



Chitosan-collagen scaffolds can regulate the biological activities of adipose mesenchymal stem cells for tissue engineering

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Abstract

Scaffolds of chitosan and collagen can offer a biological niche for the growth of adipose derived stem cells (ADSC). The objective of this work was to characterize the physico-chemical properties of the scaffolds and the ADSC, as well as their interactions to direct influences of the scaffolds on the behavior of ADSC. The methodology included an enzymatic treatment of fat obtained by liposuction by collagenase, ADSC immunophenotyping, cell growth kinetics, biocompatibility studies of the scaffolds analyzed by the activity of alkaline phosphatase (AP), nitric oxide (NO) determination by the Griess-Saltzman reaction, and images of both optical and scanning electron microscopy of the matrices. The extent of the crosslinking of genipin and glutaraldehyde was evaluated by ninhydrin assays, solubility tests and degradation of the matrices. The results showed that the matrices are biocompatible, exhibit physical and chemical properties needed to house cells *in vivo* and are strong stimulators of signaling proteins (AP) and other molecules (NO) which are important in tissue healing. Therefore, the matrices provide a biological niche for ADSC adhesion, proliferation and cells activities.

Keywords: Chitosan-collagen-genipin scaffolds, ADSC, macrophages, biological niche

Introduction

About 50 million people in the U.S. had increased in life expectancy due to tissue therapy and artificial organs, and every five people over 65 years, has benefited from tissues and organs generated "*in vitro*" [1,2]. The main cause of hospital admission and death in Brazil is due to injuries caused by accidents in general. Only in 2004, the National Health Care System has spent U.S. \$ 585 million with orthotics and prosthetics [39]. Furthermore, the American Heart Association estimates that approximately 5.7 million Americans suffer from congestive heart failure [3-6]. In Brazil, a third of the deaths are caused by cardiovascular disease, and approximately 350,000 patients suffering acute myocardial infarction per year [19]. Thus, it becomes imperative to develop new strategies to meet the demand with the development of biomaterials (scaffolds), having as main purpose the regeneration of tissues and organs and amortization costs [6,18]. These scaffolds must support cell growth, since they have mechanical properties in agreement with the tissue to be reconstructed, induce cellular responses faster or inherently possess properties directly related to tissue remodeling. A strategy to mitigate the negative impacts on the areas of quality of life, economic and social is cell therapy using adipose tissue stem cells (ADSC) and tissue engineering.

The ADSC have the advantages of low immunogenicity, pluripotency, easiness of isolation, purification, expansion "*in vitro*", and the possibility of cryopreservation [40,41,54]. Several preclinical studies [27,45] show that ASC can differentiate into

cardiomyocytes, vessels and other tissues of mesodermal origin [18,19,28,38]. Apparently, there is an important link between fat cells and the vascular network [40,41,47]. These cells represent a heterogeneous population of microvascular endothelial cells, constitute a convenient source of pluripotent cells and are not restrictive [39,44]. The understanding of the adipose tissue development and plasticity opens new perspectives for angiogenic therapy based on the administration of stem cells derived from that tissue in the treatment of several diseases [46,48,49].

The use of matrices for the adhesion and proliferation of ADSC is an important strategy in the field of tissue engineering [21-26]. Among the biomaterials used for this purpose chitosan and collagen type I are biocompatible, biodegradable and non-toxic [1-3,12,16]. This approach provides for the treated tissues supramolecular structures that ensure satisfactory spatial and functional cellular organization [34-37], as well as their systemic integration, providing a microenvironment "*in vitro*" which is very similar to the extracellular matrix (ECM) of native tissue to promote and facilitate cell differentiation into various cell lines as well as the maintenance of the differentiated phenotype [4,17,20-23]. The use of chitosan as a biomaterial has several attractive properties for tissue regeneration because their cationic nature permits electrostatic interactions with cell anionic species such as glycosaminoglycans (GAG) and proteoglycans [13,25]. It is hypothesized that the differentiation of ADSC into various cell lines occurs by paracrine, autocrine and endocrine

effects and that the use of chitosan with a high degree of deacetylation (DD=85.0%) can facilitate and accelerate tissue healing, and also improve the fitness of the biological niche at the site of injury [7-11,50-53,55-58]. The objective of this study was to analyze the physicochemical and biological interactions between and adipose stem cells (ADSC), macrophages and porous scaffolds of chitosan-collagen for tissue regeneration.

Materials and methods

Materials

The materials used to manufacture scaffolds were chitosan with a degree of deacetylation of 76.0-85.0% and molecular weight of 200 kDa, bovine tendon collagen Type I, genipin and 25% glutaraldehyde, all purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents such as cell culture medium and fetal bovine serum were obtained from Gibco Life Technologies (Van Allen Way, Carlsbad, CA, 92008, USA). The petakas for cell culture were purchased from Celartia (1555 Picardae Court Powell, OH, 43065 USA).

Experimental design

The adipose tissue was collected from 12 female patients, aged between 20 and 60 years, who underwent liposuction at the Hospital of Cardiovascular Diseases (HMC) or at the Institute of Cardiovascular Diseases (IMC), both from São José do Rio Preto, Brazil. The technique involved vacuum suction machine (Nevoni® mod 3003, SurgeryExpress, São Paulo-SP, Brazil). Samples of fat, which is a residual product of liposuction procedures, were donated for the experiment. The fat tissue was treated with collagenase type I and type IV from *Clostridium histolyticum* (Sigma-Aldrich,) for two hours, obtaining an ASC count of 3.6×10^8 . Afterwards, the cell pellet obtained by centrifugation was resuspended in 3.0×10^5 cells to 97.0% cell viability until the third cell passage when all the analysis was performed. The cell growth was analyzed up to the ninth expansion. Macrophages, derived from bone marrow donated by the hospital HMC, were isolated and characterized in the laboratory. The scaffolds were constructed based on chitosan, collagen, genipin, and glutaraldehyde. All physico-chemical and biological analysis were made after the third passage (replating) of ADSC. It was characterized the degree of chitosan deacetylation, electron microscopy of the scaffolds and the determination of the time of degradation and solubilization of the biomaterials.

