Epithelial cells from oral mucosa: How to cultivate them?

F. B. Russo · G. C. Pignatari · I. R. Fernandes ·
J. L. R. M. Dias · P. C. B. Beltrão-Braga

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Abstract Epithelial cells from oral mucosa (EOM) are responsible for important functions, like the primary protection of oral mucosa against external aggressions building a mechanical barrier against microorganisms, mechanical damage, toxic material, thermal regulation and secretion of different classes of inflammatory mediators. EOM could be an interesting tool for cellular and molecular biology research. Usually, EOM are collected by a painful and invasive process. In this study, we propose an alternative method to cultivate EOM collected by non-invasive scraping method of oral mucosa. Papanicolaou staining showed mainly two kinds of epithelial cell population after EOM scraping.

As result of the five culture methods tested here, our results revealed that the EOM were successfully cultured on a murine feeder layer. In addition, EOM could be frozen and thawed, without morphology changes and loss of viability. Our findings suggest that EOM can be considered as a good cell source for many purposes, such as genetic studies, diagnosis and cell therapy.

Keywords Oral mucosa · Epithelial cells · Epithelial cells from oral mucosa · Cell culture · Non-invasive method

Introduction

Epithelial cells from oral mucosa (EOM) are derived from ectodermal embryonic germ layer (Kolltveit et al. 2010; Queiroz et al. 2010) and have many roles in the body. EOM are the first barrier against external aggressions by microorganisms and toxic substances, perform thermal regulation and help cells from the immune system to release different classes of inflammatory mediators (Squier and Kremer 2001; Yohannes et al. 2011; Laheij et al. 2013; Bedran et al. 2014; Kullaa et al. 2014; Shiomi et al. 2015).

EOM are also a very useful cell source for molecular or DNA studies and diagnosis, concerning the easy access for collection and regeneration potential. Besides, EOM are less exposed to mutation agents compared with other skin epithelial tissues (Muotri

F. B. Russo (✉) · G. C. Pignatari · I. R. Fernandes ·
J. L. R. M. Dias · P. C. B. Beltrão-Braga
Stem Cell Lab, Department of Surgery, School of
Veterinary Medicine, University of São Paulo, 87 Prof.
Dr. Orlando Marques de Paiva Av., São Paulo 05508-270,
Brazil
e-mail: fabielerusso@gmail.com

P. C. B. Beltrão-Braga
e-mail: patriciaacbbbraga@usp.br

G. C. Pignatari · P. C. B. Beltrão-Braga
Center for Cellular and Molecular Therapy (NETCEM),
School of Medicine, University of São Paulo, 455 Dr.
Arnaldo Av., São Paulo 01246-903, Brazil

P. C. B. Beltrão-Braga
Obstetrics Department, School of Arts, Sciences and
Humanities, University of São Paulo, 100 Arlindo Bétto
Av., São Paulo 03828-100, Brazil

et al. 2004; Souto et al. 2006). EOM DNA is used to diagnose several diseases (Aidar and Line 2007), such as demonstrated to determine mutations in the Prader-Willi syndrome (PWS) (Munce et al. 2008), verifying the increasing risk for gonadoblastoma in Turner syndrome patients (Bianco et al. 2006), or analyzing segregation in DNA 3243 A>G mutation in mitochondrial diseases in children and their mothers (Uusimaa et al. 2007). Moreover, EOM smears are used for diagnosis of HNF1B mutation, a cancer related gene, to estimate cancer risk for patients using Fluorescent In Situ Hybridization (FISH) technique (Laffargue et al. 2013).

Ordinarily, EOM have been considered as a source just for single molecular or cellular analyses, usually for quick disease diagnosis, immediately discarded after their use. However, in the past few years, it was demonstrated that EOM could be cultured after biopsy procedure (Ang et al. 2006; Sen et al. 2011; Amemiya et al. 2015; Sheth et al. 2015), being successfully cultured, demonstrating reasonable proliferation potential, achieving confluence in cell culture conditions, with desmosomes junctions, intracellular cytokeratin and abundant secretion granules. Besides that, using RT-PCR and immunocytochemistry techniques, EOM cultures were considered as a heterogeneous cell culture, containing both populations of cells expressing markers from differentiated epithelial cells, such as cytokeratin, and progenitor stem cell markers (Madhira et al. 2008; Sen et al. 2011).

