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Research article

Size effect of rebamipide ophthalmic nanodispersions on its therapeutic efficacy for corneal wound healing

Noriaki Nagai [a], Yoshimasa Ito [a, *], Norio Okamoto [b], Yoshikazu Shimomura [b]

[a] Faculty of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka, 577-8502, Japan
[b] Department of Ophthalmology, Kinki University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka, 589-8511, Japan

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ABSTRACT

In a variety of tissues including gastrointestinal mucosa, rebamipide (REB) provides cytoprotection, prevents inflammation, and promotes wound healing. Clinically, REB ophthalmic dispersions are used to treat diabetic keratopathy. In this study, we investigated the optimal particle size of REB to promote corneal wound healing using a model of diabetic keratopathy, the debrided corneal epithelium from Otsuka Long-Evans Tokushima Fatty (OLETF) rats. First, we prepared three dispersions with different REB particle sizes (REB_{735}, REB_{150}, REB_{45}) by treatment with zirconia beads and Bead Smash 12 (a bead mill). The mean particle sizes of the REB_{735}, REB_{150}, REB_{45} dispersions were approximately 735 nm, 150 nm and 45 nm, respectively. Next, we measured the amounts of REB in the corneal and conjunctival tissues of rats following the instillation of the REB dispersions. The amounts of REB in the corneal and conjunctival tissues following the instillation of REB dispersions was increased by using the mill method, and the amount of REB in rats instilled with the REB_{150} dispersion was significantly higher than in rats instilled with the REB_{45} dispersion. Moreover, the corneal wound healing rate for rats instilled with the REB_{150} dispersion was significantly higher than for rats instilled with the REB_{735} or REB_{45} dispersions. In addition, these REB dispersions enhanced corneal epithelial cell growth, resulting an enhancement of corneal wound healing rate. Thus, we found that the ocular drug accumulation and therapeutic effect on corneal wound healing of REB dispersions is enhanced by preparing particles with a size of ca. 150 nm. These findings provide significant information that can be used to design further studies aimed at developing ophthalmic dispersions.

1. Introduction

The ophthalmic application of drugs is well-accepted, and represents the primary route of administration for the treatment of patients with various eye diseases. However, ophthalmic formulations, such as eye drops, are diluted approximately ten-fold by lacrimation, and the drug is lost from the tear film within 30 s to 2 min, while only a small amount remains associated with the corneal and conjunctival tissues (Schultz et al., 1988). In order to improve the retention of drugs in corneal and conjunctival tissues, various formulations including hydrogels, microparticles and nanoparticles have been developed (El-Kamel, 2002; Sultana et al., 2006; Diebold et al., 2007; Asasutjarit et al., 2011; Gupta et al., 2011; Casolaro et al., 2012; Li et al., 2012; Rafie et al., 2010; Zhou et al., 2013; Rahul et al., 2014; Nagai et al., 2015a). Numerous studies have demonstrated that viscous solutions such as those containing methylcellulose (MC) result in enhanced ocular residence time of the drug (Davies et al., 1991) so that approximately 90% of the drops are lost from the tear film within 10 min while approximately 10% remains associated with the corneal and conjunctival tissues (Schultz et al., 1988). We also have designed novel ophthalmic formulations containing drug nanoparticles, and reported that these novel formulations increase the corneal and conjunctival drug residence time and decrease direct cellular stimulation (Nagai et al., 2015a). It is expected that changes in the particle size in ophthalmic dispersions may provide an alternative strategy for increasing ocular drug residence time and penetration (Tomoda et al., 2012; Nagai et al., 2015a).

Rebamipide (REB, 2-(4-chlorobenzoylamino)-3-[2(1H)-

Abbreviations: Abs, absorbance; BAC, benzalkonium chloride; CA, commercially available; HCE-T, human corneal epithelial cell line; HPβCD, 2-hydroxypropyl-β-cyclodextrin; MC, methylcellulose; OLETF, Otsuka Long-Evans Tokushima Fatty; REB, rebamipide.

