ORIGINAL ARTICLE

Polyhydroxyalkanoate (PHA) and Wharton Jelly-mesenchymal Stem Cells (MSCs) for Corneal Regenerative Therapeutic Strategy

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ABSTRACT

Introduction: Corneal regenerative medicine is a contemporary approach involving the use of biomaterials, biological factors and stem cells to reduce dependence on native tissue supply for transplantation. This study aims to investigate cellularized polyhydroxyalkanoate (PHA) scaffold with human telomerase-immortalized cornea epithelial cells (HTCEC) in combination with Wharton Jelly-Mesenchymal stem cells (MSC) or its conditioned media (CM) for ocular surface regeneration. **Materials and methods:** The PHA employed in this investigation, P(3HB-co-4HB-co-5HV-co-3HHx), was biosynthesized from *Cupriavidus necator.* The water contact angle as a measure of scaffold wettability was determined, and in vitro PHA biodegradation by lipase enzyme was conducted. The effects of MSC on the focal adhesion proteins were determined by immunofluorescence and immune regulatory proteins were studied. Common corneal genes' expression was evaluated using qPCR. **Results:** There was a significant loss of PHA dry weight due to lipase biodegradation. MSC-conditioned media (CM) significantly improved HTCEC viability (121%) as compared to control (100%), $p < 0.005$. The effect of HTCEC/MSC co-culture on focal adhesion protein expression was significantly higher as compared to HTCEC single culture ($p < 0.05$). MSC co-culture with HTCEC showed increased secretion of IL-1β and TGF-β1 more than single HTCEC culture in a pro-inflammatory stimulated HTCEC model. Gene expressions for common corneal markers *ITGB1, ABCG2, ABCB5, CK3, CK12, CX43*, and *ΔNP63* were upregulated in the presence of CM $p < 0.05$. **Conclusion:** Cellularized PHA scaffold used in combination with MSC is a novel regenerative medicine approach which has a huge potential for anterior ocular surface diseases.
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Keywords: Cornea epithelial cells, Polyhydroxyalkanoate, Conditioned media, Mesenchymal stem cells, Therapeutic strategy

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INTRODUCTION

Ocular surface diseases involve conditions affecting the anterior surface of the eye, commonly the cornea and the conjunctiva. This covers a wide range of patho-physiologies which potentially cause blindness, affecting the quality of life and increasing the economic burden (1). The aim of tissue engineering is reducing the dependence to native tissue supply. Mesenchymal stem cells (MSCs) have become a cellular source as it is rich with bioactive molecules, possess immunosuppressive and anti-inflammatory activities, making it suitable for transplantation (2).

The combination of cellular therapy with biocompatible materials is an innovative strategy which has demonstrated beneficial effects in cardiomyocytes differentiation and cardiac stem cells expression (3). Recent findings showed evidence that MSC enhanced rejuvenation of aged cardiac stem cells in mice (4). In our previous study, Wharton jelly derived-MSCs and corneal epithelial co-cultures have improved characterization and growth kinetics of human corneal epithelial cells (2).

The cell-free therapy approach of using extracellular vesicles (EV) or exosomes from MSC has shown huge potentials in regenerative medicine (2–10). EV are bioactive molecules which mediate cell-to-cell communication, signal transduction and extracellular modelling. In the eyes, human corneal MSC-derived exosomes have been demonstrated to improve cornea

epithelial cellular migration and *in vivo* wound healing in mice (2).

Innovative biomimetic materials with unique properties are serving as *in vivo* substrates or scaffolds for many types of cellular, gene or immune therapies and drug deliveries for corneal regeneration (10). In a preliminary *in vitro* biocompatibility study, PHA showed promising outcomes with great cell adhesion, proliferation, and MSC viability (6).

This article aims to highlight a preliminary investigation into the role of Wharton jelly MSC and its conditioned media in a cellularized PHA scaffold. To the best of our knowledge, this is the first study to explore a novel PHA/HTCEC scaffold for cornea epithelial regeneration, its cytotoxicity evaluation and biodegradability in the eye. This study leverages on the growth promoting effects of MSC and its conditioned media for future ocular clinical applications.

MATERIALS AND METHODS

Ethics and research approval

Research approval was obtained, and the study followed ethical guidelines of the university and is in compliance with Universiti Sains Malaysia current safety policies for research conduct. All experiments were performed according to the Control of Substances Hazardous to Health (COSHH and BIOCOSHH) regulations. Research ethics approval was granted by Universiti Sains Malaysia's Institutional Animal Care and Use Committee (IACUC):2018/(111)(900).

