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A modified animal-free serum technique for efficient isolation and proliferation of mesenchymal stem cells from Hoffa fat pad

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ABSTRACT

We focus on this study in designing an alternative technique for obtaining mesenchymal stem cells (MSCs) from residual tissue, Hoffa fat, in arthroscopic procedures. Two males and two females were included, and underwent knee arthroscopy; a sample of infrapatellar adipose tissue was obtained with basket forceps. The primary culture was made using the explant method and the culture media: DMEM-high glucose, supplemented with 10% of inactivated human allogeneic serum. All the cellular cultures remained under culture conditions for three weeks, after that by flow cytometry the cells were characterized by MSCs antibody panel: CD105, CD73 and CD90. Subsequently, in the first pass, the MSCs were cultured in commercial human chondrogenic, osteogenic and adipogenic mediums, respectively. After primary culture, we obtained on average 95,600.00 ± 7,233.26 cells/cm^2, and the duplication time of MSCs isolate from Hoffa fat pad was established in 39 hours. By flow cytometry, we found that surface markers percentage for expanded MSCs (CD105, CD73, CD90) in primary culture significantly increased and its morphology was fibroblastic-like. After differentiation culture which was made in the first pass, by immunofluorescence, we obtained positive cell markers for three lineages of differentiation, adipocytes: LPL protein, osteocytes: RUNX2, Osteopontin, chondrocytes: SOX9, Aggrecan and COL2A1. We managed to isolate a significant number of MSCs from this source using an easy method to implement and minimal nutrient supplementation, with high potential for differentiation to mature mesenchymal tissues and potential use in basic experimental, preclinical and even clinical research.

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Introduction

Neuropathic according to the World Health Organization, osteoarthritis (OA) is a growing public health problem affecting elderly populations worldwide. The prevalence of OA is increasing, recent studies have shown that articular cartilage lesions are present in more than 60% of arthroscopic procedures, becoming one of the major disabling conditions, especially in populations with a long-life expectancy (1).

Currently, no effective treatment is available to improve or reverse the degenerative process. Therefore, the interest in the clinical use of mesenchymal stem cells (MSCs) has attracted attention due to their multilineage potential, immunosuppressive activities, limited immunogenicity, and relative ease of growth in culture (2). MSCs have the ability to differentiate into adipocytes, osteocytes, and chondrocytes while contributing to a regenerative microenvironment through the release of trophic factors (2), therefore adult MSCs have emerged as a candidate cell type with great potential for cell-based articular cartilage repair technologies (3). Many studies have demonstrated that MSCs can be isolated from multiple tissue sources including blood, adipose tissue, bone marrow, and cartilage (4).

Cell source is one factor that may influence treatment outcomes. It has been reported that adipose-derived mesenchymal stem cells (ASCs) had more rapid cell doubling times and antiapoptotic potential when compared to MSCs from other sources (5).

The infrapatellar fat pad (IPFP) is an intracapsular and extra-synovial adipose tissue structure that is located in the knee joint (6,7). It is the intraarticular inclusion that separates the anteroinferior synovial membrane from the other joint structures of the knee, therefore, is intraarticular but extra-synovial (8,9). This structure is composed of fibers with adipose tissue; the present fat has the ability to deform; in this way, it allows the adaptation and the contour change of the joint surfaces during knee motion (10). Given its anatomical location the IPFP can be easily accessed during knee arthroscopy or arthrotomy (8).

There is suggesting evidence that MSCs are present in adult human adipose tissue. Under certain conditions, MSCs show superficial cell markers associated with adipocyte and osteoblast phenotypes but they can also express superficial cell markers for chondrocytes (11). Recently, has also been shown that intraarticular injections of adipose-derived MSCs (AD-MSCs) in OA mouse and rabbit models exert anti-inflammatory and chondroprotective

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effects (9).

