Use of Topical Insulin to Normalize Corneal Epithelial Healing in Diabetes Mellitus

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Objective: To determine whether topical application of insulin normalizes delayed corneal wound healing in rats with diabetes mellitus (DB).

Methods: Diabetes mellitus was induced with streptozocin. A 5-mm corneal abrasion at 9 or 11 weeks was treated topically for 7 days (4 times daily) with 1, 2, or 5 U of insulin or with sterile vehicle (SV).

Results: Residual corneal epithelial defects of rats with DB receiving SV (hereafter called DB SV rats or animals) were approximately 35% larger than in healthy animals receiving SV (hereafter called healthy SV rats or animals). Rats with DB receiving topical insulin had wounds ranging from 19% to 60% smaller than DB SV rats, corresponding to wound sizes in healthy SV rats. Topical insulin had no effect on reepithelialization of corneal wounds in healthy SV rats. Insulin did not affect corneal thickness, ocular pressure, or serum glucose level. The corneal sensitivity of DB SV rats was markedly reduced from healthy SV rats, but rats with DB given insulin had corneal sensitivity values comparable to the healthy SV group. DNA synthesis was decreased in DB SV corneal epithelium but was comparable to that in healthy SV rats after they received insulin; apoptosis and necrosis levels were similar in all groups.

Conclusion: Topical insulin normalizes corneal reepithelialization in diabetic rats.

Clinical Relevance: Direct application of insulin may serve as an important strategy for treating diabetic keratopathy.

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Ocular complications secondary to diabetes mellitus (DB) are a well-known cause of blindness in the Western world.1-5 Diabetic keratopathy is experienced by 50% or more of diabetic patients,6 can take the form of nonhealing epithelial defects that can result from surgical (eg, vitrectomy) and nonsurgical trauma,7,8 and frequently is resistant to conventional treatment regimens. Diabetic keratopathy sequelae are infectious corneal ulcers, secondary scarring, and permanent loss of vision. Intensive systemic therapy with insulin has been found to prevent delayed wound healing of ocular surface epithelium in diabetic animals.9

The present study examined the hypothesis that topical application of insulin in rats with type 1 DB normalizes corneal wound healing.

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ANIMALS AND INDUCTION OF DB
Male Sprague-Dawley rats (approximately 165 g) were obtained from Charles River Laboratories, Wilmington, Massachusetts, and housed under standard laboratory conditions. All investigations conformed to the regulations of the Association for Research in Vision and Ophthalmology, National Institutes of Health, and the guidelines of the Department of Comparative Medicine, The Pennsylvania State University.

Type 1 DB was induced according to the method of Havel et al10 and Ahren et al.11 An intraperitoneal injection of 40 mg/kg of streptozocin (Sigma-Aldrich, St Louis, Missouri) in ice-cold 0.5M citrate buffer, pH 4.5, was administered. A second dose of streptozocin, 40 mg/kg, was injected 24 hours later. This regimen produced insulin-deficient DB in 100% of the animals within 48 to 72 hours; these 38 animals were termed DB rats. Eleven animals not receiving streptozocin, but injected with citrate buffer, were considered healthy.

Serum glucose levels were monitored from the tail vein using a glucometer (True Track Smart System glucometer; Home Diagnostics, Inc, Fort Lauderdale, Florida) immediately before receiving streptozocin and at 7 days and 8 weeks after the injection of streptozocin. Glucose levels greater than 400 mg/dL (to convert to millimoles per liter, multiply by 0.0555) were considered the minimum level compatible with a stable nontoxic diabetic state.11
CORNEAL ABRASIONS

The procedures for epithelial wounding and observation of repair followed those reported by Zagon et al.9,13,14 In brief, animals were anesthetized with a mixture of ketamine, 70 mg/kg; xylazine hydrochloride, 7 mg/kg; and acepromazine maleate, 10 mg/kg. Eyes were examined under a dissecting microscope (SZ-ET; Olympus, Tokyo, Japan), and a 3-mm-diameter circle was outlined in the center of the cornea with a disposable dermatological skin punch (Acuderm, Fort Lauderdale). The encircled corneal epithelium was removed with a No. 13 Bard-Parker scalpel blade (Becton Dickinson, Franklin Lakes, New Jersey). Care was taken not to injure the underlying corneal tissue. Wounds were created between 7:30 and 8:30 AM or between 4 and 5 PM; these time points were chosen because previous studies showed no differences in the labeling index between morning and afternoon.14 Any animal that experienced bleeding, corneal ulcerations, inflammation, or infection was not included in the study. Only 1 eye was wounded at a time in each animal. The right eye was abraded on the ninth week following injection of streptozocin. Two weeks later, the left eye was abraded. Antibiotic drops (trimethoprim sulfate and polymyxin B sulfate; Bausch & Lomb World Headquarters, Rochester, New York) were applied to the eye following surgery.

