A 3D Polymer Scaffold Platform for Enhanced *in vitro* Culture of Human & Rabbit Buccal Epithelial Cells for Cell Therapies

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Background: Buccal mucosal epithelial cells show promising application for various regenerative medicine approaches. In this study, we examined the feasibility of culturing rabbit and human buccal mucosal epithelial cells in a novel thermoreversible gelation polymer (TGP) scaffold, without feeder layers or other foreign proteins.

Methods & Results: The results of this 28-day *in vitro* culture, using the conventional technique (2D) and TGP (3D) showed that the epithelial cell morphology could be maintained only in the TGP group while cells in the 2D group de-differentiated to fibroblast morphology in both human and rabbit samples. CK3 expression, a marker for epithelial differentiation was higher in 3D-TGP cultured cells than 2D.

Conclusion: TGP based *in vitro* cell culture is a prospective methodology to culture buccal mucosal epithelial cells efficiently without using foreign biological components for tissue engineering applications.

Key words: Buccal Mucosa, Epithelial cells, Polymers, Tissue Engineering

INTRODUCTION

Buccal mucosal epithelial cells have potential tissue engineering and regenerative medical applications. These are being used to treat bilateral or total limbal deficiency [1, 2], urethral stricture repair [3], esophageal reconstruction [4, 5], bladder reconstruction [6], vocal cord repair [7], deep dermal burns [8], and repair of the palate [9]. These applications, along with the recent development of obtaining induced pluripotent stem cells from the buccal mucosa [10], widen the horizon regarding the need for buccal mucosal cell cultures *in vitro*. Both natural and synthetic scaffolds involve the amniotic membrane [11], acellular scaffolds [12], silicone, and collagen [13] etc., which have been used to culture buccal mucosal epithelial cells. Natural scaffolds, however, create the risk of biological contamination [14], while synthetic scaffolds show less biocompatibility. Also, most of these studies use feeder layers [15], although there have been reports of buccal mucosal epithelial cells cultured without them as well [16]. However, it may be possible to culture cells that do not use feeder layers, but use scaffolds instead; the cells must have a carrier to hold them *in vivo* after transplantation. Thus, an ideal methodology or scaffold would be one that allows for efficient cell culture *in vitro* and would also allow for transplantation with the cells *in vivo*.

The thermoreversible gelation polymer (TGP) is a synthetic scaffold, which is highly biocompatible, and has been used for the *in vitro* culture of different kinds of cells, including corneal limbal stem cells [17], corneal endothelial cells [18], bone marrow mononuclear cells [19], hepatocytes [20], embryonic stem cells and induced pluripotent stem cells [21]. TGP provides a three-dimensional environment and has been observed to encourage the growth of cells and stem cells with their native phenotype for an extended period of time [22]. TGP has likewise been successfully implanted in animal models [19, 22], as well as human clinical studies [23]. We have earlier reported the transplantation of *in vitro* expanded buccal mucosal epithelial cells using

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the Buccal epithelium Expanded and Encapsulated in Scaffold-Hybrid Approach to Urethral Stricture (BEESHAUS) in six patients in a pilot study [23]. Though the clinical results were very promising, we wanted to further study the *in vitro* procedures for standardization of cell culture yield, especially the grown cells in two-dimensional (2D) and three-dimensional thermo-reversible gelation polymer (3D-TGP) in terms of epithelial phenotype before moving on to large randomized clinical trials which are planned in Japan, to which this report has thrown some light on that aspect. Our focus is to examine the possibility of the *in vitro* culture of human and rabbit buccal mucosal epithelial cells, using the synthetic TGP scaffold, without the use of foreign biological components and characterize the cultured cells with H & E and RT-PCR.

