Perfusion Outflow Facility in the Rabbit Eye

Stabilization by EACA

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The time-dependent increase in apparent facility of outflow (washout effect) that occurs with prolonged perfusion of the eye has imposed limitations on the study of aqueous humor dynamics. The washout effect in postmortem in situ rabbit eyes, undergoing constant pressure perfusion with a saline perfusate, can be attenuated dramatically by adding to the perfusate the serine protease inhibitor and antifibrinolytic agent e-aminocaproic acid (EACA) at a concentration of 3.8 \times 10^{-3} \text{ molar}. Washout curves from 13 pairs of rabbit eyes, plotted as outflow facility versus time, were fitted by linear regression, and their washout slopes calculated. The washout slope of all of the 13 eyes perfused with normal saline + EACA was lower in magnitude (less washout) than the paired control eye in the same animal, perfused with a control perfusate of normal saline ± leucine. Wilcoxon signed rank test yielded \( P < 0.001 \). This suggests that a significant component of the washout effect may be mediated by fibrinolytic activity, or by some EACA sensitive component of the aqueous drainage pathway, and that addition of EACA to a saline perfusate may be useful for blunting the washout effect in prolonged perfusion studies. Invest Ophthalmol Vis Sci 26:153-158, 1985

Perfusion of the aqueous outflow channels of the eye usually leads to a slow increase in apparent facility of outflow, the so-called “washout effect.” In pioneer perfusion studies, Bárány\(^1\)-\(^4\) documented the time dependent increase in outflow facility and pointed to the importance of hyaluronic acid as an element of resistance in the outflow passages. Numerous other authors\(^5\)-\(^10\) have supported the notion of a hyaluronidase sensitive barrier to aqueous outflow. Melton and Deville\(^6\) investigated the species specificity of washout, finding greater washout in dog or cat eyes than in guinea pig or rabbit eyes. Van Buskirk and Brett,\(^3\) studying canine eyes, and Bárány and Scotchbrook\(^1\) studying cattle eyes, demonstrated significant elimination of washout following rapid intracameral infusion of hyaluronidase, yet the presence of some residual washout was noted persistently. Recent work by Knepper et al\(^10\) casts doubt on the role of hyaluronidase sensitive glycosaminoglycans as the entity responsible for such a phenomenon.

Rigorous efforts by Gaasterland et al\(^11\)-\(^12\) to stabilize monkey eyes during prolonged perfusion, have met with limited success. Despite the marked diminution of washout with the use of pooled monkey aqueous and synthetic perfusates, a residual degree of washout invariably persists.

Pandolfi and associates\(^13\)-\(^14\) and others\(^15\)-\(^16\) found plasminogen-activator activity in various regions of the eye including the aqueous outflow channels. Such activity is greatest in humans, pig, and sheep, and lower in rabbit and cow. Grant\(^7\) and others\(^17\)-\(^18\) found that infusion of plasmin (fibrinolysin) into the anterior chamber led to increased facility of outflow. These latter findings suggested to us that fibrinolytic activity might be responsible for some of the residual washout effect observed in the perfusion experiments of Van Buskirk, Gaasterland, and Knepper.

Our hypothesis was that addition of e-aminocaproic acid (EACA), a monoamino carboxylic acid of molecular weight 131 daltons, an established inhibitor of plasminogen activation and/or plasmin activity\(^19\)-\(^20\) (Fig. 1), to a saline perfusate might significantly diminish washout.

Materials and Methods

Constant pressure perfusion was from a pair of 0.1 ml pipettes calibrated in \( \mu l \) placed horizontally a measured distance above the eyes.\(^21\) Correction was made for capillary meniscus pressure, in this case 2 cm of saline. The time required for perfusion of 10 \( \mu l \) of fluid was measured periodically at approximately 15-min intervals, and facility of outflow, \( C \), in \( \mu l \)
min⁻¹ mmHg⁻¹ computed. Between measurements, the eyes were perfused from reservoirs at the same effective height as the pipettes.