The isolation of MSCs has been previously reported from this source (12). AD-MSCs are an alternative source that shares many properties with MSCs. For preclinical studies and even clinical medical research. They can be easily collected by liposuction, or biopsy by arthroscopic procedures, and their proportion is several orders of magnitude higher than that of MSCs (9). Currently, MSCs are obtained from Hoffa fat, by enzymatic digestion with collagenase type I and the cellular culture is supplemented with fetal bovine serum (13,14), recently some authors demonstrated that the use of human serum (autologous or allogeneic), for culture expansion of MSCs is feasible without compromising differentiation capacity or the MSC cell surface immunophenotype (15).

On the basis of the aforementioned findings, we designed this study based on to find an alternative technique, to be cheaper and easy to obtain during arthroscopic surgery. Therefore, this work aimed to obtain MSCs by explant culture and supplemented with human serum, in this way we seek to save economic resources something essential in emerging countries, and at the same time decrease the time obtaining these cells. We emphasize that all the used samples were remnants of arthroscopic procedures. Thereby, in the future, we can use the fat-derived MSCs of IPFP to have cellular transplant banks, and even to use them to donate cadaveric donor cells, due to their low immunity and immunoregulatory character.

Materials and Methods

Patient selection

Surgical cases were included if they were under arthroscopic anterior cruciate ligament (ACL) reconstruction or treatment of a meniscal lesion with a symptomatic cartilage lesion on the femoral condyle. Demographic factors, including gender, age, height, weight, and BMI were recorded. Exclusion criteria included any type of arthritis, previous total meniscectomy and previous treatment of chondral lesions. The patients agreed to participate willingly (expressing their will by signing the informed consent), and we had the approval of our Institution's Ethics Committee (approval number 80/17).

All patients rolled in this work signed informed consent before surgery and underwent an index surgical procedure where ACL or meniscal lesions were treated. During this time, cells were isolated and expanded in the culture at the National Institute of Rehabilitation, Mexico City.

Arthroscopic procedure

All surgeries were performed by a single surgeon with the patient in the supine position. All patients underwent spinal block and a tourniquet was used. A detailed intraoperative evaluation of the medial, lateral, and patellofemoral joint compartments was performed, and cartilage lesions were described using the International Cartilage Repair Society (ICRS) classification. After, they irrigated the compartment with at least 1 liter of saline solution and performed one or more of the following treatments: Synovectomy; debridement; or excision of degenerative tears of menisci, chondral flaps, fragments of articular cartilage, and osteophytes that prevented complete extension.

Once the planned arthroscopic treatment was completed, a sample of infrapatellar adipose tissue was obtained, using basket forceps, obtaining either piece of adipose tissue through the medial portal or lateral portal, until obtaining approximately 50 mg. The tissue was collected in a sterile bottle, to be immediately transferred to the cell culture unit located within the area of operating rooms, and to start with the planting of cell culture.

Primary cell culture

After obtaining Hoffa fat pad, the sample was mechanically disaggregated using a number 20 scalpel knife under sterile conditions. The tissue fragments were cultured like explants in a 25cm^2 flask (CORNING, cat. CLS3289-200EA) with Dulbecco's Modified Eagle's Medium (DMEM-high glucose (DMEM-HG); CORNING, USA, cat. 10-013-CVR), supplemented with 10% of inactivated human serum (HS; BIO-WEST, Inc. cat. S4190-100) and 1 % antibiotic/antimycotic (Gibco-Life Technologies, cat. 15240062). The cultures were maintained in an incubator at 37°C with 5 % CO_2, change of medium was made every other day until 90% of confluence was reached. Aliquots of 1x10^5 cells were used to obtain cellular doubling cell time, immunophenotype by flow cytometry and cell differentiation assays.

Determination of doubling cell time

Doubling cell time determination was carried out using the crystal violet staining technique as previously described (16). For this, we removed the culture medium and left the culture to air-dry. Immediately, cells were fixed with 1.1% glutaraldehyde (Sigma-Aldrich, cat. G5882) for 10 minutes, the excess fixative was removed, and the cells were washed with distilled water and left to air-dry. Then, we proceeded to stain the cells with 0.1 % crystal violet dye (Sigma-Aldrich) for 10 minutes, after we removed the excess staining by means of washes with distilled water and left to air-dry. Finally, acetic acid (Sigma-Aldrich) at 10 % was used to solubilize the dye under shaking in an orbital agitator (150 rpm) for 20 minutes. Absorbance measurements were read at 470 nm in a spectrophotometer varioskan TM LUX multimode microplate reader (ThermoFisher). Photographs of the stained cells were taken in a Zeissbrand invertoscope and analyzed with the AxioVision ver. 4.8.2 software program. To calculate the duplication time of the cells under culture, first, we calculated the growth rate (GR) utilizing equation 1 (Eq1):