Tissue culture and maintenance

Human telomerase-immortalized corneal epithelial cells (HTCEC) were a gift from Dr Winston Kao from the University of Cincinnati (USA), routinely grown in defined keratinocyte serum free medium (DKSFM, Gibco, Massachusetts, United States). Cells were passaged at 80% confluence by initial treatment with 2 ml cell dissociation buffer-enzyme free solution (Gibco, Massachusetts, United States) followed by Trypsin-EDTA 0.05%-1X solution (Gibco, Massachusetts, United States), incubated for 35 minutes until detached. Cells were collected, centrifuged at 300 x g/5 minutes/4°C), followed by re-suspension of cell pellet for continuous cultures. Changing of medium was performed every 3 days.

Wharton jelly-derived MSC was a generous gift from Cryocord (M); harvested from the discarded cord blood of consented women who underwent lower segment Caesarean section. MSC used in this study has fulfilled the minimum criteria by flow cytometric immunophenotyping and tri-lineage differentiation by histology. MSC was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Massachusetts, United States), 10% fetal bovine serum and 1% penicillin/streptomycin. For indirect co-culture, HTCEC were seeded on 24 well plates (Bio-media, SPD scientific, Singapore) as described above. MSC were seeded on 6.5 µm by 3.0 µm pore size transwell cell insert (Falcon, Corning, USA). After 4 hours, the transwell cell insert was placed over the 24 wells containing cultivated HTCEC, with the bottom part of the inset submerged in the media. As for direct co-culture, both HTCEC and MSC were cultured together in 24 well plates, incubated at 37°C in 5% CO_2 incubator (Celmate, Esco, UK).

Generation of MSC-derived conditioned medium

MSC-derived conditioned medium (CM) was generated from MSC in culture after 28 hours. Firstly, culture medium was removed and rinsed with DPBS x 3 times. 12 ml of low glucose- DMEM serum-free basal medium was added to the culture flask. Cells were incubated for 48 hours, followed by collection of the medium in a 15 ml centrifuge tube. The medium was centrifuged at 300 x g for 10 minutes to remove any residual cells; the supernatant was collected and stored at -80°C until further use.

Polyhydroxyalkanoate (PHA) biosynthesis and film preparation

P(3HB-*co*-4HB-*co*-5HV-*co*-3HHx), which is the polymer used in this study was biosynthesized from *Cupriavidus necator* governed by the enzyme PHA synthase. The gene for the enzyme was cloned into the plasmid (pBBRMCS-2) and then transformed into the bacteria, as previously described [18]. In brief, the bacterial strain was cultured for 12 hours at 30°C in nutrient rich broth medium (Sigma, USA). Cultures were moved under aseptic conditions into a media of mineral supplements, carbon source and fructose, followed by the addition of precursors Na4HB and Na5HV and kanamycin antibiotic. The full detail of nutrient rich media, mineral supplements and trace elements concentrations are provided in Supplementary Table I. The culture was left for 48 hours at 30 °C with gentle agitation and then harvested by centrifugation at 2000 g for 15 minutes at 4° C. The cell pellet was collected and washed twice with distilled water and then lyophilized by freeze drying method. 50 mg of PHA powder was dissolved in tetrahydrofuran (THF) (Merck, USA) and placed in the magnetic stirrer (US151, Stuart, UK) overnight. 80 µl from the solution was cast on 12 mm cover slips (Fisher scientific, USA) with a thickness of 0.1 mm. The films were aged in a desiccator (Japson, India) for one week, and then used for further experiments.

Supplementary Table I: Components of nutrient rich broth medium (50 ml), mineral medium supplements, mineral medium components and trace element constituents.

Component	Concentration
Nutrient rich broth medium (50 ml)	
Extract (Sigma, USA)	10 g/L
Yeast Extract (Sigma, USA)	2 g/L
Peptone (Sigma, USA)	10 g/L
Mineral medium supplements	
Fructose (Merck, USA)	10 g/L
CPKO (IOI Acid Chem, Malaysia)	5 g/L
Sodium 5-hydroxyvalerate (Na5HV) precursor	2 g/L
Sodium 4-hydroxybutyrate (Na4HB) precursor	2 g/L
Mineral medium components	
Magnesium sulphate heptahydrate, $MgSO_4.7H_2O$ (R&M, India)	0.25 g/L
Ammonium chloride, NH_4Cl (R&M, India)	0.5 g/L
Potassium phosphate, KH_2PO_4 (Bendosen, Malaysia)	2.80 g/L
Disodium hydrogen phosphate, Na_2HPO_4 (Bendosen, Malaysia)	3.32 g/L
Trace element solution (R&M, India)	1 mL/L
Trace element solution constituents	
Ferric chloride, $FeCl_3$ (R&M, India)	9.7 g/L
Calcium dichloride, $CaCl_2$ (Merck, USA)	7.8 g/L
Cobalt chloride, $CoCl_2.6H_2O$ (HiMedia, India)	0.22 g/L
Copper sulphate pentahydrate, $CuSO_4.5H_2O$ (HiMedia, India)	0.16 g/L
Nickel chloride hexahydrate, $NiCl_2.6H_2O$ (R&M, India)	0.12 g/L
Chromium chloride hexahydrate, $CrCl_3.6H_2O$ (HiMedia, India)	0.11 g/L

