

# Evaluation of *In Vitro* Human Gingival Fibroblast Seeding on Acellular Dermal Matrix

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The acellular dermal matrix (ADM) was introduced in periodontology as a substitute for the autogenous grafts, which became restricted because of the limited source of donor's tissue. The aim of this study was to investigate, *in vitro*, the distribution, proliferation and viability of human gingival fibroblasts seeded onto ADM. ADM was seeded with human gingival fibroblasts for up to 21 days. The following parameters were evaluated: cell distribution, proliferation and viability. Results revealed that, at day 7, fibroblasts were adherent and spread on ADM surface, and were unevenly distributed, forming a discontinuous single cell layer; at day 14, a confluent fibroblastic monolayer lining ADM surface was noticed. At day 21, the cell monolayer exhibited a reduction in cell density. At 7 days, about to 90% of adherent cells on ADM surface were cycling while at 14 and 21 days this proportion was significantly reduced. A high proportion of viable cell was detected on AMD surface both on 14 and 21 days. The results suggest that fibroblast seeding onto ADM for 14 days can allow good conditions for cell adhesion and spreading on the matrix; however, migration inside the matrix was limited.

Key Words: Acellular dermal matrix, cell culture, human gingival fibroblasts.

## INTRODUCTION

Autogenous grafts are commonly used on mucogingival surgeries and can be harvested from different regions of the mouth, such as the palate and the edentulous ridges. Regardless of the excellent esthetic results and the high technical predictability (1), they present some limits including donor area morbidity, amount of available of donor tissue, and maintenance of the clinical characteristics of the donor area.

The acellular dermal matrix (ADM; AlloDerm; Lifecell Corp., Branchburg, NJ, USA) was introduced in Periodontology as a substitute for the autogenous grafts. ADM is derived of human skin, from tissue banks, prepared by a carefully controlled process that removes the epidermis and the cells from the dermis without altering the basement membrane and extracellular matrix

organization leaving undamaged the collagen and elastin fibers (2). It is made from a patented process that does not damage the crucial elements of the tissue structure allowing the structural integrity of the entire extracellular matrix from dermal substrate from which dead cells are eliminated after the freeze-drying process. The whole process is important for antigen destruction, although a small risk of inflammatory or immune response by the host's recipient tissue or disease transmission exists (2).

Originally, ADM was introduced in Medicine in reconstructive plastic surgeries, for the treatment of burn victims (2). In Dentistry, ADM may be indicated in various periodontal procedures, like root coverage procedures (3,4) and keratinized tissue augmentation around teeth and implants (4).

Advantages of using ADM allograft as a substitute for the subepithelial connective tissue graft would be the

unlimited quantity of material, decreased surgical time, reduction in discomfort, due to elimination of the donor surgical procedure, and decreased risk of postoperative complications (5). However, the absence of cells and vessels makes tissue incorporation slower if compared to the subepithelial connective tissue graft. Differently from autograft, that can be revascularized based on the anastomoses between blood vessels and those pre-existing in the graft, the allograft, as an acellular and avascular structure, depends on cells and blood vessels from the recipient site to achieve reorganization (2), showing that the cell-cell interactions and the vessel structure have influence on graft maturation (6).

In an attempt to solve these difficulties, the epithelial cell (keratinocytes) and fibroblasts cultures on ADM has been investigated as an alternative to achieve early wound healing, improve incorporation, and decrease wound contraction (7-10). However, the efficiency of cell incorporation throughout the matrix is still under investigation.

ADM may act as a scaffold for host cells, and as a biologically compatible framework into which epithelial cells and fibroblasts can adhere, migrate, repopulate and facilitate the incorporation of the material into the newly formed tissue. Fibroblasts play an important role in wound healing and tissue repair. These cells accelerate the healing process because they appear at the injury site very fast and proliferate rapidly. These cells are responsible for the synthesis of a variety of growth factors, insulin growth factor (IGF), keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), and cytokines involved on the healing process, in addition to extracellular matrix deposition and epithelial cell differentiation (6).

Research on new alternatives to improve allograft incorporation and healing time reduction on soft tissue reconstruction or tissue augmentation in the mouth, is still limited. The possibility of autogenous cell seeding onto ADM before use in surgical sites has to be considered. However, the practicability and the needs for the seeding methods have to be addressed first in order to determine whether the time and complexity of these procedures is worthwhile.

