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Associação de arcabouço de polipropileno e células tronco mesenquimais para uso em engenharia de tecido*

Association of polypropylene scaffolds and mesenchymal stem cells for use in tissue engineering

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Resumo

A engenharia de tecidos substitui tecidos danificados com a manipulação de células, confecção de arcabouços e a utilização de moléculas que estimulem o tecido. As células-tronco mesenquimais (MSCs) são boas candidatas para engenharia de tecido, pois são um dos tipos celulares recrutadas para a reparação de tecidos lesionados. O arcabouço deve ser um dispositivo estrutural que forneça uma estrutura para o crescimento e a diferenciação celular no sítio, sendo a tela de polipropileno um exemplo. O objetivo deste estudo foi avaliar o cultivo de células-tronco mesenquimais de tecido de adiposo (ADSCs), isoladas de camundongos C57Bl/6 GFP+, em dois tipos de telas de polipropileno (macroporosa e microporosa) em placas de cultura convencionais e revestidas com metacrilato, durante quinze dias, para obter o melhor protocolo de interação entre a tela e as células. A escolha do melhor método foi baseada na adesão, manutenção da adesão e viabilidade durante cultivo. A quantidade de ADSCs aderidas foi verificada diariamente em contagem em Câmara de Neubauer e através de uma curva de crescimento realizada através de ensaio de MTT. As ADSCs aderidas nas telas foram visualizadas com a marcação de DAPI, panótico, hematoxilina e eosina, imumo-histoquímica (integrina) e imunofluorescência (actina). Nas duas formas de cultivo e nos dois tipos de telas de polipropileno houve aderência das ADSCs. Houve maior aderência na tela microporosa, no período de sete dias de cultivo e em placas sem metacrilato. Conclui-se que a tela de polipropileno oferece um bom arcabouço para as ADSCs se aderirem.

Palavras-chave: camundongo, células, tela de polipropileno.

Abstract

Tissue engineering replaces injured tissues by manipulating cells, making scaffolds, and using molecules that stimulate the tissue. Mesenchymal stem cells (MSCs) are good candidates for tissue engineering, as this is one of the cell types which are recruited to repair injured tissues. Scaffolds are structural devices that allow cell fixation and migration, with polypropylene meshes being an example. This study aims to evaluate the culture of adipose tissue-derived mesenchymal stem cells (ADSCs), isolated from C57Bl/6 GFP + mice, in two types of polypropylene meshes (macroporous and microporous) in conventional culture plates and plates coated with methacrylate, over a period of fifteen days. The objective was to obtain the best interaction protocol between the mesh and the cells. The choice of the best method was based on adherence, maintenance of adherence and viability during culture. The amount of ADSCs adhering was checked daily by counting in a Neubauer Chamber and by using a growth curve performed with the MTT assay. The ADSCs adhering to the meshes were visualized with DAPI, panotic, hematoxylin and eosin, immunohistochemistry (integrin), and immunofluorescence (actin). ADSCs adhere to all forms of culture and to the two types of polypropylene mesh. ADSCs adhered more to the microporous mesh, within the seven day period of culture and in the plates without methacrylate. Thus, polypropylene meshes offer a good scaffold for ADSCs to adhere to.

Keywords: mice, cells, polypropylene meshes.

Introduction

Tissue engineering aims to restore function or replace injured tissues by manipulating cells, making scaffolds and using molecules that stimulate regeneration (BELOTI et al., 2011). The low availability of tissues for transplantation results in limitations to traditional treatment techniques; hence, tissue engineering approaches offer potential to regenerative medicine (KOH & ATALA, 2004; LI et al., 2013).

Mesenchymal stem cells (MSCs) are good candidates for tissue engineering, as this is one of the cell types recruited to repair injured tissues (MOREAU et al., 2007). MSCs secrete a large number of growth factors that allow cell migration and expansion, exert immunomodulatory activities, thus improving healing (GEBLER et al., 2012; GAO et al., 2014).

