Published in final edited form as:

Exp Eye Res. 2009 April ; 88(4): 729–737. doi:10.1016/j.exer.2009.01.015.

The Washout Phenomenon in Aqueous Outflow - Why Does It Matter?

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Abstract

The washout effect is a phenomenon in which the resistance to aqueous outflow diminishes with the volume of perfusate flowing through the outflow pathways, even if the perfusate is aqueous humor itself. One intriguing aspect of this phenomenon is that it appears to occur in the eyes of all species studied to date except humans. Even non-human primate eyes exhibit washout.

Because washout does not occur in human eyes some have concluded that a greater understanding of this effect could not be relevant to the study of human primary open angle glaucoma. Those who have chosen to study this phenomenon realize that if a washout effect could be induced in the human eye, the result would be a reduction in outflow resistance and a drop in intraocular pressure – precisely the goal of all current therapy for open angle glaucoma.

This article reviews the discovery of this phenomenon, the various lines of investigation aimed at unraveling its underlying mechanisms. It concludes with recent structural and functional comparisons that point to clear differences in the connectivity between the inner wall (IW) endothelial cells of Schlemm's canal and matrix or cells in the juxtacanalicular connective tissue (JCT) between human eyes that do not exhibit washout and non-human eyes that do exhibit washout. This enhanced connectivity consisted of a more complex array of elastic fiber connections between the IW and JCT in human eyes. This enhanced connectivity may withstand the hydrodynamic forces driving separation between the IW and JCT, which occurs in non-human eyes during washout. Strategies targeting JCT/IW or JCT/JCT connectivity in human eyes might be promising anti-glaucoma therapies to decrease outflow resistance, and thus IOP.

Keywords

washout; aqueous outflow facility; outflow resistance; trabecular meshwork; Schlemm's canal; glaucoma

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Commercial Relationships: None

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What is the “washout” effect?

The “washout effect” describes a phenomenon observed when an *in vivo* or enucleated non-human eye is experimentally perfused. As perfusion continues the outflow facility of the eye progressively increases (Kaufman et al., 1988; Erickson-Lamy et al., 1990; Kaufman et al., 1988), even if perfused with aqueous humor (Gaasterland et al., 1978). Washout was originally thought to be time-dependent (Erickson and Kaufman, 1981; Kaufman et al., 1988), but more recent studies have documented that the effect is actually perfusion volume-dependent (Johnson et al., 1991; Sit et al., 1997a).

Washout was first recognized by Barany and Scotchbrook (1954), who attributed the increase in outflow facility to a “washing away” of extracellular material (ECM). In their pioneering perfusion studies, they perfused hyaluronidase into enucleated bovine eyes and when “washout” was observed, they naturally concluded that the substrate for this enzyme, hyaluronic acid, was the material being washed out and pointed to the importance of hyaluronic acid as an element of resistance in the aqueous outflow pathway (Barany and Scotchbrook, 1954; Barany and Woodin, 1955; Barany, 1962; Barany, 1964). Other investigators have supported this notion (Peterson and Jocson, 1974). However, more recent work, described below, casts doubt on this conclusion.

Only one species, human, does not exhibit washout

Washout has been reported in all non-human mammalian eyes studied to date, including bovine (Fig. 1), pig, rabbit, dog, cat and guinea pig (Barany, 1962; Barany, 1964; Epstein et al., 1982; Erickson-Lamy et al., 1988; Fourman and Fourman, 1989; Gaasterland et al., 1979; Hashimoto and Epstein, 1980; Melton and DeVille, 1960; Overby et al., 2002; Rao et al., 2001; Ruben et al., 1985; Van Buskirk and Brett, 1978; Yan et al., 1991) and even non-human primate eyes (Epstein et al., 1982; Erickson and Kaufman, 1981; Gaasterland et al., 1978; Gaasterland et al., 1979; Kaufman et al., 1988; Peterson and Jocson, 1974). Some species have a greater washout effect than others (Melton and DeVille, 1960). Age does not affect washout in either rhesus or cynomolgus monkey eyes (Kiland et al., 2005). Possibly the most intriguing aspect of the washout effect, however, is that it does not occur in the human eye (Fig. 1) (Erickson-Lamy et al., 1990; Scott et al., 2007). The absence of washout in enucleated human eyes is unlikely to be the result of postmortem changes. Organ-cultured anterior segments, enucleated eyes, and *in-vivo* monkey eyes undergo a similar magnitude of washout (Erickson-Lamy et al., 1990; Hu et al., 2006). Furthermore, since washout does not occur in the perfused human infant eye, the age of the donor cannot explain the difference in washout properties between human and other primate eyes (Erickson-Lamy et al., 1990). This being the case, some would argue that studies of washout are irrelevant to the human eye or to the pathogenesis of glaucoma in humans. But a thorough understanding of the mechanism of washout, and the reason for its absence in the human eye would likely provide important insight into the fundamental mechanisms that generate outflow resistance. Possibly most important is that by understanding washout we might be able to artificially induce a washout-like response in human eyes as a means of reducing intraocular pressure (IOP) in glaucoma.