The aim of this investigation was to verify, *in vitro*, if human gingival fibroblasts seeded onto ADM grow onto and inside the matrix and if they are viable and proliferating.

## MATERIAL AND METHODS

Three healthy patients, non-smokers, without systemic diseases, periodontal disease or any other oral infection that required periodontal surgery involving the removal of healthy gingival tissue, were selected for this study. The research protocol was approved by the institutional Research Ethics Committee (Protocol #2007.1.1234.58.5).

### Cell Culture

Human gingival fibroblasts were seeded by the explant technique. The tissue samples removed were stored on a 50 mL falcon tube (Corning Incorporated, Corning, NY, USA), containing transport medium - Dubelcco's Modified Eagles Medium (DMEM; Gibco, Gaithersburg, MD, USA) 500 µg/mL of vancomycin, 250 µg/mL of gentamicin and 250 ng/mL of fungizone. After 3 washes of 15 min each with the medium, the tissue were placed in a Petri dish, sliced into 1-mm<sup>3</sup> piece with a surgical blade and transferred to 25-cm<sup>3</sup> flask (Nunc, Roskilde, Denmark), with DMEM supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, UT, USA) and 5 µL/mL of vancomycin, 0.5 µL/mL of gentamicin and 0.2 µL/mL of fungisone in a humidified incubator at 37°C in a 5% CO<sub>2</sub> air atmosphere.

The medium was changed 3 times a week. After confluence, fibroblasts were detached from culture flasks by treatment with a solution of trypsin/EDTA at 0.05% (Gibco), followed by its inactivation with culture medium. Cells detached and inactivated were centrifuged for 3 min at 30,000 rpm and the pellet formed suspended on a new culture medium. Fibroblasts from first to fourth passages were used in the experiment.

### Tissue Culture

The ADM was cut and washed with sterile saline solution on 50 mL flasks for 10 min, according to the supplier's instructions. The specimens were washed with sterile saline solution until the protect paper flotation, after that the ADM was transferred to a new solution and this procedure repeated for 2 times more. A specimen of 20 x 40 mm was sliced into pieces of approximately 10 x 7 mm each. Before use they were adapted in the bottom of a well in a 24-well-plate and the cells were seeded at a density of 60,000 cell/well on the specimens. The specimens were carried out at 14 or 21 days post seeding.

### Fibroblast Distribution on ADM

To verify the fibroblast distribution on ADM, a direct fluorescence microscopy labeling protocol for actin cytoskeleton and the nucleus detection were processed. After 14 and 21 days, the ADM + cells specimens, were removed from the well-plates and frozen on dry ice and mounted using Optimal Cutting Temperature (OCT) compound (Tissue-Tek OCT; Miles Inc., Elkhart, IN, USA). Frozen specimens were sectioned at 10- $\mu$ m intervals in a cryostat, and the semi-serial sections were adapted on a gel coverslip. The frozen specimens were sliced at 3 different depths, namely border, transition and center, which allowed cell identification and localization, whether at the surface or inside the ADM (Fig. 1). The coverslips with the frozen specimens were washed in buffer phosphate solution (PBS) and the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, followed by blocking with 5% skimmed milk in PBS for 30 min. Incubation was proceeded with Alexa Fluor 488 (Molecular Probes; Eugene, OR, USA) (green fluorescence, 1:200) conjugated phalloidin in a humidified environment for 60 min at room temperature, for actin cytoskeleton detection/labeling. After PBS rinsing, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes) was incubated at 300 nM, for 5 min, to identify the nuclei in blue fluorescence. After mounted with an anti-fade kit (Vectashield; Vector Labs, Burlingame, CA, USA) the samples were then examined with a fluorescence microscope under epifluorescence (Leica, Benstein, Germany).