Cell viability with trypan blue

The study involved ADSC cell viability using the dye trypan blue, which marks only dead cells. From the difference between the number of alive and dead cells divided by the total number of cells, counted using a Neubauer chamber, it is possible to determine cell viability [5].

Preparation and characterization of the scaffolds

Based on the method reported by Baldwin and Kiick [1],

previously characterized chitosan (Mw = 200 kDa and degree of deacetylation (DD) 83%) was dissolved in 10.0 mL of acetic acid solution at 2.5% v/v for 24 h at room temperature. Blend A (chitosan-collagen, 1:1 m/m) was prepared by mixing both solutions in 2.5% v/v acetic acid solution for 96 h. The solutions were weighed and transferred to a 96 well plate (170µL/well). The scaffolds were prepared starting with 200 µL of chitosan-collagen solutions. The scaffolds were then intercrossed to 0.05, 0.10, 0.25, 0.50, 0.75 and 1.00% v/v glutaraldehyde and genipin. Thereafter, the mixture was frozen in liquid nitrogen (-196.0°C) and lyophilized.

Scanning electron microscopy of matrices

The matrices were analyzed by scanning electron microscopy (SEM) at the Laboratory of Microscopy and Microanalysis (Ibilce-Unesp) through the gold plating of the matrices and playback of images on the computer to obtain different images, using a range of magnifications: 300, 200, 100, 30, 20 and 10x[17].

ADSC extraction

Samples of fat tissue were extracted [5] by method: liposuction vacuum equipment (using 3.0 mm diameter cannulae), and taken to the laboratory for ADSC extraction procedures. The fat samples were homogenized and an initial volume of 120.0 mL was divided in 2.0 mL specimens. Subsequently, to three volumes were added collagenase type I and to the other three were added collagenase type IV, in a proportion of 1:3 (fat:collagenase), and the mixture was taken to an incubator at 37.0°C to 5.0% CO₂ for 2.0 hours. After this time, the mixture was centrifuged at 800 xg for 10 min at 23°C. After that, the pellet was resuspended in 10.0 mL of culture medium, and 1.0 mL was transferred to each Petaka cell culture.

Study of growth kinetics of ADSC

In order to study the kinetics of cell growth, as well as the population doubling time (PDT), cell cultures in petakas should start from an initial concentration of 2.0×10^5 [41]. Quantitation of living cells by means of trypan blue was performed on days 2-20 for each step of cell expansion.

Cell culture and scaffold seeding

For ADSC culturing [32,35], these cells were expanded in culture medium MEM ALPHA with 10.0% v/v fetal bovine serum, containing 1.0% v/v of ampicillin and streptomycin, in the Petakas. After reaching approximately 80% of confluency, the cell lines were "trypsinized" with trypsin-EDTA and then the cell were counted in a Neubauer chamber in order to know the number of cells to be seeded on the matrices. The matrices were sterilized in 70% ethanol solution (overnight) in the presence of ultraviolet light (15 min). After washing the scaffolds 6 times with sterile water, 1.0×10^5 ADSC were seeded at into scaffolds. After seeding, the matrices were transferred to an incubator at 37.0°C with 5.0% carbon dioxide for 48 h.

Optical microscopy of ADSC adhered matrices

The ADSC adhered matrices were processed in paraffin and fixed in a paraformaldehyde solution for 24 hours, followed by several steps in baths in solutions of increasing ethanol content up to absolute ethanol and, soon after, passages in xylene for complete dehydration of the scaffolds [32].

Characterization of ADSC for immunophenotyping

ADSC immunophenotyping was done using a flow cytometer and Cell Quest™ software (BD Biosciences) in order to analyze binding of specific markers in the extracellular matrix [5,39,40].

Enzyme activity of alkaline phosphatase (AP)

In order to perform the AP assay the culture medium was removed and then it was added 200 µL of solution of BCIP/NBT (5-bromo, 4-chloro, 3-indolylphosphate-nitrobluetetrazolium) (GIBCO). The reaction occurred for two hours in an incubator with 5.0% CO₂ at 37.0°C and was solubilized with 210 µL of SDS solution–10% HCl for 12 h. The control was made with 300 µL of culture medium plus 100 mL of NBT solution–BCIP and phosphate buffer. Quantification of the reactions of AP was made based on a calibration graph, and absorbance readings were done in a spectrophotometer at 595 nm [49].

Study of the interaction between the ADSC and scaffolds and cell viability mediated by nitric oxide (NO) in macrophages

For the determination of NO, we used the classic method of Griess-Saltzman [58]. Dissolve 5.0 g of sulfanilic acid in 800 mL deionized water containing 140 mL of glacial acetic acid. A small heating may be necessary to accelerate the dissolution process. To this mixture was added 20 mL of N-(1-naphthyl)-ethylenediamine 0.1% and diluted to 1.0 L. Keep the solution in amber bottle in the refrigerator. To perform spectrophotometric measurements, wait about 15 minutes to complete the reaction. The absorbance measurements were made at 540 nm [14,57].

