Perfusion-Cultured Bovine Anterior Segments as an Ex Vivo Model for Studying Glucocorticoid-Induced Ocular Hypertension and Glaucoma

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PURPOSE. To determine whether perfusion-cultured bovine anterior segments would be a suitable model for glaucoma research.

METHODS. Fresh bovine eyes were dissected and sealed on a custom-made acrylic dish with an O-ring. Perfusion medium was infused by a syringe pump at a constant infusion rate of 5 µL/min. After intraocular pressure (IOP) was stable, bovine eyes were perfused with medium containing either a vehicle control (0.1% ethanol [ETH]) or dexamethasone (DEX) for up to 7 days. IOP was recorded by a pressure transducer and a computerized system. Perfusion medium was collected for Western immunoblot analysis of myocilin (MYOC).

RESULTS. The morphology of the bovine trabecular meshwork after perfusion culture was similar to that of freshly dissected, nonperfused bovine eyes. Treatment with DEX elevated IOP in some bovine eyes, whereas others showed little change. The authors analyzed the data from 18 ETH-treated control eyes and defined 2.82 mm Hg as the threshold of ocular hypertension (OHT), which equals mean pressure change + 2× SD. Approximately 40% (12/29) of the bovine eyes were DEX responders, which is very close to the DEX-responsive rates observed in human and monkey eyes. Western blot data showed that DEX treatment induced the expression of the DEX-inducible gene MYOC only in the perfusion-cultured anterior segments with DEX-induced OHT.

CONCLUSIONS. OHT can be induced by DEX in perfusion-cultured bovine anterior segments. This is a fast, convenient, affordable, and reliable model for studying DEX-induced OHT and the mechanisms of trabecular outflow. (Invest Ophthalmol Vis Sci. 2011;52:8068–8075) DOI:10.1167/iovs.11-81353

Glucocorticoid-induced glaucoma is a subtype of secondary open angle glaucoma. Either topical or systemic administration of glucocorticoids may induce (OHT) in susceptible persons, some of whom develop optic neuropathy/glaucoma even after glucocorticoid withdrawal.

Although glucocorticoid-induced glaucoma is considered a secondary glaucoma, it is closely associated with primary open angle glaucoma (POAG). First, early studies showed that glucocorticoids induce OHT in <36% of the general population compared with >90% of POAG patients. Furthermore, glucocorticoid responsiveness is an important risk factor for POAG. Second, POAG and glucocorticoid-induced glaucoma share similar clinical presentations, including open anterior chamber angle, IOP elevation, characteristic optic neuropathy, and loss of peripheral vision. Third, the elevated IOP in both cases is primarily due to damage to the trabecular meshwork (TM). The TM plays an important role in IOP regulation. It is the key component of the conventional aqueous humor outflow pathway and contributes to the majority of outflow resistance. Compromised TM function dramatically increases outflow resistance, which leads to IOP elevation. Pathologic changes in the TM, including loss of TM cells, thickening of TM beams, deposition of plaque-like materials, excessive extracellular matrix (ECM) accumulation, and increased cross-linked actin network (CLANs) are found in glucocorticoid-induced glaucoma as well as in POAG. Therefore, studying glucocorticoid-induced glaucoma will not only help us to understand this disease but will also provide insightful information about POAG.

A number of models have been developed for studying glucocorticoid-induced glaucoma. These can be divided into in vitro, in vivo, and ex vivo models. In vitro models use cultured TM cells. These models are simple and easy to maintain, but they may not reflect in vivo conditions. Instead, in vivo models are more relevant to human glucocorticoid-induced glaucoma. Monkeys, rabbits, mice, rats, cats, cows, and sheep develop glucocorticoid-induced OHT. However, these models cost more and usually need at least 2 to 4 weeks to develop OHT. Ex vivo models combine both the pros and the cons of the two previous models. They provide better simulation of the physiological conditions than in vitro cell cultures and require less time and cost than in vivo models.

