Original Article

pISSN, eISSN 0125-5614
M Dent J 2024; 44 (3) : 131-140

A decellularized extracellular matrix hydrogel to promote the proliferation of human salivary gland cells

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Objectives: Matrigel, a raw matrix from the Engelbreth-Holm-Swarm mouse sarcoma, has been commonly used to promote salivary gland (SG) cell assembly and proliferation *in vitro*, however, it possesses limitations such as batch-to-batch variations and undefined tumor-derived components, hence, alternative matrices are lacking. This study aimed to develop porcine submandibular gland decellularized extracellular matrix (SG-dECM) hydrogels to support SG cell viability and proliferation.

Materials and Methods: SG-dECM was produced using non-ionic and ionic detergent perfusions, then digested with a pepsin-based HCl buffer to generate SG-dECM hydrogels at concentrations of 1, 5, and 10 mg/mL. SG-dECM sections were stained with hematoxylin and eosin (H&E), and rhodamine-labeled peanut agglutinin staining of glycoproteins/mucins. A human submandibular gland cell line, A253 (HTB-41™, ATCC), was cultured as a monolayer culture with SG-dECM hydrogel- or Matrigel-coated on 96-well plates and assessed for proliferation over 4 days using an ATP-dependent assay. Uncoated wells were used as negative controls. Viable and late apoptotic cells were quantified with calcein-AM and propidium iodide staining, respectively. One- and two-way ANOVA with *Tukey*'s post-hoc tests were performed with 5 biological replicates.

Results: The developed SG-dECM retained moderately glycoproteins and mucins while cellular components were effectively removed. SG-dECM hydrogels at 5 mg/mL significantly enhanced A253 cell proliferation and viability compared to Matrigel ($p<0.05$) and uncoated wells ($p<0.001$) after 4 culture days.

Conclusion: SG-dECM hydrogels at 5 mg/mL promoted the proliferation and viability of A253 cells over 4 culture days. Thus, SG-dECM hydrogel could be a viable alternative to Matrigel for future drug screening applications.

Keywords: decellularized extracellular matrix, hydrogels, submandibular gland

How to cite: Lam CB, Phan TV, Kesdangsakonwut S, Tummaruk P, Chaisuparat R, **Ferreira JN**. A decellularized extracellular matrix hydrogel to promote the proliferation of human salivary gland cells. M Dent J 2024;44(3): 131-140.

Introduction

Salivary glands (SGs) are exocrine organs that constantly produce salivary fluid, which plays a crucial role in food digestion and assists in the maintenance of oral mucosa lubrication and epithelial homeostasis [1]. Reduced saliva production, known as hyposalivation, can lead to oral health disorders such as taste loss, dental caries, oral infections, and psychological distress. Hyposalivation commonly arises from head and neck cancer chemoradiotherapy, autoimmune disorders

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Received: 9 August 2024 **Revised:** 14 September 2024 **Accepted:** 18 September 2024

(i.e., Sjögren's syndrome), glandular infectious pathologies, and the adverse effects of anticholinergic medications [2]. Existing pharmacological therapies including muscarinic agonists (e.g., pilocarpine, cevimeline) and artificial saliva substitutes mainly provide temporary relief and do not address the underlying causes of hyposalivation [3]. Therefore, ongoing research is attempting to discover novel therapeutic products or repurpose old medicines for patients with SG hypofunction.

