Fluid Transport by the Cornea Endothelium is Dependent on Buffering Lactic Acid Efflux

Shimin Li, Edward Kim, and Joseph A. Bonanno *

*Corresponding author
jbonanno@indiana.edu

Indiana University
School of Optometry
800 E. Atwater Ave.
Bloomington, Indiana USA 47405

Running Title: Water Flux linked to Lactate Flux in Cornea
ABSTRACT

Maintenance of corneal hydration is dependent on the active transport properties of the corneal endothelium. We tested the hypothesis that lactic acid efflux, facilitated by buffering, is a component of the endothelial fluid pump. Rabbit corneas were perfused with Bicarbonate-Rich (BR) or Bicarbonate-Free (BF) ringer of varying buffering power, while corneal thickness was measured. Perfusate was collected and analyzed for lactate efflux. In BF with no added HEPES, the maximal corneal swelling rate was 30.0±4.1µm/hr, compared to 5.2±0.9 µm/hr in BR. Corneal swelling decreased directly with [HEPES], such that with 60 mM HEPES corneas swelled at 7.5±1.6µm/hr. Perfusate [lactate] increased directly with [HEPES]. Similarly, reducing the [HCO₃⁻] increased corneal swelling and decreased lactate efflux. Corneal swelling was inversely related to ringer buffering power (β), while lactate efflux was directly related to β. Ouabain (100 µM) produced maximal swelling and reduction in lactate efflux, whereas carbonic anhydrase inhibition and an MCT1 inhibitor produced intermediate swelling and decreases in lactate efflux. Conversely, 10µM adenosine reduced the swelling rate to 4.2±0.8 µm/hr and increased lactate efflux by 25%. We found a strong inverse relation between corneal swelling and lactate efflux (r=0.98, p<0.0001). Introducing lactate in the ringer transiently increased corneal thickness, reaching a steady-state (0±0.6 µm/hr) within 90 minutes. We conclude that corneal endothelial function does not have an absolute requirement for bicarbonate; rather it requires a perfusing solution with high buffering power. This facilitates lactic acid efflux, which is directly linked to water efflux, indicating that lactate flux is a component of the corneal endothelial pump.

Key Words: Corneal Endothelium, Water Transport, Buffering Power, Lactate Flux, MCTs
The notion of a bicarbonate-carbonic anhydrase and monocarboxylic acid transporter (MCTs) metabolon (12) has been suggested as a means to facilitate lactic acid flux. Linkage among bicarbonate buffering, carbonic anhydrases and lactate transport has been shown in several systems, including retinal pigment epithelium (1, 29, 37), muscle (20, 70), astrocytes (60, 64), tumors (18, 31), oocyte expression systems (4, 5) and cornea (8, 43, 63). Potential coupling of lactate flux or gradients with water has been suggested in a few cases such as retinal pigment epithelium (2, 21), jejunum (68), kidney (25), and cornea (47, 48). In this study, we examine the role of bicarbonate-carbonic anhydrase buffering in facilitating lactate efflux from the cornea and the potential coupling to water flux.

Optical transparency of the cornea is dependent on maintenance of corneal stromal hydration, which is controlled by the active transport properties of the corneal endothelium, often called the “Endothelial Pump”. The Pump compensates for a passive “Leak”, which is driven by highly charged stromal GAGs (glycosaminoglycans) that exert a tissue swelling pressure of about -60 mmHg at normal tissue hydration (3.5mg H2O/mg dry wt.) (22-24, 34). When the Pump and Leak are similar, corneal hydration remains within a small range (see Figure 1A). Corneal Endothelial Dystrophies, which are characterized by cell degeneration, transport inhibition and/or disruption of the endothelial monolayer, lead to corneal edema, light scatter and loss of visual acuity.

Early studies have shown that the corneal endothelial pump is dependent on Na+/K+ ATPase activity and the presence of bicarbonate in the perfusing solution (3, 14, 16, 26, 55). Partial inhibition of the pump could be achieved by inhibition of secondary active transport processes by amiloride (41), DIDS (35, 52), as well as by Carbonic Anhydrase inhibitors (16, 26, 51). These studies and others led to the hypothesis that the corneal endothelial pump uses a bicarbonate secretory mechanism (see Figure 1B) that produces small transendothelial osmotic gradients to drive water efflux. Efforts to identify these bicarbonate transport components have found strong basolateral (stromal side) bicarbonate uptake (e.g. NBCe1) (28, 62, 69). However, apical bicarbonate permeability did not show any Na or Cl dependency and overall was found to be three times smaller than basolateral (8, 39), although apical bicarbonate permeability can be temporarily doubled via activation of CFTR or CIC-A1 (38, 61, 72). Attempts to measure net...
bicarbonate fluxes by the corneal endothelium, however have been equivocal (44, 45). More recently, an alternative electro-osmotic model of the endothelial pump incorporating bicarbonate transport has also been put forth (13, 57, 58).

Interestingly, one report indicated that the endothelial pump could be supported in the absence of bicarbonate if high concentrations of Goods buffers were included in the perfusing solution (15). The authors noted that buffering capacity was important, but did not suggest a mechanism. Their report was followed by two studies that purported to refute the findings, suggesting that bicarbonate was essential (36, 51). However, neither of these studies focused on the buffering power of the perfusing solution, which we show in the current study is a key factor in supporting the endothelial pump.

