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Thermosensitive chitosan-based hydrogels for sustained release of ferulic acid on corneal wound healing

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ABSTRACT

Oxidative damage to cornea can be induced by alkaline chemical burn which may cause vision loss or blindness. Recent studies showed that exogenous application of natural antioxidants may be a potential treatment for corneal wound healing. However, low ocular bioavailability and short residence time are the limiting factors of topically administered antioxidants. Ferulic acid (FA) is a natural phenolic compound and an excellent antioxidant. The study was aimed to investigate the effects of FA in corneal epithelial cells (CECs) under oxidative stress and evaluate the feasibility of use the thermosensitive chitosan-based hydrogel containing FA for corneal wound healing. The results demonstrated that post-treatment of FA on CECs could decrease the inflammation-level and apoptosis. In the rabbit corneal alkali burn model, post-treatment FA-loaded hydrogel may promote the corneal wound healing. The results of study suggest that FA-loaded hydrogel may have the potential applications in treating corneal alkali burn.

1. Introduction

Cornea is an avascular tissue on the ocular surface and plays a role in maintaining normal vision. Cornea is the most vital refractive media in the anterior part of eyes and responsible for two-thirds of total ocular refractive power. From the outer surface to the innermost cellular layer, cornea consists of the epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium in turn (Dua, Faraj, Said, Gray, & Lowe, 2013). Oxidative damage to cornea can be induced by ultraviolet B light and alkaline chemical burn which may cause vision loss and blindness (Black et al., 2011; Shoham, Hadziahmetovic, Dunaief, Mydlarski, & Schipper, 2008). It has been demonstrated that the reactive oxygen species (ROS) production was elevated immediately after an alkali injury (Cejkova et al., 2013; Kubota et al., 2011). Under physiological conditions, the endogenous antioxidants (including superoxide dismutase, catalase, glutathione peroxidases and reductase, glucose-6-phospate dehydrogenase, ascorbic acid, glutathione, reduced nicotinamide–adenine dinucleotide, α-tocopherol, retinol, ferritin and albumin) can maintain redox homeostasis in the cornea (Chen, Mehta, & Vasiliou, 2009). However, the endogenous antioxidant defense systems may fail to provide appropriate protection against oxidative stress in many acute and chronic diseases of the ocular surface (Chen et al., 2009; Sacca, Roszkowska, & Izzotti, 2013). In the past few years, the studies showed that exogenous application of natural antioxidants may be a potential treatment for corneal wound healing (Alio, Artola, Serra, Ayala, & Mulet, 1995; Alio, Ayala, et al., 1995; Cavet, Harrington, Vollmer, Ward, & Zhang, 2011; Chen et al., 2010; Ruszymah et al., 2012).

Polyphenols are natural antioxidants and found largely in the fruits and vegetables. The mechanism of antioxidant action of polyphenol is that phenolic nucleus and unsaturated side chain of polyphenol can form a resonance stabilized phenoxy radical. Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) (FA) belongs to the polyphenolic compounds and has been shown to prevent ROS-related diseases (Mancuso & Santangelo, 2014; Pandey & Rizvi, 2009; Rahman, Biswas, & Kirkham, 2006). However, low ocular bioavailability and short residence time are the limiting factors of

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topically administered antioxidants (Achouri, Alhanout, Piccerelle, & Andrieu, 2013).

Thermosensitive hydrogel formation by simple sol–gel transition has been increasing interest in a wide range of biomedical and pharmaceutical applications (Drury & Mooney, 2003; Nagarwal, Kant, Singh, Maiti, & Pandit, 2009). Chitosan-based thermosensitive hydrogel is currently a great deal of interest for drug and protein delivery (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005; Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013; Muzzarelli, 2009, 2011; Ruel-Gariepy & Leroux, 2004; Ta, Dass, & Dunstan, 2008). Chitosan is a cationic polymer and has been shown to possess the mucoadhesive properties due to the molecular attraction forces by electrostatic interactions with the negative charges of the mucus (Gratieri, Gelfuso, de Freitas, Rocha, & Lopez, 2011; Gratieri et al., 2010; Ludwig, 2005). Chitosan membrane has been used to promote wound healing and decreases the scar tissue formation in a rabbit corneal alkali burn model (Du, Wu, Li, Wang, & Pang, 2008). In the previous studies (Cheng, Yang, & Lin, 2011; Cheng et al., 2010), we developed a thermosensitive chitosan/gelatin/glycerol phosphate hydrogel which is liquid at room temperature but gel-formed at body temperature. Moreover, the hydrogel shows promising gelation properties and excellent biocompatibility.

In the present study, the effects of FA in corneal epithelial cells under oxidative stress and the feasibility of use the FA-loaded hydrogel for corneal wound healing were investigated. The optimal concentration of FA to treat corneal epithelial cells from the damage caused by hydrogen peroxide was evaluated by cell viability, ROS production, m-RNA gene expression and apoptosis analysis. Moreover, the effects of topical administration of FA-loaded hydrogel were examined in the rabbit corneal alkali burn model.

2. Materials and methods

2.1. Cell culture of rabbit corneal epithelial (RCE) cells

Rabbit corneal epithelial (RCE) cells (CCL-60, American Type Culture Collection (ATCC), USA) were cultured in minimum essential medium (MEM, 61100, Invitrogen, USA) containing 10% fetal bovine serum (FBS, AXB30114, Hyclone, USA). The culture medium was refreshed every 3 days.

