Standardization of Isolation and Expansion of Oral Mucosa Connective Tissue Cells

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Abstract

Objective: The aim of this study was to establish a protocol for the isolation and expansion of a fibromesenchymal cell population from the connective tissue of the oral mucosa for future bioengineering protocols.

Methods: For the isolation of fibroblasts and progenitor cells, we used pieces of surgical samples from patients with an indication for oral surgery. The protocol for isolation was as follows: the tissue was washed with Phosphate-Buffered Saline (PBS) supplemented with antibiotic-antimycotic (PSA). The tissue was placed in a test tube containing collagenase type II and was incubated overnight in the oven. After incubation, the collagenase was collected and the tissue was again washed once with PBS +PSA. Subsequently, the Colony-Forming Unit (CFU) test was performed. The cells (1.0×10^5) were plated on a 10 cm² dish containing Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, supplemented with 10% Fetal Bovine Serum (FBS). The cells were fixed with 10% formalin and stained with crystal violet before counting the colonies. The assay was performed in triplicates.

Results: The cells from all samples showed a homogeneous morphology with a characteristic stellate appearance. The only difference was in the number of colonies formed. There was a significant increase in the number of colonies formed on day 1 when compared with those formed at day 0, and a significant decrease when compared with those formed at day 2.

Conclusion: It was possible to establish a protocol for the primary culture of fibroblasts derived from human oral mucosa.

Key Words: Oral mucosa, In vitro expansion, Fibroblasts, Connective tissue

Introduction

Tissue loss due to trauma, disease, or congenital abnormalities is a health care problem. Specifically, the absence of keratinized mucosa is associated with increased risk in patients with a predisposition to periodontitis or recession [1].

Several techniques of periodontal and peri-implant plastic surgery are used for deepening vestibules, covering roots and/or implants or connections, creating papillae and increasing the edentulous ridge, to enhance the quantity and quality of keratinized mucosa. In these therapies, free or subepithelial gingival grafts are used, and a suitable donor tissue (from the palate and edentulous areas) is required. However, the limitation of available donor tissue, the involvement of painful procedures, the occurrence of residual scars, the risk of hemorrhagic processes, and the postoperative morbidity and discomfort caused by the need for surgical manipulation of additional buccal regions have prompted the search for substitutes, through the use of alloy material and tissue engineering [2-4].

Stem cells currently appear to hold a promise for repair and regeneration of lost or damaged tissues primarily due to their two distinctive features: self-renewal and differentiation into specialized cells [5].

During *in vitro* cell expansion, cell multiplication occurs through cultivation under ideal environmental conditions, resulting in high cell numbers, sufficient for use in clinical research procedures and/or standardized treatments [6,7].

In dentistry, studies have shown the successful usage of cell culture-derived keratinized mucosa for root coverage before prosthetic rehabilitation, as confirmed by macroscopic and histological analysis. In these cases, fibroblasts were cultured on a hyaluronic acid membrane as a carrier matrix, which was transferred to the patient's receptor site. Moreover, the possibility of obtaining tissue through *in vitro* cell culture may reduce trauma and surgical time [8,9].

The interest in stem cells has been steadily increasing owing to their properties and possible applications in regenerative medicine and cell therapy. Stem cells may have an embryonic origin, that is, they are isolated from the zygote or the internal cell mass of the blastocyte, whereas adult cells are derived from the adult organism. Currently, cellular therapy with adult mesenchymal cells is considered advantageous compared to other tissue repair methods, resulting in high-quality regeneration without scarring or fibrosis [10,11].

The aim of this study was to establish a protocol for the isolation and expansion of a fibromesenchymal cell population from the oral mucosa connective tissue for future tissue bioengineering applications.

Methods

The cells used in this protocol were obtained from donors at a school clinic, who needed surgery to cover the gingival recession, after signing an informed consent form approved by the local research ethics committee. The cells were under no circumstances extracted from samples obtained with procedures considered "additional" or exclusive to the project.

The inclusion criteria involved healthy subjects aged above 21 years undergoing root-surgery was used and with normal mucosa.

The exclusion criteria were subjects with habits such as tobacco chewing, smoking, and alcohol consumption; those taking drugs that could affect fibroblastic activity, e.g., calcium channel blockers, phenytoin sodium, cyclosporine, and steroids; patients having diabetes mellitus, hypertension, and ischemic heart disease.

