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Activity of the Mechanically Isolated Hybrid Stromal Vascular Fraction on Osteoarthritis

Polen Koçak[1], Serli Canikyan[2], Yağiz Denizci[3], Burak Beksaç[4], Yaren Kul[5], Tunç Tiryaki[6*]

[1]Biomedical Engineering, Istinye University, Istanbul, Turkey
[2]Genetics and Bioengineering, Yeditepe University, Istanbul, Turkey
[3]Faculty of Medicine, Medipol University, Istanbul, Turkey
[4]Private Practise Clinic, Istanbul, Turkey
[5]Bioengineering, Yildiz Technical University, Istanbul, Turkey
[6]Cadogan Clinic, London, UK
Email: *tunc@tunctiryaki.com

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Abstract

Background: Regenerative medicine holds promise for treating degenerative and inflammatory conditions like osteoarthritis (OA). However, the complex molecular mechanisms of OA and the limitations of current therapies remain challenges. Adipose-derived stem cells (ADSC) and stromal vascular fraction (SVF) are gaining attention for OA treatment due to their abundance in adipose tissue. The literature delineates two primary techniques for SVF extraction from adipose tissue: enzymatic digestion and mechanical methods. The Lipocube Hybrid SVF presents a straightforward and secure mechanical isolation method for SVF, enhancing its regenerative potential for various applications. **Purpose:** This study aims to provide valuable insights into the potential of Lipocube Hybrid SVF as a regenerative therapy for OA, contributing to the broader understanding of its applicability in addressing this debilitating condition. **Method:** To assess the effectiveness and safety of the Lipocube Hybrid SVF, we have designed a comparative study that evaluates cellular activity and viability, phenotypic characterization, and differentiation potential. The *in vitro* activity of mechanically isolated SVF is compared to the established gold standard enzymatic digestion method. After *in vitro* studies, Lipocube Hybrid mechanical isolation method was used to isolate SVF and applied in 42 knee and 7 hip joints of 28 patients with Grade II, Grade III, and Grade IV OA. **Results:** The Lipocube Hybrid group had slightly lower viable cell numbers but higher cell viability. Flow cytometry analysis showed the Lipocube Hybrid group exhibited more favorable markers for regenerative potential and reduced inflammatory response. Additionally, both groups demonstrated successful osteogenic differentiation, with the Lipocube Hybrid

group excelling in chondrogenic and adipogenic differentiation. The clinical application of the Lipocube Hybrid SVF in OA patients resulted in significant improvements in WOMAC and VAS scores across different OA grades. **Conclusions:** This comparative study was conducted to evaluate the effectiveness and safety of the Lipocube Hybrid SVF, which has shown promise in laboratory settings, for different stages of osteoarthritis. The study findings provide valuable insights into the potential of Lipocube Hybrid SVF as a regenerative therapy for OA, highlighting its suitability for addressing this debilitating condition.

Keywords

Osteoarthritis, Stromal Vascular Fraction, Enzymatic Isolation, Mechanical Isolation, Regenerative Medicine

1. Introduction

Osteoarthritis is indeed a prevalent form of arthritis, and it occurs due to the gradual wear and tear of cartilage in the joints. When cartilage breaks down, the bones can rub against each other, causing pain, swelling, and stiffness in the affected joint [1] [2]. Although the exact molecular mechanisms involved in the development of OA are not yet fully understood, research suggests that factors such as inflammation, genetic factors, and mechanical stress play key roles in the onset and progression of the disease [3]. OA is more commonly seen in older adults and athletes in the population due to the limited regenerative capacity of cartilage tissue [4]. In addition, other risk factors such as obesity, joint injuries and genetic predisposition may also increase the likelihood of developing OA. Complaints such as joint pain and stiffness in OA patients are similar to the symptoms of other types of arthritis, making it difficult to diagnose the disease. While a variety of treatment options are available to manage such OA symptoms, including pain medication, physical therapy, and surgery, these general treatment solutions do not specifically address the underlying causes of the disease [4] [5].

Regenerative treatments are new area for treatment for OA aim to promote the repair and regeneration of damaged joint tissue, such as cartilage, and to reduce inflammation and pain. One promising approach involves the use of stem cells, which can differentiate into various cell types, including chondrocytes, the cells that make up cartilage [5].

SVF is a mixture of cells that can be isolated from adipose tissue [6]. It contains ADSC, as well as other cell types, including immune cells and endothelial cells. SVF has been investigated as a potential source of stem cells for regenerative treatments for OA [7] [8] [9] [10]. SVF therapy involves isolating SVF from a patient's adipose tissue and injecting it directly into the affected joint. The stem cells in SVF may be able to differentiate into chondrocytes and regenerate

damaged cartilage, while the immune cells in SVF may help to reduce inflammation and promote healing. While early studies of SVF therapy for OA have shown promising results. It is important to note that SVF therapy is not currently approved by the FDA for the treatment of OA, and it should only be performed by qualified healthcare providers in a clinical setting [11] [12].

Isolation of SVF is an important aspect of regenerative medicine, but there are some challenges associated with this treatment approach. Enzymatic digestion is the most common method to obtain SVF, but it has negative effects in terms of both cost, safety and efficacy [12]. Various mechanical methods such as mechanical dissociation and mechanical separation procedures have been developed to overcome these challenges [13] [14]. The efficacy of these methods varies, with mechanical separation procedures having a lower cell yield than enzymatic digestion, but new methods have emerged to improve the yield and quality of mechanically digested SVF, such as Tiryaki *et al.* using the extracellular matrix (ECM) as SVM to create a "Hybrid SVF" [15] [16] [17] [18].

Lipocube Hybrid SVF offers an easy and reliable way to mechanically isolate the SVF to be applied to patients. Because SVF contains MSCs and different types of regenerative cells, they can make many reparative factors with immunomodulatory ability. In terms of stromal cell composition and viability, mechanically digested SVF was comparable to enzymatically digested SVF. Mechanical digestion allows approximately 30% - 50% more SVF to be obtained compared to enzymatic digestion with less regulatory implications [16]. Furthermore, enzymatic digestion results in a suspension of "naked" cells that lack an extracellular matrix, making the regenerative cell suspension less efficient. The sole benefit of enzymatic digestion in increasing SVF cell counts does not compensate for the other disadvantages of eliminating the entire ECM portion from the SVF [17].