The cornea is very glycolytic. Eighty-five percent of glucose consumed is converted to lactate (50). This happens predominately in the surface epithelium and stromal keratocytes, which have few mitochondria. For every 100 glucose molecules taken up by the cornea, 170 lactate molecules are produced. This sets up a large diffusion gradient with ~13 mM lactate in the cornea and ~7 mM lactate in the aqueous humor (7, 33, 54, 59). Corneal hypoxia is long known to increase corneal hydration (i.e. thickness) and this was shown to be due to increasing corneal [lactate] (33), demonstrating that lactate is an important osmolyte in the cornea. Lactate does not cross the epithelium so all of it must diffuse across the endothelium (33). Previous studies from our laboratory have shown that the presence of bicarbonate and carbonic anhydrase activity facilitate basolateral to apical lactate flux across corneal endothelium (47, 48) and that MCTs are involved in regulating corneal hydration in vivo (40). In the current study we test the hypothesis that bicarbonate in the perfusing solution, which serves as an artificial aqueous humor, is not necessary for maintenance of the corneal endothelial pump, but rather that it provides the buffering power that facilitates lactic acid efflux, which is coupled to water efflux.

MATERIALS AND METHODS

Ethical Approval. Animal procedures were approved by the Indiana University Bloomington Institutional Animal Care and Use Committee. New Zealand White rabbits (Oakwood Research
Facility, Inc, Oxford, MI USA), both males and females, age 8-12 weeks, weighing ~2.5 kg were
used for these studies. Animals were fed standard rabbit chow *ad libitum*. To collect corneas,
animals were euthanized by the following procedure: rabbits were anesthetized via intramuscular
injection of Ketamine-HCl (60 mg/kg) & Xylazine (5 mg/kg); depth was checked by toe pinch.
This was followed 3-5 minutes later by intracardiac injection of Na Pentabarbital (100mg/kg).
All animal procedures were conducted in accordance with the Guide to the Care and Use of
Laboratory Animals at Indiana University and adhered to the ARVO Statement for the Use of
Animals in Ophthalmic and Vision Research.

**Cornea Dissection and Mounting.** Isolation and atraumatic mounting of the cornea followed
essentially the method of Dikstein and Maurice (14). Briefly, a circular incision was made in the
lids around the margins of the orbit. Extraocular muscles & optic nerve were severed and the
eyeball was excised complete with conjunctiva and lids. The ocular surface with exposed cornea
was placed face down on a plastic mounting ring that was pressed onto a hollow methacrylate
rod, which was fixed to a stationary metal rod. The lids and conjunctiva were everted over the
globe and pulled down tightly to the rod. The eye was then anchored in place by a suture tied
over the everted conjunctiva and fit within a groove on the plastic mounting ring (Figure 2). An
incision was made with a scalpel blade a few mm posterior to the corneal-scleral junction and the
posterior sclera removed with scissors. The vitreous, ciliary body-iris, and lens were carefully
removed to reveal the corneal endothelial surface. The scleral rim remaining was reflected down
over the ring. A metal platform was then placed from below over the plastic rod and pressed over
the outside of the ring, which was against the anterior surface of the scleral rim. A plastic cap, to
form an artificial anterior chamber, tightly sealed the posterior surface of the scleral rim. The
plastic cap has three holes fitted with 23-gauge tubing. The central tube provided inflow and the
two side tubes provided outflow (see Figure 2). The cornea chamber was fitted into a metal
jacket with the epithelium facing up. The metal jacket had an embedded electric heater to
maintain temperature at 37°C. A thermistor embedded in the posterior plastic cap indicated
temperature equilibration with ten minutes.

**Perfusion and Solutions.** The perfusion ringer solution was driven into the corneal chamber by a
micro-pump (P720 Peristaltic Pump, Plymouth Meeting PA, [www.instechlabs.com](http://www.instechlabs.com)) at a constant
speed of 50 µl/minute. The two outflow tubes were joined behind the chamber and the outflow
tube placed 20 cm above the epithelium to simulate an intraocular pressure of 15 mmHg. Each
cornea was perfused for a period of 5 hours. The perfusate was collected for 30 minute intervals
(1.5 ml aliquots), frozen and assayed for lactate at a later time.

The corneal endothelial surface was perfused with Glutathione Bicarbonate Ringer (GBR),
which contained (mM): 110 NaCl, 2 KCl, 1 K₂HPO₄, 0.6 MgCl₂, 1.4 Ca-gluconate, 5 glucose,
0.3 reduced glutathione, 15 Na-gluconate and 28.5 NaHCO₃⁻, 5% CO₂, pH 7.5. Ringer with
varying [HCO₃⁻] (5, 10, 18, 28.5, and 44 mM) was made by equimolar substitution with Na-
gluconate. All bicarbonate solutions were equilibrated with 5% CO₂, so pH was 6.75, 7.04, 7.30,
7.5, and 7.68, respectively. HCO₃⁻ free Ringers (BF) were identical except NaHCO₃⁻ was
substituted with equimolar Na-gluconate or Na-HEPES to achieve 0, 10, 25, 40, or 60 mM
[HEPES], pH7.5 and equilibrated with air. Osmolarity was measured with a vapor pressure
osmometer (Wescor, Logan, UT USA) and adjusted to 295 mOsm/l with sucrose. GBR
containing 28.5 mM HCO₃⁻ served as a standard control in all experiments. Perfusion Ringers
solutions were maintained at 37°C in a water bath.