$$GR = \frac{Abs15 - Abs2}{Abs2} \times 100 \qquad \text{Eq 1}$$

Where Abs15 is the median absorbance value obtained on day 15 and Abs 2 is the absorbance value obtained at day two. Finally, the calculation of cellular duplication time was obtained by dividing the duplication constant by the percentage value of the growth rate.

Immunophenotype of cells isolated from Hoffa fat

An aliquot of 1x10^5 cells in primary culture was transferred to polystyrene tubes [Falcon; Becton–Dickinson (BD)], then 5µL of specific markers for MSCs was garaged: APC-conjugated CD90 (100 µg/ml, mouse IgG1κ. Miltenyi Biotec, cat. 130-114-861), PerCP-Cy™5.5-conjugated CD73 (20 µg/ml, mouse IgG1κ, BD biosciences, cat. 561260), PE-conjugated CD105 (50 µg/ml, mouse BALB/c IgG1κ, BD biosciences, cat. 562380).

The samples together with unlabeled controls were distributed for each antibody and used to obtain the percentage of fluorescence on the flow cytometer. Data were acquired in a BD FACSCalibur flow cytometer and analyzed by CellQuest™ PRO software (Becton–Dickinson) with a mean of 10,000 events. This procedure was repeated in each Hoffa's fat samples that were processed.

In vitro differentiation of infrapatellar fat pad MSCs into mesenchymal tissues

The cells in the first pass were seeded at a density of $1x10^5$, for the differentiation in monolayer cell culture, we used a commercial differentiation culture medium: adipogenic differentiation medium (StemMACS AdipoDiff Media human, Miltenyi Biotec), chondrogenic medium (StemMACS ChondroDiff Media human, Miltenyi Biotec), and osteogenic medium (StemMACS OSteoDiff Media human, Miltenyi Biotec). All the culture mediums were supplemented with 10% of inactivated human serum (HS; BIO-WEST, Inc. cat. S4190-100) and 1 % antibiotic/antimycotic (Gibco-Life Technologies, cat. 15240062). The cultures were maintained in an incubator at 37°C with 5 % CO_2, change of medium was made every two days. The cultures were maintained for a period of two weeks for osteocytes, chondrocytes and adipocytes differentiation.

Cell staining differentiation to three lineages

In the three cell lines, the coverslips (12mm diameter, cat. CLS-1760-012, Chemglass), were removed from the 12 wells plates and placed in coverslip to perform cell staining. For cartilaginous tissue, the chondrogenic differentiation of CTM in cell culture boxes results in the formation of cartilage with a typical extracellular matrix. A key molecule: in addition to type II collagen within this extracellular matrix, it is the proteoglycan aggrecan. Aggrecan can be used as an indicator of cartilage formation and can be detected with Alcian Blue, which is a dye containing dark copper blue color (17). Staining was carried out as follows:

Cells were fixed with 3% acetic acid solution for 3 minutes, then alcian blue 1% (pH 2.5) was added in a dark room for 20 minutes. Then washed with tap water for 5 minutes and rinsed with distilled water, followed by the addition of periodic acid 0.5%, dyed in a dark room for 10 minutes and washed with tap water for 5 minutes. The schiff reagent was added for 20 minutes and washed with running tap water and rinsed with distilled water, finally, we stained it for 50 seconds with hematoxylin-eosin.

