

EXTENDED REPORT

The use of human serum in supporting the in vitro and in vivo proliferation of human conjunctival epithelial cells

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Aim: To evaluate the use of human serum (HS) in supporting the in vitro and in vivo proliferation of human conjunctival epithelial cells, and compare it with fetal bovine serum (FBS) and bovine pituitary extract (BPE).

Methods: Conjunctival epithelial cells were cultivated in media supplemented with HS (5%, 10%), FBS (5%, 10%), and BPE (70 µg/ml, 140 µg/ml). The colony forming efficiency (CFE), bromodeoxyuridine (BrdU) ELISA proliferation assay, and cell generations were analysed. Cells were evaluated for keratin (K4, K19, and K3) and MUC5AC expression by immunostaining and RT-PCR. Conjunctival equivalents constructed on amniotic membranes were transplanted onto severe combined immune deficient (SCID) mice for 10 days and analysed histologically.

Results: The proliferation assays of HS supplemented cultures (CFE, 6.7% (SD 1.8%); BrdU absorbance, 0.86 (0.16)) were comparable to FBS supplemented (CFE, 9.3% (1.8%); BrdU absorbance, 1.11 (0.18)) and BPE supplemented cultures (CFE, 5.9 (1.5); BrdU absorbance, 0.65 (0.12)). Goblet cell densities for HS, FBS, and BPE supplemented media were 52 cells/cm², 60 cells/cm², and 50 cells/cm², respectively. HS supplemented cultures formed stratified epithelial sheets in vivo following transplantation.

Conclusions: The proliferative capacity of conjunctival epithelial cells cultivated in HS supplemented cultures was comparable to FBS and BPE supplemented cultures. The elimination of animal material from the culture system is advantageous when cultivating cells for clinical transplantation.

The current preferred methods for cultivating corneal, limbal, and conjunctival cells require the use of xenobiotic materials in the culture system, such as fetal bovine serum (FBS), with or without a murine feeder layer.^{1–8} Various serum free media developed to obviate the need for bovine serum have mainly been used for the study of the roles of various growth modulating factors.^{9–18} However, its use in cultivating cells for clinical transplantation has been limited because of its relatively lower efficacy for serially passaging epithelial cells compared to serum supplemented media. Bovine pituitary extract (BPE) was previously found to be an essential supplement for promoting epithelial proliferation, and has been included in most serum free culture systems.^{9–12}

In the development of tissue equivalents for transplantation, the ideal culture condition is one that is safe from disease transmission and able to support the clonal growth and serial propagation of cells. The elimination of bovine serum or animal feeder cells in the development of bioengineered tissue equivalents for clinical transplantation would be significantly advantageous, because of the reduced risks of transmission of diseases (for example, bovine spongiform encephalitis) and xenograft rejection. The ability of these ex vivo expanded cells to remain proliferative in vivo following transplantation is of paramount importance for tissue regeneration.

In an effort to eliminate the use of xenobiotic material in the culture system, we sought to evaluate the use of human serum (HS) as an alternative to animal serum, and investigated the influence of HS, FBS, and BPE on the in vitro and in vivo proliferative capacity of cultivated cells, with the aim of developing a safe and effective conjunctival epithelial equivalent for clinical transplantation.

METHODS

Human conjunctival biopsies were obtained from surgical patients after obtaining informed consent, according to the

statutes of the institutional review board of the Singapore National Eye Center. All experimental procedures conformed to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research, and the guidelines of the Declaration of Helsinki in biomedical research involving human subjects.

Harvesting of human conjunctival epithelial cells and human serum

Conjunctival biopsies were obtained from patients undergoing routine surgery for nasal pterygium or cataract. A small piece of normal conjunctiva (1×3 mm in size) was removed from the superior bulbar region, 10–15 mm from the limbus.

HS was collected from consenting healthy patients, aged 20–32 years old (mean age 29.3 years). The blood was processed by centrifugation at 2500 rpm for 20 minutes to separate the serum from cells. The serum was then stored in sterile tubes for use.

Ex vivo expansion of conjunctival epithelial cells

The conjunctiva was cut into 1 mm pieces, and cultivated as explants on 35 mm tissue culture dishes. The basal medium consisted of a 1:1 mixture of DMEM/Ham's F12 (Gibco, Carlsbad, CA, USA), which was similar to the basal media used in other reports.^{2 3 5 8} Culture conditions with the following supplements were compared: 5% HS, 10% HS; 5% FBS, 10% FBS; 70 µg/ml BPE, and 140 µg/ml BPE. Basal medium alone was used as the control. Antibiotics (100 IU/ml penicillin, and 100 µg/ml streptomycin) were added to each condition.