Current roadmap strategies for drug development research and mechanistic biomedical discoveries rely fundamentally on *in vitro* models. Despite being widely accepted, conventional two-dimensional (2D) monocultures have several limitations. The primary drawback is that cells grow as a monolayer on flat tissue flasks, offering unnatural growth patterns and cell attachments and interactions. Therefore, such platforms fail to fully mimic natural microenvironments for cell proliferation, expansion, and maturation [4]. Over the past few decades, matrix-based culture systems have received more attention as they can resemble extracellular matrix (ECM) in vivo conditions to support cell proliferation. The basement membrane extract which is known as Matrigel has been commonly used in SG cell culture. Matrigel in combination with several bioactive cues, can mimic the native mesenchymal stroma. This ECM product effectively promotes branching morphogenesis and differentiation of SG epithelial cells [5, 6]. However, Matrigel still has several undeniable limitations. As Matrigel is a raw material extracted from the Engelbreth-Holm-Swarm mouse sarcoma, it contains undefined elements associated with tumor development. Another well-known limitation is the large batch-to-batch variability, leading to inconsistent cell culture outcomes [7]. Given these drawbacks, researchers have been actively exploring alternative ECM sources that can provide more consistent and defined environments for SG cell culture. Interestingly, decellularized ECM (dECM) derived from submandibular glands (SG-dECM) offers an alternative to address these challenges owing to its native ECM components and structures. During the decellularization process, immunogenic cellular components are removed from tissues while structural and functional ECM molecules are preserved [8, 9]. The most common method for SG decellularization includes detergent perfusion or agitation combined with endonuclease treatment. Consecutive non-ionic detergent Triton X-100 and ionic detergent Sodium dodecyl sulfate (SDS) treatment has proven effective in eliminating cellular components [10-13]. Our research group investigated the effects of varying SDS concentrations on ECM preservation and cell removal efficiency during the decellularization of porcine submandibular glands and found that cellular components can be removed efficiently and ECM components such as collagen, elastin, and glycoproteins are preserved in SG-dECM perfused with 0.1% and 1% SDS solution [13]. Subsequently, SG-dECM suspension was fabricated into a porous polycarbonate track-etched membrane, and the SG-dECM with 1% SDS perfusion promoted porcine primary epithelial cell proliferation in a consistent manner [13].

Since this SG-dECM can be promptly transformed into tunable hydrogels, we envision it will have an application potential towards supporting *in vitro* drug screening organ-on-chip platforms. Though, it remains unclear whether these hydrogels can serve as a coating matrix, providing a bio-functional niche to support *in vitro*

SG primary cell attachment, viability, and expansion. Thus, this study aims to develop porcine SG-dECM hydrogels to support human SG cell viability and *in vitro* proliferation.

Materials and Methods

Production of SG-dECM hydrogel

The experimental protocols for this research were approved by the Institutional Biosafety Committee of the Chulalongkorn University Faculty of Dentistry (Certificate number: DENT CU-IBC 012/2024 dated June 5, 2024). Six porcine submandibular glands (pSGs) obtained from 2 female and 1 male 6-week-old pigs (*Sus scrofa domesticus*) were kindly provided by the Faculty of Veterinary Science, Chulalongkorn University. The decellularization process of pSGs was conducted according to our previous protocol [13]. Initially, connective and adipose tissues were meticulously removed from pSG tissues under a stereomicroscope (SZH10, Olympus, Japan). Then, samples were cut into small 1-mm pieces and washed with phosphate-buffered saline (PBS) 1X solution (HyClone™, Cytiva, US). The fragmented tissues were perfused with consecutive concentrations of 1-3% (w/v) of Triton X-100 (Loba Chemie, India) using a shaker at 80 rpm at room temperature (RT) for 24 h. Subsequently, the samples were perfused with 1% (w/v) SDS solution (Vivantis, Malaysia) for 3 h, followed by washing with PBS buffer solution containing 2% antibiotic and antimycotic solution (Gibco, Thermo Fisher Scientific, US) to wash out the remaining SDS. Then, specimens were incubated with benzonase nuclease (Sigma-Aldrich, MA, US) at 37 °C for overnight. The supernatants were discarded by centrifugation and the specimens were washed with the same PBS-based buffer. Next, specimens were sterilized with 1% peracetic acid (Solvay, Belgium) for 15 min, followed by washing with PBS buffer. To produce the SG-dECM hydrogels, the SG-dECM suspension was lyophilized and digested in 0.01 M Hydrochloric acid (HCl) solution containing 1 mg/mL pepsin (Sigma-Aldrich, MA, US) for 72 h on a magnetic stirrer. Then, 10 mL aliquots of solubilized SG-dECM were prepared and stored at -20 °C to terminate pepsin digestion. For SG-dECM hydrogel reconstitution, aliquots were thawed at 4 °C, then pH was adjusted to ~7.5 using 1 M NaOH and PBS 10X (1/9 of the pre-gel solution volume). The concentrations of SG-dECM pre-gel solution (1, 5, 10 mg/mL) were obtained by adding the desired volume of PBS 1X. Subsequently, the SG-dECM pre-gel solution was incubated at 37 °C for 10 min to produce SG-dECM hydrogels.

