REVIEW ARTICLE

Development of Oral Mucosal Organoid for Oral Disease Modeling and Management: A Review

Ambar Kusuma Astuti[1,2], Jeanne Adiwinata Pawitan[3,4,5]

[1] Doctoral Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jl. Salemba 6, Jakarta Pusat, Jakarta 10430, Indonesia
[2] Oral Medicine Department, Faculty of Dentistry Universitas Indonesia, Jl. Salemba 4, Jakarta Pusat, Jakarta 10430, Indonesia
[3] Department of Histology, Faculty of Medicine Universitas Indonesia, Jl. Salemba 6, Jakarta Pusat, Jakarta 10430, Indonesia
[4] Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital, Faculty of Medicine, Universitas Indonesia, Jl. Pangeran Diponegoro, Jakarta Pusat, Jakarta 10430, Indonesia
[5] Stem Cell and Tissue Engineering Research Center, Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia, Jl. Salemba 6, Jakarta Pusat, Jakarta 10430, Indonesia

ABSTRACT

Monolayer cultures and animal models face limitations in accurately evaluating cellular oral mucosal behavior. Oral disease modeling using tissue-engineered oral mucosa replicates the complexity of human tissue, offering improved opportunities for studying molecular mechanism. Therefore, it is important to highlight recent developments in oral mucosal organoids in wound healing, carcinogenesis, and other investigation models. This review article performed a literature search and carefully selected and summarized articles from PubMed and Scopus data base to get information on oral mucosal organoids in various fields. The keywords were: "organotypic" OR "organoid" OR "3D" AND "oral mucosa" AND "model". The search yielded 31 articles on oral mucosal organoids, which addressed various cell sources, scaffolds and techniques that can be applied to develop organoids to elucidate a certain mechanism of action. Enhancement in extracellular matrix properties and incorporation of immune and/or other oral cells could provide better understanding of oral mucosa molecular responses.
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Corresponding Author:
Jeanne Adiwinata Pawitan, PhD
Email: jeanneadiwip@gmail.com; jeanne.adiwinata@ui.ac.id
Tel: +62-213146129, +62-81295350097 (mobile)

INTRODUCTION

Three-dimensional (3D) tissue or organotypic models or organoid recently become an attractive approach to evaluate organ behavior, cell interactions, drug development, stem cell study, disease modeling, and many other scientific purposes. The 3D organoid culture can resolve the limitations of in vitro 2D monolayer culture and in vivo experiments. Compared to 2D culture, 3D models more closely mimic the structure and function of natural tissues, enabling better simulation of in vivo conditions (1). Moreover, some animal studies are not suitable for pre-clinical investigation due to the different physical properties, physiopathology and cellular receptors between animal and human (1,2).

Investigating host and microbiome interactions in oral environment direly needs an oral mucosal model with relevant physiologic qualities. The development of relevant models would ease the discovery and validation of novel pharmacologic therapy and antimicrobial selection tools (3). Such model also provides mechanistic insights of commensal and pathogenic microorganism interaction, in healthy and immunosuppressive state (e.g., chemotherapy) (3–5).

Architecture of a mucosal organoid that consists of cells with a certain arrangement and density, as well as extracellular matrix (ECM), and vascular supply will affect exposure, especially for tumor cells. Using spheroid or multilayered 3D models of oral mucosal tumor organoids help elucidate tumor pharmacokinetic profiles (6). Additionally, the tumor organoid can mimic the clinic-pathological characteristics of oral tumors such as angiogenesis properties. Thus, the effect of chemotherapeutic agent can be evaluated more accurately (7).

A safety profile and toxicology studies are very challenging to conduct in a clinical setting. Evaluation of the effect of some chemicals and drugs in oral epithelial cells still needs more comprehensive evidence (8). The

biocompatibility and oral mucosal interaction of dental material should also be assessed prior to clinical use (9). The 3D mucosal tissue models are clinically relevant, easy to be developed, and might improve cellular assessment, as well as can be conducted in either short or medium period (8, 9). Nevertheless, there are some limitations in the current organotypic models, for instance: certain structural dissimilarity, short cultivation time and contraction of the ECM (1,10,11).

This narrative review aims to describe recent advancements in the development and application of oral mucosal organoid. Oral mucosal organoid was expected to enhance the understanding of biomolecular mechanisms underlying oral diseases and to aid preclinical therapeutic studies. Therefore, this review addressed oral mucosal organoid for disease modeling, host-agent interaction model, toxicology modeling, drug development and carcinogenesis study, along with dental material biocompatibility assessment and improvement of biological properties of the organotypic model.

METHODS

Articles were selected from Pubmed and Scopus database at December 29th, 2024, and were restricted to published articles between 2017-2024. Articles from Pubmed were searched with the Boolean logic search terms as keywords ("organotypic" OR "organoid" OR "3D") AND ("oral mucosa" AND "model") NOT ("printing" NOT "review" NOT "intestinal" NOT "airway"), while from Scopus, the keywords were "organotypic" OR "organoid" OR "3D" AND "oral mucosa" AND "model". The articles were selected based on their title, then the abstract and full text. These articles were screened for duplication using a reference manager (Mendeley). The inclusion criteria were original articles in English, which exclusively reported organoid of human oral mucosa, and free full text was available. The exclusion criteria were articles reporting mucosal organoid other than oral mucosa (e.g., intestinal and airway), studies intended for animal, 3D radiology imaging or computer study, 3D printing and stem cells study without using organotypic model.

RESULT AND DISCUSSIONS

There were 58 and 200 articles in English from Pubmed and Scopus, respectively. After duplications were removed, there were 228 articles that were analyzed according to inclusion and exclusion criteria. Finally, 31 articles were used in this study (Flow chart of search strategies can be seen in Figure 1).