Perfusion-cultured human anterior segments have been frequently used as an in vivo model in glucocorticoid-induced glaucoma research. However, human donor eyes are limited by their availability and high cost. More important, because “healthy” human donor eyes are prioritized for corneal transplantation, those available for research are not of the best quality. Therefore, we were looking for eyes from other species as alternatives.

In contrast to human donor eyes, bovine eyes are inexpensive and readily available. Because of their large size, surgical manipulation and sample collection are easy. Studies using perfusion-cultured bovine anterior segments showed that the bovine TM (BTM) is directly involved in regulating the outflow pathway. Bovine TM cells share many properties with human TM cells, including the expression of myocilin (MYOC) and other markers of TM cells. Therefore, bovine TM cells may be a suitable model for studying glucocorticoid-induced glaucoma.
TM cells, including dexamethasone (DEX) induction of ECM proteins. Recently, Wade et al. reported DEX-induced CLAN formation in confluent BTM cultures, which is a unique feature of the TM. Their findings further prove the validity of this model. In addition, in vivo study showed that glucocorticoids can induce OHT in bovine eyes. Based on the advantages described here, we decided to test whether perfusion-cultured bovine anterior segments are suitable for studying glucocorticoid-induced OHT and trabecular outflow research.

**METHODS**

**Bovine Eyes**

Paired and unpaired calf eyes were obtained from local abattoirs and transported to the laboratory on ice. Eyes were processed within 6 hours of death. The bovine eyes used in this study were from mixed cow breeds. Most of them were of either the Angus or the Holstein breed.

**Anterior Segment Perfusion Culture**

We adopted the procedures reported by Johnson et al. with modifications. The experimental setup is demonstrated in Figure 1. Briefly, the extraocular tissue was removed and the integrity of the eye was evaluated. After sterilization with povidone-iodine topical antiseptic (Betadine; Purdue Products, Stamford, CT) for 1 to 2 minutes and two rinses with PBS, the bovine eye was cut at the equator to separate the anterior and posterior segments. The vitreous, uveal tract, retina, retinal pigment epithelium, and lens were carefully removed so that the TM was preserved. The remaining anterior segment, which contained the cornea, sclera, and TM, was mounted on a custom-made acrylic (Plexiglas) dish. A water-tight artificial anterior chamber was formed by clamping the anterior segment at the equator with an O-ring and four custom-made plastic screws. There were two cannulas communicating with the artificial anterior chamber. One was for medium infusion, and the other was connected to a pressure transducer for data recording. Perfusion culture medium containing Dulbecco’s modified Eagle’s medium-high glucose supplemented with 2 mM glutamine, 1% penicillin and streptomycin, and 1% amphotericin B (Thermo Scientific, Worcester, MA) was infused with a syringe pump (PHD2000; Harvard Apparatus, Holliston, MA) at a constant infusion rate of 5 μL/min.

**Glucocorticoid-Induced OHT**

Bovine anterior segments were perfusion cultured for 1 to 3 days until IOPs were stable. The eyes were then treated with either 0.1% ethanol (ETH) as a vehicle control or 100 nM DEX (Sigma-Aldrich, St. Louis, MO) in perfusion culture medium for up to 7 days.

**Data Acquisition and Analysis**

IOP was converted to electric signals by a disposable blood pressure transducer (ADInstruments, Colorado Springs, CO), amplified by a data acquisition system (PowerLab; ADInstruments) and a bridge amplifier (Octal Bridge Amp; ADInstruments) and then recorded (LabChart software; ADInstruments). IOP was sampled every minute. Data were averaged every 24 hours for analysis. We defined ΔIOP as the actual IOP averaged over 24 hours minus the basal IOP of individual eyes on certain day, and we defined mΔIOP as the maximum ΔIOP of individual eyes during perfusion culture. The outflow facility (μL/min/mm Hg) was calculated by dividing the perfusion rate (5 μL/min) by the...
eye through the angular aqueous plexus (asterisks), which is equivalent to the Schlemm’s canal in primate eyes. AC, the anterior chamber. Magnification, 100×. Experiments were performed in biological replicates, and representative data are shown.