* Corresponding author.
E-mail address: itoyoshi@phar.kindai.ac.jp (Y. Ito).

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quinolinon-4yl]-propionic acid) enhances the healing of wounded epithelial cell monolayers and the secretion of mucus glycoproteins in gastric mucosal cells, scavenges active oxygen radicals (Ishihara et al., 1992; Watanabe et al., 1998; Sakurai et al., 2005), and accelerates the healing of gastric ulcers by improving cell kinetics, and by reducing apoptosis and inflammation (Watanabe et al., 2002). The REB also provides cytoprotection, improved wound healing, and the prevention of inflammation to a variety of tissues as well as the gastrointestinal mucosa (Kashima et al., 2012; Tanaka et al., 2013; Kimura et al., 2013). Furthermore, the clinical effectiveness of REB for treatment of pulmonary (Ro et al., 2001), hepatic (Hong et al., 1998), and renal (Saad et al., 2000) inflammation, as well as stomatitis (Matsuda et al., 2003) and colitis (Kishimoto et al., 2000) has been shown. Moreover, previous studies have shown that REB enhances mucin production in corneal (Takeji et al., 2012) and conjunctival goblet cells (Rios et al., 2008). In addition, REB attenuates the structural irregularities and haze induced by ultraviolet B radiation in mice (Tanito et al., 2003), and increases the growth of corneal epithelial cells (Urashima et al., 2004). Therefore, an REB ophthalmic dispersion (Mucosta® ophthalmic suspension UD2%; Otsuka Pharmaceutical Co., Tokyo, Japan) has been used as therapy in dry eye and corneal damage in Japan since 2012.

In this study, we investigated the optimal particle size of REB for use as therapy for corneal wound healing. In addition, we demonstrate whether the capability of the commercially available REB ophthalmic dispersion (CA-REB eye drop) can be enhanced by changing the drug particle size.

2. Materials and methods

2.1. Animals

Male 7-week-old Wistar rats and 30-week-old Otsuka Long-Evans Tokushima Fatty (OLETF) rats were used in this study. The food, water intake and body weight of the OLETF rats were 37.1 ± 1.0 g/day/rat, 81.3 ± 2.1 ml/day/rat, and 668.1 ± 4.5 g, respectively (means ± S.E.). All procedures were performed in accordance with the Kinki University Faculty of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

2.2. Preparation of ophthalmic REB dispersions

Ophthalmic dispersions containing REB particles of various sizes were prepared according to our previous reports (Nagai et al., 2014, 2015a; Nagai and Ito, 2014). Conventional REB (REB coarse, 735 ± 8.2 nm, mean ± S.E., Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing MC was milled with the Bead Smash 12 (Wakanyaku Co., Ltd., Kyoto, Japan) for 30 s (3000 rpm, 4 °C), and dispersed in 2-hydroxypropyl-β-cyclodextrin (HPβCD). The dispersions were added to tubes containing 0.1 mm zirconia beads, and treated with the bead mill (5500 rpm, 30 s × 10 or 15 times, 4 °C). The 10 and 15 times-treated dispersions are referred to as the REB_{150} and REB_{45} dispersions, respectively; the dispersion containing REB coarse, MC and HPβCD is referred to as the REB_{735} dispersion (non-milled). The composition of these REB dispersions is as follows: 1.8% REB, 0.001% benzalkonium chloride (BAC), 0.1% mannitol, 0.5% MC, 5% HPβCD w/v%, pH 6.5. The precipitation of the REB_{735}, REB_{150} and REB_{45} dispersions was approximately 88.7%, 13.0%, 11.8% at 14 days after preparation, respectively (mean particle size at 14 days after preparation, REB_{735} dispersion 813 nm, REB_{150} dispersion 163 nm, REB_{45} dispersion 51 nm). A commercially available REB ophthalmic dispersion (CA-REB_{580} eye drops, Mucosta® ophthalmic suspension UD2%, preservative-free, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was also treated with the bead mill (5500 rpm, 30 s × 3 times, 4 °C), and referred to as CA-REB_{140} eye drops. The CA-REB_{580} and CA-REB_{140} eye drops precipitated by 3 days after preparation (mean particle size at 3 days after preparation, CA-REB_{580} eye drops 617 nm, CA-REB_{140} eye drops 561 nm). In this study, the REB concentration in the CA-REB_{580} and CA-REB_{140} eye drops was diluted to 1.8% by adding saline. The particle sizes were obtained using a nanoparticle size analyzer SALD-7100 (Shimadzu Corp., Kyoto, Japan; refractive index 1.60−0.01i). The REB concentration was measured by a Shimadzu LC-20AT system equipped with a column oven CTO-20A (Shimadzu Corp., Kyoto, Japan), and analyzed using the absolute calibration method. The mobile phase consisted of phosphate buffer/acetonitrile (83/17, v/v) at a flow rate of 0.25 ml/min. An Inertsil® ODS-3 (3 μm, column size: 2.1 mm × 50 mm) column (GL Science Co., Inc., Tokyo, Japan) was used, and the column temperature was 35 °C. The wavelength for detection was 254 nm.

