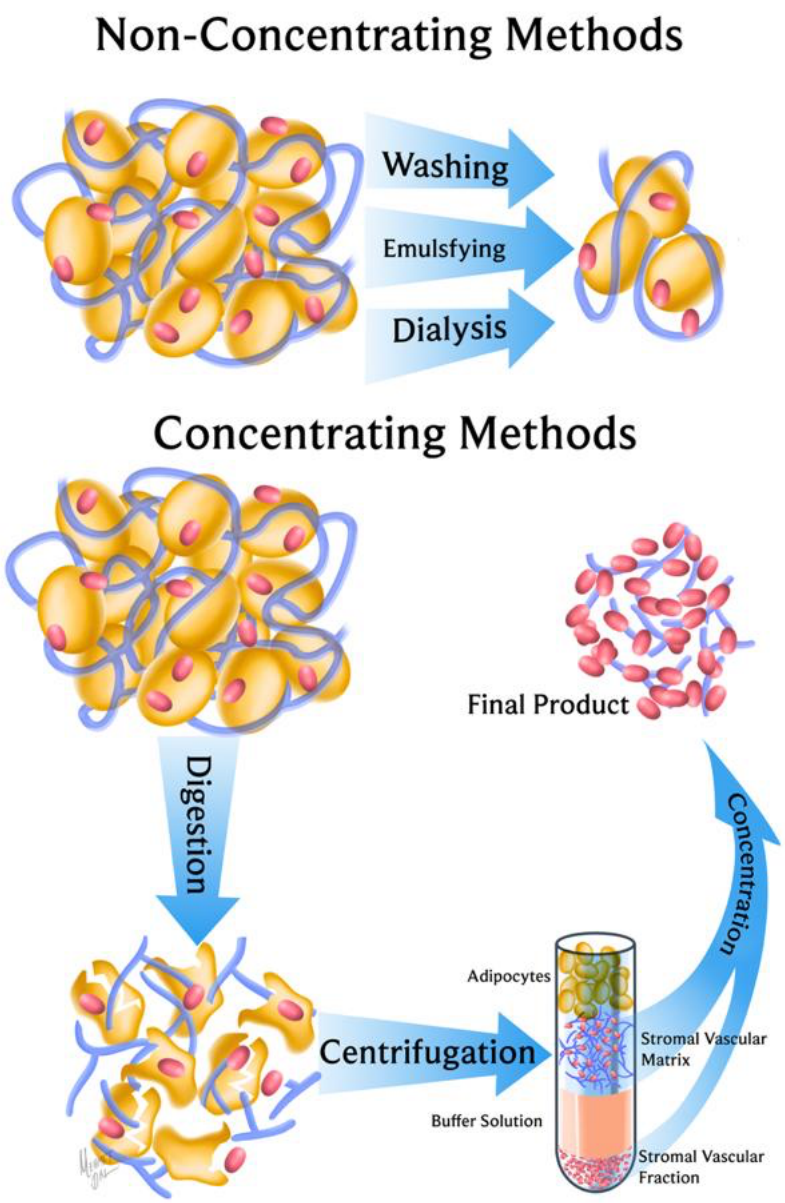


Figure 1: Schematic illustrations of Non concentrating method and Concentrating method A) Non-concentrating methods, is reducing the volume of the fat tissue by removing the liquid in the adipose tissue through filtration, or dialysis, taking advantage of the phase gradient difference. B) Concentrating method is, concentrate the regenerative cell population in the high volume adipose tissue by precipitating them by centrifugation after mechanical digestion into a very concentrated mix of regenerative cells.