IOP (mm Hg). We defined mΔC as the maximum decrease of outflow facility of individual eyes during perfusion culture.

Sample Collection after Perfusion Organ Culture
After perfusion organ culture, conditioned medium was collected, spun at 500g for 5 minutes to remove tissue debris, and stored at ~80°C until analysis. The anterior segment was cut into sectors and fixed with 4% paraformaldehyde in PBS at 4°C for 4 hours or overnight.

Histology
Fixed TM tissue was washed three times with PBS, dehydrated with ethanol, and embedded in paraffin. Samples were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) or Gomori trichrome according to conventional protocols.

Western Immunoblot Analysis
Approximately 500 μL conditioned medium was concentrated with resin (StrataClean; Agilent Technologies, Santa Clara, CA) at 1:100 (vol/vol). The resin was precipitated by centrifugation, and conditioned medium was carefully removed without disturbing the resin. After boiling the resin with the same volume of 2× Laemmli buffer, protein samples were resolved on SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk, the blot was probed with the primary antibody goat anti-MYOC (1:500; Santa Cruz, CA) or rabbit anti-fibronectin (FN; 1:500; Santa Cruz Biotechnology) or goat anti-rabbit HRP (1:10,000; Santa Cruz Biotechnology) at 1:100 dilution. The chemiluminescent signal was developed (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific, Rockford, IL) and was detected with an imaging system (FluorChem; Cell Biosciences, Santa Clara, CA).

For Coomassie blue staining, 15 μL conditioned medium was mixed with Laemmli buffer, boiled, and resolved on SDS-PAGE gel. The gel was stained with reagent (GelCode Blue Stain Reagent; Thermo Scientific) according to manufacturer’s instructions.

Statistical Analysis
Student’s t-test or one-way analysis of variance (ANOVA) was used for statistical analysis. For comparisons of categorical data, Fisher’s exact test was used. P < 0.05 was considered significant.

RESULTS

Morphology of the BTM after Perfusion Organ Culture
We first studied whether bovine anterior segments could be healthily maintained in our perfusion organ culture system. Bovine anterior segments, after perfusion culture for 9 days without any treatment, were compared to freshly dissected bovine eyes by histologic examination (Fig. 2). The morphology of the TM in both eyes was similar, which suggested that our system was suitable for perfusion culture of the bovine anterior segment.

Differential Response of Bovine Eyes to DEX
Bovine eyes were perfusion cultured with 100 nM DEX for up to 7 days. Similar to human and monkey eye studies, only some of the bovine eyes showed significant IOP elevation after DEX administration (Fig. 3A). Twelve of 29 bovine eyes were DEX responders. Our observation indicated that, as in other species, there were DEX responders and nonresponders in bovine eyes (Fig. 3B). The raw data from each eye are listed in Supplemen-
OHT in Perfusion-Cultured Bovine Anterior Segments

To define “significant IOP elevation”/OHT, we analyzed data from 18 control eyes treated with ETH. IOP was averaged every 24 hours for analysis. We determined the maximum change in IOP from the baseline IOP (m\(\Delta\)IOP; see Methods for the definitions of \(\Delta\)IOP and m\(\Delta\)IOP), which was 1.14 ± 0.84 mm Hg. We therefore defined the threshold of a significant change as the mean change in m\(\Delta\)IOP + 2× SD (1.14 + 2 × 0.84 = 2.82 mm Hg). Thus, an IOP elevation higher than 2.82 mm Hg should have been due to DEX treatment.

To confirm whether our threshold of OHT was appropriate, Student’s t-tests were performed between ETH-treated, DEX-responder, and nonresponder eyes. The m\(\Delta\)IOP of DEX-responder eyes was significantly higher than that of either ETH-treated or nonresponder eyes (both \(P < 0.01\)), whereas ETH-treated eyes had similar m\(\Delta\)IOPs as nonresponder eyes (\(P = 0.50\)) (Table 1).