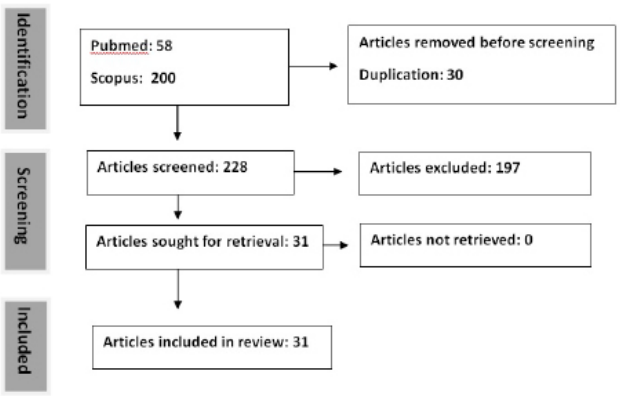

Figure 1: Flow chart of search strategies

Oral mucosal organoid

An organotypic human oral mucosa model or oral mucosal organoid or reconstituted oral mucosa tissue (ROMT) is a model that is developed to form functional tissue in vitro by providing matrix structure with viable cells within suitable environments, which are similar to in vivo condition. The well-regulated microenvironment can stimulate cell growth, organization, differentiation, formation of natural ECM and generation of a functional tissue (4).

The oral mucosal organoid provides some advantages over 2D monolayer and animal model. The 2D monolayer cannot fully represent the structural and functional characteristics of natural tissue.(1) Moreover, it is deficient of specific cell signals and responses and cannot completely describe pharmacokinetic profiles of observed condition (1,6).

Animal models have differences in pathophysiology, physical properties, and cellular receptors compared to humans, which causes difficulty in analyzing molecular mechanism of a disease (1,2) Thus, there are some limitations in extrapolation of animal studies to clinical situations such as in pharmacokinetics and drug metabolism. Organoid or 3D model can provide more insightful pharmacokinetic profiles, even in a personalized medicine approach (6). Organoid can predict the actual response of patients, supporting translational utilization of this in vitro model (12).

The essential components of various oral mucosal organoid models are similar, but the cell source, scaffold type and the methodology of epithelium and connective tissue reconstitution can be diverse (4,11). Currently there is no consensus of the kind of cells that are used to engineer the epithelial and connective layer (4). The cell source of keratinocyte and fibroblast can be derived from cell lines, primary cells, and immortalized human primary cells with various levels of epithelial stratification and differentiation. The scaffold can be made from natural (e.g., collagen, gelatin, chitosan, hyaluronan or hyaluronic acid, glycosaminoglycan, and acellular human cadaveric dermis) or synthetic materials (e.g., polycaprolactone, and polylactide) (1,11). The utilization of stable, manageable and biologically active scaffold that support cell attachment and organization similar to natural tissue will produce ideal model of

oral mucosal organoid (11). The various cells and scaffolds that were used in developing organoids, their application, advantages and disadvantages can be seen in Table I.

Disease modeling

Currently, clinicians and scientists are challenged to provide better wound management that can lower infection risk and enhance scar appearance. Damaged

Table I: Mucosal Organoid Studies' Characteristics

Reference	Cell type	Scaffold	Treatment	Application	Advantage (A)-disadvantage (D)
Koskinen Holm C, et al. (2022)	hTERT immortalized human gingival keratinocytes, fibroblasts	Collagen -genipin or cytochalasin-D cross-linked	NT	3D GTE	A: prevent matrix contraction D: no rete pegs
Rodrigues Neves C, et al. (2019)	Gingival keratinocytes, fibroblast	Collagen	Culture: 10 d, saliva treatment: 72 h	Mucosal wound closure model	D: No immune cells, microbiome, and vascularization
Buskermolen JK, et al. (2018)	hTERT-immortalized human gingiva keratinocyte and fibroblast cell line	Collagen hydrogel	Salivary microbiome (commensal, gingivitis, cariogenic: > 70 OTU) exposure: 24 h	Human organotypic gingiva - host-microbiome interaction model	D: No immune cells, saliva, and vascularization
de Carvalho Dias K, et al. (2018)	Gingival fibroblast, mucosal keratinocytes	Collagen	fungal-bacterial mixed biofilm exposure	Organotypic gingiva - host-microbiome interaction model	D: No immune cells, saliva, and vascularization
Sobue T, et al. (2019a)	3T3 ATCC cell, SCC15 cell	Collagen	5-FU and commensal microbiome treatment	Mucositis organotypic mucosa - host-microbiome interaction model	D: No immune cells, saliva, and vascularization
Joseph JF, et al. (2020)	Fibroblast, SSC-25 cell	Collagen	Docetaxel exposure	TOM models – anti cancer pharmacokinetic assay model	D: No immune cells, saliva, and vascularization
Choi S-Y, et al. (2021)	HNSCC cell line, CAF	HNSCC 3D Spheroid matrix	Chemotherapy exposure	TOM model-cancer drug study	A: presence of vascularization
Shaikh ZN, et al. (2019)	Fibroblast, oral normal and cancerous keratinocytes	Collagen gel	Flavourless ECi liquid treatment: short, medium period	3D oral mucosa – toxicology model	Nicotine concentration was not measured
Binaljadm T, et al. (2019)	Oral fibroblast, buccal carcinoma keratinocytes	Collagen type 1	Various GIC exposure	3D oral mucosal - dental material biocompatibility model model	D: No immune cells, saliva, and vascularization
Gronbach L, et al. (2020)	Human oral fibroblast, human oral keratinocytes or carcinoma cell lines	Hyalograft 3D disk, thrombin, fibrinogen	NT	TOM model – various purposes	A: better epithelial adhesion, no contraction, can be cultured 7 weeks
Sakulpaptong W, et al. (2022)	Primary human gingival fibroblasts and keratinocytes	EC, DD, Gel, Gel-R	machined titanium, SLA titanium, TiN, PEEK abutment	Human organotypic gingiva - dental material biocompatibility model	A: EC- reduced contraction, DD no contraction D: No rete ridges, distribution of fibroblast was scattered
Driehuis E, et al. (2020)	Normal human oral keratinocytes	Cultrex GF reduced BME type 2	LV treatments at various time points	MTX induced mucositis oral mucosa organoid model	D: only epithelium, no lamina propria
Wagner T, et al. (2019)	Gingival keratinocytes, fibroblast	Collagen type 1	siRNA transfection of keratinocytes	CNFN silenced gingival mucosa model	A: insight on CNFN function
Xu H, et al. (2017)	3T3 ATCC cell, OKF6/ TERT2 cells	Collagen type 1	*C. albicans, S. oralis* exposure -16 h to form mucosal biofilm	3D oral mucosa - host-microbiome interaction model	D: No immune cells, saliva, and vascularization
Krishnamoorthy AL, et al. (2020)	RHOE		*C. albicans, E. faecalis* exposure 18 h, 48 h - biofilm	Oral epithelial - host-microbiome interaction model	D: only epithelium, no lamina propria
Shang L, et al. (2019)	hTERT-immortalized human gingiva keratinocyte and fibroblast cell line	Collagen	Salivary microbiome (commensal, gingivitis, cariogenic biofilms) exposure: 24 h	RHG - host-microbiome interaction model	D: No rete ridges, immune cells, saliva, and vascularization
Shang L, et al. (2018)	hTERT-immortalized human gingiva keratinocyte and fibroblast cell line	Collagen hydrogel	Salivary commensal microbiome biofilm exposure: 1-2-4-7d	RHG -host-microbiome interaction model	D: No rete ridges, immune cells, saliva, and vascularization
Gould SJ, et al. (2023)	HGF, HaCaT cells	Collagen type 1	oral cavity *C. albicans SC5314* and *S. aureus* strain exposure – 24h	Organotypic gingiva - host-microbiome interaction model	D: No rete ridges, immune cells, saliva, and vascularization
Sobue T, et al. (2019b)	3T3 ATCC cell, SCC15 cell	Collagen type 1	5-FU exposure 16 h, *C albicans* exposure 18 h	5-FU-induced mucositis oral mucosa organoid – opportunistic infection model	D: No rete ridges, immune cells, saliva, and vascularization
Battaglia A, et al. (2017)	EpiOral™ human mucosal cells in 3D mucosal model		Various nicotine preparation exposure	3D – oral mucosa-toxicology model	D: only epithelium, no lamina propria
Konstantinova V, et al. (2017)	Normal buccal mucosa fibroblasts and keratinocytes	Collagen	nano-TiO_2 exposure: 20 min, 24 h	Organotypic buccal mucosa toxicology -model	D: no rete ridges, and prolonged time to test malignant transformation was not tested

