Normalization of Retinal Vascular Permeability in Experimental Diabetes with Genistein

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**PURPOSE.** To study the effects of genistein, a tyrosine kinase inhibitor, on retinal vascular permeability in an experimental diabetic rat model.

**METHODS.** Seventy-two rats were equally divided into four groups: (1) nondiabetic control group, (2) diabetic control group, (3) diabetic rats receiving 150 mg genistein/kg food, and (4) diabetic rats receiving 300 mg genistein/kg food. Diabetes was induced by streptozotocin injection in the three diabetic groups. Rats were fed diets with or without genistein and followed for 6 months. Retinal vascular permeability was assessed by measuring radiolabeled sucrase leakage into the retina and by Western blot analysis for total retinal albumin. Retinal phosphotyrosine levels and proliferating cell nuclear antigen (PCNA) were also evaluated by Western blot analysis.

**RESULTS.** Diabetic control rats had markedly increased retinal vascular leakage of radiolabeled sucrose compared with nondiabetic control rats. Diabetic rats receiving oral genistein had significantly less retinal vascular leakage of radiolabeled sucrose than diabetic control rats in a dose-response fashion. Diabetic control rats had increased levels of phosphotyrosine, retinal albumin, and PCNA by Western blot analysis compared with nondiabetic control rats. Rats receiving 300 mg of genistein had decreased retinal albumin by Western blot analysis. Western blot analysis demonstrated a dose-response decrease in retinal phosphotyrosine levels and PCNA in genistein-treated diabetic rats compared with diabetic control rats.


Diabetic retinopathy is the leading cause of new cases of legal blindness among working-aged Americans. The incidence of diabetes mellitus in the United States and worldwide will increase to approximately 25 million and 300 million by the year 2030, respectively. After 10 years, almost 60% of patients develop retinopathy, and after 15 years 80% develop legal blindness. In 1990 a survey estimated that there were between 4 and 6 million cases of diabetic retinopathy in the United States, with 75,000 new cases of diabetic macular edema each year. Although focal laser photocoagulation is effective at reducing the risk of moderate visual loss by approximately 50%, significant numbers of patients continue to lose vision. Focal laser treatment results in permanent paracentral scotomas, mild visual field defects, and addresses only late complications of diabetes. Currently, no preventive treatment for the ocular complications of diabetes mellitus exists.

Increased retinal vascular permeability, with subsequent development of macular edema, has been established in humans and in animal models of diabetes mellitus. Vascular endothelial growth factor (VEGF) has garnered considerable interest as an etiologic agent in the induction of vascular permeability. VEGF, an angiogenic factor and a specific mitogen for vascular endothelial cells, mediates its cellular actions through tyrosine kinase signaling pathways. The streptozotocin-induced diabetic rat is an animal model of type I diabetes mellitus that has been shown to develop increased retinal vascular permeability with increasing duration of diabetes. Numerous studies have correlated elevated retinal VEGF levels with increased retinal vascular permeability in this animal model. As such, the streptozotocin-induced diabetic rat represents an excellent animal model in which to assess the safety and efficacy of pharmacologic agents in the prevention and treatment of diabetes-associated retinal vascular permeability.

Genistein, an isoflavonoid, is a naturally occurring tyrosine kinase inhibitor that is found in soybeans. Along with its tyrosine kinase inhibitory properties, genistein has a myriad of other biological activities including antioxidant and aldose reductase inhibitory activities. Genistein has an attractive safety profile, with an IC₅₀ for endothelial cells of 12.5 μM and an LC₅₀ of >300 μM.

Our laboratory has conducted numerous investigations of genistein in animal models of retinal/choroidal disease. We have shown that genistein is an effective compound for long-term oral dosing and is effective in ameliorating the biochemical, histopathologic, and morphologic changes in experimental retinal disease states.

In this study we investigate the effect of chronic oral genistein on retinal vascular permeability in the streptozotocin-induced diabetic rat.