Sterilization of PHA films

PHA films were placed in a 24-well plate and incubated with 400 µl of ethanol (Emsure, Merck, USA) for 30 minutes in humidified CO_2 incubator. Following that, ethanol was removed, and the films were washed with DPBS 3 times. On the final wash, the films were kept in DPBS and further incubated under UV light for 30 minutes in laminar flow. 100 µl from the medium was added to the wells for the conditioning step before being ready for the experiments.

PHA water contact angle measurement

Water contact angle is a measurement of the wettability of PHA which was measured by Optical Tensiometer (Thetaflex, Biolin scientific, Finland). The films were attached to a glass slide which was a part of the machine and measurement of the contact angles was carried out with 2 µl of distilled water droplet whereby the angle was determined by the software (Attention Theta, Biolin Scientific, Finland).

PHA biocompatibility

HTCEC were cultured as single culture in DKSFM or co-cultured with MSC at 1:1 ratio in DKSFM: low glucose µDMEM. After 3 days of incubation, the media was removed and replaced by 10% Presto blue solution (Invitrogen, USA). The control cells were cultured in 24-well tissue culture plates (TCP). Teflon rings were used to weigh down the PHA films. Cell viability was measured using the microplate reader (BMG Labtech, Germany). The following formula was used to calculate for the percentage:

Cell viability (%) = [fluorescence of tested sample/ fluorescence of control sample] X 100 %

***In vitro* biodegradation of PHA**

In vitro degradation of PHA was conducted using lipase enzyme biodegradation method according to published literature (11). 2.5% PHA powder was dissolved in tetrahydrofuran (THF) before casting whereby 80 µL from the polymer solution in a borosilicate circle glass coverslip (22 mm diameter x of 0.16 mm thickness) was left in a vacuum dryer for one week for polymer aging and complete dryness for 14 days at RT. 3 mL of porcine lipase enzyme (Wako, Japan) at 500 µg/mL was used for degradation test and PBS was used as control. The films were placed in 15 mL centrifuge tubes, incubated at 37°C in an incubator shaker at a speed of 40 x g. The experiment was run in triplicates and daily weighing of the films was carried out for five days. For weighing, the films were first rinsed with 70% ethanol followed by distilled water wash. The films were left for complete drying and then weighed using a sensitive balance (Profilab, Germany). The results were calculated based on the % weight loss of the films:

Weight after test/initial weight X 100 %

Immunofluorescence

Immunostaining of vinculin and phalloidin was performed on HTCEC group alone and co-cultured with MSC (HTCEC/MSC). The cells were fixed with 4% paraformaldehyde for 10 minutes; permeabilized with 0.3% Triton™ X-100 for 10 minutes followed by blocking with 1% BSA for 1 hour at room temperature (RT). The cells were incubated with ABfinity™ Vinculin recombinant rabbit monoclonal antibody and incubated overnight at 4°C. To stain for Phalloidin, Alexa Fluor® Phalloidin (1:20) antibody was incubated for 20 minutes at RT. Alexa Flour® 488 Goat Anti-Rabbit IgG at a dilution of 1:100 for one hour at RT was used as secondary antibody. The nuclei were stained with SlowFade® Gold Antifade Mounted with DAPI (1:10000).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