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The region selected for obtaining the samples was the palate between the first distal upper molars and the first upper premolar because of its anatomical characteristics and proximity to the palatine artery. According to several scientific publications, this is the area of choice for obtaining a subepithelial connective tissue graft. Graft removal was performed according to the Harris (12) technique, using a double-blade scalpel with a 1 mm distance between the blades, a scalpel cable (Welfare, code 1071, Brazil) and the blade 15C (Swann-Morton, Brazil). The tissue was placed in a 1 mL Eppendorf tube containing Dulbecco's Modified Eagle's Medium-High Glucose (DMEM) (Sigma-Aldrich, USA, batch 210235k) supplemented with 2X antibiotic-antimycotic (PSA) (Gibco, USA, batch 1509757) and transported to the laboratory.

Cell Isolation, expansion, and morphology

All procedures for obtaining the primary culture of fibroblasts were performed under a laminar flow hood at room temperature between 25℃ and 28℃. Epithelial progenitor cells and connective tissue fibroblasts were extracted from the oral mucosa as previously described. The two cell types were expanded separately, in the presence of specific growth factors.

The isolation protocol of connective tissue cells was as follows: the tissue was washed once with Phosphate Buffered Saline (PBS) (Sigma-Aldrich, USA, batch SLBF7901V), supplemented with PSA to achieve decontamination. Subsequently, the tissue was placed in an assay tube containing collagenase type II at a concentration of 2 mg/mL and was incubated in the oven for 12 hours. The role of collagenase was to digest the connective tissue matrix and detach cells into the medium. Following the 12-hour incubation, the collagenase solution was harvested, and the tissue was washed once again with PBS+PSA to remove all cells that were not detached by the collagenase.

All liquid substances were quantified with the aid of automatic micropipettes. The time-dependent Colony-Forming Unit (CFU) assay was performed as follows: 1.0×10^5 cells were plated in a 10 cm^2 dish containing DMEM-high glucose. After one day, cells from the supernatant were harvested and rewashed. Within three days the surviving cells from day one were collected and replated again. All cells from day zero, day one and day two remained in culture for 14 days. The change in culture medium color, which indicates cellular metabolic activity and pH change, was controlled daily. After this period, the cells were fixed in 10% formaldehyde (Vetec, Brazil, batch DCBB0674) and stained with crystal violet. Cell growth was checked using an inverted phase microscope (40X to 200X, Olympus, Japan). The colonies were then counted. The assay was performed in triplicates.

Analysis of growth profile

In vitro expansion of cells depends on their attachment efficiency after passage operation and growth rate as well as proliferative population during culture. Thus, to understand the growth profile, the efficiency of fibroblasts attachment, specific growth rate and the ratio of the proliferative population were evaluated. In addition, the expansion index was also estimated to understand the folds of an increase in cell number during culture.

Statistical analysis

The statistical analysis of a one-way variance procedure was used. At each time (day 0, 1 and 2), the viable cell media of the study groups were compared. When necessary, an analysis of variance was complemented by the TUKEY test. By this test, multiple comparisons of the 2-to-2 media are performed. Considering that given a certain day, the errors of the statistical model adopted in the analysis of approximately normal distributions with zero mean and equivalent variances in relation to the groups. These errors are estimated by the difference between each count and the group average to which they belong. When considered as cell counts from the same group, but on different days, at least the measurement variables are different. However, it was evident from the data observed that the cells enlarged during this period. To roughly assess a rate of the daily average increase in cells over time, regression lines were constructed using the least-squares method. The appropriateness of this procedure was judged by Pearson's linear correlation coefficient. $P<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS Statistics Version 25.0 (IBM, Armonk, NY, USA).

Results

Were included three patients (two males and one female, between 43 and 52 years of age) with an indication of root-surgery was used.

Morphological characterization of mesenchymal cells

After culturing connective tissue cells from the oral mucosa for a period of 7 days, they formed a homogeneous population, with star-like morphology and an average of two nucleoli per cell (*Figure 1*).

Identification and quantification of mesenchymal progenitors (CFU)

The Colony-Forming Unit (CFU) assay is critical for identifying the mesenchymal progenitors [12].