CONTINUE..

Table I: Mucosal Organoid Studies' Characteristics (Continued...)

Reference	Cell type	Scaffold	Treatment	Application	Advantage (A)-disadvantage (D)
Leano SM, et al. (2024)	HGF, HaCat cells	Agarose 1%	Oral hygiene product exposure	3D HGF-keratinocyte spheroid – Toxicology model	D: spheroid did not have natural tissue structure
Dommisch H, et al. (2021)	OKG4 cells and gingival fibroblast	Collagen type 1	CMS nanocarrier exposure	Organotypic gingival mucosa model-cytotoxicity assay	D: No rete ridges, immune cells, saliva, and vascularization
Pimentel BNADS, et al (2022)	FGH, NOK-si, and THP-1 cell line	Collagen	α-$AgVO_3$, α-Ag_2WO_4, α-Ag_2MoO_4 exposure, *C albicans* exposure 24 h	3D oral mucosa model – antifungal cytotoxicity assay	A: presence of immune system cells D: No rete ridges, saliva, and vascularization
Hoque Apu E, et al. (2018)	CAF, oral carcinoma cell lines, inflammatory and endothelial cells, myofibroblast	Myogel, myoma organotypic 3D disc, Matrigel®, collagen type 1	Dsg3 mutation	TOM models-carcinogenesis study	A: Myoma organotypic 3D disc had a complete TME, and support vascular structure
Roffel S, et al. (2019)	OKG4 and gingival fibroblast cell line	Collagen hydrogel	titanium alloy abutment – 10 days or more	RHG organotypic - dental material biocompatibility model	Absence of hard tissue or bone
Barker E, et al. (2020)	OKF6/TERT2 cells. human gingival fibroblast, THP-1 cell line	Collagen type 1	TiZr-SLA, TiZr-M, ZrO2-M, PEEK-M abutment	Gingiva organotypic - dental material biocompatibility model	A: presence of immune system cells
Perduns R, et al. (2021)	OKF6/TERT2 cells. human gingival fibroblast	Collagen	CQ exposure	3D insert co-culture oral mucosa - dental material biocompatibility model	D: OKF6/ TERT2 cells were separated from fibroblast construct by insert membrane
Alamo L, et al. (2024)	NOK-Si keratinocytes, human gingival fibroblast	Collagen –	3DP resin exposure -1-3-7 d	3D Transwell co-culture oral mucosa – dental material biocompatibility model	D: NOK-Si cells were separated from fibroblast construct by Transwell membrane
Blanco-Elices C, et al. (2021)	Oral mucosal fibroblasts, dMSCs	Fibrin- agarose VII biomaterial	tranexamic acid and 1% $CaCl_2$ treatment	Organotypic oral mucosa model	A: promotion of vascularization
Ollington B, et al. (2021)	Normal oral fibroblast, hTERT-immortalized oral keratinocytes, MDMs	Collagen type 1	*E. coli* LPS stimulation	Organotypic oral mucosa model	A: presence of immune cells, more natural in vitro host-pathogen interaction

hTERT= human telomerase reverse transcriptase, NT= not test, 3D GTE= 3 dimension gingival tissue equivalent, d= days, h= hours, OTU= operational taxonomic unit, 3T3 ATCC= fibroblast cell line, SCC15 cell= cancer derived keratinocyte cell line, 5-FU= 5-fluoro uracil, SSC-25 cell= tongue cancer cell line, TOM= Tumor oral mucosa, HNSCC= head and neck squamous cell carcinoma cell line, CAF= carcinoma-associated fibroblast, 3D= 3 dimension, ECi= electronic cigarette, GIC= glass ionomer cement, EC= electrospun collagen, DD= decellularized dermis, Gel= type I collagen gels Gel-R= released type I collagen gels, SLA= sandblasted-acid etched, TiN= nitride-coated titanium, PEEK= polyether ether ketone, GF= growth factor, BME= basement membrane extract, LV= leucovorin, MTX= methotrexate, CNFN= cornifelin, OKF6/TERT2 cells= keratinocyte cell line, RHOE= reconstructed human oral epithelium from MatTek Corporation, Ashland Massachusetts, USA, RHG= reconstituted human gingiva, HGF= primary human gingival fibroblasts, HaCaT cells= keratinocyte cell line, EpiOral™= commercial oral mucosa model from Mattek life sciences (member of BICO group, Massachusetts, USA), TiO_2= Titanium dioxide, OKG4 cells= gingival keratinocyte cell line, CMS= core-multi-shell, FGH= fibroblast cell line, NOK-si= keratinocyte cell line, THP-1= monocytic cell line, α-$AgVO_3$=microcrystals silver vanadate, α-Ag_2WO_4=s ilver tungstate, β-Ag_2MoO_4= silver molybdate, Dsg3= Desmoglein 3, TME= tumor microenvironment, TiZr-SLA= titanium–zirconium alloy modified SLA, TiZr-M= machined TiZr, ZrO2-M= machined Zirconia, PEEK-M= machined PEEK, CQ= camphorquinone, 3DP= 3D printed, dMSCs= differentiated MSCs, $CaCl_2$= calcium chloride, MDMs= monocyte-derived macrophages, LPS= lipopolysaccharide.