In addition, EOM has been used in cell therapy for eye diseases, cultured both in gel or on top of cell-free amniotic membrane surface for keratoconjunctivitis scar handling and corneal epithelial reconstruction (Ang et al. 2006; Higa and Shimazaki 2008; Kanayama et al. 2009; Klingbeil et al. 2010; Bardag-Gorce et al. 2015; Sheth et al. 2015), especially for reconstruction of ocular surface in patients with bilateral limbal stem cell deficiency (LSCD), usually demonstrating good therapeutic potential (Madhira et al. 2008; Sen et al. 2011; Hirayama et al. 2012; Amemiya et al. 2015).

Here we describe a new method to culture EOM, taking advantage of simple oral mucosa swabbing with scraping, which avoids discomfort or pain for the patient during sample obtaining. This work highlights a good alternative EOM culture method, which could be useful for genetic diseases research and cell therapy for regenerative medicine.

Methods

Obtaining of EOM

This study was approved by the Ethics Committee of the University of São Paulo (Protocol n. 948/CEP) and informed consent was obtained from all subjects included in this study. Samples were collected from 3 healthy adult donors, two females and one male, ages ranging between 20 and 25 years, after oral mucosa evaluation. People with smoking history, tobacco chewing and infection or oral mucosa inflammation were discarded.

EOM were obtained by scraping palate and gum using an interdental brush, right after oral hygiene through brushing of teeth. Samples were washed in PBS with 5 % of antibiotics (500 U/mL penicillin and 500 µg/mL streptomycin—Life Technologies, Carlsbad, CA, USA) and centrifuged for 10 min at 800g for cell washing and pelleting before plating them in a cell dish (Fig. 1).

EOM viability

EOM viability after collection was analyzed by cell counting in a Neubauer chamber after trypan blue staining (Life Technologies), where live cells remain refractile and dead ones are stained in blue. Data were plotted and analyzed using GraphPad Software Prism (GraphPad Software, San Diego, CA, USA).

EOM characterization by immunofluorescence

EOM were checked for cytokeratin (CK) expression, an epithelial marker by immunofluorescence assay. Cells were washed twice in rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05 % Tween-20 (Merck Millipore, Darmstadt, Germany)), fixed with 4 % paraformaldehyde (USB Corporation, Cleveland, OH, USA) and permeabilized with 0.1 % Triton X-100 (Promega, Fitchburg, MA, USA). After blocking with 5 % of bovine serum albumin (Life Technologies), EOM were incubated with anti-Cytokeratin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:50 in PBS, overnight at 4 °C. After three times of washing with rinse buffer, secondary antibody anti-IgG mouse FITC (Santa Cruz Biotechnology) diluted 1:200 in PBS was added and incubated

Fig. 1 Schematic representation of EOM method obtaining by scraping oral mucosa

for 1 h at room temperature. Slides were mounted using Vectashield solution containing 40,6-diamidino-2-phenylindol (DAPI; Vector Laboratories, Burlingame, CA, USA) and analyzed under fluorescence microscopy (Zeiss, Oberkochen, Germany).

EOM characterization by Papanicolaou staining

EOM were collected as described and a direct smear was done on a silanized slide. Cells were fixed using 95 % of alcohol for 30 min at room temperature and subjected to immersion in alcohol 70 %, 50 % and distilled water for fixation. EOM were stained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for 8 min and then washed with water. Cells were subjected to dehydration through ten dips each of the following alcohol concentrations 50, 70, 80 and 95 % and then Orange G (Sigma-Aldrich) was added for 90 s, followed by ten immersions in 95 % ethanol. Next, cells were stained with eosin-green light (Sigma-Aldrich) for 90 s and washed ten times using 95 % alcohol. Cells were dehydrated by absolute ethanol, followed by twenty dips in xylol. Microscope slides were mounted using Permount (Fischer Scientific, Pittsburgh, PA, USA) and analyzed by light microscopy (Zeiss).

EOM culture conditions

In order to establish EOM culture, five different conditions were performed as described on Table 1. (1) Culturing EOM on top of murine fibroblasts feeder layer (NIH/3T3, ATCC, Manassas, VA, USA), previously inactivated using Mitomycin C, according to the manufacturer's suggestions (Sigma-Aldrich); (2) Culturing EOM without feeder layer; (3) Culturing EOM on Matrigel extracellular matrix, prepared according to the manufacturer's suggestions (100 μ L/mL—BD Bioscience, Franklin Lakes, NJ, USA); (4) Culturing EOM on Matrigel with conditioned medium, that was primarily prepared by culturing NIH/3T3 on fresh medium during 24 h; (5) Culturing EOM on laminin extracellular matrix prepared according to the manufacturer's suggestions (BD Biosciences).

