Effect of Topical Ginkgo biloba Extract on Steroid-Induced Changes in the Trabecular Meshwork and Intraocular Pressure

Li-Yun Jia, PhD; Ling Sun, BSc; Dorothy S. P. Fan, FRCS; Dennis S. C. Lam, MD; Chi Pui Pang, DPhil; Gary H. F. Yam, PhD

Objective: To study the effects of Ginkgo biloba extract (GBE) on dexamethasone (DEX)–induced ocular hypertension.

Methods: Rabbits aged 7 weeks received topical TobraDEX (Alcon Labs, Hünenberg, Switzerland) and/or 5 µg of GBE four times daily for 14 days. Intraocular pressure (IOP) was recorded every 3 days. After enucleation, trabecular meshwork (TM) cellularity and extracellular matrix deposition were graded. The effect of GBE on apoptosis and expression of myocilin and cell stress–related genes in DEX-treated human TM cells were studied by immunofluorescence, Western blotting, and quantitative polymerase chain reaction.

Results: Ginkgo biloba extract suppressed DEX-induced IOP elevation in rabbits. It reduced the DEX-associated accumulation of extracellular materials within the cribriform layers of the TM and achieved better TM cellularity. In cultured human TM cells, GBE substantially attenuated anti–Fas ligand–induced apoptosis and reduced DEX-induced myocilin expression. Ginkgo biloba extract modulated the expression of H9251 B-crystallin and heat-shock proteins 70 and 90 but not other stress-related genes. Furthermore, changes associated with DEX were found less in GBE-treated or GBE-primed TM cells.

Conclusion: We showed that GBE, a nontoxic, anti-apoptotic, herbal compound significantly suppressed steroid-induced IOP elevation in rabbits and it seems to prevent the adverse effects of DEX on TM cells.

Clinical Relevance: Ginkgo biloba extract could be a therapeutic agent or dietary supplement to prevent steroid-induced ocular hypertension.

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for ophthalmic therapy owing to its cytoprotective, antiapoptotic, and antioxidative properties. Clinical studies have shown that it may be useful in treating visual field damage associated with chronic cerebrovascular insufficiency, glaucomatous damage, macular degeneration, and diabetes mellitus. It also alleviates retinal impairment caused by ischemic-reperfusion injury. In rabbit corneal epithelial cells and human lens epithelial cells, GBE reduced alloxan-mediated and light-mediated stress, respectively. In this study, we investigated the effect of GBE on ocular hypertension caused by topical DEX in rabbits.

**METHODS**

**TOPICAL DEX AND GBE IN RABBITS**

The experimental procedure followed the Declaration of Helsinki and Association for Research in Vision and Ophthalmology animal statement and was approved by the Animal Ethics Committee of the Chinese University of Hong Kong. Thirty male New Zealand albino rabbits aged 7 weeks were randomly chosen for the following topical treatments: group 1 (n=7), balanced salt solution (BSS); group 2 (n=8), TobraDEX (0.1% dexamethasone and 0.3% tobramycin) (Alcon, Hünenberg, Switzerland); group 3 (n=8), 100 µg/mL of GBE; and group 4 (n=7), Tobradex and GBE. One drop was applied to the right eye 4 times daily for 14 days. For combined DEX and GBE, DEX was given 20 minutes before GBE to ensure complete drug delivery.

Intraocular pressure was recorded every 3 days with Tono-Pen XL (Solan Ophthalmic, Jacksonville, Florida) by a masked technician (W.L.). The mean (standard deviation) of 5 consecutive readings were calculated and compared by Mann-Whitney U test. A P value less than .05 was considered significant. After the rabbits were killed, enucleated eyes were cut along the nasal-temporal line, fixed in 10% neutral buffered formalin, and paraffin embedded. Ten consecutive sections were stained with hematoxylin-eosin or periodic acid–Schiff (PAS) reagents. Results were examined by 2 masked pathologists (L.-Y.J. and Ta Li Liu, FRCS [Glasg]). Cells treated with 10−8M camptothecin which contained human full-length wild-type MYOC cDNA cloned by impressive judgment as 0, no difference from the controls; 1, 5% to 20% less; or 2, more than 20% less. Thickening of the TM was categorized as 0, less than 5% less nuclei than the controls; 1, 5% to 20% less; or 2, more than 20% less. Thickening of the TM was graded by counting the nuclei in the TM and enhanced chemiluminescence (ECL) (Amersham Biosciences, Little Chalfont, England). Band intensities were quantified by Quantity One Image Analysis (BioRad, Hercules, California). Experiments were triplicated and intensities were represented as mean (standard deviation).

