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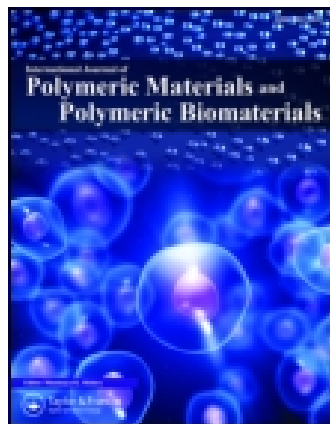

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Cellular Response of Limbal Stem Cells on Poly (Hydroxybuthyrate-co-Hydroxyvalerate) Porous Scaffolds for Ocular Surface Bioengineering

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Cellular Response of Limbal Stem Cells on Poly (Hydroxybuthyrate-co-Hydroxyvalerate) Porous Scaffolds for Ocular Surface Bioengineering

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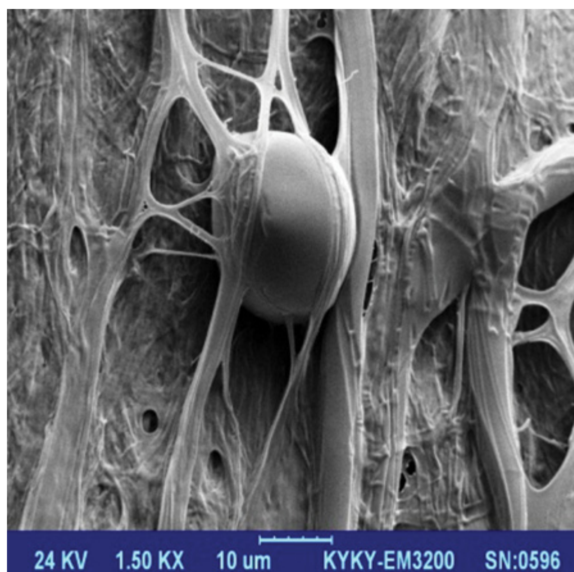
The aim of this study was to develop a modified-porous poly (3-hydroxybutyrate-co-3- hydroxyvalerate) (PHBV) scaffold for limbal stem cell (LSC) expansion that can serve as a potential alternative substrate to replace human amniotic membrane. The human limbal stem cell was used to evaluate the biocompatibility of substrates (porous scaffold, human amniotic membrane and thermo-responsive substrate) based on their viability, proliferation, and attachment ability. Biocompatibility results indicated that the all substrates were highly biocompatible, as LSCs could favorably attach and proliferate on the scaffold surface. Microscopic figures showed that the human limbal stem cell was firmly anchored to the substrates and were able to retain a normal corneal stem cell phenotype. Microscopic analyses illustrated that cells infiltrated the porous scaffold and successfully formed a three-dimensional corneal epithelium, which was viable for two weeks. Gene expression results revealed no change in the expression profile of LECs grown on scaffold when compared to those grown on human amniotic membrane or thermo responsive substrate. In addition, porous PHBV substrate provides not only a milieu supporting LSCs expansion, but also serve as a useful alternative carrier for ocular surface tissue engineering and could be used as an alternative substrate to amniotic membrane.



Keywords: Cellular analyses, LECs, limbal stem cells, modified porous scaffold, Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