Corneas were mounted in pairs to maximize utility of tissue and minimize animal use. After
mounting, the corneas were perfused with standard GBR for 90 minutes. Corneas that showed
accelerated swelling due to endothelial or epithelial damage were not included. After 90 minutes
equilibration in standard GBR the perfusing solution was switched to experimental Ringer and
perfusion continued for 210 minutes. The epithelial surface was covered with the same medium
as that in the perfusion and replaced every 15 minutes.

Corneal Thickness. The central corneal thickness (CT) was measured by optical coherence
tomography using an iVue instrument (Optovue, Inc., Fremont, CA) that was mounted above the
cornea (Figure 2). At the 15-minute interval, the epithelial Ringer solution (0.6 ml) was carefully
aspirated, three corneal thickness measurements were taken & averaged, and fresh Ringer
replaced on the epithelial surface.
Corneal Lactate Retention. After each 5-hour perfusion, the cornea was removed from the chamber and trephined to a 10 mm central button that was snap frozen in liquid N₂ and pulverized to powder using a ceramic mortar and pestle. The cornea powder was collected in a preweighed microcentrifuge tube and 0.5 ml PBS was added, vortexed for 1 minute, and centrifuged at 13,000 g for 15 minutes. The supernatant was collected and analyzed for lactate content. The remaining pellet was dried at 60°C within a vacuum centrifuge for 2 hours and weighed. The lactate content (nmoles) of the perfusate sample collected every 30 minutes and the corneal extracts (nmoles/mg dry wt) was determined by an assay kit from BioVision Research Products (Milpitas, CA).

In some experiments, inhibitors were added to the standard medium as 0.1 mM Ouabain (Sigma), 0.1 mM Acetazolamide (Sigma), 0.01 mM MCT inhibitor (HY-13248, ChemExpress, Monmouth Junction, NJ; AR-C-155858, http://medchemexpress.com/AR-C155858.html), or 0.01 mM Adenosine (Sigma).

Analysis of results. Each experimental condition included a minimum of three corneas. The results are expressed as mean ± SD and compared using t-test or regression analysis, as appropriate. Corneal thickness changes are reported as maximal rates (µm/hr), which typically occurred between the 120-180 minute perfusion period, or as total thickness change from 90-300 minutes.

RESULTS

HEPES Buffer Supports the Endothelial Pump. Figure 3A shows the general experimental sequence to test if bicarbonate free (BF) Ringer could support corneal endothelial pumping by increasing the HEPES buffer concentration. After mounting, corneas were perfused with the standard GBR Ringer for 90 minutes. During this equilibration time we observed a small decrease in corneal thickness (CT) followed by a slow increase. Decreases in CT indicate net water efflux, whereas increases in CT indicate net influx. When GBR perfusion was continued, CT increased at a fairly steady rate of 5.2±0.9 µm/hr, indicating that under the experimental conditions used the driving forces for water influx slightly exceeded those for water efflux. In
contrast, when corneal perfusion was switched at 90 minutes to BF Ringer containing 0 HEPES, the CT swelling rate increased to 30.0±4.1µm/hr. Interestingly, Figure 3A also shows that the presence of HEPES in the perfusing Ringer (10, 25, 40, and 60 mM) decreased the maximum observed swelling rate to 22±3.2µm/hr; 13±2.7µm/hr; 10±2.0µm/hr; and 7.5±1.6µm/hr, respectively. Fitting the Maximal Swelling Rate vs. [HEPES] to an exponential model yielded a significant correlation (Swelling Rate = a*e(-b[HEPES]+c, r=.985, p=0.0005). These results demonstrate that the endothelial pump can be supported in the absence of HCO₃⁻ and in the presence of increasing HEPES buffer concentration.

To test the hypothesis that lactic acid efflux across the endothelium is being buffered, we examined the [lactate] in the effluent solutions. Figure 3B shows that in general the [lactate] in the effluent decreased over the course of the experiment. However, the decrease was greatest with 0 HEPES perfusion and least with standard GBR. Total lactate efflux from 120-300 minutes was 751±36, 856±20, 983±32, 1084±36, and 1177±21 nmoles for 0, 10, 25, 40, and 60 mM HEPES, respectively. Fitting Efflux vs. [HEPES] to an exponential model yielded a significant correlation (Efflux = a*(1-e(-b[HEPES]+c), r=.976, p=0.001). Efflux was higher (1325±32 nmoles) during GBR perfusion. These data indicate that the [lactate] of the cornea at the end of the experiment (lactate retention) should be lower in those with the greatest efflux. Figure 3C shows a progressive decrease in lactate retention from 0 to 60 mM HEPES with the least amount retained following perfusion with GBR. These data indicate that increasing HEPES buffer concentration facilitates lactate efflux across the endothelium.

