*Original Article*

# Insulin Treatment Ameliorates Impaired Corneal Reepithelialization in Diabetic Rats

**Ian S. Zagon,[1] Joseph W. Sassani,[2] and Patricia J. McLaughlin[1]**

**Patients with diabetes are at an increased risk for developing corneal disorders, often as a result of surgical and nonsurgical trauma. This study investigated whether intensive treatment of diabetes with the goal of maintaining blood glucose concentrations close to the normal range could ameliorate the delayed corneal wound healing found in animals with uncontrolled diabetes. Diabetes was induced with streptozotocin, and rats were divided into groups based on the degree of blood glucose control: *1*) not treated with insulin implants (DB group), *2*) receiving insulin implants and determined to be normoglycemic (DB-IN group), and *3*) normal, nondiabetic animals serving as controls. Immediately before wounding at 9 or 11 weeks after the induction of the diabetic state, corneal thickness and corneal sensitivity of the DB and DB-IN groups were comparable with controls. DB, but not DB-IN, rats exhibited subnormal intraocular pressure. At 9 and 11 weeks after the onset of diabetes, the corneas of the right and left eyes, respectively, were abraded by mechanical scraping. The DB rats had residual corneal epithelial defects that ranged from 23% to 5.6-fold larger compared with the control group and a rate of healing that was 19% slower than control animals. The DB-IN group had healing characteristics similar to the control group. DNA synthesis in the peripheral cornea and conjunctiva, but not the limbus, of DB animals was reduced 50 and 91%, respectively, from control levels. Cell proliferation in the DB-IN group was comparable with the control group, with the exception of a 72% increase in the peripheral cornea in the DB-IN group. These results indicate that intensive therapy with insulin, which establishes normoglycemia in rats with diabetes, prevents the delay in wound healing of ocular surface epithelium observed in poorly controlled diabetic animals.**
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Ocular complications secondary to diabetes are well known and are a leading cause of blindness in the Western world (1,2). Although abnormalities of the retina and lens have been studied extensively (3,4), corneal disorders are becoming increasingly recognized as a cause of patient morbidity related to diabetes (5–8). The corneal epithelium serves as a barrier against ocular infection and contributes to the maintenance of corneal transparency and homeostasis. Prompt resurfacing of injured epithelium is needed to reestablish visual function. Unfortunately, diabetic keratopathy can take the form of nonhealing epithelial defects, which are resistant to conventional treatment regimens. These epithelial abnormalities can result from surgical (e.g., vitrectomy) and nonsurgical trauma (5–8). Among their sequelae are infectious corneal ulcers, secondary scarring, and permanent loss of vision.

Diabetic keratopathy alters the corneal epithelium with changes in epithelial morphology and fragility, abnormal basement membrane structure, poor epithelial adherence and healing rate, and ineffective epithelial barrier function. Other corneal changes resulting from diabetes include corneal neuropathy and alterations in the corneal stroma, Descemet's membrane, and corneal endothelium (9–11). It has been estimated that corneal erosions/abrasions, ranging from superficial erosions to extensive full thickness, and confluent epithelial lesions occur in 47–64% of diabetic patients during the course of their disease (6). With 1 million individuals in the U.S. reported to have type 1 diabetes, diabetic keratopathy is a serious vision-threatening disorder.

Insulin-dependent diabetes (type 1 diabetes) is a chronic disease characterized by hyperglycemia and is accompanied by long-term microvascular, neurologic, and macrovascular complications (12) that include retinopathy, nephropathy, neuropathy, and cardiovascular disease. The prevention and amelioration of these complications have been major goals of research. There is considerable evidence that the level and duration of hyperglycemia is correlated with the development of complications. For example, the landmark multicenter study, the Diabetes Control and Complications Trial (13), found that intensive treatment with insulin (insulin pump or three or more daily injections guided by frequent glucose monitoring) was effective in delaying the onset and slows the progression of diabetic retinopathy, nephropathy, and neuropathy in patients with type 1 diabetes. Compared with conventional therapy with insulin involving one or two daily injections of insulin, those with tighter control had a marked improvement in these complications. Thus, the

From the [1]Departments of Neural and Behavioral Sciences, Pennsylvania State University College of Medicine, Hershey, Pennsylvania; and the [2]Department of Opthalmology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania.