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1. Introduction

To support normal vision the renewal of the corneal epithelium is particularly important, and the source of the cells for this continuous process is found in the limbal epithelial zone surrounding the corneal periphery [1,2]. Therapeutic transplantation of the limbus has been developed for ocular surface disease and injury in which presumed stem cell deficiency has occurred; [3,4] however, in some situations healthy remaining limbal tissue may be very limited. Depletion of the limbal stem cell population is a pathologic feature of many ocular surface diseases such as Stevens-Johnson syndrome, chemical and thermal burns, ocular surface tumors, immunological conditions, radiation injury and inherited syndromes [5]. Cell culture and clonal expansion of autologous limbal cells from the opposite eye has been increasingly used to avoid the problems associated with the need to replace corneal epithelium without reverting to allografts and the risk of immune rejection [6–8]. One of the major problems associated with Stem cell therapy remains the absence of a suitable carrier for the transfer of stem cells to precise tissue locations. So far, various materials and scaffolds have been tested for the transportation of stem cells. For example, macroporous hydrogels have been used to deliver mesenchymal stem cells (MSCs) for spinal cord injury repair [9] or self-assembling peptide nanofibers have been tested for myoblast transplantation in infracted myocardium [10]. To treat severe ocular surface damage and a deficiency in limbal stem cells (LSCs), which are irreplaceable for corneal healing, various carriers for the culturing of LSCs and for their transplantation onto the recipient eye have been tested. They include fibrin glue [11], polymers or collagen sponges [12], and human amniotic membrane [13]. Among them, human amniotic membrane is the clinical standard substrate for ocular surface repair owing to its biological properties that inhibit inflammation, tissue scarring, and angiogenesis [13]. However, limitations regarding the use of amniotic membrane exist. These include relatively poor mechanical strength, semitransparent appearance, difficulty of handling, and the potential risk of disease transmission, such as human immunodeficiency virus (HIV), hepatitis B virus, hepatitis C virus, and syphilis. One of the key factors of tissue engineering is to create a three-dimensional scaffold with suitable properties also, such as degradation rate, high porosity, and interconnected pores. Typically, biodegradable polymeric scaffolds are fabricated using different methods [14,15]. In natural tissues, cells are surrounded by extracellular matrix, which has physical structural features ranging from nanometer to micrometer scale. To mimic the natural extra cellular matrix (ECM), many research groups tried to fabricate scaffolds by different methods [16–25]. Polyhydoxyalkanoates (PHAs) are polyesters produced by microorganisms under unbalanced growth conditions. PHAs are generally biodegradable, with good biocompatibility, making them attractive as tissue engineering biomaterials. We sought to deter mine whether adult tissue-specific SCs can also be grown on scaffolds and whether these scaffolds can be used as carriers for cell transplantation in tissue regeneration. In our previous study, USSCs (unrestricted somatic stem cells) were utilized as a supportive layer to support the growth of limbal stem cells [26]. In fact, USSCs are considered as the pluripotent and are one of the rare populations in umbilical cord blood. Moreover, USSCs have a high potential of proliferation and differentiation. Therefore, USSCs are a valuable source for cell therapy. These supportive cells are CD45 negative, adherent and HLA class II-negative stem cells with a long telomerse. Additionally, these cells possess a unique profile of cytokines and a high production rate of self-renewal factors [26,27].

In this study, porous PHBV scaffold was fabricated by freeze-drying and crosslink with chitosan by chemical method. The samples were evaluated by scanning electron microscope (SEM) and in vitro assays, then loaded with human limbal stem cells in the presence of mitomycin C-treated USSC feeder layer and supplemental hormonal epithelial medium (SHEM), and then LSCs transferred over thermo responsive dish, amniotic membrane, and modified PHBV scaffold, then investigated by microscopic analyses, cellular investigations, and other markers at the RNA level as well as the expressed proteins.

2. Experimental

2.1 Materials and Scaffold Preparation

In previous work, we were successfully designed thermo responsive dish [28], and modified-porous PHBV mat [29]. Briefly, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) containing 12% by mole of 3-hydroxyvalerate, were supplied by Sigma-Aldrich (USA) and were used to fabricate polymeric scaffolds. For scaffold fabrication, PHBV powder was weighed accurately and poured into a beaker (50 mL). Then an accurately measured amount of dicholoromethane was added to the tube to make a solution with a desired PHBV concentration at 10% (w/v). To obtain a homogeneous PHBV solution, the mixture was kept at 50°C in a water bath and mixed thoroughly.

2.2 Chitosan-Modified Scaffold Preparation by Chemical and Freeze-Drying Method

Chitosan (DA: 75–85%; Medium molecular weight; Sigma-Aldrich) was immobilized on the freeze-dried PHBV surfaces based on the following protocol. A weighted amount of the chitosan was rinsed with acetic acid buffer solution (50 mM, pH = 5.0). Then, the porous scaffold was submerged into the 6 M NaOH solution for 15 min. The hydrolyzed scaffold mats were rinsed into MES buffer (pH = 6.0) containing 10 mM EDC and 10 mM sulfo-NHS to activate the carboxyl groups on the surfaces. Chitosan solution (15 mg/mL in acetic acid buffer solution, 50 mM, pH = 5.0) injected into the previous solution and was shaken gently for 24 h at 4°C. Cross-linked scaffold was quenched in liquid nitrogen, then transferred to a freeze-dryer apparatus (Gamma 2–16 LSC, Martin Christ, Germany) and kept at −50°C for 24 h. The scaffolds were stored in a vacuum desiccator at room temperature for storage and for further

