Comparison of chondrogenic differentiation of mesenchymal stromal cells from human amniotic fluid and human adipose-derived tissue in chitosan-xanthan gum scaffolds

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Data Note

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Abstract

**Introduction:** After age and obesity, traumatic injuries represent the third most important risk factor for the development of osteoarthritis. Current treatments for cartilage injuries are not very effective. However, the use of stem cells, associated or not with scaffolds, has been proposed and investigated. In this study, we compared chondrogenic differentiation in human amniotic fluid mesenchymal stromal cells (hAF-MSC) and human adipose-derived mesenchymal stromal cells (hAD-MSC) grown in porous chitosan-xanthan gum scaffolds (CX) stimulated with TGF-β3, aiming at the possibility of direct implantation in the lesioned site.

**Methods:** hAF-MSC were collected from women in the second trimester of pregnancy and hAD-MSC from patients that underwent liposuction. In the case of hAF-MSC samples, CD117-positive cells were selected. The mesenchymal stromal cells (MSCs) from both sources were expanded and characterized considering their capacity to adhere to polystyrene culture flasks, by flow cytometry analysis and differentiation into cartilage, bone and fat cells. The MSCs were seeded into chitosan-xanthan gum scaffolds specially designed for use in cartilage tissue engineering and grown under TGF-β3 stimulation. Differentiation was confirmed and evaluated by scanning electron microscopy (SEM), histology, immunohistochemistry and immunofluorescence analysis.

**Results:** The results showed that MSCs from both sources exhibited high capacity for cell expansion, positivity for phenotypic markers, multipotency, chondrogenic potential and negativity for hematopoietic markers, in addition to differentiation capacity into the three above-mentioned mesenchymal lineages. Chondrogenic differentiation was confirmed by hematoxylin-eosin, alcian blue, picrosirius red and Masson's trichrome staining, indicating the presence of collagens and proteoglycans. Immunohistochemistry analysis showed positivity for collagen II and aggrecan, and immunofluorescence also showed positivity for collagen II. SEM revealed intense cell adhesion and collagen fibers adhered to the scaffold.

**Conclusions:** In summary, it was possible to differentiate *in vitro* stem cells from human amniotic fluid and human adipose tissue into chondrocytes directly in the scaffold of chitosan and xanthan in the presence of TGF-β3, with evident production of an ECM rich in collagen and PGs.

**Highlights**

- Porous chitosan-xanthan gum scaffolds were produced and their effect on mesenchymal cell chondrogenesis under TGF-β3 stimulation was assessed.
- Both human amniotic fluid mesenchymal stromal cells and those derived from adipose tissue could be differentiated into chondrocytes *in vitro* on the scaffolds, with production of extracellular matrix rich in collagen and proteoglycans.
- The strategy proposed has high potential to be used in functional studies in relevant preclinical and translational animal models.
Joint injuries are increasingly common in active individuals and have become a major focus of public health concern in recent times because they affect a large portion of the world’s population.(1) Joint injuries following trauma represent an important risk factor for the development of arthritic diseases such as osteoarthritis (OA) (2–4). Articular cartilage is a highly specialised connective tissue that lines the ends of long bones in load-bearing joints (5). It is designed for absorbing compressive loads and sheer forces associated with physical activity and joint loading, but it has a poor capacity for self-repair and regeneration, especially in older adults. The degeneration and loss of articular cartilage is a key component of the clinical syndrome of OA, which is one of the most common causes of pain and disability in middle-aged and older people (1, 6–8). OA is characterized by changes in the metabolism of chondrocytes within articular cartilage (9), increasing the expression of proteolytic enzymes and the degradation of extracellular matrix (ECM) macromolecules, resulting in the loss of cartilage tissue (10).

Although OA disease affects all joints tissues and compartments, it is the articular cartilage component which has received most of the attention, especially in the context of its role in providing low friction joint articulation and in how its degradation leads to loss of joint function (7). There is rising interest in the concept of joint preservation (11), regeneration and remodeling (12) through stimulation of cartilage repair.