Adipose tissue: red or oily stain is an assay used to detect adipogenic cultures with staining to detect mature adipocytes (18). This staining was carried out in the following way: We Prepared the solution with 300 mg of red oil and add 100 ml of 99% isopropanol. We mixed 3 parts (30ml) of the red oil O solution with 2 parts (20ml) of deionized water and left it to stand at room temperature for 10 minutes, this solution is stable for no more than 2 hours, so it was used at the time, the cells were fixed with formalin solution for 2 minutes, rinsed gently with 2 mL of sterile water each well then, we added 2 ml of 60% isopropanol to cover the lamella with cultured cells. And it was left to rest for 2-5 minutes, and rinsed with a distilled water solution, we added 2 ml of the red oil O solution, so that the cells are completely covered.

Osteogenic tissue: This staining was done in the following way: we dissolved 2 g of red alizarin S in 100 mL of distilled water, adjust pH to 4.1, filtered and stored in a dark room, the medium was aspirated, carefully washed with PBS solution (Dulbecco`s), without breaking the monolayer, fixed with formalin neutral solution (10%) covering the monolayer completely for 30 minutes then the cellular monolayer was washed with distilled water, sufficient red alizarin S solution was added to cover the monolayer, rest in a dark room for 45 minutes. The monolayer was washed 4 times with distilled water, and PBS was added for visualization under a pyramid microscope.

Characterization by immunofluorescence

After 90% of confluence was reached, adherent non-predifferenciated cells cultured in monolayer were fixed after 6 days of culture, cells were first passed to a circular coverslip and fixed with 2 % paraformaldehyde for 20 minutes Each sample was washed with 0.5 mL of PBS, followed by a solution of PBS/albumin 1%/triton 0.3% for 20 minutes to block unspecific binding sites. Subsequently, primary antibodies were incubated overnight at 4ºC at a concentration of 10:40 µL using the following antibodies specific to sheep: AGGRECAN-FITC (Novusbio, cat. NB100-74350F), SOX9 (Santa Cruz, cat. SC-17340), COL2A1 (Santa Cruz, cat. SC-7763), LPL-FITC (Biorbyt, cat. Orb14107), OPN (Santa Cruz, cat. SC-10599) and RUNX2 (Santa Cruz, cat. SC-8566). Later, was washed 2 times with PBS + 0.1 triton and the primary antibodies that needed a secondary antibody, we used: Anti-IgG-FITC (Santa Cruz, cat. SC-2356), at a concentration of 1:100 to a 37 ºC for 2h was placed and washed a time more with PBS 0.1% triton to remove excess secondary antibody. We used for isotype-controls: anti-mouse IgG2a-FITC (SANTA CRUZ cat. sc-358947), anti-mouse IgG-FITC (SANTA CRUZ cat. sc-358946), and ant-rabbit IgG-FITC (ABCAM cat. ab37406). Finally, the slides were mounted with DAPI. All the from staining and immunofluorescence images were recorded using a pyramid microscope with UV lamp Carl Zeiss Axio system image Vission 4.8.2.

Statistical analysis

All data were reported using the mean and standard deviations (SD). Statistical analysis was performed with STATISTICA StatSoft v7 data analysis program A Statistically significant difference was considered when the *p*-value was $p<0.05$. Analysis of variance was performed by means of the Mann-Whitney U test.

Results

After three weeks, the primary culture was established by Hoffa fat explant we obtained on average of 95,600.00 ± 7,233.26 cells/cm^2 (Figure 1). With Crystal violet staining (Figure 2), we determined the duplication time of HFP cells at 39h (Figure 3).

The immunophenotype for MSCs markers in the cells isolated from HFP in primary culture was: CD73: 88.42 ± 10.02 %; CD90: 95.33 ± 3.24 %; CD105: 81.71 ± 2.24 % and double staining of markers for MSCs was: CD90/CD73: 77.29 ± 8.61 %; CD90/CD105: 90.20 ± 16.44 %; CD105/CD73: 86.58 ± 10.83 % (Figure 4).