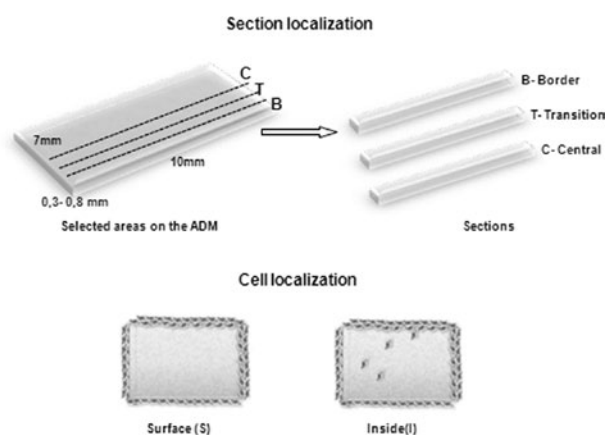


Figure 1. Schematic presentation of the specimen sections according to cell distribution and localization on ADM.

### Cell Proliferation

Indirect immunofluorescence method was used to establish the percentage of cells in cell cycling, by Ki-67 molecular localization on cellular nucleus. Histochemical proceeding for Ki-67 labeling followed the same protocol described previously, using a polyclonal human primary antibody anti-Ki-67 (Diagnostic Biosystems, Pleasanton, CA, USA) (1:70), followed by a secondary antibody goat anti-rabbit 594 (Molecular Probes) (1:200).

### Cell Viability

Cell viability was determined by flow cytometry with annexin V (Dako, Glostrup, DK) and propidium iodeto (PI; Sigma Chemical CO., St. Louis, MO, USA) staining. To cell isolation from ADM surface and inner, the specimens were submitted to 9.5 mL of trypsin in 0.25%, 500  $\mu$ L collagenase (Gibco) and 500  $\mu$ L EDTA for 15 min in a humidified incubator at 37°C in a 5% CO<sub>2</sub> air atmosphere. All process was inactivated in 2.5 mL of culture medium and centrifuged for 10 min at 2000 rpm. Cells were suspended on a 445  $\mu$ L of buffer solution ligand. Annexin V was incubated for 10 min on ice and dark. PI at 50  $\mu$ L, at 100  $\mu$ g/mL, was added to labeling non viable. Annexin V is specific for apoptosis pathway, and PI for the non-viable cells.

### Statistical Analysis

Data presented in this study are the representative results of 3 separate experiments in cell culture established from 3 different donors. All experiments were carried out in triplicate (n=3). Comparisons were performed using the nonparametric Mann-Whitney U test for independent samples at 5% significance level.

## RESULTS

### Fibroblast Distribution on ADM

Epifluorescence revealed that, at day 7, fibroblasts were adherent and spread on ADM surface, and were unevenly distributed, forming a discontinuous single cell layer (Fig. 2A-B). At day 14, a confluent fibroblast monolayer lining ADM surface was noticed (Fig. 2C-D), whose cells exhibited an elongated shape and the actin

cytoskeleton assembled into stress fibers. At this time interval, rare cells were observed inside the ADM, mostly in the border slices and in areas of low collagen fiber bundle density. At day 21, the organization of the cell monolayer did not change compared to day 14, despite the reduction in cell density (Fig. 2E).

Table 1 shows the relative proportions of adherent cells on the ADM slices, namely border, transition and central; their location (at the surface or inside the ADM) is shown in Figure 1. At day 14 a higher proportion of cells was observed inside the ADM on the border

slices compared to the central ones (41.1% and 2.5%, respectively).

### Cell Proliferation

At 7 days, about 90% of adherent cells at the ADM surface exhibited Ki-67 nuclear labeling (Fig. 2B). However, the number of Ki-67 positive cells at 14 and 21 days was significantly reduced (Fig. 2, D and F, respectively; D, arrowhead). Table 2 shows the proportions of cycling cells on ADM surface or inside