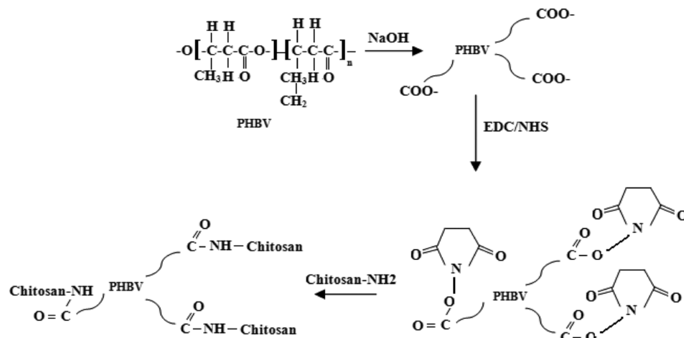

Fig. 1. Schematic illustration of the process of the chitosan grafting onto the PHBV surfaces.

removal of any residual solvent. The preparation process is shown in Figure 1.

2.3 Cellular Analyses

All cell culture reagents used were from Invitrogen-Gibco (Grand Island, NY). Cell culture plastic was from BD Biosciences (Lincoln Park, NJ). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Mouse anti-human cytokeratin 3 (K3), anti-human cytokeratin 12 (K12) antibody, Mouse antihuman connexin 43 antibody, Mmuse anti-human p63 antibody, and fluorescein isothiocyanate (FITC)-conjugated rabbit antimouse secondary antibody, antibodies were purchased from Abcam (Cambridge, England). Mounting media contained DAPI (4,6-diamidino-2- phenylindole) was purchased from Sigma-Aldrich (St. Louis, MO). Limbal stem cells isolation from the limbus biopsy first cultured on the USSC feeder layer and then transferred over amniotic membrane, thermo responsive dish, and modified porous scaffold. For preparation of USSC feeder layer; confluent USSCs were incubated with 4 μg/mL mitomycin C (MMC) for 2 h at 37°C under 5% CO_2, trypsinized and plated onto cell culture dishes at a density of 2.2×10^5 cells/cm^2. These feeder cells were used 4–24 h after plating [32,33]. Human limbal rims discarded after corneal transplantation were provided by the Iranian Eye Bank and were washed in phosphate buffer solution (PBS) containing 100 U/mL penicillin, 50 μg/mL gentamicin, and 2.5 μg/Ml amphotericin B. After careful removal of corneal epithelium, iris, excessive sclera, conjunctiva and subconjunctival tissue under surgical microscope (Zeiss, Oberkochen, Germany), the limbal rings were exposed to dispase II (1.2 IU/mL in Hanks's balanced salt solution free of Mg^{2+} and Ca^{2+}) at 37°C under humidified 5% CO_2 for 3 h. The loosened epithelial sheets were removed with a cell scraper and separated into single cells by 0.25% trypsin +0.02% ethylenediaminetetraacetic acid (EDTA) for 5 min. Cells were pelleted at 1000 rpm for 5 min and resuspended in SHEM. SHEM consisted of an equal volume of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12, supplemented with 5% fetal bovine serum, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 2.5 μg/mL epidermal growth factor, 8.4 ng/mL cholera toxin asubunit, 0.5% dimethyl sulfoxide (DMSO), 0.5 μg/mL hydrocortisone, 50 μg/mL gentamicin, 1.25 μg/mL amphotericin B, and 5 mM HEPE. Cells were plated at 10^4 cells/cm^2 on cell culture dishes containing MMC-treated USSCs feeder layer. Cultures were incubated at 37°C with 5% CO_2/95% air. Medium was changed every two days. Upon reaching 70–80% confluence, the USSCs feeder layer was removed and the limbal stem cells were subcultured to the next passage or transferred over modified porous scaffolds, amniotic membrane, and thermo responsive dish. Limbal stem cells at a plating density of 10^4 cells/well were seeded on an amniotic membrane (AM), thermo responsive dish and modified porous scaffold. After 1, 7, and 15 days of LSCs culture, for analysis of proliferation rate and viability, 20 μL of MTT (sigma) substrate (of a 2.5 mg/mL stock solution in phosphate-buffered saline [PBS]) was added to each well, and the plates were returned to standard tissue incubator conditions for an additional 4 h. Medium was then removed, the cells were solubilized in 100 μL of dimethyl sulfoxide (DMSO), and colorimetric analysis was performed (wavelength, 570 nm RAYTO Micropleat Reader). For scanning electron microscopy study, the scaffolds with cells were washed by PBS, and then fixed by glutaraldehyde (2.5%) at 4°C for 2 h.