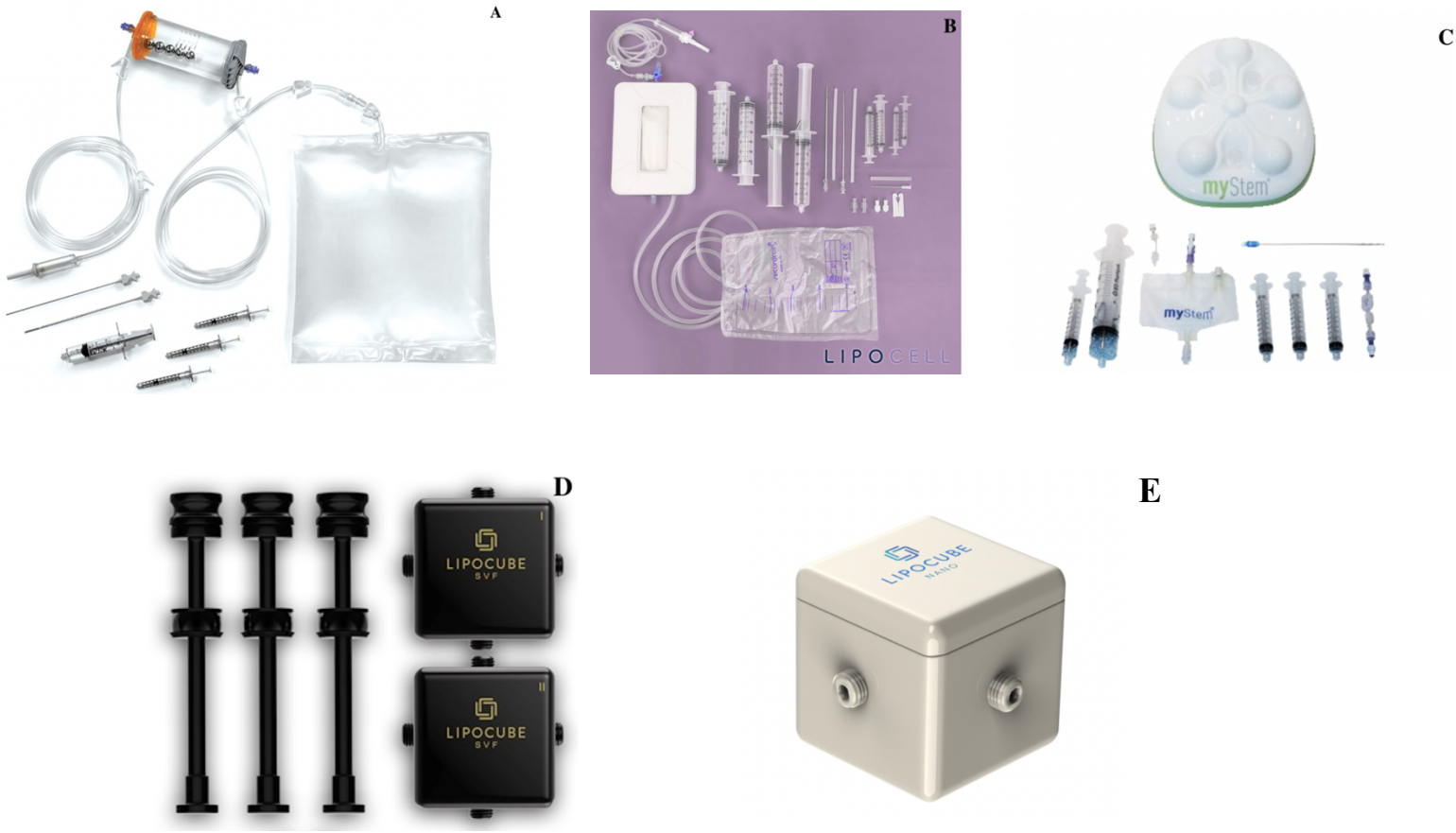
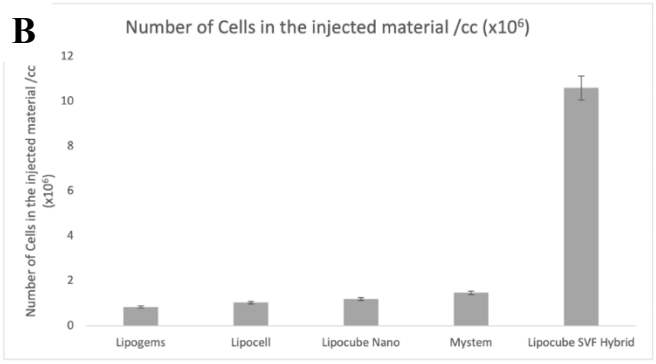