As described in the Materials and Methods section, 1.0×10^5 connective tissue cells were plated in a 10 cm^2 dish. The assay was performed in triplicate.

After 14 days in culture, the cells were stained with crystal violet and the formation of colonies was assessed. In sample 1, 2 colonies were formed on day 0, 23 colonies on day 1, and 3 colonies on day 2. The size of the colonies formed from this initial cell concentration was different at days 0, 1, and 2 ($p>0.05$).

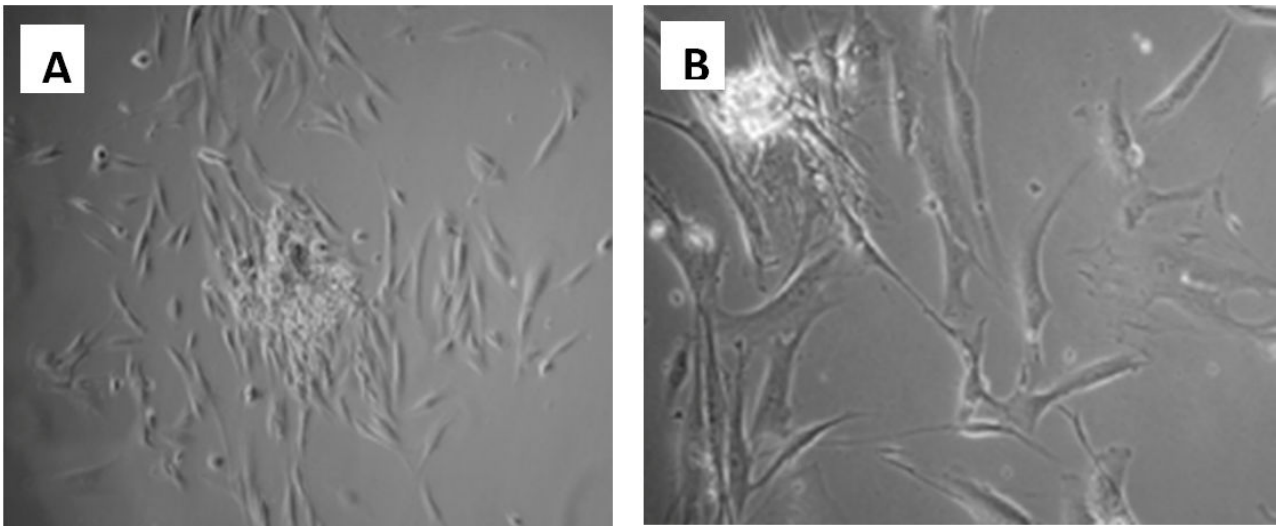

Figure 1. *Connective tissue cell in culture, with star-like morphology and an average of two nucleoli per cell Magnification A (100X) and B (200X).*

In sample 2, 6 colonies formed on day 0, 34 on day 1, and 7 on day 2. The cells exhibited a homogeneous morphology with star-like characteristics. The only difference was in the number of colonies formed ($p>0.05$). The images shown were acquired with a Nikon P500 10X zoom camera.

In sample 3, 1 colony was formed on day 0, 4 on day 1, and 2 on day 2. The cells also exhibited homogeneous morphology with star-like characteristics; the only difference was in the number of colonies ($p>0.05$).

Analysis of the number of colonies

The cells were stained as described above and the number of colonies was determined. In all three samples, there was a significant increase in the number of colonies formed on day 1 when compared to that on day 0, and a significant decrease when compared to that on day 2 (*Table 1*) ($p>0.05$).

Tabel 1. *Quantification of the number of connective tissue cell colonies. In all three samples, there was a significant increase in the number of colonies formed on day 1 when compared to that on day 0, and a significant decrease when compared to that on day 2.*

Number of Colonies			
Sample	Day 0	Day 1	Day 2
A1	2	23	3
A2	6	34	7
A3	1	4	2

Stained cells morphologic analysis

After counting the colonies, the cells were stained and their morphology was evaluated. The cells presented a large elliptical nucleus with little condensed chromatin, being several nucleoli observed. No differences in morphology were observed except for the size of the colonies formed at different days (*Figure 2*).