The lack of washout in the human eye suggests that there is some unique aspect of outflow anatomy or physiology that distinguishes human eyes from most other species, including nonhuman primate eyes despite their anatomical similarity to humans.

Possible mechanism of washout

Several hypotheses have been put forward to explain the mechanisms governing the washout effect.

1. Washing out the ECM

Originally, washout was believed to be a “washing out” of glycosaminoglycans (GAGs), particularly hyaluronic acid, from the ECM in the outflow pathway (Barany and Scotchbrook, 1954; Barany and Woodin, 1955). However, washout has been shown to be a reversible process in both bovine and monkey eyes (Overby, 2002; Sabanay et al., 2004), and reversal occurred within 1–2 hours. This timeline is less than would be necessary for secretion and organization of significant quantities of ECM (Hascall et al., 1991). This finding, combined with the findings of Knepper et al. (1984) and Johnson et al. (1993) that neither hyaluronate nor sulfated proteoglycans were depleted from the outflow pathway during washout, challenges the argument that washout results from a simple loss of hyaluronidase-sensitive GAGs from the ECM in the outflow pathway during perfusion. Additional evidence against this hypothesis is that neither a decrease in IOP nor an increase in outflow facility was found in living cynomolgus monkey eyes after removing hyaluronate or chondroitin sulfate proteoglycans with either single or multiple intracameral injections of the GAG-degrading enzymes, hyaluronidase or chondroitinase (Hubbard et al., 1997). In this regard, it should be noted that the purity of the hyaluronidase available in the late 90’s is far superior to that available to Barany in the middle 50’s.

2. Washing out plasma-derived protein

Another hypothesis has been that “washout” occurs due to a washing out of a depot of anterior segment protein located at the root of the iris and supplied by the ciliary body (Barsotti et al., 1992; Freddo et al., 1990; Johnson et al., 1993). The expected concentration of plasma-derived protein in an aliquot of aqueous humor obtained from the anterior chamber is about 1% of that in plasma. But it has been shown recently that the pathway by which plasma-derived proteins are added to the aqueous humor is via diffusion from the ciliary body stroma to the iris root (Fig. 2). From here, these proteins enter the aqueous humor adjacent to the outflow pathways (Freddo et al., 1990; Barsotti et al., 1992; Bert et al., 2006). Modelling this pathway, it was predicted that the protein being added to the aqueous humor as it enters the outflow pathways could be the equivalent of 10% of that found in plasma (Freddo et al., 1990). Building upon this result, Johnson et al. (1993) were able to significantly reduce the rate of washout in the enucleated bovine eye by supplementing the perfusion fluid with 5–15% serum [protein concentration of approximately 6–10 mg (Sit et al., 1997b)]. Kee et al. (1996) have made a similar observation in the living primate eye using perfusion fluid augmented with 5% autologous serum. These studies suggested that soluble proteins in the aqueous humor may contribute to the outflow resistance. A decreased outflow resistance during the washout may be due to decreasing protein interactions with the ECM in the trabecular meshwork. Later, in a series of perfusion experiments conducted by Sit et al (Sit et al., 1997b) on both bovine and human eyes, the effluent was collected and assayed for protein content. The influence of two major proteins of aqueous humor, albumin and γ -globulin on the rate of washout in bovine eyes was also investigated. They found that the initial protein concentration of effluent in bovine eyes (1%) was much less than the 10–15% serum solution required by Johnson et al (1993) to prevent washout, and that the decay of the protein in bovine effluent decreased at a different rate than did outflow resistance. The rate of change of outflow facility was shown to be dependent on the volume perfused rather than the protein concentration. Furthermore, neither albumin nor γ -globulin, at protein concentrations similar to that of serum solution previously used by Johnson et al (1993), affected the washout rate. These findings suggested that this result was not simply the consequence of viscosity issues because the magnitude of the outflow resistance change during washout is much greater than the increase in viscosity between serum and albumin solutions of the same concentration. These studies suggested that the reduction of washout observed in previous studies when serum protein concentrations ranging from 5–15% of that found in whole serum were perfused through bovine and monkey eyes, was not due to the general levels of dominant serum proteins, i.e. albumin and γ -globulin.

