Mechanisms for sensing increase in intraocular pressure by corneal endothelial cells.

1. Introduction

The cornea is the outermost part of the eye which is transparent and functions as a lens that focuses incident light onto the retina of the eye (Figure 1a). The cornea is a tissue of structural complexity and is made of five distinct layers, where each of them contributes to the maintenance of corneal transparency. From anterior to posterior (Figure 1b), the human corneal tissue consists of the epithelium, bowman's layer, stroma, Descemet's membrane and the endothelium[1].

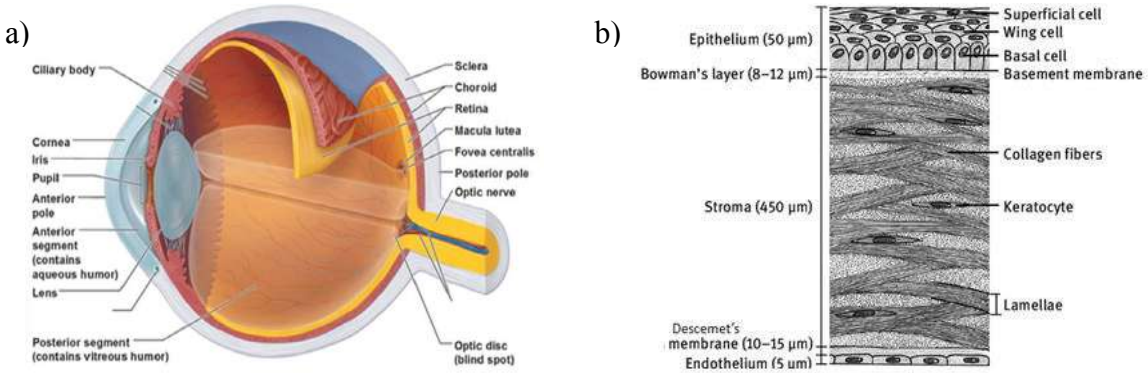

Fig. 1a: Cross-sectional representation of the human eye depicting the various parts of the eyeball (Shephard and Akay Optometrists) **1b:** Cross-sectional representation of the human cornea showing the five major layers of the cornea and their primary composition [2].

The role of the endothelium, a layer of hexagonal cells, lining the posterior cornea is two-fold: control of hydration (also called as stromal deturgescence) which it achieves by functioning as a barrier to excess fluid inflow into the stroma balanced by its active pump mechanism that moves ions which is responsible for drawing water osmotically from the stroma into the aqueous humor. The second important function of these cells is to allow nutrients to reach the avascular cornea through its leaky barrier that is permeable to nutrients from the aqueous humor. The leaky nature of the endothelial barrier is attributed to the presence of incomplete zonula occludens between adjacent cells in the endothelium. If the corneal endothelium (CE) loses its integrity and barrier function, it leaves more gaps in the monolayer leading to excess hydration, and thereby results in corneal edema/swelling [3-6].

The nutrition to the avascular cornea is supplied by the circulating fluid called the aqueous

humor that fills the anterior chamber. The outflow of the fluid from the eye is regulated by the trabecular meshwork tissue that adjoins the CE (Figure 2a). A block in the drainage of aqueous humor by the trabecular meshwork leads to the accumulation of the fluid in the anterior chamber thus increasing the pressure (intraocular pressure or IOP) within the eye (Figure 2b). The increase in pressure is shown to negatively impact the nerve cells of the retina that carry visual information from the eye to the brain resulting in vision loss and the development of a neurodegenerative condition called glaucoma (Figure 3). While the impact of increased pressure on the retinal cells is known, its impact on the neighboring cells, the corneal endothelium, is not well known.

The increase in IOP can be acute (e.g. acute angle-closure glaucoma), wherein the pressure can increase up to 70mmHg (normal population average being 15mmHg) within a short period of time. Clinically, the patients will present with pain, redness, blurred vision and nausea. The IOP is usually brought under control quickly using medications or surgical interventions and so the pressure increase lasts only for a short period of time. In another type of glaucoma, namely primary open angle glaucoma (POAG), the pressure increase is gradual and usually remains in the range of 30-40mmHg. Patients are typically asymptomatic; the pressure increase is long-standing and it is only during the later stages of the disease that treatment is sought due to loss of peripheral visual field. In both these conditions, there are reports of CE cell loss [7, 8] however, the corneal transparency is maintained until much later in the disease process.

Prior studies have shown that in patients suffering from acute increase in IOP, the corneal endothelial cells undergo morphological changes like cell enlargement accompanied by decline in endothelial cell density [8]. A similar response of the corneal endothelium was reported in Sprague-Dawley rats where the cells initially presented a disrupted morphology under acute increase in pressure, which reverted back to its native form in its absence [9]. While there is some preliminary evidence that increased intraocular pressure affects functionality and morphology of CE cells [10], the mechanisms through which these cells identify the pressure increase or adapt to the same such that they continue to maintain the corneal transparency remains unknown.

a) b)

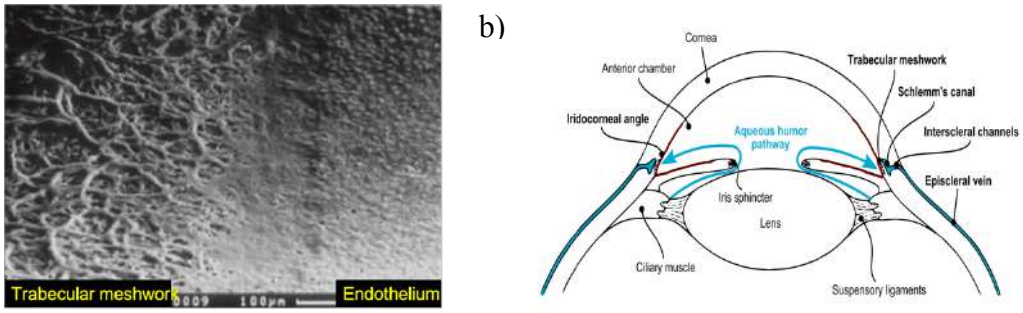

Figure 2: a) Shwalbe's line separating the endothelium and the trabecular meshwork [Anatomy of the angle by Wallace L M Alward MD and Reid A Longmuir MD] b) Aqueous humour (AH) outflow pathway (Blue – AH outflow pathway, Red – iridiocorneal angle) [11].