For cornea epithelial gene expression, the steps involved RNA extraction, RNA quantification, reverse transcription, and qRT-PCR. A total of 1 X 10^6 cells were harvested as per manufacturer's instructions. RNA extraction was performed using RNeasy plus mini kit (Qiagen, USA). The g DNA removal was done by adding the volume correspondent to 1 µg of RNA to 2 µL of genomic DNA removal mix and completing the

volume into 15 mL by RNAse free water in a PCR tube followed by an incubation for 2 minutes at 45°C, and later followed by reverse transcription. The expression of the corneal genes was detected using SsoAdvanced Universal SYBR Green Supermix (BioRad, USA). The reaction was prepared using 96-well PCR plates (Applied Biosystems, USA) on ice. Each well was filled with 1 µL (5 ng) of cDNA template, 5µL SsoAdvanced™ Universal SYBR® Green Supermix (1x), 0.5 µL (500 nM) of forward and reverse primers, and 3 µL of RNAse/DNAse-free water. The PCR plate was centrifuged at 750 x g for 2 minutes at 4°C. The qRT-PCR was carried out using QuantiNova SYBR green kit (Qiagen, USA). The PCR was done for 35 cycles; 95°C for 5 minutes as initial denaturation step, denaturation at 95°C for 5 seconds, and finally combined annealing and extension step for 5 seconds. Results were analyzed using delta delta CT method. ACTB was used as a housekeeping gene. The list of PCR primers is available in Supplementary Table II. Statistical analysis was done using Graph Pad Prism 8.0.2. Statistical analysis was done by one-way ANOVA test followed by Tukey's Post Hoc test, $p \leq 0.05$.

Supplementary Table II: List of QPCR primers

Gene name	Sequence (5'-3')	Accession number	Product size (bp)
CK3	F: GACTC-GGAGCT-GAGAAGCAT R: CAGGGTCCT-CAGGAAGTTGA	NM_057088.1	145
CK12	F: TGGAAATGC-CCAGCTCCTCTT R: CGCAGGGC-CAGTTCATTCTC	NM_000223.4	90
NP63	F: TGTGTTG-GAGGGAT-GAACCG R: CAC-CGTTCTTTGT-GCTGTCC	NM_001329150.1	143
CX43	F: TCTGAGTG-CCTGAACTTG-CC R: CCCTCCAG-CAGTTGAG-TAGG	NM_000165.5	171
ITGB1	F: CCGCGCG-GAAAAGAT-GAAT R: GAATTTGTG-CACCACCCACA	NM_002211.4	165
ACTB	F: AGAGCTAC-GAGCTGCCT-GAC R: AGCACT-GTTGGCGTA-CAG	NM_ 001101.3	184
ABCB5	F: GCAGAAGAA-CAGC-CAAAACTGA R: GGCTCCATT-GACCAGTGATG	NM_001163941.2	123
ABCG2	F: CGCGA-CAGCTTCCAAT-GACC R: AGGATGG-CGTTGAGAC-CAGG	NM_001257386.2	174

CONTINUE

Abbreviations: *CK3: Cytokeratin-3, CK12: Cytokeratin-12, NP63 : Delta Np63-alpha, CX43: Connexin-43, ITGB1: Integrin-beta-1, ACTB: Beta-actin, ABCB5: ATP binding cassette-B5, ABCG2: ATP binding cassette-G2*

RESULTS

PHA biosynthesis and characterisation

PHA (3HB-*co*-4HB-*co*-5HV-*co*-3HHx) was successfully biosynthesized, extracted, filtered, collected, and dried. Monomers were analysed by gas chromatography, cell dry weight was 3.1± 0.01 g/L, PHA content was 64±4% (Table I). Polymer contains 80.3% 3HB, 9.1% 4HB, 3% 5HV, and 7.6% 3HHx as had been published previously (12).

Table I: PHA content analysis

Carbon source and precursors	Cell dry weigh (g/L)	PHA content (%)	3HB content (%)	4HB content (%)	5HV content (%)	3HHx content (%)
CP-KO+Na4H-B+Na5HV	3.1±0.01	64±4	80.3	9.1	3	7.6

PHA wettability

The water contact angle (WCA) for P (3HB-*co*-4HB-*co*-5HV-*co*-3HHx) surface was tested to determine its wettability using the telescope goniometer method (Fig. 1). The average from the five samples was 96.6° which showed that this polymer was hydrophobic.

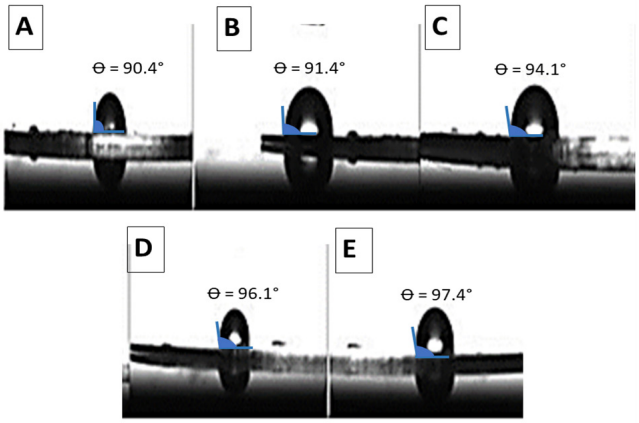

Fig. 1: Water contact angle of PHA. A - E show representative samples' water contact angles were between 90.4° to 97.4°; the configuration of the water droplet relies on the nature and surface tension of the polymer.

