Experimental Eye Research 89 (2009) 686–692

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Naltrexone and insulin are independently effective but not additive in accelerating corneal epithelial healing in type I diabetic rats

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ARTICLE INFO

Article history:
Received 17 May 2009
Accepted in revised form 23 June 2009
Available online 1 July 2009

Keywords:
naltrexone
insulin
diabetes
cornea
wound healing
opioid antagonist

ABSTRACT

Patients with diabetes are at increased risk for developing corneal disorders, termed diabetic keratopathy. Treatments for diabetic keratopathy are limited. Preclinical studies have demonstrated that topical administration of either naltrexone (NTX) or insulin (INS) accelerates corneal re-epithelialization in type I diabetic rats. This study determined whether the combination of NTX and INS would have additive effect(s) on the re-epithelialization of corneal abrasions in diabetic male Sprague–Dawley rats beyond either agent alone. Type 1 diabetes (DB) (glucose levels $>$ 400 mg/dl) was induced with streptozotocin; glycemic levels were not controlled with INS. Eight weeks after induction of diabetes, a 5 mm diameter circular abrasion was created in the center of the cornea in one eye of each rat. Eye drops (0.05 ml) of INS [1 U ($\sim$6 nM)] and NTX (10^{-5} M) in Vigamox were administered separately 4 times daily for 7 days (NTX/INS); DB control rats received drops of sterile vehicle (DB SV) 4 times daily. Two other groups of rats were given only NTX (DB NTX) or only INS (DB INS). Re-epithelialization was monitored by fluorescein staining, and images were recorded with a CCD camera. Areal measurements were made using Optimas software, and the percentage of epithelial defect over a 40 h period was calculated. Twenty-four hour after formation of an abrasion ($\sim 21.7 \pm 0.4$ mm^2 area), corneal wounds in DB rats treated with NTX, INS, or NTX/INS were significantly smaller ($p < 0.001$) than those in DB SV rats, with reductions in the size of the defect ranging from 24 to 84%. DB rats treated with NTX or INS alone also were observed to have reductions in wound size of 22 and 29%, respectively, from subjects in the DB SV group at 16 h. At 16 h both the DB NTX and DB INS groups had defects that were 13 and 27%, respectively, smaller than those for the DB NTX/INS group, and at 40 h the DB INS animals had 78% smaller corneal wounds than in the DB NTX/INS group. Therefore, the DB NTX/INS group exhibited some slight delays in wound repair compared to the DB NTX and DB INS groups. Topical application of NTX and/or INS to the cornea had no effect on non-invasive measures that included ocular morphology, intraocular pressure, or corneal thickness. These data demonstrate that although NTX or INS accelerates wound healing, concomitant application of NTX and INS to corneal abrasions in diabetic animals does not have an additive effect on re-epithelialization.

1. Introduction

Diabetic keratopathy has been estimated to occur in 46–64% of diabetic patients during the course of their disease (Schultz et al., 1981). With an estimated 1 million individuals having type 1 diabetes (Diabetes Control and Complications Trial Research Group, 1993), 500,000 or more patients may experience diabetic keratopathy at some time in the course of their disease. Moreover, corneal transplantation, removal of the corneal epithelium during vitrectomy in the course of treatment for diabetic retinopathy, and procedures such as laser photocoagulation, cataract surgery, and refractive surgery are risk factors for abnormal corneal epithelial healing in diabetic patients (Kaji, 2005; Schultz et al., 1981; Sanchez-Thorin, 1998; Cisarnik-Fredenburg, 2001). Some corneal disorders associated with diabetic keratopathy include nonhealing epithelial defects, infectious corneal ulcers, and secondary scarring, which may result in a decreased or even permanent loss of vision (Kaji, 2005; Schultz et al., 1981; Sanchez-Thorin, 1998; Cisarnik-Fredenburg, 2001). Unfortunately, these diabetes-related epithelial defects may be resistant to conventional treatment regimens (e.g., bandage contact lens, lubricants and antibiotics) (Cavallerano, 1992; Kabosova et al., 2003). Moreover, none of these therapies address the

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doi:10.1016/j.exer.2009.06.010

fundamental biological processes in corneal healing that are perturbed secondary to the pathophysiology of diabetes (Kabosova et al., 2003). Recently, topical administration of autologous serum has been investigated as therapy for corneal epithelial abrasions in diabetic patients following vitrectomy (Schulze et al., 2006).

Naltrexone hydrochloride (NTX), an opioid antagonist, when applied topically in rabbits (Zagon et al., 1998a), systemically in normal and diabetic rats (Zagon et al., 1998b, 2002a), or included in organ cultures of human (Zagon et al., 2000) or rabbit (Zagon et al., 1998a) corneas, has been demonstrated to markedly accelerate epithelial DNA synthesis and corneal re-epithelialization. The mechanism of action of NTX is by blockade of the opioid growth factor (OGF) interaction with the OGF receptor (OGFr) (Zagon et al., 2002a,b). The OGF-OGFr axis serves as a tonically repressive pathway that regulates cell proliferation through cyclin-dependent inhibitory kinases and nucleocytoplasmic transport (Cheng et al., 2009). The repercussions of NTX application in the homeostatic (i.e., normal, untreated, unwounded) cornea include a decreased epithelial transit time from the basal layer to the suprabasal layers, and increases in linear thickness of the epithelium, basal cell proliferation, and packing density of suprabasal cells secondary to a decrease in cell diameter (Zagon et al., 2006a).