**Endothelial Pump Varies with Bicarbonate Concentration.** The perfusing Ringer buffering power can also be varied by changing the [HCO₃⁻]. Figure 4A shows that reducing [HCO₃⁻] to 5, 10, and 18.5 mM produced maximal swelling rates of 20±3.8, 13.7±3.3 and 8.3±2.8 µm/hr, respectively. Whereas increasing [HCO₃⁻] to 44 mM yielded 4.3±2.2 µm/hr, relative to standard 28.5 mM GBR, 5.2±0.9 µm/hr. The swelling rate was significantly correlated with [HCO₃⁻], (Maximal Swelling Rate = a*e(-b[Bicarb]+c, r=.967, p=0.004). Figure 4B shows that lactate efflux was greatest at 44 mM and least at 5 mM [HCO₃⁻]. Total lactate efflux from 120-300 minutes was 991±14, 1082±21, 1205±36, 1325±32, and 1397±36 nmoles for 5, 10, 18.5, 28.5 and 44 mM [HCO₃⁻], respectively. Efflux was significantly correlated with [HCO₃⁻], (Efflux = a*(1-e(-b[Bicarb]+c, r=.956, p=0.006). Lactate retained in the cornea was greatest with 5 and least with 44
mM HCO₃⁻ (Figure 4C). In the [HCO₃⁻] experiments, the pH of the perfusing solutions varied because they were all equilibrated with 5% CO₂. To test that the results were not simply a reflection of varying pH, we repeated the most acidic pH perfusion (5 mM [HCO₃⁻], 5% CO₂, pH 6.75) and paired it with perfusion of 5 mM [HCO₃⁻], pH 7.5. We achieved pH 7.5 by bubbling with 5% CO₂ while measuring the pH of the Ringer. When the pH of the bicarbonate solution decreased to 7.5, bubbling was stopped. The solution pH was then checked every 10 minutes and bubbling recommenced and halted as needed to keep the pH at 7.5±0.03. From the Henderson-Hasselbach equation, 5 mM HCO₃⁻, pH 7.5 would contain 0.9% CO₂. When the experiment reached the 90-minute point, this solution was perfused. Figures 4D-F show that the corneal swelling rates, lactate efflux, and lactate retention were not significantly different at pH 6.75 and pH 7.5 (paired t-tests, p>0.05). The absence of a major effect of pH is in agreement with previous work (15). In sum, these data indicate that corneal swelling decreases, while lactate efflux increases, with increasing [HCO₃⁻].

Corneal Hydration and Lactate Efflux Facilitated by Solution Buffering Power. The data presented in Figures 3 and 4 indicate that bicarbonate perfusion is not necessary to maintain the endothelial pump, but that buffering power is important. Figure 5A and 5B plot total corneal swelling between 90-300 minutes and lactate efflux over the 120-300 minute period versus buffering power. Buffering power (β mM/pH) of HEPES at pH 7.5 (pK7.5) was assumed to be 0.58*[HEPES] and bicarbonate β=2.3*[HCO₃⁻] (30, 56). Corneal swelling decreased exponentially with increasing β (Swelling =a*exp(-b*β)+c, r=.969, p=0.0005). The HEPES and bicarbonate data partially overlapped and were relatively similar. Conversely, total lactate efflux from 120-300 minutes increased exponentially with β and there was good overlap between HEPES and bicarbonate (Efflux =a*(1-exp(-b*β))+c, r=.99, p=0.0001). These data indicate that the buffering power of the perfusing solution has a strong influence on corneal hydration and lactate efflux. Facilitating lactate efflux reduces corneal hydration.

Lactate Flux & Corneal Thickness Linked During Endothelial Pump Modulation. Next, we examined other conditions that affect the corneal endothelial pump and measured concomitant lactate fluxes. The Na⁺/K⁺-ATPase inhibitor ouabain causes the maximum amount of corneal swelling of all the transport inhibitors that have been tested (55). Figure 6A shows that 0.1 mM ouabain induced 30.1±4.1 μm/hr maximal swelling and 88±9.2 μm total swelling at 300 minutes,
as compared to 5.2±0.9 \( \mu \text{m/hr} \) and 23±1.7 \( \mu \text{m} \) for the GBR control. Figure 6B shows that lactate efflux decreased significantly with ouabain. Total lactate flux from 120-300 minutes was: 621±53 for ouabain compared to 1324±33 nmoles for GBR over the 120-300 minute time period. Lactate retention was 24.0±4.4 nmoles/mg with ouabain, twice that of the control. In the presence of ouabain, lactate efflux over the last 3 hours decreased by a factor of 2.13 relative to control and lactate retention, measured at the fifth hour, increased by a factor of 2.0 relative to the GBR control, indicating that lactate production by the cornea is not significantly altered by the presence of ouabain.

Carbonic anhydrase inhibitors (CAIs) are also known to produce moderate inhibition of the endothelial pump (16, 26, 51). CAIs also reduce bicarbonate buffering capacity (67) and thereby slow Lactate:H\(^{+}\) cotransport (47, 64, 70). Figure 6A shows that 0.1 mM acetazolamide produced 16.8 \( \mu \text{m/hr} \) maximal swelling and 47±1.5\( \mu \text{m} \) total swelling. Total lactate efflux (Figure 6B) was 967±47 nmoles and lactate retention 17.6±47 nmoles/mg, commensurate with the intermediate level of pump inhibition.

Facilitated transport of lactate is via monocarboxylate Lactate:H\(^{+}\) cotransporters (MCTs) (19). MCT1, 2, and 4 are expressed in corneal endothelium (40, 48). We used HY-13248 (AR-C155858) (10 \( \mu \)M) a relatively new and specific Lactate:H\(^{+}\) cotransport inhibitor of MCT1 and 2 activity, but not MCT4 (49). Figure 6A shows that partial block of MCTs by HY-13248 produced 16.3±2.0 \( \mu \text{m/hr} \) maximal swelling and 43.3±4.0 \( \mu \text{m} \) total swelling. Total lactate efflux (Figure 6B) was reduced to 973±46 nmoles and lactate retention (Figure 7C) was 16.3±3.0 nmoles/mg, indicating that lactate efflux occurs through MCTs and inhibition of MCTs slows water efflux.

