Purpose. To find the role of nitric oxide (NO) in streptozocin-induced corneal toxicity in rats.

Methods. Sprague-Dawley rats were injected intraperitoneally with streptozotocin (65 mg/kg). For exposure to light, each rat cage was placed in a box surrounded with aluminum foil and illuminated for 6 hours per day with two 20-W fluorescent lamps at a distance of 50 cm. When not exposed to light, each cage was placed in a dark room. Some animals with and without light exposure also were treated with and without streptozocin treatment. Control animals did not receive streptozocin and were housed in a dark room 24 hours a day. Each group contained 15 rats. After 1, 3, 7, and 10 days of light exposure, concentrations of nitrite and nitrate, stable oxidation products of NO, were measured in the aqueous humor. Corneal changes also were examined by electron microscopy after 10 days. To examine specific NO-induced histopathologic changes, several rats were injected subconjunctivally with a balanced saline solution containing the NO-generating agent (S-nitroso-N-acetyl-D,L-penicillamine or (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1, 2-diolate).

Results. Concentrations of nitrite and nitrate were highest in the streptozocin-injected rats irradiated while under the fluorescent lamp. On the 10th day of the streptozocin injection, the concentrations of nitrite and nitrate in streptozocin-treated rats irradiated while under the fluorescent lamp was almost two-and-a-half times greater than that of nontreated rats reared in the dark (111.37 ± 7.47 μM, 45.43 ± 3.91 μM, respectively). Slit-lamp biomicroscopy showed that the corneas swelled gradually and opacified by the third day in the irradiated streptozocin-injected group. The corneas became hazy to the point of indistinguishable detail structures by the 10th day, although those of the other rats were relatively clear at the same time. Histopathologically, ultrastructural changes included the remarkable swelling of intracytoplasmic organelles, including mitochondria, and denaturation of collagen fibril was shown in the streptozocin-injected-irradiated rats by the 10th day. The corneas injected with two NO-generating agents, S-nitroso-N-acetyl-D,L-penicillamine and (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1, 2-diolate, showed similar but more severe changes.

Conclusions. Nitric oxide can cause damage to the mitochondria, the most important energy source of the cell, and induce ultrastructural damage to the corneal endothelium and fibroblast. The authors suggest that NO is associated with the development of corneal cytotoxicity and that NO production and subsequent cytotoxicity can be prevented by blocking photoactivation. Invest Ophthalmo Vis Sci. 1997; 38:995-1002.
Streptozocin consists of a 2-deoxyglucose substituted by N-methyl-N-nitrosourea: It is known to be a strong alkylating agent. In pancreatic islets, STZ has been shown to cause the impairment of mitochondrial oxidative processes such as glucose and glutamine oxidation as well as inhibition of protein synthesis and proinsulin-insulin release. An action of STZ not yet identified but different or additional to alkylation has been postulated repeatedly. Recently, Kwon et al. showed that STZ can produce nitric oxide (NO) through at least two different mechanisms: first, photolysis induced by light emitted from a fluorescent lamp at neutrality and, second, proton-dependent reactions in acid. It also was obtained from Alexis Corporation (Laufelfingen, Switzerland) because it was placed 50 cm above cages contained in aluminum foil box. Streptozotocin solution was prepared with 0.02 M phosphate buffer in pH 4.5. Glucose levels were measured from tail blood using a glucometer (AMEX glucometer; Milex, Elkhart, IN) after 8 hours of fasting.

For comparison to the STZ-treated irradiated group, 10 rats were injected subconjunctivally with a balanced saline solution, 10 rats with S-nitroso-N-acetyl-D-L-penicillamine (SNAP), and 10 rats with (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO) (0.1 ml, 0.1 mg/ml, 3 times every other day). All were placed under normal light conditions to evaluate the effect of NO on the corneal tissues.

**Ocular Surface Observation**

Before exposure, each eye was examined thoroughly with the biomicroscope. Two observers independently determined the damage status and classified each eye. The conditions of the lens and the cornea, and the staining pattern with the fluorescein solution, were observed while under a red-free slit lamp after the pupils were dilated with 2.5% phenylephrine and 1% atropine sulphate. Pictures of the cornea and lens were taken with a zoom photograph slit lamp (model SM-50F; Takagi, Nakano, Japan).