Degree of cross-linking with glutaraldehyde and genipin

The degree of cross-linking with glutaraldehyde and genipin between chitosan and collagen was determined by the ninhydrin assay and was defined as the ratio of the amino groups consumed in the matrix by crosslinking and the free amino groups of the matrices [9]. Was used as a standard L-arginine at different concentrations (1.0 to 5.0 mg mL⁻¹) for the calibration graph. The absorbance was read at 570 nm in a spectrophotometer.

Matrix degradation with pepsin and collagenase

The degradation index (DI) was determined in triplicate samples that were dried in an incubator at 40°C and weighed [35,36]. Afterwards, the samples were placed in containers with pepsin and collagenase solution, obeying a relationship of 0.1 cm⁻¹ between surface area and volume of solution. The samples

were kept at 37°C in a water bath for 24 hours. Subsequently they were removed from the container and dried at 40°C. Their weight was recorded after reaching stabilization, allowing to calculate degradation index, where M_{id} represents the initial dry weight after dehydration, M_i represents the initial weight before dehydration and M_{fd} represents the final weight after degradation process.

$$M_{id} = M_i \times (100 - (\% \text{humidity}/100)) \quad (\text{equation 1})$$

$$DI = \left[\frac{M_{id} - M_{fd}}{M_{id}} \right] \times 100 \quad (\text{equation 2})$$

Solubility of the matrix

The matrices were left immersed in a solution containing MEM ALPHA medium with 10% fetal bovine serum for 1,3,7 and 15 days. To determine the degree of solubility equations 1 and 2 were also used item 2.16. The solubility of the matrices was determined by mass difference before and after the films were immersed in the solutions. The scaffolds were dried at 25°C for 16h, cooled in a desiccator for 30 minutes and weighed [3,35].

Statistical analysis

The 36 of 2.0 mL fat samples from 120 mL were taken from 12 patients (triplicate) obtained from patients using cannula of 3.0 mm as described previously were treated by simple randomization before being treated by collagenase I and IV. ADSC quantifications by Immunophenotyping were analyzed by Pearson correlation. This procedure, as well as descriptive statistics, was performed using Minitab 15 (Minitab Inc., USA).

Results

Characterization sampling technique of ADSC

There was no statistically significant difference in the quantification of ADSC extracted from twelve patients, $p < 0.05$. The technique used allows a higher fat fractionation of samples, facilitating adipose tissue digestion by collagenase. For the following studies such as immunophenotyping, chose a sample of ADSC that best adhered and proliferated in culture in Petakas.

Cell viability and quantification with trypan blue

Shortly after ADSC extraction with type IV collagenase the, viability was 97.0±3% and the total cell number was 6.0 x 10⁶ to 2.0 mL and 3.6 x 10⁸ to 120 mL proportionally. After the third passage, cell viability was 99.0±4% with a total of 2.3 x 10⁸ cells to 120 mL, 80% confluent on the third pass. However, with the type I collagenase, the cell viability was 96.0±3% with 4.3x10⁵ to 2.0 mL and 2.5x10⁷ cells to 120 mL proportionally, immediately after extraction, and cell viability of 97.0±4% with 1.5x10⁷ cells, 80% confluent on the third pass.

Study of ADSC growth kinetics

The study of cell growth kinetics analyzed population doubling time (PDT) of ADSC involving a considerable increase in the

rate of cell growth as the number of cell passages ranging from the 1st to the 9th expansion. The values of PDT (in days) decreased from the first to the ninth expansion: (Figure 1) 7, 7, 7, 6, 6, 4, 4, 2, 2. There was also greater in relation to cell growth that ADSC were extracted with collagenase type IV collagenase instead of type I, whose values of PDT in days were the first to ninth expansion, equal to 6, 5, 5, 3, 3, 2, 2, 1, 1 (Figure 2).

and CD73, being ASC positive for the majority of the labeled antibodies (Figure 3).

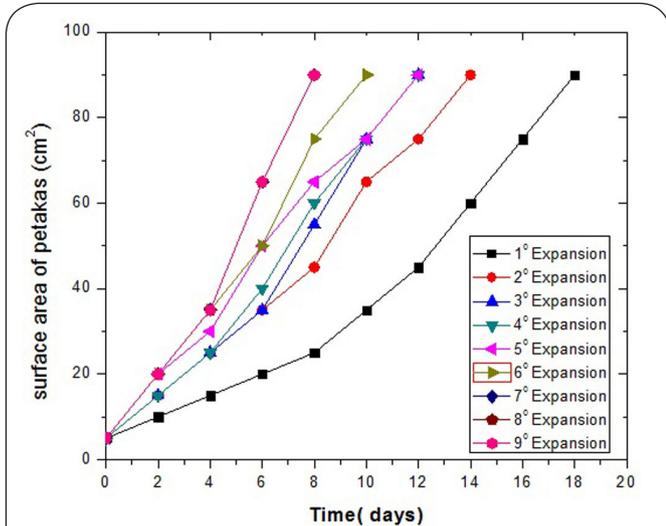


Figure 1. Curve growth kinetics of ADSC within the first to ninth Petakas expansion, following the cell doubling time with type I collagenase.

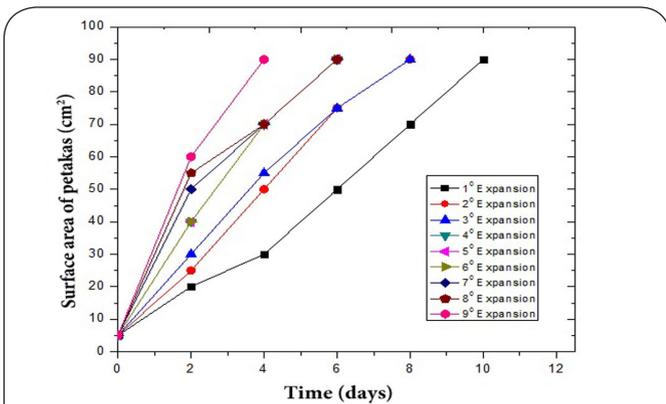


Figure 2. Curve growth kinetics of ADSC within the first to ninth Petakas expansion, following the cell doubling time with type IV collagenase.