Address correspondence and reprint requests to Dr. Ian S. Zagon, Department of Neural and Behavioral Sciences, H109, The Milton S. Hershey Medical Center, Penn State College of Medicine, 500 University Dr., Room C3729, Hershey, PA 17033. E-mail: isz1@psu.edu.

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STZ, streptozotocin.

establishment of euglycemia has been advocated to prevent diabetes complications. Unfortunately, glycemic control often is not optimal in patients with diabetes (14–16).

The present study examined the hypothesis that establishment of normoglycemia in rats with type 1 diabetes facilitates corneal wound healing. Using streptozotocin (STZ)-induced diabetic rats as a model system for type 1 diabetes, ocular surface epithelium of the cornea was abraded from inner limbal margin to inner limbal margin. One group of animals received insulin implants and was confirmed to be normoglycemic. To contrast the effects of proper control of blood glucose with a lack of control relative to the repair of corneal abrasions, a second group of rats were rendered diabetic but were maintained in a hyperglycemic state. A third group of animals that were not diabetic or receiving insulin implants served as control subjects. The outcome measures of the experiment included ocular pressure, corneal thickness, corneal topography, corneal sensitivity, the size of the corneal defect, rate of wound repair, incidence of complete reepithelialization, and DNA synthesis. Our results demonstrate clearly that the establishment of normoglycemia in diabetic animals results in significantly improved corneal epithelial healing compared with test subjects with poor glycemic control and suggest that depressed DNA synthesis in the hyperglycemic diabetic animals may help explain these findings.

## RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing 135 g were purchased from Charles River Laboratories (Wilmington, MA) and housed under standard laboratory conditions; water and Purina 5010 Rodent Chow were continuously available. All investigations conformed to the regulations of the Association for Research in Vision and Ophthalmology, the National Institutes of Health, and the guidelines of the Department of Comparative Medicine of Pennsylvania State University.

Type 1 diabetes was induced by the method of Havel et al. (17), which has been shown to not induce renal failure or loss of animals from hypoglycemia secondary to insulin release associated with β-cell destruction (18). An intraperitoneal injection of 40 mg/kg STZ (Sigma, St. Louis, MO) in ice-cold 0.5 mol/l 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 48–72 h; these animals were termed DB rats.

Blood glucose levels were monitored from the tail vein using a True Track Smart System glucometer (Home Diagnostics, Ft. Lauderdale, FL) immediately before receiving STZ, at 7 days after injection of STZ, and every 4 weeks after STZ administration. Glucose levels of ≥400 mg/dl were considered to be the minimum blood glucose level compatible with a stable nontoxic diabetic state (19).

Seven days after STZ injection, one-half of the DB rats received subcutaneous insulin implants (Linplants; LinShin Canada, Scarborough, Toronto, ON, Canada). The implants were 7 mm in length and 2 mm in diameter, and they released insulin (in microrecrystalized palmitic acid) at ~2 units/24 h for ~50 days. The implants were inserted into the right abdominal region of anesthetized (isoflurance) rats using a trocar; no sutures were required to hold the implants in place. Within 24–72 h, DB rats with insulin implants had glucose levels of ~150 mg/dl; these animals were considered to be normoglycemic and designated as the DB-IN group. Linplants were replaced every 6 weeks in the DB-IN group.

**Corneal abrasions.** The procedures for wounding and observation of repair followed those reported by Zagon et al. (20). In brief, animals were anesthetized with a mixture of ketamine (70 mg/kg), xylazine (7 mg/kg), and acepromazine (10 mg/kg). 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 produced 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 1600 and 1700. Any animal that experienced bleeding, corneal opacities, ulcerations, inflammation, or infection was not included in the study. Only one eye was wounded at a time in each animal. Antibiotic drops (trimethoprim sulfate and polymyxin B sulfate; Bausch & Lomb) were applied to the eye following surgery. The right eye was abraded on the 9th week following injection of STZ. Two weeks later, the left eye was abraded.

**Photography.** For photography of corneal abrasions, animals were anesthetized in a Plexiglas chamber attached to an isofluorane 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 (charge-coupled device) camera at ×1.5 magnification. Photographs of rat eyes were taken immediately after abrasions (0 h) and 24, 32, 40, and 48 h later. No animal was photographed at intervals <12 h in order to prevent disruption of the healing process. The area of defect was determined using Optimas software and was calculated as the percentage of residual epithelial defect.