There are currently no effective pharmacological disease-modifying OA drugs (13–15). Treatments to slow or prevent both the degradation of cartilage and other joint tissues following joint injuries and the development of joint diseases have not been fully established. Currently recommended treatments are generally based on symptom relief (16). However, a number of biological drugs and stem cell-based therapies are emerging for the treatment of OA (17–19). In the context of joint injuries and cartilage repair, the use of stem cells represents a promising prospect for future clinical applications. These cells can be combined with polymer-based scaffolds, which are capable to prevent cell dispersion and loss by increasing the number of cells close to the site of injury, with improved regeneration and immunomodulation potential (20). In addition, the scaffolds can also support cell development considering issues related to tissue architecture.

The development and use of scaffolds for stimulation of chondrogenic differentiation of mesenchymal stromal cells requires meeting a large set of requirements of the biomaterial (21–23), including: biocompatibility; biodegradable or bioresorbable features, with controllable degradation and resorption rates; adequate chemical composition, with suitable surface chemistry for cell attachment, proliferation, and differentiation; capacity to mimic the natural microenvironment of the ECM; stability in culture medium for in vitro studies and in the physiological environment after implantation; suitable porosity and roughness; pores with appropriate architecture regarding shape and distribution, size and interconnectivity; mechanical properties matching those of the tissues at the site of implantation, in addition to easy processability to form a variety of shapes and sizes, capacity to resist to sterilizations procedures and competitive costs. (24, 25) Several biomaterials have been analyzed for this purpose,
mainly those composed of polysaccharides, such as agarose, alginate, xanthan gum and particularly, chitosan (22, 23, 26).

Different sources of stem cells are studied for use in cartilage cell therapy (27, 28). However, some studies have shown that amniotic fluid stem cells have ideal characteristics for various applications in cell therapy, due to the ease of obtaining, expanding, isolating, maintaining and differentiating them (29–31). It is well known that amniotic fluid is composed by different cell types that can be differentiated into other lineages. Among them, multipotent cells characterized by the expression of the CD117 antigen (c-kit) show significant promise (29).

Another widely used source of mesenchymal stromal cells is the human adipose tissue (hAD-MSC), that are also characterized by their high capacity for expansion and differentiation (32, 33). The ability to proliferate and differentiate into various mesenchymal lineages makes hAD-MSC a potentially less invasive substitute for hBM-MSC in cell-based therapeutic applications. (34)

However, to the best of our knowledge, there are no studies comparing the chondrogenic differentiation process of hAF-MSC with that of hAD-MSC. In this study, we assessed the potential of hAF-MSC and hAD-MSC for chondrogenic differentiation directly into chitosan-xanthan gum scaffolds designed for cartilage tissue engineering, under TGF-β3 stimulation, a growth factor that is involved in chondrogenesis in vivo and is responsible for maintaining the stability of the ECM that is formed, preventing its calcification and degeneration (35, 36).

2. MATERIALS AND METHODS

2.1. Harvesting, isolation and expansion of human amniotic fluid mesenchymal stromal cells

All studies were conducted in compliance with recognized international standards, including the guidelines of the World Medical Association (WMA) and the principles of the Declaration of Helsinki. For this study (approved by the Ethical Committee of the University of Campinas, São Paulo, Brazil CAAE no.: 31984414.6.0000.5404), hAF-MSC samples were obtained from pregnant women in the second trimester of pregnancy submitted to amniocentesis under formal medical indication from the Center for Integral Attention to Women's Health at the University of Campinas (Campinas, SP), after signing the Free and Informed Consent (FIC) form. Samples containing 10 mL of amniotic fluid were submitted to the laboratory, centrifuged to obtain a cell pellet, which was resuspended in 5 mL of alpha minimum essential medium (α-MEM) (GIBCO), supplemented with 20% fetal bovine serum (FBS) (GIBCO) and 1% penicillin-streptomycin-glutamine (P/S) (GIBCO) and maintained in an incubator at 37°C with a humid atmosphere at 5% CO₂. The cells were removed from the culture flask with 0.25% Trypsin-EDTA (GIBCO) and submitted to immunomagnetic separation (MACS-Miltenyi Biotec®) for CD117 (c-kit) according to the manufacturer's protocol. Cells were expanded up to the fourth passage for characterization and application in chondrogenic differentiation experiments.
2.2. Sourcing, isolation and expansion of human adipose-derived mesenchymal stromal cells