MATERIALS AND METHODS

The study was done in accordance with the declaration of Helsinki, following all prevailing guidelines and regulations. The study was approved by the ethical committee of National Defence Medical College, Japan (Ethics Committee Approval number: 4154 (9 April, 2020). Three (n = 3) human buccal tissue samples were obtained from adult patients undergoing biopsy for buccal mucosal graft urethroplasty; informed consent was given for the collection of all samples, which are redundant after use in surgery. Three (n = 3) rabbit buccal tissue samples were obtained by punch biopsies from the cheek of rabbits under light sedation.

The TGP in this study is a copolymer, composed of poly (N-isopropyl acrylamide-co-n-butyl methacrylate) and poly (NIPAAm-co-BMA)], which form the thermo-responsive polymer component, along with [Polyethylene glycol (PEG)], forming the hydrophilic block component. TGP exhibits thermoreversible gelation becoming a liquid at temperatures lower than sol-gel transition temperatures and becoming a gel at higher temperatures [24]. The lyophilized TGP (1 g) vial was obtained from Nichi-In Biosciences (P) Ltd through M/s GN Corporation, Japan. The sol-gel transition temperature of TGP used in this study was 20°C, which was reconstituted with 10 ml of Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco BRL, Gaithersburg, MD, USA). Moreover, this reconstituted TGP was incubated at 4°C until use.

The buccal mucosal epithelial tissues were transported to the laboratory in phosphate buffer saline (PBS) (Invitrogen, Carlsbad, CA, USA) at 4°C. The tissues were subjected to enzymatic digestion using 1 ml of Digestion medium consisting of 1,000 PU/ml Dispase I (Oenon, Japan) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) at 37°C overnight and then the epithelial layer was peeled off, minced and subjected to 0.5 ml of Accutase (Sigma) digestion for 15 min at 37°C. The digested cells were washed twice at 1500 rpm for 10 min. These cells were counted with the Trypan Blue dye exclusion method.

For each sample, the cells were equally seeded in two groups. For group I (two dimensional-2D), the cells were seeded in 12-well Tissue Culture (TC) Plates (Greiner Bio-one, Greinerstraße 70, Austria) and overlaid with DMEM with 10% autologous serum for human samples & 10% FBS for rabbit samples. For group II (3D TGP), the cells were mixed with reconstituted cold liquified TGP and added to 12-well TC plates in cold conditions. The TGP with the cells was allowed to solidify for 1 min. After the TGP was solidified, DMEM with 10% autologous serum for human samples & 10% FBS for rabbit samples was added over the TGP. Both the groups were cultured in 5% CO_2 at 37°C for a maximum of 28 days. The polymer remains a gel at 37°C while the culture is performed [23–28]. The polymer does not degrade under *in-vitro* conditions; the gel stays the same during the *in vitro* culture. Cell observation and replenishment of culture media was done every three days.

After culture, the cells were harvested from both groups and fixed in 10% formalin, followed by embedment in paraffin. Serial sections were made, deparaffinized, and stained with Hematoxylin and Eosin (H&E). The human buccal mucosal epithelial cells cultured cells were subjected to qRT-PCR for the CK3 marker, specifically found in the corneal epithelium and buccal mucosal epithelium [29]. mRNA expression in cell lysate of 2D and 3D-TGP was analyzed. Total RNA was isolated using Nucleospin RNA XS kit (Macherey-Nagel). RT-PCR was performed using One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) (Takara, Japan) and Thermal Cycler Dice® Real Time System Lite (TP700, TaKaRa).

The primers employed were
GAPDH:
Forward: GCACCGTCAAGGCTGAGAAC
reverse: TGGTGAAGACGCCAGTGGA
CK3:
Forward: GGGAACAGATCAAGACCCTCAAC
Reverse: TGTGCCTGAGATGGAACTTGTG

For mRNA quantification, we used the delta-delta Ct method which is an approximation method that measures the relative levels of a mRNA between two samples by comparing them to a second RNA which serves as a normalization standard. GADPH was used as normalization standard.

All data were analyzed using Excel software statistics package analysis software (Microsoft Office Excel®); Student's paired t-tests were also calculated using this package; P-values < 0.05 were considered significant.