**Noninvasive measurements of cornea.** Three measures of corneal integrity were assessed on both eyes in the week before wounding. These included observations with a hand-held slit lamp (Zeiss HSO 10 Hand Slit Lamp; Dublin, CA) to examine general overall morphology and pathology (e.g., cataracts). Corneal thickness was determined by a pachymeter (DGH 550 Pachette 2; Pro Forma, Exton, PA), and intraocular pressure was measured by a tonopen (Tono-Pen XL Tonometer; Medtronic, Jacksonville, FL). In addition, corneal sensitivity was determined by an aesthesiometer with nylon threads (Cochet and Bonnet Aesthesiometer; Richmond Products, Boca Raton, FL). The tonopen and aesthesiometer were used on unanesthetized rats, whereas the slit lamp and pachymeter were utilized under isoflurane anesthesia; measures with the slit lamp were conducted before and after dilation. Examinations using the hand-held slit lamp were conducted by two independent observers for each eye. Ocular pressure was obtained as 4 readings per eye, corneal thickness was recorded as 20 readings per eye, 4 readings per eye were acquired with the tonopen, and 4 readings per eye were utilized for the aesthesiometer.

**DNA synthesis.** Procedures for measurements of DNA synthesis in the corneal epithelium were assessed by BrdU labeling as modified from Zagon et al. (21). Three weeks following corneal abrasions, BrdU (100 mg/g body wt) was injected intraperitoneally 3 and 6 h before euthanasia. Rats were killed by an intraperitoneal injection of 100 mg/kg sodium pentobarbital and decapitated; eyes were proptosed, enucleated, placed in formalin for 24 h, and prepared for paraffin embedding. Sections (8 μm) that included the entire corneal surface, limbus, and conjunctiva were stained with anti-BrdU antibodies (Roche) and counterstained with hematoxylin and eosin.

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 two sections per rat and five rats per experimental group were assessed.

**Data analysis.** Body weights and glucose measurements were subjected to a two-way ANOVA, with treatment and time as factors. Glucose measurements were log transformed before two-way ANOVA testing. All data were subsequently analyzed using Newman-Keuls tests. Data from noninvasive measurements and BrdU labeling experiments were analyzed with a one-way ANOVA and Newman-Keuls tests. The area of defect was analyzed at each time point using ANOVA and Newman-Keuls tests. Because healing of the cornea does not occur in a linear manner (Crosson et al. [22]), rates of healing were calculated using both monophasic and biphasic models of exponential decay.

## RESULTS

**Induction of diabetes and insulin pellet implantation.** Each group (DB, DB-IN, and control) had 10–15 rats. During implantation of the second insulin pump, two rats in the DB-IN group died.

All rats weighed 186 ± 2 g at the time of STZ injections (Fig. 1*A*). Control rats gained ~400 g over the course of 15 weeks. Rats in the DB group were comparable in body weight to control animals until 7 weeks after injection of STZ. At this time, the DB group had a 21% reduction ($P < 0.05$) in body weight relative to control animals. DB rats weighed significantly less (~24–29%) than control rats beginning on week 9 and continuing throughout the course of the 15-week study. DB-IN rats were similar in body weight to control animals for 13 weeks after receiving STZ and exhibited 14 and 13% reductions in body weight from the control group at 13 and 15 weeks, respectively. DB