hAD-MSC were obtained from liposuction material of patients at the Clinic Hospital of the University of Campinas, after signing the FIC form. The samples were processed according to the protocol of Zuk et al. (37), which consists in repeated washes and enzymatic digestion with 0.075% type II collagenase (Sigma-Aldrich, St. Louis, MO). The stem cells obtained were maintained in the same culture conditions as above with low glucose DMEM (GIBCO) medium, supplemented with 10% FBS (GIBCO) and 1% P/S.

2.3. Characterization of mesenchymal stromal cells

For morphological analysis, adherent cell cultures from both sources were systematically monitored by optical microscopy to maintain 70% confluence.

For differentiation into the three mesenchymal lineages, we used StemPro® Adipogenic Differentiation Kit (Gibco™), StemPro® Chondrogenic Differentiation Kit (Gibco™) and StemPro® Osteogenic Differentiation Kit (Gibco™) commercially available, in accordance with the manufacturer’s instructions. Cell differentiation into adipocytes, chondrocytes and osteocytes was evaluated after 21 days of culture with Oil Red O, Alcian Blue and Alizarin Red staining, respectively.

For immunophenotypic analysis, hAF-MSC and hAD-MSC were incubated with anti-CD90, anti-CD105 and anti-SSEA4 antibodies conjugated to FITC, anti-CD73, anti-HLA-DR and anti-CD34- conjugated to PercP-Cy5.5, anti-CD19, anti-CD25 and anti-CD117 conjugated to PE-Cy7, anti-CD166, CD151 and CD49c conjugated to PE (Biolegend, San Diego, CA), for 20 min followed by fixation (2% paraformaldehyde) and data acquisition in flow cytometry (BD FACS Verse, BD Biosciences). The analysis was performed using the FlowJo cytometric data analysis software.

2.4. Production and characterization of the chitosan-xanthan scaffolds

The chitosan-xanthan scaffolds (CX) used were produced and characterized according to Westin et al. (21), using medium molecular weight chitosan with deacetylation degree around 75% (Sigma-Aldrich Co.), xanthan gum from Xanthomonas campestris (Sigma-Aldrich Co.), Poloxamer 188 solution at 10% (Sigma-Aldrich Co.) and acetic acid (Synth).

Briefly, 200 mL of a 1% chitosan solution (w/v) prepared in 1% (v/v) acetic acid were mixed to 215 mL of a 0.93% xanthan gum aqueous solution (w/v) at pH 7.7 containing 7% of Poloxamer 188 under controlled flow rate (10 mL/min), temperature (25°C) and stirring (1000rpm). The final suspension was poured into two polystyrene Petri dishes (15 cm in diameter), dried at 37°C for 48 h and washed with deionized water to remove the Poloxamer 188 surfactant and the residual acetic acid. The scaffolds were immersed in 200 mL of 10 mM HEPES buffer for 30 min, dried at 37°C, cut in the shape of discs of 1.5 cm in diameter, placed in 24-well plates and sterilized by exposure to ethylene oxide.
The scaffolds were characterized regarding morphology (by visual inspection, optical microscopy and SEM using a Leica microscope model LEO 440 operating at 10 kV e 50 pA), thickness (using a Mitutoyo micrometer, model MDC-25S, Japan) and toxicity to the tested cells by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT-Sigma) colorimetric assay.