RESULTS

The average initial cell count was 0.5 x 10^6 cells in each group, while the average cell count post culture in group I (2D) was 0.3 x 10^6 and in group II (3D-TGP) it was 0.7 x 10^6. Among rabbit buccal mucosal epithelial culture samples, the average cell count obtained in 2D groups was 1.26 x 10^6 cells; In 3D-TGP the average cell count obtained was 0.07 x 10^6 cells. Thus, it was observed that the average cell counts of the human buccal mucosal epithelial cells after culture were relatively higher in group II (3D-TGP), compared to group I (2D); however, the difference was not statistically significant (p-value = 0.1543). In case of rabbit samples, the cell counts in 2D groups were higher than the 3D-TGP groups which was also not statistically significant (p-value = 0.2184). The cell counts after *in vitro* culture have been presented in Fig. 1.

Culture images of human buccal mucosal epithelial cells in groups I (2D) and II (3D-TGP) are seen in

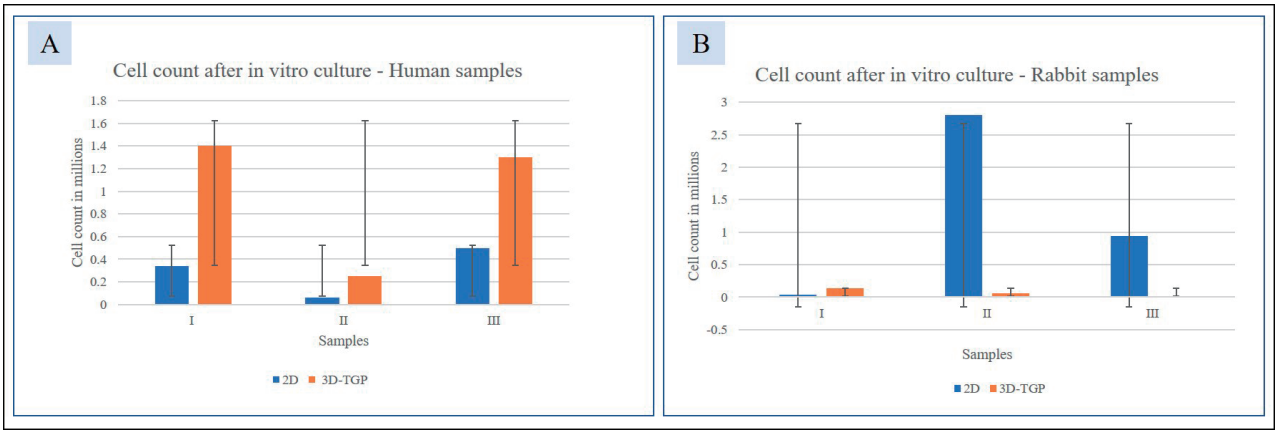


Fig. 1 Cell counts obtained after *in vitro* culture in two-dimensional (2D) versus three-dimensional 3D-TGP scaffold-based culture, showing relatively higher cell counts in 3D-TGP in human samples (n = 3) (A); while in the rabbit samples (n = 3), 2D culture gave rise to relatively higher cell counts (B); Error bars indicate standard deviation, $p < 0.05$, paired t-test.