Histological analysis to evaluate removal of nuclear content and retention of ECM proteins

Porcine SG and SG-dECM specimens were fixed in 4% paraformaldehyde (PFA) for 24 h, then dehydrated through graded series of ethanol and embedded in paraffin. The specimen blocks were cut into 5 µm slices, followed by a deparaffinization step with xylene. The sections were stained with hematoxylin and eosin (H&E) and rhodamine-conjugated peanut agglutinin (Rho-PNA) (Vector Laboratories, US), followed by counterstaining with nuclear dye (Hoechst 33342, Thermo Fisher Scientific, US). Finally, histochemical and fluorescence micrographs were taken using an EVOS FL Auto II fluorescence microscope (Thermo Fisher Scientific, US).

Cell culture

The use of human cells for this study was approved by the Ethics Committee of Chulalongkorn University Faculty of Dentistry (Certificate number: HREC-DCU 2024-042 dated June 7, 2024).

Human submandibular epithelial cell line, A253 (HTB-41™, ATCC, US), was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) (Gibco, US) supplemented with 1% Glutamax (Gibco, US), 10% fetal bovine serum (FBS) (HyClone™, Cytiva, US), and 1% solution containing antibiotic and antimycotic at 37 °C and 5% CO_2. The medium was replaced every two days. When reaching 70-80% confluency, A253 cells were collected by trypsinization (TrypLE, Gibco, US) for subsequent experiments.

Hydrogel coating method

Three different concentrations (1, 5, 10 mg/mL) of SG-dECM hydrogels and Matrigel (Corning, US, catalog number 356231) were diluted in serum-free medium (1:100) at 4 °C to obtain the pre-gel solution. Subsequently, 100 µL of pre-gel solution was added into each well of 96-well tissue culture-treated plates (Life Sciences, Corning, US) and incubated for 1 h at RT. After incubation, the unbound solution was aspirated, and A253 cells were seeded onto coated wells at a density of $2x10^3$ cells/well. Uncoated wells were used as negative controls.

Cytotoxicity and viability assay

To determine the number of viable and late apoptotic cells, A253 cells were cultured on 96-well plates with or without hydrogel coatings. Calcein-AM and propidium iodide (PI) (Thermo Fisher Scientific, US) were used according to the manufacturer's instructions. On day 4 of culture, A253 cells were incubated with Calcein-AM, PI, and nuclear dye (Hoechst 33342) solution for 30 min at 37 °C. Subsequently, fluorescent micrographs were acquired from 5 culture wells per each experimental or control group using a fluorescence microscope, with 3 randomly selected regions of interest (ROIs) for each condition. These micrographs were then analyzed with ImageJ software (Version 1.54, NIH, US). The percentage of cell viability and cell death was calculated by normalizing the number of viable and late apoptotic cells to the total number of nuclei, respectively.

ATP-dependent cell proliferation assay

A253 cell proliferation was assessed at baseline, on day 2, and day 4 using ATPLite™ 1 step (PerkinElmer, MA, US) in according to the manufacturer's instructions. Briefly, at each time point, 50 µL of the reagent was thoroughly mixed with 50 µL of media in each well, followed by shaking for 2 min on a shaker at 700 rpm. The plate was then incubated at RT for 10 min. Subsequently, 50 µL of the medium was transferred to an opaque plate for bioluminescence quantification using the Glomax Discover microplate reader (Promega Corporation, Madison, WI, US).

Statistical analysis

The numerical data demonstrated a normal distribution by the Shapiro-Wilk test. Data were displayed as mean ± standard deviation (SD) and a p-value<0.05 was considered as statistical significance. Differences between groups were evaluated using one-way ANOVA or two-way ANOVA analysis followed by *Turkey's* post-hoc test. Statistical analyses were performed using Prism version 9.5.3 (GraphPad, San Diego, CA, US).

Results

Efficiency of nuclear content removal and ECM protein retention of porcine SG-dECM

After decellularization, purplish blue-stained nuclear components were entirely removed as observed in H&E-stained specimens, while acellular eosinophilic structures were

preserved within the SG-dECM (Figure 1a). Additionally, sections of pSG biopsy and SG-dECM were stained with rhodamine-conjugated PNA, which binds to galactosyl (β-1,3) *N*-acetyl galactosamine structure of membrane-associated glycoproteins or mucins. The fluorescence micrographs confirmed matrix glycoproteins or mucins were moderately retained and the stained nuclei with Hoescht 33342 were absent in the SG-dECM, in comparison with the pSG biopsy specimens (Figure 1b).