The interaction between the scaffold and hAF-MSC and hAD-MSC was also assessed using SEM.

2.5. Chondrogenic differentiation of mesenchymal stromal cells in chitosan-xanthan scaffold

The CX scaffolds were pre-equilibrated in the culture medium for up to 7 days prior to contacting the cells. The hAF-MSC and hAD-MSC were injected in the scaffolds with a 1 mL syringe at the concentration of 1 to 2x10^6 cells in 0.5 mL of high glucose DMEM (GIBCO) culture medium supplemented with ascorbic acid (50 µg/mL, Sigma-Aldrich Co.), proline (40 µg/mL, Sigma-Aldrich Co.); insulin-transferrin-selenium (ITS+1−1%, Sigma-Aldrich Co.), dexamethasone (0.1 µM Sigma-Aldrich Co.) and 10 ng/mL TGF-β3 (R&D Systems). The 24-well plates were maintained in a CO2 incubator at 37°C and humid atmosphere for 7 and 21 days.

2.6. Confirmation of chondrogenic differentiation

After the differentiation period, the samples were fixed for analysis by SEM with a scanning electron microscope (Jeol, model JSM 5800 LV) according to the protocol described by Westin et al. (23).

For histological analysis, Hematoxylin-Eosin (HE), Masson’s Trichrome (MT), Picrosirius Red (PR) and Alcian Blue (AB) staining procedures were performed (38). Images were acquired using an optical microscope (Leica, DM2500) coupled with a digital camera and analysed using the Leica Application Suite LAS software, version 4.6.2.

For molecular analysis, we used immunohistochemistry markers for type II collagen (bs-0709R-BIOSS®) and aggrecan (bs-11655R-BIOSS®). The 5 µm cut-outs on silanized slides of the samples were treated according to the Trilogy protocol (Cell Marque®) followed by endogenous peroxidase blocking and overnight incubation with antibodies. We used HiDef Detection™ HRP Polymer System kit (Cell Marque®) and DAB for detection and amplification and counter-staining with Mayer’s Hematoxylin. A slide without primary antibody was used for negative control of the reaction.

For immunofluorescence analysis, the material was deparaffinized according to the protocol cited above (28), followed by washes with PBS 1x (pH 6.8), glycine 0.1M, BSA block (10 g/L) and incubation with primary antibody for collagen II 1:100 (bs-0709R-BIOSS®) overnight in a humid chamber. Following, the samples were incubated for 1 hour with Alexa 647 conjugated secondary antibody at room temperature and in a humid chamber. Finally, blocking with BSA (10 g/L) was performed, marking with DAPI, fluorescent mounting medium (DAKO) to preserve the fluorescent signal and storage at 4°C, without exposure to light, until confocal microscope analyses (Leica, model SP8).
3. RESULTS

3.1. Morphological and phenotypic characterization of mesenchymal stromal cells derived from human amniotic fluid and adipose-derived tissue

Morphological evaluation and characterization of MSCs were carried out in accordance with the recommendations established by the International Society for Cell Therapy for the characterization of mesenchymal stem cells (39). During their expansion phase, hAF-MSCs (Fig. 1a) and hAD-MSCs (Fig. 1d) exhibited a fibroblast-like morphology, forming characteristic structures resembling fibroblast-like colonies.

Both hAF-MSCs and hAD-MSCs demonstrated differentiation potential into the three expected mesenchymal lineages. Adipogenic differentiation was evident through Oil Red staining, revealing the formation of lipid droplets. Chondrogenic differentiation was confirmed via Alcian Blue staining, indicating the presence of cartilaginous ECM components. Osteogenic differentiation was detected through Alizarin Red staining, providing evidence of calcified ECM formation (Figs. 1b and e).

