The Effect of Aminoguanidine on the Blood–Retinal Barrier in Streptozocin–Induced Diabetic Rats

Ho Kyun Cho, MD
Hitoshi Kozu, MD
Gholam A. Peyman, MD
Gareth J. Parry, MD
Bahram Khoobehi, PhD

ABSTRACT
The effect of aminoguanidine hydrochloride (AG) on the blood–retinal barrier was studied in rats with streptozocin–induced diabetes. Half of the rats were given AG (100 mg/kg/day) while the remainder received no treatment. Vitreous fluorophotometry was performed on all rats before STZ injection and 2 weeks after induction of diabetes mellitus. Two weeks after STZ injection the mean 60-minute vitreous fluorescein concentration following dye injection had increased more in the nontreated group than in the AG-treated group, suggesting that AG may have some beneficial effect on the breakdown of the blood–retinal barrier.

Diabetic retinopathy is primarily a microvascular disorder characterized by capillary microaneurysms, capillary obstruction, retinal hemorrhages, and fibrovascular proliferation. One of the earliest detectable changes is the breakdown of the blood–retinal barrier, resulting in leakage of circulating macromolecules into the vitreous. The exact pathogenesis of the retinal microangiopathy is unknown, but nonenzymatic protein glycosylation probably plays an important role. Stable end-products of nonenzymatic protein glycosylation accumulate within insulin dependent cells and interstitially, on cell membrane proteins, structural proteins, and circulating proteins. These advanced glycosylation end-products (AGE products) are irreversible and may contribute to the degenerative processes of aging both in health and disease. Aminoguanidine (AG) has been shown to inhibit the formation of AGE products and may have a role in the prevention of diabetic complications, including diabetic retinopathy. We therefore evaluated the ability of AG to prevent increased capillary leakage of fluorescein in diabetic rats, using a technique of vitreous fluorophotometry recently developed for use in rats.
TABLE 1
Vitreous Fluorescein Concentration in Aminoguanidine and Non-Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Time After Fluorescein Administration</th>
<th>Number of Eyes</th>
<th>Before Streptozocin Injection (ng/ml)</th>
<th>2 Weeks After Streptozocin Injection (ng/ml)</th>
<th>T-Test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoguanidine</td>
<td>5 min</td>
<td>9</td>
<td>31.91 ± 7.87</td>
<td>33.08 ± 8.98</td>
<td>P = .87</td>
</tr>
<tr>
<td>Treated Rats</td>
<td>60 min</td>
<td>9</td>
<td>50.19 ± 10.58</td>
<td>59.71 ± 23.15</td>
<td>P = .52</td>
</tr>
<tr>
<td>Non-treated Rats</td>
<td>5 min</td>
<td>9</td>
<td>24.53 ± 6.12</td>
<td>38.33 ± 15.29</td>
<td>P = .20</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>9</td>
<td>46.11 ± 8.97</td>
<td>64.18 ± 9.45</td>
<td>P = .01</td>
</tr>
</tbody>
</table>

*P > .05: Statistically not significant
P < .05: Statistically significant
** Dosage of fluorescein was 30 mg/kg body weight

MATERIALS AND METHODS

Twenty-four Long Evans male rats (150 to 200 g) were injected intraperitoneally with streptozocin (STZ, 65 mg/kg) after an 18-hour fast. Fasting capillary blood glucose was measured 3 days later and nonfasting blood glucose was measured at 4 and 10 days with a commercial glucometer (Accu-Check, BMC). For this study, diabetes was defined as a fasting blood glucose concentration greater than 120 mg/dL and a nonfasting level above 450 mg/dL. The 19 rats that developed diabetes were matched for fasting blood glucose concentration and divided into treated and untreated groups. Treated rats (group I) received an aqueous solution of AG (100 mg/kg/day, 5 days/week) for 2 weeks, starting the day after STZ injection. Untreated rats (group II) received no placebo. All vitreous fluorophotometry was performed by the same individual, without knowledge of the treatment status of the rats. Both groups of rats were given free access to food and water and maintained in a 12 hours on/12 hours off light cycle. None of the rats in either group received insulin.