TOPICAL ADMINISTRATION OF INSULIN

Bovine insulin (Sigma-Aldrich) was prepared in moxifloxacin hydrochloride ophthalmic solution (Vigamox; Alcon, Inc, Fort Worth, Texas) every second day; moxifloxacin ophthalmic solution has been documented to have no negative effect on corneal wound healing.13,15 Eyedrops of insulin at a final dose of 1, 2, or 5 U were administered to unanesthetized animals at 7:30 AM, 10:30 AM, 1:30 PM, and 4:30 PM for 7 consecutive days. Solutions of insulin were prepared every second day. Compounds were given as a single drop (20 µL) from the slitlamp. Two weeks later, the left eye was abraded. Antibiotic drops (trimethoprim sulfate and polymyxin B sulfate; Bausch & Lomb) were applied to the eye following surgery.

PHOTOGRAPHY

Animals were anesthetized in an acrylic plastic chamber attached to an isoflurane vaporizer, and the residual epithelial defect was stained with topical fluorescein (Fluor-I-Strip; Ayerst Laboratories, Philadelphia, Pennsylvania). Rat eyes were viewed using a dissecting microscope with a tungsten light source and a gelatin Wratten No. 47 filter and photographed with a CCD camera (Sony Corporation, San Diego, California) at ×1.5 magnification. Photographs were taken immediately after epithelial debridement (0 hours) and 16, 24, 32, and 40 hours later. No animal was photographed at intervals less than 12 hours to prevent disruption of the healing process. The area of defect was determined using Optimas software (Meyer Instruments, Inc, Houston, Texas) and was calculated as the percentage of the original epithelial defect.

NONINVASIVE CORNEAL MEASUREMENTS

Three measurements of corneal integrity were assessed on both eyes in control and DB rats the week before wounding, and in animals receiving topical treatment with SV or 1 U of insulin 2 weeks after abrasion. These measurements included observations with a handheld slitlamp (HSO 10 Hand Slit Lamp; Zeiss, Dublin, California) to examine general overall morphological and pathological (eg, cataracts) features. Corneal thickness was determined by a pachymeter (DGH 550 Pachette 2; Pro Forma, Exton, Pennsylvania), and intraocular pressure was measured by an applation tonometer (Tono-Pen XL Tonometer; Medtronic, Jacksonville, Florida). In addition, corneal sensitivity was determined by an aesthesiometer (Cochet and Bonnet Aesthesiometer; Richmond Products, Boca Raton, Florida). The applation tonometer and aesthesiometer were used on unanesthetized rats, whereas the slitlamp and pachymeter required animals to be anesthetized with a mixture of ketamine, 70 mg/kg; xylazine hydrochloride, 7 mg/kg; and acepromazine maleate, 10 mg/kg. Evaluation with the slitlamp was conducted before and after dilation by 2 independent observers (M.S.K. and J.W.S.) for each eye. Ocular pressure and sensitivity values were obtained from 4 readings per eye, whereas corneal thickness was recorded as 20 readings per eye.

DNA SYNTHESIS

DNA synthesis in the corneal epithelium was assessed by bromodeoxyuridine (BrdU) labeling, as modified from Zagon et al,9 in DB animals receiving SV (hereafter called DB SV animals or rats) and animals with DB receiving 1 U of insulin, and in healthy animals receiving SV (hereafter called SV animals or rats). Forty-eight hours following corneal abrasions, BrdU, 100 mg/kg body weight, was injected intraperitoneally 3 and 6 hours before euthanasia by intraperitoneal injection of 100-mg/kg sodium pentobarbital. Animals were decapitated, and eyes underwent propososis and enucleation and were placed in formalin for 24 hours and prepared for paraffin embedding. Sections (8 µm) that included the entire corneal surface, limbus, and conjunctiva were stained with anti-BrdU antibodies (Roche Applied Science, Indianapolis, Indiana) and counterstained with hematoxylin.