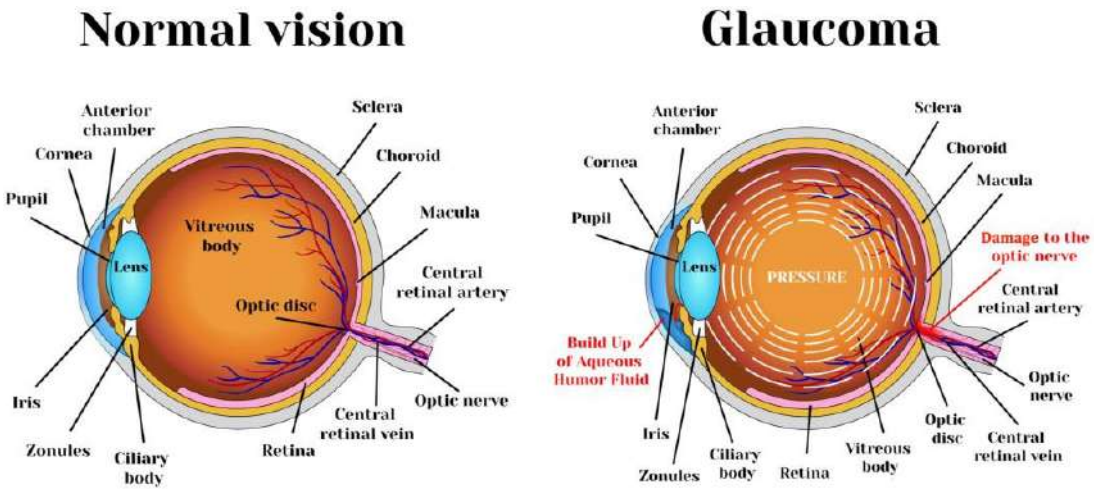

Fig. 3: Graphical representation of elevated IOP in the human eye (InMed Pharmaceuticals https://www.inmedpharma.com/learn/what-is-glaucoma/).

Transient receptor potential (TRP) channels are a group of channels involved in calcium signaling and are usually localized to the plasma membrane of various cell types. These channels are considered to be potential mechanosensors in relaying many transduction pathways. These ion channels are broadly classified into two major groups: Group 1 and Group 2. The subtypes in Group 1 are the TRPC (canonical), TRPV (vallinoid), TRPVL (vallinoid-like), TRPM (melastatin), TRPS (soromelastatin), TRPN (no mechanoreceptor potential) and TRPA (ankyrin). TRPP (polycystic) and TRPML (mucolipin) are included in Group 2. The subtypes in Group 1 are the TRPC (canonical), TRPV (vallinoid), TRPVL (vallinoid-like), TRPM (melastatin), TRPS (soromelastatin), TRPN (no mechanoreceptor potential) and TRPA (ankyrin). TRPP (polycystic) and TRPML (mucolipin) are included in Group 2. The presence of a few of these channels in the corneal endothelium have been reported

[12-17], however the need for characterization of these channels in the corneal endothelium and their involvement in sensing increased IOP remains to be explored. Therefore, the aim of the proposal is to understand the role of TRPV channels in sensing an increase in intraocular pressure by the corneal endothelial cells.

2. Literature Review

Damage to the corneal endothelium is permanent because corneal endothelial cells (CECs) have a no proliferating potential in vivo [18, 19]. The age related CEC loss can be exaggerated by intraocular surgery, trauma, or illnesses such as diabetes and glaucoma [20-25]. When the pump and barrier function of the endothelium is lost, decompensation of the endothelium is observed due to which the neighboring cells spread and/or migrate to compensate for cell loss in the early stages of endothelium injury, resulting in an increase in cell size and/or a change in cell shape. In POAG patients, a significant decrease in endothelial cell density (ECD - 2757±262 cells/mm^2) was observed when compared to healthy eyes which was used as a control (ECD - 2959±236 cells/mm^2) and average cell size was significantly greater (364±34.05 µm^2) than the control group (322.23±54.69 µm^2) [7]. In another study, the authors assessed three different categories of acute angle closure glaucoma (PACG) i) *sub-acute cases*: the patient on at least three occasions, had a shallow anterior chamber with intraocular pressure (IOP) less than 21 mmHg and no visual field changes were experienced. ii) *acute PACG:* where the patients presented with pain, corneal edema, decreased visual acuity with IOP greater than 40mmHg and iii) *chronic IOP:* where on at least three occasions the patients IOP was greater than 21mmHg resulting in optic nerve head changes with the presence or absence of visual field changes. The ECD in sub-acute cases had a mean of 2396±271 cells/mm^2, acute cases 1597±693 cells/mm^2, chronic cases 2229±655 cells/mm^2 which was significantly lower than the control group 2461±321 cells/mm^2. The mean of the central thickness of the cornea was measured which was highest in acute cases: 567.9±37.3 µm, followed by sub-acute cases: 531.4±25.3 µm, chronic cases: 526.4±31.9 µm against the control: 525.0±12.6 µm [26]. This data proves that an acute increase in IOP causes significant morphological changes in CECs and resulting in decreased ECD and increased corneal edema, however, in chronic cases of IOP, there is an increase in cell size, a decrease in ECD but no corneal edema. These significant morphological changes drive us to ponder upon the mechanisms involved in sensing an increase in IOP.

When there is a gradual build in IOP in POAG patients, it is known that there is a decrease in CEC density but that does not necessarily lead to decompensation of the endothelium [10]. In acute cases, a study conducted in monkey eyes, revealed that an increase in IOP (33-44 mmHg for 3-7 hours) caused significant morphological changes like flattening of cells, blebbing of cellular surface, intracellular vacuolisations, nuclear expulsion (excaryocytosis) and complete endothelial disorganization or disappearance of the corneal endothelium [27]. Whereas, in yet another study conducted in albino rabbits, the corneal endothelium was observed under SEM and revealed small opacities at 1-2 hours, which consisted of nuclear expulsion, cell rupture and endothelial disintegration. Mitochondrial and ER swelling were observed in thin sections, pointing towards damage of vital metabolic processes. At 3-4 hours, corneal opacification which led to diffuse oedema was seen along with the defects mentioned earlier [28]. It was reported that acute ocular hypertension (AOH) severely disrupted zonula occludens-1 (ZO-1) and F-actin which made the endothelial layer leakier and disrupted the cell morphology respectively [9]. These findings demonstrate a suggestive proof that an increase in IOP affects the corneal endothelium resulting in the initial morphological changes exhibited by the CECs.