In terms of phenotypic marker expression, both cell types displayed high levels of mesenchymal markers (CD73, CD90, and CD105), minimal or no expression of hematopoietic lineage markers (CD19, CD34, CD45, and HLA-DR), and positive markers associated with chondrogenic potential (CD49c, CD151, and CD166). Additionally, hAF-MSCs also exhibited positive results for pluripotency markers (SSEA4 and CD117) (Fig. 1c and f).

3.2. Characterization of the chitosan-xanthan scaffold

When wet, the scaffold had thickness between 887 and 969 µm, and were flexible and opaque, showing a foam-like structure. SEM analysis of the dried scaffold cross-section revealed a wavy surface of the biomaterial (Fig. 2a) and cavities in its inner structure (Fig. 2b). Toxicity assessments using MTT analysis indicated that the scaffolds were non-toxic to the cells (data not shown).

3.3 Confirmation of chondrogenic differentiation in the Scaffold
After 21 days of implantation of stem cells (SCs) into the scaffolds, macroscopic examination unveiled the presence of cell agglomerates distributed throughout the material. SEM results provided insights into the interaction between MSCs from both sources and the porous chitosan-xanthan (CX) scaffold (Fig. 2). The cells displayed robust viability, adhered effectively, proliferated, and contributed to the production of extracellular matrix (ECM) following inoculation into the scaffold.

Histological analysis offered further confirmation of chondrogenic differentiation, illustrating the synthesis of collagen and glycosaminoglycans (GAGs), particularly within the group stimulated with TGF-β3 (Fig. 3).

Immunohistochemistry results affirmed the production of collagen II and aggrecan by both cell sources. Samples were counterstained with hematoxylin to mark cell nuclei in blue, and negative controls utilized samples lacking primary antibodies (Fig. 4).

Immunofluorescence findings exhibited positive staining for collagen II, corroborating the chondrogenic differentiation of stem cells from both sources (Fig. 5). Intact cells, marked by cell nuclei, were observed along with a significant production of ECM. **Fig. 3.** Comparative chondrogenic differentiation of hAF-MSC and hAD-MSC after 21 days into CX scaffold without and under TGF-β3 stimulation. The histological images of the group without TGF-β3 show the beginning of cellular condensation close to the scaffold and discrete ECM production (a-h). Hematoxylin and Eosin (HE) staining the intact cell nuclei stained in purple and the ECM in pink (a and e), Alcian Blue (AB) staining showing cell nuclei in pink and ECM in blue (b and f) with low ECM production, while Picrosirius Red (PR) (c and g) and Masson’s Trichrome (MT) (d and h), with specific affinity for collagen, stained the ECM in shades of pale yellow to intense red and blue, respectively. hAF-MSC (c and d) show small collagen production in red and blue, while PR and MT of hAD-MSC (g and h) show only the beginning of condensation, without ECM production. The group under TGF-β3 stimulation HE (i and m) shows a well-structured cell condensation with intense ECM production; AB (j and n) shows ECM rich in GAGs stained in blue; PR (k and o) and MT (l and p) indicate the presence of collagens.

### 4. DISCUSSION

The global incidence of chronic joint diseases such as OA is steadily increasing due to an aging population and rising obesity worldwide (40). The inherent poor ability of articular cartilage to repair, regenerate, and remodel is one of the factors believed to facilitate the development of OA (41–43). There are currently no effective pharmacological treatments for OA, and in many advanced cases joint arthroplasty is the inevitable treatment for patients who have not responded to any other recommended therapies (44, 45). Consequently, there is an urgent medical need for the development of new therapies.
for OA such as stem cell therapies (46, 47), using different MSCs sources as well as using different sources of MSC, as well as with different biomaterials to be used as scaffolds (48).