In vitro biodegradation of PHA

In vitro biodegradation of the PHA (3HB-*co*-4HB-*co*-

5HV-*co*-3HHx) was determined by measuring the weight loss of the cast films in five days after the treatment with porcine lipase enzyme. On day 5, the films had degraded, losing up to 77% of the initial weight. The experiment was performed in triplicate and the average of the results was taken (Fig. 2).

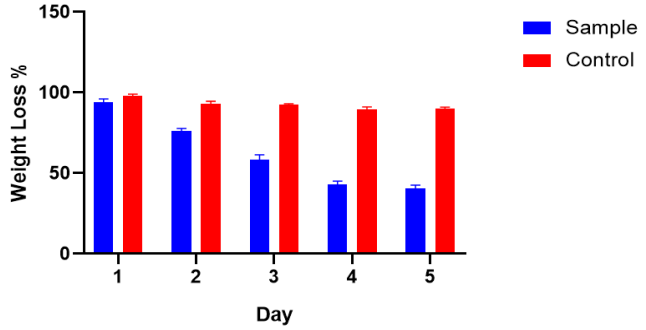

Fig. 2: PHA biodegradation by porcine lipase enzyme at 37°C. There is a significant weight loss between the sample and control from day 1 to day 5, ($p < 0.05$), N=3. Blue = PHA films with lipase, Red = Control (PHA in DPBS).

Role of MSC and conditioned medium on PHA biocompatibility

Presto blue cell viability assay was used to test the PHA biocompatibility of HTCEC; with and without MSC and CM (Fig. 3). Cell viability for both PHA and TCP are similar (100%), while in the presence of CM the cell viability of HTCEC on PHA has increased significantly.

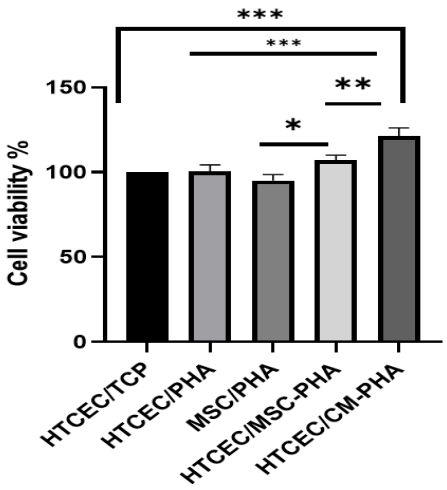

Fig. 3: Mean cell viability of HTCEC on PHA with and without MSC and addition of CM. HTCEC viability on PHA was similar to TCP control (100%). As for MSC effects, addition of MSC to the HTCEC/PHA construct has significantly increased the viability to 110%, compared to its control, MSC/PHA (95%). Addition of CM to the HTCEC/PHA gave the best results, surpassing the viability of HTCEC/MSC-PHA group, (*p<0.05), ($p \leq 0.05$), and (*** $p \leq 0.001$), N=3.**

Role of MSC on corneal adhesion proteins

MSC role in the expression of focal adhesion proteins (phalloidin and vinculin) of HTCEC was investigated by immunofluorescence as shown in Fig. 4. HTCEC cocultured with MSC has increased the expression of both molecules significantly ($p < 0.05$).

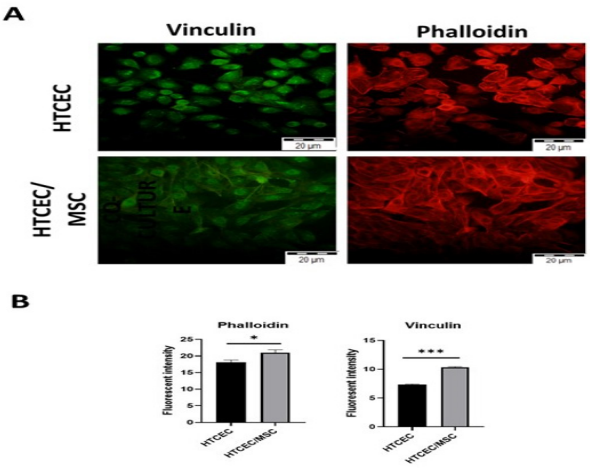

Fig. 4: The expression of corneal adhesion molecules phalloidin and vinculin by immunofluorescence. A) Expression of both proteins increased in HTCEC/MSC cocultures compared to HTCEC single cultures. B) Levels of colour intensity by image J showed the significant difference, (*$p < 0.05$, *** $p \leq 0.001$), N=3.