Conversely, increasing endothelial [cAMP] is known to produce a modest stimulation of the endothelial pump (17, 52). Figure 6C shows that 10 \( \mu \text{M} \) adenosine reduced the maximal swelling rate in GBR from 5.2±0.9 to 4.2±0.8 \( \mu \text{m/hr} \) (t-test, \( p=0.025 \)). Moreover, Figure 6D shows that total lactate efflux was 1648±13 nmoles with adenosine, which was significantly greater than with GBR (1324±33 nmoles, independent t-test, \( p=0.001 \)).
Figure 7 summarizes the ΔCT, Total Lactate Efflux and Lactate Retention data due to these transport modifiers and compares them to GBR control and 0 HEPES perfusion. The data clearly indicate that blocking primary active transport causes: a) maximal corneal swelling (Fig. 7A), b) the least lactate efflux (Fig. 7B), and c) the most lactate retention (Fig. 7C). This compares closely with the effects of 0 HEPES perfusion, which has the lowest buffering capacity (<1 mM/pH due to phosphate). Intermediate levels of swelling show intermediate inhibition of lactate flux and lactate retention, while stimulation of the pump with adenosine reduces swelling with maximal lactate efflux and the least lactate retention. Figure 7D plots the change in corneal thickness (ΔCT) vs Lactate Efflux for all experiments (BF & BR perfusion, Ouabain, acetazolamide, HY-13248, and adenosine) and also shows a fit of the data to an exponential model (ΔCT=ae^-b*Efflux +c, r=0.98, p<0.0001), indicating a strong association between water flux and lactate flux.

Lactate in Perfusing Ringer Stabilizes Corneal Thickness. In all cases, the [lactate] gradient from cornea to perfusate was infinite since the perfusing Ringer has zero lactate. Although this has been the customary perfusion setup, the [lactate] of the aqueous humor in vivo is about 5-10 mM (7, 54, 59). If lactate efflux is linked to water efflux, then adding lactate to the perfusing Ringer will reduce the gradient, slow lactate efflux and increase corneal thickness. To test this we switched GBR perfusion to include 5 mM Na-lactate (substituted for Na-gluconate). The osmolality of the solutions were the same. Figure 8 shows that when lactate was introduced at 90 minutes, the corneas swelled. Within 90 minutes however, corneal thickness reached a steady-state with no swelling (0±0.6 μm/hr), whereas the corneas perfused without lactate have not reached a steady-state. Because of the large [lactate] in the perfusing solution, efflux was not measured. Not surprisingly, lactate retention in the cornea was almost twice (22.2±2.1 nmoles/mg) that of the GBR control. This data confirms that lactate gradients affect corneal hydration and suggests that fixing the lactate concentration at the endothelial surface may stabilize the transendothelial lactate gradient, thereby slowing background swelling.

DISCUSSION
The earlier study of Doughty and Maurice had suggested that the corneal endothelial pump can be supported by high concentrations of Good’s buffers in the absence of bicarbonate (15). Although several studies appeared to refute this notion (36, 51), none focused on buffering power as a potential perfusing solution property that could be the basis for the observations. The data presented here, now show that endothelial pump activity can be supported in the absence of bicarbonate if sufficient solution buffering power is present. A close examination of the study by Riley et al. (51) showed maximal swelling in a bicarbonate-free phosphate (9.6 mM) buffered ringer ($\beta \approx 5$ mM/pH) and 25% less swelling with a 25 mM HEPES ringer ($\beta \approx 12.5$ mM/pH), consistent with our hypothesis that endothelial pump function varies directly with $\beta$. Endothelial pump function in vivo is maximally supported due to the presence of very robust buffering power provided by the HCO$_3^-$-CO$_2$-Carbonic Anhydrase buffering system.

We tested the hypothesis that lactic acid efflux was being buffered and that lactate efflux was linked to water efflux. Increasing buffering power increased lactate efflux under bicarbonate or bicarbonate-free conditions (Figures 3 & 4). Lactate efflux was directly related to buffering power (Fig 5B), whereas corneal swelling (Leak>Pump) was inversely related to buffering power (Fig. 5A), indicating that lactic acid efflux was being buffered and this was directly associated with water efflux. A similar relation between corneal swelling and lactate efflux was observed by inhibiting primary active transport with the Na$^+$/K$^+$ ATPase inhibitor ouabain, even though solution buffering power was high, indicating that transendothelial lactate flux is dependent on active transport. We also show that the carbonic anhydrase inhibitor acetazolamide, which produces moderate corneal swelling, led to moderate decreases in lactate efflux. For the corneal endothelium, carbonic anhydrase enzymes are located on both basolateral (CA12) and apical (CA4) membranes as well as in the cytoplasm (CA2). Acetazolamide is cell permeable, so all isozymes were inhibited. Carbonic anhydrase inhibitors slow the hydration and dehydration of CO$_2$, which in turn slows the consumption or production of H$^+$ and lowers effective buffering power. While buffering is facilitating lactic acid flux, MCTs provide the transporter route for transcellular flux. Partial inhibition of corneal endothelial MCTs by HY-13248, directly inhibiting MCT1 and MCT2 lactate:H$^+$ cotransport (but not MCT4), slows lactate and water efflux concomitantly. Conversely, stimulating endothelial function by increasing cAMP, decreased corneal swelling and increased lactate efflux. The excellent correlation
between water flux (ΔCT) and lactate flux under all conditions tested (Fig 7D) is strong evidence that lactate efflux is directly linked to water efflux.