We demonstrate the cellular differentiation of HFP

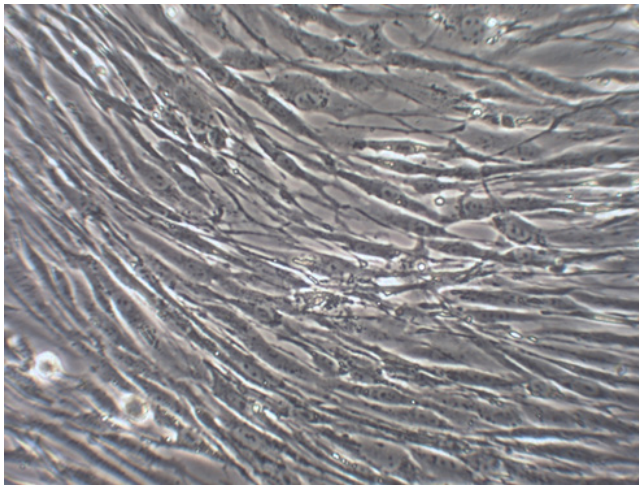

Figure 1. Microphotographs under visible light, in which we can appreciate: a) the morphology of the cells isolated from Hoffa fat pad. At day 2 of Primary culture (PC), b) fibroblast-like cells at week 2 of PC were predominant over the rounded cells.

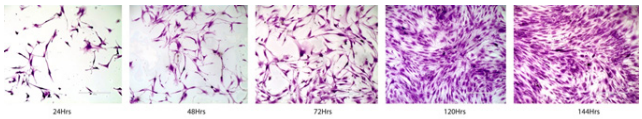

Figure 2. Morphology and proliferation potential by crystal violet staining at 24,48,72,120 and 144 hours of in vitro culture of IFFP.

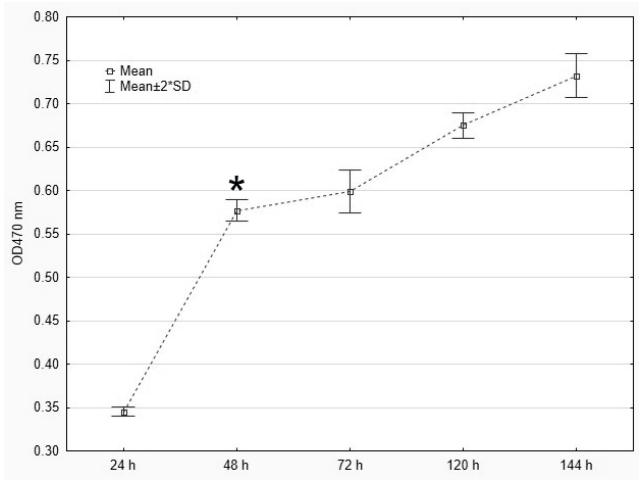

Figure 3. Cell proliferation curve from 24 hours of culture and up to 144 hours, where an important positive correlation is observed. Reaching confluences greater than 90% after 6 days of cultivation. Proliferation in vitro cells isolated from IFFP MSCs by Crystal violet staining through absorbance measurement at 470 nm.

cells to the osteoblastic, adipogenic and cartilage lineages, by positive staining for alkaline phosphatase for bone, oily red for adipocytes and alcian blue for chondrocytes (Figure 5). In addition, by immunofluorescence, the positive expression of the LPL protein was observed for differentiated cells to adiposities, RUNX2 and OPN in cells differentiated to osteoblasts and proteins COL2A1, Aggrecan and SOX9 in cells differentiated to chondrocytes (Figure 6).

Discussion

Our results show that isolation of MSCs from IPFP using the cellular culture by explant is an alternative source. The IPFP is an ideal source for biomedical research, preclinical and clinical; it has been proposed as a source for a broad gamma of applications, such as tissue engineering, combine with cadaveric donations from specialized tissue procurement banks.

MSCs cell therapies have already been tested in clinical trials at the safety and efficacy phases, with remarkable restorative activity in the treatment of OA (4). Besides It has been reported that MSCs, can be isolated from several tissues (19). Adipose tissue is more accessible than bone marrow and previous studies have demonstrated that there are considerable differences in growth and differentiation between different sources (5,20,21). MSCs derived from adipose tissues have been shown to be more effective in their multipotent potential. The MSCs derived from adipose tissue had a low population doubling time for cell proliferation and higher anti-apoptotic potential than mesenchymal cells derived from bone marrow and cartilage (5). In the knee, the IPFP is resected during joint arthroscopy in patients with acute OA and is a suitable source of fat tissue, for the autologous treatment of additionally affected joints.

