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Evaluation of the Biological Behavior of Mucograft® in Human Gingival Fibroblasts: An *In Vitro* Study

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Mucograft® is a resorbing porcine matrix composed of type I and type III collagen, used for soft tissue augmentation in guided tissue bony regeneration procedures. This *in vitro* study aimed to evaluate the biological behavior of Mucograft® in human gingival fibroblasts, as well as the ability of the matrix to induce production of extracellular matrix. Six resorbing Mucograft® matrices (MCG) were cut into 3 x 2 mm rectangles and 5 x 5 mm squares and were placed in 96- and 24-well plates, respectively. The control group (CTRL) consisted of cells plated on polystyrene without the MCG. After one, two, three and seven days, cell proliferation and viability were assessed using the Trypan exclusion method and MTT test, respectively. Type III collagen (COL 3A1) and vimentin (VIM) expression were also evaluated at 10 and 14 days, using Western blotting. Statistical analysis, using ANOVA with post hoc Bonferroni test, revealed that human gingival fibroblasts from MCG showed similar results ($p>0.05$) for proliferation and viability as the cells cultured on CTRL. After 14 days, a significant decrease in COL 3A1 expression ($p<0.05$) was observed when cultured with the MCG. VIM expression showed no significant difference at any time period ($p>0.05$). Although no increase in extracellular matrix secretion was observed in this *in vitro* study, Mucograft® presented cellular compatibility, being an option for a scaffold whenever it is required.

Key Words: gingival tissue graft, Mucograft® collagen matrix, cell culture, guided tissue regeneration.

Introduction

Periodontal soft tissue grafts are primarily used for root coverage, to thicken a gingival site or improve the crestal volume in pre-prosthetic surgery. Soft tissue grafts are also advised to create a favorable environment for peri-implant mucosa (1). Soft tissue grafts may be harvested from the palate, retromolar pads or edentulous sites. Disadvantages of harvesting the graft from the retromolar pad and edentulous sites are the minimal amount of tissue availability, as well as recuperation of thinner grafts only. Therefore, the preferred site for harvesting soft tissue grafts is the palate (1), which requires a second surgical site, increasing morbidity in terms of post-operative discomfort and procedure time (2).

Biomaterials are being used as a replacement for palatal tissue harvest, with the aim of reducing morbidity. Biomaterials may be considered as such when it allows for adequate tissue integration, without inducing an immune response, chronic inflammation or sensitivity that may interfere with healing and, hence, harm the patient. The advantages of biomaterials are their unlimited availability, decreased surgical time, reduced discomfort due to lack of a donor site and fewer post-operative complications (3).

Among the biomaterials used for periodontal tissue regeneration, collagen matrices have received significant attention. Mucograft® is a resorbing porcine matrix composed of type I and type III collagen, which is used for soft tissue augmentation in both guided tissue and bone regeneration procedures. It is composed by a porcine collagen bilayer structure (4,5). The compact layer, which consists of compact collagen fibers with occlusive cellular properties, allows tissue adherence as a prerequisite for favorable wound healing. This layer not only protects against bacterial infiltration during open healing conditions, it also contains adequate elastic properties to accommodate suturing. The second layer consists of a thick, porous, spongy collagen structure, which is placed next to the host tissues to facilitate organization of the blood clot and promote neoangiogenesis and tissue integration (5,6).

Although clinical and histological studies with Mucograft® have demonstrated the induction of a mild tissue reaction, excellent tissue integration was observed (5). A recent study comparing Mucograft® with BioGuide®, which is another biomaterial, demonstrated that the former facilitated cell proliferation and promoted early tissue reaction *in vitro* and *in vivo*, respectively (7). This matrix has been extensively studied in clinical setting (4,8-10), with several studies showing promising esthetic results (4,9-12). Additional studies that report on cell proliferation and viability, and the potential to induce connective tissue synthesis are, however, lacking.

The aim of this *in vitro* study was to evaluate the

biological behavior of Mucograft® on human gingival fibroblasts, as well as its ability to induce production of extracellular matrix.

Material and Methods

Specimen Preparation

In order to investigate the action of Mucograft® (MCG) on human gingival fibroblast viability, as well as its capacity to induce cellular proliferation and protein expression, six resorbing Mucograft® matrices (Geistlich Biomaterials, Wolhusen, Switzerland) measuring 3 x 2 mm and 5 x 5 mm were placed in 96- and 24-well plates for the proliferation and viability tests, and Western blotting, respectively. The control group (CTRL) consisted of cells plated on polystyrene without the Mucograft® collagen matrix.

Cell Cultures

Gingival fibroblasts were obtained from explants of healthy attached human gingiva from three different donors, obtained from periodontal surgery for crown lengthening (13,14). This study was approved by the São Leopoldo Mandic Institute and Research Center Institutional Review Board (IRB - #2012/0308).