Our study aimed to describe an office-based combined processing method for mechanically isolated adipose-derived SVF application in Grade II, Grade III, and Grade IV OA indications. Comparison studies were carried out between mechanical SVF and E-SVF in terms of cell number, viability, differentiation capacity and gene expression levels. Subsequently, to examine the effectiveness of the procedure on different degrees of OA, SVF was applied to the hips and joints of patients. Overall, this study highlights the importance of developing efficient and safe methods for isolating SVF for regenerative medicine purposes. It also emphasizes the need for *in vitro* evaluation of the isolated SVF to ensure its quality and regenerative potential.

2. Material and Methods

2.1. Study Design

This study aims to investigate the clinical application of SVF obtained through the Lipocube Hybrid device in patients with varying grades of OA. The *in vitro* efficacy of the Lipocube Hybrid method is evaluated by comparing it with the

established enzymatic digestion technique. SVF isolation was performed on 42 knees and 7 hip joints from 28 patients with Grade II, Grade III, and Grade IV OA. Outcomes were assessed using physical examinations and standard questionnaires, including the Visual Analogue Scale (VAS) and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), both pre-operatively and at multiple post-intervention time points.

2.2. Patient Selection and Inclusion Criteria

Patients referred to the department due to persistent knee pain lasting for more than six months and diagnosed with knee osteoarthritis (Kellgren-Lawrence grade 1 - 4) were considered eligible for inclusion. Inclusion criteria comprised an age range of 18 to 70 years, the capability to attend rehabilitation sessions and follow-up examinations, and the ability to provide informed consent. Exclusion criteria included knee varus or valgus malalignment exceeding five degrees, knee cruciate ligament rupture, and a BMI exceeding 35 kg/m^2. Patients were informed about the study verbally and in writing, followed by a 24-hour reflection period before obtaining written consent, ensuring voluntary participation.

2.3. Adipose Tissue Harvesting

The harvesting of adipose tissue was conducted under sterile conditions in the operation room. Procedures were performed following the policies approved by the Institutional Review Boards to characterize *in vitro* SVF material. Patients were positioned supine, and an area just below the umbilicus measuring approximately 10 cm (craniocaudal) by 25 cm (laterally) was marked on the skin using a surgical marker. Before tissue collection, local anesthesia was applied with a mixture of xylocaine (10 mg/ml), adrenaline (0.005 mg/ml), and a solution containing 110 ml SF, 60 ml xylocaine, and 60 ml adrenaline under sterile conditions. Adipose tissue was harvested using a 200 mm-long multi-hole cannula with a thickness of 3.0 mm and a pore size of 2.0 mm. Cannula was attached to a 20-ml syringe for the procedure. For each knee injection, an average of 100 cc tissue was aspirated. After decantation of the lipoaspirate, approximately 50 cc of adipose tissue was obtained per patient. 40 cc of adipose tissue underwent processing with the Lipocube Hybrid device, while the remaining 10 cc was allocated for *in vitro* experiments. Regarding hip injections, an average of 60 cc adipose tissue was aspirated. After decantation, approximately 30 cc tissue was obtained per patient. Following decantation, 20 cc of adipose tissue was processed using the Lipocube Hybrid device, and the remaining 10 cc was utilized for *in vitro* experiments. All procedures performed in this study were under the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Study participants and/or their legal guardian(s) had signed a written consent form and agreed to participate in the study before surgery.

2.4. Processing of Adipose Tissue with Lipocube Device

The mechanical SVF isolation process utilized the LipocubeTM system, which replaces standard pistons with detachable pistons with concave, cell-adhesive gaskets from the kit (**Figure 1(a)**). The lipoaspirate was transferred into syringes and connected to the Lipocube^{TM} device, a closed unit with three sets of blade grids on three luer-lock ports situated along a rotating canal. As in the protocol previously described by Tiryaki *et al.* [17], the lipoaspirate was introduced through the first port and passed back and forth 10 times through the initial 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 to ensure complete dissociation. Pistons were detached, and syringes containing the dissociated lipoaspirate were centrifuged at 2000 g for 10 minutes (**Figure 1(b)**), with Luer-lock tips inward to collect SVF in concave gaskets. The pistons were reattached, and the supernatant was removed until the SVM component was obtained. The adipose SVM part with high ECM content and the SVF part were resuspended.

2.5. *In Vitro* Analysis of Processed Adipose Tissue

This study involved the comprehensive *in vitro* characterization of the isolated SVF from Lipocube Hybrid SVF samples. E-SVF, recognized as the gold standard for SVF extraction, was employed to establish a foundational reference. The analysis encompassed the evaluation of cell viability, cell counts, and surface marker expression using flow cytometry. Moreover, cell differentiation assays were carried out to determine the potential for adipogenic, osteogenic, and chondrogenic differentiation.

(a) (b)

Figure 1. Mechanical SVF Isolation Process using the Lipocube^{TM} System (a) Illustration of the Lipocube^{TM} system, highlighting the detachable pistons with concave, cell-adhesive gaskets from the kit, replacing standard pistons. (b) The isolated components from Lipocube Hybrid, including the adipose Stromal Vascular Matrix (SVM) part with a high extracellular matrix (ECM) content and the Stromal Vascular Fraction (SVF) part.

For the *in vitro* experiments, an average of 20 ml of lipoaspirate per patient was required. The adipose tissue was divided into equal 10 ml portions for the isolation of Hybrid SVF and E-SVF.

The Lipocube Hybrid protocol was employed for the Hybrid SVF isolation, while the control group underwent the enzymatic digestion method. This technique involved the utilization of collagenase NB6 enzyme (SERVA Electrophoresis GmbH, Heidelberg, Germany) at a concentration of 0.1 U/ml and a 1:1 (v/v) ratio adhering to good manufacturing practices (GMP). After enzymatic digestion, the material was washed, and centrifuged twice at 300×g for 5 minutes, and the resulting pellet was resuspended and drained.