We have demonstrated improved proliferative, chondrogenic, and osteogenic rates from the IPFP cells derived, showing a promising and improved source for efficient cell therapeutic resolution of joint degeneration (22).

The IPFP cell amplification *in vitro* with autologous serum was always better than with fetal bovine serum, previous reports from subcutaneous adipose tissue have shown detrimental proliferative activity of MSCs derived

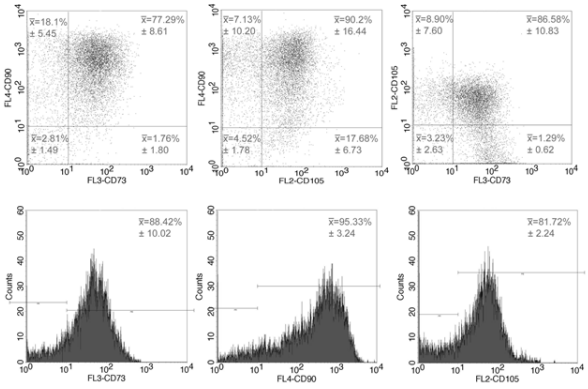

Figure 4. Immunophenotype of mononuclear cells present in Hoffa fat pad, in primary culture, for CD90+, CD105+, CD73+. The results are represented by the percentage of fluorescence of each fluorochrome conjugated with each antibody, respectively, against the number of events counted. In the histograms.

Figure 5. Differentiation of MSCs isolated from Hoffa fat from knee joint, to the three cell lines chondrocytes with alcian blue staining, osteocytes with alkaline phosphatase staining, oil red staining adipocytes.

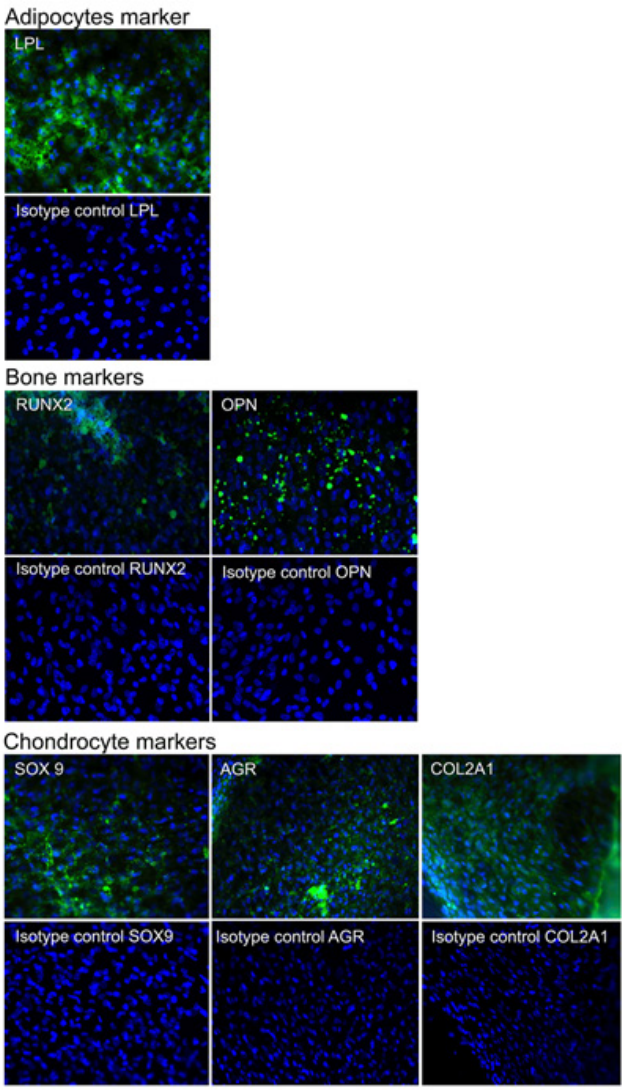

Figure 6. Inmunofluorescence in cells isolated from Hoffa Fat Pad, in first pass at 2 weeks of differentiation to adipocytes, osteocyte and chondrocyte. The nuclei of each cell appear in blue (stained with 4`6-diamidino-2-phenylidole (DAPI) and in green marking indicates positivity for each cell differentiation lineage is observed the fluorescein isothiocynate (FITC) fluorochorme-labeled antibodies.