**QUANTITATIVE POLYMERASE CHAIN REACTION**

Total RNA was extracted by RNasy Kit (Qiagen, Valencia, California) and on-column RNase-free DNase digestion (Qiagen). After reverse transcription, complementary DNA were amplified for β-crystallin (CRY), interleukin 6 (IL-6), metallothionein (MET), Copper/zinc superoxide dismutase (SOD), heat shock protein 70 (Hsp70), heat-shock protein 90a (Hsp90a), β-actin (eTable available at http://www.archophthalmol.com) with QuantiTect SYBR Green PCR kit (Qiagen). Target gene expression relative to β-actin was analyzed by 2−ΔΔCT method.

**RESULTS**

**EFFECT OF GBE AND DEX ON IOP IN RABBITS**

The mean (SD) baseline IOP of the rabbits was 10.6 (1.2) mm Hg (Figure 1A). The rabbits demonstrated IOP changes after topical Tobradex. In preliminary experiments, we compared the marketed Tobradex with self-prepared 0.1% DEX in Tears Naturale II (Alcon). Both agents showed similar efficiency of IOP induction in rabbits. No complications or discomfort were observed with Tobradex. To eliminate the risk of infection, we used Tobradex. Three rabbits (1 in group 2 and 2 in group 4) died of natural causes unrelated to the topical treatments. Intraocular pressure was monitored throughout treatment. The DEX–treated rabbits (n=7) showed higher IOPs than the BSS controls (n=7). The increment range was 3 to 5.5 mm Hg (Figure 1B) and was significant (P=.04) when compared with the BSS controls. In contrast, when rabbits were given Tobradex followed by GBE (n=5), mild changes in mean IOP (<2 mm Hg) were observed (Figure 1A and B). By Mann-Whitney U test, the difference between DEX only and combined DEX and GBE treatment was significant at the examination intervals (P=.04). Rabbits with topical GBE only (n=8) did not show distinct IOP changes when compared with pretreatment values (Figure 1A and B).
microscopy showed no gross changes in anterior tissues (including cornea, conjunctiva, and eyelids) after topical DEX and/or GBE treatment. In the TM, PAS staining showed increased extracellular materials within the cribriform layers after DEX treatment (Figure 1D, H, and L) compared with the controls (Figure 1C, G, and K). After the combined DEX and GBE treatment, less extracellular matrix material was noted (Figure 1E, I, and M). No distinct alteration of the TM was found after GBE application (Figure 1F and J, and N). Moreover, DEX resulted in an alteration of cellularity, suggesting structural changes that would affect aqueous humor outflow. While strict counts were not conducted, impression by ocular pathologists revealed mild TM thickening with more PAS-positive material and fewer cells in the TM regions of rabbits treated with DEX (Tables 1 and 2, Figure 1H). These changes were fewer in rabbits who had received topical combined DEX and GBE (Figure 1I). Alone, GBE produced no observable changes (Figure 1J), similar to the controls (Figure 1G).

**REDUCTION OF DEX-INDUCED MYOC EXPRESSION IN CULTURED HUMAN TM CELLS BY GBE**

Trabecular meshwork cells at confluence showed more MYOC staining after 5-day treatment with 100nM DEX (DEX5 group) (Figure 2B) when compared with untreated cells (Figure 2A). This was corroborated by Western blotting (Figure 2E). Densitometry indicated a significant increase in MYOC expression in DEX5 cells, about
ATTENUATION OF ANTI-Fas–INDUCED APOPTOSIS OF MYOC-TRANSFECTED TM CELLS BY GBE

Trabecular meshwork cells were transfected for FLAG-tagged MYOC. Anti-Fas treatment substantially increased the number of apoptotic cells, as revealed by the frequent appearance of fragmented nuclei by the combined immunofluorescence for FLAG and DAPI. An approximately 3-fold increase in the apoptosis rate was found in MYOC-expressing cells after anti-Fas incubation compared with nontransfected cells (Figure 3A). Subsequent GBE lowered the apoptosis rate in a dose-dependent manner. Incubation with 100 µg/mL of GBE for 48 hours resulted in a 75% decrease in apoptosis, approaching the level without anti-Fas. Lower GBE concentrations had lesser preventive effects against apoptosis. Similar results were obtained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis. Incubation of anti-Fas–treated cells with 100 µg/mL of GBE for 2 days resulted in a 3-fold decrease in TUNEL-positive cells from a mean (SD) of 43.6% (10.7%) to 12.6% (3.7%). Controls (6.7% [1.6%]) and GBE-treated cells (8.7% [2.4%]) had similar mean (SD) apoptosis rates. Moreover, anti-Fas–induced activation of caspase-3 was greatly suppressed by GBE (Figure 3B). The active caspase-3 at about 17 kDa was found in MYOC-expressing cells after anti-Fas treatment as well as in camptothecin-treated cells, which served as positive controls. The 30-kDa precursor form was shown in Figure 3B. When anti-Fas treatment was given with 100 µg/mL of GBE incubation for 24 hours, less active caspase-3 was present. Band densitometry of caspase-3 revealed a significant de-