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St Louis, MO, USA) supplemented with 1% antimycotic-antibiotic solution (10,000 units of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per mL, in 0.9% sodium chloride; Sigma), containing 10% donor calf serum (DCS; GIBCO, Buffalo, NY, USA), plated in 60 mm diameter plastic culture dishes and incubated under standard cell culture conditions (37 °C, 100% humidity, 95% air, and 5% CO_2). Once the cells reached subconfluence, they were detached using 0.05% trypsin and subcultured at a density of 110 cells/mm^2. The cells were used at subculture levels 3 or 4 for all experimental assays.

Cell Proliferation and Cell Viability Assays

Cells were grown on 24 and 96 wells plates (Costar®, Corning, NY, USA) at an initial concentration of $1.9x10^4$ cells/mL and $0.42x10^4$ cells/mL per well, respectively for the proliferation and viability tests. After 1, 2, 3 and 7 days, the cells were detached using 0.05% trypsin and counted in a Neubauer chamber to calculate proliferation indices. In a different set of plates under the same conditions, 10 µL of MTT solution (5 mg/mL in PBS) and 90 µL of base medium were added to each well. Cells were incubated for 3 h at 37 °C, 5% CO_2, 95% air and complete humidity. After 3 h, the MTT solution was removed and replaced with 100 µL of dimethyl sulfoxide (DMSO). The plate was further incubated for 15 min at room temperature (RT) and the optical density (OD) of the wells was determined at a wavelength of 590 nm in a SpectraMax Plus microplate reader (Molecular Devices). The experiments were repeated twice under the same conditions to ensure accuracy.

Western Blotting

Cells were grown on the membranes for 10 and 14 days, homogenized and centrifuged at 15,000 g for 15 min at 4 °C. The protein concentration was measured by a BCA assay (Pierce, Rockford, IL, USA). Protein extracts were separated on 15% sodium dodecylsulfate–polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Hybond; Amersham Biosciences, Piscataway, NJ, USA), exposed for 1 h to the primary antibodies anti-vimentin (VIM, mouse, 1:1000, Dako Corp., Carpenteria, CA, USA) and anti-type III collagen (COL3A1, mouse, 1:1000, Abcam, Cambridge, UK), and diluted in TBST and 5% low fat milk. The primary antibody GAPDH was used as an endogenous control (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the secondary monoclonal antibody of either mouse or rabbit origin (1:2500), the reaction was developed using Bio-Rad Laboratories (Hercules, CA, USA) Western blotting chemiluminescent detection reagents (Opti-4CN) onto x-ray films (GE Healthcare, Fairfield, CT, USA). Measurements of optical density were performed using the NIH Image J 1.37 (National Institutes of Health, Bethesda, MD, USA) for scanned membranes.

Statistical Analysis

Data were first examined for normality using the Shapiro-Wilk test. Two-way analysis of variance with *post hoc* Bonferroni test was then applied to all assays, at a significance level of 0.05. The results were expressed as mean ± standard deviation.

Results

Mucograft® biological behavior was evaluated using cell proliferation counts and the Trypan vital exclusion method. In the control group (CTRL), human gingival fibroblasts, which were cultured in DMEM only, presented similar results to the cells cultured on the Mucograft® resorbing matrices at 1, 2, 3 and 7 days, demonstrated by the lack of a significant difference between the groups ($p=0.7582$) (Fig. 1).

The MTT assay for cell viability revealed no significant difference between CTRL and MCG ($p=0.7003$), as shown in Figure 2.

Cytoplasmic collagen III (COL 3A1) and Vimentin (VIM) content are shown in Figure 3. At 10 days, a higher expression of COL 3A1 was observed for the cells cultured on the MCG when compared to the CTRL, but it was not statistically significant ($p>0.05$). At 14 days, however, a significant decrease in COL 3A1 expression was observed when the cells were cultured on MCG ($p<0.05$). This

finding demonstrated that the matrix was not capable of inducing COL 3A1 synthesis in 10 days, but a considerable reduction of collagen expression occurred after 14 days when compared to the control group.

A numeric decrease in Vimentin (VIM) expression was observed when the cells were cultured on the matrix when compared to CTRL, although it was not statistically significant (p>0.05).

Discussion

Biomaterials have been developed as tissue substitutes, using the concept of guided tissue regeneration (GTR) (15,16). An adequate resorbing material for oral procedures should allow cell adhesion, proliferation and migration in order to prevent exposure to oral microorganisms (17,18). Collagen is one of the most researched resorbing materials, being the main component of the periodontal connective tissue matrix, with significant hemostatic properties, thus aiding in early stabilization of the surgical wound.

