Retroperfusion Studies of the Aqueous Outflow System
Part I: Evaluation of Technique Using N-Ethyl Maleimide

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Purpose. The goal of this study was to develop a new technique to deliver drugs or other agents to the lumen of the angular aqueous plexus/Schlemm's canal (AAP/SC) while bypassing the trabecular meshwork, thereby gaining insight into AAP/SC inner wall function.

Methods. The anterior chamber is held at a small negative pressure and fluid is allowed to flow retrograde from the limbal vessels, through the collector channels, and into the AAP/SC ("retroperfusion"). Facility measurements are combined with histologic and tracer studies in bovine eyes.

Results. (1) Retroperfusion with a saline solution does not alter facility or change outflow pathway morphology; (2) fluid is able to move retrograde from the scleral surface and enter the lumen of the AAP; and (3) retroperfusion with N-ethyl maleimide causes a dose-dependent increase in washout rate and concomitant inner wall breaks.

Conclusions. It is hypothesized that the observed increase in washout is due to leakage of extracellular materials through breaks in the inner wall. Invest Ophthalmol Vis Sci 1993;34:385-394.

Essential to an improved understanding of the etiology of primary open-angle glaucoma (POAG) is identification of the pathologic conditions leading to decreased outflow facility in the glaucomatous eye. Although the majority of total outflow resistance is thought to reside in the juxtacanalicular tissue (JCT) and/or the inner wall of Schlemm's canal,1 a definitive breakdown of the relative importance of these two tissues remains elusive. A number of studies2-4 have indicated that inner wall pore resistance is directly responsible for only 10% (or less) of total outflow resistance. Other observations, however, suggest that the inner wall endothelium is important in determining outflow resistance. First, loss of inner wall integrity, resulting either from artificially increased intraocular pressure (IOP),5 cytochalasin-B,6 7 sulfhydryl-active agents,8 EDTA,9 or α-chymotrypsin,10 is correlated with a significant increase in facility. Second, perfusion with a fixative solution, which presumably stiffens the inner wall, leads to a decreased outflow facility.11 Third, a mechanism whereby inner wall structure may indirectly influence outflow resistance by altering flow patterns "upstream" within the JCT has been proposed.12 Finally, Allingham at al13 have shown a significantly decreased inner wall pore density in POAG eyes.

Unfortunately, it is not possible to draw definitive conclusions about the influence of the inner wall on outflow facility from the perfusion studies mentioned above, since in these and all such studies the delivery of materials to the inner wall of Schlemm's canal requires that these materials pass through the trabecular
meshwork (TM). The result is that alterations in inner wall structure are invariably accompanied by changes in JCT and TM morphology. Hence, it is not known whether changes in outflow facility observed during conventional perfusion studies are directly due to disruption of the inner wall, are due to leakage of ground substance and/or other extracellular materials through breaks in the inner wall, or are due to other unknown factors. We therefore suggest that a method of altering inner wall properties while minimally modifying the structure of the TM would be of great benefit in advancing our understanding of the mechanisms of aqueous outflow, and in particular in determining how the inner wall of Schlemm's canal influences aqueous outflow resistance.

In the current study we present a new method developed to deliver agents to the inner wall of Schlemm's canal, while avoiding the TM. Because such an approach has not been reported previously in the literature, we describe in detail the basic procedure, capabilities, and limitations of the technique, as well as preliminary results determined from a series of drug and tracer studies in bovine eyes. In a subsequent report, we will describe results obtained using human eyes.

MATERIALS AND METHODS

Overview

The retroperfusion method described below was designed to deliver agents into the lumen of the angular aqueous plexus (AAP) in bovine eyes while bypassing the TM. (The AAP plays the role of a multilumenal Schlemm's canal in the bovine eye.13) A fluid-filled well was formed at the limbus of an enucleated eye and IOP was held at a small negative value to induce a controlled retrograde flow into the AAP from the aqueous veins and collector channels, similar to a procedure described by Johnstone and Grant.14 Outflow facilities were measured before and after retroperfusion with vehicle or N-ethyl maleimide (NEM), and changes in inner wall and TM ultrastructure were analyzed by light and transmission electron microscopy (TEM). NEM was selected as the retroperfusate because it has been shown to cause an increase in facility and a change in inner wall structure when forward-perfused into bovine eyes.815 Cationized ferritin (CF) was used as a tracer to determine the extent of penetration of the retroperfused fluid.