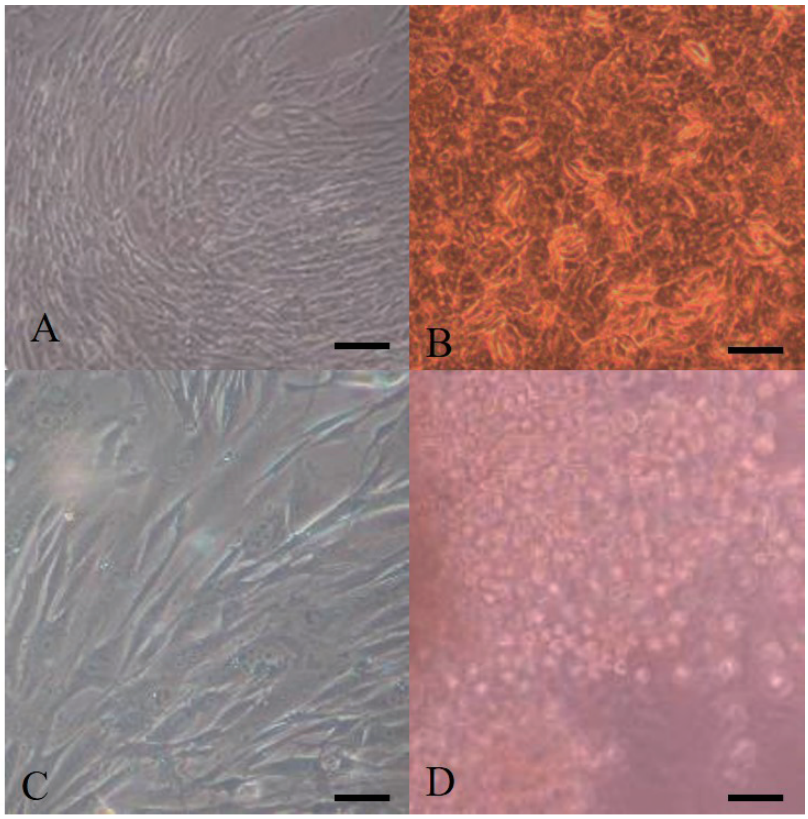


Fig. 2 Buccal mucosal epithelial cells culture images. A & B: Human Buccal mucosal epithelial cells; C & D: Rabbit mucosal epithelial cells; All scale bars = 100 μm.

Fig. 2A & B and rabbit buccal mucosal epithelial cells in Fig. 2C & D. It can be observed that the epithelial cell morphology was maintained only in the 3D-TGP groups during *in vitro* culture (Fig. 2B, D) while there was de-differentiation to fibroblast morphology in 2D samples (Fig. 2A, C) in both human and rabbit oral mucosal epithelial samples. Further, H & E showed positivity for buccal mucosal epithelial cell morphology for both the groups in human and rabbit samples (Fig. 3). Human buccal mucosal epithelial cells in 2D and 3D-TGP were positive for CK3 expression in RT-PCR characterization but the expression was relatively higher in 3D-TGP cultured cells (Fig. 4).

DISCUSSION

Cultured buccal mucosal epithelial cells are suitable in various regenerative medical and tissue engineering applications. Our main focus is the application of these cells for corneal surface reconstruction [29, 30] and treating urethral strictures with the Buccal epithelium Expanded and Encapsulated in Scaffold-Hybrid Approach to Urethral Stricture (BEES-HAUS) procedure [23]. Although several types of scaffolds are used to enhance the culturing of these cells, most of them use feeder layers or animal proteins, which can pose a risk during clinical translation.