For all five conditions, EOM were cultured using DMEM-High glucose (LGC Biotechnology, Cotia, SP, Brazil) supplemented with 15 % of calf serum (Thermo Scientific, Waltham, MA, USA), 1 % antibiotic–antimycotic solution (Sigma-Aldrich) and maintained in an humid atmosphere with temperature at 37 °C and of 5 % CO₂. Cells were split weekly or when necessary using a standard enzymatic method; the cells were

Table 1 EOM culturing conditions and viability

	Conditions	Time of EOM viability in culture (days)
EOM	Feeder layer (NIH3T3)	45 or more
EOM	No feeder layer	6
EOM	Matrigel	6
EOM	Matrigel + conditioned media	8
EOM	Laminin	12

washed with PBS, incubated with 1 mL of trypsin (Invitrogen) by 5 min at 37 °C, washed again and centrifuged for 5 min at 800g. Last step, cells were resuspended in medium and maintained in the same conditions as described before.

EOM cryopreservation

Cryoviability process was analyzed by freezing EOM cultured using the first condition. Cryopreservation solution was composed of 30 % of DMEM-High Glucose (LGC Biotechnology), 60 % of FBS (Life Technologies) and 10 % of DMSO (Life Technologies) and cells were gradually submitted to low temperature conditions. After 30 days in liquid nitrogen, cells were thawed and counted using trypan blue (Life Technologies) staining to check viability.

EOM proliferation rate analysis by MTT assay

For MTT assay, 1×10^3 cells were plated in triplicate into 96 well/plate under two cell culture conditions described before (Fernandes et al. 2014). First, EOM were cultured on top of feeder layer and second, without feeder and matrigel or laminin matrix. Starting after 24 h of cell plating, 5 mg/mL of MTT (Thiazolyl Blue Tetrazolium, Sigma-Aldrich) was added every 48 h followed by 3 h of incubation at 37 °C. After incubation, MTT product was sedimented by centrifuge procedure (800g, 10 min) and solubilized using 50 µL/well of DMSO (Life Technologies). The product originated, formazan, was measured in a spectrophotometer (I-Quant Plate Reader, BioTek Instruments Inc., Winooski, VT, USA) at 550 nm as described by Fernandes et al. (2014). Data were plotted and analyzed using GraphPad Software Prism (GraphPad Software).

EOM culture characterization by transmission electron microscopy

Cultured cells were scraped from the dish by cell scraper and centrifuged for 5 min at 800g for pellet formation. Pellet was fixed using a 3 % glutaraldehyde (Merck Millipore, Billerica, MA, USA) solution, washed in PBS and incubated for 16 h at 4 °C. The next day, post-fixation was performed with 1 % of osmium tetroxide for one hour at room temperature. Dehydration was carried out gradually in a series of ethanol 50, 70, 90 and 100 %. In addition, resin was mixed with propylene oxide in 1:1 ratio and left in a rotary mixer for 4 h. Neat resin was added for 12–18 h, followed by paraffin embedding. Ultrathin sections were performed using an ultra-microtome. Cells were visualized by transmission electron microscope (FEI Morgagni 268d—FEI, Hillsboro, OR, USA).

Molecular characterization of EOM by RT-PCR

RNA was extracted from EOM using TRIzol reagent (Life Technologies) and cDNAs were synthesized using Revert Aid M-MuLV Reverse Transcriptase (Fermentas Life Science, Amherst, NY, USA) according to the manufacturer's instructions. Primers used for molecular characterization were CK13, CK12, connexin-43, Oct3/4, Nanog, Vimentin and GAPDH, a housekeeping gene (primers sequences are presented in Table 2). PCR conditions were denaturation at 94 °C for 4 min followed by 40 cycles at 94 °C for 1 min, annealing for 1 min at 61 and 72 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were separated by 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transilluminator.

Statistical analyzes

All experiments were done in triplicate and analyzed by GraphPad Software Prism (GraphPad Software).

Table 2 Primers used in this study

Gene/size (bp)	Sense	Anti-sense
Oct3/4 (120)	5'GACAGGGGGAGGGGAGGAGCTAGG3'	5'CTTCCCTCCAACCAGTTGCCCCAAAC3'
Nanog (120)	5'CCTATGCCTGTGATTTGTGG3'	5'CTGGGACCTTGTCTTCTCTTT3'
Vimentin (204)	5'AAGCAGGAGTCCACTGAGTACC3'	5'GAAGGTGACGAGCCATTTC3'
CK13 (289)	5'GATCCAGGGACTCATCAGCA3'	5'AAGGCCTACGGACATCAGAA3'
CK12 (150)	5' ACATGAAGAAGAACCACGAGGATG3'	5'TCTGCTCAGCGATGGTTTCA3'
Con43 (145)	5'CTTCTTGCTGATCCAGTGGTA3'	5'ACCAAGGACACCACCAGCA3'
GAPDH (463)	5'ACCACAGTCCATGCCATCAC3'	5'TCCACCACCCTGTTGCTGTA3'