Role of MSC on IL-1β and TGF-β1 in pro-inflammatory stimulated HTCEC

IL-1β and TGF-β1 levels were determined in IFN-γ stimulated HTCEC to evaluate the effects of MSC on wound healing in an inflammatory model. For IL-1β, the expression was higher upon the addition of MSC to the HTCEC-stimulated cells as compared to untreated HTCEC and treated HTCEC without MSC, although the differences were not statistically significant ($p > 0.05$). Upon the addition of MSC to the stimulated HTCEC, the level of TGF-β1 has increased significantly as compared to the levels found in the untreated HTCEC and treated HTCEC without MSC groups, $p \leq 0.001$, Fig. 5.

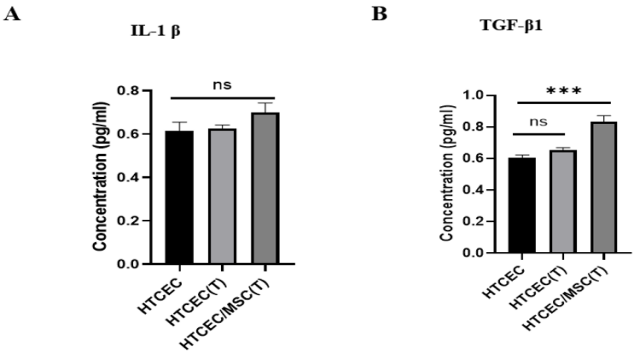

Fig. 5: Effects of MSCs on IL-1β and TGF-β1 in HTCEC. (A) After 48 hours co-culture, addition of MSC has increased secretion of IL-1β in the IFN-γ -stimulated group HTCEC/MSC(T) and untreated HTCEC groups, although the difference is not statistically significant ($p > 0.05$) (B) IFN-γ stimulated HTCEC/MSC(T) co-culture showed a significant increase in TGF-β1 level compared to treated cells without MSC, HTCEC(T) and un-treated HTCEC, $p \leq 0.001$, N=3.

Role of MSC and conditioned media on corneal markers' gene expression

Significantly high gene expression of Integrin β1 (ITGB1) was demonstrated in the presence of conditioned media (CM) in cultured HTCEC on PHA scaffold as compared to control, $p < 0.0001$ (Fig. 6). Similar effects of CM were

evident in the expression of the putative transcription factor DeltaNp63 gene as compared to control, $p < 0.001$, ABCB5 gene, $p < 0.01$, connexin 43 gene, $p < 0.01$, and ABCG2 gene, $p < 0.05$. The presence of CM has also significantly expressed epithelial differentiation markers CK3 and CK12, $p < 0.0001$ and $p < 0.01$, respectively.

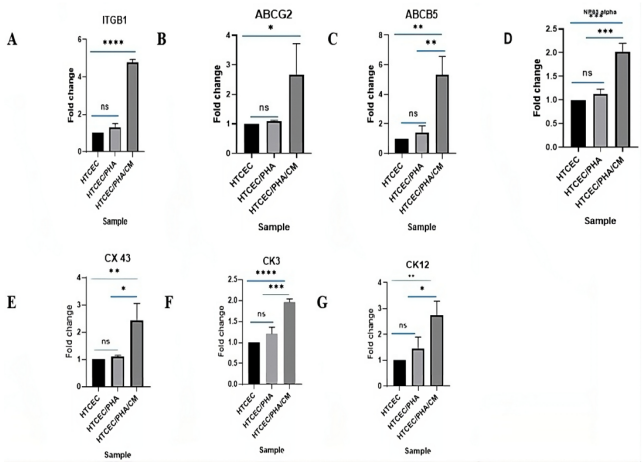

Fig. 6: The expression of corneal epithelial markers on different surface and culture conditions by qPCR. There is no significant difference in the expression levels between control group HTCEC/TCP, and HTCEC cultivated on PHA (HTCEC/PHA). However, upon addition of CM treatment (HTCEC/PHA/CM), gene expressions for *ITGB1, ABCG2, ABCB5, ΔNp63α, Cn43, CK3* and *CK12* showed significant upregulation. Data normalized to *ACTB* levels. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

DISCUSSION

P(3HB-*co*-4HB-*co*-5HV-*co*-3HHx) are the monomers chosen based on its potential biomedical applications. Mechanical properties and biodegradability are the most desirable characteristics for designing a corneal scaffold from PHA. In this study, PHA was biosynthesized using *Cupriavidus necator* bacterium from carbon sources which are structurally related i.e., 4-hydroxybutyric acid and 1,4-butanediol. P(4HB) has an excellent biodegradation rate and the biodegradation products become normal metabolites in the human body. P(4HB) is also FDA-approved as biocompatible and appropriate for medical applications such as suture materials (13).