The cells were incubated at 37°C, under 5% CO₂ and 95% air, with media change carried out every 2 days. Following

Abbreviations: BPE, bovine pituitary extract; BrdU, bromodeoxyuridine; CFE, colony forming efficiency; FBS, fetal bovine serum; HAM, human amniotic membranes; HS, human serum; PAS, periodic acid Schiff; SCID, severe combined immune deficient

subculturing, cells were plated onto culture dishes at a seeding density of 4×10^3 cells/cm².

Clonal growth assay

The clonal growth ability of cultured cells was determined by the colony forming efficiency (CFE). Cells were plated at a clonal density of 1000 cells onto 60 mm culture dishes. A colony was defined as a group of eight or more contiguous cells. The colonies were fixed on day 10, stained with rhodamine B, and counted. The CFE was defined as follows:

CFE (%) = (colonies formed at end of growth period \times 100%)/total number of viable cells seeded.

Bromodeoxyuridine (BrdU) ELISA cell proliferation assay

The proliferative capacity of passage 1 cells was determined by a BrdU ELISA cell proliferation assay. Analysis was performed on the sixth day of passage. Cultured cells were incubated with 10 μ M BrdU labelling solution (Amersham Biosciences, Freiburg, Germany) for 2 hours and incubated with 100 μ l of monoclonal antibody against BrdU for 30 minutes. The BrdU absorbance was measured directly using a spectrophotometric microplate reader at a test wavelength of 450 nm and a reference wavelength of 490 nm. This gave us a measure of the degree of proliferation of cells, which we termed the proliferation index. Each sample was cultured in quadruplicate.

Number of cells generations

The long term proliferative capacity of cultured cells was determined by passaging these cells till senescence in their respective media. The number of cell generations, x , was calculated as follows:

$$x = \log_2(N/N_0)$$

where N is the total number of cells harvested at subculture, and N_0 is the number of viable cells seeded.

Development and xenotransplantation of conjunctival epithelial equivalents

Human amniotic membranes (HAM) were obtained from mothers who had undergone cesarean section delivery, and prepared as described previously.¹⁹ The HAM was incubated with Dispase II 1.2 U/ml (Gibco) for 2 hours and gently scraped to remove any amniotic epithelial cells. The denuded HAM was placed basement membrane side up on a nitrocellulose filter paper support (Millipore, Bedford, MA, USA).

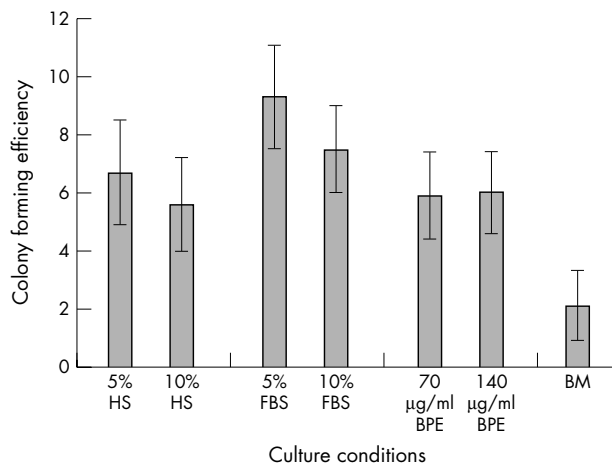


Figure 2 Colony forming efficiency of conjunctival epithelial cells cultivated in HS, FBS, and BPE supplemented media ($n = 12$). HS: human serum; FBS: fetal bovine serum; BPE: bovine pituitary extract; BM: basal medium.

Conjunctival specimens were cut into 1 mm pieces and inoculated onto the basement membrane side of the HAM. When a confluent layer of cells was obtained after 10 days, the calcium concentration was increased to 1.2 mM for 4 days with calcium chloride solution to promote differentiation and stratification.

Conjunctival epithelial equivalents were then xenografted onto the subcutaneous tissue of severe combined immune deficient (SCID) mice, aged 7–9 weeks old. Dorsal skin incisions were made to create a skin flap that was raised to expose the underlying dorsal muscle fascia. The conjunctival equivalent was placed epithelial side up over the muscle fascia and the skin flap sutured in place with 8/0 silk sutures. Ten days following grafting, the mice were killed and the tissues were excised for analysis.