Instead it was concluded that these results were more likely due to interactions of a particular protein(s) (Sit et al., 1997b). Of interest, human eyes, which do not exhibit washout, showed no statistically significant correlation between the volumes perfused and outflow resistance. In human eyes, a significantly higher level of initial protein concentration in the perfusate leaving the eye and a much longer decay time were found compared with that of the perfusate leaving bovine eyes treated in the same manner (Sit et al., 1997b). This was likely due to the lower flow rate through the human outflow pathways. These investigators concluded that the outflow facility would be controlled by a resistance causing mechanism other than the bulk level of aqueous humor proteins.

When microporous filters with pore sizes similar to those found within the juxtacanalicular connective tissue (JCT) were perfused with aqueous humor, they became progressively more resistive to flow (Johnson et al., 1986). This blockage occurred during perfusion with aqueous humor but not with a serum solution at the same protein concentration, suggesting a particular aqueous humor protein was responsible for it. Importantly, this resistance was not relieved when the system was treated with a GAGase, but was relieved when the system was treated with a protease. It would be interesting to determine whether the same protein(s) is responsible for both the reduction of washout effect in both bovine and monkey eyes and blockage of microporous filters. This line of research has led some investigators to probe whether it is small, perhaps hydrophobic, proteins acting like “fines” in a column that are the principal interactive proteins that contribute to impeding flow thus inhibition of washout (Russell et al., 1993).

Consistent with a possible role for proteins in the washout effect are the results of studies on postmortem, *in-situ* rabbit eyes by Ruben, et al (Ruben et al., 1985). These investigators demonstrated that the washout effect can be reduced dramatically by adding a serine protease inhibitor and antifibrinolytic agent epsilon-aminocaproic acid (EACA) to the perfusate. Although the precise mechanism of outflow stabilization by EACA in rabbit eyes remains unclear, using this protease inhibitor, protein levels might remain more stable and this would be consistent with the effect of proteins playing a role in reducing washout. In contrast to the rabbit, the same concentration of EACA does not inhibit the washout effect in cynomolgus monkey eyes (Menage et al., 1995). No further studies have been done to explain this difference.

3. Mechanical disruption of connectivity between the inner wall and JCT

Several studies have localized the primary site of outflow resistance to within the region near the JCT and the inner wall endothelium of SC (Grant, 1958; 1963; Maepea and Bill 1989; 1992). Morphological studies focus on the inner wall and JCT region have been conducted recently to correlate with increase outflow facility during washout.

a). Morphological correlates for increasing outflow facility during washout in nonhuman eyes

Studies in Bovine Eyes: Two studies in bovine eyes (Overby et al., 2002; Scott et al., 2007) have provided evidence suggesting that the structural correlate for the increase in outflow facility observed during washout is the degree of separation between the JCT and basal lamina of the inner wall endothelium of the aqueous plexus (bovine equivalent of Schlemm’s canal) (Fig. 3). A significant positive correlation was found between the extent of inner wall/JCT separation and the absolute value of outflow facility in bovine eyes (Fig. 4)

This separation was proposed to increase outflow facility by disrupting a hydrodynamic interaction between the inner wall and JCT known as a “funneling” effect (Johnson et al., 1992). The funneling theory states that the patterns of outflow through the JCT are confined to those regions nearest the pores in the inner wall, and this flow confinement reduces the

filtration area through the JCT, thereby increasing the outflow resistance (Fig 5A). Studies by both Overby et al (2002) and Scott et al (2007) suggested that washout might result from a disruption in the connectivity between the basal lamina of the inner wall endothelial cells and the matrix in the JCT, which decreased outflow resistance in the separated region by eliminating the funneling effect (Fig 5B).