Scaffolds are a structural device that defines the geometry of the replaced tissue and provides signals for the promotion of

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tissue regeneration (AHMED & HINCKE, 2010). It must be biocompatible, provide a platform to which cells can adhere to and proliferate on, have mechanical stability and be easy to manufacture, sterilize and manipulate during surgery (MOREAU et al., 2007). Furthermore, interconnectivity between pores is desirable for uniform cell seeding, the distribution and diffusion of nutrients, and the removal of metabolites (LIU & MA, 2004; LIAO et al., 2006; ZHANG et al., 2009).

Polypropylene meshes are the most commonly used material in hernia repair surgeries due to their low cost, non-biodegradability, and extensive tissue incorporation (HUBER et al., 2012). They are woven with monofilament thread and interspersed with pores, having a rough surface that allows the infiltration of fibroblasts and the production of collagen (VAZ et al., 2009; ARAÚJO et al., 2010). A polypropylene mesh provides a functional scaffold for MSCs, which is able to promote fibroblast growth, collagen deposition, neovascularization, and natural host defenses (DOLCE et al., 2010).

This study evaluated the adhesion of MSCs to polypropylene meshes during fifteen days of culture. Two types of polypropylene mesh were used to evaluate whether pore diameter influences cell adhesion, in two types of culture plates: conventional and coated with methacrylate. The objective was to obtain the best conditions of interaction between the mesh and the cells, the best culture time and the best concentration of MSCs on the mesh. We expect the largest number of MSCs to be able to adhere to the polypropylene mesh, forming a three-dimensional system for use in tissue engineering.

Materials and Methods

All animals were maintained and handled according to the rules of the Brazilian College of Animal Experimentation and the Ethics Committee on the Use of Animals, based on Law no. 11,794, of October 8, 2008. This study was evaluated and approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA), where it is registered under protocol 15-104.

Isolation, expansion and characterization of ADSC

The isolation of adipose tissue-derived MSCs was performed from inguinal fat of C57Bl/6 GFP + mice. After the animals were euthanized by anesthetic overdose by isoflurane, the fat was removed and processed in a laminar flow hood. The collected adipose tissue was digested with a type I collagenase solution (1mg/mL) and the cell suspension obtained was centrifuged and then plated in six-well plates cultured with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 9 mM HEPES (Invitrogen), 1% antibiotic solution of penicillin and streptomycin, and 20% fetal bovine serum (FBS) (Invitrogen), kept in an oven at 37°C and 5% CO_2. After 24 hours of culture, the culture medium was aspirated and fresh medium was added. When the cell culture showed an approximate 80% confluence, the adherent cells were removed with a 0.05% trypsin-EDTA solution for subsequent subculture in DMEM supplemented with 20% FBS (complete medium). When the fourth passage was reached, the cells were characterized according to the standards of the *International Society for Cell Transplantation*, with *in vitro* differentiation in the chondrogenic, osteogenic, and adipogenic lineages (MEIRELLES & NARDI, 2003).

Osteogenic differentiation was induced by culturing ADSCs for up to 4 weeks in DMEM supplemented with 15 mM HEPES; 10% FBS; 20 nM dexamethasone (Alfa Aesar), 0.05 mN ascorbic acid 2-phosphate (Sigma-Aldrich), 10 mM b-glycerophosphate (Sigma-Aldrich) and 1% antibiotic penicillin and streptomycin solution. To observe calcium deposition, cultures were washed once with PBS, and stained for 5 min at room temperature with Alizarin Red S stain (Nuclear). Excess stain was removed by several washes with distilled water.

For chondrogenic differentiation, ADSCs were cultured in DMEM supplemented with 9 mM HEPES, 6.25 µg/mL insulin, 10 ng/mL TGF beta-1 and 50 mM of ascorbic acid 2-phosphate for 21 days. Differentiation was detected by staining with Alcian Blue, which has an affinity for the anionic groups present in the glycosaminoglycans of the extracellular matrix.

To induce adipogenic differentiation, ADSCs were cultured in DMEM supplemented with 9mM HEPES, 10^{-8} mol/L dexamethasone, 5 g/mL insulin and 50 µg/mL indomethacin (Sigma). Adipogenic differentiation was detected 21 days after the start of the differentiation assay by staining with Oil Red, which stains the fat deposits in vacuoles.