oral mucosa heals faster with less scar development. It might be related to the presence of saliva that helps regulate wound healing along with the oral mucosal cells itself. Therefore, Rodrigues Neves et al (2018) (2) generated an in vitro oral mucosa model to evaluate the potential therapeutic effects of saliva in improving wound healing in skin and oral tissues. The study used freeze blisters in 3D organotypic gingiva model that resembled a blistering wound. The organotypic 3D model of gingiva was constructed from cells that were obtained from a healthy donor dental implant surgery surgical waste. Keratinocytes were cultured and isolated from gingival waste. Subsequently, the human gingival wound model was constructed using a fibroblast-populated hydrogel method. Firstly, fibroblasts were mixed with collagen solution and incubated overnight, later on, keratinocytes were seeded. The reconstructed gingival mucosa was cultured for 10 days at the air liquid interface, to produce oral mucosal organoid (Fig. 2). Stratum corneum in gingival model had visible nuclei (parakeratinized epithelium). A full thickness freeze blister wounds were made following 10 days of air exposed culture. Undiluted pooled saliva was given and after 24 hours the supernatant was collected for ELISA analysis of inflammatory cytokines. Histopathological analysis was conducted to evaluate the wound re-epithelialization (2).

The organotypic gingival model showed total wound closure 72 hours after saliva application at the blister wound, but the wound margin could not adequately identified. Another limitation of this study highlighted the importance of inflammatory status, cell infiltration, microbiome, and vascular effects. The variation of donor saliva (age of donor, saliva composition that was related to trauma) should be extensively investigated before proceeding into phase 1 study (2).

Human oral epithelium forms a barrier that provides protection from the external environment, and has gene expressions that are involved in epidermal differentiation and intercellular connections. One of the intercellular adhesive junctions is called desmosome that can resist physical and chemical disturbance, and modification of desmosomes produces cornifelin (CNFN) (13).

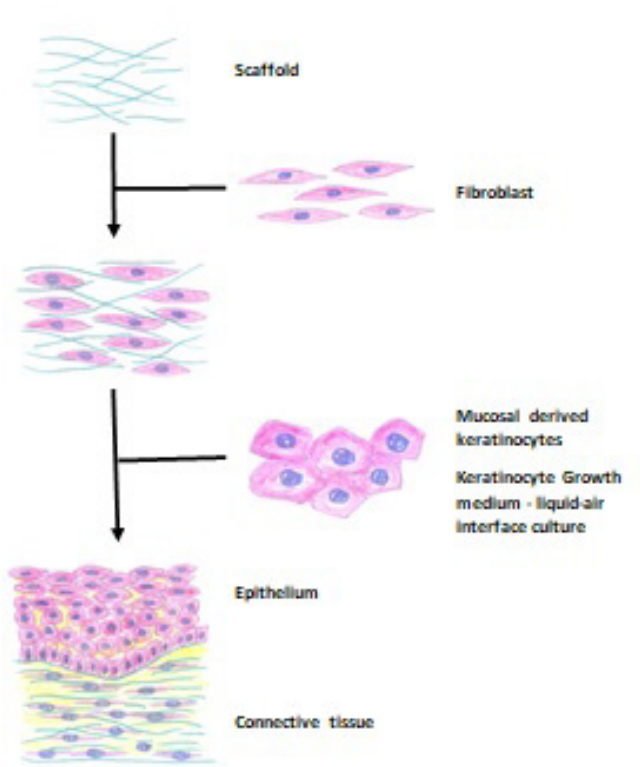

Figure 2: Oral mucosal organoid tissue engineering

Wagner et al., (2019) (13) constructed an organotypic oral mucosal model to help demonstrate that desmosomes was an essential molecule in the superficial layers of the oral mucosa. The presence of CNFN in the epithelial layers was observed with immunofluorescence staining. These models successfully demonstrated that the deficiency of CNFN as a component of cell adhesion structure in the oral epithelium led to extensive defect in the barrier integrity. There was a substantial loss of cell-to-cell contacts and acantholysis in suprabasal keratinocyte of oral mucosa. In conclusion, CNFN is a component of cell-cell adhesion system in oral mucosa (13).

Host-agent interaction model

Monolayer cultures limit the investigation of bacterial invasion due to their weaker barrier function compared to differentiated multilayered epithelium. The secretion of in vitro inflammatory cytokine is also synergically affected by the various cell types within a tissue (e.g., keratinocytes and fibroblast) (3). A reliable observation of host and microbiome in human oral mucosa or gingiva can be achieved by exposing the organotypic model with different types of oral biofilm or certain pathogens within a certain period (3-5,14–18). In organotypic model, growth of microorganisms, their penetration ability, effect of commensal to epithelial barrier integrity and the degree of damage can be investigated (4,17). Furthermore, the model can elucidate the main histopathological characteristics of certain diseases, the role of oral commensal and pathogenic bacteria and the mechanistic molecular event of the host response against tested microbes (5,16-18).

Multiple species biofilm exposure

A method to observe the interaction of host and complex microbiome, an oral organotypic model with direct contact to multispecies biofilms, which was derived from saliva, was developed. After 24 hours of commensal, gingivitis, and cariogenic salivary microbiome exposure, a dense biofilm was formed and disrupted the epithelial upper layer. The elafin expression and protease activity were increased. Early innate immune response towards oral biofilms showed that there was a release of pro-inflammatory cytokines that were strongly associated with periodontitis. Uniquely, the cytokines' level was higher in commensal than the pathogenic group. This result indicates that the pathogenic bacteria tends to cause tissue invasion before the immune response is activated (3).