Figure 2: Five different commercial mechanical isolation SVF kit (A), Lipogems procedure kit (Lipogems International Spa, Italy (B) Lipocell procedure kit (Tiss'You, Domagnano, Republic of San Marino) (C) Mystem procedure kit (MyStem LLC, Wilmington, Del.) (D) Lipocube™ SVF Device (Lipocube Biotech, London) (E) Lipocube Nano Device (Lipocube Biotech, London)

A

Mechanical Isolation Methods	Product Used (N=3)	The Amount of Adipose Tissue Processed (ml)	Number of Viable Cells in the injected material /cc ($x10^6$)	Cell Viability (%)
Volume Reduction Method	Lipogems	20	0,84± 0,06	94,03 ± 0,9
	Lipocell	20	1,04 ± 0,12	96,36 ± 1
	Mystem	20	1,48 ± 0,08	43,5 ± 0,8
	Lipocube Nano	20	1,2 ± 0,21	99,3 ± 0,4
Concentration Method	Lipocube SVF Hybrid	20	10,6 ± 0,1	99,2 ± 0,6

B



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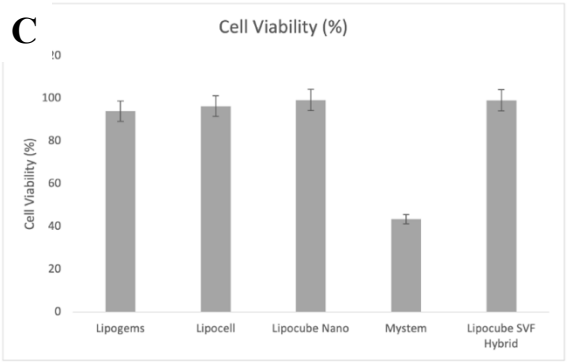

Figure 3: The Cell viability and Nucleated cell number evaluation after the mechanical SVF isolation. (A) The table of the summary of cell viability and cell number (B)Total nucleated cell number obtained at the end of the processes. (C) The Cell Viability of the isolated cells with different kits.

Cell Type	Clusters of Differentiation (CD)
Adipose Derived Stem Cell	CD90+/CD73+, CD90+/CD45-
Endothelia Cell	CD45-/CD31+
Macrophage/Monophage	CD45+/CD14+