Based on this value, we classified bovine eyes into DEX responders (m\(\Delta\)IOP > 2.82 mm Hg) and nonresponders (m\(\Delta\)IOP < 2.82 mm Hg) (Table 2, Fig. 4). Our data suggested that approximately 41% (12/29) of the bovine eyes used in this study were DEX responders.

To better describe the IOP change of the three groups of eyes (ETH treated, DEX responders, and nonresponders), we plotted them based on mean ± SEM over time (Fig. 5). ANOVA showed that IOP elevation in DEX-responder eyes was significantly higher than in nonresponder eyes or ETH-treated eyes (\(P < 0.05\)) from one day after treatment, and persisted throughout the 7 days of perfusion culture (Fig. 5).

DEX-Induced MYOC Expression in Perfusion-Cultured Bovine Eyes

The glaucoma gene MYOC\(^{30}\) is a secreted glycoprotein of unknown function expressed in TM cells and other ocular tissues.\(^{30,31}\) MYOC expression is inducible by DEX in TM cells, which is often considered a standard for TM cell identification.\(^{10,31}\) Similarly, the ECM protein FN is DEX-inducible in the TM.\(^{7,8}\) FN deposition in the TM is a contributor to increased outflow resistance and IOP elevation in glaucoma.

We collected conditioned medium at the end of perfusion culture and compared MYOC and FN expression between ETH- and DEX-treated eyes. Because of the lack of a protein marker as a loading control for conditioned medium, the SDS-PAGE gel was stained with Coomassie blue after electrophoresis and as a loading control for conditioned medium, the SDS-PAGE gel showed equal amounts of total proteins between the corresponding samples (Fig. 6A). Western blot analysis showed that MYOC was upregulated by DEX in 6 of 8 pairs of DEX-responder eyes (75%) (Fig. 6B). In contrast, none of the six pairs of nonresponder eyes examined showed DEX-induced MYOC elevation (Fig. 6B), and the difference was statistically significant (Table 3; \(P < 0.001\)). Quantitative analysis of MYOC expression by densitometry showed an average 2.00 ± 0.57 (mean ± SD) fold elevation in the six pairs of DEX responders that demonstrated MYOC induction, which was statistically significant as shown by paired t-test (Fig. 6C; \(P < 0.01\)). However, similar differential induction of FN was not observed (Table 3; \(P > 0.500\)).
Morphology of the BTM after DEX Treatment

Besides biochemical changes, we investigated morphologic changes of the BTM after DEX treatment by H&E staining and Gomori trichrome staining; the latter is a commonly used technique to differentiate nuclei (black), cytoplasm/keratin/muscle fibers (red), and collagen (green or blue). The morphology of the BTM of DEX responders was similar to that of nonresponders, except that there was intense staining of collagen fibers in DEX responders (Fig. 7), which indicated DEX-induced excessive ECM deposition in the TM of DEX-responder eyes.

**DISCUSSION**

Human, monkey, porcine, and bovine eyes are the frequently used glaucoma models. The similarities and differences in the TM of the four species are summarized in Table 4. One of the characteristics that significantly affect IOP measurement is the washout effect. The washout effect is the increase of outflow facility during perfusion culture of the eye. This effect has been observed in all species studied except humans and mice. Recent findings suggest that washout is due to the separation of the juxtaocular tissue from the inner wall of Schlemm’s canal. The time-rate-of-change of facility (washout rate) is approximately 20% per hour in the bovine eye during the initial 2 hours. However, our model is not suitable for studying this effect. Whether the washout effect is time-dependent or volume dependent is still controversial, although most of the studies reported this effect within the initial several hours of perfusion culture of the whole eye. In the anterior segment perfusion culture model, the bovine anterior segment was initially underinflated to avoid rupture of the TM. The eye usually requires overnight culture to become inflated and stabilized, during which the washout effect is masked. Therefore, the bovine anterior segment perfusion culture model is not suitable for the study of this washout effect.