Previous studies from our laboratory are consistent with this hypothesis. We have shown that bicarbonate and carbonic anhydrase activity support lactate flux across cultured bovine corneal endothelial cells (43, 47). Lactate fluxes were inhibited by the carbonic anhydrase inhibitor acetazolamide, the anion transport inhibitor DIDS, and Na⁺:2HCO₃⁻ cotransporter (NBCe1) specific siRNA knockdown. Moreover, ouabain, DIDS, or Carbonic anhydrase inhibition in the in vivo rabbit cornea produced corneal edema with a concomitant increase in corneal [lactate] (48). NBCe1 shRNA in vivo produced variable knockdown, which overall showed no increase in corneal thickness(43). However, if topical AZOPT (a carbonic anhydrase inhibitor) was added, increased corneal edema was seen in those eyes that received the NBCe1 shRNA (43). When a subset of corneas that showed high levels of NBCe1 knockdown were analyzed, the ΔCT measured was associated with a concomitant increase in corneal [lactate] (48). These observations point out the robustness of the in vivo cellular bicarbonate buffering system. Lastly, shRNA silencing of CD147, the MCT1 & 4 chaperone, resulted in stromal lactate accumulation and corneal edema in the in vivo rabbit cornea (40), consistent with the actions of HY-13248 in the current study.

Whereas solution buffering power supports endothelial pumping, active transport is also necessary. The Na⁺/K⁺ ATPase provides the Na⁺ gradient that drives Na⁺:2HCO₃⁻ cotransport and Na⁺/H⁺ exchange, two transporters that are essential for pHᵢ regulation. Dysregulation of pHᵢ will likely perturb basolateral to cellular as well as cellular to apical surface pH gradients, which in turn will inhibit lactate:H⁺ cotransporter fluxes at each membrane. In Figure 9A, we show a transport model for transendothelial lactate flux across the corneal endothelium. At the basolateral (stromal side) membrane, MCT1 & 4 provide lactate:H⁺ cotransport influx. The influx of protons is buffered by bicarbonate and facilitated by CA2. NBCe1 provides for continual bicarbonate influx. At the apical membrane MCT2 provides lactate:H⁺ efflux. The efflux of protons is buffered by aqueous humor bicarbonate and facilitated by CA4. Apical anion channel activation, e.g. CFTR, by A2b receptor agonist adenosine induced increases in cAMP.
(66), can provide a modest boost in $[\text{HCO}_3^-]$ at the apical surface (61, 63), enhancing lactic acid buffering.

Our data shows that other buffers at the apical surface can also facilitate lactate efflux. In the absence of bicarbonate however, the basolateral buffering system, NBCe1-CA2, will be weakened. Because of the leakiness of the endothelial cell tight junctions (25 Ωcm²), it is likely that HEPES can diffuse into the basolateral space to provide buffering. Nevertheless, cytosolic buffering would have to rely on NHE1 activity or other unidentified proton transporters where the ejected protons are buffered by HEPES. Previous studies have shown that the absence of bicarbonate lowers pHᵢ in endothelial cells (10) and this will activate NHE1 (9, 65), providing additional cytosolic buffering. Moreover, it is possible that non-enzymatic H⁺ shuttling via carbonic anhydrases could facilitate transport activity of MCT1 & 4 (6) at the basolateral membrane and by CA4-MCT2 (32) at the apical membrane in the absence of bicarbonate.

Model for Linking Water Flux to Lactate. In addition to lactate flux through transporters, there is potentially a small non-ionic lactate flux and a larger paracellular flux, as illustrated by the continual efflux of lactate in the presence of ouabain. Also, our data show large decreases in lactate efflux concomitant with very little change in corneal thickness during the first 90 minutes of perfusion. This is most likely due to paracellular diffusion of stromal lactate driven by the infinite gradient for lactate across the endothelium. Presumably, it is the flux through the transporters that has the greatest potential for linkage to water flux. One possibility is direct coupling of water molecules with lactate by the MCTs, as has been suggested for the retinal pigment epithelium (21). Another possibility is the presence of a standing lactate gradient that drives water by osmosis across the cells and is facilitated by AQP1. The cornea is a source of lactate and the steady-state [lactate] is higher in the stroma than in the aqueous humor. To achieve the needed gradient to drive water osmotically the apical surface [lactate] must be a little higher than the basolateral [lactate]. This can be realized if the basolateral influx of lactate is greater than the rate of diffusion in the stroma creating a slight drop in [lactate] in the basolateral space. In addition, lactate efflux via MCT2 into the apical unstirred layer provides a slight increase in the local [lactate] such that the transendothelial lactate gradient is sufficient to drive water flux. This is illustrated in Figure 9B. Adding lactate to the perfusing solution, at a level
approximating that in vivo, had an interesting effect. As expected, it produced a transient increase in CT since this will slow lactate efflux from the cornea. However, within 90 minutes, CT stabilized such that CT did not change during the last hour of perfusion whereas the control continued to show small increases in CT. This is consistent with the model in that lactate free perfusion could reduce or destabilize apical unstirred layer lactate accumulation that helps drive water osmotically, while having a fixed amount of lactate in the bulk solution could stabilize the lactate gradient. The notion that production of lactate can produce a physiological osmotic gradient is not limited to the cornea. Lactate gradients have been suggested as a urine concentrating mechanism within the renal inner medulla (25). Moreover, pathophysiologic water flux induced by lactate production in brain edema has also been documented (11, 42, 46).