3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) are less acidic, easily metabolized in the body in one hour or less, and the byproducts are also naturally found in the human body (14). 5-hydroxyvalerate (5HV) is less toxic to the body as compared to lactic and glycolic for example. 3HHx monomer was chosen as it has a good tensile strength, and particularly the aligned ultrafine fibres (15) has high elasticity value and hemocompatible (16). Hydroxybutyrate and hydroxyvalerate monomers are also safe from tumor induction as demonstrated by Peng and colleagues (17). This data supported the selection of PHA monomers used in this study.

Cellular interaction with the scaffold would determine the cell behavior, functions, and capacity for tissue repair. The tensile strength of a fabricated corneal cell construct is important for easy handling during transplantation; a key characteristic which is easily found in a PHA polymer, as well as being light transmissible (18).

Wettability of the polymer affects the cell-to-polymer interaction and this is influenced by the roughness of the polymer surface and its functional groups (19). In the current study, the polymer showed slight hydrophobicity. It is understood that mammalian cells prefer polymers with moderate hydrophilicity (more than 5° water contact angle). WCA which falls below 90° is considered as hydrophilic and hydrophobic when WCA is above 90°(11). Superhydrophobic polymers with contact angle above 150° are not generally suitable for cell growth (20,21). To overcome this limitation, PHA is amenable for further refining to obtain a more acceptable wettability to fulfil the biomedical requirements.

One of the most crucial concepts is the assurance that the polymer is biodegradable as they can break down naturally and safely, causing no harmful effects to the body system. In this current study, P(3HB-*co*-4HB-*co*-5HV-*co*-3HHx) was a highly biodegradable PHA especially synthesized and designed for ocular application. The *in vitro* biodegradation study using lipase enzyme showed a high degradation rate, attributed to the 3HB and 4HB monomers incorporated in the polymer. As the mode of PHA biodegradation is dependent on lipase, there is an abundant level of the enzyme in many body organs including the eye (22) whereby lipase modulates the function of the lipids in the tear fluid and the corneal epithelial cells (23).

In corneal regenerative strategies, cellular attachment, proliferation, and migration are essential processes involved to ensure successful tissue integration and wound repair. Stem cells activation in its microenvironment heralds a specific cell differentiation from a multipotent cells source usually coordinated by a complex signaling network (24). Vinculin and phalloidin (F-actin) have been widely proposed as potential attachment proteins which link the focal adhesions and the actin cytoskeleton. A study revealed that the migrating corneal epithelial cells elaborate actin filaments lay off at cell-to-substratum adhesion complexes (25). In the migrating sheet of corneal epithelial cells, there was an evidence of vinculin abundance. Taken together, actin and vinculin are important for corneal epithelial cell migration and adhesion (26).

In this study, MSC/HTCEC coculture demonstrated increased expression of corneal adhesion proteins. As shown in Fig. 4, there is a strong presence of skeletal proteins as evidenced by immunofluorescence studies. This finding supports MSC as key factors in cellular

migration and adhesion via upregulation of vinculin and phalloidin (F-actin) expression. The role of MSC in corneal wound healing has been described previously, where differentiation of MSC into myofibroblasts was indicated by the upregulation in the smooth muscle actin expression in a rat alkali burn model (27). Azmi and colleagues also reported that the role of human umbilical cord MSC had improved HTCEC migration and protected them from early senescence (28).

IFN-γ stimulation model is relevant to exploring the immunoregulatory pathways in corneal wound healing, especially in potentially blinding and painful eye conditions due to infection, dry eye syndrome, connective tissue disease or chronic cicatricial conditions and others. The IFN-γ stimulated co-culture model of HTCEC/MSC showed a major upregulation in TGF-β1 and an increased level of IL-1β, although the latter was not statistically significant (29).

Both TGF-β and IL-1β are master regulators of corneal wound healing. There are multiple interactions between IL-1 and TGF-β, as well as other cytokines and trophic factors in modulating wound injury response in the cornea. These communications could be regenerative in nature or fibrotic, leading to corneal scarring. IL-1β modulates healing functions, such as keratocyte apoptosis, chemokine production by corneal fibroblasts, and release of hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) by keratocytes and corneal fibroblasts. It is also involved in modulating corneal epithelial cell migration, proliferation and differentiation to heal epithelial defects (30).