As such, we studied the feasibility of culturing hu-

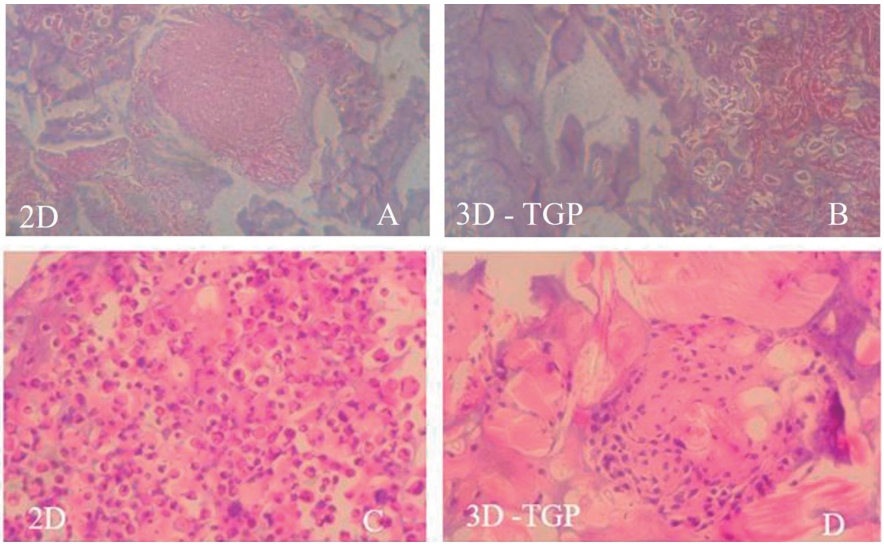
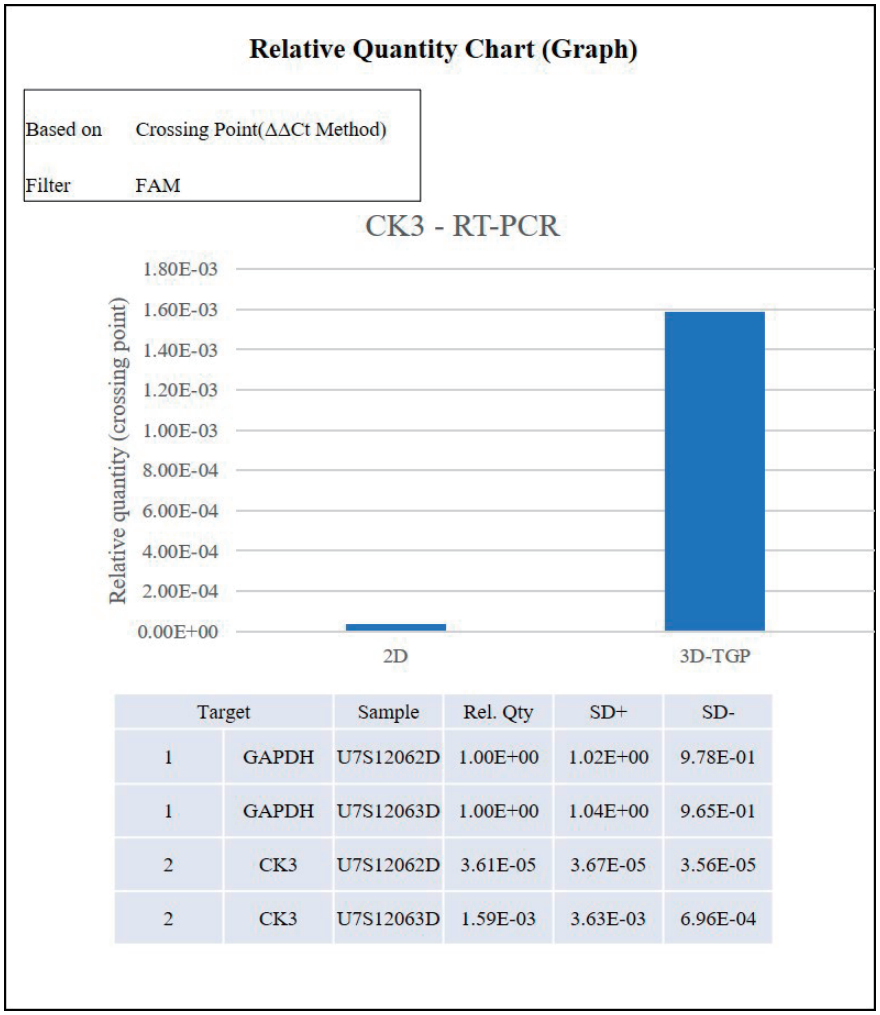


Fig. 3 H & E staining images of cultured buccal mucosal cells showing positivity for epithelial cell morphology; A & B: Human samples; C & D: Rabbit samples.