This hypothesis and the funneling theory emphasize the role of cell and extracellular matrix adhesions, rather than soluble matrix in maintaining outflow resistance by altering the connectivity between the inner wall and JCT in the face of an opposing pressure gradient. Interference with this connectivity thereby influences outflow resistance by controlling the local hydrodynamic patterns of outflow (See more detailed review by Overby et al in this issue). Washout is a reversible process (Overby, 2002; Sabanay et al., 2004). The reversibility of washout was found correlated with a decrease in the separation between the inner wall endothelium and the JCT (Overby, 2002).

Studies in Monkey Eyes: Despite their greater anatomical similarity to human eyes than bovine eyes, monkey eyes also exhibit washout. As with bovine eyes, the JCT region undergoes distention or separation (McMenamin and Lee, 1986; Zhang et al, 2007). But in monkey eyes, the plane of separation most often occurs below the first layer of JCT cells that are attached directly to the inner wall (Figure 6). By comparison, in bovine eyes, separation is between the basal lamina of the inner wall and the JCT matrix (Figure 3). The possible reason might be that the connections between the first layer of JCT cells and basement membrane of IW cells may be stronger than the connection between the rest of the JCT cells in monkeys. The separation between the basal lamina of the inner wall and JCT as reported in bovine eyes (Overby et al, 2002; Scott et al, 2007) was also observed, which was usually near the collector channel ostia region where without the outer wall limitation, the inner wall cells had room to extend greatly. However, both these morphologic changes increased available area for aqueous outflow in the separated area resulting in an increase in outflow facility (Scott et al, 2007, Zhang et al 2008).

b). Morphological correlate for the lack of washout in human eyes—In human eyes, outflow facility was not increased during prolonged anterior chamber perfusion (Erickson-Lamy et al., 1990) or enucleated eye perfusion (Scott et al., 2007). No significant morphologic differences, specifically inner wall/JCT separation or expansion, were observed between long- and short-duration perfusion of human eyes (Fig. 7).

Morphologic analysis revealed that the previously described “cribriform plexus” of elastic-like fibers was far more extensive in the JCT of human eyes (Fig 7) compared to bovine eyes (Fig 3), appearing to form numerous connections to the inner wall endothelium. The cribriform plexus appears to function as a mechanical tether that maintains inner wall/JCT connectivity in human eyes by opposing hydrodynamic forces generated during perfusion. This alone could potentially explain the lack of washout in human eyes.

c). Similar morphological changes were found comparing washout and the effect of certain drugs that disrupt the cytoskeleton—Drugs that disrupt the cytoskeleton or cytoskeletal contractility have been shown to reduce aqueous humor outflow resistance and IOP. Common morphologic features reported after drug-induced facility increase include expansion or “ballooning” of the subendothelial ECM within the JCT, apparent separation between the JCT and the inner wall, and distension of the inner wall endothelium. These morphological changes were observed following Y-27632 perfusion in porcine and bovine eyes (Rao et al., 2001; Lu et al, 2008), H-7 (Sabanay et al., 2000, 2004) and latrunculin-B (Sabanay et al., 2006) in monkey eyes. These morphological changes are similar to that observed during “washout” in both bovine (Overby et al., 2002; Scott et al.,