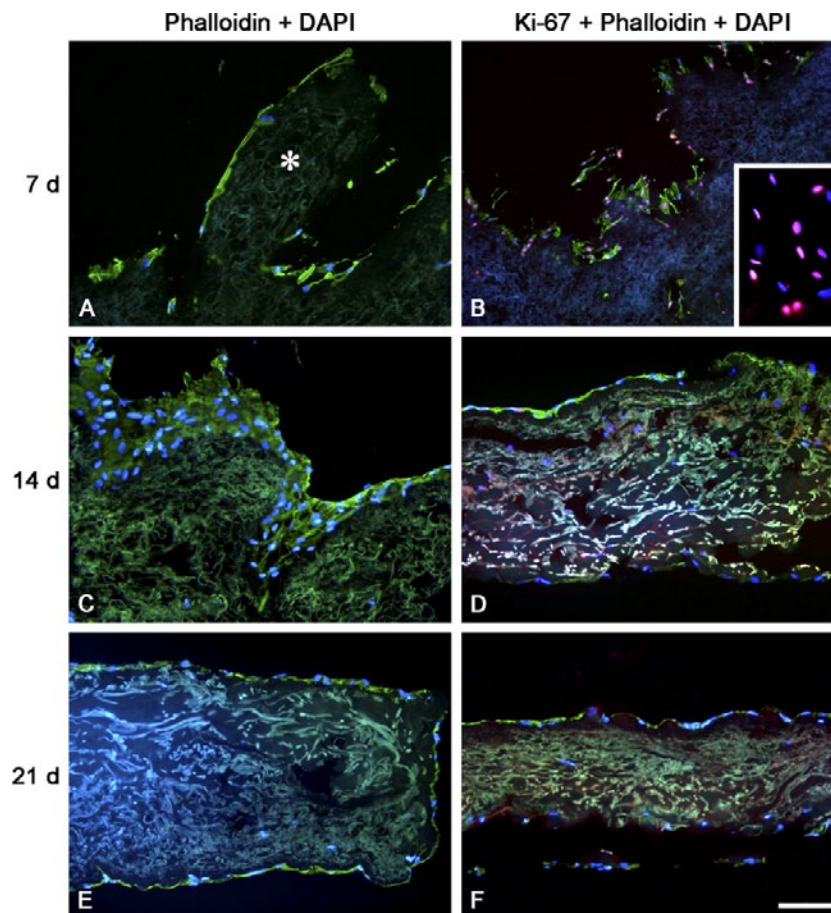


Figure 2. Epifluorescence of human gingival fibroblast cultures on ADM (indicated by an asterisk on A) at 7 days (A and B), 14 days (C and D) and 21 days (E and F) stained by phalloidin (green fluorescence) and DAPI (blue fluorescence) for actin cytoskeleton and nucleus observation, respectively. In panels B, D and F, Ki-67 nuclear labeling, represented in magenta as a result of the superimposition of red (Ki-67) and blue (DAPI) fluorescence. Note: on A and B, fibroblasts were adherent and spread on ADM surface, and were unevenly distributed, forming a discontinuous single cell layer. In panels C and D, a confluent fibroblastic monolayer lining the ADM surface was observed. In panels E and F, lower cell density is seen. Detail on panel B shows the prevalence of cells at the cell cycling (positive to Ki-67) at day 7, which were just occasionally seen at day 14 (D and F, respectively; D arrowhead) and at day 21. Objectives from 20 $\times$  (A, C, D, E, F), 10 $\times$  (B) and 30 $\times$  (detail). Bar of equal value of 100  $\mu$ m (A, C, D, E and F), 50  $\mu$ m (B) and 75  $\mu$ m (Detail) [Color figure can be viewed in the online issue, which is available from [http://www.scielo.br/scielo.php?script=sci\\_issues&pid=0103-6440&lng=en&nrm=iso](http://www.scielo.br/scielo.php?script=sci_issues&pid=0103-6440&lng=en&nrm=iso)].

the matrix. No statistically significant difference was observed between 14 and 21 days.

**Cell Viability**

High proportions of viable cells were detected on ADM surface at days 14 and 21 (96.4% and 94.9%, respectively; Fig. 3), with no statistically significant difference ( $p>0.05$ ) between both time points. No

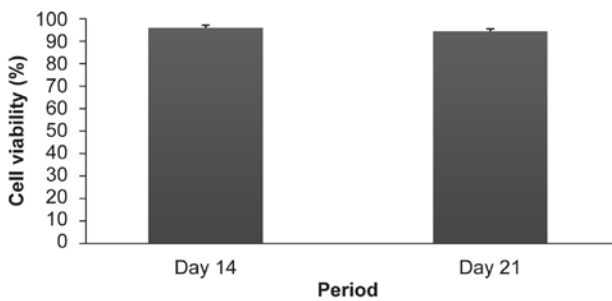


Figure 3. *In vitro* cell viability in human gingival fibroblasts derived from human gingival tissue cultured on ADM evaluated at 14 and 21 days. Data are reported as mean and standard deviation (n=3).

significant differences ( $p>0.05$ ) as to the apoptotic index were observed between days 14 and 21 (19.5% and 22.7%, respectively, Fig. 4).