Table 1. Grading of Cell Loss in TM Region After Topical TobraDEX<sup>a</sup> and/or GBE Treatments<sup>b</sup>

<table>
<thead>
<tr>
<th>Treatment,</th>
<th>No./Total</th>
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<th>1</th>
<th>2</th>
</tr>
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<tbody>
<tr>
<td>BSS (control)</td>
<td>4/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>DEX</td>
<td>0/7</td>
<td>5/7</td>
<td>2/7</td>
<td></td>
</tr>
<tr>
<td>DEX and GBE</td>
<td>3/5</td>
<td>2/5</td>
<td>0/5</td>
<td></td>
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<tr>
<td>GBE</td>
<td>4/4</td>
<td>0/4</td>
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Abbreviations: BSS, balanced salt solution; DEX, dexamethasone; GBE, *Ginkgo biloba* extract; TM, trabecular meshwork.

<sup>a</sup>TobraDEX (Alcon Labs, Hüniken, Switzerland) comprises 0.1% DEX and 0.3% tobramycin.

<sup>b</sup>Grading scale: 0, no difference from controls; 1, mild TM thickening and PAS reactivity; 2, marked TM thickening and PAS reactivity.

Table 2. Grading of Trabecular Thickening After Topical TobraDEX<sup>a</sup> and/or GBE Treatments<sup>b</sup>

<table>
<thead>
<tr>
<th>Treatment,</th>
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<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>BSS (control)</td>
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<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>DEX</td>
<td>2/7</td>
<td>5/7</td>
<td>0/7</td>
<td></td>
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<tr>
<td>DEX and GBE</td>
<td>4/5</td>
<td>1/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>GBE</td>
<td>4/4</td>
<td>0/4</td>
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</table>

Abbreviations: BSS, balanced salt solution; DEX, dexamethasone; GBE, *Ginkgo biloba* extract; PAS, periodic acid-Schiff staining; TM, trabecular meshwork.

<sup>a</sup>TobraDEX (Alcon Labs, Hüniken, Switzerland) comprises 0.1% DEX and 0.3% tobramycin.

<sup>b</sup>Grading scale: 0, less than 5% less cell loss than controls; 1, 5% to 20% cell loss; 2, more than 20% cell loss.

10-fold more than in the control cells (P = .01, t test; n = 3) (Figure 2E). This MYOC induction was similar to that of the 10-day DEX treatment (data not shown). During DEX withdrawal, the elevated MYOC level was maintained for 5 days and then reduced gradually (Figure 2F).

The high MYOC expression level was reduced by more than 60% when cells were incubated with 100 µg/mL of GBE for another 5 days (DEX5 + GBE5 group) (Figure 2E). This dosage of GBE was optimized to have minimal effect on cell proliferation. Using immunofluorescence (Figure 2C), weak MYOC staining was noted in the GBE5 group. Moreover, we preincubated TM cells with 100 µg/mL of GBE for 5 days prior to DEX (GBE5 → DEX5 group). A weak band of immunoreactive MYOC was observed that had the intensity of about one-fifth that of the DEX5 cells (P = .02, t test; n = 3) (Figure 2D and E). Low MYOC expression was similarly found in cells pretreated with combined GBE and DEX for 5 days before DEX incubation (GBE5 + DEX5 → DEX5 group) (P = .02, t test; n = 3) (Figure 2E).