Cell culture on mesh

After the characterization of ADSCs in the fourth passage, they were plated onto two types of polypropylene mesh: macroporous, with diameter pores from 1.1–1.3 mm (SulMedical), and microporous, with diameter pores from 0.09–0.6 mm (IntraCorp, Venkuri), to evaluate whether the pore diameter affects cell adhesion. They were also cultured in two types of 24-well plates: conventional and coated with methacrylate (Adper Scotchbond), so that the cells do not adhere to the bottom of the plate. Three samples were created and used for each procedure performed. The meshes were scissored aseptically to obtain 0.5 x 0.5 cm pieces, placed in the plates and, afterwards, $1x10^4$ ADSC and 500 µL of DMEM supplemented with 1% antibiotic and 20% FBS was added, being maintained in an oven at 37°C and 5% CO_2. The culture medium was replaced every three days for 15 days, and the cells adhering to the mesh were counted in a Neubauer Chamber daily, in order to establish the best time. To perform this count, the mesh was removed from the well and placed in a new plate, 200 µL of 0.05% trypsin-EDTA was added for 1 minute at room temperature. Then, 1 mL of fresh culture medium was added; the entire contents, including the mesh, were aspirated and placed in a tube for centrifugation. After centrifuging at 775G for five minutes, the supernatant was discarded and 100 µL of culture medium was added, resuspending the *pellet* formed by the cells. 20 µL was aspirated and the cell suspension was quantified in a Neubauer Chamber under an optical microscope (Nikon Eclipse) with a 10X microscope objective.

MTT Assay

To confirm the number of viable cells adhering to the meshes, a growth curve was performed by means of the mitochondrial activity evaluated by the MTT assay (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide) (Sigma), with known amounts of ADSC. Twelve different concentrations of cells were plated on 24-well plates ($1x10^2$; $8x10^2$; $1x10^3$; $1.5x10^3$; $2x10^3$; $5x10^3$; $1x10^4$; $1.4x10^4$; $3x10^4$; $4.4x10^4$; $5.9x10^4$; $7.4x10^4$), with 1 ml of complete medium. The plates were placed in an oven at 37°C

and 5% CO_2 for 12 hours. One hundred and eleven microliters of MTT were added to each well and the plates, wrapped in aluminum foil, were re-incubated for a further four hours. After removing the culture medium with MTT, 300 µL of DMSO (dimethylsulfoxide) was added for 30 minutes under gentle agitation every three minutes. Then, 100 µL of this solution was transferred to a 96-well plate and the reading was performed on a spectrophotometer (Spectramax M3 software Pro 6.22) with an absorbance of 550 nm. The optical density reading was then plotted as a function of the cell number to obtain the growth curve based on mitochondrial activity.

The MTT assays on the meshes cultured with the ADSC were carried out on days 3, 7, 11, and 15, with three samples for each day. To remove the cells adhered to the meshes, the same procedure described for counting in a Neubauer Chamber was performed. To obtain the reading of optical density, the same procedure described for the growth curve was performed.

DAPI nuclear staining

Nuclear staining with DAPI (4′,6-diamidino-2-phenylindole) (Sigma) was used to visualize and highlight the cells adhering to the polypropylene meshes. The meshes were removed to a new plate and washed with PBS. Two drops of DAPI were added and the plate was covered with aluminum foil for 20 minutes. Afterwards, it was washed twice with PBS and the reading was performed under a fluorescence (Olympus IX71) and confocal (Leica TCS SP5) microscope.

Hematoxylin and Eosin (HE) and Panotic Staining

HE and Panotic (LB Laborclin) staining were performed directly on the mesh to view the adhered ADSCs. For the fast panotic staining, the mesh was first washed with PBS and then the following reagents were used, in the following order: 0.1% triarylmethane solution, 0.1% xanthene solution, and 0.1% thiazine solution. Each mesh was submerged in each solution for five seconds and afterwards they were washed with water and visualized under an optical microscope (Nikon Eclipse).

For the HE staining, the following protocol was used: fixing the ADSCs to meshes with 10% buffered formaldehyde for eight hours, hydrating the sample with decreasing concentrations of ethyl alcohol, washing the sample with water, staining with hematoxylin for three minutes, washing the sample with water, staining with eosin for seven minutes, washing the sample with water, and visualizing the sample under an optical microscope.