Viability and cytotoxicity of A253 cells cultured with SG-dECM hydrogel

After 4 culture days, the effects of varying concentrations of SG-dECM hydrogels on A253 cell viability and cytotoxicity were assessed by real-time fluorescence microscopy with Live/Dead staining. The experimental groups treated with 1, 5, and 10 mg/mL SG-dECM hydrogels displayed relatively fewer late apoptotic cells than the uncoated wells (CTL) (Figure 2a). This observation was further confirmed by quantifying cell death percentage using ImageJ software (Figure 2c). Additionally, significantly higher cell viability in SG-dECM hydrogel groups was consistently observed when compared to the CTL ($p<0.001$) (Figure 2b). It is noteworthy that SG-dECM hydrogel at the concentration of 5 mg/mL significantly promoted cell viability (99.11% ± 0.27%) compared to Matrigel (97.36% ± 1.35%) ($p<0.05$). There was no statistical difference in cell viability and cell death between Matrigel and CTL groups.

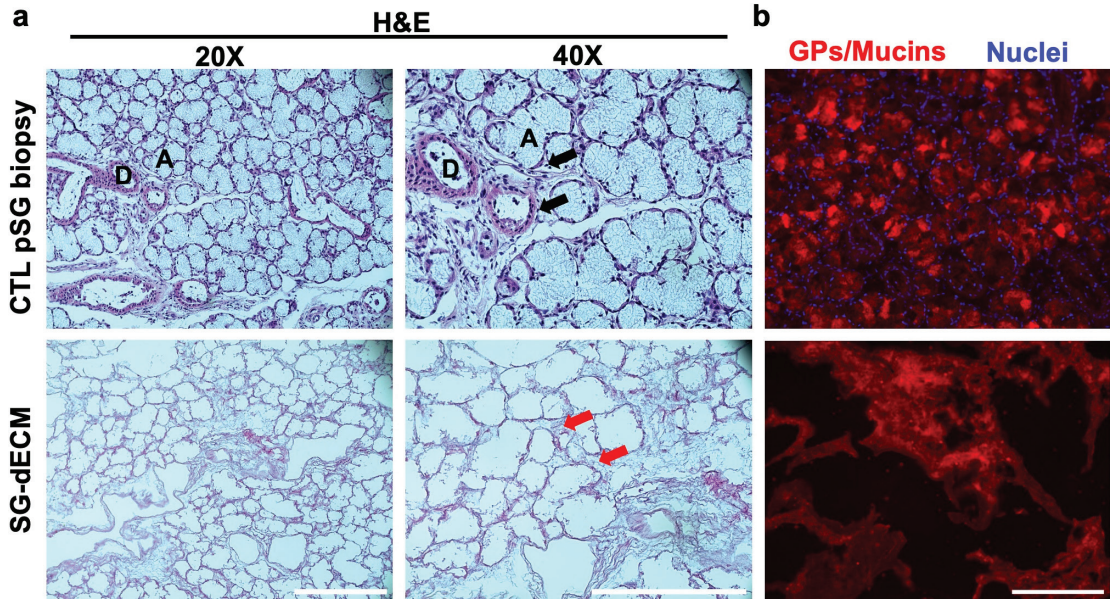

Figure 1 Nuclear contents were removed, and matrix glycoproteins (GPs)/mucins remained moderately intact in SG-dECM. (a) Hematoxylin-eosin (H&E) staining showed exocrine compartments including ducts (D) and acini (A) with blue-stained nuclear components (black arrowhead) in the native pSG biopsy, while only acellular eosinophilic structures (red arrowhead) were found in the SG-dECM section (left scale bar: 150 um; middle scale bar: 100 um). (b) Rhodamine-PNA staining of GPs/mucins counterstained with the nuclear dye Hoechst 33342 in pSG biopsy and SG-dECM sections with scale bar: 150 um.