The numbers of BrdU-positive cells were counted in basal and suprabasal layers of the cornea, limbus, and conjunctiva. A labeling index was computed as the number of labeled cells divided by the total number of cells with nuclei, times 100. At least 2 sections per rat and 3 rats per experimental group were assessed.

APOPTOSIS AND NECROSIS

Apoptosis was assessed using a kit (DermaTACS In situ Apoptosis Detect Kit; Trevigen, Gaithersburg, Maryland), following previous methods,15 whereas necrosis was determined in hematoxylin-eosin-stained sections. At least 2 sections per rat and 3 rats per experimental group were assessed.

DATA ANALYSIS

Body weights and blood glucose measures were analyzed by the t test. Noninvasive measures were analyzed by 1-way analysis of variance. The area of residual defect was analyzed at each time point using 1-way analysis of variance. Data for noninvasive measures and the area of residual defect were subsequently analyzed using Newman-Keuls tests. Because healing of the cornea does not occur linearly, rates of healing were calculated using monophasic and biphasic models of exponential decay.9

RESULTS

INDUCTION OF DB

The rats weighed a mean ± SEM of 165 ± 3 g at the streptozocin injections (Figure 1A). Healthy rats gained approximately 300 g over 12 weeks. Rats in the DB group...
were comparable in body weight to healthy animals until 2 weeks after the injection of streptozocin, when the DB group had a 20% reduction (P < .001) in body weight relative to healthy animals. Rats with DB weighed significantly less (approximately 19%-32%) than healthy rats beginning at week 2 and continuing throughout the 12-week study.

Baseline glucose readings were a mean±SEM of 165±3 mg/dL for all rats (Figure 1B), and these values were consistent in the healthy group throughout the study. Rats receiving streptozocin became hyperglycemic within 5 days (Figure 1B) and had glucose levels greater than 450 mg/dL. Serum glucose levels remained the same for each group throughout experimentation.

CORNEAL REEPITHELIALIZATION

The 5-mm trephine demarcated the entire corneal region of the rat eye but did not encroach on the limbus or conjunctiva. Wound healing occurred in a manner consistent with previous studies9,13-15 on healthy rats, rabbits, and humans, and diabetic rats. The initial area of the abrasion ranged from 19.2 to 25.4 mm² and corresponded to corneal injuries of 4.9 to 5.6 mm in diameter. No differences in the size of the initial abrasions were noted between groups.

Healthy SV rats had corneal wounds that reepithelialized significantly faster than DB SV animals at 16, 24, and 32 hours (Figure 2), with the DB rats exhibiting residual defects that were 32% to 37% larger than defects in the healthy group. At 16 and 24 hours, DB rats treated topically with 1, 2, or 5 U of insulin or sterile vehicle (SV) (n=9-11), whereas healthy (nondiabetic) rats received SV (n=6) (original magnification ×1.5). In part B, significant differences vs the DB SV group are indicated by the asterisk (P < .05), dagger (P < .001), and double dagger (P < .01).

Healthy SV rats had corneal wounds that reepithelialized significantly faster than DB SV animals at 16, 24, and 32 hours (Figure 2), with the DB rats exhibiting residual defects that were 32% to 37% larger than defects in the healthy group. At 16 and 24 hours, DB rats treated topically with 1, 2, or 5 U of insulin had wounds that were 19% to 37% smaller than DB SV animals. At 32 hours, DB rats treated with 1 U of insulin had defects that were 53% smaller than DB SV rats; wounds of DB rats receiving 2 or 5 U of insulin were not significantly different in size from animals in the DB group exposed to SV at 32 hours. At 40 hours, despite a 60% smaller wound in the DB animals subjected to 1 or 2 U of insulin compared with DB SV rats, the results were not significantly different because of the few animals having a residual defect in each group. No differences were observed in the size of the defects at 16, 24, or 32 hours between rats in the 1-, 2-, or 5-U insulin groups.
Further analysis of healing rates using monophasic and biphasic models of exponential decay revealed similar half-lives for the healthy SV animals and the DB animals receiving 1, 2, and 5 U of insulin (Table). However, in the first 24 hours of the biphasic model, the DB SV rats had a 2.6-fold longer half-life with respect to healing rates than the healthy SV group, and approximately a 2-fold longer half-life than the DB rats receiving 1, 2, or 5 U of insulin.

