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Development and characterization of a 3D oral mucosa model as a tool for host-pathogen interactions

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ABSTRACT

The aim of this study was to (i) design, develop and validate a practical and physiologically relevant reconstituted *in vitro* oral mucosa tissue model and (ii) to assess its applicability in *in vitro* host-pathogen interactions with *C. albicans* and *S. aureus*. Co-culture organotypic constructions were created by incorporating specific numbers of keratinocytes (NOK-si) onto cellularised, collagen gel scaffolds containing human gingival fibroblasts incubated in KGM media and cultured for 14 days. The detection of the appropriate oral mucosa/epithelial structure was evaluated by histology (hematoxylin and eosin (HE), periodic acid–Schiff (P.A.S.) and Picrosirius red), and immunocytochemistry (cytokeratin 13, cytokeratin 14, Ki-67 and collagen IV) compared to a normal human gingiva. The morphology of the reconstituted tissue was analyzed by Transmission Electron Microscopy. To further quantitate tissue damage, lactate dehydrogenase (LDH) was measured in the tissue supernatant. NOK-si grown upon a gingival scaffold provided an organotypic model in an *in vitro* setting and exhibited structural characteristics typically associated with normal oral mucosa. Immunocytochemistry revealed the detection of epithelial cytokeratins 13 and 14, Col IV and Ki-67 in the reconstituted oral mucosa model. Infection was detected after 8 h and 16 h. This study presents an *in vitro* cellularised, organotypic model of reconstituted oral mucosa, which enables close control and characterization of its structure and differentiation over a mid-length period of time in culture.

1. Introduction

Reconstituted oral mucosa tissue (ROMT) represents a model providing suitable matrix support structure in conjunction with viable cells coupled with an optimal growth environment that allow the development of functional tissue *in vitro*. The basic premise of ROMT is that controlled manipulation of the extracellular microenvironment can influence the ability of cells to organize, grow, differentiate, form a functional extracellular matrix (ECM) and, ultimately, generate new functional tissue (Scheller et al., 2009). ROMT has a broad applicability and it has been used as an alternative to human and animal testing of drugs, and for pharmacological and clinical applications (Brohem et al., 2011). Moreover, three-dimensional culture exhibits cells growing in an environment that closely mimics the *in vivo* environment (Edmondson et al., 2014).

Although the components of ROMT are basically the same, different methodologies to reconstitute epithelium and connective tissue have been reported. There is no consensus in relation to the kind of keratinocyte and fibroblast cells used to reconstitute epithelial- and connective-like layers, respectively, and a variety of scaffolds have been employed (Boelsma et al., 1999). The model proposed in this paper is composed of an epithelial- and connective-like layers formed by immortalized normal human oral keratinocytes (NOK-si), and a collagen matrix formed by human gingival fibroblasts (FGH) in a rat tail collagen type I as scaffold. The use of established cell lines allows homogeneous and unlimited access by passaging and cryopreservation and may also improve the reproducibility and consistency of 3D models, thereby allowing specific pathways or variables to be identified and assessed (Boelsma et al., 1999).

A similar oral mucosa model developed by Dongari-Bagtzoglou and

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Kashleva (Dongari-Bagtzoglou & Kashleva, 2006) used a line of normal keratinocytes immortalized in a collagen and fibroblast matrix, as in the present study. However, the authors used 3T3 fibroblasts from mice in the dermal layer, which do not present the same dermal layer contraction pattern as achieved by our research group with human gingival fibroblasts, meaning our model is closer to human oral mucosa. In addition, the advantages of using stablished cell lines are that donor samples is not needed and interindividual differences do not influence experiments (Kehe et al., 1999). Furthermore, other studies that develop ROMT use the corneal dermis or even synthetic polymers as scaffolds, which also fail to adequately reproduce the desired in situ conditions. Commercial models do not always have a dermal layer and most of them are developed with tumor cell lines which do not provide the same realistic healthy oral mucosa as proposed in this study.

As a tool for host-pathogens interactions, ROMT has been used to evaluate the potential of microorganisms to grow on, penetrate and damage oral mucosa and to elucidate the mechanism of local infection. It has been suggested that *Candida albicans* improves its ability to penetrate across the oral mucosa and to promote tissue destruction leading to focal infection when it is associated with *Staphylococcus aureus* (Shirtliff et al., 2009). *C. albicans* can colonize the cavity alone or in combination with other microorganisms (Coronado-Castellote & Jiménez-Soriano, 2013), and it is the most frequently isolated microorganism (64.4%) in denture bases (Ribeiro et al., 2012). *C. albicans* has numerous virulence factors which allow it to invade and infect host cells; for example, polymorphism (Jacobsen et al., 2012; Mayer et al., 2013), presence of adhesins (Mayer et al., 2013; Garcia et al., 2011), ability to form biofilm (Mayer et al., 2013; Finkel & Mitchell, 2011), and phospholipase and protease enzymes (Lyon & Resende, 2006; Pinto et al., 2008; Zago et al., 2015).