Immunohistochemistry integrin

Immunohistochemistry was performed directly on the mesh to view the adhered ADSC. These were fixed in methanol and were then submitted to antigenic recovery, performed in 95ºC water bath for one hour in citrate buffer. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution in methanol for 30 minutes. The cells were incubated overnight at 4ºC, with primary antibody CD29/Integrin (BD Transduction Laboratories) in a 1:100 dilution. After incubation, the secondary antibody detection system *goat anti-mouse* IgG-HRP (Santa Cruz Biotechnology) was applied and the reaction was visualized with Liquid Dab (Dako), according to the manufacturer's recommendations. After visualization, the cells were counterstained with Harris' hematoxylin and differentiated in 2% ammoniacal water.

Immunofluorescence actin

Immunofluorescence was performed directly on the mesh to view adhered ADSCs. These were fixed in methanol and were then washed with PBS three times. Endogenous peroxidase activity was blocked with a 2% BSA solution in PBS for 30 minutes. The cells were incubated overnight at 4°C, with the primary anti-actin antibody Ab-5 (BD Transduction Laboratories) in a 1:100 dilution. After incubation, cells were washed with PBS three times and incubated with the secondary antibody *anti-mouse* IgG F (ab')2 fragment-R-Phycoerythrin (Sigma-Aldrich) for three hours, being washed with PBS three times. The nuclei were stained with DAPI for 20 minutes and were then washed with PBS three times.

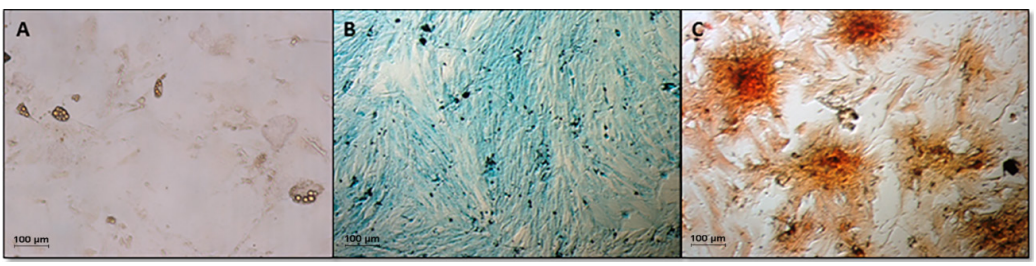
Statistical Analysis

For statistical analysis, IBM SPSS Statistics V22.0 was used. The MTT data were compared by ANOVA complemented by the Tukey's test. The level of significance considered was 5%.

Results

There was differentiation of ADSC in the chondrogenic, osteogenic, and adipogenic lineages in the *in vitro* differentiation induction assay, seen in Figure 1.

Figure 1: Characterization of the ADSC obtained from inguinal adipose tissue of C57Bl/6 GFP + mice. (A) Adipogenic differentiation detected by Oil Red O staining at 100X magnification; (B) Chondrogenic differentiation detected by Alcian Blue staining at 100X magnification; (C) Osteogenic differentiation detected by Alizarin Red staining at 100X magnification.



The daily count in a Neubauer chamber showed that the seventh day of culture was the period in which the ADSCs adhered most to both meshes and that the mesh that obtained the largest number of adhered cells was the microporous one (Figure 2). The growth curve generated using the evaluation of mitochondrial activity by the MTT assay obtained the same results, thus confirming the data (Figure 3A). In the statistical analysis of the MTT assay, there was a statistical difference ($p < 0.05$) on the seventh day between the microporous and macroporous meshes, and also between the seventh day with the other days on both meshes (Figure 3B).

Figure 2: Count of the ADSC adhered to the microporous (A) and macroporous (B) meshes in a Neubauer Chamber during cell culture in conventional culture plates and in plates with methacrylate coating.