One reservoir and pipette contained 0.9% NaCl (pH 6.3), and the other 0.9% NaCl to which 3.8 × 10⁻³ M EACA (Sigma Chemical; St. Louis, MO) was added (pH 6.4). Normal saline was selected as the simplest perfusate of near physiologic osmotic activity known to produce washout consistently.¹ The minimal effective plasma concentration for systemic use of EACA has been shown to be 0.1 × 10⁻³ M.¹⁹

In two experiments, 3.8 × 10⁻³ M L-leucine (Sigma) MW 131 daltons, a molecule of similar molecular weight and structure to EACA without known antifibrinolytic activity (Fig. 1), was added to the control saline perfusate.

All perfusions were at room temperature (23.5°C). Laboratory white rabbits were selected for study because of consistency of outflow facilities between paired eyes, low cost, and ready availability. Each animal was placed in a restraining box and killed with intravenous injection of pentobarbital. The eyes were left in situ in order to minimize manipulation-induced "massage effect" previously described by Bárány.² Each globe was stabilized by a suture gently passed under the superior rectus muscle. The animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

A 23-gauge needle was attached to each perfusion reservoir via PE 50 polyethylene tubing (Fig. 2). Prior to each experiment, hydrodynamic symmetry of the perfusion apparatus was tested by measuring facilities of outflow for both the saline, and EACA + saline halves of the apparatus (with needles attached and needle tips open to barometric pressure). Virtually no difference was detected. Calculated resistance for the perfusion apparatus was noted to be close to 100 orders of magnitude lower than the resistance of the aqueous outflow pathway of the rabbit eye. Variation from postmortem changes in each animal were minimized by running the perfusion as near simultaneous as possible. A mandatory 5–15-min desynchrony was incurred as a result of the time delay between needle insertions.

Thirteen paired perfusion experiments were performed. In the first four animals, the perfusion needle was passed through the cornea into the anterior chamber, and the chamber was allowed to deepen. In the last nine animals, the needle was advanced between iris and lens until the tip lay in the posterior chamber. Care was taken to insure the integrity of the anterior lens capsule. The cornea was kept from drying with repeated applications of either saline or mineral oil. To assess for any pressure or viscoelastic induced alterations in apparent facility of outflow, our perfusions were performed at 30 mmHg for the first nine pairs of eyes, and at 15 mmHg for the last four pairs of eyes. Perfusions were run for 300 min or until corneal edema was noted (at which time accelerated perfusion rates occurred).

Results

Experimental data were presented to a statistician for analysis. Plots of the data points yielded curves which varied in shape from animal to animal. Some curves appeared near linear as shown in Figure 3A, while others were shaped more as a "sideways J," implying a linear relationship with an added quadratic component (Fig. 3B).

Least-squares regressions using both linear alone, and linear plus quadratic components were generated on all experimental pairs. The relative goodness of fit achieved by linear regression alone was in general quite good, only minimally improved in a few cases.
by addition of a quadratic component, and hence
deemed sufficient for the aims of this investigation.

Results are shown in Table 1 where slope, intercept,
and various statistical parameters are compared be-
tween EACA perfused and control eyes. As facility
increased nearly linearly with time, the best approx-
imation of initial facility is the intercept of the facility
curve at zero time. The many high $r^2$ values imply a
good general fit of the line to the data points. Unusu-
ally low $r^2$ values, as seen in experiments 8E, 9E,
and 11E, result from the lack of variance. Standard
errors in these cases are of comparable magnitude to
the remainder of the experiments where $r^2$ is relatively
high, and thus the low $r^2$ is not indicative of poor fit.

Slopes and intercepts of fitted linear regressions are
statistically compared within each experimental pair
by invoking the t-distribution with the indicated
degrees of freedom to derive a $P$-value. For all 13
pairs of eyes, the slopes of outflow facility versus time
in the eye to which EACA was added to the perfusate
was less than the control eye perfused with normal
saline alone. Within 12 of 13 pairs of eyes, the smaller
slope of the EACA eye was statistically significant to
$P < 0.04$, and ten of thirteen pairs were significant
to $P < 0.001$. A Wilcoxon signed rank test yielded $P
< 0.001$. A plot comparing fitted slopes of the washout
curves is shown in Figure 4. Seven of 13 of the EACA
treated eyes had a negative slope, indicative of a
complete absence of washout, compared with only
one of thirteen of the saline controls.