2.3. Body weight and blood test parameters for diabetes mellitus in OLETF rats

Body weights and some blood test parameters for diabetes mellitus were measured for 30-week-old OLETF rats according to our previous methods (Nagai et al., 2009a). Blood was taken without anesthesia from the tail vein of each rat fasted for 15 h. The plasma glucose and triglyceride levels were measured by an Accutrend GCT (Roche Diagnostics, Mannheim, Germany). Total cholesterol and insulin levels were determined with a Cholesterol E-Test Kit (Wako, Osaka, Japan) and an ELISA Insulin Kit (Morinaga Institute of Biological Science Inc., Kanagawa, Japan), respectively. Plasma glucose, triglyceride, total cholesterol and insulin levels of the OLETF rats were 230.9 ± 3.9 mg/dl, 389.2 ± 10.8 mg/dl, 185.0 ± 12.6 mg/dl, and 322.6 ± 10.3 ng/dl, respectively (means ± S.E., n = 38).

2.4. Measurement of REB content in corneal and conjunctival tissues of rats

The REB (30 μl) was instilled, and the eyes were kept open for about 1 min after instillation to prevent the REB from being washed out for the stable *via* drug equilibrium in the dispersions. Wistar rats with a normal ocular surface were killed 30 min after the instillation of a REB dispersion or eye drops under deep isoflurane anesthesia, and the corneal and conjunctival tissues were collected. The samples were homogenized in N,N-Dimethylformamide on ice, and centrifuged at 15,000 rpm for 15 min at 4 °C. The content of REB in the supernatant was analyzed by the HPLC method described above. We previously reported that the peak of drug concentration in cornea and conjunctiva was 15−30 min after the instillation of dispersions containing drug nanoparticles (Nagai et al., 2015b). Therefore, we selected one point (30 min after instillation) to evaluate the amounts of REB in the cornea and conjunctiva of rats.

2.5. Measurement of corneal wound healing rate in rats

The therapeutic effects on corneal wound healing by REB dispersions or eye drops were analyzed according to our previous reports (Nagai et al., 2009a, 2009b). Rats were anesthetized with isoflurane, and a patch of corneal epithelium was removed with a BD Micro-Sharp™ (blade 3.5 mm, 30°, Becton Dickinson, Fukushima, Japan). The areas of debrided corneal epithelium in OLETF rats were as follows: saline, 10.54 ± 0.41 mm^2; vehicle (5% HPβCD solution containing 0.001% BAC, 0.1% mannitol, 0.5% MC), 10.88 ± 0.47 mm^2; REB_{735} dispersion, 11.01 ± 0.50 mm^2; REB_{150} dispersion, 10.82 ± 0.41 mm^2; REB_{150} dispersion, 10.91 ± 0.56 mm^2;