2.5.1. Cell Count and Viability Assay

The total nucleated cell count and the viability of all groups were determined using the Muse Cell™ Analyzer, following the manufacturer's protocol after lysing red blood cells. The Muse Cell™ Analyzer is specifically designed for the rapid and easy analysis of cell viability and concentration in various cell types.

Cell pellets obtained through Lipocube Hybrid and enzymatic isolation were re-suspended and seeded in culture flasks using 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% CO_2. Cell suspensions were cultured in T-75 flasks (Corning, Milan, Italy), with medium changes every four days. At confluence, cells were detached with trypsin-EDTA and re-suspended for cell differentiation (Sigma-Aldrich).

2.5.2. Flow Cytometry Analysis for Phenotypic Characterization

Cells were obtained through the digestion of lipoaspirate with both mechanical and enzymatic methods. Characterization of ADSCs (CD45−/CD90+, CD73+/CD90+), endothelial cells (CD45−/CD31+), macrophages, and monocytes (CD45+/CD14+) was performed. Staining was conducted using 5 µl of monoclonal antibodies (BD Biosciences, Le Pont de Claix, France). Cells were analyzed using a flow cytometer (FACSCalibur, BD Biosciences), collecting 10,000 events, and the data were analyzed using FACSCalibur Software (BD Biosciences).

2.5.3. *In Vitro* Differentiation

Adipogenic differentiation was induced by seeding 1×10^4 cells/cm^2 (passage number 3) in a 12-well plate and conducting differentiation using the StemPro Adipogenesis Differentiation kit. Medium was replaced every three days for three weeks. Osteogenic differentiation was initiated similarly, involving seeding and differentiation through the utilization of the StemPro Osteogenic Differentiation kit. Comparable to this, chondrogenic differentiation was induced using the StemPro Chondrogenic Differentiation kit.

2.5.4. Histological Staining

Adipogenic differentiation was assessed through Oil red-O staining solution

(Sigma Aldrich Chemical Co, USA) for 5 minutes. Cells were then washed with 1 × cold PBS three times. Osteogenic differentiation was assessed through Alizarin Red staining solution (Sigma Aldrich Chemical Co, USA) for 40 minutes in the dark. For chondrogenic differentiation evaluation, samples were stained with Alcian Blue (Sigma Aldrich Chemical Co, USA) for detecting glycoproteins in the ECM. Subsequently, cells were washed with 1 × cold PBS three times. All samples were examined by using a phase-contrast microscope.

2.5.5. Gene Expression Assay

Gene expression profiles were examined for adipocyte-specific Adiponectin and Lipoprotein lipase (LPL) genes, chondrogenic-specific SRY-box transcription factor 9 (SOX9) and Collagen Type 2 (COL2) genes, and osteogenic-specific Osteocalcin (OCN) and Collagen Type 1 (COL1) genes. Primers were designed using Primer-BLAST software from the National Center for Biotechnology (Bethesda, MD). Total RNA isolation from differentiated cells of both groups was performed according to the manufacturer's protocol (Total RNA Purification Plus Kit, Norgen, CAN). Hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), IGF, and TGF-β were also evaluated.

2.6. Lipocube Hybrid Injection

The harvested Hybrid SVF through the Lipocube Hybrid device was injected under sterile conditions. For hip joint injections, the patient was positioned laterally on the unaffected side with the hip slightly flexed. Injection sites were determined using palpation of the greater trochanter and the anterior superior iliac spine, guided by ultrasound. An average of 1 cc Hybrid SVF was injected in a single knee and 2 ml in a single hip.

2.7. Follow-Up Visits and Outcome Measures

Follow-up assessments were conducted at three, six, and twelve months, led by a designated project nurse. Data collection involved patient-reported questionnaires and a combination of physical examinations and standardized questionnaires, including VAS and WOMAC. These assessments occurred both before and at intervals of 6 weeks, 3 months, 6 months, and 1-year post-intervention. During in-person visits, the senior investigator collected VAS and WOMAC scores, while patients were educated about potential adverse effects and instructed to report any experiences promptly.

2.8. Data Analysis

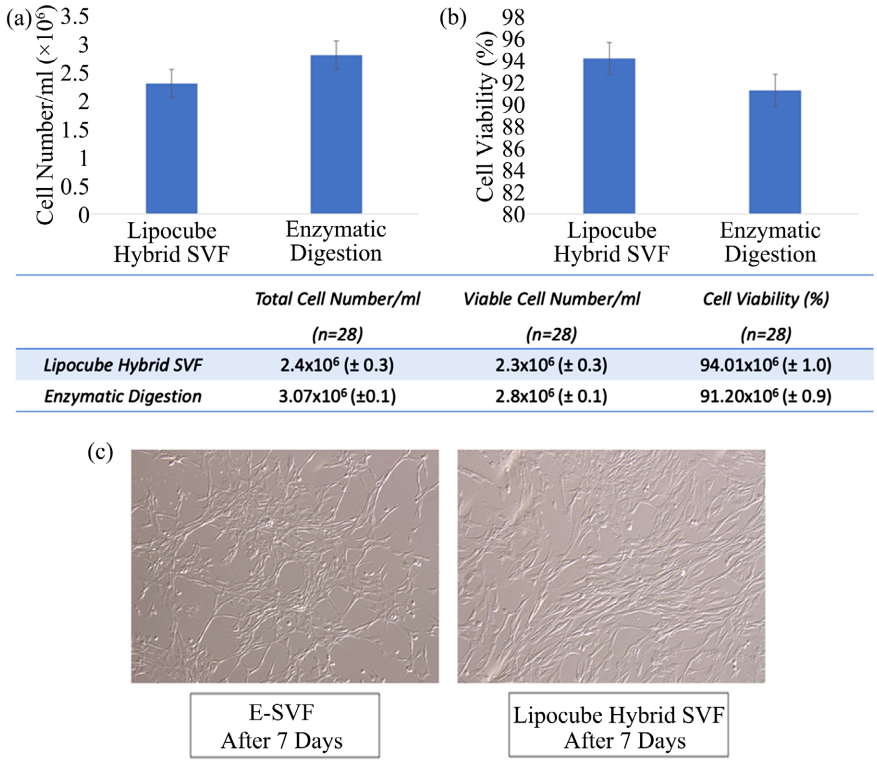
Data analysis was performed using GraphPad Prism 8 software. Results were presented as mean ± standard deviation (SD) or median (range). Statistical significance was determined through paired t-test or Mann-Whitney U test, with $p < 0.05$ considered significant. GraphPad Prism (Version 9; GraphPad Software, San Diego, CA, USA) was employed for calculations.