**Aqueous Humor Sampling**

On the 1st, 3rd, 7th, and 10th days, experimental animals were killed by an overdose of pentothal sodium after sedation with an intramuscular injection of ketamine hydrochloride. All of the eyes were enucleated immediately after death and frozen at −70°C. The anterior chamber was exposed by an incision on the limbus, and the aqueous humor was collected with a 27-gauge needle using an operating microscope for measurement of NO. Aqueous humor was centrifuged in a refrigerated (4°C) centrifuge at 3000 rpm for 10 minutes to remove cellular debris.

**Measurements of the Concentrations of Nitrite and Nitrate**

Measurement of NO is difficult because of the instability of NO in the presence of oxygen. Measurement of the concentration of nitrite plus nitrate, the accumulating stable oxidation products of NO, is a standard method in the NO assay. For measuring the concentration of nitrite plus nitrate, aqueous humor was mixed with an equal volume of Griess reagent (mixture of a part of 0.1% naphthylethylenediamine dihydrochloride in water and a part of 1% sulfanilamide when not exposed to the fluorescent lamp. Although the power of fluorescent lamps is 0.02 J/cm² when irradiated 2 m from lamps, the power of this lamp was 2.16 J/cm² (UVRI radiometer; Topcon, Tokyo, Japan), because it was placed 50 cm above cages contained in aluminum foil box. Streptozotocin solution was prepared with 0.02 M phosphate buffer in pH 4.5. Glucose levels were measured from tail blood using a glucometer (AMEX glucometer; Milex, Elkhart, IN) after 8 hours of fasting.

**MATERIALS AND METHODS**

**Material**

Ammonium chloride, naphthylethylenediamine dihydrochloride, sodium borate, sodium phosphate, STZ, sulfanilamide, and N-acetyl-D,L-penicillamine were obtained from Sigma (St. Louis, MO). DETA NON-Oate [NOC-18, DETA/NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate] was obtained from Alexis Corporation (Laufelfingen, Switzerland). S-nitroso-N-acetyl-D,L-penicillamine was prepared by the method of Field et al. Other chemicals used in this study were reagent grade.

**Experimental Animals**

Sprague-Dawley albino male rats (230 to 250 g), handled and cared for according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, were housed with free access to water and food. They were divided into 4 groups with 15 rats in each group. Group A was placed in a dark room. Group B was exposed to two fluorescent lamps (20 W) for 6 hours a day, which are hung in the box surrounded with aluminum foil. Group C received STZ (65 mg/kg) intraperitoneally and was placed in a dark room. Group D was treated with STZ and exposed to fluorescent lamps for 6 hours a day in the box. Groups B and D were housed in a dark room for 6 hours a day, which are hung in the box.
TABLE 1. Nitrite and Nitrate Concentrations in Four Groups

<table>
<thead>
<tr>
<th>Group*</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
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<tbody>
<tr>
<td>A</td>
<td>43.17 ± 5.21</td>
<td>59.24 ± 2.92</td>
<td>40.26 ± 4.45</td>
<td>45.43 ± 3.91</td>
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<tr>
<td>B</td>
<td>42.49 ± 5.41</td>
<td>52.60 ± 9.26</td>
<td>62.84 ± 4.50</td>
<td>85.74 ± 8.73</td>
</tr>
<tr>
<td>C</td>
<td>48.18 ± 6.20</td>
<td>54.16 ± 7.17</td>
<td>63.82 ± 4.70</td>
<td>81.29 ± 7.70</td>
</tr>
<tr>
<td>D</td>
<td>60.70 ± 7.37</td>
<td>69.29 ± 5.84</td>
<td>70.19 ± 5.10</td>
<td>111.37 ± 7.47</td>
</tr>
</tbody>
</table>

Statistical significances with days: day 1: BACD; day 3: ABCD; day 7: ABCD; day 10: ACBD. Statistical significances with groups: Group A: 3rd 7th 1st 10th; Group B: 1st 3rd 7th 10th; Group C: 1st 3rd 7th 10th; Group D: 1st 3rd 7th 10th. The underlines indicate that the connected groups are not statistically significant; *P < 0.05 (by modified Z-tests: LSD).