**DISCUSSION**

This study evaluated the seeding of gingival fibroblasts onto the ADM, aiming to create *in vitro* a hybrid scaffold that could be able to reduce the wound

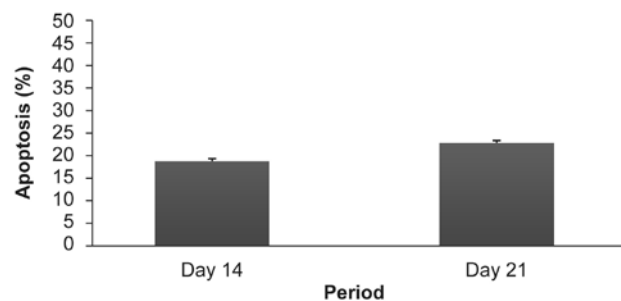


Figure 4. *In vitro* cell apoptosis rate of human gingival fibroblasts derived from human gingival tissue cultured on ADM evaluated at 14 and 21 days. Data are reported as mean and standard deviation (n=3).

Table 1. Relative proportions (%) of fibroblasts adherent to the surface of and migrated into the ADM scaffold at days 14 and 21.

	Day 14		Day 21		p value
	Mean	Median (range)	Mean	Median (range)	
BI	16.5	10.2 (8.7 - 30.6)	13.3	14 (8 - 18)	0.5
BS	41.1	33 (28.4 - 62)	29.7	24.3 (24 - 41)	0.2
TI	5.1	5 (4 - 6.4)	7.6	6 (4 - 13)	0.42
TS	20.6	19.6 (16.1 - 26)	29	31 (24 - 32)	0.1
CI	2.5	3.4 (0.7 - 3.5)	4.3	4 (4 - 5)	0.05*
CS	14.1	10.4 (5 - 27)	15.6	11 (8 - 28)	0.35

Mann-Whitney U-test. \* Statistically significant at  $p<0.05$ . B= border; T= transition; C= center; S= surface; I= inside .

Table 2. Relative proportions (%) of fibroblasts in the cell cycle by Ki-67 immunostaining on ADM.

	Day 14		Day 21		p value
	Mean	Median (range)	Mean	Median (range)	
BI	0.7	0.6 (0.4 - 1)	0.7	0.6 (0 - 1.6)	0.57
BS	2.3	2.5 (1.3 - 3.5)	1.9	1.1 (0 - 4.7)	0.35
TI	0.5	0.5 (0.4 - 0.8)	0.4	0.5 (0.2 - 0.5)	0.35
TS	0.9	0.6 (0.4 - 1.8)	1.2	0.7 (0.3 - 2.7)	0.5
CI	0	0 (0 - 0.2)	0.4	0.5 (0.2 - 0.6)	0.07
CS	0.3	0 (0 - 0.9)	0.7	0.7 (0 - 1.4)	0.35

Mann-Whitney U-test. \* Statistically significant at  $p<0.05$ . B= border; T= transition; C= center; S= surface; I= inside .

healing events *in vivo*, ultimately improving tissue reorganization and neoangiogenesis. While at day 7 fibroblasts were adherent and spread on ADM surface, forming a discontinuous single cell layer, at day 14 the monolayer was confluent, exhibiting mostly non-cycling cells with an elongated shape. At day 21, the cell monolayer exhibited a reduction in cell density. Only rarely were cells observed inside the ADM throughout the culture interval.

Some studies have evaluated the effect of fibroblast seeding on ADM, with promising results. Novaes et al. (8) found that the addition of gingival fibroblasts to the matrix enhanced vascularization *in vivo* in early stages of healing. Recently, Jhaveri et al. (7) showed no significant differences between ADM seeded with autogenous gingival fibroblasts and subepithelial connective tissue autograft for root coverage and keratinized tissue increase in the treatment of gingival recession. However, the internal distribution of cells was not evaluated in none of these studies.