Fungal-bacterial biofilm

Initially, *Candida albicans*, *Candida glabrata*, *Staphylococcus aureus* and *Streptococcus oralis* are oral mucosa commensals. Candida species are commonly present in the epithelial tissue of healthy subjects (15). *S. aureus* is normally found in high number on the oral mucosa of the denture wearer (4). *S. oralis* is found abundantly as a commensal in healthy people's oral mucosa surfaces (14). *E. faecalis* has been identified to cause infections, such as endocarditis, bacteremia, and nosocomial infections in immunocompromised host. *E. faecalis* can colonize the epithelial surface, but usually cannot breach intact mucosal layer. Moreover *E. faecalis* are antagonistic to *C. albicans* in abiotic surface (15).

Fungi and bacteria can synergize and generate greater damaging effects. Under immunosuppression, dysbiosis could occur, which promote overgrowth of certain bacterial species (15). In developing mucosal organoid construct of mixed biofilms models, various cell sources and methods can be employed (4,5,14-19). The oral mucosal organoid fungal-bacterial mixed biofilms model provides mechanistic understanding into the interplay between *C. albicans* with certain bacterial species (4, 14-16, 18). The role of mixed fungal-bacterial biofilm can be evaluated by the signaling pathway, colony numbers, pattern of gene expression and other host response (4, 14-16, 18). Chemotherapeutic agents often cause mucositis. Therefore, in chemotherapy model, besides observing the host-agent interaction, the effect of chemotherapeutic agents in modifying the inflammatory response to microorganism can also be investigated (5).

Toxicology modeling

Oral mucosa is significantly more permeable than epidermis, thus making toxicology studies better observed. Smoking cessation methods comprises the use of electronic cigarettes (EC) and nicotine replacement therapy such as Tincture of Benzoin (TOB) (8,20). A widely used biomaterial, TiO2 has a hidden toxicology

risk that has been rarely explored, especially on the oral mucosa (21). In addition, various oral hygiene products may have adverse effects on oral mucosa (22). Therefore, the safety level and cytotoxic effects of those substances to the oral mucosa need to be elucidated (8, 20-22). Multilayer 3D mucosal organotypic models may give a better illustration of the chemical's exposure effect to full thickness oral mucosa over time in a different concentration.

Electronic cigarettes effect to oral mucosa

Shaikh et al. (2019) (8) investigated the effect of flavorless EC liquid on normal and cancerous cells, over short and medium durations. The study utilized monolayer culture and 3D models of human oral mucosa. However, a future study needs to incorporate aerosols form of EC with various nicotine concentration and flavors and do a comparative studies with cigarette smoke (8).

Nicotine safety assessment of Tincture of Benzoin (TOB)

Nicotine replacement therapy (NRT) should have a sustained effect to prevent continuing use of the product and reduce the harmful effects of nicotine. TOB can be combined with nicotine as a mucosal protectant. It can form an adherent bioprotective film for two hours and help nicotine sustained release to the oral mucosa. The ability of TOB to promote transmucosal absorption of nicotine, and at the same time deliver safe and sustained nicotine to the oral mucosa was assessed by a commercial 3D human oral mucosal model, (EpiOralTM) from Mattek life sciences (member of BICO group, Massachusetts, USA), which was exposed to nicotine in saline, nicotine in ethanol 79% and nicotine in TOB. The nicotine in TOB was able to reduce toxic effects in oral mucosa, compared to nicotine in saline or ethanol (20).

Nano-Titanium dioxide (TiO2) exposure to oral mucosa

Titanium dioxide (TiO2) has desirable physical properties and good biocompatibility and has been widely used in biomedical field. However, recurrent exposure of TiO2 may develop toxicological effects. Buccal mucosa is potentially exposed to various sources of TiO2 from food additives, cosmetics, and oral products. The infiltration of nano-TiO2 into oral mucosa can be assessed by exposing nano-TiO2 with various concentrations into a 3D organotypic mucosa model. The nano-TiO2 was added into the center of the model and examined after 20 minutes and 24 hours. After exposure, the penetration of nano-TiO2 into organotypic human epithelium occurred immediately. The particles of nano-TiO2 were mostly located at the upper epithelium and the penetration was dependent on the particle shape, concentration, and time of exposure. The thickness of the epithelium remained the same despite the desquamation after extended exposure periods and increased concentrations of nanoparticles. The repeated exposure of nano-TiO2 potentially induced mucosal alterations related to malignant transformation, if occurred in a prolonged time, but prolonged time to test malignant transformation was not tested. (21)

Drug Development

Experimental approaches that facilitate investigation of drug delivery, pharmacokinetics, biocompatibility, and biosafety of a newly proposed drug are needed. Topical and systemic route for treating oral mucosa disease that is related to inflammatory conditions, infections and oral tumor has different challenges (6,23,24). Cytotoxicity, pharmacokinetics, and bioavailability examinations in 2D culture did not provide enough evidence, since the dynamics of multilayered mucosa and tumor architecture could not be fully reproduced. Cells that grow in 3D organoid models provide improved metabolic function, immunology response, cell proliferation and spatial arrangement (6,24).

Nanocarrier

Topical drug application to oral mucosa has some limitations that are related to the reduced bioavailability and low adherence, which is caused by the application methods or frequency and timing of drug. Oral mucosa inflammatory diseases need an improvement of a safe topical drug with better bioavailability. A core-multishell (CMS) nanocarrier is a new carrier system that can enhance the bioavailability of topical steroid. A 3D gingival equivalent was used to evaluate the efficiency of the ester-based CMS nanocarrier in the penetration ability, its influences on epithelial cell's inflammatory reactions, metabolic activity, and rate of proliferation. The in vitro experiments showed that the newly developed CMS nanocarrier could penetrate the epithelial tissue by transcellular route in a short time. Additionally, it did not interfere with mucosal integrity, and at low concentrations did not trigger significant cell proliferation or cell metabolic activity and did not upregulate inflammatory cytokines expression. These results suggest that the CMS nanocarrier does not cause cell toxicity to oral mucosa and represents a promising new approach for treating oral inflammatory diseases (23).