Of the three major anions, Cl⁻, HCO₃⁻, and lactate, our data indicate that lactate is a component of the endothelial fluid pump mechanism. Whereas chloride has been shown to accumulate within endothelial cells above electrochemical equilibrium through the action of NKCC1 (27), the lack of constitutively active apical Cl⁻ channels or transporters and the fact that the NKCC1 inhibitor bumetanide had no effect on corneal hydration (53), ruled out a direct role for a Cl⁻ secretory mechanism in the endothelial pump. A direct role for a HCO₃⁻ secretory mechanism is also ruled out since we show that HCO₃⁻ is not an absolute necessity for pump maintenance. While there is robust basolateral HCO₃⁻ influx via NBCe1 (62), the apical membrane HCO₃⁻ permeability is three times smaller and no Na or Cl⁻ dependent HCO₃⁻ transport could be found (39). However, under conditions where apical anion channels are activated, a small apical HCO₃⁻ flux can be measured (71, 72). We show that apical anion channel activation by Adenosine increases lactate efflux suggesting that the apical HCO₃⁻ flux is buffering apical lactic acid. As for lactate, there are several remaining questions: 1) is lactate flux effective in de-epithelialized corneal preparations? Previous studies have shown that the corneal epithelium can be removed and replaced with silicone oil and the stromal-endothelial preparation can maintain hydration(55, 58). This removes a major source of lactate production. Of course, lactate is still being produced by the stromal keratocytes and endothelial cells, but is that sufficient to form the needed concentration gradient or MCT linked water flux? 2) is there a link between lactate and water flux directly via MCTs?; and 3) do the modeled basolateral to apical lactate gradients, as shown in Figure 9B, exist?
In summary, this study has shown that perfusing solutions of high buffering power can support the active transport properties of the corneal endothelial fluid pump. Lactic acid efflux across the endothelium is being buffered and the lactate flux via MCTs is directly related to water flux. The system works well in vivo due to the presence of high aqueous humor bicarbonate concentrations, robust basolateral bicarbonate transport and multi-isozyme carbonic anhydrase activity, which impart a high buffering power. The data suggest that enhancing lactate efflux in patients with Corneal Endothelial Dystrophies could improve corneal clarity and vision, as long as an intact endothelial monolayer is present. Lastly, the results indicate that efforts to engineer an endothelial cell replacement that could substitute for traditional donor transplant tissue, must have the requisite lactate:H⁺ cotransport activity to be effective.

**Funding**

This work was funded by a grant from the National Institutes of Health (USA) NIH RO1EY008834 to JAB.

**Disclosures:** S. Li, None; E. Kim, None; J. Bonanno, None

**REFERENCES**


33. Klyce S. Stromal lactate accumulation can account for corneal oedema osmotically following epithelial hypoxia in the rabbit. *J Physiol (Lond)* 321: 49-64, 1981.


19. Liebovitch LS, and Fischbarg J. Effects of inhibitors of passive Na+ and HCO3-
fluxes on electrical potential and fluid transport across rabbit corneal endothelium. *Curr

20. Lindinger MI, Leung MJ, and Hawke TJ. Inward flux of lactate(-) through
monocarboxylate transporters contributes to regulatory volume increase in mouse muscle


22. Maurice DM. Passive ion fluxes cross the corneal endothelium. *Curr Eye Res* 4: 339-

23. Mayes KR, and Hodson S. Local osmotic coupling to the active trans-endothelial

24. Mori S, Morishima S, Takasaki M, and Okada Y. Impaired activity of volume-
sensitive anion channel during lactacidosis-induced swelling in neuronally differentiated

25. Nguyen TT, and Bonanno JA. Bicarbonate, NBCe1, NHE, and carbonic anhydrase
activity enhance lactate-H+ transport in bovine corneal endothelium. *Investigative

26. Nguyen TT, and Bonanno JA. Lactate-H(+)-transport is a significant component of
the in vivo corneal endothelial pump. *Investigative Ophthalmology & Visual Science* 53:

27. Ovens MJ, Manoharan C, Wilson MC, Murray CM, and Halestrap AP. The
inhibition of monocarboxylate transporter 2 (MCT2) by AR-C155858 is modulated by the

28. Riley M. Glucose and oxygen utilization by the rabbit cornea. *Experimental Eye

29. Riley M, Winkler B, Czajkowski C, and Peters M. The roles of bicarbonate and
CO2 in transendothelial fluid movement and control of corneal thickness. *Invest Ophthalmol

30. Riley M, Winkler B, Starnes C, and Peters M. Adenosine promotes regulation of
corneal hydration through cyclic adenosine monophosphate. *Invest Ophthalmol Vis Sci* 37:
1-10, 1996.