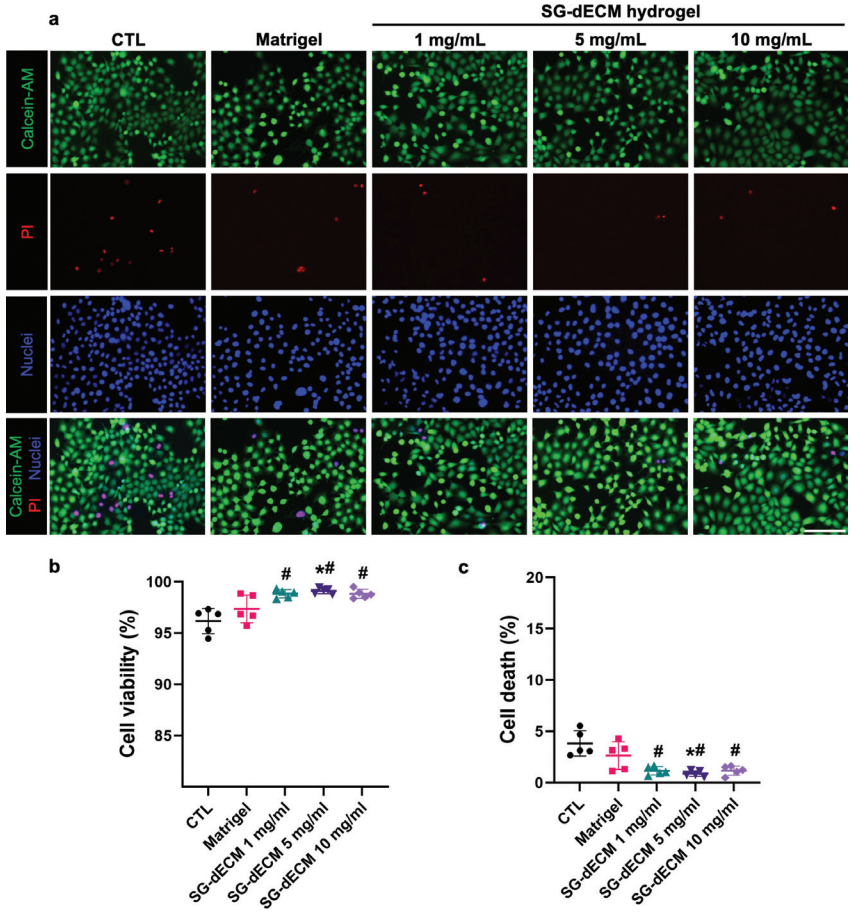

Figure 2 SG-dECM hydrogels supported the viability and decreased late apoptosis of human SG A253 cells. (a) Representative fluorescent micrographs of A253 cells for viability (Calcein AM staining), late apoptosis (PI staining), and nuclear counterstaining (Hoechst 33342) at day 4 of culture. Cells were compared in wells coated with SG-dECM hydrogels versus Matrigel and uncoated control (CTL), scale bar: 150 um. Quantitative analysis of (b) A253 cell viability and (c) A253 cell death using Image software. Data were normalized to total nuclei and are presented as mean SD (n=5). One-way ANOVA with Tukey's post-hoc test was performed: #p<0.001 when compared to CTL, *p<0.05 when compared to Matrigel.

ATP-dependent proliferation of A253 cells cultured with SG-dECM hydrogel

A253 cell proliferation was investigated over 4 culture days by an ATP-dependent assay. On day 2 of culture, SG-dECM hydrogel at the concentration of 1 mg/mL enhanced A253 cell proliferation compared to the CTL (Figure 3a). On day 4, cell proliferation was markedly higher in all SG-dECM hydrogel groups compared to the CTL. No significant difference in cell proliferation was observed between Matrigel and CTL over the 4-day culture period. Interestingly, 5 mg/mL of SG-dECM hydrogel exhibited a greater proliferation rate of the cell than Matrigel after 4 days of culture. This trend was also revealed in brightfield micrographs, which showed 100% cell confluency on day 4 in groups coated with 1 and 5 mg/mL SG-dECM hydrogels (Figure 3b).