* Groups: A = control; B = only fluorescent lamp irradiated; C = only STZ-treated; D = STZ and fluorescent-treated.

in 5% phosphoric acid) by repeated pipetting, and the absorbance at 543 nm was measured immediately in a Gilford spectrophotometer. The concentration was measured by an automated procedure using a Cu²⁺-Cd column to reduce nitrate to nitrite.27

Histopathologic Examination

Eyes obtained on the 10th day of treatment were immersed in 2% glutaraldehyde in 0.1 M phosphate buffer, dissected while under the operating microscope to make tissue blocks of 3 × 3 mm. Each block contained cornea in full thickness. The tissue blocks were placed in 2% glutaraldehyde in 0.1 M phosphate buffer for 90 minutes at 4°C, and postfixed in 1% osmium tetro oxide for 90 minutes at 4°C. After fixation, the specimens were dehydrated, serially sectioned, stained with uranyl acetate and lead citrate, and examined with either the scanning or transmission electron microscope (JEM 200 CX; JEOL, Tokyo, Japan).

Statistical Analysis

Results are expressed as mean ± standard deviation. Statistical significance was determined by analysis of variance followed by a modified t-test (least significance difference) to determine specific differences among groups and times.

RESULTS

To examine light-dependent NO generation in the aqueous humor of eye after STZ administration, concentrations of nitrite plus nitrate, accumulating oxidation products of NO, were measured in the aqueous humor of rats treated with STZ and irradiation. In the absence of STZ-light treatment, 39 ~ 45 μM nitrite plus nitrate was found. In animals illuminated with a fluorescent lamp for 10 days (6 hours a day) after STZ treatment, the concentration increased to ~111 μM (Table 1). Although NO generation was enhanced by either the light treatment or STZ alone, much higher levels were noted in STZ–light treatment. These results indicate that light-dependent NO generation associated with STZ administration can occur in vivo.

The effects of NO generated from STZ were examined by biomicroscopy and electron microscopy. Cataract formation was observed surrounding the Y-suture on the 3rd day of light exposure after STZ injection in 12 of 15 rats. This cataract formation was not observed by STZ treatment alone nor light exposure alone. Subepithelial opacities with edema appeared subsequently after 7 days in eight of nine rats treated with STZ–light (Fig. 1A). On the 10th day, cortical lens opacities with water vacuoles appeared. Furthermore, the corneas became hazy and the iris indistinguishable in five of the rats (Fig. 1B). In rats treated with light or STZ alone, corneal epithelial punctate erosions were seen on the 10th day; however, corneal opacities and lens changes were not observed. When SNAP, another NO-generating reagent, was injected subconjunctivally, similar but more severe corneal changes were observed. Corneal epithelial erosion and edema appeared on the first day of injection, and corneal and lens opacities were visible on the third day of injection. These changes were not observed in rats treated with N-acetylpentenidicillamine, a parent compound of SNAP not containing NO moiety, and balanced salt solution (data not shown). Similar changes were observed when DETA/NO, another NO-generating agent, was injected subconjunctivally. Thus, NO generated either from SNAP, DETA/NO, or from the photolysis of STZ appears to be linked to these corneal changes in rats.

Blood glucose levels of either irradiated or nonirradiated STZ-treated rats showed that rats became hyperglycemic by the third day after STZ injection (Table 2). However, there were significantly different morphologic changes between the STZ alone-treated and STZ–light-treated groups. Therefore, this indicates that the biomicroscopic changes observed the
FIGURE 1. Morphologic changes of cornea in streptozocin-treated rats with the fluorescent lamp irradiation. Edematous cornea shows on the seventh day (A). Hazy cornea with cataractous lens is shown on the 10th day (B) (photographed with zoom photograph slit lamp).

cornas of the STZ-light treatment group were not caused by the secondary effects of a high blood glucose levels.