A group of ion channels that are commonly located on the plasma membrane of animal cells called as Transient Receptor Potential channels (TRP channels) are thought to be of importance in relaying mechanical signals. They play a vital role in recognition of sensory stimulus and its role in mechanotransduction has been extensively researched over the years. TRP channels have proven to be essential in various ocular tissues for health maintenance and visual processing [29]. These channels are responsible for sensing the stresses in the environment and convert them into signalling cascades which control the various adaptive responses of cells that are needed during such challenges [29]. TRP channels get activated by stretch-induced membrane tension, actinomyosin- induced tension and mechanosensitive complex proteins may affect the opening of these channels [30]. TRPV 1-4 expression was reported in human corneal endothelium cell line by RT-PCR and immunofluorescence studies. The authors have also revealed their involvement in calcium signaling and studied their potential thermosensitive roles in the hCECs. The osmosensory function of TRPV4 was identified in human CE cells as intracellular Ca^{+2} ion-channel and TRP-like currents in these cells increased when exposed to a hypotonic

environment [15, 16]. The TRPM2 channel expressed putatively in the corneal endothelium is activated by oxidative stress while TRPM8 and TRPV-3 expression are linked to their thermosensitive role [12-15, 17]. Further, in the trabecular meshwork cells, studies in cases of intraocular pressure have reported that TRPV4 is activated by mechanical stress (e.g., pressure, swelling, and tissue distension) and the formation of arachidonic acid (AA), which is a substrate for cytochrome P450 leads to the production of eicosanoid metabolites (EETs), which are TRPV4's ultimate activators. TRPV4 activation might also be aided by TRPV1 channels and/or cell swelling, both of which are mediated by aquaporin 1 (AQP1) channels (Figure 4) [31-34]. In another study, impaired activity of the TRPV4-eNOS (Nitric oxide is known to modulate the cell morphology [35-37]) signaling was observed in glaucomatous patients suggesting that the disruptive activity of the TRPV4 channel is involved in the dysfunction of the trabecular meshwork cells and increased IOP in glaucoma [38]. While the involvement of TRPV channels has been reported in the trabecular meshwork cells, understanding the role of TRPV channels in the corneal endothelium, will enable us to understand their contribution towards sensing an increase in IOP in cases of glaucoma.

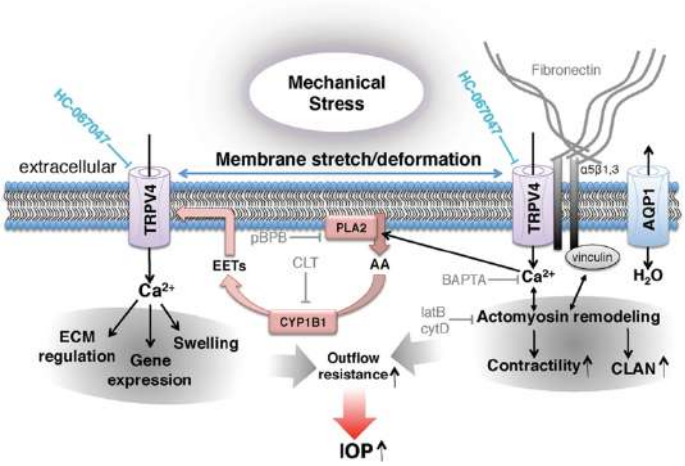

Fig. 4: Model for trabecular meshwork signaling in response to mechanical stress [31].

3. Gaps identified

a) The response of CE cells to increased pressure is a topic that has been scarcely researched.

b) The distribution and characterisation of TRP channels in the human endothelial cells

have been poorly understood.

c) The role of TRP channels in the CECs and the downstream signalling in response to increased pressure has not been reported much.

4. Objectives of the study

Hypothesis: Based on existing literature, we hypothesize that the TRP channels function as mechanosensors in CE cells and aid in regulating the cells response to increased IOP.

The main objective of the study is to determine the role of the TRP channels in regulating the response of the CE cells to increased pressure and the underlying molecular mechanisms involved in the same.

Rationale of the study: Corneal endothelial cells (CECs) have a limited proliferative capability in vivo [23, 24], hence damage to the endothelium is irreversible. Intraocular surgery, trauma, and diseases like diabetes and glaucoma can also result in CEC loss [25-30]. In the early stages of endothelium damage, neighboring cells spread and/or migrate to compensate for cell loss, resulting in cell size and/or shape changes**.** Increase in IOP is known to cause a definite CE cell loss. However, the cell loss does not always cause endothelial decompensation which would result in corneal edema. And based on the available information in the literature, the CE cells seem to respond differently to an acute increase versus the chronic increase in IOP. This indicates that the CE cells adapt to the prolonged presence of the stress, in this case increased IOP. Therefore, understanding the machinery that helps the cells identify an increase in the IOP and determining the molecular mechanisms that regulate the cell response would serve two purposes: 1) understand a very interesting and basic aspect of the CE cell biology and 2) help with developing potential molecules that can help reduce the cell loss in cases of elevated intraocular pressure.

Objective 1: To characterize the expression of TRP channels in corneal endothelial cells.

Objective 2: To determine the short-term and long-term effect of increased IOP on cell morphology and function.

Objective 3: To determine the role of TRPV channels in the remodeling of actin

cytoskeleton in the corneal endothelial cells in response to increased pressure.

5. Detailed methodology

Objective 1: To characterize the expression of TRP channels in corneal endothelial cells.

The expression of and TRP channels was assessed using PCR, immunostaining and western blot in fresh cells and cultured CE cells of humans and rats as detailed in the methodology below. Establishing the basal expression levels and patterns of TRP channels in the CE cells is necessary for conducting all further experiments.