2007) and monkey eyes (Zhang et al, 2008) (Figure 8). Although the separation between the inner wall and JCT was different between bovine (between the basal lamina of the inner wall and the extracellular matrix of the JCT, i.e. matrix-matrix separation) and monkey eyes (between the JCT cells, i.e. cell-cell separation or between JCT cell and matrix i.e. cell-matrix separation) after treatment with agents disrupting cytoskeleton and washout, both these morphologic changes increased outflow facility (Lu et al, 2007; 2008; Zhang et al, 2008). In short term (30 min) perfusion studies of the effect of Y27632 on three species (bovine, monkey, and human), an increase in outflow facility was found in both bovine and monkey but not in human eyes under a similar experimental condition (Lu et al 2007, 2008). However, previous studies in enucleated human eyes did show a facility increase following H-7 (37%, 100 μ M) (Bahler et al., 2004) or latrunculin-B (64%, 1 μ M) (Ethier et al., 2006) and demonstrated a diminished IW/JCT separation after long-term perfusion (more than 2 hours) compared to non-human primates that exhibit a 2 to 4-fold facility increase following perfusion with similar drug concentrations (Tian et al., 1999; Sabanay et al., 2000; Sabanay et al., 2004; Sabanay et al., 2006). These differences may be related to the finding that human eyes do not exhibit washout induced outflow facility increase and do not exhibit inner wall/JCT separation after prolonged perfusion (Erickson-Lamy et al., 1990; Scott et al., 2007). These results suggest an enhanced connectivity between the IW and JCT in human eyes, which may withstand the hydrodynamic forces driving separation between the IW and JCT. A more complex array of elastic fiber connection (cribriform plexus) may be responsible for the enhanced connectivity between the IW and JCT in human eyes. Therefore, strategies targeting JCT/IW or JCT/JCT connectivity in human eyes may be promising anti-glaucoma therapies to decrease outflow resistance, thus IOP.

d). Positive correlation between the inner wall separation and available area for aqueous outflow labeled by fluorescent tracer—Recently, the relationship between effective filtration length and related changes in the morphology and outflow facility after washout and Y27632 treatment was investigated in enucleated bovine and monkey eyes. To identify changes in outflow patterns, fluorescent microspheres were perfused to outline aqueous outflow patterns, followed by light and electron microscopic examination on the same tissues (Lu et al., 2007; Lu et al., 2008; Zhang et al., 2008). A significant positive correlation was found between percent effective filtration length and percent separation length in both washout and Y27632 treated eyes (Figs. 9, 10), suggesting that as connectivity between the JCT and IW decreases the available area for aqueous humor drainage increases.

These findings support the hypothesis that an increase in the outflow facility during washout is associated with an increase in the effective filtration area for aqueous outflow, which is regulated by a loss of the connectivity between the inner wall and JCT.

4. Possible Mechanisms involved in the inner wall/JCT separation

Cytoskeleton disrupting agent, Y-27632, is a protein kinase inhibitor selective for Rho associated kinase (ROCK) (Davies et al., 2000; Ishizaki et al., 2001; Uehata et al., 1997), which regulate the phosphorylation of the regulatory myosin light chain (MLC) to promote actomyosin-driven cell contractility. Inhibiting ROCK with Y-27632 decreases MLC phosphorylation by promoting MLC-phosphatase activity (Kaibuchi et al., 1999; Rosenthal et al., 2005), leading to cell relaxation and disassembly of actin stress fibers and focal adhesions in many cell types (Rao et al., 2001), including human trabecular meshwork (TM) and Schlemm's canal endothelial cells in vitro (Honjo et al., 2001; Rao et al., 2001). H-7, a serine-threonine kinase inhibitor, induced cellular relaxation in the TM. This effect was primarily attributed to its MLC kinase inhibition (Volberg et al., 1994; Tian et al., 1998). However, in the absence of an obligatory change in the concentration of intracellular Ca^{2+} , smooth muscle contraction can be enhanced by G-protein-mediated Ca^{2+} sensitization, in which ROCK, a

member of serine/threonine kinase family (Leung et al., 1995), plays a key role (Kureishi et al., 1998; Iizuka et al., 1999). Therefore, it is also hypothesized that H-7-induced cellular relaxation in the TM may be partially related to its Rho kinase inhibition (Tian et al., 1998). Latrunculins, macrolides isolated from the marine sponge *Latrunculia magnifica*, are specific and potent actin-disrupting agents that sequester monomeric G-actin, leading to the disassembly of actin filaments. (Coue et al., 1987; Lyubimova et al., 1997). The two most common latrunculins, latrunculin (LAT)-A and -B, cause reversible dose- and incubation time-dependent destruction of actin bundles and associated proteins in several types of cultured cells, including HTM cells (Coue et al., 1987; Epstein et al., 1999; Lyubimova et al., 1997; Peterson et al., 1999; Spector et al., 1989; Spector et al., 1983). In living monkeys, both LAT-A and LAT-B increase outflow facility by up to fourfold, probably by disrupting the actin cytoskeleton in TM cells, in turn relaxing the TM and separating cell-cell and cell-extracellular matrix adherens junctions within it (Peterson et al., 1999; 2000).