Conjunctival epithelial cell differentiation

For immunocytochemistry, cultivated conjunctival epithelial cells were incubated for 1 hour with monoclonal antibodies to keratins 4, 19, and 3 (AE-5 antibody) and MUC5AC. The cells were detected with the mouse immunoperoxidase detection kit (Vectastain Elite Kit) and stained with DAB substrate. MUC5AC was detected by immunofluorescence, by

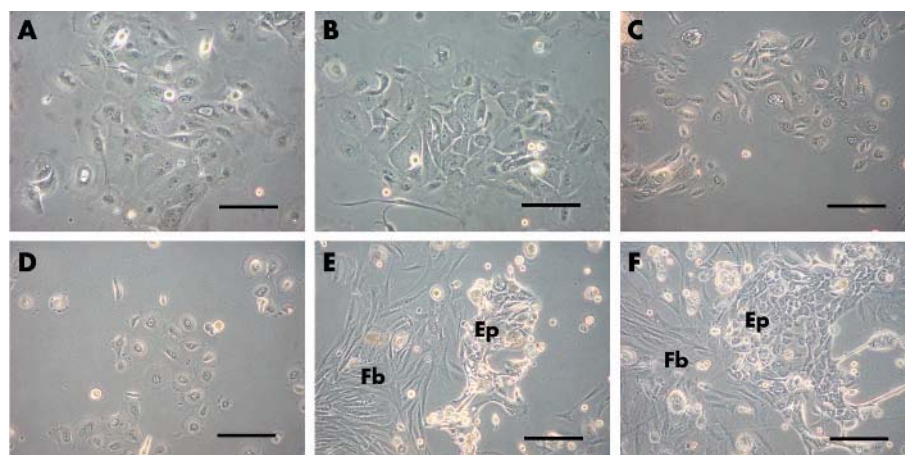


Figure 1 Phase contrast appearance of conjunctival epithelial cell cultures. (A) Passage 2 conjunctival epithelial cells in 5% HS supplemented media, 6 days after plating. Colonies consisted of a collection of round or ovoid cells, surrounded by larger elongated cells. (B) 5% FBS supplemented culture, passage 2 conjunctival cells. (C) BPE supplemented (70 μ g/ml) culture, and (D) basal medium, 6 days after plating (passage 2). Fibroblast proliferation was noted in (E) HS supplemented and (F) FBS supplemented cultures in passage 3. Ep: Epithelial cells; Fb: Fibroblasts. Bar = 125 μ m.