When segregated by perfusion pressure, EACA
diminished washout in all nine of nine eyes perfused
at 30 mmHg and completely eliminated washout in
three of the nine. All four of the four eyes perfused
at 15 mmHg demonstrated complete obliteration of
washout with addition of EACA. Likewise, positioning
of the needle in the anterior chamber (experiments
1-4 in Table 1) did not alter the observation that
EACA leads to attenuation of washout. Interestingly,
slopes of all four EACA perfused eyes with needles
in the anterior chamber were positive, as opposed to
positive slopes in only two of the nine eyes in which
the needle was in the posterior chamber, a finding
consistent with chamber deepening with time.7

Both experimental pairs that had leucine added to
the control, exhibited washout in the control eye and
an absence of washout in the EACA perfused eye.

Comparison of the Y-intercepts derived from
regression analysis yielded a trend of higher values
for the saline perfused control eye. Overall, six of the
13 paired intercepts showed a significant ($P < 0.01$)
difference, with five of the six from the nine pairs of
eyes perfused at 30 mmHg, and one from the four
pairs perfused at 15 mmHg. However, the mean
initial facilities derived from raw data are 0.223
± 0.060 and 0.257 ± 0.055 for EACA and control
eyes, respectively, suggesting that the difference be-
tween Y-intercepts may represent extrapolative artifact
rather than a veritable difference in initial outflow
facility.

Discussion

Changes in apparent facility of outflow with time
can be influenced by a host of variables, confounding
interpretation of perfusion data. Species specificity,6
antemortem versus postmortem state, degree of ma-
ipulation,1 changes in anterior chamber depth,7 lens
position,22 perfusate composition,11,12 perfusate vis-
cosity,23 temperature,23,24 perfusion pressure,25-29 ocu-
lar viscoelasticity,30,31 and structural composition of
the conventional and unconventional aqueous drain-
age pathways, all may influence apparent washout
and account for much of the discrepancy between
perfusion studies in the current literature.

The present study was designed to minimize vari-
ability. Near simultaneous perfusions using identical
equipment under similar conditions allowed accurate
comparisons within each pair of eyes differing only
by composition of perfusate.

The existence of a washout effect in rabbit eyes
has been noted by Pandolfi12 and Knepper10 using
normal saline and Heps buffered Ringers lactate,
respectively, as perfusates and is confirmed by our
observation of a positive washout slope in 12 of 13
Table 1. Linear regression analysis of constant pressure perfusion

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Perfusion pressure (mmHg)</th>
<th>n</th>
<th>Variance ×10^4</th>
<th>Standard error × 10^3</th>
<th>r^2</th>
<th>Slope (μl/min/mmHg) ×10^4</th>
<th>t</th>
<th>P</th>
<th>Intercept (μl/min/mmHg) ×10^3</th>
<th>t</th>
<th>P</th>
<th>df</th>
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<td>C 1</td>
<td>30</td>
<td>8</td>
<td>58.40</td>
<td>33.40</td>
<td>0.819</td>
<td>1.179</td>
<td>4.45</td>
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<td>0.10</td>
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<td>E 2</td>
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<td>10</td>
<td>26.20</td>
<td>7.42</td>
<td>0.979</td>
<td>0.147</td>
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<td>C 3</td>
<td>30</td>
<td>9</td>
<td>1.35</td>
<td>7.41</td>
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<td>0.129</td>
<td>4.18</td>
<td>0.0009</td>
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<td>11</td>
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<tr>
<td>E 6</td>
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<td>1.77</td>
<td>2.38</td>
<td>0.968</td>
<td>0.380</td>
<td>1.77</td>
<td>0.048</td>
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<td>C 7</td>
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<td>11.20</td>
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<td>-0.137</td>
<td>3.36</td>
<td>0.010</td>
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<td>*</td>
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<td>1.33</td>
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<td>0.58</td>
<td>4.44</td>
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<tr>
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<td>11</td>
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<td>*</td>
<td>0.98</td>
<td>0.34</td>
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<tr>
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<td>11</td>
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<td>9.19</td>
<td>0.686</td>
<td>-0.117</td>
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<td>*</td>
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<tr>
<td>C + L</td>
<td>15</td>
<td>12</td>
<td>4.12</td>
<td>3.90</td>
<td>0.963</td>
<td>0.282</td>
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<td>*</td>
<td>0.98</td>
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<tr>
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<td>7.70</td>
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<td>-0.050</td>
<td>6.67</td>
<td>*</td>
<td>0.28</td>
<td>0.78</td>
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</table>