It is clear that certain drugs that disrupt the cytoskeleton or cytoskeletal contractility appear to cause morphological changes of inner wall/JCT separation similar to those resulting from washout. However, washout induced outflow facility increase takes a longer time and depends on the volume perfused. Decreased outflow resistance in the separated area and increase in the available area for outflow may account for the increase in outflow facility induced by either washout or the drugs that disrupt the cytoskeleton or cytoskeletal contractility. Whether they act through similar or different pathways remains unknown but clearly it is possible to induce changes that mimic the effects of washout.

Summary

In summary, the washout effect appears to be less the result of washout of a soluble matrix than to identifiable changes in the JCT and inner wall connectivity. Whether certain soluble proteins participate in initiating these changes remains to be established. Based upon studies using various chemical agents known to disrupt the cytoskeleton or cytoskeletal contractility thus alter cell-cell and cell-matrix connectivity, it would appear that their effect of increasing outflow facility occurs through a mechanism similar to that found for washout but in a shorter time. Further studies are needed to determine whether washout is regulated by a specific pathway(s). As we begin to understand the mechanisms underlying washout, the possibility of inducing washout in eyes with POAG, as a means to reducing IOP, draws closer.

Acknowledgments

Grant Support: National Glaucoma Research, a program of the American Health Assistance Foundation, NIH EY-09699 and The Massachusetts Lions Eye Research Fund, Inc.

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Figure 1. “Washout” in bovine eyes but not in humans

Anterior segments from human eyes ($n = 13$) and neonatal calf eyes ($n = 9$) were perfused at 15 mmHg with Dulbecco’s modified eagle medium (DMEM) in a 5% CO_2 environment at 37° C. A progressive increase in facility of outflow over baseline facility (%CO) with time was noted in the calf eyes but not in the human eyes. *Erickson-Lamy et al: Absence of time-dependent facility increase (“washout”) in the perfused enucleated human eye. Invest Ophthalmol Vis Sci 31:2384–2388, 1990*

Figure 2. The principal route of entry for plasma-derived proteins into the aqueous humor of the normal eye

Note the route of passage delivers protein in close proximity to the trabecular meshwork and aqueous outflow pathway. Morrison, J. C. and Freddo, T. F. (1996). *Anatomy, microcirculation and ultrastructure of the ciliary body*. In: *The Glaucomas*. 2nd ed. Ritch, R., Shields, B and Krupin, T., eds., Mosby/W.B. Saunders, St. Louis, MO., U.S.A., Cptr. 6, p. 137.

Figure 3. Morphologic comparison between long- and short-duration perfusion in bovine eyes

A. Transmission Electron Micrograph of Bovine Aqueous Plexus and underlying JCT region after Long-Duration Perfusion Significant separation between the basal lamina (BL) of the inner wall (IW) of the bovine aqueous plexus (AP) and underlying extracellular matrix of the JCT region is observed (double headed arrow). Scale bar: 5μm

B. Transmission Electron Micrograph of Bovine Aqueous Plexus and underlying JCT region after Short-Duration Perfusion No distention of the JCT or separation of the IW lining of the aqueous plexus (AP) was observed. Giant vacuoles (GV) were seen along the inner wall. Scale bar: 5μm (Scott PA, Overby DR, Fredde TF, Gong H: Comparative studies between species that do and do not exhibit the washout effect. *Exp. Eye Res*, 84:435–443, 2007)

Figure 4. Correlation of Absolute Value of Pre-Fixation Facility and JCT/Inner Wall Separation Scores

The absolute value of outflow facility measured prior to fixation correlated with the extent of inner wall/JCT separation in all bovine eyes ($p=0.0024$). Closed circles = long-duration, open circles = short-duration. (Scott PA, Overby DR, Freddo TF, Gong H: *Comparative studies between species that do and do not exhibit the washout effect. Exp. Eye Res*, 84:435–443, 2007.)

Figure 5. An illustration of the streamlines of aqueous flow through the JCT before (A) and after (B) washout, as predicted by the funneling hypothesis

A. To enter Schlemm's canal, aqueous must converge to pass through discrete openings along the inner wall, which decreases the available area for flow and increases outflow resistance.