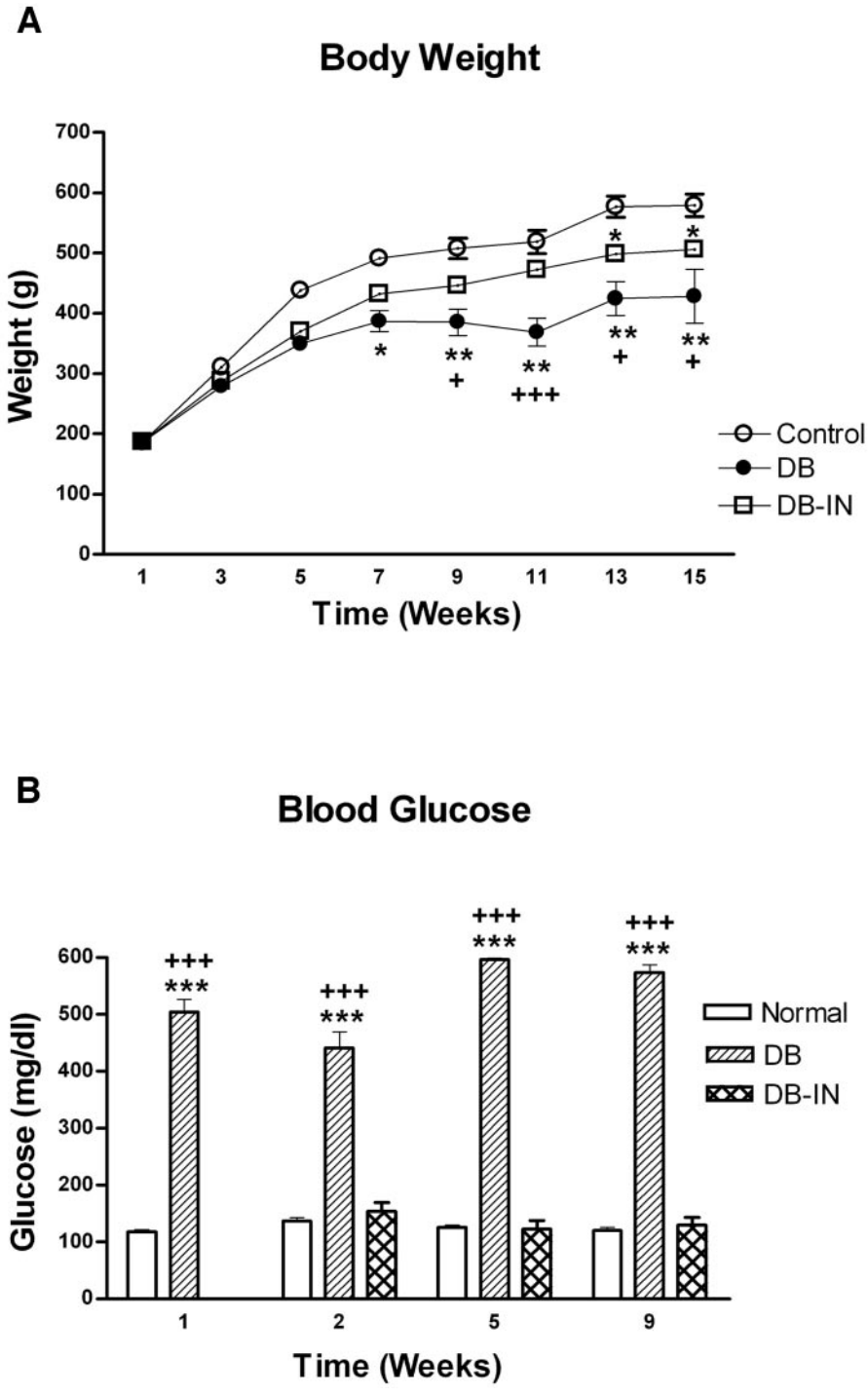


**FIG. 1. Body weights (*A*) and glucose levels (*B*) of rats rendered diabetic with STZ (DB), diabetic animals receiving insulin (DB-IN), and untreated rats receiving saline (Control or Normal). *A*: Body weights were recorded at the time of STZ injection (week 1) and every 2 weeks thereafter. *B*: Blood glucose levels were recorded within 1 week after administration of STZ (week 1) and at 2, 5, and 9 weeks thereafter. Insulin pellets were implanted in some animals 7 days after injection of STZ. Values represent means ± SEM for at least eight animals/group at each time point. Significantly different from control rats at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Significantly different from DB-IN rats at +$P < 0.05$ and +++$P < 0.001$.**

animals weighed markedly less than DB-IN rats beginning at 9 weeks, with reductions of 14–22% noted.

Baseline glucose readings were 105–126 mg/dl for all rats (Fig. 1*B*), and these values were consistent in the control group throughout the study. Rats receiving STZ became hyperglycemic within 5 days (Fig. 1*B*) and had glucose levels >450 mg/dl. DB animals receiving insulin were normoglycemic within 5–7 days of implantation (week 2) and had blood glucose levels of 154.3 ± 15 mg/dl; control rats at this time point had blood glucose levels of 136.7 ± 6 mg/dl. Blood glucose levels remained the same for each group throughout the duration of experimentation. DB rats had significantly higher glucose levels (2.8- to 4.8-fold) than either the control or DB-IN animals.