The samples were dehydrated by methanol (20% [5 min] → 40% [5 min] → 60% [5 min] → 80% [5 min] → 100%[30 s]) and then kept with tetraoxide osmium vapors at 4°C for 2 h. The samples were kept in desiccators, coated with gold, and investigated by a scanning electron microscope (Cambridge Stereo-scan, S-360, Wetzlar, Germany).

2.4 Gene Expression

Total RNA was isolated from the cells using an RNA extraction kit (Fermentas International, Burlington, Canada). RNA samples were treated with DNase I (Fermentas International, Burlington, Canada) in order to avoid the genomic DNA contamination. RNA quantity was assessed by spectrophotometry (Nano Drop, Thermo, Wilmington, USA). For Reverse transcription 2 μg of total RNA was used with the Revert Aid-first strand cDNA synthesis kit (Fermentas International, Burlington, Canada). RNA extracted from limbus tissue was used as a positive control. Real time PCR of K3, K12, and P63, ABCG2 genes after 7 and 15 days co-culture LSCs on modified porous scaffold, amniotic membrane, and thermo responsive dish. Real Time-PCR (Rotor-Gene Q Real-Time PCR System, Qiagen, USA) reaction was performed with SYBR Premix Ex Taq (TAKARA BIO, INK, Japan) which uses Taq Fast DNA Polymerase, SYBR Green I dye to detect double-stranded DNA. The reaction was performed with following program; 5 min of 95°C for enzyme activation, initial denaturation for 20 s at 95°C, annealing temperature for 40 s, and extension at 72°C for 1 min, followed by 40 cycles with a final extension at 72°C. The final stage comprises the analysis of the melt curve through a denaturing step (15″ at 95°C) followed by annealing (1′ at 60°C) and ramping to 95°C with 0.3°C increment/step. Levels of mRNA for tested genes were quantified using ΔΔCT method and normalized against

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human β-actin as a housekeeping gene. Data were expressed as Log 10 mean. Statistical analysis was performed using ANOVA. A P value 0.05 was considered to be significant. The level of candidate genes in different sample types was compared by the Fisher LSD test.

2.5 Immunocytochemistry (ICC)

LSCs after 15 days cocultured on Amniotic Membrane, Modified PHBV Scaffold and Thermo Responsive Dish at 60–80% confluence were fixed with 4% paraformaldehyde for 10 min at room temperature. After blocking with 3% bovine serum albumin (BSA)/0.3% Triton X-100/PBS for 30 min at room temperature, cells were incubated for 2 hours at room temperature with primary antibody in 1% BSA/PBS at the following dilutions: K3 1:100, K12 1:100, ABCG2 1:100, and p63 1:25. After staining with proper secondary antibody, the cells were mounted with DAPI containing media.

3. Results and Discussion

Figure 2 shows the SEM images of the modified porous scaffold, the thermo responsive dish, and the amniotic membrane. Cell growth slowed significantly and only small cone-shaped colonies were observed under the electron scanning microscope. Figure 3 shows the MTT assay for samples. The results showed a high viability for the all samples. In addition, bio viability was similar between the porous scaffold and the other substrates.

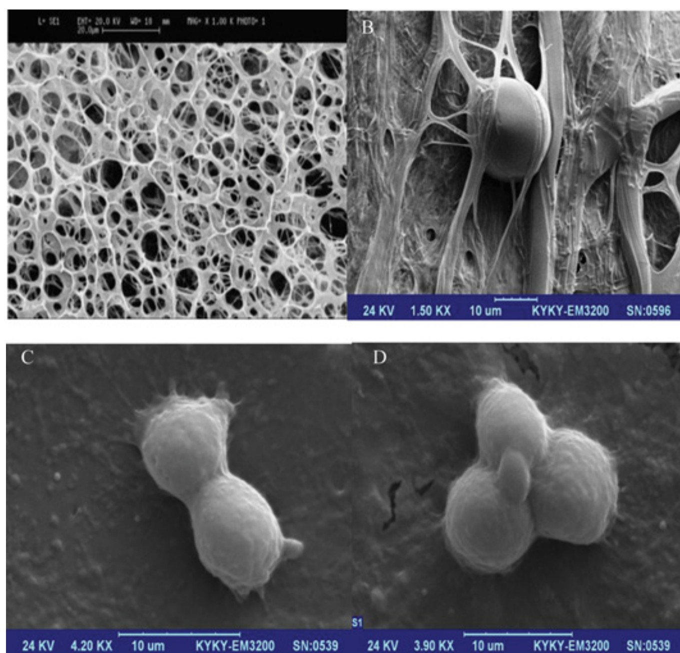

Fig. 2. SEM images of the designed substrates on the (A) modified porous scaffold, (B) cultured limbal stem cells on modified porous scaffold, (C) thermo responsive dish, and (D) amniotic membrane.

