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In situ polymerizable hydrogel incorporated with specific pathogen-free porcine platelet-rich plasma for the reconstruction of the corneal endothelium

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ABSTRACT

Corneal inflammation and injury result in scarring and the loss of transparency of the cornea, accounting for a million cases of blindness worldwide each year. Hence, tissue engineering for the reconstruction of the corneal endothelium is a potential therapy. In this study, hydrogels composed of various ratios of hyaluronic acid (H) and Pluronic F-127 (F) were synthesized. These hydrogels can transit between liquid below 15 °C and hydrogel at 37 °C and were injectable for treating corneal wounds via *in situ* polymerization. The hydrogel preparations were assessed by Fourier transform infrared spectroscopy and scanning electron microscopy, and their physical–chemical properties, including transparency, viscoelastic properties, biodegradation and swelling properties, were also evaluated. The results demonstrated that the two ratios of hydrogels, H:F = 1:15 (H1F15) and 1:20 (H1F20), exhibited thermal responsive rheological properties, biodegradation and swelling properties. Specially, the H1F15 hydrogel incubated with 10,000 U of a lysozyme solution still retained over 70% of its remaining weight for 7 days. Moreover, this study further compared the characteristics of bovine corneal endothelial cells (BCECs) cultured on the hydrogels with and without porcine platelet rich plasma (P-PRP) using cytotoxicity, migration, apoptosis/necrosis and cell cycle assays. The results showed that the H1F15 hydrogel with P-PRP displayed higher cell viability and cell migration. In addition, the incorporation of P-PRP positively affected the hydrogel to reduce apoptosis and to enhance the cell cycle of BCECs. Taken together, these results confirmed that the H1F15 hydrogel lacks cytotoxicity and is biodegradable. BCECs can survive, grow and retain normal characteristics in the H1F15 hydrogel with P-PRP. These results provide an opportunity for corneal endothelium reconstruction using a tissue engineering approach with the H1F15 hydrogel with P-PRP.

Abbreviation list

BCECs	bovine corneal endothelial cells
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EDC	carbodiimide
F	Pluronic F-127
FBS	fetal bovine serum
FTIR	Fourier transform infrared
H	hyaluronic acid
HOBt	hydroxybenzotriazole
IGF	insulin-like growth factor
KGF	keratinocyte growth factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI	propidium iodide
P-PRP	porcine platelet rich plasma
SEM	scanning electron microscopy
TGF	transforming growth factor

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1. Introduction

The adult cornea has five layers: the epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium [1]. The important characteristics of a healthy cornea, such as thickness, transparency and its refractive properties, are primarily determined by its avascularity and deturgescence [2]. Unlike most tissues in the body, the cornea contains no blood vessels to nourish or protect it against infection. Instead, the cornea receives its nourishment from the tears and aqueous humor filling the chamber behind it. The three essential requisites for a natural cornea are shielding of insubstantial intraocular contents, the development of an absolute optical interface to refract light onto the retina and transparency to visible light [3]. When the corneal endothelium is damaged, corneal transparency is decreased, and its thickness increases [4].

As age increases, the corneal endothelial cells of some species degenerate at a substantial rate over time. Usually, this gradual loss does not affect endothelial function, but accelerated loss caused by trauma, corneal dystrophy or previous corneal transplant can dramatically alter endothelial functions [5]. Furthermore, the destruction of the corneal endothelial cells is increased in a number of pathological conditions, like corneal endotheliopathies such as Fuch's dystrophy and inflammation, hypoxia, glaucoma, prolonged UV exposure, iatrogenic causes involving a number of drugs, intraocular surgery and many laser surgical procedures [6]. These corneal endothelial diseases leading to endothelial dysfunction may cause blindness and affect millions of people worldwide. If damaged or diseased, these cells will not regenerate but be repaired by migration. Unlike the corneal epithelium, corneal endothelium cells do not regenerate. Thus, many surgical procedures, including traditional keratoplasty and corneal transplantation, may save vision in patients, but the standard therapy of corneal endothelial dysfunction of corneal transplantation with full thickness is prominent today [7,8]. However, corneal transplantation from allograft regularly faces problems, such as insufficient donor corneas, primary immune rejection and sequential graft failure.