Another important parameter worth further investigation is the perfusion rate. Ideally, the perfusion rate should be identical with the bovine aqueous humor formation rate, which can be measured by fluorophotometry. However, to our knowledge, the bovine aqueous humor formation rate has not been reported. The 5 μL/min perfusion rate was therefore selected based on our experience and preliminary studies. The aqueous humor formation rate can also be estimated by the Goldmann equation IOP = F/C + EVP or F = C(IOP − EVP), where F is the aqueous humor formation rate, C is the outflow facility, and EVP is the episcleral venous pressure. The mean IOP of in vivo bovine eyes is approximately 16 mm Hg. Perfusion culture studies with the whole bovine eye reported a basal outflow facility ranging from 1.06 to 1.54 μL/min/mm Hg. In the human eye, the EVP is approximately 8 to 9 mm Hg. Although no bovine EVP data are available, because of the high blood pressure of the cow (160/110 mm Hg), we speculate that the EVP of the bovine eye may be higher than that of the human eye. Combining these factors, the actual bovine aqueous humor formation rate may be close to 5 μL/min. More important, the fact that the morphology of the bovine TM was well maintained and the successful induction of glucocorticoid response suggest that this perfusion rate is appropriate. Nevertheless, it would be optimal if a perfusion rate matching the physiological aqueous humor formation rate could be adopted in this model.

With the perfusion rate of 5 μL/min, we found that the basal outflow facility of the 47 bovine eyes was 1.01 ± 0.08 μL/min/mm Hg (mean ± SEM) (Supplementary Table S2 legend, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8135/-/DCSupplemental), a value very close to that reported by Scott et al. in eight perfusion-cultured bovine whole eyes (1.06 ± 0.06 μL/min/mm Hg). Although several bovine eyes demonstrated an outflow facility <0.5 or higher than 2 μL/min/mm Hg in this study, considering our large sample size (n = 47), these “off-center” outflow facility values were possibly physiologically significant.

**TABLE 3.** Comparisons of MYOC and FN Induction between DEX Responder and Nonresponder Eyes

<table>
<thead>
<tr>
<th></th>
<th>DEX Responders</th>
<th>Nonresponders</th>
<th>Fisher’s Exact Test</th>
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<tr>
<td>MYOC</td>
<td>6/8</td>
<td>0/6</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>FN</td>
<td>3/8</td>
<td>1/6</td>
<td>P &gt; 0.500</td>
</tr>
</tbody>
</table>
Differential glucocorticoid responsiveness has been reported not only in primates but also in a number of other species, including mice and rabbits.\textsuperscript{13,40,41} In contrast to our observations, Gerometta et al.\textsuperscript{18} reported a 100% DEX-responder rate in an in vivo study of 12 Bradford cows treated with prednisolone for 4 weeks. This apparent discrepancy with responder rates in our present study may result from differences in animal strains, experimental methods, glucocorticoids used, and sample sizes. We speculate that an animal strain difference is the most likely reason. Furthermore, unlike cows raised on the same ranch, which are often of the same strain, bovine eyes from abattoirs are usually from mixed cow strains. A recent study by Whitlock et al.\textsuperscript{13} showed heterogeneity in DEX responsiveness in mice of a mixed genetic background, which further suggests the importance of animal strains in glucocorticoid-induced OHT. The second possible cause is experimental methods. The two experiments were carried out in different systems (ex vivo vs. in vivo) with different glucocorticoids (perfused DEX vs. topical predniso-

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**FIGURE 7.** Morphology of the BTM after DEX treatment. The TM of a DEX-responder eye (A, B) or a nonresponder eye (C, D) was subjected to H&E staining (A, C) or Gomori trichrome staining (B, D) after perfusion culture with DEX. Asterisks: angular aqueous plexus. SC, sclera. Magnification, 200×. Experiments were performed in biological replicates, and representative data are shown.