**Noninvasive measurements of corneal integrity.** At the beginning of the 9th week following injection of STZ, rats were examined with a hand-held slit lamp. Subjects in the control and DB-IN groups were comparable except for a cataract in one eye of a DB-IN animal. Four rats in the DB group had cataracts in one eye. Animals with corneal opacities were not used for corneal wounding studies. Following examination with a hand-held slit lamp, rats were evaluated by pachymetry, tonometry, and aesthesiometry.

**Pachymetry.** No significant differences in corneal thickness were noted between the control (156 ± 3 μm), DB (151 ± 4 μm), and DB-IN (157 ± 4 μm) groups.

**Tonometry.** Intraocular pressures of control and DB-IN rats were 24.7 ± 1.0 and 22.1 ± 1.4 mm, respectively. DB

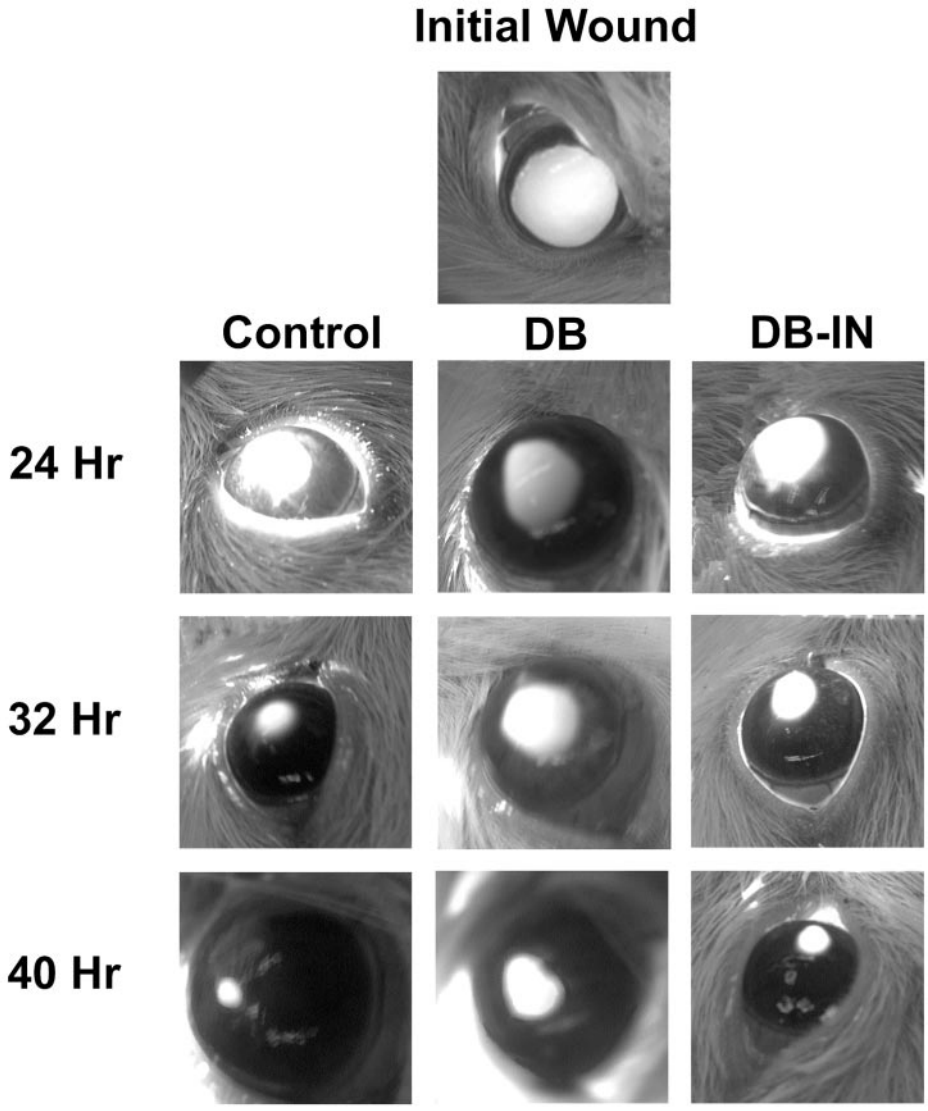


**FIG. 2. Photographs of the rat eye stained with fluorescein immediately stained after the initial wound or 24, 32, or 40 h after creation of a 5-mm corneal abrasion. Rats were either untreated (nondiabetic) (Control), diabetic (DB), or diabetic and receiving insulin (DB-IN). Magnification ×1.5.**

rats had mean intraocular pressure readings of 19.4 ± 1.1 mm. The mean pressure of DB animals differed significantly from control subjects at $P < 0.01$ but did not differ from the DB-IN rats.