Reepithelialization of corneal wounds in healthy rats monitored at 16, 24, 32, and 40 hours (Figure 3) treated topically with 1 U of insulin was similar to animals receiving only SV.

NONINVASIVE MEASUREMENTS OF CORNEAL INTEGRITY

At the beginning of the ninth week following the injection of streptozocin, rats were examined with a handheld slitlamp. Ocular morphological features were comparable between all groups of animals. However, cataracts were present in 100% of the DB rats, but no cataracts were recorded in any of the healthy subjects.

Two weeks after wounding, healthy and DB rats in the 1-U-of-insulin or SV group were evaluated by slitlamp, pachymetry (Figure 4A), tonometry (Figure 4B), and aesthesiometry (Figure 4C).

For slitlamp evaluations, insulin treatment had no effect on ocular surface morphological features. Animals in the DB SV group, DB group given 1 U of insulin, healthy SV group, and healthy group receiving 1 U of insulin had comparable results.

For pachymetry evaluations, no significant differences (P=.20) in corneal thickness were noted between...
the healthy (mean±SEM, 127±11 µm) and DB (mean±SEM, 120±2 µm) groups before or 2 weeks after wounding, and for any treatment modality.

For tonometry evaluations, the intraocular pressures of healthy and DB rats were a mean±SEM of 20.2±2.6 and 19.7±0.5 mm Hg, respectively, before wounding. No differences in intraocular pressure were recorded between healthy and DB rats treated with 1 U of insulin or SV, or from values before wounding.

For aesthesiometry evaluations, the mean±SEM corneal sensitivity measures of healthy animals before or after wounding, and of those receiving topical treatment with either 1 U of insulin or SV for 1 week after abrasion, did not differ (0.51±0.08 g/mm²). Before wounding, DB animals (mean±SEM, 1.52±0.25 g/mm²) had a 2.6-fold decrease in corneal sensitivity compared with healthy animals (mean±SEM, 0.57±0.03 g/mm²). After corneal abrasion, rats in the DB SV group did not differ from levels before wounding. However, DB animals topically exposed to 1 U of insulin had sensitivity values comparable to healthy animals, and significantly (P=.009) better than pretreatment levels.

**DNA SYNTHESIS AND APOPTOSIS AND NECROSIS**

The number of BrdU-labeled cells located in the basal layer of the peripheral cornea, limbus, and conjunctiva of DB SV rats was decreased by 96%, 85%, and 97%, respectively, from healthy SV rats (Figure 5). The number of cells undergoing DNA synthesis in these 3 regions of the ocular surface epithelium in DB animals receiving insulin was comparable to the number in healthy SV subjects and differed significantly (P<.001) from the DB SV group.

The labeling index of suprabasal cells in the peripheral cornea, limbus, and conjunctiva of all groups was 1%, and no differences between groups were recorded. Programmed cell death in basal or suprabasal layers of the peripheral cornea, limbus, and conjunctiva of all groups was less than 1%. Necrotic cells were not observed in hematoxylin-eosin–stained sections of any region of any treatment group.

**GLUCOSE MEASUREMENTS AFTER TOPICAL INSULIN EXPOSURE**

Serum glucose levels monitored after 2, 6, 10, and 14 topical exposures to 1 U of insulin revealed no changes in plasma glucose values in DB or healthy rats (Figure 6).

**COMMENT**

The present study demonstrates that topical insulin treatment of corneal wounds in diabetic animals prevents the delay in ocular surface epithelial wound healing observed in poorly controlled diabetic animals. Insulin concentrations ranging more than 5-fold did not differ from one another in efficacy and were not toxic to the cornea as determined by ocular surface morphological features, intraocular pressure, and corneal thickness. However, the hyposensitivity of the cornea in diabetic rats was restored to normal by insulin exposure. This change in corneal sensitivity suggests that the hyposensitivity observed in diabetic animals is not permanent and can be reversed at least in early stages of the disease. Topical insulin treatment did not alter reepithelialization of the cornea of healthy rats, demonstrating that the insulin therapy is specific for diabetic animals. Finally, the data show that topical exposure of the abraded cornea to insulin does not influence serum glucose levels, implying that insulin action is directly at the cellular level rather than sys-
temically driven. Moreover, this result suggests that the immediate environment of the corneal epithelium in diabetic animals is responsive to the pathophysiological mechanisms in diabetic keratopathy. Thus, to our knowledge, we have shown for the first time that topical application of insulin can normalize repair of the ocular surface epithelium in type 1 diabetic animals. Future studies are needed to define whether topical insulin will also be a treatment for type 2 DB.