3. Results

This study aimed to compare the effectiveness of mechanical isolated SVF compared with gold standard enzymatic digestion method and the efficiency of mechanically isolated SVF in osteoarthritis. We have performed analyses of cellular activity, phenotypic characterization, differentiation potential, and gene expression profiles. The clinical evaluation, performed by applying these techniques in OA patients, culminates the study, providing valuable insights into pain relief, joint functionality, and overall patient outcomes.

3.1. Cell Counts and Viability

The SVF yield was determined by calculating the number of viable nucleated cells in SVF per ml of the end product. The quantity of fat harvested from the patients was consistent for all groups. According to the isolation methods, total nucleated cell number and viability data are shown in **Figure 2(a)**. The viable nucleated cell number per ml of the E-SVF group was 2.8×10^6 (±0.1) and the Lipocube Hybrid SVF group was 2.3×10^6 (±0.3). The average cell viability was 94.01% (±1) by Lipocube Hybrid SVF and 91.20% (±0.9) by enzymatic digestion. The comparison of cell viability across all utilized methods did not yield statistically significant differences (n = 28, p > 0.005).



	Total Cell Number/ml (n=28)	Viable Cell Number/ml (n=28)	Cell Viability (%) (n=28)
Lipocube Hybrid SVF	$2.4x10^6$ (± 0.3)	$2.3x10^6$ (± 0.3)	$94.01x10^6$ (± 1.0)
Enzymatic Digestion	$3.07x10^6$ (±0.1)	$2.8x10^6$ (± 0.1)	$91.20x10^6$ (± 0.9)

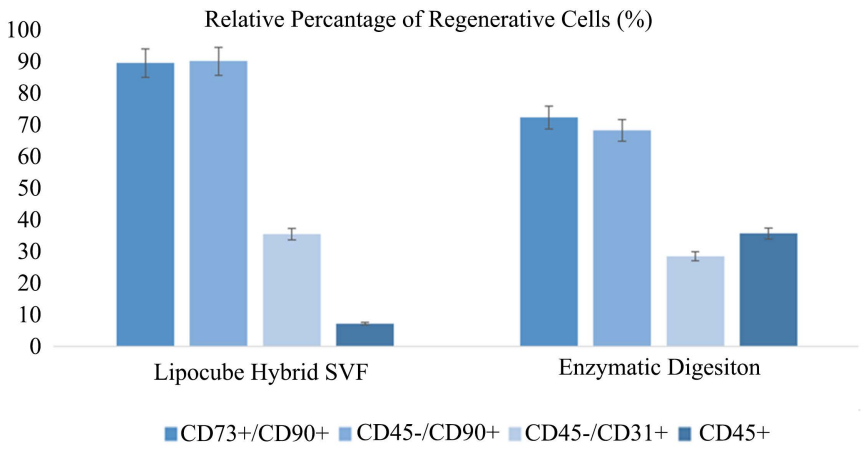
Figure 2. Evaluation of cell viability and nucleated cell number after Hybrid SVF and enzymatic isolation. (a) Summary table of cell numbers obtained using different SVF isolation methods. (b) Comparison of cell viability at the end of the isolation process using two different isolation methods. (c) Comparison of cell culture after 7 days among isolated cells using different isolation methods (n = 28, p < 0.05).

Cells that reached passage number 3 were transferred to tissue culture plates to be cultured for 7 days, and then examined under a light microscope. Cells that were directly seeded into the flasks after isolation underwent evaluation for their growth and confluence over the 7-day period. The observations revealed a significant difference between the two groups. Cells in the Lipocube Hybrid group exhibited a more rapid and prolific growth rate and confluence than cells in the E-SVF group as shown in Figure 2(b).

3.2. Cellular Activity and Phenotypic Characterization

The expression of stem cell markers in fresh lipoaspirate products obtained by E-SVF and Lipocube Hybrid (n = 28) methods was examined by flow cytometry analysis. and the results are reported in Figure 3.

The results revealed prominently higher expression levels for CD90+/CD73+ and CD90+/CD45− markers, indicative of cells with ADSC characteristics, within the Lipocube Hybrid group compared to the enzymatic digestion group (89.31% ± 0.9%, 72.2% ± 1.2%/90.01% ± 1.5%, 68.1% ± 1.3%, respectively). Moreover, the Lipocube Hybrid product demonstrated a significantly elevated portion of CD45−/CD31+ endothelial (progenitor) cell pattern elements (35.4% ± 0.9%) in contrast to the E-SVF group (28.4% ± 1.5%). Furthermore, the Lipocube Hybrid group exhibited a significantly diminished percentage of macrophages/monocytes positive for CD45 and CD14 (7.2% ± 2%), as opposed to the E-SVF group (35.6% ± 2.2%).



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

Figure 3. Percentage of CD surface markers of cells isolated with using different isolation methods (n = 28, $p < 0.05$).

3.3. Differentiation and Gene Expression Studies

The study investigated the differentiation and gene expression ability of different SVF isolation techniques under controlled culture conditions. The Lipocube Hybrid SVF group demonstrated superior outcomes in chondrogenic and adipogenic differentiation compared to the E-SVF group, whereas a comparable outcome was observed in osteogenic differentiation ability (**Figure 4**).