Methodology:

Donor corneas were obtained from Ramayamma International Eye Bank (RIEB), Hyderabad, India. Donor tissues unsuitable for transplantation will be used for experiments and the following criteria will be applicable to the tissues:

- Cell count 2000 cells/mm^2 and above
- Tissues free of infections or scarring
- Paired corneas where applicable to reduce biological variability

Descemet's Membrane (DM) with intact endothelium layer was dissected under stereo microscope. Briefly, DM with the cells were peeled and allowed to recover overnight in medium (OptiMEM- Invitrogen, Grand Island, US) containing 8% fetal bovine serum. On recovery the peels were either fixed in formaldehyde for immunostaining or kept in Trizol for RNA isolation. For culture, recovered cells were collagenase (1mg/ml) treated for 2-3 hours at 37ºC. Followed by collagenase treatment, the cells were treated with 0.25% trypsin-EDTA to obtain single cell suspension. At confluence the cells were sub-cultured using 0.25% trypsin-EDTA where cells in passage 1 were used either for immunostaining or RNA isolation.

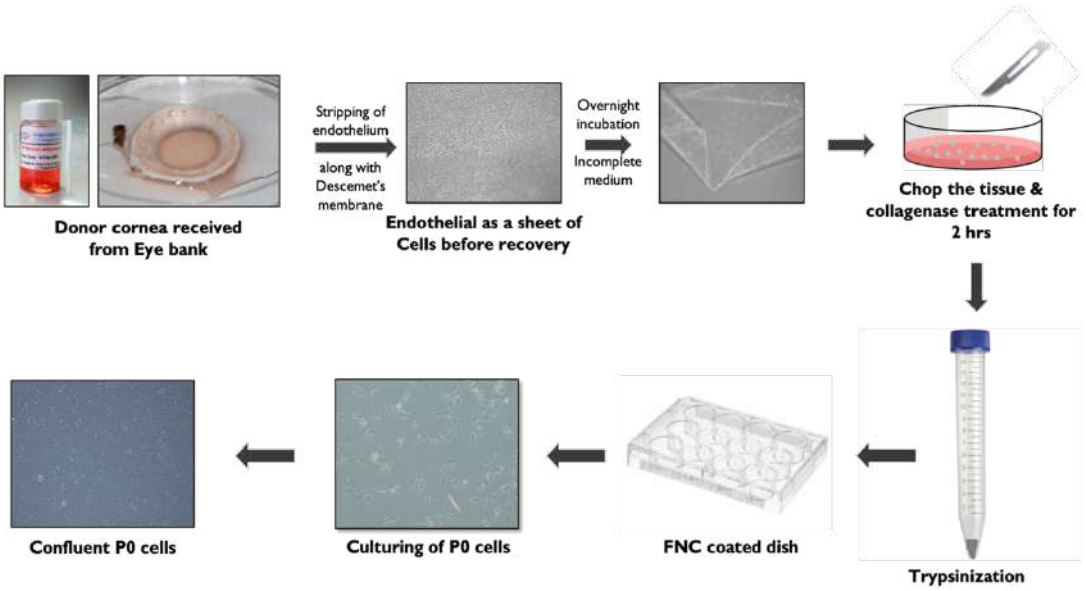

Fig. 1: *Schematic representation of culturing for primary cells.*

a) RT PCR: Expression of TRP channels in the corneal endothelium was studied where RNA was isolated from fresh tissue and primary cells using Trizol method. The RNA concentration was determined using nanodrop and 1ug of RNA was converted to cDNA using Superscript III kit (Invitrogen, Grand Island, US). 50ng of cDNA was used to screen TRP channels in fresh tissue and cultured cells (P1 cells).

b) Immunofluorescence: Staining was performed to understand localization and the expression of TRP channels in corneal endothelial cells. The Descemet's membrane and the corneal endothelium complex were peeled and left for recovery overnight in Opti-MEM. The tissues were fixed with 4% formaldehyde followed by permeabilization by using 0.1% Triton X for 5 minutes. Blocking was done with 2.5% BSA for one hour. Primary antibodies were diluted in 1% BSA and incubated at 4°C overnight. The tissues were washed with PBS followed by incubation with their respective secondary antibodies (1:500 dilution). Finally, the tissues were counterstained with DAPI for 10 minutes to stain the nucleus and mounted using glycerol.

c) Western blot: TRP channels were detected using their specific antibodies. The proteins were extracted from P1 cells using RIPA buffer with protease inhibitor and heated using beta mercaptoethanol. The samples were then loaded onto the polyacrylamide gel and transferred from the gel onto a PDVF membrane. The membrane was blocked using 5% non-fat milk, incubated in primary antibody overnight at 4 ºC and washed twice (trys buffered saline, TBS

or phosphate buffered saline, PBS with 0.1 percent tween 20) followed by incubation with appropriate secondary antibodies. The membrane was washed and developed by chemiluminescence.

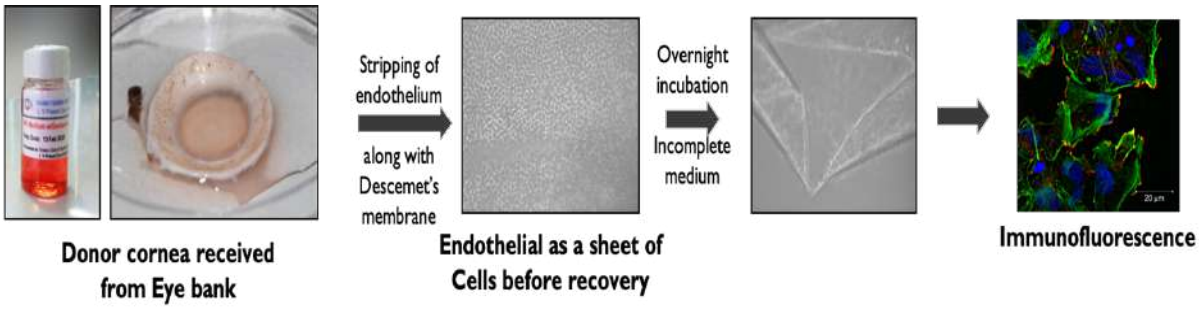

Fig. 2: *Flowchart of immunostaining for fresh tissue.*

d) Fraction separation of protein and western blot analyses: Confluent cell monolayers grown on 35mm tissue culture plates were solubilized for 10 minutes on ice in 0.5% Triton X in 1X PBS, after which it was collected and added to 4X Laemmli sample buffer. This constitutes the soluble (cytoplasmic) fraction of the protein isolated. The insoluble (membrane) material was resuspended in 4X Laemmli sample buffer with the help of a cell scraper. For each fraction 30µl of protein sample were loaded on 4% stacking gel and 10% running SDS-PAGE gel.

e) Image analysis by ImageJ for colocalization: After imaging, the images were processed and converted into tiff form using Zen software. Specific regions of interest (ROIs) were chosen having the membrane proteins as references to look for colocalization of the TRPV channels with the membrane proteins using the polygon tracing tool and analyzed with the help of the JACoP plugin and the corresponding scatterplot was plotted with the use of ScatterJn_v1_0 plugin.

f) Live cell calcium imaging: The human (HCECs) were seeded on 25mm coverslips for all live-cell experiments. After 36 hours, the cells were loaded with Ca^{+2} sensitive dye – Fluo-4 AM (Invitrogen) and incubated for 30 minutes at 37^{o}C. Imaging was done at 60X using an Olympus confocal microscope and images were acquired at every 1.08 seconds. The drugs (agonists and antagonists of TRPV1 and TRPV4) were added at frame 20. Images obtained were processed and analyzed by using Fiji (Image J) software and the corresponding graph was plotted using Graphpad Prism 9 software.