from IPFP in presence of fetal bovine serum (23). The current study shows that human MSCs isolated from IPFP of the knee have the ability, under lineage-specific culture conditions. In addition, modulation of characteristic gene markers for each of the three mesodermal lineages was observed under the appropriate culture conditions. We consider to IPFP as an optimal source for MSCs, since this tissue is considered a biological waste, so no damage is caused to the patient; the current findings indicate that the IPFP of the adult knee harbors a source of stromal cells with multilineage potential similar to those of cells derived from bone marrow stroma (18,24,25), or from subcutaneous adipose tissue (26,27). Although the function of these multipotent progenitor cells in the fat pad is not fully understood, their presence is consistent with previous reports identifying undifferentiated cells within various adult tissues.

The IFPF is a heterogeneous and fibrous structure that differs significantly from the tissue recovered in liposuction (11). Histologic analysis with hematoxylin and eosin staining showed that a proportion of the fat pad is dense collagenous tissue. The fad pad isolates are a heterogeneous population of cells that may contain small amounts of other cells such as pericytes and endothelial cells (28).

In general, the population from the pad share adhesion and receptor molecules (18,29,30). Three of these common surface makers CDk105, CD90, and CD73, have been used to define the bone marrow population called mesenchymal stem cells that are capable of adipogenesis, chondrogenesis, osteogenesis, and hematopoietic bone marrow mesenchymal stem cells (31-35), have been reported to produce collagen types I, II, and X by immunostaining (25, 32). In the present study, collagen type II was observed intracellularly and in the pericellular region of the fat pad-derived cells. This finding is consistent with studies of marrow-derived mesenchymal stem cells, which do not produce significant amounts of collagen type II at least the second week of *in vitro* culture (32). In normal cartilage, this molecule is localized to the pericellular matrix of chondrocytes and is thought to play an important role in the extracellular matrix.

Chondrogenic culture conditions significantly improved and sustained the biosynthetic activity of the fat pad-derived cells, with consequent increases in the expression of protein and proteoglycan. These findings are similar to those studies reported on subcutaneous adipose tissue (26), These findings may suggest that a portion of these fat pad-derived cells is producing genes associated with chondrocytes, adipocytes, and osteocytes without growth factor stimulation.

Our findings corroborate the presence of cells with multipotent phenotype in the stroma of the knee fat pad. Given it locations and accessibility, the fat pad may prove to be a readily accessible source of MSCs for specialized tissue banks, tissue engineering or other cell-based therapies. For example. The fat pad could provide and autologous source of cells for chondral or osteochondral lesions and repair them with the same knee, and also be a source for preclinical or even clinical studies.

The objective of the work was achieved using an easy technique to implement and minimal nutrient supplementation (using human serum). The MSCs, that we isolated from Hoffa fat pad in humans have the ability to differentiate into three lineages: chondrocytes, osteocytes and adipocytes. We think, that these cells have a high potential for the development of new therapeutic strategies to repair focal lesions of the articular cartilage.

Using the explant method, we don´t need to use any kind of enzymatic method to disaggregate the cells, inasmuch as the MSCs present in Hoffa Fat Pad, have the ability to migrate since the fragments tissue to the flasks growing area.

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Interest conflict

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Consent for publications
The author read and proved the final manuscript for publication.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contribution
AOM undertook managed data and wrote the manuscript. VHCS conceived and designed methods. ABLA wrote the manuscript. RAG wrote the manuscript and conducted data analysis and data processing. KMF and YZC wrote the manuscript and data verification and contributed analysis. CLS wrote the manuscript, conceived the study and review the design. All authors read and critically revised the manuscript and approved the final version.

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Ethics approval and consent to participate
The patients agreed to participate willingly (expressing their will by signing the informed consent), and we had the approval of our Institution's Ethics Committee (approval number 80/17).

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