Microcrystal antifungal drugs

Overuse of antifungal might cause drug resistance. Therefore, development of a new antifungal drug is needed to anticipate such circumstances. The microcrystals silver vanadate (α-AgVO3), silver tungstate (α-Ag2WO4), and silver molybdate (α-Ag2MoO4) were recognized to have antimicrobial activities against C. albicans and S. aureus. Based on in vitro monolayer studies, those microcrystals were not cytotoxic. However, because monolayer culture could not resemble the dynamics of living tissues, there might be a false positive result. To improve the limitations of previous studies, a 3D culture model with three cell lines, i.e. fibroblasts (FGH), keratinocytes (NOK-si), and monocytes (THP-1) was constructed to investigate the biocompatibility, antifungal activity, and the mucosal inflammatory response. An antibiofilm activity towards C. albicans

biofilm was only found in α-Ag2WO4 with similar result to Fluconazole as standard control. Microcrystal has a known activity against C. albicans in a planktonic form, but in biofilm form, the microorganisms are more resilient. Thus, the required dosage of antifungal drugs would be greater in biofilm than in planktonic form. Besides having antifungal activity, α-Ag2WO4 did not affect cell viability, was able to preserve epithelial cell thickness and prevented fungal invasion reaching into the deeper collagen matrix layer. Cell viability using MTT assay showed that α-Ag2WO4 did not show significant difference compared to untreated control (24).

Pharmacokinetic of anticancer

Preclinical evaluation of anticancer doses using an in vitro 2D model has a low translational level into patient's efficacy. The drug exposure is affected by tumor's spatial cell arrangement, cell density, protein of ECM, and blood perfusion. Spheroid or 3D multilayered tumor model gives better opportunities to understand the pharmacokinetic profiles of an anticancer. A proof-of-concept in vitro study investigated the tumor pharmacokinetic in a real time measurement as a personalized medicine approach. Tumor oral mucosa (TOM) models were generated with sample port integration. The docetaxel absorption was measured automatically in a squamous cell carcinoma 3D model using ultra-high-performance liquid-chromatography-mass spectrometry (UHPLC-MS/MS) method. The UHPLC-MS/MS methods in tumor organotypic model exhibited a real time monitoring of pharmacokinetic analysis without sample preparation. It demonstrated the uptake of docetaxel in the inner layers of tumor and the degradation product formation. The method will provide a valuable test to analyze novel indications of approved medication and help develop personalized medicine (6).

Effect of leucovorin in methotrexate toxicity

About 20% of acute lymphoblastic leukemia (ALL) patients with high-dose methotrexate develop oral mucositis, which cause treatment delay and lower their quality of life. Administration of leucovorin (LV) after MTX chemotherapy is recommended to decrease mucositis development, but the optimal dose and timing of LV regimen needs to be optimized. Therefore, optimization of different dosing and timing of LV regimen in high dose-MTX treatment were evaluated in human-derived oral mucosal organoid. Oral mucosa organoid was given a different dosage of MTX and concentrations of LV at various time points. LV administration caused a significant reduction in cell death that was related to MTX treatment in a dose-dependent manner. The degree of LV rescue was related to the schedule of LV administration. The earlier the administration, the higher was the viability of the whole cells. This result suggests that LV pretreatment might lessen the oral mucosal toxicity by MTX, but clinical safety needs to be performed to ascertain whether LV might reduce the anti-leukemic effect of MTX (12). Therefore, animal studies are needed to provide information on the timing, dose, safety profile and drug interaction, before conducting a clinical trial.

Carcinogenesis study

The tumor microenvironment (TME) has a vital role in carcinogenesis, cancer invasion and metastasis (7,25). TME consisted of inflammatory cells, carcinoma-associated fibroblast (CAF), ECM and vascularization (25). CAF was reported to modulate tumor proliferation and invasion by secreting molecules that change ECM and oncogenic signals (7). Different expressions of an adhesion molecule, Desmoglein 3 (Dsg3) and various kinds of ECM might result in diverse cancer cell adhesion and migration behavior. Dsg3 has an unclear contribution in cancer metastasis and progression, even though many studies showed its role in cell cohesion. Therefore, several oral carcinoma cell lines were developed to assess the Dsg3 role in squamous cell carcinoma (SCC) cell movement. The 3D models, which were developed to study the role of Dsg3 in cell invasion and migration, used soluble myogel, myoma organotypic 3D disc, Matrigel®, and rat tail type I collagen. Myogel contains growth factor and growth factor receptor that induce invasion of cancer cells. The myoma disc has ECM structural protein, inflammatory and endothelial cells, as well as myofibroblast, which support vascular structure that are important for tumor development and invasion. Results of the experiment indicated that besides matrix stiffness, tumor invasion and migratory behavior were also modified by the composition of tumor microenvironment. Different expressions and functions of Dsg3 affected cellular attachment into 3D tumor microenvironment model matrices. Mutant Dsg3 cells with loss of Dsg3 actions demonstrated increased migration and invasion ability in TME matrix. Human derived Myogel allowed faster invasion of human cancer cells than rat derived Matrigel (25).

Solid tumor growth usually has a necrotic region that is caused by oxygen and nutrients' access disruption. Uniquely, it is not common in oral squamous cell carcinoma (OSCC), where only 10% of cases have a necrotic region, as it has different stromal characteristics with increased vasculature. Moreover, investigation of 346 OSCC cases showed that most of the tumor diameter was smaller than 4 cm (76%), while only 6% that was bigger than 6 cm. The anticancer effect on OSCC can be more precisely predicted if the tumor microenvironment is similar to the nature of OSCC with nicely distributed vasculature, creating less necrosis. Therefore, a head and neck SCC (HNSCC) 3D spheroid cells combined with CAF was used to develop mice xenograft model to evaluate the effect of chemotherapy on OSCC. The tumor model could also be used to confirm the role of exosomes in tumor angiogenesis. One of tumor microenvironment's major components is CAF, which lacks of CAF may influence cisplatin resistance. Recent

advances in personalized chemotherapy are rarely related to oral cancer, therefore the 3D spheroid model combined with CAF may be used to study cisplatin resistance (7).

Dental material biocompatibility

Besides osteointegration, attachment between the surface of dental implant or abutment to the soft tissue is essential to support connective and hard tissue health and prevent failure of dental implant (26). There are various kinds of abutment materials with different characteristics such as metal, ceramic, and plastic. All of them need good mechanical properties and biocompatibility (26,27). Modification of implant/abutment surface can contribute to the good implant and soft tissue attachment (27). Numerous surface modifications can be employed to abutment surface, like titanium surface anodization (26). Some bioactive materials are considered to be combined with glass ionomer cement (GIC) as a cervical restorative material to enhance its biological and mechanical properties (9). In addition, camphorquinone, which is a common material for composite resin polymerization (28), as well as 3D printed resins, which are used as restoration materials (29), need to be evaluated. Each novel implant material, surface modification and bioactive material needs to be evaluated thoroughly before introduction into clinical setting.