31. Riley M, Winkler B, Starnes C, and Peters M. Fluid and ion transport in corneal

32. Riley MV. Intraocular dynamics of lactic acid in the rabbit. *Invest Ophthalmol* 11:
600-607, 1972.

33. Riley MV, Winkler BS, Peters MI, and Czajkowski CA. Relationship between fluid
transport and in situ inhibition of Na(+)-K+ adenosine triphosphatase in corneal


35. Sanchez JM, Cacace V, Kusnier CF, Nelson R, Rubashkin AA, Iserovich P, and
Fischbarg J. Net Fluorescein Flux Across Corneal Endothelium Strongly Suggests Fluid


Figure 1. Schematic Diagrams Illustrating the Corneal Endothelial Pump. Figure 1A shows a schematic anterior-posterior section of the cornea to illustrate that stromal GAGs (glycosaminoglycans) exert a passive swelling pressure that drives water across the endothelium (also from the tears across the epithelium to a lesser extent), which is called the LEAK. The endothelial monolayer (en face view shown below) exerts an equal and opposite active transport dependent driving force, called the PUMP, such that the hydration of the cornea is maintained at a level that minimizes light scatter. B, shows a simplified model of the prevailing notion that the endothelial PUMP is a bicarbonate secretory mechanism where there is Na⁺ dependent HCO₃⁻ uptake at the basolateral membrane, facilitated by carbonic anhydrases (CA), and HCO₃⁻ secreted across the apical membrane (yellow box) by anion channels or transporters generating a small transendothelial voltage, driving Na⁺ across the tight junctions, yielding a small transendothelial NaHCO₃⁻ gradient that drives water osmotically and is facilitated by water channels.

Figure 2. Left, shows the initial arrangement of the eyeball on the mounting ring. Middle, after dissection the isolated cornea is perfused from the bottom and placed within a warming ring (not shown). An optical coherence tomography imager is placed above the cornea and produces cross-sectional images (right) at 5 μm resolution.

Figure 3. Bicarbonate-Free HEPES Buffer Perfusion. A, Corneal Thickness change vs. time. All corneas were perfused with GBR for 90 minutes. At the arrow perfusion was switched to 0, 10, 25, 40, or 60mM HEPES in the absence of bicarbonate. B, Lactate content in 1.5 ml samples of perfusion effluent. C, lactate remaining in the cornea after 5 hours of perfusion. Data from 18 corneas, n=3 per each condition.

Figure 4. Bicarbonate-Rich Perfusion. A, Corneal Thickness change vs. time. All corneas were perfused with GBR for 90 minutes. At the arrow perfusion was switched to 5, 10, 18.5, or 44mM bicarbonate. B, Lactate content in 1.5 ml samples of perfusion effluent. C, lactate remaining in the cornea after 5 hours of perfusion. D, Corneal Thickness change vs. time, comparing
perfusion with 5 mM HCO₃⁻ at pH 7.5 and pH 6.75. E, Lactate content in 1.5 ml samples of 
perfusion effluent from experiment in D. F, Lactate retained in corneas from experiment in D.
Data is from 21 corneas, n=3 per each condition.

Figure 5. Effect of Solution Buffering Power on Corneal Thickness and Total Lactate Efflux. A, 
Corneal Thickness change (swelling) from 90-300 minutes vs β, buffering power for 
bicarbonate-Rich and HEPES buffered perfusion. Line is fit to exponential model \[ \text{Swelling} = a \cdot e^{-\beta \beta} + c, \quad r=0.969, \quad p=0.0005 \]. B, Total Lactate Flux over 120-300 minutes vs β. Line is fit to 
exponential model (Efflux = a*(1-e^{-\beta \beta}) + c, \quad r=0.99, \quad p=0.0001).

Figure 6. Effect of Transport Modulators on Corneal Thickness and Total Lactate Efflux. A, 
Corneal Thickness change vs. time showing effects of 100 μM Ouabain, 100 μM Acetazolamide, 
and 10 μM HY-13248 (AR-C155858). B, lactate content of effluent samples. C, Effect of 10μM 
Adenosine on corneal thickness and D, effect of Adenosine on Lactate efflux.

Figure 7. Summary of Corneal Thickness, Total Lactate Efflux & Retention. A, Comparison of 
GBR, BF (0 HEPES), acetazolamide-CA inhibition (ACTZ), MCT inhibition (HY), and 
adenosine perfusion on Corneal Thickness change between 90 and 300 minutes of perfusion. B, 
Comparison of Total Lactate Efflux between 120 and 300 minutes of perfusion. C, Comparison 
of Lactate Retention in the cornea at the end of each experiment. D, Regression analysis for all 
experiments (BF & BR perfusion, Ouabain, acetazolamide, HY-13248, and adenosine; ΔCT vs. 
Lactate Efflux, ΔCT=ae^{-\beta \beta} + c, \quad r=0.98, \quad p<0.0001). Symbols show mean±SD of each 
experiment.

Figure 8. Effect of Lactate in the Perfusion Ringer. A, Corneal Thickness change vs. time for 
5mM lactate and GBR control. Switched to lactate containing ringer at arrow.

Figure 9. Model for in vivo Corneal Endothelial Lactate and Water Flux. A, Cellular transport 
model: Basolateral MCT 1 & 4 provide lactate:H⁺ cotransport uptake facilitated by bicarbonate 
buffering (NBCe1 cotransport and CA2) and supported by the Na⁺/H⁺ exchanger, NHE1. Lactate 
efflux at the apical membrane is facilitated by CA4-bicarbonate buffering. Transcellular water
flux is facilitated by AQP1. B, Possible model for a transendothelial lactate standing gradient.

Schematic cornea cross-section showing lactate concentration gradient (red line), mean stromal [lactate] of 13 mM, and anterior chamber [lactate] of 7 mM. Expanded area below shows perturbation of lactate gradient in the basolateral space due to robust lactate: H\(^+\) uptake and in the apical unstirred layer due to lactate: H\(^+\) efflux, creating a transendothelial lactate gradient that drives water flux.