The majority cell-based cartilage repair models available on the market have high costs and relatively short lifespan in terms of efficacy (49). Furthermore, to the best of our knowledge, there are no studies comparing the chondrogenic differentiation process of hAD-MSC with that of hAF-MSC. Therefore, in the present study, we assessed the potential of hAF-MSC and hAD-MSC for chondrogenic differentiation directly into chitosan-xanthan gum scaffolds designed for cartilage tissue engineering, under TGF-β3 stimulation, analysing the suitability of a seldom used but abundant and easily obtained source of MSCs, combined with a biocompatible material easily produced, economically accessible and that does not induce inflammatory response, comparing several aspects to establish the most adequate cell source for clinical application. While hAF-MSC are younger cells embryologically closer to embryonic stem cells (ESCs), hAD-MSC are adult stem cells. In recent years, hAF-MSC has been considered a promising option to obtain MSCs, owing to the positive aspects that stimulate its application in clinical practice, such as ethical concerns, unlike the controversial use of embryonic stem cells, hAF-MSC, but also because they are easy to obtain, easy to grow and exhibit no tumorigenicity when compared to adult stem cells (50, 51).

A short time after harvesting the MSCs from both sources, the cells showed rapid adhesion and proliferation, with fibroblastic formimng compatible with the characterization standards of stem cells required by the International Society for Cell Therapy (39) (Fig. 1a and d). In the flow cytometry analysis, hematopoietic lineage markers such as CD34, CD45 and CD19 were present at levels above 2%, differently from the consensus established by Dominici (39). However, as observed previously by our group and by others, CD34 expression was positive in up to 50% of cells (52, 53). hAF-MSC contains several cell types derived from the development of the fetus and, as these cells express different markers according to their lineage and gestation time, it is believed that this peculiarity is responsible for the findings in cytometry. Therefore, it is possible to assume that the minimum criterion proposed by the International Society for Cell Therapy.

Considering that hAF-MSC are less mature and more variable and that this could negatively interfere with cell differentiation, immunomagnetic separation was performed after the second passage of cell expansion. A subpopulation of cells positive for CD117 (50) was selected, representing approximately 1% of the cultured cells (29). After this additional selection step, flow cytometry analysis showed high levels of SSEA-4 and other markers of chondrogenic potential (Fig. 1c and f).

After the fourth passage of the SCs from both sources, it was possible to demonstrate the potential of these cells for differentiation into three mesenchymal lineages: adipogenic, chondrogenic and osteogenic, according to the pre-established consensus (39). The cells showed high ECM production in the differentiation process into three mesenchymal lineages. In the analysis of adipogenic differentiation, we observed the formation of lipid droplets within the newly differentiated adipocytes, in addition to ECM stained in different shades of orange by Oil Red staining. When evaluating chondrogenic
differentiation, the intense production of ECM was visualised by staining with Alcian Blue, confirming the presence of proteoglycans (PGs). For osteogenesis analysis, the calcified ECM formed was stained red by Alizarin Red staining, showing the presence of calcification in significant quantities (Fig. 1b and e).

After characterization, MSCs from both sources were seeded into the scaffold. Interestingly, it was observed that, although cells from both sources differentiated into chondrocytes, cells from hAF-MSC adapted better to the scaffold than those from hAD-MSC. This lower affinity of hAD-MSC cells to the scaffold was observed by SEM, with a dispersed cell population, with fewer cells distributed over the scaffold and formation of looser fibers. Regarding TGF-β3 stimulation, in stimulated cells, both from hAF-MSC and hAD-MSC, after 21 days of culture it was possible to observe chondrogenic differentiation, with intense cell condensation and ECM production. In contrast, in groups without stimulation, there was little ECM formation (Fig. 2).