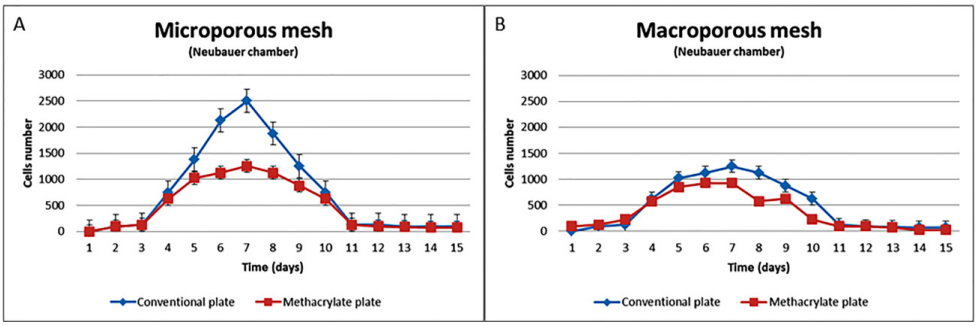
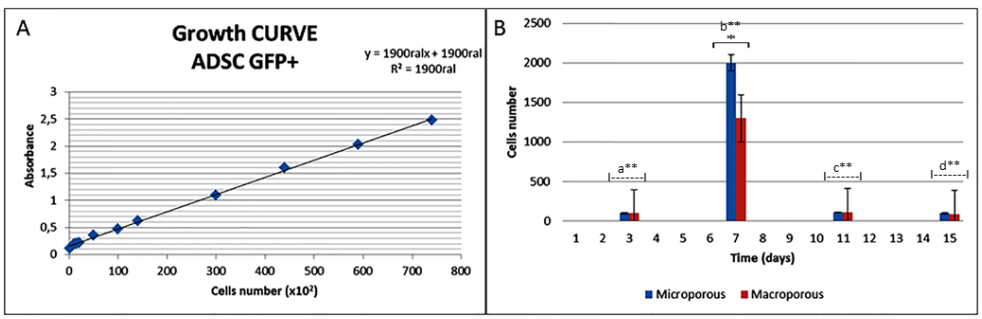


Figure 3: (A) Growth curve generated using the evaluation of mitochondrial activity by the MTT assay with known amounts of ADSC; (B) Amount of ADSC adhered to the microporous and macroporous meshes during days 3, 7, 11, and 15 of cell culture in the conventional culture plate. Horizontal bar represents statistically significant differences (*p<0.05), and statistically significant differences (**p<0.05) between the seventh day (b) with the other days on both meshs (a,c,d), ANOVA complemented by the Tukey's test.



The successful incorporation of ADSCs in the prosthetic was also confirmed by microscopy, which allowed the visualization of adherence and cell growth on the meshes. Panotic, HE, and immunohistochemistry with anti-integrin antibody staining (Figure 4), as well as DAPI, immunofluorescence with anti-actin antibody staining and GFP + cell expression, visualized under fluorescence and confocal microscopy (Figures 5 and 6), allowed a better observation of the ADSCs on meshes, showing adherence with an irregular pattern of these cells on the surface of meshes.

Figure 4: Microscopy of the ADSC adhered to the polypropylene mesh at 200X magnification (A) Panotic staining; (B) HE staining; (C) Immunohistochemistry with anti-CD29/Integrin staining.

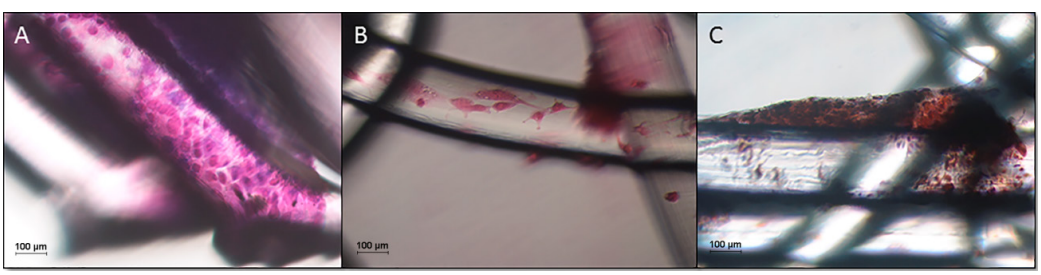


Figure 5: Fluorescence microscopy of the ADSC adhered to the polypropylene mesh with DAPI staining. (A) ADSC adhered to the macroporous mesh at 100X magnification; (B) ADSC adhered to the microporous mesh at 100X magnification; (C) ADSC adhered to the microporous mesh, in confocal microscope at 200X magnification.