Target		Sample	Rel. Qty	SD+	SD-
1	GAPDH	U7S12062D	1.00E+00	1.02E+00	9.78E-01
1	GAPDH	U7S12063D	1.00E+00	1.04E+00	9.65E-01
2	CK3	U7S12062D	3.61E-05	3.67E-05	3.56E-05
2	CK3	U7S12063D	1.59E-03	3.63E-03	6.96E-04

Fig. 4 Expression of Keratin 3 (CK3) in the *in vitro* cultured human buccal mucosal epithelial cells cultured in 3D-TGP using RT-PCR.

man and rabbit buccal mucosal cells *in vitro* without feeder layers or animal proteins, only using TGP. TGP has been employed for the culture of several cell types, such as corneal limbal stem cells, retinal pigmented epithelium, hepatocytes, and neural cells [17–22]; it provides (3D) environment in which cells can grow for longer without losing their viability or undergoing alterations in morphology [22]. TGP is also a synthetic polymer that is biodegradable and safe; it has been applied *in vivo* in animal models [19, 20] and humans [23, 25] with proven assurance.

TGP was demonstrated to support cell growth without altering gene expression profiles or karyotypes [21, 31]. Although statistically this is not significant,

the present study shows that the TGP group in human samples has a relatively higher cell number compared to cells cultured without TGP. It is likely that TGP will be a valuable scaffold for cell cultures of buccal mucosal epithelial cells, eliminating the need for biological scaffolds or feeder layers.

Cell counts in this study are equivalent to those reported in the literature [2, 30, 32]. However, in our previous study on application of *in vitro* expanded for buccal mucosal cells for BEES HAUS application in a pilot clinical study [23], the cell counts were higher than the present study. The essential difference between the present study and the previous study is in the cell isolation and methodology especially the digestion process. In the present study, the tissues were subjected to enzymatic digestion using Dispase I overnight and then the epithelial layer was peeled off, minced and subjected to Accutase digestion. In the previous study, Dispase II was used at 37°C for 1 h. The tissues were then treated with trypsin – ethylenediaminetetraacetic acid (0.25% solution) for 15 min [23]. Since after overnight digestion (present study), we are able to obtain a. separate epithelial layer that can be peeled off, there were no tissue bits left after filtration. In the previous study, the one-hour digestion with Dispase II gave rise to tissue bits which were also seeded into culture to allow them grow as explants and then during the process of culture were digested and re-seeded. All the cells together were used for transplantation [23]. In the previous study, though clinical results were promising, we wanted to study the *in vitro* procedures for deriving epithelial cell phenotype, instead of a mixed cell phenotype and if there is difference between 2D and 3D-TGP cultures in terms of epithelial cell expression. Hence, we performed the present study on discarded human buccal mucosal samples and rabbit buccal mucosal samples. Further, the overnight digestion process in the present study gave rise to more epithelial cells especially in 3D-TGP which is observed by higher CK3 expression in 3D-TGP cultured cells and hence though the cell count is lower, the intended outcome of deriving epithelial cells for transplantation could be achieved. This decrease in cell count compared to the previous study [23] can also be attributed to the fact that mature or differentiated cells die rapidly, due to apoptosis during *in vitro* cell culture [33], while their precursors are maintained for relatively longer periods of time. This was also shown in a mice study, in which cerebral infarcts with transplanted cells, using TGP, had more neural progenitors than mice, where cells were transplanted without a scaffold [34]. Perhaps, there were more precursors in 3D-TGP group in this study. However, this would necessitate further validation which we are planning to undertake by further experiments on characterization of various populations including the progenitor cells present among the cultured cells, along with assessment of their viability in 3D-TGP compared to 2D.

CONCLUSION

In vitro culture of human and rabbit buccal mucosal epithelial cells, without feeder layers of animal/human origin or other foreign proteins, was accomplished with innovative TGP. After more validation is established, this methodology is expected to have increased applications, as cells cultured with the TGP can be used for corneal surface reconstruction and treatment of urethral strictures: Buccal epithelium Expanded and Encapsulated in a Scaffold-Hybrid Approach to Urethral Stricture (BEES-HAUS).

POTENTIAL CONFLICT OF INTERESTS

Dr. Abraham and Dr. Katoh are inventors of several patents on biomaterials including the one described in the manuscript.

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