6. Results

Expression of TRP channels at the gene level in human corneal endothelial cells:

The transient receptor potential channels are of 28 types that are expressed in mammals which are divided into six families. They are of the following types: TRPV1-6 (vanilloid), TRPM 1-8 (melastatin), TRPC 1-7 (canonical), TRPP 1-3 (polycystic), TRPML 1-3 (mucolipin) and TRPA1 (ankyrin). While many of them are found in various cell types, our aim was to evaluate the presence of these channels in the corneal endothelial cells. TRPV1-6, TRPM1-8, TRPC1-6, TRPML 1-3, TRPP1-3 and TRPA1 channels were screened out of which TRPV1-4, TRPV6, TRPM7, TRPM8, TRPC1, TRPP1, TRPP3 and TRPML1 were positive in fresh tissue (Figure 3 A, C, E) and TRPV1-4, TRPV6, TRPM1, TRPM3, TRPM7, TRPM8, TRPC1 and TRPML1-3 were positive in cultured cells (Figure 3 B, D, F) which were confirmed by Sanger sequencing. We observed a positive expression of a greater number of TRP channels in primary cells and not in fresh tissue due to environmental cues including, secreted proteins, temperature, and oxygen which are examples of cell-extrinsic variables that control expression. As we are adding serum (whose constituents are not clearly defined), growth factors and antibiotics to our culture media, and maintaining it at 37ºC it is very likely that these conditions may be responsible for the expression of these channels in primary cells and not fresh tissue.

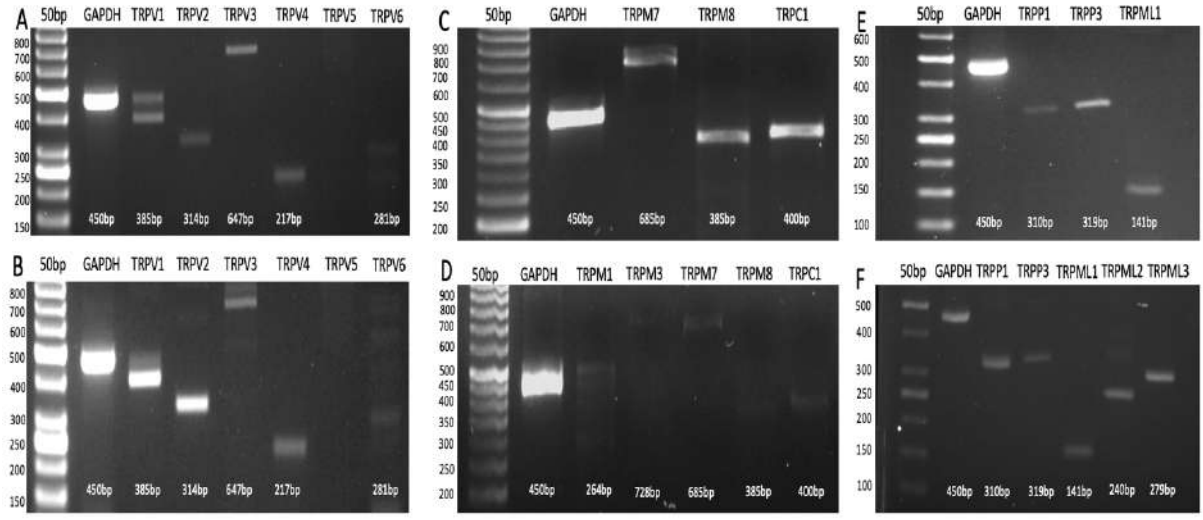

Fig. 3: *Representative image of human corneal endothelial cells expressing TRP channels in fresh tissues (A,C,E) and cultured cells (B,D,F).*

Protein expression of TRPV1-4 and TRPV6 channels in HCECs

A positive expression of TRPV1- 4 and TRPV6 proteins was noted in fresh tissues and primary cells by immunostaining, where a cytoplasmic expression of TRPV1, TRPV4 and TRVP6 (Figure 2A-a,d,e) and membrane expression of TRPV2 and TRPV3 (Figure 2A-b,c) were observed in fresh tissues, while in cultured cells, all the channels were localized to the cytoplasm with some proportion of it being expressed along the membrane (Figure 2B, f-j). Similarly, a positive expression of these channels was observed by western blot analyses where distinct bands were observed for each of the channels.