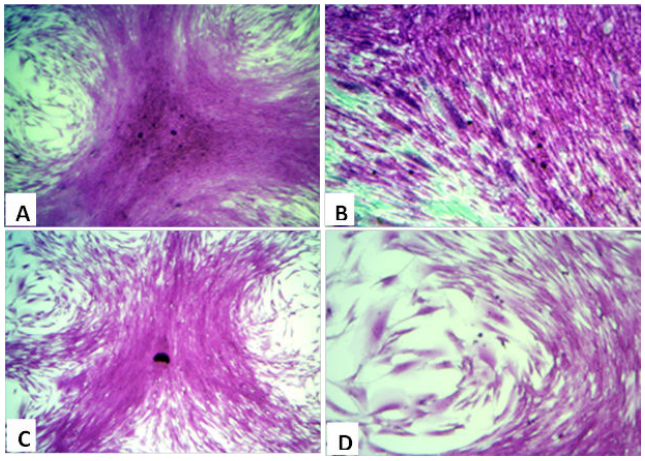

Figure 2. *Sample 1 and 2, Stained connective tissue cells, no morphological differences were observed except the size of the colonies formed on different days. Magnification A and C (40X) and B and D (100X).*

Discussion

In this study, we established a preliminary protocol for the primary culture of fibroblasts from human oral mucosa originating from a buccal site, for future tissue bioengineering applications.

One of the most important considerations for surgery to correct the pathology of the oral mucosa is to cover the bare and injured area. An autogenous gingival graft of partial-thickness is considered the most suitable graft material; however, its availability is a limiting factor. Cell culture technology, originally described for skin grafts and subsequently for mucosal grafting, has been a new era in the field of buccal reconstruction surgery. The main advantage of cell culture is the expansion within a few weeks of a small sample of the biopsy in tissue transplantation of the mucosa [13].

Despite the advances in the use of stem cells to facilitate the treatment of numerous diseases, many unanswered questions remain. Thus, it is possible to argue that a great concern about the factors that may determine the success or failure of the various cell culture procedures is related to the number of cells obtained in the collections, and their differentiation and viability in the media used, especially when dealing with cells with potential of repair [14].

Currently, the "key" focus of scientific research is the mechanism of *in vitro* differentiation of laboratory-grown stem cells. For this purpose, combinations of exogenous substances, such as growth factors have been used. Among them are Bone Morphogenetic Proteins (BMP), Fibroblast Growth Factors (FGF), and Neurotrophic Growth Factors (NGF). In the future, the signaling pathways regulating cell differentiation are expected to be better understood, enabling the control, targeting, and differentiation of cells capable of regenerating injured tissues [15].

In this study, type II collagenase was used, when cells are isolated from donor tissue, they can be maintained in various forms. A small fragment of tissue that adheres to the growth surface, either spontaneously or mechanically, such as clots or an extracellular matrix component (collagen), usually supports cell growth [16].

DMEM (Sigma, USA) was used as a medium for cell growth and maintenance. This culture medium contains nutrients (vitamins, proteins, growth factors, etc.) necessary for cell growth and maintains pH and osmolarity compatible with cell viability [10]. PSA was used to wash the sample and perform tissue decontamination, thus avoiding colony contamination. The Phosphate Buffered Saline (PBS) contains a mixture of salts that promote the removal of organic and cellular debris. Therefore, it is suitable for washing cells. Moreover, it has a pH around 7.0 [17,18].

Fetal Bovine Serum (FBS) (Gibco, USA) was added to the culture medium at a concentration of 10% to facilitate the maintenance and proliferation of the cultured cells. This one is the most commonly used animal serum in cell culture medium supplements owing to its relative availability, ease of storage and high concentrations of growth factors [19].

The study of the dynamic behavior and adhesion between cells and substrates in tissue engineering is of major importance to predict the final biological properties of tissue implants. The adhesion of cells on the substrate influences morphology, proliferation, and cellular viability. In a study where fibroblasts were cultivated on the membranes in a DMEM medium supplemented with 10% of FBS at 37°C, containing 5% of CO_2, significant morphologic differences were observed in the cells. Although the fibroblasts well adhered to the membrane, they maintained a round-shape, different from the findings of this research [15].