For vitreous fluorophotometry, the animal was preanesthetized with halothane inhalation, followed by intraperitoneal injection of ketamine hydrochloride (65 mg/kg body weight) and xylazine hydrochloride (15 mg/kg). The pupil was dilated with 1.5% phenylephrine hydrochloride and 1% tropicamide. Corneal dehydration was prevented by application of hydroxypropyl methylcellulose (Gonik, Akorn Inc) prior to the fluorophotometric examination. All rats were injected via the jugular vein with 5% fluorescein sodium at a dose of 30 mg/kg body weight. Five minutes and 60 minutes after fluorescein injection, vitreous fluorescein was measured with the fluorophotometer (Fluorotron Master, Coherent, Palo Alto, Calif) in one eye using a specially designed rat adapter lens (Coherent, Palo Alto, Calif). With the rat adapter connected to the fluorophotometer, its optical system is coupled to the optics of the eye, and no contact lens is necessary to image objects as posterior as the retina. A reading is obtained starting at a position appearing to be posterior to the retina and ending at a position anterior to the cornea. The instrument made 34 discrete measurements between these two positions. A measurement taken 0.7 mm in front of the peak of the chorioretinal curve was considered to be the fluorescein concentration in the midvitreous. The fluorescein concentration was reported by the instrument. A check of the calibration of the instrument revealed that the reported values underestimated the actual concentration of a standard solution in a cuvette. The values reported in Table 1 are corrected values obtained using the calibration curve shown in the Figure.
**TABLE 2**

Correlation Between Fasting Blood Glucose and Change in Fluorescein Concentration In Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Fasting Blood Glucose (mg/100 mL)</th>
<th>Number of Rats</th>
<th>Time After Fluorescein Injection</th>
<th>Mean ± SD Differences (ng/mL)†</th>
<th>Spearman’s* Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoguanidine Treated Rats</td>
<td>334.3 ± 58.297</td>
<td>9</td>
<td>5 min</td>
<td>1.17 ± 12.18</td>
<td>r = .865</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 min</td>
<td>9.52 ± 24.74</td>
<td>r = .700</td>
</tr>
<tr>
<td>Non-treated Rats</td>
<td>333.4 ± 43.628</td>
<td>9</td>
<td>5 min</td>
<td>13.80 ± 12.28</td>
<td>r = .831</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 min</td>
<td>18.07 ± 8.69</td>
<td>r = .557</td>
</tr>
</tbody>
</table>

* r > .05: Statistically not significant. r < .05: Statistically significant.
† Fluorescein concentration between before and 2 weeks after streptozotocin injection

Fluorophotometric data from the nine rats in each group which survived for 2 weeks were analyzed using the protocol supplied by the manufacturer. Statistical analysis was done using student’s t-test and Spearman correlation. Probability values of less than 5% were considered statistically significant.

**RESULTS**

The prediabetic, mean vitreous fluorescein (VF) concentration 5 minutes after intravenous fluorescein injection was 31.9 ng/mL in AG-treated rats and 24.5 ng/mL in untreated rats. At 60 minutes, VF had increased to 50.2 and 46.0 ng/mL respectively (Table 1). There was no significant difference between the two groups for these values. After 2 weeks of diabetes in untreated rats, the VF concentration at 5 minutes had increased from 24.5 to 38.3 ng/mL, but this difference was not statistically significant (P = .20). However, in the AG-treated rats, the VF concentration at 5 minutes was completely unchanged (33.1 ng/mL). After 2 weeks of diabetes in untreated rats, the 60 minute VF concentration had increased from 46.1 to 64.2 ng/mL (P < .05). In AG-treated rats, the 60 minute VF concentration had also increased from 50.2 to 59.7 ng/mL (P = .52).