Topically applied NTX for 7 days (4 times per day) has been shown to be an efficacious and safe treatment for abraded corneas of animals that were normal (non-diabetic), diabetic and hyperglycemic, and diabetic but normoglycemic through the use of insulin (Zagon et al., 2006b; Klocek et al., 2007). These findings were determined using a variety of non-invasive and invasive measures, and the results suggest that NTX is safe for topical use across a wide range of biological conditions.

Topically applied insulin (1 U/0.05 ml) for 7 days (4 times a day) also has been shown to be efficacious in facilitating healing abraded corneas of hyperglycemic diabetic rats (Zagon et al., 2007). Topical insulin (INS) did not alter corneal thickness, intraocular pressure, or serum glucose. Diabetic rats had markedly decreased corneal sensitivity compared to non-diabetic rats, but rats treated with topical INS had sensitivity readings comparable to that of non-diabetic rats.

The present study was designed to examine the efficacy of concomitant administration of NTX and insulin in facilitating corneal wound healing when applied topically. Streptozotocin (STZ)-treated rats were used as a model system for type 1 diabetes. In a standard, reproducible manner, the ocular surface epithelium of the cornea was abraded from inner limbal margin to inner limbal margin, and rats were exposed to NTX, INS, NTX/INS, or vehicle 4 times per day for 7 days. The experiments assessed the size of the defect to examine whether NTX/INS can further accelerate re-epithelialization. The outcome measures of the experiments included the size of the defect, and rate of repair as well as a wide range of non-invasive measures (intraocular pressure, corneal thickness, and corneal topography) and invasive parameters (histopathology, apoptosis/necrosis, and DNA synthesis in the peripheral cornea, limbus, and conjunctiva). Our results demonstrate that topical application of both NTX and INS does not significantly increase corneal re-epithelialization beyond the efficacious properties of either NTX or INS alone.

2. Materials and methods

2.1. Animals and induction of diabetes

Six-week old male Sprague–Dawley rats weighing ~135 g were purchased from Charles River Laboratories (Wilmington, MA) and housed under standard laboratory conditions; water and food were continuously available. All investigations conformed to the regulations of the Association for Research in Vision and Ophthalmology, National Institutes of Health, and the Institutional Animal Care and Use guidelines of the Department of Comparative Medicine of The Pennsylvania State University.

Type 1 diabetes was induced by the method of Havel et al. (2000), which has been shown to avoid renal failure or loss of animals from hypoglycemia secondary to insulin release associated with β-cell destruction (Ahren et al., 1995). An intraperitoneal (i.p.) injection of 40 mg/kg STZ (Sigma, St. Louis, MO) in ice-cold 0.5 M citrate buffer (pH 4.5) was administered. A second dose of STZ (40 mg/kg) was injected 24 h later. This regimen produced insulin-deficient diabetes in 100% of the animals within 3–5 days; these animals were termed DB rats. Another group of animals received citrate buffer only, and were considered Normal.

Blood glucose levels were monitored from the tail vein using a True Track Smart System glucometer (Home Diagnostics, Inc., Ft. Lauderdale, FL) immediately prior to receiving STZ, and at 1 and 8 weeks after injection of STZ. Glucose levels of $\geq$400 mg/dl were considered to be the minimum blood glucose level compatible with a stable non-toxic diabetic state (Nakamura et al., 1997).

2.2. Corneal abrasions

The procedures for wounding and monitoring repair followed those reported earlier (Zagon et al., 2002a; Klocek et al., 2007). In brief, animals were anesthetized with a mixture of ketamine (70 mg/kg), xylazine (7 mg/kg), and acepromazine (10 mg/kg); Proparacaine Hydrochloride Ophthalmic Solution 0.5% (Baush & Lomb Inc., Tampa, FL) was administered topically to the eye immediately prior to surgery. Eyes were examined under a dissecting microscope (SZ-ET; Olympus, Tokyo, Japan), and a 5 mm diameter circle in the center of the cornea was marked with a disposable dermatological skin punch (Acuderm, Ft. Lauderdale, FL). The encircled corneal epithelium was removed with a No. 15 Bard-Parker scalpel blade. Care was taken not to injure the underlying corneal tissue. Wounds were created between 0730 and 0830 or 1630 and 1730. Any animal that experienced bleeding, inflammation, or infection was not included in the study. The right eye was abraded on the 9th week following injection of STZ.

2.3. Photography

For photography of corneal abrasions, animals were anesthetized in a Plexiglas chamber attached to an isoflurane vaporizer, and the residual epithelial defect was stained with topical fluorescein (Fluor-I-Strip; Ayerst Laboratories, Philadelphia, PA). Rat eyes were viewed using an Olympus dissecting microscope with a tungsten light source and a gelatin Wratten no. 47 filter, and photographed with a Sony CCD (charged couple device) camera at $\times$1.5 magnification. Photographs of rat eyes were taken immediately after abrasions (0 h) and 16, 24, 32, and 40 h later. No animal was photographed at intervals $<$8 h in order to prevent disruption of the epithelial healing process. The area of defect was determined using Optimas software and was calculated as the percentage of the original residual epithelial defect. Rates of healing (mm^2/h) were calculated for only the first 24 h, because healing of the cornea does not occur in a linear manner (Crosson et al., 1986).

2.4. Topical administration of naltrexone and insulin

NTX (10^{-5} M, Sigma, Indianapolis, IN) and bovine insulin (1 U, Sigma–Aldrich) were prepared in Vigamox (moxifloxacin hydrochloride ophthalmic solution, Alcon, Inc. Ft. Worth, TX); these dosages were selected based on data from earlier reports (Zagon et al., 2007; Klocek et al., 2007). Compounds were given separately