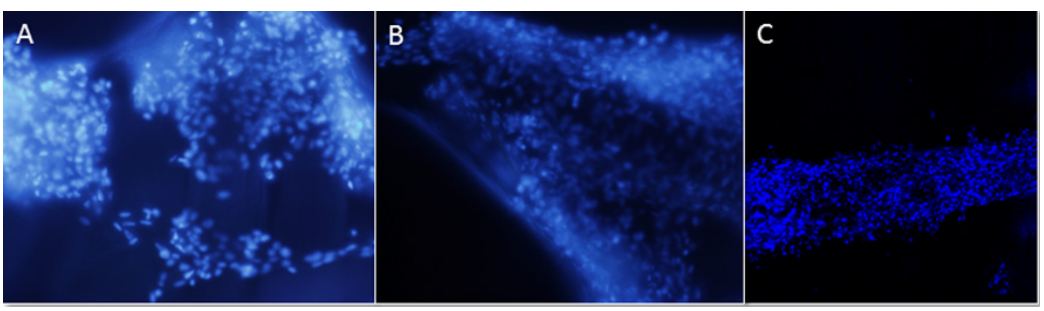
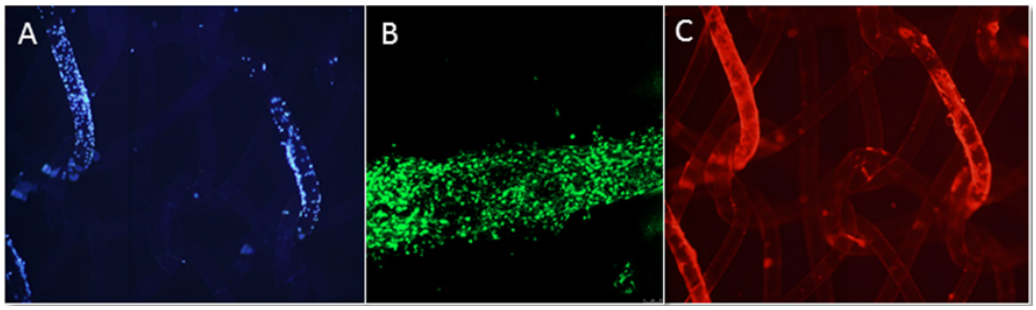


Figure 6: Fluorescence microscopy of the ADSC adhered to the polypropylene mesh. (A) DAPI nuclear staining at 100X magnification; (B) GFP+ cell expression at confocal microscope at 200X magnification; (C) Nuclear staining with primary anti-actin antibody and secondary anti-IgG conjugated to R-phycoerythrin at 100X magnification.



Discussion

This study demonstrated the feasibility of incorporating ADSCs in two types of polypropylene mesh (macroporous and microporous) for use in tissue engineering.

The polypropylene mesh was used because it is the most commonly used material in the making of meshes for hernia repair, as it provides a high mechanical reinforcement for the weakened abdominal wall and provides a rapid growth of connective tissue and extensive tissue incorporation (HUBER et al., 2012; BIKHCHANDANI & FITZGIBBONS, 2013). Other characteristics of polypropylene meshes that allowed us to carry out this study include the fact that they are woven with monofilament threads and are interspersed with pores, having a rough surface that allows the infiltration of fibroblasts, collagen, stem cells, and the internal growth of tissue after implantation in humans (VAZ et al., 2009; DOLCE et al., 2010).

The choice to use MSCs was due to the fact that therapy with these cells has gained a lot of attention nowadays, not only for their easy isolation and culture, but also due to their potential for adhesion in plastic and ability to differentiate and produce growth factors and cytokines (PEREIRA et al., 2008; AMORIN et al., 2012; MAXSON et al., 2012; HASSAN et al., 2014).