Perfusion Experiments

Freshly enucleated bovine eyes (2–3 hr postmortem) were acquired from a local abattoir and transported to the laboratory in saline and ice. To the best of the authors' knowledge, methods for securing animal tissue were humane and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Orbital connective tissue was trimmed off the eyes and the limbal conjunctiva was gently excised with a razor blade. A 2 × 14 cm acetate plastic strip was glued to the eye circumferentially at the equator with cyanoacrylate adhesive (Krazy Glue; Dupont Instruments, Newtown, CT) to form a fluid tight "fence"
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After 60 to 120 min, when a stable baseline facility had been obtained, the forward perfusion was halted by closing a valve, and the retroperfusion procedure was carried out as follows. The perfusion needle was withdrawn several millimeters from the posterior chamber until the tip was in the anterior chamber. After drying the limbus, 1 to 2 ml of the fluid to be retroperfused (the “retroperfusate”) was pipetted into the acetate fence to form a well of fluid overlying the limbus. By means of a three-way valve, the perfusion needle was connected to a reservoir located 1 cm below the surface level of the fluid in the limbal area to create an effective negative IOP of approximately —0.75 mmHg (Fig. 1). This negative pressure was maintained for 30 min. (Preliminary experiments indicated that extended retroperfusion times or larger negative pressures caused inner wall rupture.) It was not possible to measure facility during the retroperfusion procedure.

For NEM retroperfusion studies, the retroperfusate was 1, 2.5, 5, 10, or 20 mmol/l NEM (Sigma Chemical Co., St. Louis, MO) dissolved in DPBS/G vehicle. The contralateral control eye received an osmotically equivalent glucose + DPBS/G solution. For tracer studies without NEM, the retroperfusate was a solution of sonicated, undiluted cationized ferritin (10.4 mg/ml; Sigma) in isotonic saline vehicle (both eyes). For tracer studies with NEM, undiluted CF was dissolved in isotonic saline vehicle with 1, 2.5, 5, 10, or 20 mmol/l NEM present (one eye), whereas the contralateral eye received CF in vehicle only.

At the end of the retroperfusion period, the three-way valve was closed to isolate the perfusion needle from the negative pressure reservoir. Retroperfusate was removed from the sclera, and eyes then underwent forward perfusion with DPBS/G as described above for an additional 2 to 3 hr with the infusion needle in the posterior chamber, during which time facilities were again measured. It should be emphasized that during these experiments NEM was deliv-

### Table 1. Summary of Retroperfusion Results for Various Concentrations of N-ethyl Malemeide (NEM) in Pairs of Enucleated Bovine Eyes

<table>
<thead>
<tr>
<th>NEM Concentration (mmol/l)</th>
<th>Change in Washout Rate Due to Retro (ΔW)</th>
<th>Significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEM Retro</td>
<td>Saline Retro</td>
</tr>
<tr>
<td>20 (n = 10)</td>
<td>0.92 ± 1.06 %/min</td>
<td>−0.08 ± 0.21 %/min</td>
</tr>
<tr>
<td>10 (n = 11)</td>
<td>0.92 ± 0.64 %/min</td>
<td>0.14 ± 0.27 %/min</td>
</tr>
<tr>
<td>5 (n = 10)</td>
<td>0.95 ± 0.51 %/min</td>
<td>−0.26 ± 0.29 %/min</td>
</tr>
<tr>
<td>2.5 (n = 12)</td>
<td>0.55 ± 0.39 %/min</td>
<td>0.00 ± 0.17 %/min</td>
</tr>
<tr>
<td>1 (n = 9)</td>
<td>0.15 ± 0.18 %/min</td>
<td>0.04 ± 0.24 %/min</td>
</tr>
</tbody>
</table>

In columns 2 and 3, the change in washout rate due to retroperfusion ΔW is calculated as described in text. Column 4 is column 2 minus column 3. Values shown are mean ± standard deviation. P values refer to significance of net ΔW (column 4). n, number of pairs of eyes perfused. NS, not statistically significant.
FIGURE 3. Micrographs of selected tissues from bovine eyes retroperfused with vehicle only (fixation at 8 mmHg pressure). (A) Low-magnification overview of trabecular meshwork (TM) and angular aqueous plexus (AAP). Note nerve bundles (arrows). (Original magnification X660.) (B) Typical TM cell from mid-meshwork. Note glycogen present in cytoplasm. (Original magnification X13,200.) (C) Inner wall of angular aqueous plexus (AAP) and subendothelial tissue. Note giant vacuole (GV), cell-to-cell junctions in inner wall (arrowheads), and inner wall attachments to subendothelial connective tissue. (Original magnification X7000.)

Microscopy

Eyes that were not immersion-fixed immediately after retroperfusion were perfusion-fixed for 1 hr at a pressure of 8 mmHg after the second forward perfusion.