**METHODS**

**Animals**

Seventy-two 4-week-old, Sprague-Dawley albino male rats were obtained from Harlan Sprague-Dawley Inc. (Frederick, MD). The animals were divided into four groups, with 18 rats in each group. The four groups were as follows: (1) nondiabetic control group (non-DM Control), (2) diabetic control group (DM Control), (3) diabetic rats receiving 150 mg genistein/kg food (DM 150), and (4) diabetic rats receiving 300 mg genistein/kg food (DM 300). General conditions of all rats were monitored during the study. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction and Maintenance of Diabetes Mellitus with Streptozotocin

Rats in the DM Control, DM 150, and DM 300 groups were injected with streptozotocin at the start of the study to induce diabetes mellitus. Under intraperitoneal or intramuscular ketamine (50 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and xylazine (5 mg/kg; Phoenix Pharmaceutical) anesthesia, streptozotocin (65 mg/kg; Sigma, St. Louis, MO) in 1 mM sodium citrate buffer (pH 4.5) was injected through the penile vein using a tuberculin syringe with a 30-gauge needle. Animals were declared diabetic when their plasma glucose level exceeded 13.7 mM 4 days after streptozotocin injection. Plasma glucose levels were measured every other week using One Touch Basic (Lifescan, Milpitas, CA) until they were killed. Percent glycohemoglobin was measured using the Glicatrend Hemoglobin Kit (Sigma) at the time they were killed. Diabetic animals received 2 to 3 units of NPH insulin (Lilly, Indianapolis, IN) weekly to prevent ketosis.

Diet Administration with or without Genistein

Synthetic genistein (99% purity) was obtained from IC Laboratories (Woburn, MA). Genistein was incorporated into Purina 5008 rat diet in the amounts of 150 and 300 mg genistein/kg diet by Bioserv (Frenchtown, NJ). Rats in the non-DM control and DM control groups were fed ad libitum food with 300 mg of genistein/kg of food. DM 300 rats were fed ad libitum a Purina 5008 diet without genistein. DM 150 rats were fed ad libitum food with 150 mg of genistein/kg of food, and DM 300 rats were fed ad libitum food with 300 mg of genistein/kg of food. Rats received the same diet within each of their respective groups for the duration of the study.

The weight of each rat was measured at baseline every other week. Average daily intake of food and genistein per rat were calculated every other week by measuring the remainder of a known quantity of food and dividing by the number of rats in that cage.

Measurement of Blood–Retinal Barrier Permeability

The use of radiolabeled sucrose to measure the blood–retinal barrier (BRB) permeability was adapted from a previously reported technique. This technique is based on the assumption that the amount of tracer contained within the retinal vasculature is constant. Increased carbon-14 (\(^{14}\)C)-sucrose counts in the retina reflected tracer that had leaked from the vessels into the retinal parenchyma. \(^{14}\)C counts of the Sucrose Test

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Non-DM Control (g)</th>
<th>DM Control (g)</th>
<th>DM 150 (g)</th>
<th>DM 300 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>444.0</td>
<td>319.3</td>
<td>329.8</td>
<td>323.4</td>
</tr>
<tr>
<td>6</td>
<td>503.7</td>
<td>356.2</td>
<td>342.1</td>
<td>336.5</td>
</tr>
<tr>
<td>12</td>
<td>550.0</td>
<td>424.6</td>
<td>359.0</td>
<td>352.3</td>
</tr>
</tbody>
</table>

Values are expressed as kg-food/kg-body weight/rat/d.

**RESULTS**

Development of Diabetes

Rat body weights, food consumption, blood sugar levels, and hemoglobin A1C levels were consistent with the development of clinical diabetes mellitus in the streptozotocin rat groups (Tables 1 through 4). A total of 39 of 72 rats survived to 6 months. Table 5 shows the survival rate for each group. Table 6 shows the calculated average consumption of genistein per rat per day in each of the two rat groups receiving oral genistein.