Figure 2. Effect of *Ginkgo biloba* extract (GBE) on myocilin (MYOC) expression. A-D, Immunofluorescence of MYOC. A, Controls; B, Rabbits treated with 100 nM dexamethasone for 5 days (DEX5); C, 100 nM DEX for 5 days followed by 100 µg/mL of GBE for 5 days (DEX5 → GBE5); D, 100 µg/mL of GBE for 5 days followed by 100 nM DEX for 5 days (GBE5 → DEX5). E, Western blot analysis of MYOC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in trabecular meshwork (TM) samples treated with 100 nM DEX and/or 100 µg/mL of GBE. F, Western blot analysis of MYOC and GAPDH in TM samples with 100 nM DEX for 5 days and measured for 10 days after DEX removal. GBE5 + DEX5 → DEX5 indicates cells pretreated with combined GBE and DEX for 5 days before DEX incubation; scale bars, 10 µm; *P < .05.
crease in caspase-3 activation after GBE treatment in anti-Fas-treated MYOC-expressing cells (P = .01, paired t test; n = 3). Cells treated with Ginkgo biloba extract alone had very low levels of active caspase-3.

SAFETY OF GBE FOR TM CELLS

Ginkgo biloba extract in concentrations from 10 to 1000 µg/mL were administered to TM cells. Growth kinetics were determined and the mean of triplicate measurements showed that cells treated with GBE in concentrations of less than 100 µg/mL had similar growth rates to untreated cells (cell doubling with 10 µg/mL of GBE, 40.3 hours; 100 µg/mL GBE, 41.5 hours; 1000 µg/mL GBE, 60 hours; controls, 38.1 hours). Similar results were observed for CHO-K1 cells (data not shown).

We analyzed the expression of genes associated with stress responses (oxidative, heat-shock, and metal-induced) in TM cells treated with 100nM DEX followed by 100 µg/mL of GBE and cells treated only with 100 µg/mL of GBE. A 5-day DEX treatment was chosen, as we aimed to examine early gene induction. First, for 5 days, cells treated with DEX upregulated MYOC expression (7.7-fold more than untreated cells) (Figure 4); this elevated level was maintained after DEX withdrawal for 2 days (Figure 2F). Subsequent GBE treatment gradually reduced MYOC expression by 10% after 60 minutes and 50% after 120 minutes. For cells treated with GBE only, steady-state RNA levels of stress-related genes (CRY, IL-6, MET, and SOD) were not different from those of the controls (Figure 4). Quantitative polymerase chain reaction revealed no significant alteration. However, a 5-day DEX treatment induced an approximately 3-fold increase in CRY expression (Figure 4). Additional incubation with GBE for 60 minutes further increased CRY RNA levels, which decreased after 120 minutes of incubation with GBE. The levels of IL-6, MET, and SOD RNA were not significantly altered by treatment with DEX and/or GBE (Figure 4; eFigure). Moreover, Hsp70 and Hsp90α were upregulated after incubation with DEX and/or GBE (Figure 4).

COMMENT

This study demonstrated the potential effect of GBE on DEX-induced ocular hypertension. We showed with a rabbit model that GBE suppressed IOP elevation caused by topical DEX. Ginkgo biloba extract exhibited antisteroid action by reducing DEX-induced MYOC expression in cultured human TM cells. We further showed that GBE caused MYOC-expressing cells to be less prone to apoptosis induced by anti-Fas ligand method. Ginkgo biloba extract at our application dosage had no apparent toxicity for TM cells. It moderately upregulated heat-shock responses and molecular chaperones, but did not affect other stress-related genes. This suggests that GBE has the potential to
treat IOP-associated glaucoma. In addition, priming TM cells with GBE before treatment with DEX greatly reduced MYOC expression, indicating that either dietary GBE or supplemental GBE in eye drops could reduce the risk of developing steroid-induced ocular hypertension.

Medicinal use of GBE can be traced back 5000 years in China. Today this herbal compound is one of the most widely sold herbal dietary supplements in the United States and Europe. It is known to have antiapoptotic, antioxidative, antiplatelet aggregation, antitumor, antiaging, and nitric oxide inhibitory effects on various cell types. The standard extract of GBE, EGb761, is commonly prescribed as an herbal remedy for peripheral vascular, cardiovascular, and cerebrovascular diseases. It reduces memory loss and symptoms of cognitive disorders, including Alzheimer disease and dementias. Recently, the antiinflammatory effect of GBE relieved asthma in patients. It is proposed to be a natural therapeutic agent for glaucoma, in particular the non–pressure-dependent type. Dietary intake of GBE improved the intrinsic glutathione level of retinal glial cells in older guinea pigs. In a phase 1 trial, orally administered GBE enhanced ocular blood flow. This shows the neuroprotective role of GBE in glaucomatous neuropathy. In this study, we established a novel feature of GBE, ie, the antisteroid effect, in suppressing DEX-induced IOP elevation. From various clinical and animal studies, steroids, in particular DEX, are known to upregulate MYOC expression in a time course similar to the induction of ocular hypertension. Elevated IOP due to perturbation or blockage of aqueous humor flow along the TM and Schlemm canal is a strong risk factor for glaucoma. Sustained high IOP causes progressive damage to retinal ganglion cells, causing loss of bilateral vision. In a rat model of chronic IOP-associated glaucoma, GBE was shown to promote the survival of retinal ganglion cells.