Characterization of ADSC for immunophenotyping

After the third passage, the cells analyzed by flow cytometry displayed expression of ADSC through specific markers shown in (Figure 2), mesenchymal cell marker panel containing CD45, CD14, CD51, 61, CD54, CD44, CD49e, CD34, CD13, CD31, CD166, HLA-DR, HLA-ABC, CD29, CD146, CD90, CD105, CD106

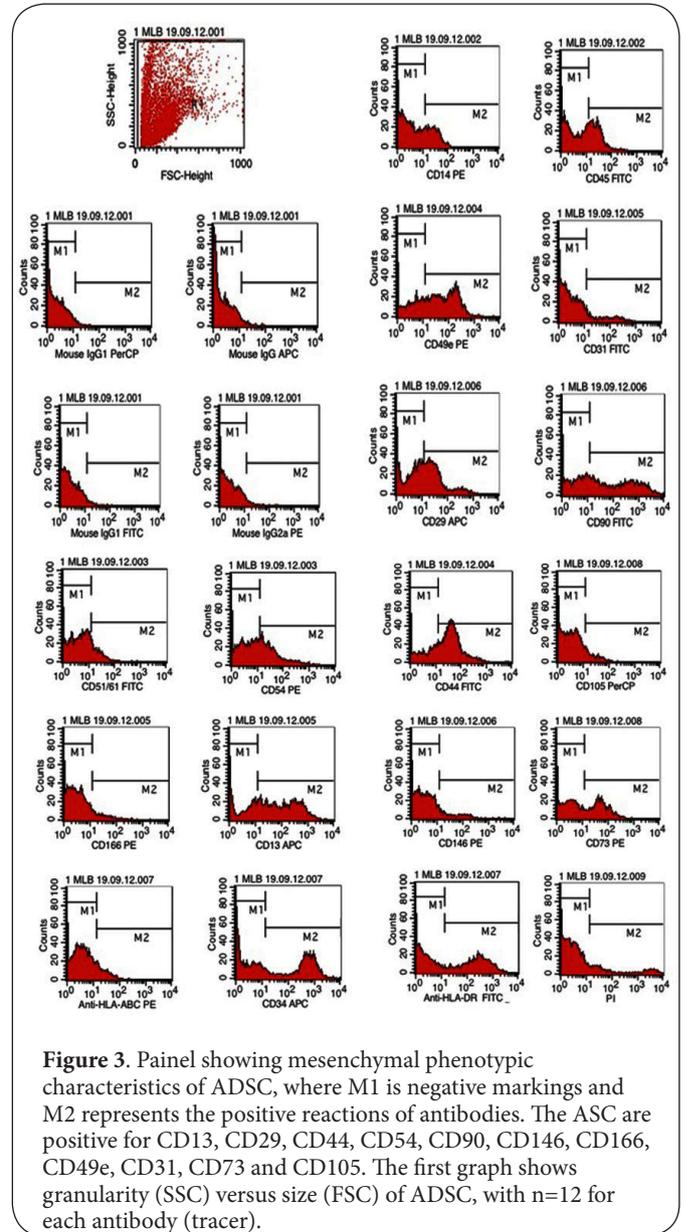


Figure 3. Pannel showing mesenchymal phenotypic characteristics of ADSC, where M1 is negative markings and M2 represents the positive reactions of antibodies. The ASC are positive for CD13, CD29, CD44, CD54, CD90, CD146, CD166, CD49e, CD31, CD73 and CD105. The first graph shows granularity (SSC) versus size (FSC) of ADSC, with n=12 for each antibody (tracer).

Characterization of scaffolds-degree of degradation datrices with pepsin and collagenase and solubility of matrices

The treatment with pepsin induced greater degradation of the blends compared to type IV collagenase under both the same teperature to 37°C (Figure 4), knowing that the melting point of collagen is between 27°C-34°C. Although the reaction of IV collagenase degradation of the matrices being performed above the melting point of the collagen, that is, at 37°C, the enzymatic activity of pepsin was added to the degradation of the matrices at the same temperature.

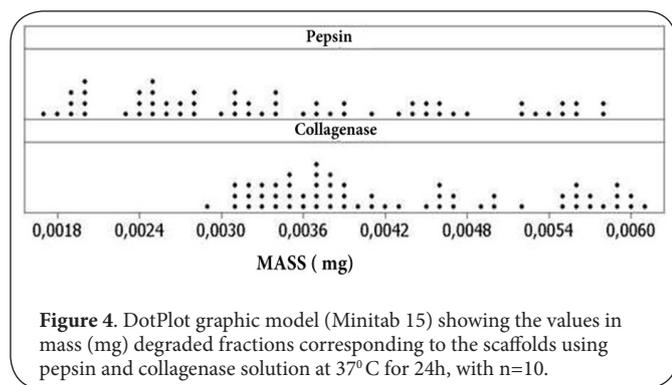


Figure 4. DotPlot graphic model (Minitab 15) showing the values in mass (mg) degraded fractions corresponding to the scaffolds using pepsin and collagenase solution at 37°C for 24h, with n=10.

The degradation index values were obtained by equation. The solubility of the blends culture in MEM-Alpha medium and fetal bovine serum (pH=7.4) was determined by means of equations 5 and 6. The Increased solubility to the degree of crosslinking with genipin decreased (**Figure 5**).