**Aesthesiometry.** Mean corneal sensitivity measures did not differ between the control, DB, and DB-IN groups.

**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 studies on normal rat, rabbit, and human as well as diabetic rat (20,23,24). The initial area of the abrasion ranged from 17.3–21.6 $mm^2$ and corresponded to corneal injuries of 4.6- to 5.1-mm diameter. No differences in the size of the initial abrasions were noted between groups.

DB rats had corneal wounds that reepithelialized significantly more slowly than those in control rats at 24, 32, and 40 h (Figs. 2 and 3), with the DB rats exhibiting residual defects that were 23% to 5.6-fold larger than defects in the control group. The percent epithelial defect was 23% greater in the DB rats than the DB-IN animals at 24 h and 67% greater at 32 h.

Analysis of healing rates between 0 and 24 h revealed that the control group had a mean rate of reepithelialization of 0.53 ± 0.02 $mm^2$/h in comparison with the DB (0.43 ± 0.03 $mm^2$/h) and DB-IN (0.52 ± 0.03 $mm^2$/h) groups. Comparison of the rates of wound healing between the control and the DB groups, as well as the DB and the DB-IN groups, differed at the $P < 0.05$ level. No differences in the rate of reepithelialization were detected between animals in the control and the DB-IN groups. Further analysis of healing rates using monophasic and biphasic models of exponential decay revealed similar half-lives for the control and DB-IN groups (Table 1). However, the DB group had 25–50% longer half-life with respect to healing rates than the control or the DB-IN

TABLE 1
Analysis of rate of healing rates for control, diabetic (DB group), and diabetic-insulin (DB-IN group) animals over a 48-h period

| | Half-life (h) | | |
|---|---|---|---|
| | Monophasic | Biphasic (0–24 h) | Biphasic (24–48 h) |
| Control group | 9.0 | 16.8 | 4.9 |
| DB group | 12.7 | 21.2 | 7.6 |
| DB-IN group | 10.8 | 16.6 | 6.6 |

Data are exponential decay values generated by both monophasic and biphasic models.

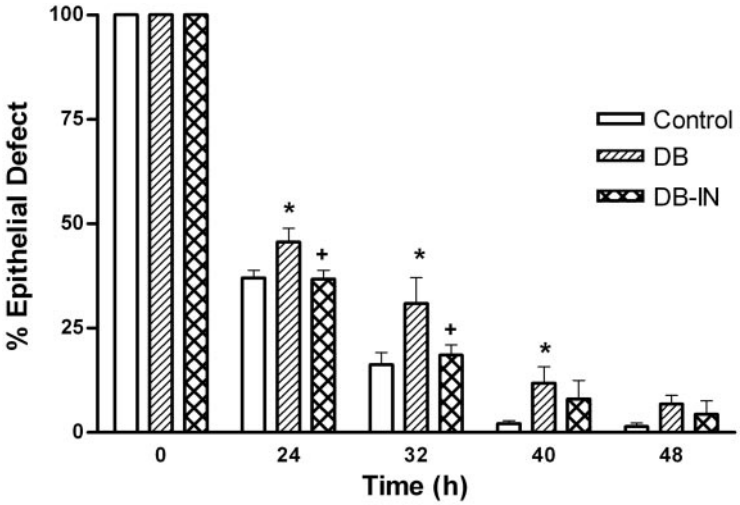


**FIG. 3. Histograms of residual epithelial defect (%) in rat corneas after formation of a 5-mm wound and followed for 48 h. Three groups of rats were included: nondiabetic controls (Control), diabetic (DB), and diabetic receiving insulin (DB-IN). Photomicrographs of the fluorescein-stained corneas were captured with a Sony CCD camera (Olympus), and areas were analyzed by Optimas software. Residual epithelial defects are presented as percentage of the original wound. Data are expressed as means ± SEM. Significantly different from control at *$P < 0.05$ and from DB rats at +$P < 0.05$.**

groups. All groups of animals were followed on a weekly basis for a total of 4 weeks beyond complete wound closure. No residual or recurrent corneal epithelial defects were
identified.