In this study, epifluorescence revealed fibroblasts adherent and spread on ADM surface, exhibiting actin stress fibers. It is well known that cell-matrix interactions regulate cell growth, migration, differentiation, survival, tissue organization and matrix remodeling (11). The formation of a cell monolayer lining the ADM surface at day 14 was probably due to the reduced migration throughout the ADM scaffold. The dense architecture of the ADM seemed to limit cell penetration in its structure and, therefore, cell proliferation and migration took place mostly on its outer surface, where cells could adhere and spread. Similar results were described by Ojhe et al. (12) using a dermal equivalent reminiscent of ADM. However, these characteristics are not restricted to dermal matrices. Hillmann et al. (13), evaluating biodegradable synthetic collagen matrices as a cell carrier, observed that cell migration was lower on matrices with dense collagen fibrillar network, whereas cells were distributed homogeneously throughout the scaffold with a loose fibrillar structure.

Maasser et al. (14) showed that fibroblast migration in 3-D collagen matrices requires cell adhesion and matrix remodeling. In the present study, although cells adhered and spread on ADM surface, the low number of cells inside the matrix, especially at the central slices, suggests that during the culture interval cells were unable to alter the dense organization of the collagen bundles, which acted as a physical barrier. Even if long-term cell cultures could allow the cells to modify

the collagen structure and penetrate the ADM, it should be taken into consideration that the cell contact inhibition that takes place as the fibroblast monolayer reaches confluence triggers the apoptosis pathway (15), thus reducing the number of viable cells. Moreover, relevant phenotypic changes can occur in long-term cell culture (16), which could have an impact in the graft quality.

In cell cultures, increasing cell number depends on the establishment of cell to cell interactions, which limits the proliferation of fibroblasts and induces their exit from the cell cycle (17). Indeed, while at day 7 most non-confluent fibroblasts were cycling cells, at days 14 and 21 the monolayer exhibited reduced cell proliferation rate, which is consistent with previous results (18). Schor (19) reported that the reason for the slower growth of fibroblasts within the collagen matrix could be due to several factors, including the availability of nutrients. Thus, the low porosity and poor pore interconnectivity in the ADM could decrease diffusion of nutrients and consequently impair cell migration throughout the matrix, restricting the cell growth to the surface.

ADM is an important source of collagenous and non-collagenous extracellular matrix proteins, which has been used as a scaffold for fibroblasts to adhere, spread, proliferate, and migrate. In addition, the growth of fibroblasts on ADM *in vitro* allows these cells synthesizing and releasing growth factors, which can be sequestered into the matrix and eventually made available *in vivo* (20), thus contributing to ADM revascularization and wound healing. However, for tissue engineering purposes, fiber bundle arrangement does not allow the repopulation with autogenous cells throughout the entire matrix.

Aiming to allow cell migration and colonization onto the matrix surfaces, the 14-day culture of gingival fibroblasts on ADM seems to be ideal to support cell growth as well as the formation of a confluent, but not saturated cell monolayer on matrix surface, characterized by mostly viable, non-cycling fibroblasts.

Within the limits of the present study, the results suggest that fibroblast seeding on ADM for 14 days can provide good conditions for cell adhesion and spreading on the matrix surface. However, the presence of cells inside the matrix is limited and unpredictable.

## RESUMO

A matriz dérmica acelular (MDA) foi introduzida na Periodontia como um substituto para enxertos autógenos, os quais se tornaram restritos devido à quantidade limitada de tecido doador. O objetivo

deste estudo foi verificar, *in vitro*, a distribuição, proliferação e viabilidade de fibroblastos gengivais humanos cultivados em MDA. Fibroblastos gengivais foram cultivados sobre MDA por até 21 dias. Os seguintes parâmetros foram avaliados: distribuição, proliferação e viabilidade celular. Os resultados revelaram que, aos 7 dias, os fibroblastos estavam aderidos e espalhados na superfície da MDA, e estavam distribuídos de forma desigual, formando uma camada celular descontínua; aos 14 dias, uma monocamada confluyente de fibroblastos revestindo a superfície da MDA foi observada. Aos 21 dias, a monocamada celular exibiu uma redução na densidade celular. Aos 7 dias, cerca de 90% das células aderidas na superfície da MDA estavam no ciclo celular, enquanto que aos 14 e 21 dias esse número reduziu significativamente. Uma maior proporção de células viáveis foi detectada na superfície da MDA tanto aos 14 quanto aos 21 dias. Os resultados sugerem que fibroblastos cultivados sobre a MDA por 14 dias permitem boas condições de adesão e espalhamento das células sobre a matriz, porém, a migração de células para o interior da matriz foi limitada.

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