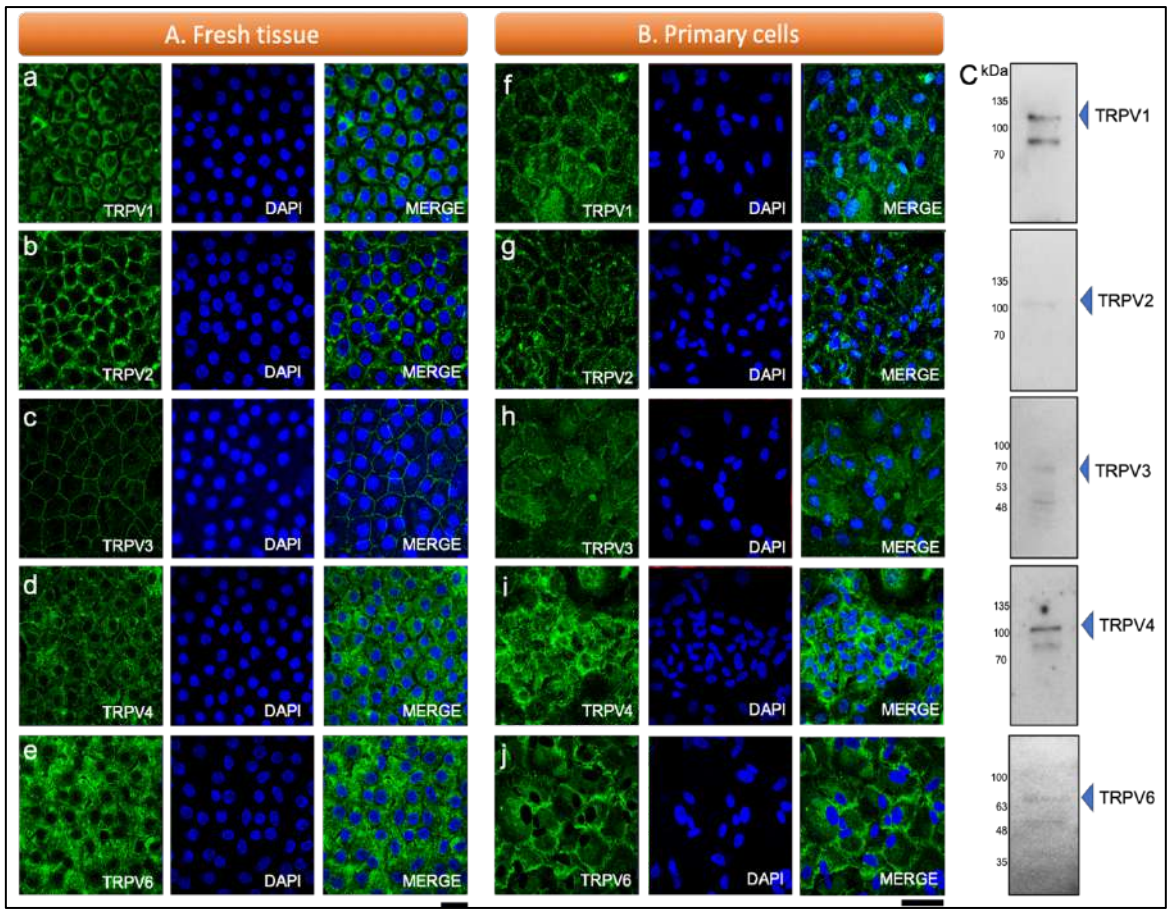

Figure 2: Protein expression of TRPV channels in the corneal endothelium. A positive expression of TRPV1-4 and TRPV6 was noted in the fresh tissues (panel A) and cultured cells (panel B) of the corneal endothelium, which was confirmed by western blot (panel C).

Fraction separation and protein expression by immunoblotting

Similarly, western showed positive expression of TRPV1-4 and TRPV6 channels in primary cultured cells (Figure 3), where two distinct bands (~95-110kDa) and a faint band (~30kDa) were observed for TRPV1 in the soluble fraction with a faint upper band (~110kDa) being expressed in the insoluble fraction of the protein. For TRPV2, the protein seemed to be

equally distributed between the insoluble and the soluble fractions (~100-105kDa) with the lower band (~50kDa) being more intense in the soluble fraction. A distinct band was observed for TRPV3 in the soluble fraction at ~55kDa. A clear intense band was observed in the soluble fraction for TRPV4 at ~100kDa with faint bands seen at ~75kDa and ~48kDa. TRPV6 expression was observed in both soluble and insoluble fractions where a faint band was observed at ~50kDa in the insoluble fraction and three bands were observed in the soluble fraction at ~100kDa, ~50kDa and ~20kDa. Beta-actin served as control. These results are in concordance with our immunofluorescence data (Figure 2 and Figure 4).

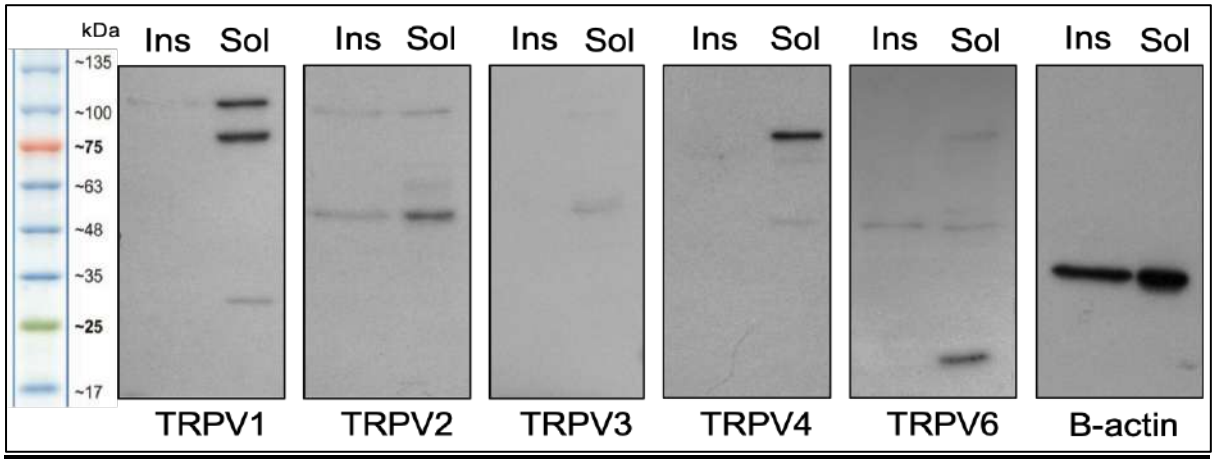

Figure 3: Distribution of TRPV channels in the corneal endothelium. A positive expression of TRPV1-4 and TRPV6 was noted in primary cells by western blot after fraction separation of the HCECs using 0.5% Triton X.

Co-localization of TRPV channels with N-cadherin, ZO-1 and actin cytoskeleton

While TRPV channels are known to localize to the membrane of the cells as seen in Figure 2, its location along the membrane is unknown. Therefore, we used N-cadherin, ZO-1 and phalloidin as reference proteins to establish their approximate location in the cell with the help of colocalization analyses using ImageJ. The scatterplot gives us information on the degree of colocalization between the two channels; the more linear the plot, the more the two channels directly overlap with each other. Pearson's coefficient ranges from -1 to +1 where 1 indicates complete positive localization between two channels and -1 indicates complete separation of the two channels.