The combined effect of *C. albicans* with other microorganisms may result in synergism and increase the pathogenicity of both microorganisms (Zago et al., 2015; Morales & Hogan, 2010; Peters et al., 2012). It has been estimated that 27% of nosocomial *C. albicans* bloodstream infections are polymicrobial, with *S. aureus* as the third most common isolated organism (Harriott & Noverr, 2009). *S. aureus* is a Gram-positive bacterium and can be found on the skin and mucosa surfaces of human beings (VandenBergh et al., 1999). Studies have described the high prevalence of *S. aureus* on the oral mucosa in denture prosthesis wearers, suggesting that *S. aureus* is a normal colonizer of the oral cavity (Baena-Monroy et al., 2005; Cuesta et al., 2011). In addition, the association between *S. aureus* and *C. albicans* in the colonization of oral mucosa and dental prosthesis wearers with denture stomatitis has been reported (Ribeiro et al., 2012; Baena-Monroy et al., 2005). Not only the microorganisms themselves, but the secreted factors from their metabolisms can promote cell death and inflammatory response in monolayer cell culture (de Carvalho Dias et al., 2017). The secreted factors from the biofilm of mixed-species *C. albicans* and *S. aureus* cultures were more damaging to the monolayer epithelial cells than the secreted factors from the biofilms of single *C. albicans* and *S. aureus* cultures (de Carvalho Dias et al., 2017).

The objectives of this study were to develop and validate a practical and physiologically relevant reconstituted oral mucosa model using immortalized cell lines and to evaluate the developed ROMT as a tool for host-pathogen interaction, both in infected and non-woven tissue exposed to only the secreted factors of single and mixed *C. albicans* and *S. aureus* pathogens.

2. Materials and methods

2.1. ROMT construction

Fibroblasts were obtained from Rio de Janeiro Cell Bank (FGH, cod. 0089), which were derived from human primary cell line established from biopsies of healthy patients' gingiva. Fibroblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, USA), antibiotic, antimycotic solution (Sigma, St. Louis, MO, USA). Collagen gel was produced by mixing rat tail collagen type I (First Link (UK) Ltd.) with DMEM, and FBS at 4°C. The solution was neutralized with 1 M NaOH, and a fibroblasts suspension (3.0 x 10 (Dongari-Bagtzoglou & Kashleva, 2006) cells/ml) was added to the mixture. The dermal layer of fibroblast-containing collagen solution was placed in 24 well plates. After 24 h of contraction, the dermal layer was gently removed with the aid of a small spoon immersed in culture medium and washed twice with Hank's Balanced Salt Solution containg calcium and magnesium without phenol red (Gibco, Grand Island, NY). NOK-si keratinocytes were seeded upon the dermal layer (2.0 x 10^6 cells/ml). NOK-si (Castilho et al., 2010) were previously cultivated in DMEM supplemented with 10% FBS and antibiotic antimycotic solution. After NOK-si seeding, tissues were grown until the epithelial cells reached confluence. Then, the neotissues were raised to an air-liquid interface for 14 days in KGM-Gold medium (Lonza, Walkersville, MD USA) supplemented with 0.5 ml hydrocortisone, 0.5 ml transferrin, 0.25 ml epinephrine, 0.5 ml gentamicin sulfate amphotericin-B, 2.0 ml bovine pituitary extract, 0.5 ml epidermal growth factor human and 0.5 ml insulin. The medium was changed every other day. The tissues were prepared in duplicate for each experimental condition and three independent experiments were performed.

2.2. Histological evaluation

The tissues were fixed in 4% formaldehyde buffered at pH 7.2 with 0.1 M sodium phosphate for 24 h at 4°C. Subsequently, the tissues were dehydrated and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin (HE) and submitted to the periodic acid-Schiff (P.A.S.) histochemical method. Some sections were also stained with Picrosirius-red method and analyzed under light microscope BX-51 (Olympus, Japan) equipped with filters to provide polarized illumination. As control, normal human gingiva was used. Slices of human oral tissue samples were kindly provided by Prof. Dr. Éricka Silveira (Department of Dentistry, Federal University of Rio Grande do Norte-UFRN, Brazil) from the histopathological collection of the institution.

2.3. Immunohistochemical reactions

In the present study, we used the following primary antibodies: rabbit anti-Ki-67 polyclonal antibody (Abcam; ab833, 1/200), rabbit anti-collagen IV polyclonal antibody (Abcam; ab6586, 1/500), rabbit anti-cytokeratin 13 polyclonal antibody (Abcam; ab154346, 1/1000) and mouse anti-cytokeratin 14 monoclonal antibody (Abcam; ab7800, 1/400) (mouse monoclonal). Immunohistochemical reactions were performed using rabbit specific HRP/DAB detection IHC kit (Abcam, ab64261) for Ki-67, collagen IV and cytokeratin 13. The sections were incubated with biotinylated secondary antibody (Dako-K0690; Dako Universal LSAB Kit) for cytokeratin 14.

Tissue sections (4 μm) were deparaffinized, rehydrated and submitted to heat-induced epitope retrieval by microwave treatment for 2 x 5 min in 0.001 M sodium citrate buffer (pH 6.0) (Ki-67, collagen IV and cytokeratin 14), or trypsin/0.1% calcium chloride (cytokeratin 13). After washing with Phosphate-buffered saline - PBS (pH 7.3), sections were treated with 5% hydrogen peroxide (H_2O_2) to block endogenous peroxidase for 10 min at room temperature. After washing, the sections were incubated for 20 min with 2% bovine serum albumin (BSA) and sodium azide/triton at room temperature. Then sections were incubated with primary antibody overnight in the humidified chamber at 4º C. Subsequently, the sections were incubated in biotinylated secondary antibody (Abcam) for 20 min at room temperature and streptavidin for 30 min. The reaction was revealed by using 3,3'-diaminobenzidine (DAB) (Dako, Carpinteria, CA, USA) for 3 min and the sections were counterstained with hematoxylin and mounted. For each