It is believed that this involves upregulation of the matrix metalloprotease-9 (MMP-9) via the NF-K β and AP-1-Dependent pathways (31). TGF-β1 and TGF-β2 from corneal epithelial cells are important in the development of myofibroblasts from their precursors, giving rise to wound contraction and scar formation in the corneal stroma (32,33). Here, our findings are important for future research directions, specifically in the role of MSC as an immunomodulator in the TGF-β pathway associated with potentially blinding inflammatory eye conditions.

In this study, we demonstrate CM was superior to HTCEC/MSC co-culture in the growth of HTCEC on PHA. The latter was comparable to the HTCEC growing on TCP control. The cell viability assay reflects the biocompatibility of PHA as a scaffold for cornea epithelial regeneration. There is an increasing evidence on the use of CM as a cell-free therapy, manipulating its repertoire of growth factors and cytokines in xenografts as well as allografts (34) such as evidence from bone marrow-CM accelerating wound healing by promoting pro-collagen synthesis and skin hydration (35). CM contains many components like vascular endothelial growth factor (VEGF) which has neurotrophic and proangiogenic factors for endothelial cells proliferation, pigment epithelium-derived factor (PEDF), and hepatocyte growth factor (HGF); which has immunomodulating function as well as possessing angiogenic and anti-apoptotic functions (36).

A panel of corneal gene markers was used to study the effects of CM on HTCEC growth with or without PHA. We found that the addition of CM upregulated corneal genes expression at varying significance levels (Fig. 6). Among these molecular markers, integrins and delta Np63 showed the highest significant levels of upregulation. Integrins are cell adhesion molecules which play a significant role in mediating the mechanical stress, cell-to-cell and cell-to-extra cellular matrix interaction, in particular, the role of the alpha and beta subunits integrin receptors (37–40). Integrin beta 1 (ITGβ1) and fibronectin are actively involved in the corneal wound healing process. The loss of ITGβ1 resulted in loss of epithelial layer, increased gaps between the collagen fibrils, subsequent thinning of the cornea and loss of cornea integrity (37).

Nuclear transcription factor p63 gene controls the cell proliferation and renewal of corneal epithelial cells. Delta Np63α is the main p63 isoform in the process of ectoderm development, and it is also critical for tissue homeostasis and cancer formation (41–43). ABCG2 and ABCB5 are among members of the family ATP binding cassette (ABC) transporters. ABCG2 gene was first identified as a molecular marker of bone marrow stem cells. ABCG2 protein is found in the human limbus. Its specific location is the basal epithelial cells in both membrane and cytoplasm. It is expressed by corneal epithelial cells. ABCB5 gene is important in the limbal stem cells maintenance. It has a role in the development of cornea as well as corneal repair. It controls the cell fusion, growth, and differentiation process via an anti-apoptotic function which is very crucial for the maintenance and persistence of stem cells (44).

Connexin 43 is a gap junction protein belonging to the connexins family, assembled with many other proteins to form the gap junctions (45). It is a cell surface membrane structure connected to adjacent cells' cytoplasm. It has been found in many epithelial tissues including corneal epithelium (46). Results of this study proved that HTCEC on PHA expressed *CX43* and the expression was significantly upregulated on addition of CM.

Cytokeratins are used in the differentiation between corneal epithelial cells and other cells. More than 20 cytokeratins known up-to-date (47). These cytokeratins are expressed in different type of cells, but there are two specific types which are only expressed in matured corneal epithelial cells: CK3 and CK12 (48). Rabbit bone marrow-MSC retained the cobblestone morphology of the epithelial cells and the cells that were treated with CM expressed CK3 gene which is a corneal epithelium

specific gene (49).

CONCLUSION

PHA has useful physicochemical and mechanical properties that are adjustable according to the composition and type of monomeric units for specific biomedical applications. The use of PHA films for ocular application is highly potential as demonstrated by the biocompatibility assay and *in vitro* biodegradation study. HTCEC growth, viability, and corneal gene markers' expressions on PHA were better than control on addition of MSC or CM. MSC also showed immunomodulatory effects in IFN-γ stimulated cornea epithelial cells which provides a good inflammatory eye model involving TGF-β1 and IL-1β pathways. This study presented preliminary data for a novel tissue-engineered approach using biodegradable PHA supplemented with MSC or CM for corneal epithelial wound healing and regeneration. .

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