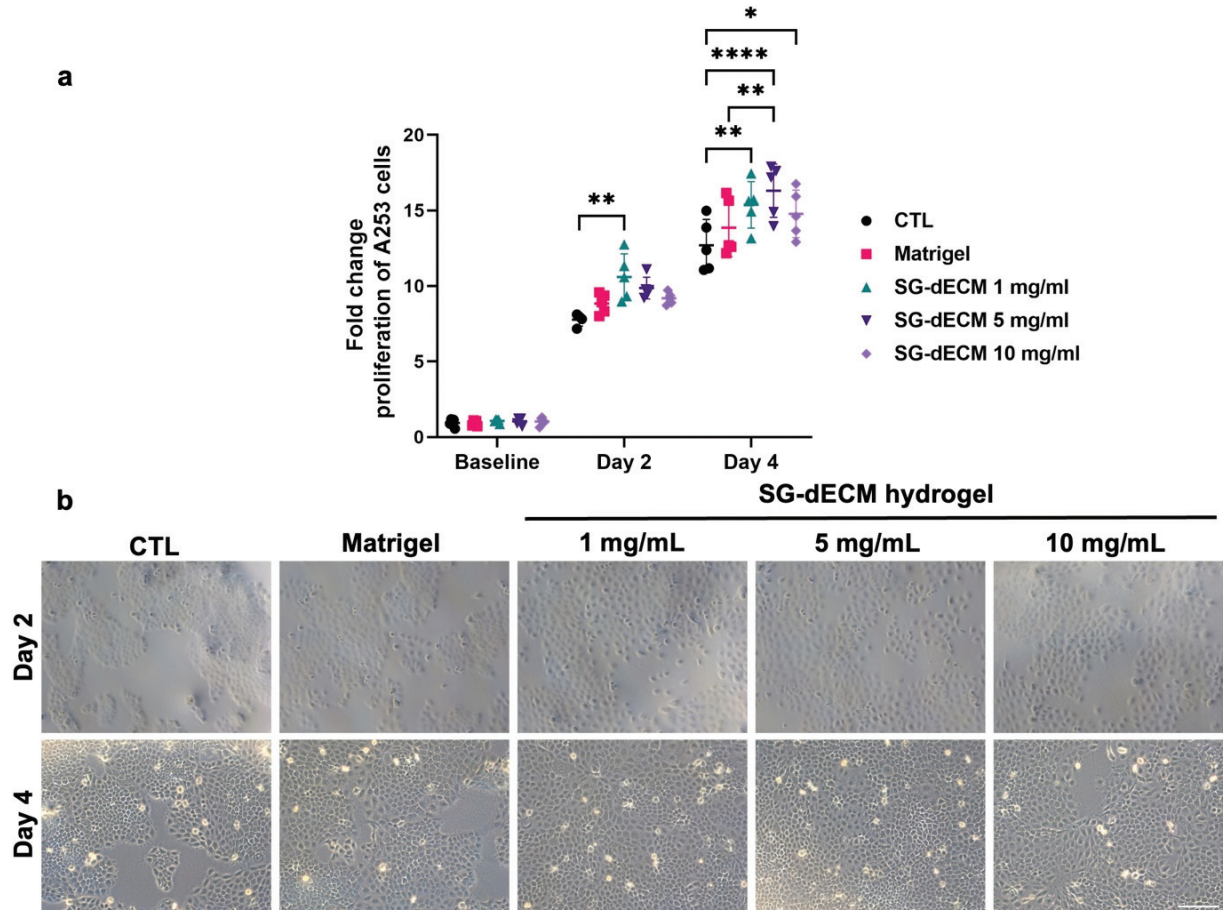

Figure 3 SG-dECM hydrogels enhanced human SG A253 cell proliferation during 4 culture days. (a) ATP-dependent proliferation of SG A253 cells. The fold change was determined by normalizing to baseline. Data are presented as mean # SD (n=5) and two-way ANOVA with Tukey's post-hoc test was performed: *p<0.05, **p<0.01, ****p<0.0001. (b) Representative micrographs with the adherent viable cells on day 2 and day 4 were observed under light microscopy with phase contrast. Scale bar: 200 um.

Discussion

In this study, we manufactured the porcine SG-dECM hydrogels which appeared to provide a matrix-based microenvironment to support the viability and proliferation of the human SG epithelial cell line A253.

To date, a wide range of decellularization methods have been developed involving physical, chemical, enzymatic techniques, and the combination of these approaches. The selection of the appropriate decellularization method depends on the type and size of the source tissues [8, 14]. Physical methods, such as freeze-thaw cycles, can effectively induce cell lysis by creating intracellular ice crystals, but excessive ice formation can be detrimental to the ECM structures [8]. In contrast, the common method for SG decellularization involves the combination of chemical and enzymatic techniques [10-13]. Particularly, the agitation of ionic detergents such as SDS and sodium deoxycholate (SDC) is crucial in solubilizing cellular membranes and nuclear components.