Breakdown of the BRB as Assessed by Carbon-14 Sucrose Test

Figure 1 is a boxplot of D/C ratio by treatment group with results of pairwise comparisons using repeated-measures of ANOVA. D/C is the calculated ratio of \(^{14}\)C-sucrose in the retina overnight at 50°C in a water bath. Glacial acetic acid, 30 μl, was added to the vial, and 0.5 ml of blood sample was added to 6 ml of NCS-II and heated at 50°C in a waterbath until clear. Benzyol peroxide solution, 2 ml, was added and heated for a further 30 minutes at 50°C. The digests were then counted in a scintillation counter (Wallac, Inc., Gaithersburg, MD).

**Western Blot for Phosphotyrosine, Retinal Albumin, and Proliferating Cell Nuclear Antigen**

Primary antibodies used for Western blot analysis were rabbit anti-rat phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:400 with blocking solution, rabbit anti-rat albumin antibody (Kirkgaard and Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:400, and rabbit anti-rat proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology) diluted 1:400.

Twenty-one eyes were enucleated, and the anterior segment was removed (4 non-DM control, 5 DM control, 6 DM 150, 6 300 DM). The vitreous was removed with forceps, and the retina was peeled away from the choroid. The retinal tissue was lysed in 100 μl of the lysis buffer (0.1% sodium dodecylsulfate [SDS] in 0.1 M Tris-HCL, pH 7.4). Aliquots (30 μg of protein) of sample lysates were boiled for 5 minutes at 90°C in the sample buffer containing 0.1% SDS. The samples were then subjected to NuPAGE 10% Bis-Tris gel (Novex, San Diego, CA) and transferred onto Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Either broad range SDS-PAGE biotinylated or prestained SDS-PAGE molecular weight standards (Bio-Rad) were used. The membranes were incubated in a blocking solution (5% skim milk, 0.1% Triton X-100, 0.02% NaN3, and 10 mM Tris-HCL, pH 7.4) overnight at 4°C to avoid nonspecific immunoreaction. The membranes were then incubated for 60 minutes at room temperature with a primary antibody solution. The membranes were washed four times with wash solution (0.1% Triton X-100 in Tris-buffered saline) for 10 minutes at room temperature. As a secondary antibody, we used horseradish peroxidase–conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology) diluted 1:10,000 for 60 minutes at room temperature. After washing four times with wash solution for 10 minutes each at room temperature, membranes were immobilized with ECL Western blot detection reagents (Amersham) and exposed on x-ray film (Eastman-Kodak, Rochester, NY).
to $^{14}C$-sucrose in the blood. The D/C ratio serves as an index of retinal vascular permeability (a higher ratio indicates greater permeability). Overall, there were significant differences in retinal vascular permeability between treatment groups ($P = 0.0002$). The non-DM control group had significantly less vascular leakage compared with the DM control group ($P < 0.0001$), but did not differ significantly from the DM 150 or DM 300 groups ($P = 0.083$ and $P = 0.46$, respectively). The DM control group had significantly greater retinal vascular permeability compared with the DM 150 and DM 300 groups ($P = 0.0065$ and $P < 0.0001$, respectively). The DM 150 group had marginally greater vascular leakage compared with the DM 500 group ($P = 0.022$).

### Western Blot Analysis for Phosphotyrosine

A representative Western blot stained with anti-rat phosphotyrosine antibody is shown in Figure 2. The DM control rats showed an overall increase in tyrosine-phosphorylated proteins (lanes 9, 10, and 11) when compared with non-DM control eyes (lanes 1, 2, and 3). Rats in the DM 150 group (lanes 6, 7, and 8) and DM 500 group (lanes 4 and 5) demonstrated a dose–response decrease in staining for phosphotyrosine to near non-DM control rat levels (lanes 1, 2, and 3).

### Western Blot Analysis of Retinal Albumin

A representative Western blot stained with anti-rat albumin antibody is shown in Figure 3. The DM control group showed a dose–response decrease in staining for albumin compared with the non-DM control group. The 80-kDa band was less prominent in the DM 300 group.