The fixative solution consisted of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/l Sorensen’s buffer, pH 7.3. The eyes were bisected and fixed by immersion for an additional 24 to 48 hr. Radial segments of the limbus were dissected and postfixed in 1% osmium tetroxide with 1.5% potassium ferricyanide, dehydrated, infiltrated, and embedded in Epon-Araldite. For TEM, ultrathin sections were cut on a Sorvall Porter-Blum Ultramicrotome MT2 (Borden Co., Toronto, ON), stained with uranyl acetate and...
lead citrate, and viewed with a Hitachi (Tokyo, Japan) H-7000 transmission electron microscope. For light microscopy, 1 μm sections were cut and stained with toluidine blue.

Data Analysis
Because of the washout effect, measured facility typically changes throughout the course of the experiment. It is therefore convenient to express perfusion results in terms of both facilities and washout rates, where washout rate is defined as the rate of change of facility with time divided by initial facility. Washout rate was determined by calculating the slope of a line linearly regressed to the appropriate portion of the facility versus time curve and dividing by initial facility. Data from the first 30 min after initiation of perfusion were excluded from this calculation to avoid the filling transient associated with pressurizing the eye. The initial facility was estimated by extrapolating the regressed line (before retroperfusion) back to the beginning of the perfusion. To evaluate the effects of retroperfusion on washout rate, the quantity AW was calculated as: \( AW = (\text{washout rate after retroperfusion}) - (\text{washout rate before retroperfusion}) \), for each control and experimental eye. AW values were statistically analyzed using a paired two-sided student t-test.

RESULTS

Facility Studies
The initial facility and washout rate (before retroperfusion) for all eyes (n = 104) were 0.67 ± 0.25 μl/min/mmHg and 0.19% ± 0.27% per min. Outflow facility was essentially unchanged by saline retroperfusion (Fig. 2), as was washout rate (\( AW = -0.03\% ± 0.26\% \) per minute for all control eyes; \( P = 0.44; n = 52 \). Outflow facility in eyes retroperfused with NEM increased significantly, however (Fig. 2). This increase did not occur immediately after retroperfusion; rather, it appeared as an increased washout rate that led to a time-dependent gradual facility increase. The increase in washout was statistically significant at the 2.5, 5, 10, and 20 mmol/l NEM concentrations, but was not statistically significant at 1 mmol/l (Table 1). The increased washout rates were approximately three to five times their baseline level of 0.19% per minute.

Morphologic Findings

Control Retroperfusion. By light microscopy and TEM, the TM of saline retroperfused eyes (controls) appeared essentially normal, exhibiting a more reticular and less lamellar structure than found in human or primate TM13,16 (Fig. 3A). TM cells appeared healthy, with some minor cell swelling (Fig. 3B) and occasional loss of intracellular glycogen into the extracellular space.* The AAP was continuously lined with endothelial-like cells. Cell-to-cell and cell-to-substrate attachments in the inner wall of the AAP and in the TM appeared normal in saline retroperfused eyes (Fig. 3C), as did collector channel structure.

Cationized Ferritin Retroperfusion. After 30 min of CF retroperfusion, tracer was seen decorating the ex-
terior scleral surface in the limbal region, the luminal surface of cells lining collector vessels, and the luminal surface of cells lining the AAP (Fig. 4). CF staining was very dense on the exterior scleral surface, but became less pronounced in regions closer to the AAP. Within the lumen of the AAP, CF occasionally was absent from a section, or when present, was sometimes seen in aggregates rather than being uniformly distributed. CF was detected on the luminal side of the interendothelial junctions, but did not appear to pass through these junctions (Fig. 4B).

N-ethyl Maleimide Retroperfusion. Eyes retroperfused with NEM showed marked ultrastructural changes. At concentrations of 10 and 20 mmol/L NEM, frank disruptions of cell-to-cell junctions in the inner wall of the AAP, diffuse detachment of the inner wall from the subendothelial tissues, and inner wall cell swelling were observed (Fig. 5A). Inner wall vacuoles were present, but often appeared markedly enlarged. In addition, cell-to-cell and cell-to-substrate detachment was observed in the endothelia of aqueous collector channels and episcleral vessels. Areas of the
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JCT adjacent to inner wall breaks showed moderate cell swelling, and disruption of the filamentous components of the extracellular matrix. Cells in the TM that were in close proximity to inner wall breaks also showed some swelling, focal membrane ruptures, and, in some cases, loss of cell-to-cell and cell-to-beam attachments. TM cells not adjacent to inner wall breaks appeared normal, however (Fig. 5B).

At the lower concentrations of 5, 2.5, and 1 mmol/l NEM, the ultrastructural alterations were not as extensive as those described above. Occasionally, breaks in the inner wall and focal detachments of inner wall cells from the subendothelial tissues were noted (Fig. 6). Cells lining both the aqueous collector channels and the AAP showed moderate to severe cell swelling, but TM demonstrated little or no cell swelling.