While SDC is highly efficient in eliminating cellular components, it tends to cause more disruption to the ECM structure compared to SDS [9]. In this study, the decellularization process involved perfusions of Triton X-100 and SDS in combination with endonuclease treatment, previously developed by our group [13]. Triton X-100, a non-ionic detergent, effectively removes DNA and nuclei without compromising the structure and alignment of collagen. However, due to its minimal impact on the tissue structure, Triton X-100 is insufficient to eliminate completely cellular contents. As a result, this detergent requires additional ionic detergent SDS to ensure the thorough removal of cellular components [8]. Additionally, an endonuclease was employed post-detergent perfusion to assist in the removal of nuclear debris and rinsing away residual detergents. The efficacy of cell removal and ECM preservation with this decellularization process was confirmed by H&E and rhodamine-PNA staining. These observations were consistent with previous studies that aim to minimize the potential immunogenicity while maintaining the functional molecular units of the native ECM [11-13].

Following decellularization, SG-dECM hydrogels were produced through two main steps: solubilization and controlled neutralization based on temperature and/or pH, as previously described [15]. Several hydrogels have been produced with components of ECM, such as collagen, hyaluronic acid, and Matrigel. Unlike hydrogels made from individual ECM components or tumor-derived Matrigel, dECM hydrogels preserve the complete biochemical complexity of native SG tissues. The manufacturing process for the dECM hydrogel involves a self-assembly process of collagens, which is partly modulated by other ECM proteins like glycosaminoglycans and proteoglycans. Therefore, the polymerization kinetics are influenced by the concentration of proteins that remain after decellularization and solubilization [16]. In this study, three different concentrations of SG-dECM hydrogel (1, 5, 10 mg/mL) were tested for *in vitro* cytocompatibility and whether such hydrogels could support cell proliferation and expansion. Our findings demonstrated that 1 mg/mL of SG-dECM hydrogel facilitated A253 cell proliferation by day 2 compared to the CTL. The initial proliferation observed with the 1 mg/mL concentration may be attributed to its ability to provide a favorable environment for early-stage cell proliferation, particularly when cell density is low. However, this concentration may not be sufficient to support sustained proliferation with higher cell density. In contrast, 5 mg/mL of SG-dECM hydrogel emerged as the optimal concentration for cell viability and proliferation over 4 culture days, surpassing both uncoated wells and Matrigel substrate. This concentration may provide a more robust environment that supports ongoing cell growth and expansion over time. These findings were consistent with a previous study that found SG-dECM hydrogel at 5 mg/mL promoted primary SG cell viability and proliferation during 7 days of culture [12]. Additionally, A253 cells grown on Matrigel substrate exhibited similar cell proliferation rates to those cultured on polystyrene substrate, consistent with previous findings of SG cell line [17,18]. Altogether, these findings suggested that 5 mg/mL closely mimics the native SG ECM microenvironment, providing essential functional molecules and growth factors necessary for cell viability and proliferation over 4 culture days.

Despite gaining new insights into SG-dECM hydrogels, this study has limited findings since it is mainly focused on 2D SG cell culture with a classic hydrogel substrate coating method.

One promising approach is incorporating SG-dECM hydrogels into robust culture platforms such as microfluidic systems. This innovative strategy offers various advantages, including the creation of small-scale culture environments that closely mimic physiology. Additionally, by enabling highly controlled experimental setups, microfluidic systems can robustly enhance the sensitivity and accuracy of various biological and chemical assays [19]. Therefore, our future steps will focus on the potential integration of SG-dECM with SG organ-on-a-chip systems to create physiologically relevant *in vitro* models for high-throughput drug screening applications.

Conclusion

Our study produced porcine SG-dECM hydrogels that effectively support the *in vitro* viability and proliferation of human SG epithelial cell line A253 for 4 days of culture. Thus, SG-dECM hydrogels may constitute a promising alternative to Matrigel for future *in vitro* drug screening applications.

Acknowledgements

This project is funded by the National Research Council of Thailand (NRCT) and Chulalongkorn University with project number: N42A670176 to JNF (main PI) and RC (Co-I). This project is funded in part by Thailand Science Research and Innovation Fund and Chulalongkorn University (Project ID: 197190) to JNF. Center of Excellence and Innovation for Oral Health and Healthy Longevity is funded by the Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University. CLB studies were supported by a Chulalongkorn University scholarship from the Graduate Scholarship Program for ASEAN or Non-ASEAN Countries. This research is also supported by the 90th Anniversary of Chulalongkorn University Scholarship under Ratchadapisek Somphot Endowment Fund provided to CLB. This research project is supported by the Second Century Fund (C2F), Chulalongkorn University provided to TVP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We would like to give a special thanks to Mr. Somchai Yodsanga from the Department of Oral Pathology for his assistance in preparing and staining the tissue sections for histology.

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