Scanning and transmission electron microscopic examinations were performed to examine the histopathologic nature of NO-dependent changes in the cornea of rats treated with STZ and light for 10 days. There were no specific changes among control rats, STZ alone-treated rats, and rats exposed to fluorescent light alone. However, there were numerous bead-like intracytoplasmic protuberances found in the scanning electron micrograph of the corneal endothelium in the STZ-fluorescent light-treated group (Fig. 2D). Similar changes but more severe appearances were noted in SNAP-treated rats. There were not bead-like intracytoplasmic protuberances but rather punched-out lesions (Fig. 2E). These protuberances were found in rats neither treated with STZ alone nor exposed to fluorescent light alone. The protuberances in scanning electron micrograph and the vacuolization, and cleft formation in transmission electron micrograph of corneal endothelium suggested degenerated intracellular organelles (Fig. 3D). A magnified picture of these lesions showed intracytoplasmic myelin figures, which is a characteristic of cellular degeneration (Fig. 3E). These intracytoplasmic vacuolizations are coincident to the bead-like protuberance in scanning electron micrograph of corneal endothelium. These specific findings also were observed in rats treated with SNAP (Fig. 3F) and DETA/NO (data not shown). In the endothelium of either light alone or STZ alone-treated rats, there also was an increased number of mitochondria and swelling of mitochondria on the 10th day. These ultrastructural changes caused biomicroscopic changes in these groups. Although the concentration of nitrite and nitrate in these groups was elevated by the 10th day, the biomicroscopic and ultrastructural changes were not remarkable. The nucleus compressing perinuclear vacuolization also was observed in the corneal endothelium and fibroblast in the stroma of the STZ-light treated rats (Fig. 4A). In the SNAP-treated group, there were similar intracellular degenerative changes that were more severe than that of STZ-light-treated rat (Fig. 4B). These similar morphologic changes of the STZ-light treatment group and SNAP- and DETA/NO-treated group give evidence that NO might play a role in corneal toxicity. In addition, there were abnormally denatured collagens shown in rats treated with STZ and light (Fig. 4C).

**DISCUSSION**

To study ocular complications of diabetes mellitus, a few experimental approaches are available. The use of animal models that are spontaneously developing diabetes is limited by the availability of the animals. A streptozocin-induced diabetic model has been the method used most commonly. Streptozocin is believed to damage pancreatic β-cells, induce hypoinsulinemia, generate hyper-

<table>
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<th>Group*</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
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<tbody>
<tr>
<td>A</td>
<td>93 ± 5</td>
<td>109 ± 5</td>
<td>105 ± 4</td>
<td>95 ± 3</td>
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<tr>
<td>B</td>
<td>92 ± 5</td>
<td>92 ± 6</td>
<td>82 ± 4</td>
<td>85 ± 8</td>
</tr>
<tr>
<td>C</td>
<td>248 ± 6</td>
<td>354 ± 7</td>
<td>365 ± 4</td>
<td>381 ± 7</td>
</tr>
<tr>
<td>D</td>
<td>240 ± 7</td>
<td>369 ± 6</td>
<td>370 ± 8</td>
<td>383 ± 7</td>
</tr>
</tbody>
</table>

Statistical significances with days: day 1: ABCD; day 3: ABCD; day 7: A; day 10: ABD. Statistical significances with groups: Group A: 1st 2nd 7th 10th; Group B: 1st 2nd 7th 10th; Group C: 1st 2nd 7th 10th; Group D: 1st 2nd 7th 10th. The underlines indicate that the connected groups are not statistically significant, P < 0.05 (by modified tests. LSD).

*Groups: A = control; B = only fluorescent lamp irradiated; C = only STZ-treated; D = STZ and fluorescent-treated.
FIGURE 2. Scanning electron micrograph of the corneal endothelium of the four groups. Control group (A). Fluorescent lamp irradiated group (B). Streptozocin (STZ)-treated group reared in darkness (C). Streptozocin and fluorescent lamp-treated group (D). Magnified figure of STZ and fluorescent lamp-treated group (E). S-nitroso-N-acetyl-D,L-penicillamine (SNAP)-treated group (F). In STZ and fluorescent lamp-treated group, there are bead-like intracytoplasmic protuberances seen (arrow). In SNAP-treated group, similar but more severe changes, including punched-out lesions, are seen (arrowhead).