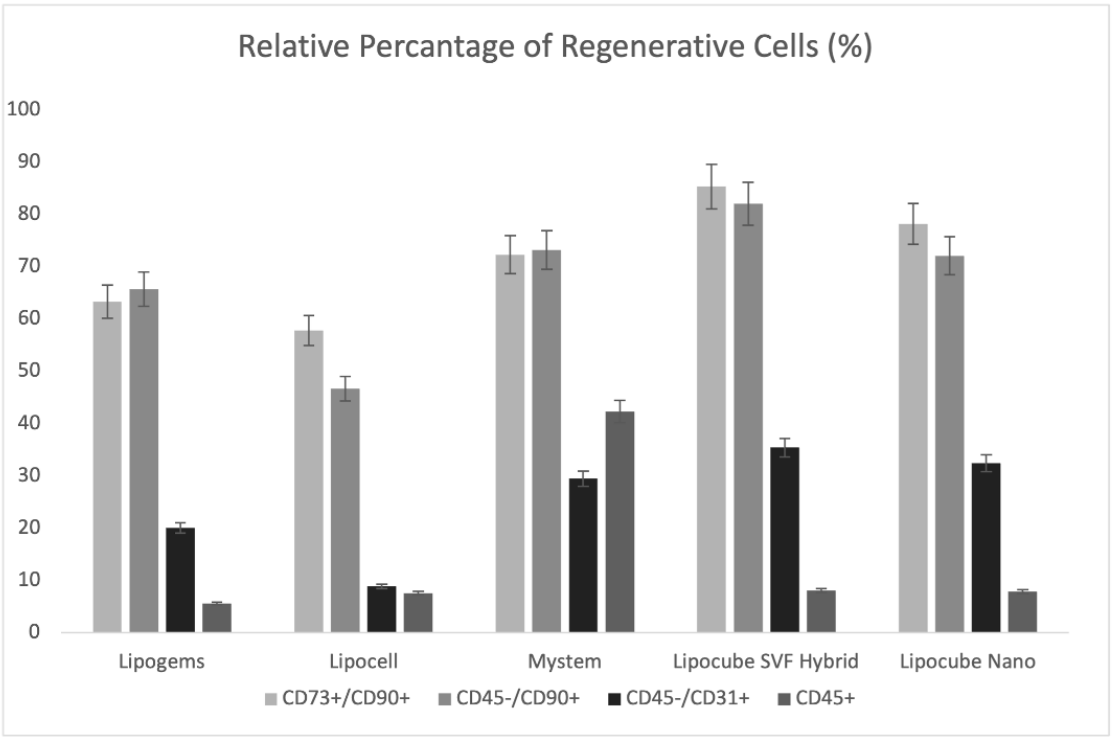

Figure 4: Percentage of CD surface markers of cells (as a sample average) isolated with each method and technique examined. Lipogems: CD73 (+)/CD90(+) 63,31%, CD45(-)/CD90(+) 65,7%, CD45(-)/CD31(+) 20,04%, CD45(-) 5,61%; Lipocell: CD73 (+)/CD90(+) 72,3%, CD45(-)/CD90(+) 73,2%, CD45(-)/CD31(+) 29,5%, CD45(-) 7,6%; Mystem: CD73 (+)/CD90(+) 57,8%, CD45(-)/CD90(+) 46,7%, CD45(-)/CD31(+) 8,9%, CD45(-) 42,3%; Lipocube^{TM} SVF Hybrid: CD73 (+)/CD90(+) 85,31%, CD45(-)/CD90(+) 82,01%, CD45(-)/CD31(+) 35,4%, CD45(-) 8,1%