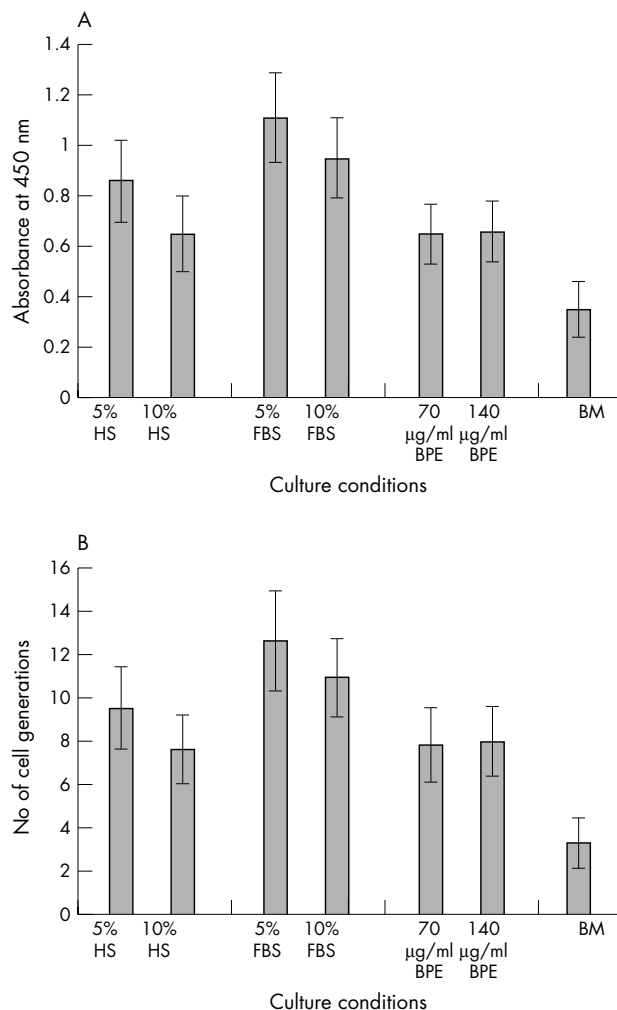


Figure 3 (A) BrdU ELISA cell proliferation assay of cells cultivated in HS, FBS, and BPE supplemented media ($n = 16$). The bars represent the mean values of BrdU absorbance in each culture condition. There was greater incorporation of BrdU in cells cultivated in FBS supplemented media, compared to HS or BPE supplemented media. (B) The number of cell generations achieved in the various culture conditions. Cells cultivated in FBS supplemented media demonstrated the greatest number of cell generations, followed by 5% HS supplemented media ($n = 16$). HS: human serum; FBS: fetal bovine serum; BPE: bovine pituitary extract; BM: basal medium.

incubation with FITC conjugated secondary antibody, followed by counterstaining with propidium iodide at 2.5 $\mu\text{g}/\text{ml}$.

The goblet cell density was determined by the number of goblet cells present in six representative high power fields. The ratio of goblet cells to non-goblet epithelial cells was determined by the number of goblet cells divided by the total number of cells stained with propidium iodide within the same area.

The expression of conjunctival goblet cell mucin, MUC5AC, was determined by RT-PCR, using the methods described previously.^{19,20} The primer sequences were as follows: MUC5AC sense primer, 5'-TCCACCATATACCGCCACAGA-3'; and antisense primer, 5'-TGGACCGACAGTCACTGTCAAC-3'. The amplification reaction was performed as follows: 3 minutes at 96°C, followed by 30 cycles of denaturation for 45 seconds at 96°C, amplification for 1 minute at 55°C, and extension for 1 minute at 72°C. The predicted length of the PCR product was 103 bp.^{19,21} Amplified cDNA was analysed by electrophoresis on a 1% agarose gel and viewed by ethidium bromide staining.

Morphological analysis

Cultivated cells were stained with periodic acid Schiff (PAS). Xenografted conjunctival equivalents were paraffin embedded, and 4 μm sections were cut and stained with haematoxylin and eosin.

RESULTS

Morphology and propagation of cells cultivated in serum free and serum supplemented media

In both HS and FBS supplemented media, epithelial cells migrated from the explants by day 1, and over the next few days, formed an epithelial sheet with a well defined advancing edge. Following subculturing, the colonies in HS and FBS supplemented cultures consisted of ovoid and round cells, with some elongated cells (fig 1A, B). Increasing the concentration of HS and FBS to 10% resulted in a greater proportion of large and elongated cells compared to the corresponding cultures at 5% concentration.

Cells cultivated in BPE supplemented medium began to migrate from the explants by the second day. Following subculturing, the colonies comprised fewer cells compared with serum supplemented cultures at corresponding time intervals (fig 1C). Large elongated cells were noted in cultures by day 6. Cells cultured in basal medium alone were large and elongated, forming small colonies with limited proliferative capacity (fig 1D).

Fibroblast proliferation was noted in both FBS and HS supplemented media (figs 1E, F) by passage 3 of culture. These were removed by mechanical scraping and incubation with 0.02% EDTA for 5 minutes before subculturing.

Clonal growth and proliferation

HS supplemented cultures had CFEs of 6.7% (SD 1.8%) and 5.6% (1.6%) for 5% and 10% concentrations, respectively. Cells cultivated in FBS supplemented media had the highest CFE (5% FBS, 9.3% (1.8%)) (fig 2). Increasing the concentration of FBS to 10% resulted in a decrease in CFE (7.5% (1.5%)). BPE supplemented cultures had a CFE that was similar to that of HS supplemented cultures (70 $\mu\text{g}/\text{ml}$, 5.9% (1.5%); 140 $\mu\text{g}/\text{ml}$, 6.0% (1.4%)).

Five per cent HS supplemented cultures had a proliferation index of 0.86 (0.16), while increasing the concentration of HS to 10% resulted in a decline in proliferation index to 0.65 (0.15) (fig 3A). FBS supplemented cultures had proliferation indices of 1.11 (0.18) and 0.95 (0.16) for 5% and 10% concentrations, respectively. BPE supplemented cultures had a lower proliferation index compared to 5% HS cultures. These differences, however, were not statistically significant (t test, $p > 0.05$).