**DNA synthesis.** The number of BrdU-labeled cells located in the basal layer of the peripheral cornea and conjunctiva of DB rats was decreased by 50 and 91%, respectively, in comparison to subjects in the control group (Fig. 4). Moreover, the number of BrdU-positive cells in the basal layer of the peripheral cornea and conjunctiva in DB rats was decreased by 71% ($P < 0.01$) and 90% ($P < 0.05$), respectively, from the DB-IN group. The number of cells undergoing DNA synthesis in the peripheral cornea of the DB-IN group was increased 72% from control levels. No alteration in DNA synthesis of cells in the basal epithelial layer of the conjunctiva of the DB-IN group was recorded in comparison to control levels. Labeling indexes in the limbus were too variable to permit distinction of significant effects. Moreover, the low rate of labeling in the suprabasal layer of any region and treatment group (<4%) precluded meaningful comparisons.

## DISCUSSION

A major finding in this study is that maintenance of a normoglycemic state in diabetic animals is vital to efficient repair of corneal epithelial defects. Thus, in comparison to the marked delays in wound healing experienced by rats with uncontrolled hyperglycemia recorded in this and previous studies (25), animals that are diabetic but receiving insulin to maintain euglycemia had the size of the corneal defect, rate of repair, and incidence of complete reepithelialization comparable with levels recorded in control rats. These data support the thesis set forth in previous reports (1,13) regarding systemic complications of diabetes in humans, which demonstrated that intensive therapy with insulin delays the onset and slows the progression of diabetic retinopathy, nephropathy, and neuropathy in type 1 diabetic patients. For the first time, we have extended this principle to include wound healing of the corneal epithelium.

In addition to wound healing, the present study has made a number of observations regarding noninvasive measures with respect to hyperglycemia and normoglycemia in diabetic rats. Slit lamp examination revealed that some diabetic animals, but not controls, had cataracts. There was a fourfold increase in the incidence of cataracts in the group that did not receive insulin. The presence of cataracts in some diabetic rats parallels the situation with diabetic humans (26), showing that the diabetes induced in the DB animals was of sufficient degree and duration to be analogous to that in diabetic humans after many years of the disease. These data also support the thesis that strict metabolic control of the hyperglycemic state in diabetes reduces the incidence of cataracts (26).

In the course of these studies, we found that corneal thickness and sensitivity in diabetic rats, with or without insulin treatment, did not differ from control subjects. These findings are in agreement with some reports involving diabetic patients (27). Nevertheless, other investigators have reported changes in one or the other of these parameters (10). It is interesting to note that only diabetic rats that did not receive insulin exhibited a marked decrease in ocular pressure. Whether dehyrdation (despite having access to water ad libitum) contributed to the subnormal ocular pressures in this group of animals is unclear.

Cell proliferation has been reported to be decreased in the basal epithelial layer of the peripheral cornea, limbus, and conjunctiva in STZ-induced diabetic rats, which are not rendered normoglycemic with insulin (25), and monitored 8 weeks after induction of diabetes. In the present investigation, rats were examined 14 weeks after induction of diabetes. The results showed that DNA synthesis was markedly subnormal in the basal epithelial cells of the peripheral cornea and the conjunctiva but not the limbus of rats with uncontrolled diabetes. These data supplement the results of our earlier study on homeostatic corneal epithelium (25) and demonstrate that poorly controlled diabetes depresses DNA synthesis in corneal epithelial cells that should be undergoing active proliferation. Because cell proliferation is an essential step in the corneal epithelial healing process (28), it could be postulated that compromising DNA synthesis under diabetic conditions contributes to the impaired corneal wound healing in diabetic individuals. If this is indeed the case, it is interesting to note that insulin treatment to restore the normoglycemic state in diabetic rats prevented subnormal rates of DNA synthesis. Specifically, the basal corneal epithelium of diabetic, insulin-treated, normoglycemic rats exhibited an increased number of proliferating cells compared with untreated control subjects. Thus, these