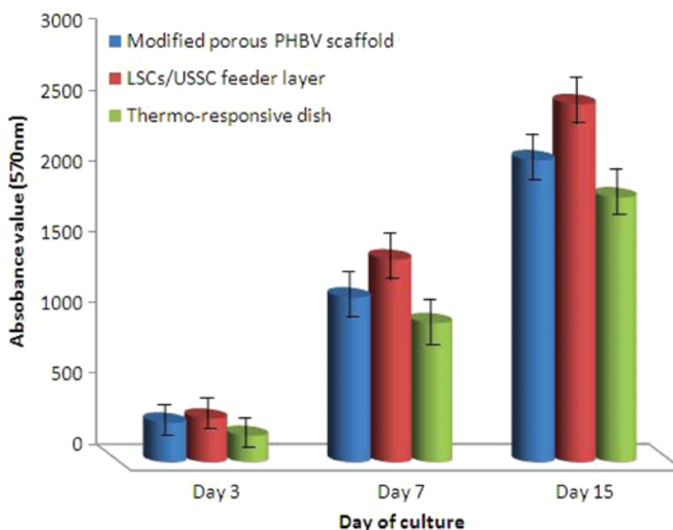

Fig. 3. The MTT assay result for rate of proliferation of LSCs after coculture on USSC feeder layer for modified porous scaffold, thermo responsive dish, and amniotic membrane in 3, 7, and 15 days of continuous culture.

3.1 Gene Expression Analysis

Thermo responsive dish, modified porous scaffold and amniotic membrane were analyzed for expression of KRT 3/12, P63, and ABCG2. GAPDH was used as an endogens control. Expression level of ABCG2 and P63 in amniotic membrane and thermo responsive dish were higher than modified porous scaffold. Furthermore, modified porous scaffold and amniotic membrane do have expression of KRT3, but in addition, the expression level KRT12 for modified porous scaffold is lower that amniotic membrane (Figure 4).

3.2 Immunocytochemistry

An immunocytochemistry assay was also carried out for KRT3/12, ABCG2, and p63. All of the limbal stem cells on thermo responsive dish, modified porous scaffold and amniotic membrane were positive for ABCG2, and p63. Furthermore there are negative for KRT3/12, but were better for amniotic membrane and thermo responsive substrate than modified porous scaffold (Figure 5).

In recent years, many carriers have been used to culture limbal stem cells; however, the majority of previous studies have used amniotic membrane as a choice of carrier due to its low-cost procurement, easy availability, promote epithelialization, and reduced inflammation and scarring [30,31]. Nonetheless, due to its biologic origin, amniotic membrane carries inherent risks such as disease transmission and infection that cannot be totally avoided [32]. The use of synthetic biomaterial can eliminate the risk factors associated with biologic materials. Various natural and synthetic extra cellular matrixes have been used previously for ocular surface bioengineering [33], such as fibrin [34], collagen [35], amniotic membrane [36], temperature responsive cell culture surfaces [37], human anterior capsule [38], chitosan [39], Mebiol gel [40], and silicon [41]. Here, our aim is to develop

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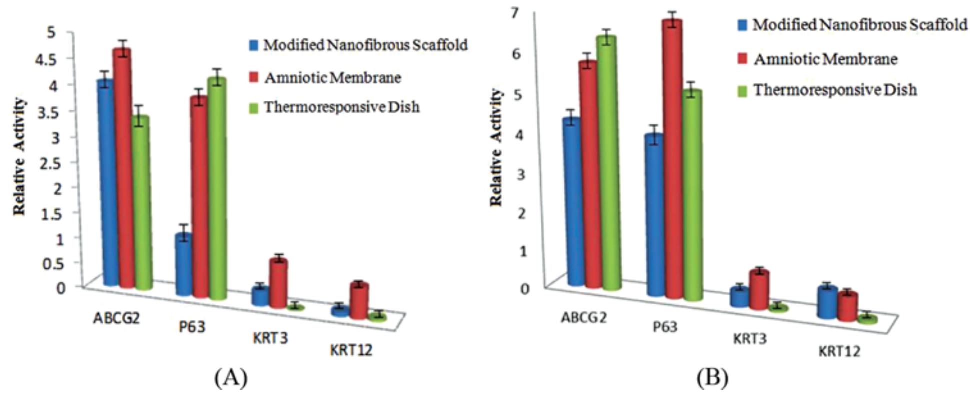

Fig. 4. The expression level of KRT3/12, ABCG2, and p63 in amniotic membrane, thermo responsive dish, and modified porous scaffold after 7 (A) and 15 (B) days ($p < 0.001$).