Histological analysis showed very similar results that confirmed the chondrogenic differentiation of MSCs from both sources, because in addition to intense cell condensation and ECM production, the stains used indicated the presence of collagen and proteoglycan. The greatest difference that could be observed in the histology results was in relation to the group stimulated with TGF-β3 and the group without stimulation (Fig. 3). These data enable confirming the influence of the stimulation strategy used (31, 54). The differentiation even without TGF-β3 stimulation can be explained by the high concentration of cells injected together into the scaffold, mimicking the condensation of chondrogenic differentiation that occurs in embryonic development (55, 56). In such conditions, hAD-MSC may recapitulate favorable cellular microenvironment. The analysis of the ECM formed in the cell-scaffold set by immunohistochemistry (IH) showed the presence of type II collagen and aggrecan, thus confirming chondrogenic differentiation from the SCs from both sources (Fig. 4). The results of the IH analysis of hAF-MSC showed greater cell condensation and greater ECM production compared to the IH of hAD-MSC SCs, which confirms the lower affinity of hAD-MSC to the CX scaffold, corroborating the results obtained in the other analyses, as well as the immunofluorescence results (Fig. 5).

Despite this study does not include quantitative analysis of cell differentiation, as well as a functional analysis of the set consisting of scaffold, cells and TGF-β3 stimulation, it is possible to infer that hAF-MSC have a favorable potential for use in clinical practice in the treatment of chondral lesions, in association with a scaffold with greater accessibility. Further studies should be carried out to confirm our findings.

5. CONCLUSIONS

Despite the few studies comparing human amniotic fluid stroma cells for the purpose of chondrogenic differentiation and considering the complexity of hAF-MSC due to their variability, the results of this study confirm that it is possible to differentiate in vitro stem cells from human amniotic fluid and human adipose tissue into chondrocytes directly in the scaffold of chitosan and xanthan in the presence of TGF-β3, with evident production of an ECM rich in collagen and PGs. Taking the findings from the current
study into account, we believe that future studies should assess the efficacy of this approach in chondrogenic differentiation, as well as functional results in chondral lesions in relevant preclinical and translational animal models. These efforts will help to confirm the utility of hAF-MSC for clinical use in cartilage repair and regeneration.

**Abbreviations**

AB
Alcian Blue
BSA
Bovine Serum Albumin
c
Kit-Receptor tyrosine kinase protein
CX
chitosan-xanthan gum scaffolds
DAB − 3,3'
Diaminobenzidine
DAPI − 4',6'
diamino2-fenil-indol
DMEM
Dulbecco's Modified Eagle Medium
ECM
extracellular matrix
ESC
embryonic stem cells
FBS
fetal bovine serum
FIC
Free and Informed Consent
FITC
Fluorescein isothiocyanate
GAG
glycosaminoglycans
hAD
MSC-human adipose-derived mesenchymal stromal cells
hAF
MSC-human amniotic fluid mesenchymal stromal cells
hBM
MSC-human bone marrow-derived mesenchymal stromal cells
HE
Hematoxylin-Eosin
HEPES − 4
(2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid
HLA
DR Human leucocyte antigen-DR isotype
ITS
Insulin-transferrin-selenium
MSC
mesenchymal stromal cells
MT
Masson's Trichrome
MTT − (3
(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
OA
osteoarthritis
P/S
penicillin-streptomycin
PBS
Phosphate Buffered Saline
PR
Picrosirius Red
SC
stem cells
SEM
scanning electron microscopy
SSEA
4-Stage-specific embryonic antigen-4
TGF
β3-Transforming Growth Factor Beta-3
WMA
World Medical Association
α
MEM-alpha minimum essential medium

Declarations

6. ACKNOWLEDGMENTS

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7. ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All studies adhered to recognized international standards, including the guidelines of the World Medical Association (WMA) and the principles outlined in the Declaration of Helsinki. This particular study received approval from the Ethical Committee of the University of Campinas, São Paulo, Brazil, with registration number CAAE no. 31984414.6.0000.5404. The study involved the collection of hAF-MSC samples from pregnant women in the second trimester of pregnancy. These samples were obtained through amniocentesis procedures performed under formal medical indication at the Center for Integral Attention to Women's Health at the University of Campinas in Campinas, SP, Brazil. Participants provided their informed consent by signing the Free and Informed Consent (FIC) form.

Consent for Publication

Publication consent is not applicable to this study.