Ideally, a model to evaluate abutment and soft tissue attachment should allow measurement in molecular level, such as examination of seal strength and attachment strength (26). In vivo models of engineered human gingiva have been utilized to evaluate the oral mucosa and implant interactions, material development and prevention or treatment of peri-implantitis (11). An in vitro organotypic model can be developed to characterize the attachment or abutment of an implant to soft tissue and analyze the expression of protein markers (26). The engineered 3D models can also be modified with immune cells like monocytes to generate inflammatory response and simulate inflammation following implant placement. Various kinds of implant surfaces might have differences in implant-soft tissue attachment in inflamed gingival condition (27). The engineered 3D oral mucosa matrix often uses collagen type I as a scaffold. The optimization of matrix scaffold for engineered lamina propria in organotypic model is needed to improve the cellular and implant attachment (11). The organotypic or engineered mucosa can also be employed to evaluate the mechanical and biocompatibility of biomodified glass ionomer to the oral soft tissue (9).

Dental implant-soft tissue attachment

To study the implant abutment-soft tissue attachment, Roffel et al. (2019) (26) utilized an organotypic model to form a reconstituted human gingiva (RHG). Two types of titanium alloy abutment, an unmodified surface and anodized surface, were studied to assess the attachment with RHG. Abutments were put into the 3D model by initially puncturing the model with 3 mm tissue punch biopsy. The soft tissue attachment was observed after 10 days. The histomorphology analysis of RHG models demonstrated a down-growing epithelium that was similar to human sulcular and junctional epithelium. In the SEM analysis, the epithelium was closely contacted with both titanium oxide abutment surfaces. The presence of important protein expressions of keratins 4 and 19 were comparable to the expression pattern in human gingiva. Basement membrane protein collagen IV and laminin 5 were present in the interface of collagen matrix and down-growing epithelium, but were absent in the interface between the abutment surface and sulcular and junctional epithelium. In that case, the internal basement membrane might have not formed, or the cells that were producing the protein were ruptured following the implant removal, or the basement membrane failed to be deposited into the implant surface. The depth of sulcus and junctional epithelium were slightly shorter than native gingiva, which might be caused by the short experiment time and limited hydrogel height. The RHG model showed less keratinocyte proliferation due to the short cultivation time, but if cultivation time is longer, the junctional epithelium depth will be significantly longer and become non-physiologic. Future study is warranted to set experimental condition that is suitable for implant tissue attachment and migration. Another drawback of this model is the absence of hard tissue or bone as the point of reference like in the clinical studies. In general, the RHG model provides an equal characteristic of native gingiva and is able to evaluate the implant abutment and soft tissue attachment. After 10 days of insertion, both surfaces of titanium oxide (original and anodized) had a good soft tissue attachment and keratinocyte spreading; therefore, comparison in different clinically relevant conditions need to be investigated. An improved quantification of functional parameters of attachment strength would be very valuable (26).

Modification of implant surface contributes to the good implant and soft tissue attachment. Various kinds of implant surfaces (metal, ceramic and polymer) have been utilized to evaluate implant-soft tissue attachment in inflamed gingiva. Titanium (Ti) is very biocompatible and has a good integration with bone. To enhance mechanical strength and corrosion resistance, titanium is often combined with other alloys such as zirconia. However, titanium alloy has low aesthetic properties. Zirconium and PEEK provide more aesthetic features. PEEK is lightweight, has a high biocompatibility, easy to polish but highly resistance to friction (27).

The 3D models can be added with immune cells besides fibroblast and keratinocytes to generate inflammatory response. In a study, implant materials, which were tested, were titanium–zirconium alloy modified with sandblasting and acid etching (TiZr-SLA), machined TiZr

(TiZr-M), machined Zirconia (ZrO2-M), and machined PEEK (PEEK-M). All those implant rods were inserted into organotypic oral mucosa. To induce inflammation, the Escherichia coli's LPSs and TNFα cytokine were added into the culture medium. Qualitative endpoints were measured with histology and SEM; meanwhile quantitative biological assay was performed by tissue viability pull test. SEM images showed that all implant surfaces provided attachment to oral mucosal model; however, there were distinct differences of oral mucosal cell attachment in different implant materials. The TiZr-SLA provides better soft tissue attachment compared to TiZr-M, ZrO2-M, and PEEK-M surfaces, and displayed the largest cells attachment. Moreover, TiZr-SLA surface showed higher cell viability (almost two fold significant increase in viability) compared to other types of materials, indicating that numerous cells attached to the surface producing a good environment for cell growth (27).

Peri-implantitis might have resulted from weak epithelial barrier after implant placement and failure to create satisfactory attachment between the gingiva and implant. In vivo models of engineered human gingiva have been utilized to evaluate the oral mucosa and implant interactions, material development and prevention or treatment of peri-implantitis. Organotypic gingiva models were constructed using different scaffold. An electrospun collagen (EC), decellularized dermis (DD), type I collagen gels (Gel) and released type I collagen gels (Gel-R) were evaluated to identify the most suitable scaffold. The engineered organotypic gingiva model was similar to native tissue. The interaction of implant material to the gingival models such as cell attachment and penetration were also investigated. The EC had uniform free spaces, so that cells were able to penetrate deeper into the upper quarter to third of the scaffolds. Collagen gel displayed an extensive tissue contraction, limiting its long-term utilization. The EC matrix efficiently lowered tissue contraction compared to collagen gel matrix. In the other hand, DD did not show any contraction. Due to the high cell viability, deeper cell penetration ability and relatively affordable material, EC could be considered as an optimal scaffold for engineered lamina propria. The EC using gingival model had a differentiation and stratification of the epithelial layer and lamina propria like native gingival tissue. Moreover, it expressed similar proteins of human gingiva such as laminin-332, collagen type IV that showed basement membrane formation, as well as cytokeratin-4, 5, and 10, which showed epithelial differentiation. However, no rete ridges were seen in the gingival models, and the distribution of fibroblast was scattered. The engineered gingiva also did not demonstrate a large bundle of collagen as quick as the native gingiva did in the culture. It might be related to the less rapid matrix remodeling in the in vitro culture (11).