Adiponectin and LPL gene expression levels were measured to investigate adipogenic differentiation-related gene expression, while OCN and COL1 were measured to check osteogenic differentiation [19] [20]. SOX9 and COL 2 were evaluated to check chondrogenic differentiation [21]. The results revealed notable differences in gene expression between the groups ($n = 28$, $p > 0.005$). In the context of adipogenic differentiation genes, the Lipocube Hybrid group exhibited significantly higher expression levels of adiponectin, with a 2.8-fold increase compared to the enzyme group. Similarly, the expression of the LPL gene, was remarkably elevated in the Lipocube Hybrid group, being 2.9 times higher than in the enzyme group. For COL2 and SOX9 osteogenic differentiation genes, the Lipocube Hybrid group demonstrated higher expression levels than the enzyme group, with 2.8 and 3.3-fold increases, respectively. Regarding chondrogenic differentiation, the Lipocube Hybrid group exhibited notably higher expression levels of COL1 and OCN, with a 2.3 and 2.1-fold increase, respectively, compared to the enzyme group. Gene expression results are summarized in **Figure 5**.

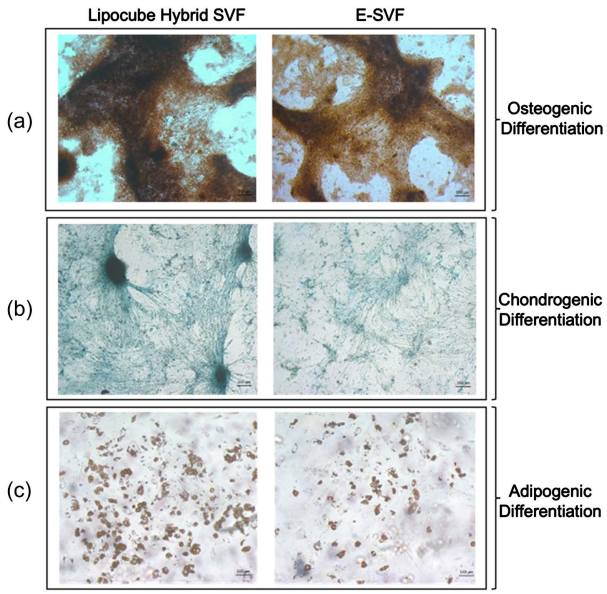


Figure 4. *In vitro* differentiation capability of two different isolation method; Lipocube Hybrid and enzymatic digestion. *In vitro* differentiation of cells (as described in the Materials and Methods section). (a) The formation of mineralized matrices, as demonstrated by Alizarin red staining, was evidence of osteogenic differentiation. (b) For Chondrogenic Differentiation cells were stained with lcian blue (Sigma) for detecting glycoproteins in the extracellular matrix. (c) Oil red O staining for lipid droplets revealed adipogenesis ($n = 28$, $p < 0.05$).

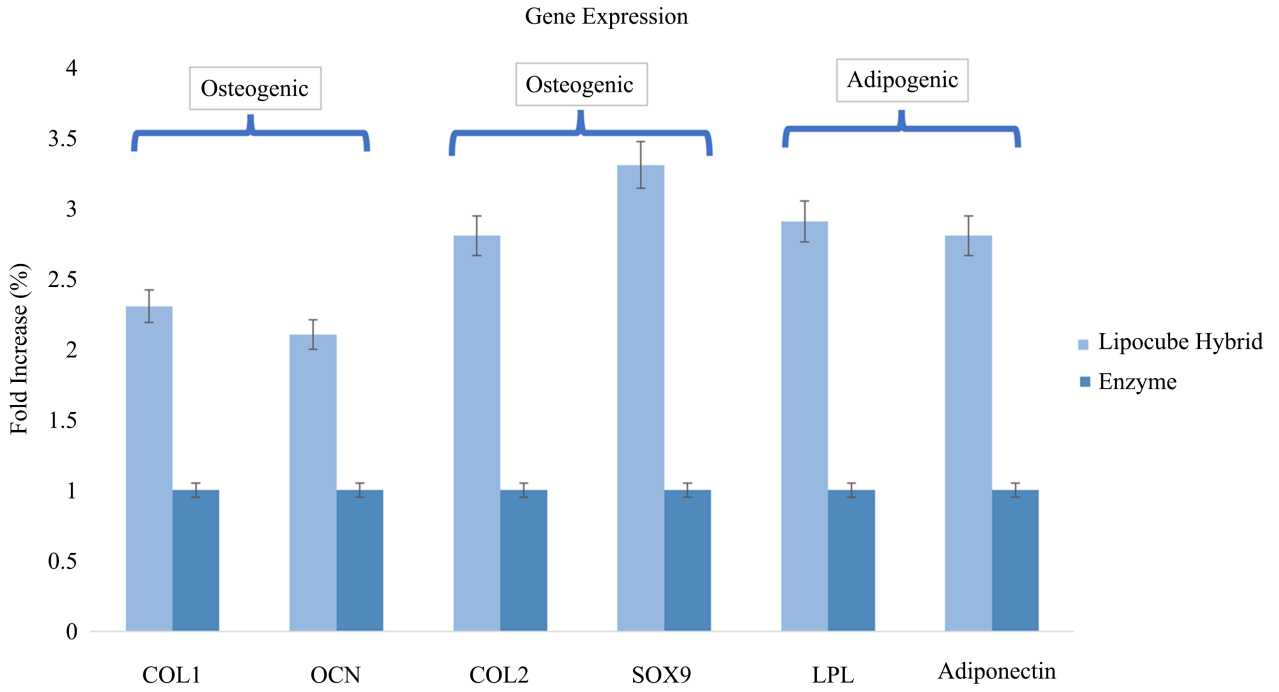


Figure 5. *In vitro* differentiation capability of two different isolation method; Lipocube Hybrid and enzymatic digestion. Comparative analyses of gene expression patterning of adipogenic, chondrogenic and osteogenic differentiation genes. Adipocyte specific Adiponectin and LPL genes were examined, as were chondrogenic specific SOX9 and COL2 genes, and osteogenic specific OCN and COL1 genes ($n = 28$, $p < 0.05$).