Results

EOM sample viability, characterization and morphology

As in this work, we collected EOM by scraping oral mucosa, usually considered superficial dead cells. Before proceeding cell culture protocols, we checked cell viability score using trypan blue staining. Unlike typically expected result, collected samples contained 45 % of live cells beside of 55 % of dead cells (Fig. 2a). Cellular phenotype was rapidly characterized by immunofluorescence assay, being positive for cytokeratin-pan (Fig. 2b). Epithelial tissue usually presents three types of cell population, like parabasal, intermediate and superficial cells (Fig. 2c), but our Papanicolaou staining evidenced two population of cell, intermediate (stained in bluish-green) and superficial cells (stained in orange-pink) (Fig. 2d).

EOM culturing

In the first condition, EOM attached right after seeding onto feeder layer at day 0 and survived until day 45, the last day of experiment (Fig. 3a).

In the second condition, EOM also attached immediately after seeding (day 0). Cell proliferation was observed until day 2, but despite of this growth, EOM died 6 days after cell culture (Fig. 3b). The same results were observed for the third condition (Fig. 3c).

For the fourth condition, results were very similar to condition two and three, but with a higher proliferation rate. Even though, EOM just survived for 8 days (Fig. 3d).

The last condition also favored cell attachment. However, despite of less proliferation rate on day 2, cells were able to survive until day 12 (Fig. 3e).

EOM cryopreservation

Just the first condition was tested. EOM cells were viable after 30 days of freezing and apparently kept their ability to proliferate and preserved their cell morphology (Fig. 4a, white arrows).

EOM proliferation rate analysis by MTT assay

MTT assay was carried out by day 9. First condition revealed a better growth rate of EOM, with cell death in the beginning and an exponential growth rate after 5 days (Fig. 4b). In the second condition tested, a slight cell growth was observed on the first 5 days, giving rise to a sudden and constant cell death (Fig. 4c).

Transmission electron microscopy

Analysis by transmission electron microscopy revealed that cultured EOM presented juxtaposed cells, with large nucleus/cytoplasm (N/CT) ratio (Fig. 4d). In addition, some mitochondria (MT) were observed, denoting cell activity. Secretory granules (SG), characteristic for epithelial cells were observed, as well as rough endoplasmic reticulum (RER) (Fig. 4d).

EOM molecular characterization

Cultured EOM expressed CK13 and Vimentin and did not express CK12, a corneal marker, Oct3/4 and Nanog, known as pluripotent markers. For Connexin 43, a gap junction marker, expression was detected just for the first condition culture, but unfortunately, based on this result, we cannot attribute this expression to EOM (Fig. 5).

Fig. 2 EOM sample viability and morphology. **a** Cell viability after EOM obtaining by scraping oral mucosa. Collected samples contained 45 % of live cells beside of 55 % of dead cells. **b** Immunofluorescence assay revealed EOM positive for cytokeratin-pan, in green (FITC) and nucleus staining with

DAPI, in blue. **c** Representative scheme of EOM differentiation/development. **d** Photomicrograph of EOM cells by Papanicolaou stain evidenced two populations of cells, intermediate (stained in bluish-green) and superficial (stained in orange-pink). Scale bar **b** 20×, **d** 100 µm. (Color figure online)

Discussion

Collecting samples for biological investigation is something that will always be necessary, but non-invasive and non-painful methods should be explored and applied. Scraping epithelial cells from oral mucosa is considered a non-invasive and non-painful method. Here we presented, for the first time, a protocol for culturing EOM cells after a non-invasive method of collection, avoiding pain and discomfort, which represent a useful tool for patients in general, opening opportunities for new applications. Surprisingly, after scraping, almost half of the cells collected were still alive and viable to culture (Janardhanam et al. 2012).

Phenotypical analyses revealed epithelial cell markers and cell morphology corresponding to epithelial mucosa oral tissue. Usually, epithelial mucosal tissue is constituted of three types of cells, varying according to the cell cycle (Fig. 2c), as was observed for genital mucosa (Castro et al. 2009). However, our results

showed only two types of cells, the more superficial ones, probably due to the depth of the localization of parabasal cells in oral mucosa.