Mucograft® is a collagen matrix of porcine origin used as a substitute in cases of loss of the connective tissue structure (4,8-11,19). It has been used to replace the connective tissue graft from the palate, as well as for recession coverage and regeneration of keratinized mucosa around teeth and implants (4,12,20). Additionally, Mucograft® has shown promising results for use as a graft for socket seal in ridge preservation procedures (21). Its mechanism of action is the creation of a three-dimensional scaffold that allows the ingrowth and repopulation of fibroblasts, blood vessels and epithelium from surrounding tissues, eventually transformed into keratinized tissue. Despite its extensive use in clinical procedures, few studies have demonstrated *in vitro* the biological behavior of the Mucograft® collagen matrix. The present study aimed to investigate whether human gingival fibroblasts would increase their proliferation potential and extracellular matrix protein expression in the presence of Mucograft®, The results revealed no significant difference in cell growth or viability on the Mucograft® surface when compared to the control group.

A pre-clinical trial in mice used two prototype collagen matrices, namely 1 (CM1) and 2 (CM2), to study their tissue integration, biodegradation and new blood vessel formation (20). These matrices were composed of native porcine

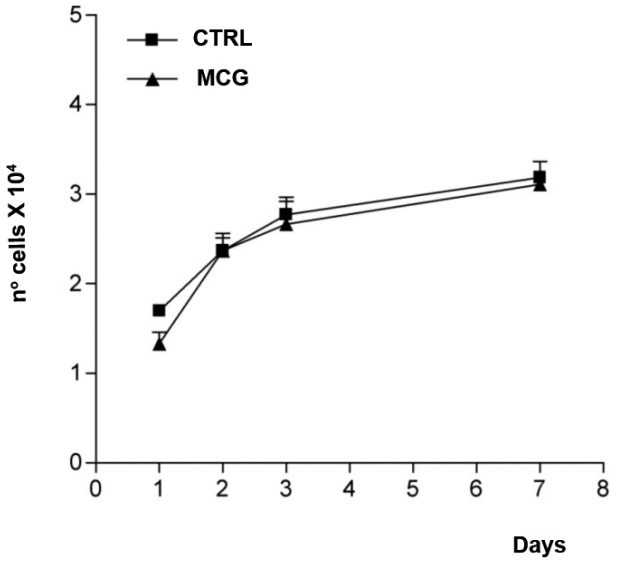

Figure 1. Evaluation of cell proliferation via the Trypan exclusion method for human gingival fibroblasts at 1, 2, 3 and 7 days. The curve was based on biological triplicates with values expressed as the mean (±SD). Two-way ANOVA and Bonferroni test (p=0.7582).

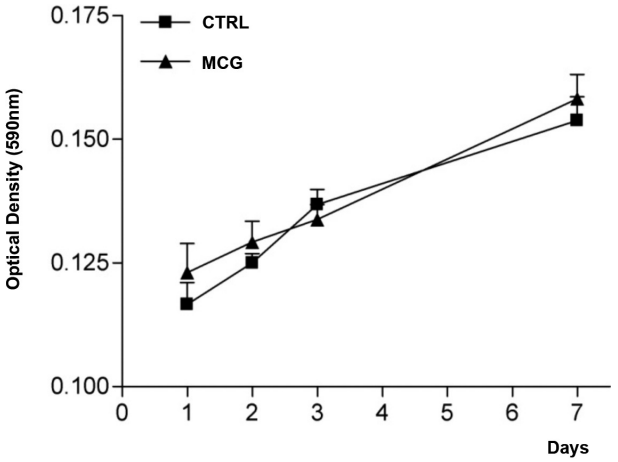

Figure 2. Cell viability test (MTT) in human gingival fibroblasts at 1, 2, 3 and 7 days. The curve was based on biological triplicates with values expressed as the mean (±SD). Two-way ANOVA and Bonferroni test (p=0.7003).

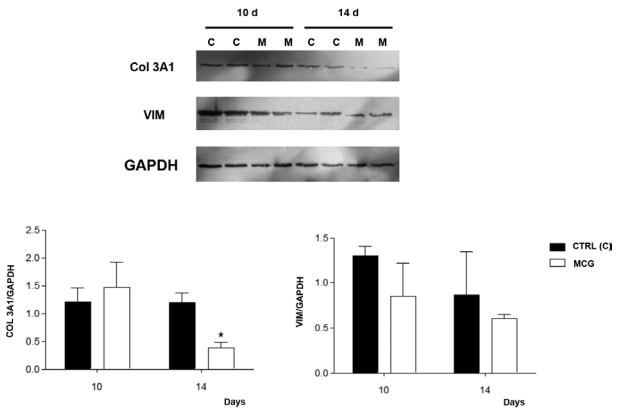

Figure 3. Semi-quantitative analysis of cytoplasmic expression of collagen III and vimentin in human gingival fibroblasts cultured on Mucograft (MCG) and polystyrene (CTRL, C) for 10 and 14 days. The data are presented as the mean ± standard deviation of the duplicated experiment. *indicates statistical significance (p<0.05). Two-way ANOVA and Bonferroni test.