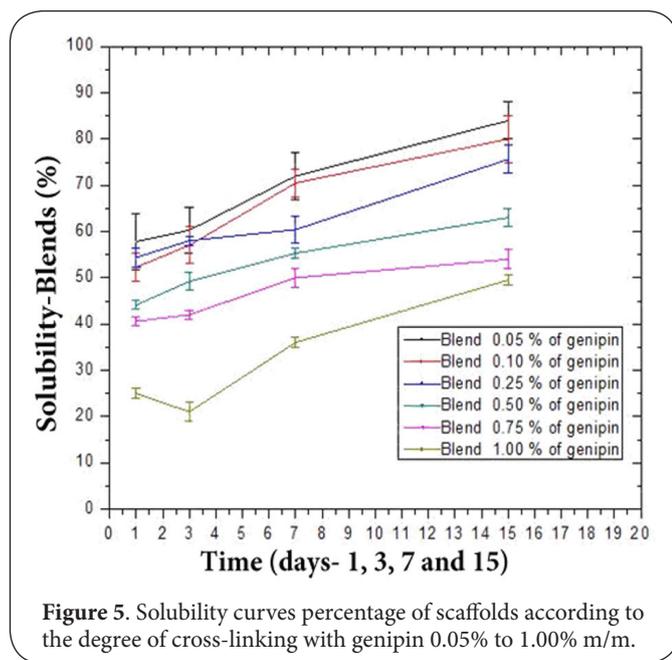


Figure 5. Solubility curves percentage of scaffolds according to the degree of cross-linking with genipin 0.05% to 1.00% m/m.

Determination of the degree of cross-linking with glutaraldehyde and genipin

It was found that as the amino groups of chitosan and collagen were being consumed by increasing genipin concentration and glutaraldehyde (**Figure 6**), the absorbance values decreased, since the amino groups did not react with ninhydrin.

Scanning electron microscopy of the matrix of chitosan and the blend of images and ADSC

Images A and B (**Figure 7**) obtained from previous work of Zotarelli et al., 2011 show the morphology and structure of the chitosan and chitosan-collagen

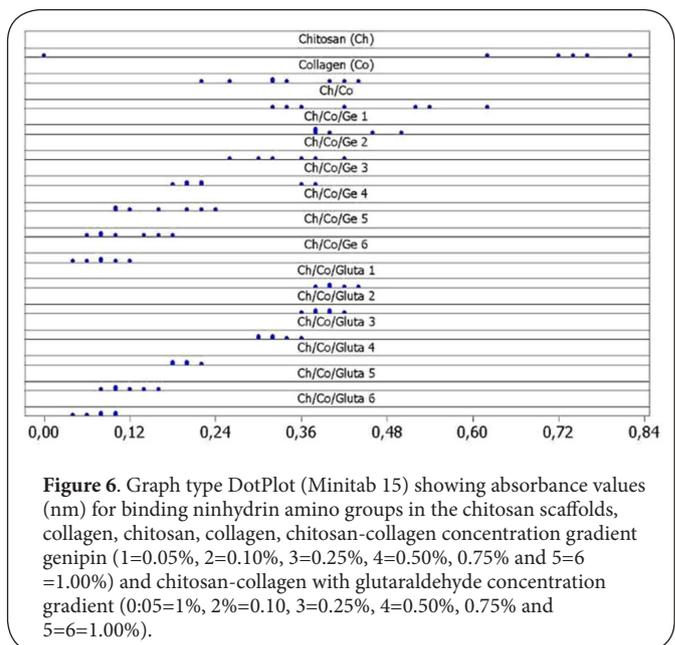


Figure 6. Graph type DotPlot (Minitab 15) showing absorbance values (nm) for binding ninhydrin amino groups in the chitosan scaffolds, collagen, chitosan, collagen, chitosan-collagen concentration gradient genipin (1=0.05%, 2=0.10%, 3=0.25%, 4=0.50%, 0.75% and 5=6=1.00%) and chitosan-collagen with glutaraldehyde concentration gradient (0:05=1%, 2%=0.10, 3=0.25%, 4=0.50%, 0.75% and 5=6=1.00%).

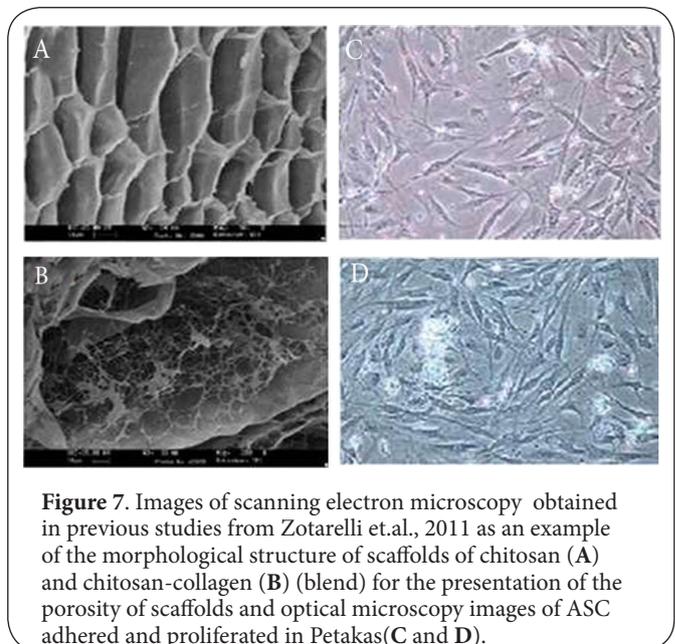


Figure 7. Images of scanning electron microscopy obtained in previous studies from Zotarelli et al., 2011 as an example of the morphological structure of scaffolds of chitosan (A) and chitosan-collagen (B) (blend) for the presentation of the porosity of scaffolds and optical microscopy images of ASC adhered and proliferated in Petakos (C and D).

matrices and show the porosity of the scaffolds. Images C and D show the adhesion and proliferation of ASC in Petakos, emphasizing cellular morphology.