As shown in figure 4 A, TRPV1 colocalised more with N-cadherin (Aa-d, with its corresponding pearson's correlation(r) in panel B-r=0.72±0.03) than phalloidin (Aa''-d'', B-

r=0.68±0.06) and ZO-1 (Aa'-d', B-r=0.56±0.04) suggesting that it is located towards the basoletral region of a cell. A similar pattern was observed with TRPV2 where it colocalised more with N-cadherin (Ae-h, B-r=0.80±0.05) than phalloidin (Ae''-h'', B-r=0.73±0.04) and least with ZO-1 (Ae'-h', B-r=0.50±0.08). TRPV3 was seen to be colocalising more with ZO-1 (Ai'-l', B-r=0.71±0.05) than phalloidin (Ai''-l'', B-r=0.66±0.05) and N-cadherin (Ai-l, B-r=0.54±0.06) suggesting that it is present towards the apical region of a cell. Highest colocalization was observed with TRPV4 and ZO-1 (Am'-p', B-r=0.86±0.03) and N-cadherin (Am-p, B-r=0.81±0.03) than phalloidin (Am''-p'', B-r=0.59±0.06) which is suggestive of its presence throughout the cell membrane (apical and basolateral region). Almost equal proportion of TRPV6 was seen to be colocalised with N-cadherin (Aq-t, B-r=0.63±0.04), phalloidin (Aq''-t'', B-r=0.61±0.07) and ZO-1 (Aq'-t', B-r=0.56±0.03) which is suggestive of its partial presence along the membrane of a cell. As the TRPV channels colocalise with membrane markers such as N-cadherin, ZO-1 and the actin cytoskeleton, this data points towards a potential mechanosensory function of these channels in regulating the cell morphology in the HCECs.

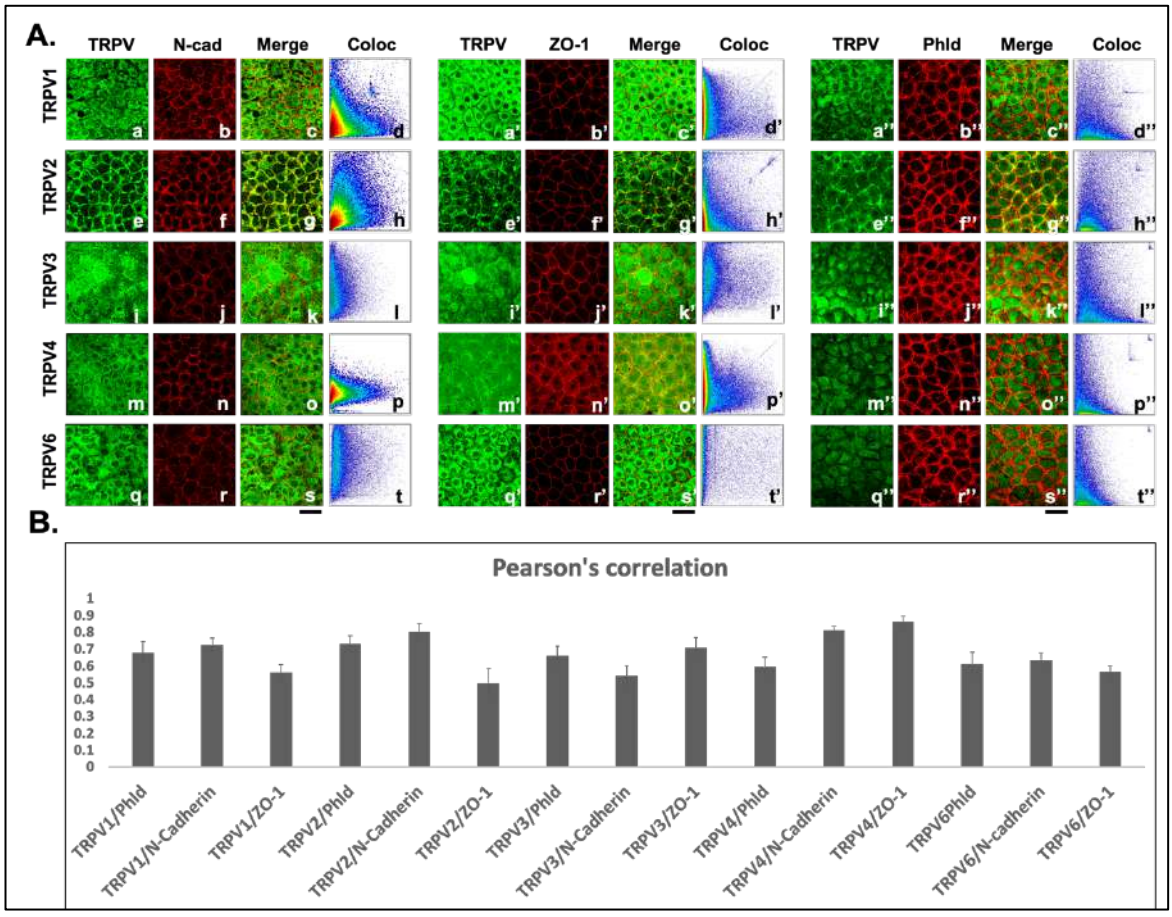

Figure 4: Representative image showing colocalization of TRPV channels with reference

proteins. Panels A shows immunofluorescence of TRPV channels (green) with N-cadherin/ZO-1/ Phalloidin ZO-1 (red) (Images taken at 63X, Scalebar: 20 μm). Panels d-d'', h-h'', l-l'', p-p'' and t-t'' show the scatterplot of the channels based on Pearson's coefficient. Panel B is the Pearson's coefficient of the TRPV channels with reference to the known membrane proteins.