collagen I and III, which differed by the degree of additional chemical cross-linking, with CM1 and CM2 presenting a denser and looser network structure, respectively. Results from the histological analysis demonstrated that the level of cross-linking had a significant influence on the amount of new blood vessel and connective tissue formation, as well as on degradation of the collagen network. The less dense CM2 did indeed offer an improved angiogenic pattern and enhanced connective tissue formation compared to the denser network of CM1. More recently, Willershausen et al. (7) performed a detailed surface and morphological ultrastructure analysis of Mucograft® and compared it with BioGuide® (BG). Cellular growth patterns and proliferation rates of human fibroblasts on Mucograft® and BG were analyzed *in vitro*. The early tissue reaction of CD-1 mouse on these materials was also analyzed by histological and histomorphometrical techniques. The results demonstrated that both matrices facilitated *in vitro* cell proliferation. *In vivo*, these two materials induced a comparable early tissue reaction. The present *in vitro* study did not demonstrate an increase in cellular proliferation and viability. Mucograft works simply as a scaffold, since it did not induce significant changes in the expression of collagen III and Vimentin.

Different barrier membranes, growth and differentiation factors and soft tissue substitutes have been used to promote healing and soft tissue regeneration. When used for the treatment of localized gingival recessions, barrier membranes have provided improved histological outcomes in terms of reduced epithelial attachment and greater amounts of new cementum, connective tissue attachment and bone. However, these improved histological outcomes had limited clinical significance and not predictably present in all studies (15). Some studies have investigated the clinical and histological outcome of Mucograft® for procedures surrounding teeth and dental implants (4,8,10,12). The matrix showed acceptable tissue integration, even in open healing conditions. Application of the collagen matrix significantly reduced the time spent in the surgical chair when compared with autologous grafting (2). Areas of regeneration have shown a similar appearance to that of the surrounding natural soft tissues, both in terms of texture and color, which makes its use preferable in esthetic areas that are difficult to match with palatal transplants (10,12,22). Additionally, the use of collagen matrix removes the need for painful tissue harvesting procedures and significantly reduces postoperative pain (4,19). However, despite the advantages presented by Mucograft®, the gold standard for esthetic soft tissue management for teeth and dental implants is still the subepithelial connective graft (9,22).

It is difficult to establish a suitable comparison between *in vivo* and *in vitro* studies, since laboratory conditions allow good control of the variables for the latter, while the former comprises greater structural, cellular and tissue complexity, making isolated analysis of the regeneration or stimulation potential of the matrices more difficult. Therefore, it may be concluded that although no increase in extracellular matrix secretion was observed, Mucograft® presented cellular compatibility, therefore being a viable option as biomaterial when a scaffold is required.

Resumo

A Mucograft® é uma matriz reabsorvível, de origem suína, composta de colágenos do tipo I e III, utilizada para aumento de tecido mole em regeneração óssea guiada. Este estudo *in vitro* teve como objetivo avaliar o comportamento biológico da Mucograft®, em fibroblastos gengivais humanos, bem como a indução da síntese de matriz extracelular. Seis matrizes reabsorvíveis de Mucograft® (MCG) foram cortadas em retângulos e quadrados medindo 3 x 2 mm e 5 x 5 mm e alocadas em placas de 96 e 24 poços, respectivamente. O grupo controle (CTRL) consistiu no plaqueamento celular em poliestireno, sem MCG. Após um, dois, três e sete dias, a proliferação e a viabilidade celular foram avaliadas utilizando o corante vital azul de Trypan e o teste MTT, respectivamente. Além disso, a expressão de colágeno tipo III (COL 3A1) e vimentina (VIM) foi avaliada após 10 e 14 dias, por meio de Western-blotting. Após análise estatística (Anova e pós teste de Bonferroni), pode-se observar que os fibroblastos gengivais humanos, cultivados sobre MCG, apresentaram proliferação e viabilidade semelhantes em comparação às células que foram cultivadas apenas no poliestireno (CTRL). Após 14 dias, notou-se uma diminuição significativa da expressão de COL 3A1 ($p<0,05$) quando as células foram cultivadas sobre a MCG. A expressão da VIM não mostrou diferença significativa em nenhum dos períodos estudados ($p>0,05$). No presente estudo *in vitro* pode-se concluir que apesar de não ter sido observado aumento da síntese de matriz extracelular, a Mucograft® apresentou compatibilidade celular, sendo uma opção de biomaterial em casos que o arcabouço é necessário.

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