Cationized Ferritin + N-ethyl Maleimide Retroperfusion. When 10 mmol/l NEM was added to the CF retroperfusate, tracer particles were distributed as described above. Additionally, CF often was seen decorating the subendothelial structures within the JCT immediately below breaks in the inner wall (Fig. 7).
Although in some cases it was difficult to estimate, CF penetration through the inner wall was limited to approximately 10 μm from the observed break.

**DISCUSSION**

We have described a new technique ("retroperfusion") designed to deliver drugs or other agents to the lumen of the AAP in bovine eyes while bypassing the TM. For this technique to be useful, it must satisfy two requirements: (1) it should be able to reliably deliver agents to the lumen of the AAP through the collector channels; and (2) the delivery procedure itself cannot alter or damage aqueous outflow pathway tissues. Below we evaluate the technique in light of these requirements, and then discuss results obtained from NEM retroperfusion studies.
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Evaluation of the Retroperfusion Technique

CF can be used as a tracer to delineate fluid flow within the outflow system. Because CF was seen decorating the lumenal surface of endothelial cells lining the aqueous veins, collector channels, and the AAP, we conclude that the retroperfusion technique described herein is a viable method for delivering fluid from the exterior limbal region into the AAP through the collector channels and aqueous veins. CF decoration of the inner wall of the AAP was variable from section to section, most probably indicating an uneven retrograde filling of the AAP.

Retroperfusion for 30 min at an IOP of 0.75 mmHg does not alter the functional anatomy and physiology of the outflow pathway in bovine eyes. This conclusion is based on (1) the normal morphologic appearance of the tissue after retroperfusion with vehicle; (2) the unaltered outflow facility and washout rate observed after retroperfusion with vehicle; and (3) the integrity of inner wall endothelial junctions after retroperfusion with vehicle, as demonstrated by their apparent ability to exclude CF. Retroperfusion is therefore a viable method of delivering agents into the lumen of the AAP.

N-ethyl Maleimide Retroperfusions

The effects of NEM retroperfusion were similar to those previously observed in forward perfusions using this agent. Morphologically, disruption of the inner wall was observed, which was correlated with a concomitant increase in outflow facility and a threefold to fivefold increase in washout rate. Experiments in which both CF and NEM were retroperfused indicated that there was limited penetration of CF into the JCT adjacent to breaks in the inner wall. Although NEM, due to its lower molecular weight, may reach sites inaccessible to CF through such breaks, ultrastructural alterations characteristic of NEM also were localized to the immediate area adjacent to inner wall breaks.

We hypothesize that penetration of NEM through the inner wall breaks resulted in the observed cell-to-substrate detachments.

Our original aim in developing the retroperfusion technique was to alter inner wall structure without affecting the JCT. This has not proven to be possible with NEM retroperfusion, which disrupts inner wall integrity, because it appears that inner wall breaks represent high-conductivity pathways for further retrograde flow. Thus, it is not possible to conclude that all facility increases that we observed resulted from inner wall disruption. It does appear, however, that facility increases were initiated either in the inner wall or in the approximately 10-μm thick region of the JCT adjacent to the inner wall. This is consistent with the findings of Bill and Määpea, who claimed that approximately 50% of total outflow resistance is located within 30 μm of the inner wall in cynomolgus monkeys.

Interestingly, the increase in outflow facility after NEM retroperfusion took the form of an elevated washout rate, rather than an abrupt increase in facility immediately after retroperfusion. One possible interpretation of this observation is that the inner wall and/or JCT were weakened by NEM retroperfusion, and were then progressively damaged during subsequent forward perfusions, resulting in a gradual facility increase. We believe that a more likely interpretation of our findings, however, is that observed gradual facility increases were a result of the washout of resistive extracellular material through NEM-induced inner wall breaks, whereas the relatively small increase in facility immediately after retroperfusion was due to formation of breaks in the relatively low flow resistance inner wall. This interpretation is consistent with previous suggestions that increased washout is due to loss of extracellular material from within the TM and with the gel-filled meshwork model and with data from other species showing a small direct inner wall resistance. If true, it would imply that the inner wall can markedly influence total outflow resistance even though it has only a small flow resistance itself.

In summary, the retroperfusion technique we have developed in bovine eyes appears to be a viable method of delivering fluids into the lumen of the AAP through the aqueous collector channels, and may therefore be useful in further studies investigating the role of the inner wall of Schlemm’s canal in aqueous outflow resistance. We are extending this retroperfusion technique to human eyes.

Key Words
aqueous outflow pathway, angular aqueous plexus, bovine eye, morphology, Schlemm’s canal, washout.

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References