3.4. Clinical Evaluation

The clinical evaluation of this study did not show any negative effects from the techniques used for mechanically processing adipose tissue and hybrid SVF preparation. The mean number of purified SVF cells was $20.4 \times 10^6 \pm 1.2$ from 40 ml lipoaspirate for knee injection in 2 ml end product and $10.2 \times 10^6 \pm 1.3$ from 20 ml lipoaspirate for hip injection in 1 ml end product, respectively. The mean hybrid SVF cell viability was reported as 94.01% ± 1.0% (Table 1). Patients reported some pain and swelling at both injection and fat harvesting areas, but these effects were short-lived and well-controlled with prescribed painkillers. No other potential treatment-related adverse reactions were reported during the study.

The study cohort comprised 28 patients, with an average age of 42.5 ± 5.4 years, encompassing 42 knee and 7 hip joints. According to the Kellgren-Lawrence classification, no patients were categorized as Grade I. The investigation revealed remarkable improvements in total WOMAC scores across all grades, with sustained enhancements observed over a one-year period. Preoperative VAS scores were consistent for both knee and hip injections, with significant improvements evident between the six-week and six-month follow-up visits for both WOMAC and VAS scores (Figure 6).

Table 2 presents a detailed overview of the clinical scores and Kellgren-Lawrence classifications associated with knee and hip injections in the study participants. Preoperative assessments revealed that the WOMAC scores were 59 ± 20.4 for

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

	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 ($\times 10^6$)	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	-

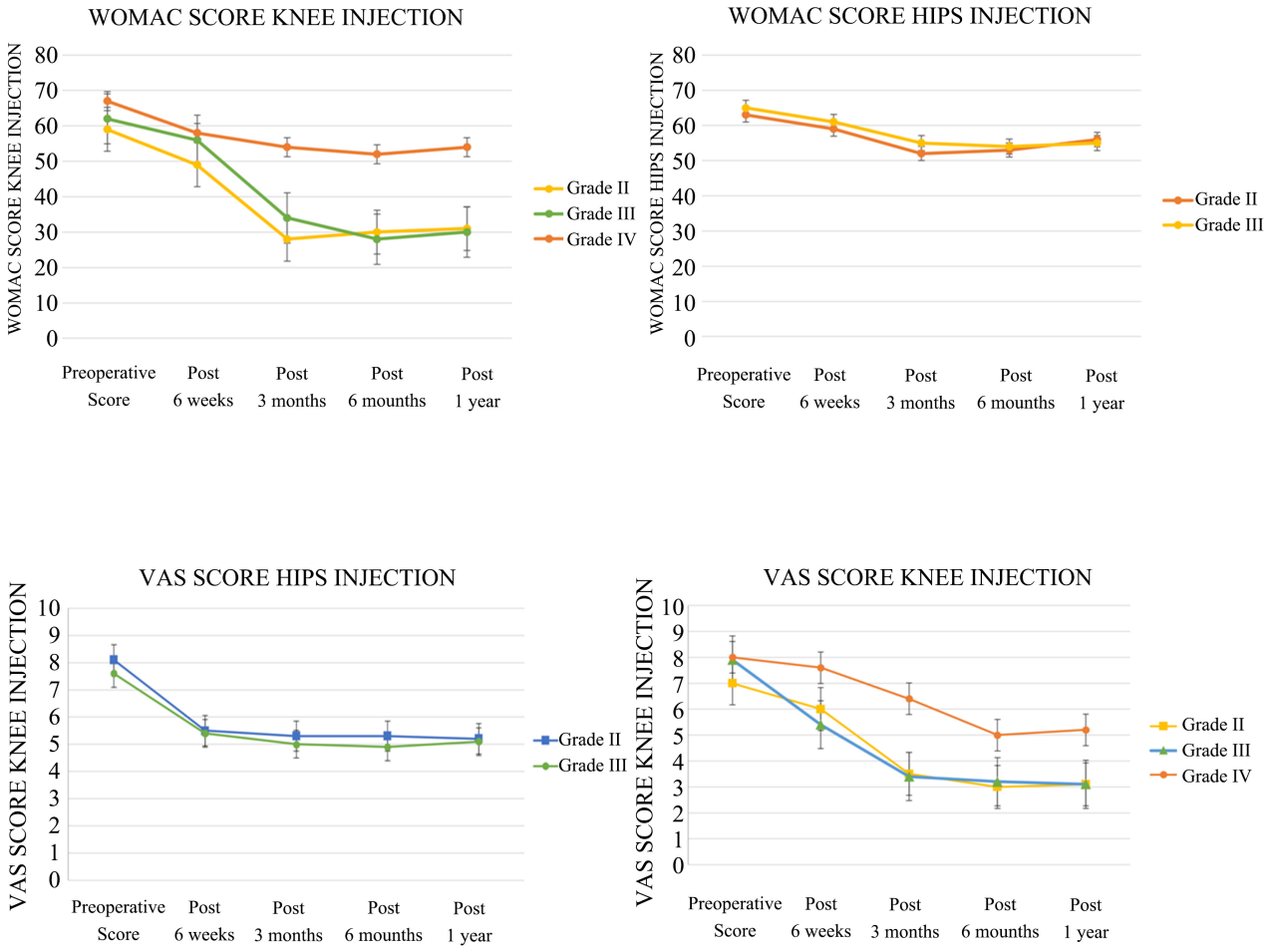


Figure 6. After hybrid SVF treatment, the clinical evolution of treated patients was assessed using the WOMAC, and VAS scales. At 12 months postoperatively, clinical scores of WOMAC and VAS for Grade II and Grade III were significantly higher than for Grade IV injection in knee, there is no significant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

Table 2. Improvement rate from baseline to 12-month postoperatively in WOMAC and VAS for pain scores among Kellgren-Lawrence classifications.