Gingiva models were then inserted with implant posts of different materials: machined titanium, sandblasted-acid etched (SLA) titanium, nitride-coated titanium (TiN), and PEEK. Tissue adhesion to implant abutment was different in each kind of material been tested. An epidermal and stromal attachment were demonstrated in machined Titanium and PEEK groups, meanwhile SLA titanium surface only displayed epithelial attachment. Attachment was completely absent in TiN, probably caused by the incompatibility of the material with epithelium. Gingiva models would be useful to evaluate tissue-material attachment and interactions and might also be used for drug assessment, which was related to the mucosal integrity (11).

Improvement of cervical restoration material

Tooth restoration for cervical carious or non-carious lesion has some challenges to overcome such as occlusal loads that lead to fracture and debonding. There were biomechanical and safety requirements that must be achieved for cervical restorative materials. Polymerization shrinkage, toxicity of unpolymerized monomers from resin-based materials and compromised attachment of gingival soft tissues were some issues of cervical restorations. Glass ionomer cement (GIC) material would be an excellent restorative material for class V restorations if the biological properties were improved. To create a long-term success of cervical restoration, the material should be well attached into hard and soft oral tissue. Some bioactive materials were considered to be incorporated into GIC to develop a bio-modified restoration material. Hydroxyapatite, bioglass, processed bovine dentine, chitosan, chondroitin sulphate and gelatin were chosen due to their bioactivity and biocompatibility. 3D organotypic mucosal models were used to evaluate the interaction of biomodified glass ionomer materials to the oral soft tissue, and to test their biocompatibility by evaluating the viability and restoration-soft tissue attachment. The 3D mucosal model and the GIC specimens were prepared by placing them in the middle of culture inserts using NiTi wires. Bovine dentine, bioglass, and gelatin lowered the viability of 3D oral mucosal model, meanwhile chitosan and chondroitin sulphate did not significantly influence 3D models viability (9).

Improvement of biological properties

Vascularization capacity

There is an increasing need of bioartificial, or 3D tissue engineered oral mucosa for treatment of oral disease, but it has limitation in promoting vascularization. Vascularization is very important for biocompatibility and viability of engineered oral mucosa to host tissue. The combination of human umbilical vein endothelial cells (HUVEC) and nanofilm biomaterials were able to form capillary blood vessels in oral mucosa, but it has a low proliferation and tends to induce immunogenic rejection. Conversely, mesenchymal stem cells (MSCs)

have a high proliferation rate, low immunogenicity, and are able to express some angiogenic factors and induce immediate vascularization. Novel human engineered oral mucosa models were developed from three sources of MSCs (adipose tissue (ADSC), bone marrow (BMSC), and dental pulp (DPSC)) to generate pre-vascularized oral mucosa. The model was used to observe the vascularization properties of each type of MSCs and their ability to produce vascularization in vivo. Four models of artificial oral mucosa were used, a model that contained oral mucosal fibroblasts as a negative control, human oral mucosal fibroblasts and HUVEC (HOM-HUVEC) as a positive control, oral mucosal fibroblasts and non-differentiated MSCs, and oral mucosal fibroblasts and differentiated MSCs (dMSCs). The vascularization potential of all MSCs was determined in vivo by grafting the artificial oral mucosa into athymic mice. The mice showed no signs of complication that were related to graft, which implied that the human oral mucosa-MSCs models were biocompatible. A micro-vessel density (MVD) assay was performed to evaluate the oral mucosa capacity to form blood vessels. The MVD was increased significantly in positive control HOM-HUVEC, but the differentiated MSCs were also able to increase MVD, particularly for HOM-dBMSC and HOM-dDPSC. However, the non-differentiated MSCs induced vessels' formation in a lesser capacity (30). Therefore, in the future, the use of dBMSC or dDPSC is recommended in the development of organotypic oral mucosa.

Prevention of Collagen Matrix Contraction

One of the widely used biomaterials to construct 3D organotypic models is collagen. However, uncontrolled contractions often occurred. Genipin can be mixed with collagen and has become a promising candidate to prevent contraction. Genipin-cross-linked hydrogels are able to increase cell proliferation and/or differentiation of various cells as well as show lower cytotoxicity over mixture of scaffold and another cross-linker. The strength of hydrogel can be adjusted by modifying genipin concentration, but various cells may respond to genipin differently. In addition, an inhibitor of cytoskeleton, Cytochalasin D is shown to induce depolymerization of actin filaments quickly, thus hindering collagen gel's cell-based contraction (1).

Incorporating immune cells into organotypic models

Innate immune cells are important to protect oral tissues. Macrophages are essential innate immune cells that protect host form pathogens. Activation of macrophages can be achieved by subjecting them to bacterial LPS. Incorporating immune cells in human oral mucosa might improve studies on immune responses. Previously, immune oral mucosal models were developed from primary monocytes, peripheral blood mononuclear cells or myeloid cancer cell lines. Primary monocytes often demonstrate different character and function, peripheral blood monocytes will be transformed quickly into macrophages, and some technical issues limit the use of myeloid cancer cell lines. Therefore, using primary macrophages is preferred to depict innate immune system of tissue (31).

Some oral diseases have constant TNFα levels such as oral lichen planus and periodontitis. Since macrophages are involved in the disease pathogenesis, utilization of 3D model of disease that incorporates monocyte derived macrophage (MDM) would assist in understanding the disease progression and therapeutic approaches, as MDM provides functional immune response by secretion of TNFα that is related to inflammatory stimulation (31). For clinician and researchers, oral mucocal organoids, which mimic natural mucosa by having vascularization and immune cells, are valuable for disease modeling and drug development.

CONCLUSION

Oral mucosal organoids or organotypic models allow profound molecular investigation of oral tissue responses. Organotypic models have similar histopathologic and physiologic characteristics of native oral tissues, thus able to explore novel oral mucosal drug and biomaterials. The key components to construct various organotypic models might be similar, using various sources of cells and matrices, and each technique in developing organoid construct has a certain purpose depending on the research objective. The biological properties of oral mucosal organoid can be further modified and enhanced by advances in tissue engineering.

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