Biodegradable hydrogels are significant biomaterials for drug delivery and tissue engineering. *In situ* hydrogels are utilized in corneal regenerative medicine [5]. Several previous studies have explored and validated the practice of using thermo-reversible hydrogel for repairing corneal wounds [5,9]. Hence, this means formulated hydrogels are important alternative materials for the regeneration and transplantation of the corneal endothelium. Some studies have proposed the need for a unique synthetic biomaterial that would act as a biomimetic corneal bandage and have also shown that hydrogels possess a suitable gelling profile and inimitable biocompatibility properties, making it a potential treatment for corneal wound repair [5,9]. Our previous study developed a thermoresponsive hydrogel comprised of hyaluronic acid and Pluronic F-127, it is able to transition between liquid (below 15 °C) and semi-solid (37 °C) and injectable through 25 gauge needle. These hydrogels are more hydrophilic, persistent and providing better biocompatibility than silicone oil and suitable for ophthalmological applications [10].

Platelet-rich plasma (PRP) is blood plasma that has been enriched with platelets [11]. Within the platelets, there are granules containing clotting and growth factors. During the healing process, platelets are activated and aggregate together. They then release granules, which contain growth factors that stimulate the inflammatory cascade and healing process [12]. PRP anchorages have a high concentration of growth factors related to the elevation of wound healing. This aspect of PRP, having a high growth factor concentration, is a very efficient and distinctive advantage for its use in treating chronic ocular surface disorders [13]. However, there is still much to discover, not only about the growth factors but also about all the biochemical mediators and cytokines involved in the processes of tissue regeneration. The treatment of corneal ulcers with autologous PRP was found to be effective [14].

In our previous studies, experimental data showed that the growth factors liberated from porcine platelet-rich plasma (P-PRP) were identified as TGF-β, IGF, KGF and PDGF [15,16]. These reports also demonstrated that P-PRP increased the attachment rate, proliferation rate and viability of human mesenchymal stem cells [15] and human hair follicle dermal papilla cells [16] more than fetal bovine serum (FBS)-supplemented media. As prior research has demonstrated that PRP applied on corneal injury, we hypothesized that PRP may further enhance the effects of thermal-responsible hydrogel.

Summation of these enhancing factors supports of PRP and hydrogels, we tried to optimize the application of PRP and hydrogels on the reconstruction of the corneal endothelium. We examined cross-linked hydrogels containing various ratios of hyaluronic acid and Pluronic F-127 and incorporated with P-PRP on corneal endothelial cells to test their potential for use for corneal wound repair. The objectives of this research are:

(1) Study the properties of hydrogel, such as the mechanical properties of hydrogels usually characterized by means of the shear modulus through rheological measurements in previous studies [10], which are important to various tissue engineering applications.
(2) Examine the effects of hydrogel with P-PRP on cell toxicity and functions by MTT assay.
(3) Examine P-PRP associated with hydrogel-stimulated cell migration. This hypothesis was further examined using a migration assay. This assay works by placing the cells on top of a filter, allowing them to incubate and migrate through a porous filter.
(4) Apoptosis is a physiologically active, tightly regulated process in which cell death follows a programmed sequence of events. This study hypothesized that apoptosis in the corneal endothelium plays a major role in cell loss under various conditions. Understanding the loss of corneal endothelial cells is thus critical and could provide the basis for numerous important clinical aspects related to aging and other disease processes involved in the cornea. To test this hypothesis, the apoptosis percentage of BCECs with different hydrogels with P-PRP is compared.
(5) During one's lifetime, there is a physiological reduction in the cell density of the corneal endothelial cells that cannot be replaced, as corneal endothelial cells are arrested in the G_1/G_0 phase of the cell cycle. After that, they do not show proliferation *in vivo* but undergo tangential cell enlargement to replace lost or damaged cells. We performed a cell cycle study with hydrogels containing P-PRP to investigate cell arrest in BCECs.

2. Materials and methods

2.1. Reagents

Hyaluronic acid (sodium salt, Mw 2,000,000) was purchased from Shiseido (Tokyo, Japan). Pluronic F-127, carbodiimide (EDC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), FBS, penicillin/streptomycin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). DAPI/Antifade solution and cell culture plate inserts for the migration assay (12-well Millicell, 0.4 μm pore size) were purchased from Millipore (Billerica, MA, USA). Hydroxybenzotriazole (HOBt) was purchased from Chem Impex Intl. (Wood Dale, IL, USA). A Cell Meter™ Annexin V binding apoptosis assay kit and propidium iodide (PI) were purchased from AAT Bioquest (Sunnyvale,