Dolce et al. (2010) reported the first study on the incorporation of MSCs in three types of prosthetics: polypropylene (Marlex), polyglactin 910 (Vicryl), and polyglecaprone together with polypropylene (Ultrapro). These researchers demonstrated that such incorporation varies depending on the type of mesh. The mesh that obtained the greatest cell adhesion was Vicryl, followed by the polypropylene mesh, while the Ultrapro mesh obtained the lowest number of adhered cells. One of the relations noted by these researchers about the amount of MSCs adhered may be due to the pore size of each mesh. Vicryl was the mesh with the smallest pore, with an average of 0.5 mm, followed by Marlex (1–2 mm), and Ultrapro (3–4 mm). These data are in accordance with that which was found in our study, since the microporous polypropylene mesh (0.09–0.6 mm) obtained a greater number of adhered cells compared to the macroporous mesh (1.1–1.3 mm). Furthermore, other authors mention that the size and interconnectivity of scaffold pores are important points that promote vascularization, growth, the supply of nutrients, and cellular differentiation processes (MITTAL et al., 2010). For application in tissue engineering, the ideal scaffold should generally have high porosity and a high surface area (ATALA, 2007). The cell adhesion process to the scaffold is influenced by the physical and chemical properties of the matrix surface. This process is mediated by proteins, one of them being integrin, which bind to the collagen present in the extracellular matrix providing resistance to the matrix, and the fibronectin responsible for interceding and facilitating the process of fixing cells to the matrix. Cell-matrix interactions influence the processes of cell growth, proliferation and differentiation, as well as morphological changes in the cells adhered. Poor or even impaired cell adhesion to the matrix can lead to an apoptotic process, leading to cell self-destruction (CHEN et al., 2013).

In another study that used polypropylene meshes, the viability of the adherence of human fibroblasts to these meshes was shown. This study demonstrated the irregular adherence of fibroblasts to polypropylene meshes observed by the expression of Ki67 when compared to the culture of these cells in polystyrene culture plates, emphasizing that these plates are treated for better cell adhesion and that polypropylene meshes do not have special culture properties (KAPISCHKE et al., 2005). We observed the same results; the MSCs adhered more to the culture plates and fixed in a focal manner on the meshes, not forming a homogeneous adhesion across the mesh, according to data obtained with HE, panotic, DAPI, immunohistochemistry, and immunofluorescence staining.

Hence, for the ADSCs not to adhere to the plate, we also used plates coated with methacrylate, so that the cells would have only the mesh as an option for their adherence. However, the use of these plates promoted lower adhesion, visualized more on the microporous mesh, since the cell adhesion on macroporous mesh was low due to the distance of the pores. It is known that most cells require adhesion to an extracellular matrix (ECM) for survival and growth. In addition, cells are vulnerable to apoptosis after cell detachment and dissociation. These suffer extensive cell death, particularly after complete dissociation, decreasing cloning efficiency (VILLA-DIAZ et al, 2009). As there are a smaller number of cells to expand on the mesh, a smaller amount of chemical mediators are available to stimulate the proliferation of cell colonies. There are studies that use a method of dynamic cell seeding to culture MSCs with scaffolds. Some authors, in studies that used stem cells on a defatted bovine bone matrix structure, suggest superior results when using a centrifuge in a bioreactor to favor the infiltration of culture medium between the pores of the scaffold (ORSI et al., 2007).

There are also other examples of research that investigate the improvement in cell adhesion in scaffolds. Kay et al. (2002) showed that the poly (lactic-co-glycolic acid) (PGLA) polymer

is hydrophobic, thus limiting the adhesion and proliferation of osteoblasts and chondrocytes. More recent studies have chemically modified the PLGA polymer with NaOH treatment to improve cell adhesion (PARK et al., 2005), or the use of growth factors associated with the polymer to promote cell proliferation and differentiation (LIU et al., 2007). Casado et al. (2014) also noted the optimization of MSC adhesion in PLGA composite meshes when pretreated with poly-L-lysine. Another study used a physiological inorganic polymer (polyP) together with a collagen matrix on a polypropylene mesh and found that this coating improved cell attachment to the polypropylene mesh and greatly increased the growth of fibroblasts (ACKERMANN et al., 2017).

Conclusion

The results obtained demonstrate that ADSCs can successfully adhere to polypropylene meshes; the microporous mesh cultivated for seven days in conventional culture plates was the best result found in this study. Therefore, the polypropylene mesh with smaller pores offered a good scaffold for ADSCs to adhere to. However, further studies are needed to better understand the factors that increase or inhibit stem cell growth in prosthetics and whether that growth can be induced or driven by other forms of culture, as well as the use of growth factors or techniques that improve the adherence of cells to meshes.

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