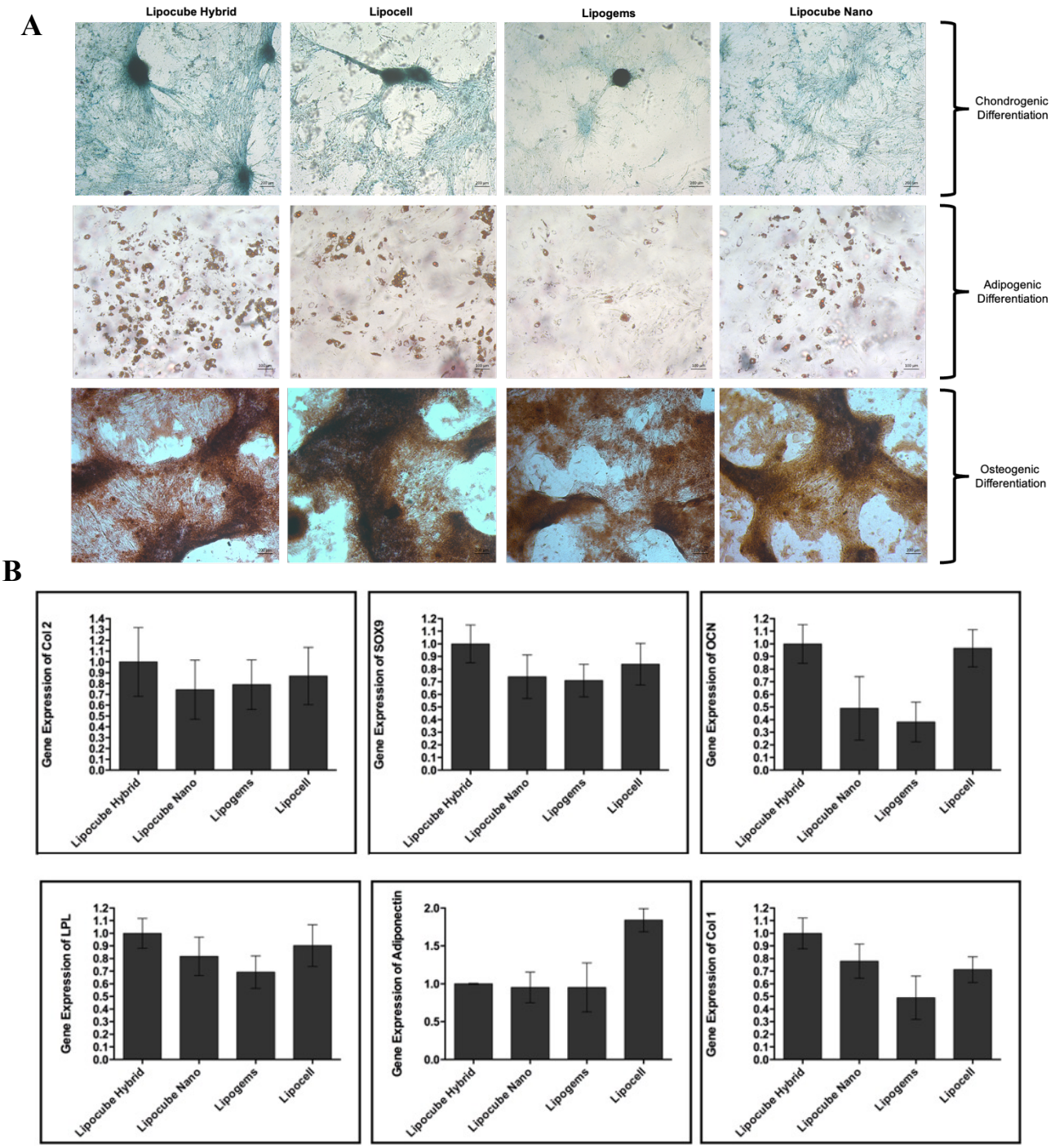

Figure 5: In vitro differentiation capability and related gene expression profile of four different commercial device; Lipocube Hybrid, Lipogems, Lipocell and Lipocube Nano. **A)** In vitro differentiation of cells (as described in the Materials and Methods section). Oil red O staining for lipid droplets revealed adipogenesis. The formation of mineralized matrices, as demonstrated by Alizarin red staining, was evidence of osteogenic differentiation. For Chondrogenic Differentiation cells were stained with lcian blue (Sigma) for detecting glycoproteins in the extracellular matrix. **B)** Comparative analyses of gene expression patterning of adipogenic, chondrogenic and osteogenic differentiation genes. Adipocyte-specific Adiponectin and Lipoprotein lipase (LPL) genes were examined, as were chondrogenic specific Sox9 and collagen type II (COL2) genes, and osteogenic specific Osteocalcin (OCN) and collagen type I (COL1) genes.