2.2. Cytotoxicity of FA on RCE cells

Cytotoxicity of FA on RCE cells was performed by crystal violet (C3886, Sigma, USA) assay at days 1 and 3. FA was dissolved in dimethyl sulfoxide (DMSO, D2438, Sigma, USA) and then added to MEM. RCE cells were seeded on the 96-well cell culture plates with the density of 5000 cells per well and cultured in MEM. After 18 h, cells were washed with phosphate-buffered solution (PBS); the 5, 50, 100, 200 and 400 μM of FA (46280, Fluka, USA) in MEM were then separately added to the culture well (200 μl per well) as culture medium throughout the culture. Crystal violet (C3886, Sigma, USA) was dissolved in the 10% (v/v) ethanol (E7148, Sigma, USA). At days 1 and 3, cells were washed with PBS, and 50 μl of 0.2% (w/v) crystal solution was added in the culture well for 10 min. The crystal violet dye was carefully washed in running water, and then the 100 μl of 33 (vol%) acetic acid was added. The absorbance was measured by the enzyme-linked immunosorbent assay reader (ELISA, Sunrise remote, TECAN, USA) at the wavelength of 570 nm. The relative cell viability (expressed in percent) was compared to the untreated control cells.

Table 1
Primers for relative gene expression using quantitative real-time-PCR.

Gene		Forward (F) and reverse (R) primer sequence
GAPDH	F	5′-GTCACCAGGGCTGCTTTTAACTC-3′
	R	5′-CGTGGGTGGAATCATACTGGAAC-3′
IL-1α	F	5′-CAAAGTCCCTGATCTGTTTGAAGA-3′
	R	5′-GAAGTGGTTCATAGCTTGCATCAT-3′
TNF-α	F	5′-AGTATGATCCGGGACGTCGAGCTGG-3′
	R	5′-GAAGTGCAGCAGGCAGAAGAGCGTG-3′
TGF-β1	F	5′-ATGCGACTGCTGCTGCCTCTGCTGT-3′
	R	5′-CGACGGCCTGGATGTGCTGTTGTCT-3′
MMP-9	F	5′-CCACCTTGGTGGTCTTCCCAGGAGA-3′
	R	5′-TGCTGGCTACGCGGGTGTAACCA-3′
TIMP-2	F	5′-AAGCGGTCAGCGAGAAGGAGGTGGA-3′
	R	5′-CCGAGGACGGCGCTGTGTAGATGAA-3′

2.3. Induction of oxidative stress on RCE cells

The RCE cells were seeded in the 6-well cell culture plates with the density of 2×10^5 cells per well and cultured in MEM. After incubated for 18 h, oxidative stress on RCE cells was induced by H_2O_2 (216763, Sigma, USA). The RCE cells treated with 50, 100 and 200 μM of H_2O_2 was designed and abbreviated as H50, H100 and H200 group, respectively. The cell viability was evaluated by crystal violet assay (as described in Section 2.2) at 2, 4 and 6 h.

2.4. Chemiluminescence assay

The antioxidant properties of FA on H_2O_2-induced oxidative stress were determined by chemiluminescence (CL) assay using 0.2 mM of luminol (09253, Fluka, USA). The CL measurement was performed by Tecan's Infinite® M1000 spectrophotometer. Oxidative stress was induced by 200 μM of H_2O_2 and the 5, 10, 25, 50, 75, 100 and 200 μM of FA was then separately added to the 24-well plates. Then, 1 ml of luminol was added. The result of reactive oxygen species (ROS) production was expressed as CL counts. The sample without treatment was abbreviated as control group. H_2O_2 induced oxidative stress without FA further treatment was designed and abbreviated as H group. Oxidative stress induced by 200 μM of H_2O_2 and then further treated with 5, 10, 25, 50, 75, 100 and 200 μM of FA would be abbreviated as HFA10, HFA25, HFA50, HFA75, HFA100 and HFA200, respectively.

2.5. RNA extraction and gene expression of RCE cells

In the FA post-treatment model, the RCE cells were incubated with 200 μM of H_2O_2 for 30 min and 25 μM of FA was then added. After 5.5 h, the RCE cells were collected and total RNA was extracted by TRIzol® Reagent (15596-018, Ambion, USA) according to manufacturer's instructions. Total RNA yield was quantified by the GE NanoVue Plus spectrophotometer at the wavelength of 260 and 280 nm. The ratio of 260–280 nm was between 1.8 and 2.0. RNA was treated in RNase-free water and stored in −80 °C for reverse transcription polymerase chain reaction (RT-PCR) assay. The complementary DNA (cDNA) was synthesized by SuperScript™ III First-Strand Synthesis System (18080-051, Invitrogen, USA) for RT-PCR. Quantitative PCR was performed with power SYBR green PCR master mix (1210076, ABI, USA) according to manufacturer's instructions. The target genes of real-time PCR are listed in Table 1. Reaction was performed by Applied Biosystems step one fast real-time PCR System. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous housekeeping gene. The relative expression of each target gene was calculated using $2^{-\Delta\Delta Ct}$ method. The RCE cells without any treatment and treated with 200 μM of H_2O_2 only were designed and abbreviated as control and H group, respectively. Cells incubated with 200 μM of H_2O_2 and