The topical ocular route of insulin administration was reported by Christie and Hanzal in 1931, with the focus of attention being a search for alternative pathways to control serum glucose levels. Unfortunately, eye drops containing insulin in an isotonic sodium chloride solution formulation were ineffective at reducing systemic D-glucose levels in humans and animals. The present results stand in agreement with the inability of topical insulin to influence serum glucose levels. More recently, Woost and colleagues have reported an increase in the tensile strength of wounds in healthy rabbits with full-thickness, perforating, corneal incisions following 3 daily applications of insulin. Shanley and coworkers documented an increase in cell migration using an in vitro model of an immortalized simian virus 40 human corneal epithelial cell line treated with insulin. Thus, to our knowledge, this is the first study showing that the topical ocular route of insulin can modulate wound healing of the cornea in diabetic animals. The fact that topical insulin does not alter serum glucose levels in these diabetic animals points to the correction of delays in reepithelialization in the diabetic cornea at the cellular level rather than the systemic level.

The mechanism of the effects of accelerating reepithelialization of corneal wounds in diabetic animals by topical treatment with insulin remains to be fully elucidated. In earlier reports, a decrease in cell proliferation of the corneal epithelium, limbus, and/or conjunctiva compared with healthy (nondiabetic) rats was recorded 2 weeks after creating a corneal abrasion, but neither apoptosis nor necrosis was abnormal in these diabetic corneas. Normoglycemia in diabetic animals, induced by systemic treatment with insulin, restores the decreased levels of DNA synthesis in ocular surface epithelium to normal values when examined 3 weeks after wounding. In the present investigation, direct application of insulin to the injured ocular surface restores the decreased levels of DNA synthesis of basal epithelial cells to normal values, as measured 48 hours after wounding—a time of active cell proliferation. Because DNA synthesis of cells in the undamaged peripheral cornea is critical to the pace of reepithelialization, this finding may indicate that at least 1 mechanism for normalization by topical insulin treatment may be related to cell proliferation.

Given the critical role of the corneal epithelium in maintaining vision, the frequency of corneal complications related to DB (diabetic keratopathy), and the problems occurring in diabetic individuals postoperatively (eg, vitrectomy and cataract extraction), in whom the ocular surface epithelium is disturbed, an effective treatment to facilitate corneal epithelial wound healing in diabetic individuals is needed. Unfortunately, conventional thera-

pies, such as artificial tears and bandage contact lenses, often fail. The present study demonstrates that topical insulin to accelerate corneal reepithelialization has many advantages to offer, including cost-effectiveness, increased patient compliance, ease of delivery, independence of systemic serum glucose levels, specificity for diabetic keratopathy, avoidance of first-pass metabolism, fast rate of absorption and effectiveness, minimal immunological reactions, and no tolerance or adverse effects. Moreover, insulin administered long-term to the human eye at concentrations of up to 100 U/mL for 8 weeks in isotonic sodium chloride solution has been shown not to be toxic. Finally, an additional advantage of using topical ocular administration of insulin in corneal epithelial wound healing of diabetic cases is that it avoids difficulties in converting the dosage needed for the rat to the human based on such factors as surface area, blood volume, and body mass. Thus, the novel findings in the present study showing that topical insulin is efficacious for restoring normal reepithelialization in the rat support the need for clinical trials using this strategy.

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REFERENCES

15. Yee RW, Setabutr P, Foltermann MO, Sami MS, Chuang AZ. The effects of topical moxifloxacin 0.5% ophthalmic solution and gatifloxacin 0.3% solution on corneal healing after bilateral photorefractive keratectomy. Cornea. 2006;25(9) (suppl 2):S8-S11.

From the Archives of the Archives

Forty years ago . . . when an attempt was made to show almost any prominent American ophthalmologist a microscopic section, he would approach the microscope with his hands behind his back, displaying no desire to change the focus of the instrument or to adjust the slide. In fact, he would approach the instrument as though he feared it might bite him. You may expect me to say that conditions have now vastly changed, but I must disappoint you. American ophthalmologists sufficiently familiar with ophthalmic pathology to interpret or describe a microscopic section of a diseased eye are still extremely few.