	Clinical score	Kellgren-Lawrence classification for knee injection			Kellgren-Lawrence classification for hip injection	
		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 ± 1.87	5 ± 1.9
6 Months Score	WOMAC	30 ± 20.9	28 ± 18.7	52 ± 19.5	53 ± 1.71	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 ± 1.85	55 ± 17.9
	VAS	3.1 ± 1.1	3.1 ± 0.9	5.2 ± 1.1	5.2 ± 1.8	5.1 ± 1.3

knee injections and 63 ± 22.6 for hip injections, accompanied by VAS scores of 7 ± 0.9 and 8.1 ± 2.2, respectively. After a six-week interval, the WOMAC scores showed improvements to 49 ± 20.4 for knee injections and 59 ± 18.2 for hip injections, with VAS scores reduced to 6 ± 2.0 and 5.5 ± 1.2, respectively. Notably, these improvements continued at the three-month mark, with WOMAC scores reaching 28 ± 17.7 for knee injections and 52 ± 18.7 for hip injections, while VAS scores dropped to 3.5 ± 1.7 and 5.3 ± 1.9, respectively. Similarly, at the six-month interval, WOMAC scores were reported as 30 ± 20.9 for knee injections and 53 ± 1.71 for hip injections, with corresponding VAS scores of 3 ± 2.0 and 5.3 ± 1.7. These trends persisted over a one-year period, as demonstrated by WOMAC scores of 31 ± 21.9 for knee injections and 56 ± 1.85 for hip injections, along with VAS scores of 3.1 ± 1.1 and 5.2 ± 1.8, respectively. This cumulative data indicates the noteworthy improvements achieved through the study interventions, thereby underscoring the potential effectiveness of the examined treatments for individuals with OA. The study findings indicate that similar positive results, including improvements in pain relief and joint functionality, are applicable to Grade III OA cases. However, the results are less promising for Grade IV knee cases. These Grade IV patients did not experience the same level of significant improvement as observed in Grade II and Grade III cases.

This comprehensive clinical evaluation underscores the safety and efficacy of the mechanical processing of adipose tissue and the preparation of hybrid SVF, providing valuable insights into the potential benefits of these methodologies for osteoarthritis treatment.

4. Discussion

SVF injection is one of the important therapy methods for regenerative medicine, but there are several challenges regarding the isolation of SVF cells from adipose tissue [19] [20]. The enzymatic method, which is accepted as the gold standard for SVF isolation, is costly and requires special equipment and qualified personnel for application. Although non-enzymatic methods are cheaper and faster, the cell viability of SVF isolated with this method is lower. Furthermore, mechanical isolation devices that take less time to process adipose tissue and produce fewer cells may not be suitable for clinical applications [21] [22] [23]. To overcome this situation, we developed the hybrid SVF concept that achieves a head-to-head cell yield in comparison with the enzymatic SVF isolation method [17].

Lipocube Hybrid is a sterile, closed system that permits high-throughput isolation of progenitor cells without the use of enzymes. Using a sharp blade based system, Lipocube Hybrid isolates cells from a small amount of adipose tissue by mechanical techniques. In this study, we evaluated mechanical and enzymatic methods to isolate SVF from subcutaneous abdominal fat. *In vitro* experiments were conducted to compare cell number, cell activity, flow cytometry, and cell differentiation capacity of SVF cells isolated mechanically by concentration and non-concentration methods [24]. The present research observed that the Lipocube Hybrid demonstrates a comparable count of viable nucleated cells as the enzymatic method. Nonetheless, the proportion of progenitor cells within the SVF prepared using Lipocube Hybrid was found to be greater. After *in vitro* studies, the Lipocube Hybrid mechanical isolation method was used to isolate SVF and applied in 42 knee and 7 hip joints of 28 patients with Grade II, Grade III, and Grade IV OA.

This study involved conducting cellular characterizations of the SVFs acquired through the Lipocube Hybrid approach and subsequently comparing them with the enzymatic method, which is considered the gold standard. As shown in **Figure 2(a)**, in the E-SVF group, the number of viable nucleated cells per milliliter was approximately 2.8×10^6. Comparatively, the Lipocube Hybrid SVF group had around 2.3×10^6 viable nucleated cells per milliliter. In terms of cell viability, the average viability rate was 94.01% for the Lipocube Hybrid SVF method and 91.20% for the enzymatic digestion method. Normally, there are significant differences in the results of cell viability and yield between the enzymatic method and mechanical isolation methods [16]; however, the Hybrid SVF technique increases the effectiveness of mechanical isolation methods.

Cellular activity and phenotypic characterization of the Lipocube Hybrid SVF and E-SVF were investigated through flow cytometry analysis. As shown in **Figure 3**, Lipocube Hybrid group displayed notably higher expression levels of CD90+/CD73+ and CD90+/CD45− markers associated with ADSC characteristics, as well as an elevated presence of CD45−/CD31+ endothelial (progenitor) cell patterns [25]. This robust phenotypic profile suggests the enriched regenera-

tive potential of Lipocube Hybrid SVF. Furthermore, the diminished presence of macrophages/monocytes in the Lipocube Hybrid group compared to enzymatic digestion points to a potentially reduced inflammatory response associated with mechanical isolation.

The differentiation and gene expression studies provided critical insights into the potential of the mechanically isolated SVF for osteogenic, chondrogenic, and adipogenic differentiation. Differantiation results revealed that Lipocube Hybrid SVF cells successfully performed osteogenic, chondrogenic and adipogenic differentiation.

Both E-SVF and Lipocube Hybrid SVF groups demonstrated comparable osteogenic differentiation capabilities, as demonstrated by **Figure 4(a)**. The osteogenic differentiation is evidenced by the deposition of calcium-rich extracellular matrix, visible as dark regions in the Alizarin Red stained sections, signifying a presence of calcium deposition and mineralization. This indicates the ability of the cells to undergo mineralization and form bone-like structures. The staining intensity and presence of bone-like structures were similar between the two isolation methods, suggesting that the potential for osteogenic differentiation remains relatively consistent regardless of the isolation approach.