The choice to establish the primary culture conditions of EOM was based on the demand for epithelial cell culture (Thomson et al. 1998; Navarro-Alvarez et al. 2008). The feeder layer condition to support epithelial cells was the one which presented the best results and exhibited exponential proliferation ability and good cell viability for weeks, as observed by the MTT assay. This behavior is probably due to the similarity of the first condition with the epithelial tissue architecture found in vivo, whose cells from stratified squamous epithelium are deposited on basal layer and connective tissue, which is rich in fibroblasts (Kullaa et al. 2014). Similar results, with the necessity of feeder as a support layer for EOM culturing, were related for biopsied human oral epithelial cells, where cells were cultured on NIH/3T3 feeder layer, on the amniotic membrane (AM) or a mixture of these two and the best

Fig. 3 EOM culturing. **a** EOM cultivated on support layer (NIH/3T3) on the collection day (day 0), after 2 days and on day 45. **b** EOM growing without support layer, on day 0, day 2 and day 6. **c** EOM cells growing on Matrigel on the collection day,

day 2 and day 6. **d** EOM growing on matrigel with medium conditioned on NHI3T3 cells on day 0, day 2 and day 8. **e** EOM growing on laminin on day 0, day 2 and day 12. Scale bar **a–e** 20×

result was obtained when the cells were cultured on NIH/3T3 feeder layer (Hashemi et al. 2009). Despite of fibroblasts production and releasing of important

factors, vital for cell health (Scafetta et al. 2015), just the presence of factors seems to be insufficient to guarantee cell maintenance and growth, as

Fig. 4 EOM characterization. **a** EOM showed viability after cryopreservation process. NIH/3T3 are indicated by *black arrows*. EOM are indicated by *white arrows*. **b** Proliferation rate of EOM growing on feeder layer (NIH/3T3) and feeder

layer alone, by MTT assay. **c** Proliferation rate of EOM growing without feeder layer by MTT assay. **d** Transmission electron microscopy of EOM growing on feeder layer (NIH/3T3). Scale bar **a** 20 $\times$, **d** 10 and 2 μ m

demonstrated by the fourth condition, where cells lived no more than 1 week, supporting the necessity of feeder layer contact for cell survival. This kind of behavior can also be observed for other cells, like pluripotent stem cells (PSC). For PSC culture, the presence of feeder cells on the first days of culture seems to be essential (Takahashi et al. 2007), since just after some weeks it is possible to keep the cells feeder-free, but using media supplemented with exogenous factors (Beltrão-Braga et al. 2011). Finally, our data revealed similar results for all cultures conditions without feeder layer contact, leading to cell death approximately after 1 week in culture.

Cultured EOM analyses by ultrastructure characterization revealed all the relevant characteristics founded for epithelial cells, like large amount of mitochondria, secretory granules associated with maintenance of humidity and viscosity of the environment and juxtaposed cell architecture (Ye et al. 2011). These findings suggest interesting features to note, considering that also in culture, EOM preserved the features founded in vivo. This is very important,

especially for disease modeling, where it is interesting to find a model that mimics the conditions found in their natural environment.

In addition, EOM phenotype analyses also confirmed the cell nature, since epithelial markers were expressed in the cultured cells (Coulombe and Omary 2002). On the other hand, EOM did not express corneal marker, such as CK12, pluripotent stem cells markers, Oct3/4 and Nanog (Jung et al. 2011; Khatri and Saif 2011). This result confirms the superficiality of the collection method used here, since stem cells are more deeply localized in the oral mucosa.

Our data confirm that the EOM cells collected by non-invasive and superficial method can be cultured using a support cell layer for maintenance and growth in vitro, allowing its further use for many protocols, including material for diagnosis of genetic diseases. The protocol described here provided an easy, pain-free, discomfort-free or stress less way to EOM obtaining, especially important for collecting samples from children or patients with special requirements.

Fig. 5 EOM gene expression by RT-PCR. *Line 1:* EOM day 0—human epithelial cells from oral mucosa subjected to RNA extraction immediately after obtaining; *Line 2:* EOM day 2—RNA extraction from EOM in culture 2 days after obtaining; *Line 3:* NIH/3T3—Murine fibroblast cells used as a support layer; *Line 4:* EOM cultivated on murine feeder layer (NIH/3T3); *Line 5:* Negative control used in all reactions; *Line 6:* Molecular weight marker (M)—Gene Ruler 1 Kb DNA Ladder

Conclusion

In this work we demonstrated that EOM can be easily collected by oral scraping being a painless and non-invasive method and the cells obtained are viable and able to proliferate in a support feeder cell layer.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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