Study of the production of alkaline phosphatase by ADSC

The production of alkaline phosphatase increased from the third to the eighth cell expansion. The production of this enzyme increased in the presence of the chitosan matrix, decreased in the presence of collagen, was slightly higher in the presence of the scaffold with

genipin, and showed the most significant increase in the presence of the scaffold with glutaraldehyde (**Figure 8**).

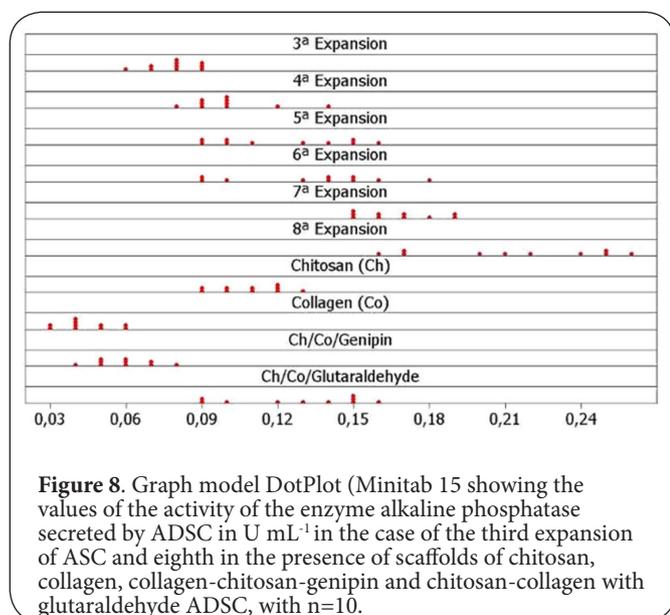


Figure 8. Graph model DotPlot (Minitab 15 showing the values of the activity of the enzyme alkaline phosphatase secreted by ADSC in U mL⁻¹ in the case of the third expansion of ASC and eighth in the presence of scaffolds of chitosan, collagen, collagen-chitosan-genipin and chitosan-collagen with glutaraldehyde ADSC, with n=10.

Study of the interaction of scaffolds and ADSC mediated by nitric oxide (NO) in the presence of macrophages

The study of the interaction between scaffolds and ADSC-mediated NO in scaffolds of chitosan, collagen and blends both in the presence of macrophages or macrophages and ADSC showed increased production of NO in the presence of a larger number of protonated-NH₂ in chitosan and in the presence of genipin, suggesting that cations may stimulate NO production (**Figure 9**). Cell viability was higher in these

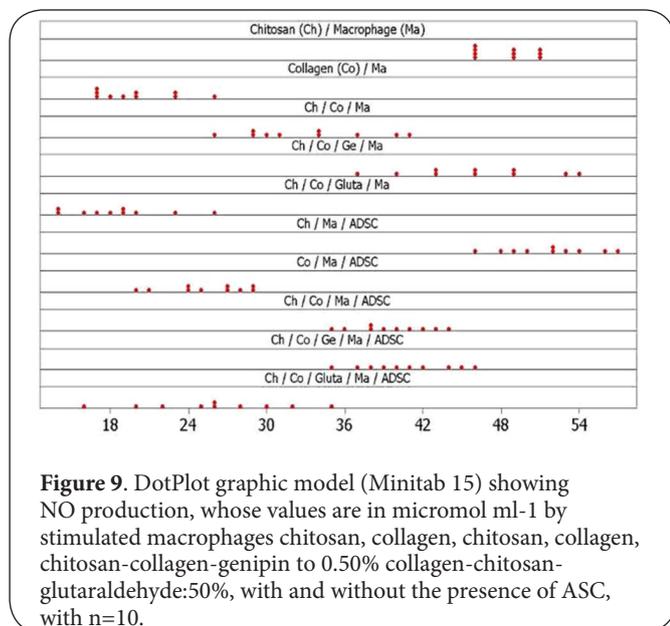


Figure 9. DotPlot graphic model (Minitab 15) showing NO production, whose values are in micromol ml⁻¹ by stimulated macrophages chitosan, collagen, chitosan, collagen, chitosan-collagen-genipin to 0.50% collagen-chitosan-glutaraldehyde:50%, with and without the presence of ASC, with n=10.

samples, reaching around 92.0%, compared to cell viability in scaffolds with glutaraldehyde samples, around 70.0%.

Study of biocompatibility of the blends for the adhesion and proliferation of ADSC

After seeding 4.2x10⁴ cells on the scaffolds at 37°C and 5.0% CO₂ for a period of 48 h, the ASC showed good adhesion and proliferation on the scaffolds, as shown in (**Figure 10**).