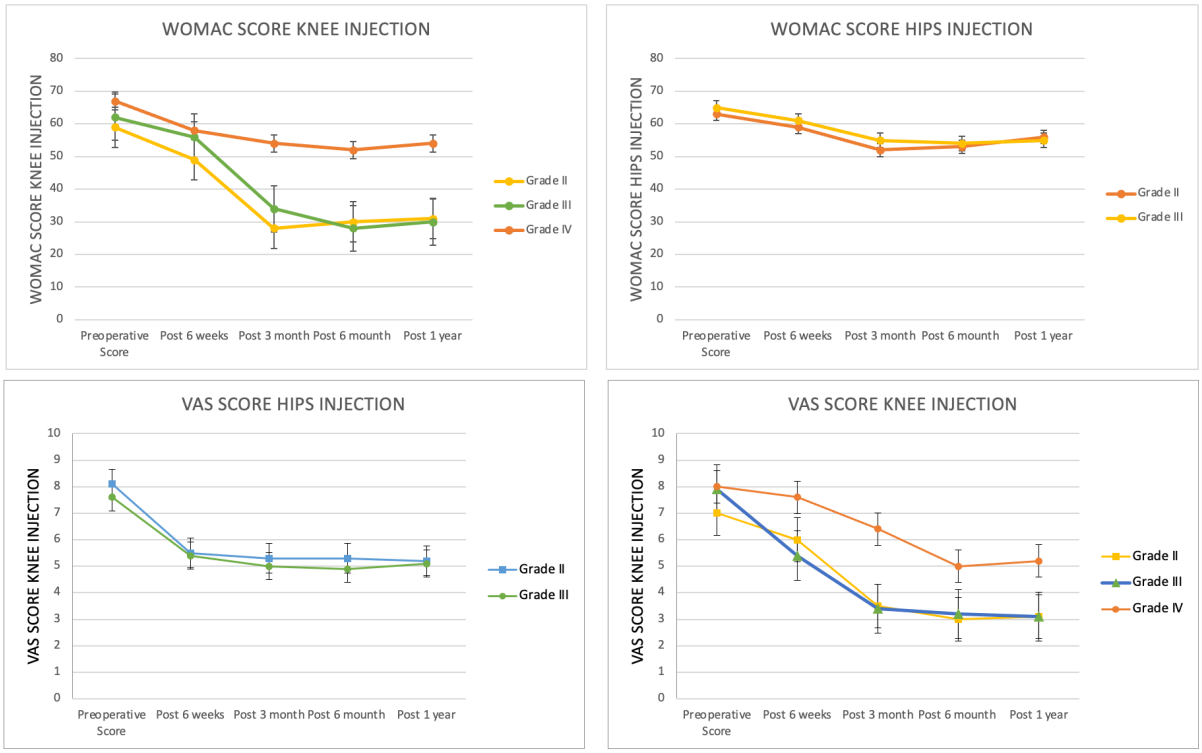

Figure 6: After SVF treatment, the clinical evolution of treated patients was assessed using the WOMAC, and VAS scales. At 12 months postoperatively, clinical scores of WOMAC and VAS for Grade II and Grade III were significantly higher than for Grade IV injection in knee, there is no significant difference in hub injection.

	Knee Treatment with SVM (N= 42)	Hub Treatment with SVM (N= 7)
Age (mean ± standard deviation);	42,5± 5,4 (20-65)	53,5± 4,6 (42-65)
Body mass index	23.0 ± 2.2 (19.0-28.4)	25.0 ± 1.9 (21.0-29.2)
SVF Cell Density (x10^6)	20,4 ± 1,2	10,2 ± 1,3
SVF Injection Volume (ml)	2 ± 0,2	1± 0,1
SVF Cell Viability (%)	94,1 ± 0,3	94,1 ± 0,4
Kallgren-Lawrence Grade, n		
Grade II	20 ± 1,2	2 ± 0,5
Grade III	16 ± 1,4	5 ± 0,7
Grade IV	6 ± 0,8	-

Table 1: Patient characteristics and total injected SVF volume and the cell number

		Kellgren-Lawrence classification **for knee injection**			Kellgren-Lawrence classification **for hip injection**	
	Clinical score	Grade II (20 patients)	Grade III (16 patients)	Grade IV (6 patients)	Grade II (2 patients)	Grade III (5 patients)
Preoperative score	WOMAC	59 ± 20.4	62±18,2	67±21,1	63 ± 22.6	56±18,5
	VAS	7± 0,9	7,9±1,2	8±1,1	8,1 ± 2,2	7,6±2,3
6 Weeks Score	WOMAC	49 ± 20.4	56±18,2	58±24,1	59 ± 18,2	61±17,7
	VAS	6 ± 2,0	5,4±1,8	7,6±1,5	5,5 ± 1,2	5,4±1,7
3 Months Score	WOMAC	28±17,7	34±18,9	58±24,1	52±18,7	55±17,9
	VAS	3,5±1,7	3,4±1,3	6,4±1,3	5,3±18,7	5±1,9
6 Months Score	WOMAC	30±20,9	28±18,7	52±19,5	53±17,1	54±16,7
	VAS	3±2,0	3±1,2	5±1,6	5,3±1,7	4,9±1,6
1 Year Score	WOMAC	31±21,9	30±20,9	54±21,6	56±18,5	55±17,9
	VAS	3,1±1,1	3,1±0,9	5,2±1,1	5,2±1,8	5,1±1,3

Table 2: Improvement rate from baseline to 12-month postoperatively in Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), visual analog scale (VAS) for pain scores among Kellgren-Lawrence classifications