FIGURE 3. Transmission electron micrograph of the corneal endothelium of the four groups. Control group (A). Fluorescent lamp-irradiated group (B). Streptozocin (STZ)-treated group reared in darkness (C). In STZ and fluorescent lamp-treated group, there is intracytoplasmic mitochondrial swelling, and intracytoplasmic cleft formation was shown. These indicate the cellular degeneration (D). Magnified figure of the corneal endothelium in STZ and fluorescent lamp-treated group (E). There is an intracytoplasmic myelin figure, which is a characteristic of cellular degeneration. These lesions are coincident to the bead-like protuberance in scanning electron micrograph of corneal endothelium. Transmission electron micrograph of corneal endothelium of group subconjunctivally injected with S-nitroso-N-acetyl-D,L-penicillamine (F). There were similar but more severe ultrastructural findings shown than those of (E). Original magnifications, ×4100 (A through D), ×8200 (E), ×5000 (F).
glycemia, and, thus, finally produce diabetic ocular complications. However, STZ is a toxin with alkylating activity. Furthermore, Kwon et al. showed photolytic generation of NO recently, a toxic and bioregulatory radical, from STZ with responsible wavelength peaks 300 to 310 nm and 410 to 420 nm. Light within these wavelength regions can influence the cornea and evoke NO generation from STZ.

In the current study, we show cataract formation (in preparation) and various degenerative changes in corneal endothelium and stromal fibroblast along with increased accumulation of nitrite and nitrate, oxidation products of NO, in the aqueous humor of rats exposed to fluorescent light for 10 days after STZ administration. Similar microscopic changes were observed in SNAP- and DETA/NO- (another NO-generating reagent) -treated rats. In light alone- or STZ alone-treated rats, corneal epithelial superficial punctate erosions showed on the 10th day. No lens opacity or corneal opacity was seen in these groups, although the concentrations of nitrate and nitrate in these groups showed increased with time. We suggest that there is a critical limit of NO concentration and time to cause the morphologic changes. These results suggested strongly that NO is responsible for corneal damage by STZ administration.

Physiologic half-life of STZ is short, although NO generated from STZ has influence for at least 10 days. We suppose that NO can bind with S-nitrosothiols, especially those of albumin and existing storage form (S-NO). On light exposure, photolytic cleavage of the S-NO bond induces production of NO. This causes the elevation of nitrite and nitrate concentration at 10 days. The photoactivatable store of NO may play a role in the elevation of nitrite and nitrate concentration in light alone-treated group. The light source that we used in this experiment was, in the box-surrounded aluminum foil, different from routinely housed conditions that did not show the elevation of the concentration of nitrite and nitrate. This excessive light potentiates the STZ toxicity in the current experiment.

One of the prominent microscopic changes in NO-exposed corneas was mitochondrial swelling. This also was found in corneal endothelium and might be caused by inhibition of Na+-K+ pump in mitochondrial membrane because of deprivation of ATP. The ATP depletion can be caused by NO, because NO has been shown to inactivate many mitochondrial enzymes involved in energy production such as cis-aconitase, NADH:ubiquinone oxidoreductase, and NADH:succinate oxidoreductase. Bubble-like intracytoplasmic protuberance and cytoplasmic vacuolization occurred in the corneal endothelium of rats treated with light—STZ or SNAP and DETA/NO. These degenerative changes have not been found in any other pathologic conditions and thus could be NO-specific changes.

Here we show for the first time the NO-induced histopathologic changes of the cornea in STZ-, SNAP-, and DETA/NO-treated rats. These results, however, do not limit the usefulness of STZ in studying the ocular complications of diabetes, but they suggest that NO-mediated effects should be considered in the interpretation of experimental results from STZ-treated animals, and they support placing STZ-treated animals in dark rooms to exclude the effects of NO.

**Key Words**

cellular degeneration, corneal endothelium, nitric oxide, photoactivation, streptozocin
Corneal Toxicity by Streptozocin

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