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**TABLE 4.** Comparisons of the Human, Monkey, Porcine, and Bovine Models

<table>
<thead>
<tr>
<th>Eye Model</th>
<th>Anatomic Landmarks of the TM</th>
<th>Morphology of the TM</th>
<th>Drainage</th>
<th>Washout Effect</th>
<th>Differential Glucocorticoid Responsiveness</th>
<th>Cost and Availability</th>
<th>Biohazard Risk Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Schwalbe’s line and scleral spur</td>
<td>Meshwork-like</td>
<td>Schlemm’s canal</td>
<td>No</td>
<td>Yes</td>
<td>High and limited</td>
<td>High</td>
</tr>
<tr>
<td>Monkey</td>
<td>Schwalbe’s line and scleral spur</td>
<td>Meshwork-like</td>
<td>Schlemm’s canal</td>
<td>Yes</td>
<td>Yes</td>
<td>High and limited</td>
<td>High</td>
</tr>
<tr>
<td>Porcine</td>
<td>Unclear</td>
<td>Reticular</td>
<td>Angular aqueous plexus</td>
<td>Yes</td>
<td>Not reported</td>
<td>Affordable and readily available</td>
<td>Low</td>
</tr>
<tr>
<td>Bovine</td>
<td>Unclear</td>
<td>Reticular</td>
<td>Angular aqueous plexus</td>
<td>Yes</td>
<td>Yes</td>
<td>Affordable and readily available</td>
<td>Low</td>
</tr>
</tbody>
</table>
nisolone) and treatment times (5–7 days vs. 4 weeks). Finally, our ex vivo model enabled us to have a larger sample size.

In addition to differential IOP changes, MYOC expression seemed to be associated with DEX responsiveness. DEX-induced MYOC expression was found only in DEX-responder eyes but not in nonresponder eyes. We would like to emphasize that the “DEX-inducible” and “glaucoma” gene MYOC was used as a marker to determine overall DEX responsiveness. MYOC induction should not be interpreted as the cause of IOP elevation because previous studies already showed that wild-type MYOC does not contribute to IOP elevation. Nevertheless, it is still a good indicator of DEX responsiveness.

Another DEX-inducible gene, FN, did not demonstrate differential induction or correlation with DEX responsiveness. We believe that this could have been due to two reasons. First, FN is one of the most abundant ECM genes expressed in the TM, and this abundance may mask any slight change in FN expression. Second, there are more than 12 FN isoforms, and the antibody used in the study may be able to detect only some of them. Therefore, further studies of FN are required to elucidate its role in DEX-induced OHT.

In contrast to biochemical changes, light microscopy morphologic analysis of the BTM after DEX treatment revealed little difference between DEX-responder and nonresponder eyes except intense staining of the DEX-responder TM with Gomori trichrome. Although this may suggest excessive ECM deposition in the TM, other research techniques, including electron microscopy, will be used in future studies to compare the BTM of DEX responders and nonresponders.

The mechanism involved in glucocorticoid responsiveness is unclear. However, glucocorticoid receptor α (GRα) and its alternatively spliced form GRβ may play a key role. In the human GR pathway, glucocorticoids bind to GRα, translocate into the nucleus, and change gene expression after binding to the glucocorticoid response element. In contrast, GRβ does not bind to glucocorticoids but remain in the nucleus as a dominant negative inhibitor. Many studies have suggested that the GRα/GRβ ratio determines glucocorticoid responsiveness in TM cells. In addition to humans, GRβ has been found in zebrafish and mice. Although the existence of bovine GRβ has not been confirmed, the alternative splicing site and the GRβ coding sequence have been predicted by computational analysis. If GRβ can be cloned, our model will become an invaluable tool for GR pathway research as well. In summary, perfusion-cultured bovine anterior segments are a quick, convenient, affordable, and reliable model for studying glucocorticoid-induced OHT and glaucoma.

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References