Chondrogenic differentiation is evident through the presence of well-defined, intensely stained cartilage-like matrix distributed within certain areas. This demonstrates the capacity of cells to generate chondrocyte-like cells and produce cartilage-specific components [26]. As shown in **Figure 4(b)**, Lipocube Hybrid SVF group led to more prominent chondrogenic differentiation, as evidenced by strong Alcian Blue staining, indicative of abundant glycosaminoglycan (GAG) production within the extracellular matrix. GAGs are essential components of cartilage tissue, and their presence suggests robust chondrogenic potential [27]. In contrast, E-SVF group exhibited relatively weaker staining, indicating reduced cartilage matrix synthesis potential.

Moreover, the adipogenic differentiation is marked by the accumulation of lipid droplets within the cells, appearing as clear vacuoles in the Oil red-O-stained sections. This suggests the successful transformation of SVF cells into adipocyte-like cells capable of storing lipid reserves [28]. As shown in **Figure 4(c)**, Lipocube Hybrid SVF group demonstrated a clear advantage in adipogenic differentiation compared to E-SVF. The Oil Red-O staining of Lipocube Hybrid SVF displayed a vivid red coloration, indicating a high accumulation of lipid droplets within the cytoplasm. This intense staining pattern is indicative of robust adipogenic differentiation, suggesting that the cells have successfully transformed into adipocytes and accumulated lipids, a hallmark of mature fat cells. In contrast, E-SVF staining was less intense, and the presence of lipid droplets appeared limited, implying that these cells had a lower propensity to undergo adipocyte differentiation.

Overall, the histological staining and gene expression results provide compelling evidence of the successful differentiation of Lipocube Hybrid SVF cells into osteogenic, chondrogenic, and adipogenic lineages. These findings hold signifi-

cant promise for regenerative medicine applications, showcasing the cells' multipotency and potential to contribute to various tissue repair and regeneration strategies. Furthermore, while the Lipocube Hybrid method has shown good differentiation potential towards these lineages, more research is needed to fully assess its potential for clinical applications. It is important to conduct further studies to evaluate the safety and efficacy of this method in different clinical settings and to compare it to other isolation techniques.

In the clinical domain, the study's application of mechanically processed adipose tissue and the preparation of hybrid SVF in OA patients underscored the safety and efficacy of these interventions. 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 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.

The improvements in WOMAC scores and VAS scores across all grades and the sustained enhancements observed over a one-year period are indicative of the potential benefits of the examined treatments. The notable reductions in WOMAC and VAS scores signify improved pain relief, joint functionality, and overall patient outcomes.

However, it is essential to acknowledge certain limitations in this study. The absence of control groups, the lack of clinical and imaging assessments during the study, and the unexplored correlation between SVF cell dosage and outcomes represent areas for future investigation. Furthermore, the clinical evaluation included transient pain and swelling as side effects, suggesting the need for further exploration into potential adverse reactions associated with the interventions.

In conclusion, the comprehensive results presented in this study provide a strong foundation for the consideration of mechanically isolated SVF as a potential therapeutic approach for OA treatment. The viability, cellular activity, phenotypic characterization, differentiation potential, and clinical outcomes collectively demonstrate the potential benefits of mechanically isolated SVF, particularly through the Lipocube Hybrid SVF method. These findings encourage fur-

ther research and exploration to elucidate the full scope of SVF's regenerative capabilities and its applicability in addressing osteoarthritis and related conditions.

5. Conclusions

This study delves into the potential of using autologous SVF injection as a minimally invasive approach for treating orthopedic disorders, specifically focusing on OA. The study comprehensively evaluates the viability, cellular activity, phenotypic characterization, differentiation potential, and clinical outcomes of mechanically isolated SVF, particularly through the Lipocube Hybrid method. The investigation begins by comparing the effectiveness of mechanically isolating SVF with the gold standard enzymatic digestion method. Although the count of viable nucleated cells in the Lipocube Hybrid SVF is slightly lower compared to enzymatic digestion, the difference is not statistically significant, indicating the viability of mechanical isolation.

Through flow cytometry analysis, the Lipocube Hybrid SVF demonstrates elevated expression of stem cell markers and a diminished presence of inflammatory cells, suggesting its enhanced regenerative potential and reduced inflammation. The study highlights the remarkable differentiation capacity of Lipocube Hybrid SVF across adipogenic, osteogenic, and chondrogenic lineages. These findings underline the therapeutic potential of mechanically isolated SVF for regenerative applications. In the clinical evaluation, osteoarthritis patients who received Lipocube Hybrid SVF injections experienced significant improvements in pain relief and joint functionality, as evidenced by improved WOMAC and VAS scores over one-year period.

In conclusion, the study lays the groundwork for considering mechanically isolated SVF, particularly through the Lipocube Hybrid method, as a potential therapeutic option for OA. The comprehensive evaluation of viability, cellular activity, differentiation potential, and clinical outcomes provides a compelling rationale for further research to unlock SVF's regenerative potential in orthopedic disorders and beyond.

6. Limitation of the Study

Several limitations are present in this study, encompassing the lack of a clinical application control group and the absence of imaging assessments throughout the study period. Additionally, there exists no correlation established between the dosage of intra-articular SVF cell injection and resultant clinical or structural outcomes, and the study only entailed a singular treatment administration. Future studies should investigate the potential benefits of multiple injections and the correlation between SVF cells and other intra-articular interventions. Nonetheless, this study provides a promising new approach for the treatment of knee and hip OA, offering patients a viable alternative to traditional treatment methods.

Another limitation of the study concerns to the relatively modest sample size, especially in the context of hip injections. Additionally, the follow-up period is relatively short at just 12 months. This timeframe might not offer a comprehensive understanding of the long-term efficacy of Hybrid SVF application. Future studies with larger sample sizes and longer follow-up periods may provide additional insight into the efficacy of this treatment.

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Author Contributions

P. K. and S. C. designed the experiments and wrote the manuscript, P. K. performed and analyzed the staining experiments, helped with the FACS analysis and RT PCR. T. T. performed the liposuction procedure. Y. D. helped to write the manuscript and provided critical feedback to the manuscript. Y. K. helped to write the manuscript. B. B. provided the feedback of the study and check the data and all manuscript during the submission. All authors have given approval to the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author Dr. Tiryaki upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interests with respect to the authorship and/or publication of this manuscript.

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