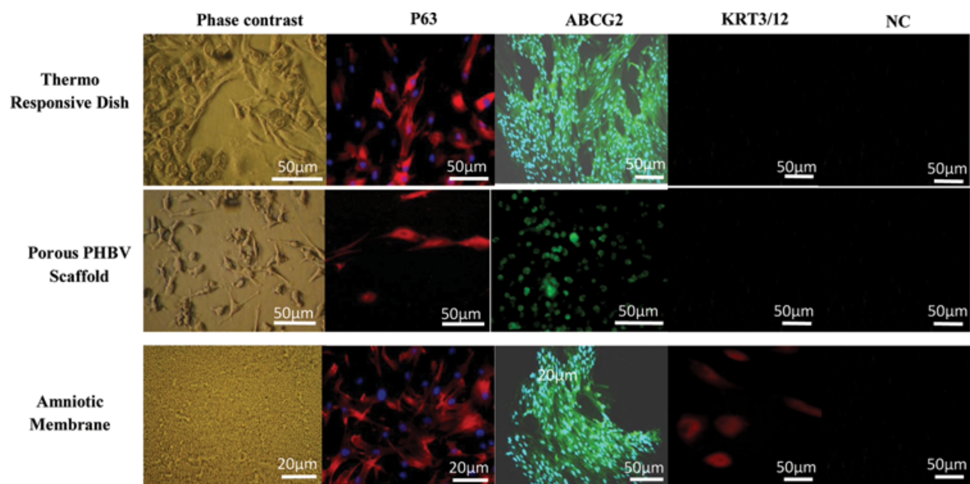

Fig. 5. Immunocytochemistry of KRT3/12, ABCG2, and p63 for LSCs after coculture on the amniotic membrane, thermo responsive dish, and modified porous scaffold.

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a biocompatible synthetic substrate that can work as an excellent scaffold for limbal stem cells expansion and should share a close resemblance to the native extra cellular matrix. In this study, we have evaluated the adhesion, growth, motility, and phenotype of the limbal stem cells cultured on cross-linked porous PHBV scaffold. SEM findings substantiate the hypothesis that the structural properties and architecture of the cross-linked porous PHBV scaffold are able to create natural extra cellular matrix for cell growth and have an effect on cellular attachment and proliferation. High cell viability suggests that under our fabrication conditions, porous PHBV scaffold could provide favorable growth conditions for cell survival and offer optimum nutrient and gas exchange for cell growth, even when cells have penetrated the scaffolds. 3D architecture of the scaffold increases the cell packing capacity, which in turn increases the load of stem cells inside the scaffold for limbal stem cell transplantation (LSCT) as compared to conventional LSCT using amniotic membrane as a scaffold. MTT assay showed that cells cultured on a porous PHBV surface were metabolically active; however, their activity was less compared with cells cultured over USSCs feeder layer. The intensity of purple-colored formazan crystals increased with time, indicating that cellular proliferation was enhanced. This implied that the scaffolds were suitable for supporting cell growth. ABCG2 has been proposed as a universal marker of stem cells [42]. De Paiva et al. [43] immunolocalized the expression of ABCG2 in the cell membrane and cytoplasm of the human limbal basal epithelial cells. Corneal epithelial cells retained the normal phenotype and differentiation properties when cultured on scaffold and other samples, as demonstrated by the progenitor cell marker p63 and differentiation marker KRT3/12 expression [44]. Our results suggest that scaffolds prepared from PHBV by can serve as a convenient matrix for the growth of limbal stem cells and for their transfer to treat ocular surface injuries.

4. Conclusion

In conclusion, our study showed that human limbal stem cells isolated from limbal fresh biopsy were able to proliferate in vitro. These cells, when cocultured with mitomycin

C-treated USSC in SHEM, after transfer LSCs to amniotic membrane, thermo responsive dish and modified porous scaffold, maintained the features of limbal stem cells. It is further suggested that modified porous scaffold would be useful for the clinical application of limbal cell culture as well as the study of limbal stem cell mechanisms.

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