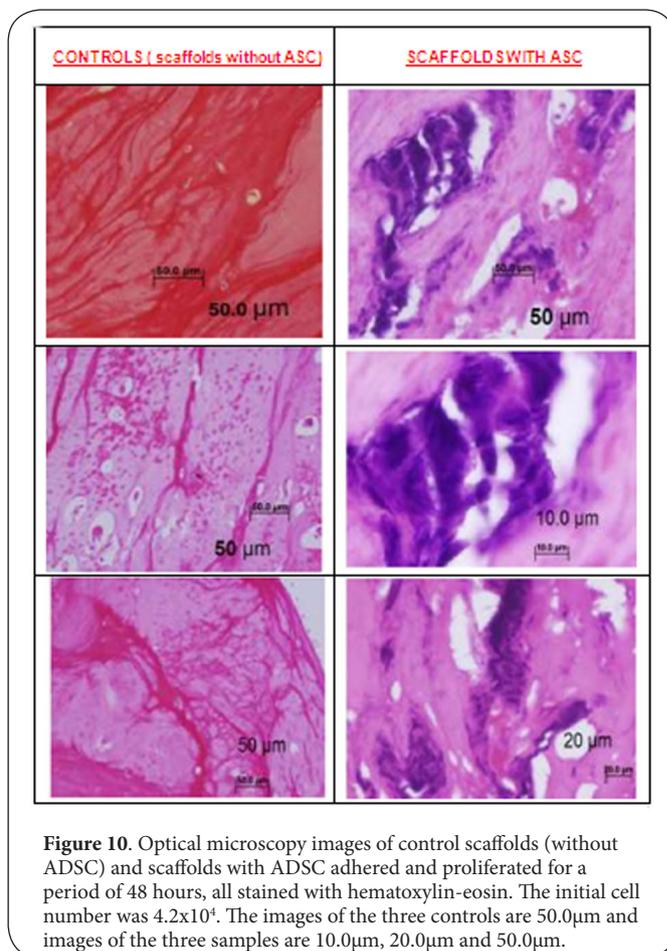


Figure 10. Optical microscopy images of control scaffolds (without ADSC) and scaffolds with ADSC adhered and proliferated for a period of 48 hours, all stained with hematoxylin-eosin. The initial cell number was 4.2x10⁴. The images of the three controls are 50.0µm and images of the three samples are 10.0µm, 20.0µm and 50.0µm.

Discussion

Study of extraction and characterization of ADSC

The study of the sampling techniques of lipoaspirate using apparatus for vacuum directed to the use of samples with more quality and ADSC as shown in (**Figure 11**). The amount of ADSC obtained by the 36 samples of 2.0 mL (from 120 mL) was similar with critical significance level of p < 0.05 = 0.008 for type IV collagenase and type I collagenase p < 0.05 = 0.02. The technique it allowed to obtain a higher surface area of fractions of lipoaspirate, facilitating increased activity of the enzyme collagenase types I and IV to extract the ADSC and also enabled a larger number of adipose mesenchymal stem cells for the use of this cannula allowed the extraction of such cells that are present around blood vessels. In this study the

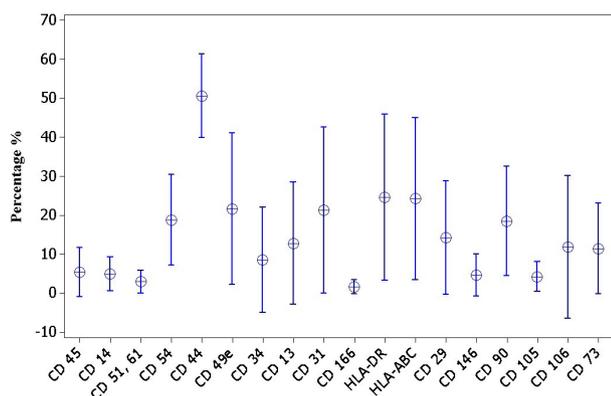


Figure 11. Percentage values ADSC analyzed by quantitative immunophenotyping with the markers CD45, CD14, CD51, 61, CD54, CD44, CD49e, CD34, CD13, CD31, CD166, HLA-DR, HLA-ABC, CD29, CD146, CD90, CD105, CD106 and CD73 and were extracted using the technical equipment for the vacuum for sampling, with n=18 markers.

number of ADSC extracted with the use of collagenase type IV was 3.6×10^8 cells, whereas with type I collagenase was 2.5×10^7 cells, immediately after extraction. The results were better especially using collagenase IV compared to other studies reporting the use of collagenase I and wider cannulae, because the amount of extracted ADSC was approximately 2.0×10^7 [39,40].

On the other side, comparing the population doubling time (PDT) of cell growth from the 1st to the 9th expansion, our interpretation is that PDT decreased because of the higher purity of the cellular environment and also elevated metabolic activity and ADSC expression. It could facilitate the occurrence of autocrine effect (more interaction cell-cell) between ADSC for cell growth, similar to the studies reported by Nardi [5]. The study of adherence and proliferation of the ADSC Petakas proved to be relevant, because it was possible to obtain a greater number of cells in less time compared to the traditional cultivation bottles, lowering the consumption of culture medium and also the incubation time.

Although the results were representative of immunophenotyping ADSC is, Mainly because of CD 44 + the phenotype of these cells results is not conclusive, since most of these markers are expressed at different levels in strains of fibroblasts. Therefore, assays of enzymatic activity of alkaline phosphatase were conclusive for the characterization of ADSC, as reported also by Planat Bernard [39].

Characterization of scaffolds and their interactions with ASC and macrophages

Degradation of the scaffolds of chitosan-collagen-genipin in the presence of pepsin was higher compared to the enzyme collagenase because of enzyme action on chitosan and collagen. Although the reaction of IV collagenase degradation of the matrices being performed above the melting point

of the collagen 34°C, that is, at 37°C, the enzymatic activity of pepsin was added to the degradation of the matrices at the same temperature compared to the studies from Chiono et al., [10]. The solubility of the scaffolds in order from highest to lowest degree of cross-linking genipin was greater due to the increased vulnerability to dissolve the scaffold because it increased the degree of dissociation of chitosan and collagen due to the protonation of the amino groups, showing that the best concentration of genipin for the fabrication of scaffolds with resistance and malleability was of 0.50%, in agreement with results reported by Baldwin and Kiick [1]. That concentration was the best choice for crosslinking since when genipin concentration is less than 0.5% the biomaterial is very fragile, with extremely low resistance against the solution, and when the concentration is greater than 0.5%, the biomaterial breaks as flakes for being too rigid.