### Western Blot Analysis of PCNA

A representative Western Blot stained with anti-rat PCNA antibody is shown in Figure 4. The DM control rats showed an increase in the 36-kDa band corresponding to PCNA compared with non-DM control rats. A dose–response decrease in the 36-kDa band was noted in the DM 150 and DM 300 groups.

### Discussion

This study investigated the effect of long-term oral genistein on the retina in the streptozotocin diabetic rat. We quantitatively assessed the effect of oral genistein on retinal vascular permeability and qualitatively assessed retinal albumin, phosphotyrosine, and PCNA by Western blot analysis.

We assessed retinal vascular permeability in two ways: indirect measurement of BRB permeability using radioiodinated sucrose and Western blot analysis for total retinal albumin. Markedly increased retinal vascular permeability was observed in diabetic control rats compared with non-diabetic control rats ($P < 0.0001$). Rat food containing genistein significantly reduced retinal vascular permeability in diabetic rats to near-control levels, in a dose–response fashion. Rats receiving 300 mg genistein had decreased retinal albumin on Western blot, supporting amelioration of retinal leakage.

Western blot analysis demonstrated a dose–response decrease in retinal phosphotyrosine levels and PCNA in genistein-treated diabetic rats compared with diabetic control rats. PCNA is a nuclear protein that appears in the S phase of the cell cycle and is a known marker for vascular endothelial cell proliferation in the retina. $^{25-27}$ The Western blot findings are thus consistent with genistein’s inhibitory effects on tyrosine kinase activity and on endothelial cell proliferation.

Leuenberger et al. $^{28}$ has described the histopathologic changes of the streptozotocin rat. Streptozotocin rats that had been diabetic for 6 months developed cataract, neovascularization of the iris, loss of retinal capillary endothelial and mural cells, focal basement membrane thickening, and variations in capillary diameter, resulting in the appearance of fusiform microaneurysms. Several authors have described the breakdown of the BRB that occurs in the streptozotocin rat. $^{5-7,9,10}$ Ishibashi et al. $^{6}$ noted that the increased permeability in retinal capillaries of streptozotocin rats preceded the thickening of the basement membrane and seemed to play an important role in the development of diabetic retinopathy.

In vitro, animal, and clinical investigations support a role for VEGF in the induction of increased retinal vascular permeability. $^{5-10,29-34}$ Murata et al. $^{9,10}$ have correlated increased VEGF protein expression with increased BRB breakdown in streptozotocin diabetic rat eyes. The rate of both BRB breakdown and VEGF immunoreactivity increased in proportion to the duration of diabetes. VEGF immunoreactivity was distributed in all layers of the retina, especially near the optic disc and around large vessels. These two regions coincide with the sites wherein BRB breakdown is clinically detected by fluorescein angiography in diabetic patients. Mathews et al. $^{8}$ demonstrated in postmortem human diabetic eyes that VEGF immunoreactivity along retinal vessels was correlated with increased vascular permeability to macromolecules such as human serum albumin and heparin sulfate proteoglycan. Using ADPase flat-embedded fellow retinas, they observed that the increased VEGF immunoreactivity and vascular permeability occurred before morphologic changes in the vasculature. These findings are in line with those of Ishibashi et al., which suggested that vascular permeability is increased before the onset of diabetic retinopathy and may play an important role in its development. The intracellular mechanisms underlying VEGF’s actions on endothelial cell permeability are not fully understood but ap-

### Table 3. Average Blood Sugar Levels

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Non-DM Control (mM)</th>
<th>DM Control (mM)</th>
<th>DM 150 (mM)</th>
<th>DM 300 (mM)</th>
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<tr>
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### Table 4. Hemoglobin A1C Levels

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<th>Non-DM Control</th>
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<th>DM 150</th>
<th>DM 300</th>
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<tbody>
<tr>
<td>Hb A1C Level</td>
<td>3.9</td>
<td>7.2</td>
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<td>6.9</td>
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</table>

Values are expressed as percentages. Hb A1C levels were measured at time animals were killed (6 months).