The number of cell generations achieved in 5% and 10% concentrations of HS supplemented conditions were 9.5 (1.9) and 7.6 (1.6) respectively (fig 3B). These were lower than that of FBS supplemented conditions (5% FBS, 12.6 (2.3); 10% FBS, 10.9 (1.8)). The number of cell generations achieved in BPE supplemented cultures was similar to that of HS supplemented cultures.

Conjunctival epithelial cell differentiation

The keratin expression of cells cultivated in HS, FBS, and BPE supplemented cultures were consistent with that of normal human conjunctiva. The cultivated conjunctival epithelial cells demonstrated a positive immunoreactivity for antibodies to K4 and K19 (fig 4A, B). The cornea associated cytokeratin, K3, was not expressed by the cultured cells.

Goblet cell differentiation and proliferation

PAS positive goblet cells were observed in HS, FBS, and BPE supplemented cultures (fig 4C). These cells also stained positively for MUC5AC goblet cell mucin (fig 4D–F). Goblet

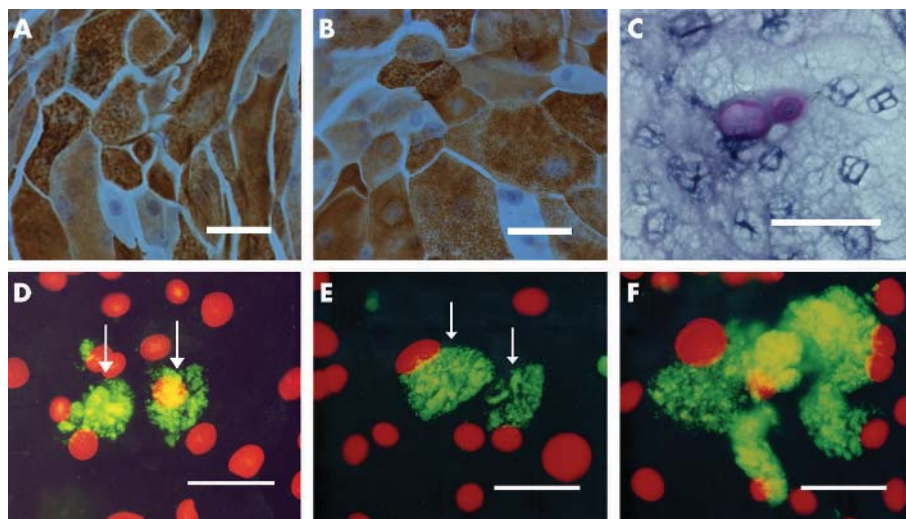


Figure 4 Immunocytochemistry of cultivated conjunctival epithelial cells. Cells cultivated in HS supplemented media stained positively for (A) keratin 4 and (B) keratin 19. (C) HS supplemented culture, showing a pair of goblet cells stained positively with PAS. Immunofluorescence demonstrating a pair of MUC5AC positive cells (indicated by arrows) in (D) HS supplemented and (E) FBS supplemented cultures. (F) A cluster of MUC5AC stained goblet cells in HS supplemented media. (A–C) Bar = 125 μm. (D–F) Bar = 63 μm.

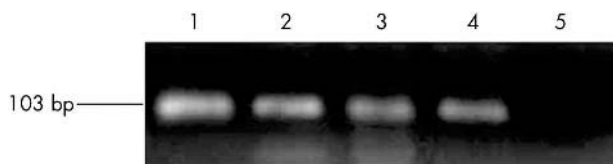


Figure 5 Identification of MUC5AC transcripts in normal conjunctiva and cultivated conjunctival epithelial cells by RT-PCR. MUC5AC transcripts were detected in cDNA from human fornical conjunctiva (lane 1), conjunctival epithelial cells cultured in FBS supplemented media (lane 2), HS supplemented media (lane 3), and BPE supplemented media (lane 4). PCR was performed with water replacing cDNA as the negative control (lane 5). The similar signal for MUC5AC was present in the three culture conditions.

cells were present at a mean density of 52 cells/cm², 60 cells/cm², and 50 cells/cm² for 5% HS, 5% FBS, and BPE supplemented cultures, respectively. The ratio of goblet cells to non-goblet epithelial cells was approximately 1:60, 1:50, 1:65 for HS, FBS, and BPE supplemented cultures, respectively. These differences were not statistically significant (p>0.05). Goblet cells were mainly observed after 5 days in culture, and were found to occur singly, as doublets, or as small clusters of up to 6–10 cells.