There was no difference between the two groups in fasting or nonfasting blood glucose concentration and no correlation between blood glucose and changes in vitreous fluorescein concentration (Table 2).

After 2 weeks of diabetes, some lens opacification appeared as tiny vacuoles in the cortex or as radial incipient opacities in the equatorial region. These changes were progressive and made it impossible to repeat vitreous fluorophotometry beyond 2 weeks of diabetes. The lens opacities occurred with equal frequency in AG-treated and untreated rats (12 eyes in treated and 14 eyes in untreated).

**DISCUSSION**

These studies show that 2 weeks of diabetes produces a marked increase in fluorescein leakage into the vitreous, 60 minutes after intravenous injection. There was also an increase in the vitreous fluorescein concentration 5 minutes after injection, but the difference from the prediabetic values did not achieve statistical significance. These results are in accordance with the findings of Smith et al in different experimental models of diabetes. However, Vine et al found that the absolute vitreous fluorescein concentration was significantly reduced after 2 weeks of diabetes in rats. Nonetheless, in their studies, the ratio of vitreous to serum fluorescein concentration was significantly increased, indicating excessive fluorescein leakage from retinal capillaries. Therefore, regardless of the absolute vitreous fluorescein concentration, all investigators agree that one of the earliest changes in the retina in experimental diabetes is excessive leakage of fluorescein into the vitreous.

Treatment of diabetic rats with AG appeared to have a favorable effect on vitreous fluorescein concentration. The slight increase seen in untreated rats at 5 minutes was abolished by AG treatment and the more severe changes, seen at 60 minutes, were reduced. These results indicate that AG may reduce the breakdown of the blood–retinal barrier, with increased retinal capillary permeability, which occurs in untreated diabetics. However, in our study the level of statistical significance is not strong; thus the results need further confirmation before recommendation of the use of AG in diabetics.

Pharmacologically, AG prevents the formation of advanced glycosylation end products by producing non–reactive Amadori products during nonenzymatic glycosylation. In experimental diabetic rats, AG prevented diabetes–induced formation of fluorescent advanced nonenzymatic glycosylation products and cross–linking of arterial wall connective protein. Nonenzymatic glycosylation occurs as a normal aging process, but is accelerated in diabetes, suggesting that glucose–induced cross–linking may contribute to the development of diabetic complications as
well as the physical changes of aging. Cross-linking alters the tissue protein and reduces its susceptibility to physiologic degradation by enzymes. The progressive occlusion of diabetic vessels may result from excessive formation of glucose-induced cross-linking. The alteration of proteins is related to arterial stiffening, decreased joint mobility, and the severity of microvascular complication in diabetes mellitus. Although most protein in living systems turns over rapidly to avoid non-enzymatic browning, some long-lived proteins are vulnerable.

Thickening of the retinal vascular basement membrane and renal glomerular mesangial thickening has been found in diabetes. Staining of the glomerular basement membrane for IgM and IgG has been reported. Also, deposition of IgM and albumin in the renal tubular extracellular membrane of severe diabetic patients has been found. These results may reflect structural changes in the renal extracellular membranes that permit entrapment of serum proteins. Similar pathologic changes have been found at the autonomic plexus of choroid and retinal vessels and the retinal pigment epithelium in experimentally induced diabetic rat eyes. Recently, Shires et al demonstrated nonenzymatic glycosylation of the vitreous collagens in STZ-treated rats. In rats with STZ-induced diabetes, vitreous fluorescein concentrations have been seen to increase 4 to 8 days after a single dose of STZ, with retinal pigment epithelial lesions observed in the same rats. These reports show that a close relationship exists between functional and structural changes, and that those structural changes may alter the normal blood-retinal barrier and cause diabetic complications.

Mean fasting blood glucose level after STZ was identical in the two groups. There was no correlation between fasting blood glucose levels and vitreous fluorescein concentrations in either group (Spearman Correlation Test). Thus, AG does not appear to exert its effect through alteration in glucose homeostasis.

REFERENCES