Fibroblasts are cells found in abundance in connective tissue. In a cell culture population obtained from connective tissue explants by enzymatic or mechanical disintegration and maintained in culture media supplemented with serum, fibroblasts proliferate rapidly and become the predominant cell type. After a few passages, the only surviving proliferative cells remaining are usually fibroblasts. One reason is that most available culture media optimize and favor fibroblast cell lines [20].

In this research, after primary cultivation, cells derived from the human oral mucosa showed fibroblastic appearance, with elongated or stellate morphology and long cytoplasmic prolongations, compatible with the findings of other studies. The Shape of the cells observed showed that this isolation protocol is efficient, with a cell morphology defined as standard by the International Society Cell Therapy [20-22]. Other studies obtained similar results in establishing and characterizing a continuous lineage of human periodontal ligament (LP) derived cells. These cells were obtained by the tissue explant technique and they were removed by scraping the middle third of the roots of third molars extracted from three healthy patients. Subsequently, the cells were characterized by light microscopy, growth pattern analysis, and immunohistochemical, histochemical, and enzymatic tests. Morphologically, they had a fusiform or stellate appearance, compatible with fibroblastic cells [23].

Based on clinical and immunohistological evidence, that autogenous oral mucosa grafting, obtained by tissue engineering, can be used to cover oral cavity wounds. Complete tissue healing and normal epithelial differentiation could be observed in the grafted area within a 6-month postoperative period [23,24]. It is possible to get success in the use of mucosa grafts in patients through autologous oral mucosa cell cultures. Three weeks after the placement of cultured mucosa grafts, usually, the sites are keratinized without signs of infection and a healthy mucosa it's found after three months. Therefore, cultured mucosa grafts are useful in correcting large oral mucosa defects, especially in pathological conditions [13].

The findings of this study corroborate with other findings in the literature, in the sense that cellular isolation goes through an initial stage of digestion of the extracellular matrix. It is a critical step and must be controlled so that the extracellular matrix is degraded, however, there can be no loss of cell viability. Therefore, it is necessary to standardize the type of enzyme and the concentration used, the quantity and size of tissue fragments and the incubation periods during the process [16,20].

The application of *in vitro* generated oral mucosa grafts to oral cavity defects, peri-implant surgeries, prosthetic procedures, and vestibulopathy has been used successfully [13,25]. Technological and scientific advances, occurring in educational institutions and especially in companies focused on the production of materials for cell culture, have helped researchers to generate more effective isolation and culture protocols. These materials, such as temperature and humidity-controlled incubators, flasks, and culture media, are crucial in generating a suitable *in vitro* environment, resembling *in vivo* conditions, so that cells can successfully proliferate and differentiate into specific lines [15].

The continuing study and development of tissue engineering play an important role in increasing the quality of life and comfort during oral surgeries and rehabilitation. During the formation of the periodontal soft tissues (gingiva and periodontal ligament), fibroblasts are the responsible cells for the formation and maintenance of the extracellular matrix

and production of collagen fibers of the periodontal ligament. Therefore, since tissue engineering aims at forming a tissue similar to the one originally formed, the first step in the future application of tissue engineering involving soft tissues is the cultivation, *in vitro*, of human fibroblasts [13,20].

In addition, the future possibility of using tissue engineering is envisaged, with the purpose of obtaining gingival and peri-implant tissues, according to aesthetic, functional and hygienic needs [15,21]. The proposed protocol permitted to establish a human fibroblast cell lineage with compatible cell viability similar to other results found in the literature. The results obtained in this study indicate that it is possible to use these cells for future research [25].

Based on the results described in this study, we hope to develop and improve tissue engineering technologies focusing on the oral cavity, which can follow the conditions dictated by the relevant regulatory agencies, and be evaluated in clinical protocols, in partnerships with biotechnology companies.

Conclusion

It was possible to establish a preliminary protocol for the culture of fibroblasts from the human oral mucosa, originating from a buccal site. The cells from all the samples, exhibited homogeneous morphology with star-like characteristics, only changing the number of colonies formed. In all three samples observed, there was a significant increase in the number of colonies formed on day 1 when compared with those formed on day 0, and a significant decrease when compared with those formed on day 3.

Data Availability

All data included in our manuscript are available.

Conflict of Interests

The authors declare that they have no conflict of interests.

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All authors gave final approval and agree to be accountable for all aspects of the work.

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