The mRNA for MUC5AC goblet cell mucin was detected as a 103 bp band by RT-PCR (fig 5). The expression of MUC5AC

mRNA was found to be similar in HS, FBS, and BPE supplemented cultures.

Xenotransplantation of conjunctival epithelial equivalents in SCID mice

Conjunctival epithelial equivalents cultured in basal medium alone were only 2–3 layers in thickness 10 days after transplantation (fig 6A). Xenografted conjunctival equivalents derived from HS supplemented media formed stratified epithelial sheets 4–7 layers in thickness (fig 6B). Conjunctival epithelial sheets derived from FBS supplemented media were 5–8 layers in thickness, while those derived from BPE supplemented cultures were 4–6 layers in thickness (fig 6C, D).

DISCUSSION

The use of bovine material in the culture media, such as FBS or BPE,^{9–16} is a major concern throughout Europe as bovine spongiform encephalitis cannot be detected by any known *in vitro* tests. As such, European regulatory authorities prefer that cells cultured for clinical use avoid the use of bovine and other animal derived products, so as to reduce the risk of disease transmission. The use of HS as an alternative to bovine serum would help eliminate the need for bovine material in the culture process.

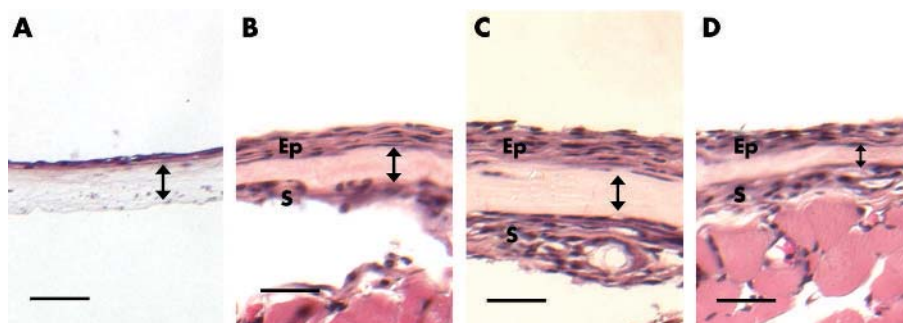


Figure 6 Human conjunctival epithelial equivalents 10 days following transplantation into SCID mice. (A) Basal medium. The conjunctival epithelial sheet consisted of 2–3 layers of cells. (B) HS supplemented culture, demonstrating a multilayered stratified epithelial sheet comprising 4–7 layers of cells. (C) FBS supplemented culture, demonstrating an epithelial sheet consisting of 5–8 layers of cells. (D) BPE supplemented culture, demonstrating 4–6 layers of flattened cells. The HAM is indicated by the area within the double headed arrow. Ep: epithelial sheet; S: stroma of SCID mouse. Bar = 100 μm.

The ability of culture media to support the continued proliferation of cultivated cells in vivo is important for ensuring tissue regeneration following transplantation. Previous reports on epithelial equivalents have mainly focused on obtaining differentiated, stratified tissue constructs.^{5-8, 22-25} However, terminally differentiated cells have limited proliferative potential, and few reports have focused on the ability of cultured cells to continue to proliferate in vivo.²⁶⁻²⁹ We found that conjunctival epithelial cells derived from HS supplemented cultures had comparable in vitro proliferative capacities compared to FBS and BPE supplemented cultures. In addition, transplanted HS derived conjunctival epithelial equivalents underwent a similar degree of stratification and proliferation compared to those derived from FBS and BPE supplemented cultures. These findings provide valuable information regarding the regenerative potential of these cells following transplantation.

HS supplemented medium was also found to support the proliferation of goblet cells. Goblet cells mainly appeared after several days in culture and formed clusters of up to 10 cells. These clusters suggest that bipotent conjunctival progenitor cells present in culture were able to retain their propensity towards goblet cell differentiation after several cell divisions.

The elimination of animal material from the culture process has significant advantages over existing serum supplemented culture methods. The use of HS to replace FBS in the culture process would help to reduce the risk of zoonotic infection. This raises the possibility of using autologous HS for cultivating cells for clinical use. These findings bring us one step closer towards the development of a safe and effective xenobiotic free bioengineered tissue equivalent for clinical transplantation.

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The authors have no competing interest in any of the material discussed in the paper.

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Ethical approval from the institutional review board of the Singapore National Eye Center was obtained for this study.

Written informed consent was obtained from all patients who participated in this study.

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