### Table 5. Rat Survival Rates

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Non-DM Control (n = 9)</th>
<th>DM Control (n = 9)</th>
<th>DM 150 (n = 11)</th>
<th>DM 300 (n = 10)</th>
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<tr>
<td>Survival rate at 6 months</td>
<td>50%</td>
<td>50%</td>
<td>61%</td>
<td>56%</td>
</tr>
</tbody>
</table>

Each group consisted of 18 rats at the start of the experiment.

### Table 6. Average Genistein Intake

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>DM 150</th>
<th>DM 300</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>17.7</td>
<td>42.9</td>
</tr>
</tbody>
</table>

Values are expressed as mg genistein/kg-body weight/rat/d.
pear to involve tyrosine kinase, nitric oxide, and protein kinase C (PKC) pathways.\textsuperscript{35,36}

Genistein is a naturally occurring protein tyrosine kinase inhibitor\textsuperscript{14} that has been shown to inhibit in vitro angiogenesis.\textsuperscript{18} Despite the fact that genistein has a myriad of biological activities, it has a wide therapeutic index with no known toxicity at extremely high doses.\textsuperscript{19,37} Because of its tyrosine kinase inhibitory activity, its natural occurrence in nature, and its attractive safety profile, our laboratory has investigated its efficacy in numerous animal models of ocular disease. In all these experiments, we have demonstrated that genistein inhibited retinal tyrosine phosphorylation and ameliorated the retinal disease process in animals.\textsuperscript{20–23}

Our present study provides additional preclinical evidence that genistein may be a useful pharmacologic agent in the prevention and treatment of the ocular manifestations of diabetes mellitus. Our Western blot analyses provide supportive evidence that protein tyrosine kinase (PTK) pathways are activated in the streptozotocin diabetic rat retina. Genistein’s ability to dose-dependently reduce retinal vascular permeability and inhibit the PTK pathways supports the purported pathophysiologic mechanisms of VEGF-activated PTK pathway induction of vascular leakage in the streptozotocin diabetic rat. We believe that genistein, by inhibiting VEGF-activated PTK pathways, leads to inactivation and downregulation of nitric oxide synthase with subsequent amelioration of retinal vascular leakage.

Our data indicate that chronic oral genistein can significantly reduce retinal vascular leakage in an animal model of diabetic retinopathy. The clinical development of a safe and effective compound for the prevention of the leading cause of vision loss in diabetes, macular edema, would have a major public health impact. Studies by Ishibashi et al.\textsuperscript{6} and Mathews et al.\textsuperscript{8} have suggested that retinal vascular permeability may be the single greatest factor that leads to the development of subsequent diabetic retinopathy. If this is true, then the phar-

\begin{figure}
\includegraphics[width=\textwidth]{fig1}
\caption{Boxplot of D/C ratio (index of retinal vascular permeability). Mean values for each group: (1) non-DM control, 0.537 ± 0.144; (2) DM control, 1.266 ± 0.345; (3) DM 150, 0.797 ± 0.316; (4) DM 300, 0.437 ± 0.104.}
\end{figure}

\begin{figure}
\includegraphics[width=\textwidth]{fig2}
\caption{Western blot for phosphotyrosine. The 116-kDa band corresponds to phosphotyrosine. Staining with anti-phosphotyrosine antibody is greater in DM control (lanes 9, 10, 11) compared with non-DM control (lanes 1, 2, 3). Staining in DM 150 (lanes 6, 7, 8) and DM 300 (lanes 4, 5) demonstrates a dose-response decrease in staining to near non-DM control (lanes 1, 2, 3) levels.}
\end{figure}

\begin{figure}
\includegraphics[width=\textwidth]{fig3}
\caption{Western blot for retinal albumin. The 80-kDa band corresponds to albumin. Staining with antialbumin antibody is greater in DM compared with non-DM control. Staining is less prominent in DM 300.}
\end{figure}
macrodlogic development of a compound, such as genistein, would have an even larger impact on altering the natural history of diabetic eye disease.

Acknowledgments

The authors thank Rhonda Grebe for her invaluable support and assistance with this study.

References