Availability of Data and Materials

The datasets utilized and/or analyzed during the course of this study are accessible from the corresponding author upon reasonable request.

Competing of Interest

The authors declare no conflicts of interest. Funders played no role in the study's design, data collection and analysis, decision to publish, or manuscript preparation.

Authors’ contributions

ID performed the experiments, analysed the data and wrote the manuscript; CZ performed cells cultures and histologic analysis; KA performed the obstetric indication and amniocentesis; PK performed the liposuctions; CW and AM produced and characterized the scaffold; AM was responsible for suggestions and for the final version of the manuscript. IC contributed for the study conception and design, financial support, and was responsible for analyzing the results and approval of the final version of the manuscript. All authors have read and approved the final submitted manuscript.

References


Figures
Characterization of human mesenchymal stromal cells derived from amniotic fluid and adipose-derived tissue. Phase contrast microscopy morphological characterization of cells in culture exhibiting fibroblast-like aspect and cell adhesion hAF-MSC (a) and hAD-MSC (d). Differentiation into the three mesenchymal lineages (b and e). Adipogenic differentiation, with formation of lipid droplets stained in orange by Oil Red, chondrogenic differentiation with GAGs in blue for Alcian Blue stained; osteogenic
differentiation, with calcified ECM stained in red by Alizarin Red. Immunophenotype analyses by flow cytometry (c and f) indicating the expression of the positive markers of MSCs (CD90, CD105, CD73), negative markers of hematopoietic lineage (CD34, HLA-DR, CD19 and CD45) and positive markers chondrogenic potential (CD49c, CD151 and CD166) in both cellular sources. In hAF-MSC pluripotency markers appear positive (SSEA-4 and CD117).

Figure 2

Scanning electron microscopy of chitosan-xantan gum scaffold without cells, surface (a) and transversal view showing interconnected pores (b). Chondrogenic differentiation of hAF-MSC (c, d and e) and hAD-MSC (f, g and h) in the scaffold respectively. Control group without TGF-β3 stimulation with 7 days of culture showing some attached cells on the surface of the scaffold (c and f). The group under TGF-β3 stimulation after the same period showed a greater number of adhered cells (d and g) and after 21 days, there production of fibrillar structures compatible with ECM production was observed.
Figure 3

Comparative chondrogenic differentiation of hAF-MSC and hAD-MSC after 21 days into CX scaffold without and under TGF-β3 stimulation. The histological images of the group without TGF-β3 show the beginning of cellular condensation close to the scaffold and discrete ECM production (a-h). Hematoxylin and Eosin (HE) staining the intact cell nuclei stained in purple and the ECM in pink (a and e), Alcian Blue (AB) staining showing cell nuclei in pink and ECM in blue (b and f) with low ECM production, while Picrosirius Red (PR) (c and g) and Masson’s Trichrome (MT) (d and h), with specific affinity for collagen, stained the ECM in shades of pale yellow to intense red and blue, respectively. hAF-MSC (c and d) show small collagen production in red and blue, while PR and MT of hAD-MSC (g and h) show only the beginning of condensation, without ECM production. The group under TGF-β3 stimulation HE (i and m) shows a well-structured cell condensation with intense ECM production; AB (j and n) shows ECM rich in GAGs stained in blue; PR (k and o) and MT (l and p) indicate the presence of collagens.
Figure 4

Immunohistochemistry analysis showing chondrogenic differentiation under TGF-β3 stimulation induced from hAF-MSC and hAD-MSC with positive marking for type II collagen (a and c) and aggrecan (b and d) when compared to the negative control (e).
Figure 5

Confocal immunofluorescent analysis of hAF-MSC and hAD-MSC differentiated into chondrocytes into CX scaffold. (a and e) are control images. Cells nuclei (DAPI, green) staining (b and f). Positive staining for Collagen II (bs-0709R-BIOSS®) conjugated with Alexa 647 secondary antibody in the ECM formed in red (c and g). Merge image (d and h).