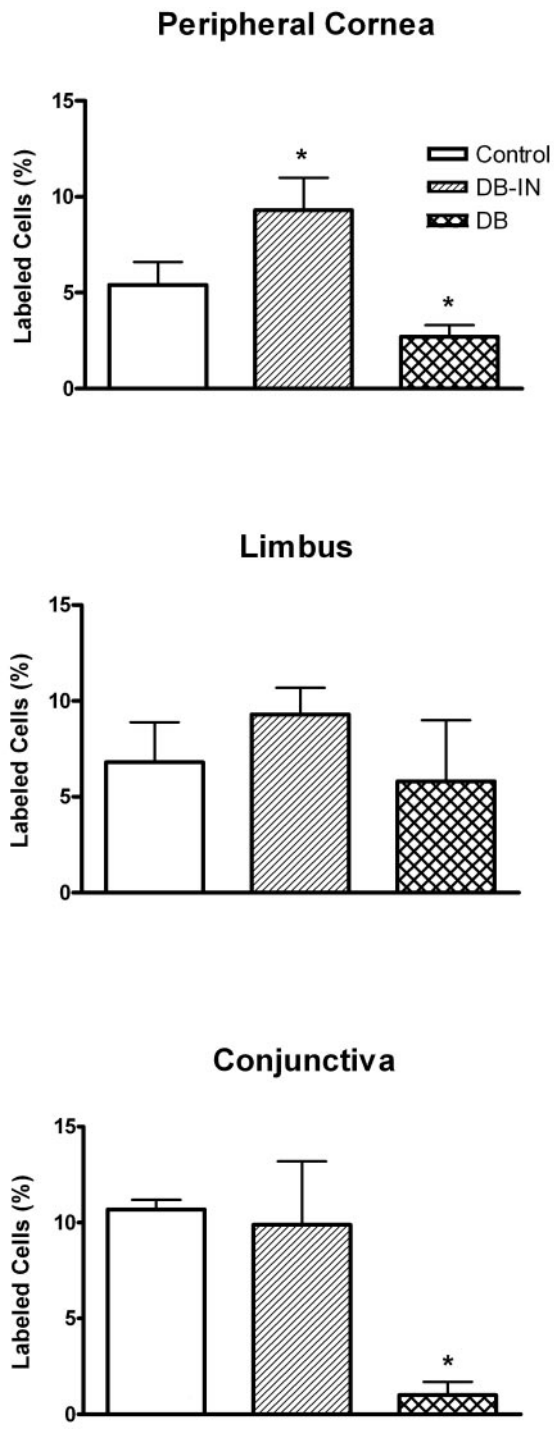


**FIG. 4. Histogram of the labeling indexes of rat basal epithelial cells in the peripheral cornea, limbus, and conjunctiva as measured by BrdU incorporation 3 weeks after wounds were created. Three groups of rats were included: nondiabetic controls (Control), diabetic (DB), and diabetic receiving insulin (DB-IN). Six and 3 h before being killed, animals received injections of BrdU (100 mg/g body wt). Data represent means ± SEM. Significantly different from control at *$P < 0.05$.**

data, demonstrating depressed DNA synthesis in hyperglycemic rats, provide a mechanism by which corneal epithelial wound healing could be depressed in poorly controlled diabetes.

Our finding that the rats in the DB group weighed substantially less than animals in the control and the DB-IN groups, raises the question of whether body size is related to the process of corneal wound healing. Specifically, are the delays in wound healing displayed by the DB group compared with controls and the DB-IN groups related to the reduced body weights in the DB group? However, examination of the data in this study and those performed earlier (20,25) with animals of less body weight showed no differences in the temporal course of wound healing or the rate of corneal reepithelialization of rats despite marked differences in body weights at the time of corneal abrasion. Therefore, body weight differences between the DB and control or DB-IN groups were not related to the changes in corneal reepithelialization.

In summary, this report supplements our previous research involving corneal epithelial homeostasis in diabetic rats by demonstrating that corneal epithelial reepithelialization also is significantly depressed in hyperglycemic diabetic rats and offers a mechanism involving depressed rates of DNA synthesis to explain this finding. Given the vital role of the corneal epithelium in maintaining vision, and the frequency of corneal complications related to diabetes (diabetic keratopathy), the present data supports the work of other researchers regarding systemic diabetes complications in humans and the need for the maintenance of the normoglycemic state. As a corollary, our findings suggest that poor glycemic control in diabetes could result in a higher risk for abnormalities involving corneal epithelial wound healing. The information derived from these studies also may have implications for diabetic individuals postoperatively (e.g., vitrectomy, cataract extraction) in which the ocular surface epithelium is disturbed. Strict control of hyperglycemia in patients with diabetes could offer more favorable outcomes and a reduction in complications related to surgical procedures. Further studies are indicated to confirm this hypothesis.

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