B. Separation of the inner wall from the JCT eliminates the funneling pattern, increases the available area for flow, and increases outflow facility during washout. (Overby D, Gong H, Freddo TF and Johnson M: *The mechanism of increasing outflow facility during washout in the bovine eye. Invest Ophthalmol Vis Sci.* 43:3455–3464, 2002.)

Figure 6. Morphologic change in monkey eyes during washout

Separation between the JCT cells and the JCT cells and matrix were often observed (double arrows). This micrograph was from a tracer study, microspheres were seen in the JCT (*). (From Dr. Haiyan Gong)

Figure 7. Morphologic comparison between long and short during perfusion in human eyes
A. Transmission electron micrograph of human Schlemm's canal and underlying JCT region after short-duration perfusion. **B.** Transmission electron micrograph of human Schlemm's canal and underlying JCT region after long-duration perfusion. No separation is observed between the inner wall (IW) of Schlemm's canal (SC) and the underlying JCT in both short- and long-duration perfusion (A and B, respectively). Giant vacuoles (GV) are seen along the IW of SC with a similar distribution in both short and long-duration perfusion. Compared to bovine eyes (Fig. 3A and B) there is a more complex array of elastic fibers (EL) in the JCT region (single headed arrows), especially in the area immediately underneath the inner wall (IW). Scale bars: 3 μm. (Scott PA, Overby DR, Freddo TF, Gong H: *Comparative studies between species that do and do not exhibit the washout effect. Exp. Eye Res*, 84:435–443, 2007.)

Figure 8. Morphological comparison between washout effect and the effect of certain drugs that disrupt the cytoskeleton

Similar morphological changes were observed between washout (A) and Y27632 treatment (B) in bovine eyes and between washout (C) and latrunculin-B treatment (D) in monkey eyes. In A and B, separations were seen between the inner wall and JCT (double arrow). In C and D, separations between the cell-cell and cell and matrix were seen in the JCT regions (double arrow). SC, Schlemm's canal; IW, inner wall; JXT (JCT), juxtacanalicular region; OW, outer wall; P, SUB, sub-canalicular cells

(A from Overby D, Gong H, Freddo TF and Johnson M: *The mechanism of increasing outflow facility during washout in the bovine eye. Invest Ophthalmol Vis Sci.* 43:3455–3464, 2002; B from Lu Z, Overby DR, Scott PA, Freddo TF, Gong H: *The mechanism of increasing outflow facility by rho-kinase inhibition with Y-27632 in bovine eyes. Exp Eye Res*, 86: 271–281, 2008; C from Dr. Haiyan Gong; and D from Sabanay, I., B. Tian, B.T. Gabelt, B. Geiger, and P.L. Kaufman. 2006. *Latrunculin B effects on trabecular meshwork and corneal endothelial morphology in monkeys. Exp Eye Res.* 82:236–46).

Figure 9. Confocal (A, B) and Light microscopic (C, D) analysis

A. In a control eye, a segmental distribution of tracers (pink color) was seen along the inner wall of aqueous plexus (AP). B. In an Y27632-treated eye, the tracer distribution (pink color) was more uniform along the inner wall of AP. C. No separation was found between the inner wall of aqueous plexus (AP) and juxtacanalicular connective tissue (JCT) in a control eye. D. Separation was found between the inner wall of AP and JCT (double arrow) in an Y27632-treated eye. TM = trabecular meshwork. (Modified from Lu Z, Overby DR, Scott PA, Freddo TF, Gong H: The mechanism of increasing outflow facility by rho-kinase inhibition with Y-27632 in bovine eyes. *Exp Eye Res*, 86: 271–281, 2008)

Figure 10. Correlation between percent separation length (PSL) and percent effective filtration length (PEFL)

Plotting PSL versus PEFL for each eye revealed two populations corresponding to the control and Y27632-treated eyes, and both populations fall approximately along the line of unit slope indicating a one-to-one correspondence between two variables. (Lu Z, Overby DR, Scott PA, Freddo TF, Gong H: *The mechanism of increasing outflow facility by rho-kinase inhibition with Y-27632 in bovine eyes. Exp Eye Res, 86: 271–281, 2008*)