In this study, GBE was effective in relieving IOP elevation induced by DEX in rabbits aged 7 weeks. Ginkgo biloba extract (5 µg) was topically applied 20 minutes after DEX and the procedure was performed 4 times daily. Measurement of IOP showed that combined DEX and GBE caused much milder IOP fluctuation than DEX only. Increased extracellular materials within the TM layers were observed after DEX treatment was reduced. This indicates the action of GBE in the maintenance of a clear and open outflow channel for the aqueous humor. Moreover, GBE treatment was associated with less apparent cell loss in the TM region.

The antisteroid effect of GBE could be mediated by downregulating DEX-induced MYOC expression in TM cells. Studies have shown that MYOC in TM cells was stimulated by stress, such as hydrogen peroxide and mechanical stretches, elevated IOP in organ culture of anterior segment, or steroids. Adding to this list, GBE suppresses MYOC expression in the context of the elevated level induced by steroids. Thus, our results demonstrated a potential antisteroid capability of GBE in human TM cells. Ginkgo biloba (10 to 100 µg/mL) was not cytotoxic and did not affect cell growth. The cause of the slight lengthening of cell-doubling time after 1000 µg/mL of GBE treatment needs further investigation. In addition, GBE showed a protective effect against DEX as evidenced by the low MYOC expression in cells preincubated with GBE. Either priming TM cells with GBE or combined DEX and GBE resulted in a reduced effect of DEX on MYOC induction by about 80%. This result suggests that GBE is a suitable priming agent to suppress DEX-induced MYOC expression and could be envisaged as an ocular nutrient supplement.

Trabecular meshwork cells are stimulated to undergo apoptosis via the Fas:Fas ligand pathway as first demonstrated by Agarwal et al. Moreover, low MYOC expression was an advantage toward better TM cell survival. In our experiments, immortalized TM cells instead of primary TM culture were used to provide a minimal background of spontaneous apoptosis. When transfected with FLAG-tagged MYOC, our TM culture was prone to apoptosis when induced by anti-Fas. Dexamethasone-induced apoptosis was also observed in another TM cell line and in chondrocytes. Ginkgo biloba extract, with its suppressive action of DEX-induced MYOC expression, maintained a low apoptosis rate for TM cells when exposed to anti-Fas. This was well demonstrated by the low percentage of cells with fragmented nuclei and cells that were TUNEL positive. We further illustrated that a possible mechanism for GBE reduction of TM cell death could be associated with activation of αβ-crystallin, Hsp70, and Hsp90α, which are molecular and heat-shock chaperones. Also, αβ-crystallin protects TM cells from damage due to oxidative stress and mechanical stretching. Heat shock protein 70 promiscuously recognizes substrate proteins with nonnative conformation and, via interaction with Hsp90, promotes protein folding. Their upregulation by GBE would be expected to protect TM cells from MYOC induced by DEX. Moreover, GBE did not cause other stress-related changes like that of IL-6 (a marker for inflammation or occurrence of reactive oxygen species), metallothionein (induced by metals, glucocorticoids, inflammatory, and oxidative stress), and copper/zinc superoxide dismutase (induced by oxidative stress). Similar antiapoptotic capability of GBE was observed in other cell types.

Loss of TM cells was reported in glaucoma patients. This could reduce the phagocytic capability along the TM and the Schlemm canal, leading to accumulation of extracellular substances that result in blockage of outflow pathways and IOP elevation. In this study, GBE showed a potential antisteroid ability to reduce DEX-induced MYOC expression and an antipapoptotic effect to maintain the TM cell population. Similar effects could be obtained either by priming cells before DEX or rescuing DEX-treated cells. This could allow better TM function. Hence, GBE could be envisaged as a potential drug for treating IOP-associated glaucoma or it could be used as a food supplement to reduce the risk of developing steroid-induced glaucoma.

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Author Contributions: Li-Yun Jia, PhD, and Ling Sun, BSc, contributed equally to the study.

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