Facility = b + aT; a = slope; b = intercept; T = time (minutes); C = control perfusate (normal saline); C + L = control perfusate (normal saline with added L-leucine); E = perfusate of normal saline with added EACA; n = number of data points, each set; df = degrees of freedom, 2 sets = (n - 2) 2; P = significance level of comparison between control and treatment groups; t = "t-statistic"; r^2 = coefficient of determination for linear regression; and *connotes P < 0.0001.

pairs of the control rabbit eyes. This contrasts with earlier findings by Melton that rabbit eyes perfused for 120 min with Ringer-locke solution fail to demonstrate a discernible washout. There is no clear-cut explanation for these conflicting results.

The comparison of washout slopes between EACA perfused eyes and the control eyes illustrates the profound stabilizing effect of EACA on apparent facility of outflow in the rabbit. Observation of statistically significant diminution of washout by EACA in 12 of 13 pairs of eyes, and elimination of washout as demonstrated by a negative washout slope in seven EACA treated eyes, implies that EACA can exert a powerful outflow stabilizing effect during prolonged perfusion experiments. This effect is observed at perfusion pressures of both 15 and 30 mm of mercury and is thus unlikely to be an entirely pressure or viscoelastic related phenomenon.
The precise mechanism of outflow stabilization by EACA and the exact role of inhibition of fibrinolytic activity in producing such an effect remains to be elucidated. The prevention of fibrinolysin activation and/or direct inhibition of fibrinolysin activity in the aqueous outflow channels represents an attractive hypothesis for the mode of action of EACA, yet the current literature does not entirely support such a postulate. While high levels of fibrinolytic activity have been demonstrated in the region of Schlemm’s canal and collector channels in man and monkey, little activity is seen in rabbit eyes. Furthermore, in contrast to primate and human eyes, addition of plasmin to a perfusate does not alter outflow resistance in the rabbit eye, nor does rabbit primary aqueous contain significant amounts of plasminogen, plasminogen activator activity, or inhibitors of plasminogen activation. The latter contradicts the hypothesis that perfusate washout of some endogenous fibrinolytic inhibitor is responsible for the washout effect. Failure to detect tissue activator activity may result, in part, from species specificity of the assay methods that utilize bovine fibrin rather than rabbit fibrin.

Perhaps EACA, as a protease inhibitor, may act to prevent proteoglycan dissolution. Van Buskirk found decreased washout in canine eyes following hyaluronidase treatment, implying that a significant component of the washout effect may be attributable to proteoglycan washout from activation of endogenous hyaluronidase. However, data from a series of hyaluronidase treated rabbit eyes gathered by Melton, demonstrates a time-dependent increase in outflow facility that persisted after hyaluronidase treatment, and Knepper has discussed eloquently the stability of glycosaminoglycans during prolonged perfusion of the rabbit eye. Furthermore, EACA is a relatively potent and specific serine protease inhibitor, and it is improbable that such a molecule would exert an effect stabilizing hyaluronidase, which catalyzes dissolution of proteoglycans by cleavage between sugar groups.

One must further consider the possibility that EACA may stabilize apparent facility of outflow by action at other possible outflow channels such as the ciliary body, posterior pole, or cornea. Fibrinolytic activity is generally noted to be high in vascular structures such as the choroid, but when Peterson added fluorescein to saline perfused primate eyes, outflow was noted only from the aqueous veins draining Schlemm’s canal, and no staining of the choroid, retina, or vitreous was noted. Several authors have proposed that inhibition of fibrinolysis by tranexamic acid (Fig. 1) may aid in the maintenance of corneal deturgescence. We noted development of corneal edema after prolonged perfusion in four of the control eyes and two of the eyes perfused with EACA—a suggestive but not statistically significant finding.

In conclusion, the precise mechanism or mechanisms responsible for the washout effect remain to be elucidated. The data presented herein suggests that in rabbits, addition of the antifibrinolytic agent e-aminocaproic acid to a saline perfusate, allows stabilization of the time-dependent apparent facility of aqueous outflow for studies involving long perfusion times.

Key words: Outflow facility, washout effect, e-aminocaproic acid (EACA), fibrinolysis

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References