On the other side, the morphology of the scaffold structure as well as its porosity were determined by scanning electron microscopy, showing the biocompatibility of chitosan-collagen scaffolds-genipin for adhesion and growth of the ADSC, allowing to induce differentiation of these cells into various cells line, as already demonstrated by Planat Bernard et al., [39,40]. Furthermore, Chitosan, being a polyelectrolyte as shown by the degree of deacetylation in, stimulated *in vitro* expression and activity of ADSC by several factors such as paracrine and autocrine effects, alkaline phosphatase activity and nitrogen oxide (NO) production by macrophages. Collagen is bioadherent by site-specific arginine-glycine-aspartate (RGD). The RGD group promotes cellular adhesion by binding to integrin receptors, thereby promoting growth and cell differentiation. Type I collagen is prominent in use in regenerative medicine for sustaining the most tissues and also by anchoring proteins and cells in the extracellular matrix. Studies report that the physical unit of type I collagen designated as "D period" is involved with the control of biological activities that keeps the biological niche in the tissues. The miscibility of chitosan is due to the electrostatic interactions in the form of ion-ion, ion-dipole and dipole-dipole interactions, Van der Waals, p-electrons and charge transfer complexes, forming ionic bonds, hydrogen and covalent among the polymeric components, producing a Gibbs free energy ($\Delta G < 0$) in the negative mix, despite the high molecular weight polymers. Groups of hydroxyproline (OH-) collagen are hydrogen bonds between the chains and the interactions with other side groups are important in collagen fiber formation. These side groups also form hydrogen bonds with-OH and NH_2 of chitosan. Furthermore, the terminal groups-COOH and- NH_2 collagen can also form hydrogen bonds with-OH and- NH_2 chitosan. The two polymer chains can tangle collagen-chitosan, forming a complex with increased viscosity. Mixtures of polycations (chitosan) and polyanions (collagen) leading to spontaneous aggregation and release of counter ions, causing a gain in entropy ($\Delta S > 0$). In this work, the presence of counter ions, as well as other

cations and anions of the individual polymer chains stimulated cellular activities of both ADSC with the production of alkaline phosphatase (AP) and the macrophages to produce nitric oxide (NO).

The increased production of the enzyme AP during the process of cell expansion was associated with the increased purity cell culture medium, increased intercellular interaction and also increase cell stress due to the increasing number of passes and intense rate of cell division. Similar results were obtained by Planat Bernard et al., [40] and Rodriguez et al., [41]. The reduction in alkaline phosphatase enzyme activity in the presence of media chitosan (Ch) and chitosan-collagen-genipina (Ch-Co-Ge), compared to the increased synthesis of NO and decrease in cell proliferation may be indicative of the ADSC initiated cell differentiation, as reported by studies Domard Taravel and [16]. In the presence of arrays intercrossed with glutaraldehyde, AP values were elevated activity compared with the matrix Ch-Co-Ge, considering the effect of the cytotoxic aldehyde groups of glutaraldehyde.

NO production and tissue regeneration

The ADSC secrete a number of cytokines and growth factors with paracrine and autocrine activities, such as macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor-macrophage (GM-CSF), macrophage inflammatory protein (MIP-1 α /CCL3), as demonstrated by Sibille [56] and Cerqueira [55]. These secreted factors produce a series of responses in the local immune system, enhance angiogenesis and induce the proliferation and differentiation of tissue stem cells. Moreover, the ADSC induce the expression of junction proteins, increase microvascular integrity and NO production. The paracrine effect of ADSC was observed in co-culture with activated macrophages, with the production of NO. The macrophages are essential for effective tissue regeneration since they regulate the recruitment, proliferation and differentiation of the target cells, such as cardiomyoblasts, fibroblasts and mesenchymal stem cells. (Figure 11) shows the intense production of NO in the presence of Ch-Ma, Ch-Co-Ge-Ma, Ch-Ma-ADSC, Ch-Co-Ma-ADSC and Ch-Co-Ge-Ma-ADSC revealing the importance of the presence of chitosan, collagen, ADSC genipin and in the process of tissue regeneration, because these scaffolds strongly stimulated NO production that may be responsible, *in vivo*, by vasodilation present in physiological angiogenesis, in addition to function as a mediator in wound healing, and as an inhibitor of leukocyte adhesion in post capillary microvessels. This was also demonstrated by Chiono [10]. Nitric oxide(NO) is an important antimicrobial agent, plays an important role in the decontamination site of implantation of scaffolds, and regulates the secretion of collagen by fibroblasts, influencing the mechanical strength of the new tissue formation.

Conclusion

There is a greater amount of adipose stem cells around blood

vessels, and the relative amount of these cells in tissue. The interaction and adhesion of ADSC was facilitated due to the protonated amino groups of chitosan and the collagen RGD groups that enabled the anchoring ADSC, macrophages and other molecules in the counter-ions of the matrix of chitosan-collagen-genipin. Also enabling increased alkaline phosphatase activity in the absence of macrophages, as well as decreased activity of alkaline phosphatase in the presence of macrophages, which meant a possible differentiation process of ADSC induced by scaffolds chitosan-collagen-genipin particularly.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	IJZF	LFF	OTG	JDA	AB	ENK	RVA	GOBR
Research concept and design	✓	✓	✓	✓	✓	✓	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	✓	✓	✓	--
Data analysis and interpretation	✓	--	--	--	--	--	--	✓
Writing the article	✓	--	--	✓	--	--	--	✓
Critical revision of the article	✓	--	--	--	--	--	--	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	✓	--	--	--	--	--	--

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