Functional assessment of the TRPV1, TRPV3 and TRPV4 channels in HCECs

The ability of an ion channel to let ions pass through it dictates the channel's functionality. TRPV1 is a nonselective cation channel that may be opened by a wide range of physical and chemical stimuli, both exogenous and endogenous. Resiniferratoxin (RTX) is a well-known TRPV1 channel activator, while 5'-Iodoresininferratoxin (IRTX) is its inhibitor. Similar to this, it is thought that TRPV4 is crucial for controlling how cells operate since it mediates Ca2+ influx, which allows for the regulation of several intracellular proteins necessary for sustaining various physiological activities. TRPV4 is known to be activated by GSK1016790A, whereas RN1734 blocks the channel. Similarly, farnesyl pyrophosphate (FPP) is an activator of the TRPV3 channel and 2,2-diphenyltetrahydrofuran (DPTHF) is its inhibitor. The functionality of these channels was assessed with the help of Fluo 4 AM (1μM). We assessed the functionality of these channels by live cell imaging using Ca^{2+} sensitive dye Fluo4-AM (1μM) that makes it possible to measure the amount of Ca^{2+} inside cells. RTX (100nM) elicited TRPV1 activation-mediated rise in the intracellular Ca^{2+} in HCECs, a reaction that ultimately declined as the cell regained the homeostatic level of the ions. TRPV1 channel was inhibited by 5' IRTX (1μM) as there was no change in the intracellular Ca^{2+} levels. Similarly, GSK1016790A (1μM) evoked a spike in the intracellular Ca^{2+} levels in HCECs and no change was observed when RN1734 (10μM) was added to the cells. Pre-incubation of the cells with their respective antagonists (5'IRTX and RN1734) for one hour revealed blocking of the respective channels. This was confirmed when calcium influx was abolished when their respective agonists were added. As DMSO/Ethanol were used to dissolve the drugs, they were used as controls to establish the baseline calcium present in the cells. This data is suggestive of the functional role of the TRPV1 and TRPV4 channels in the HCECs.

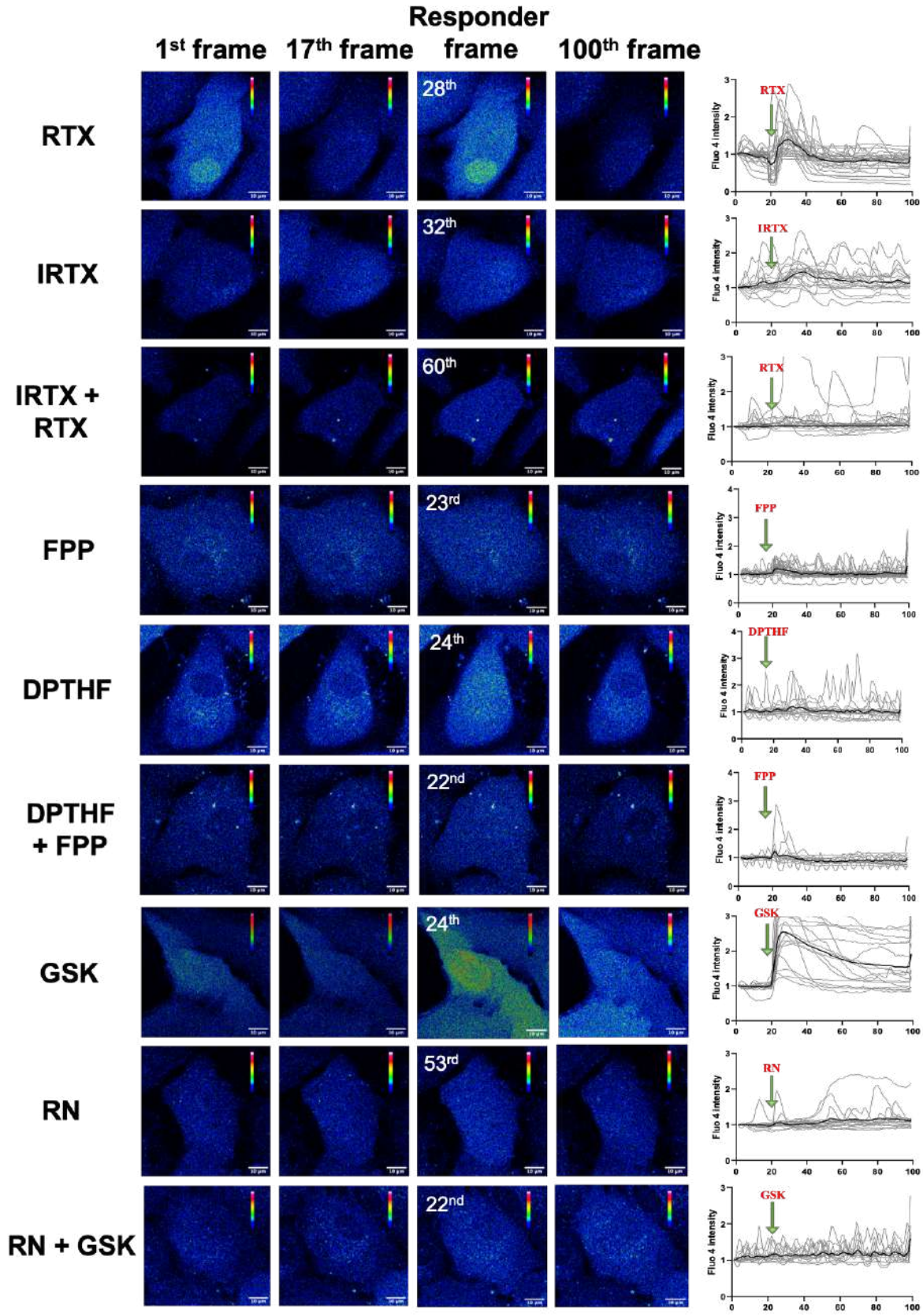

Figure 5: *Functional characterization of TRPV1, TRPV3 and TRPV4 channels in the HCECs.* RTX (TRPV1 agonist) induced calcium influx was noted, which was absent when

5'IRTX (TRPV1 antagonist) was added. Similarly, FPP (TRPV3 agonist) elicited a small spike in the calcium influx which was inhibited by DPTHF (TRPV3 inhibitor. Similarly, GSK1016790A (TRPV4 agonist) elicited a spike in the calcium influx, which was not seen when RN1734 (TRPV4 antagonist) was added. The graphs corresponding the drug indicates the fluorescent intensity of calcium, where each grey trace represents a cell (n=40) and the black trace is the average value. The red arrows represent the time when the drug was added (frame 20).

Increase in IOP disrupts the actin cytoskeleton tight junctions in the corneal endothelium (proof of concept)

As previous literature has revealed that the CE cells adapt under increased IOP, we subjected the corneas to increased IOP for a short period (acute – 50mmHg) to evaluate the cells morphology, adhesion and barrier properties. The cadaveric corneas were placed onto the anterior chamber and locked to avoid any leaks. The anterior chamber in-turn was connected to a peristaltic pump to maintain a constant flow rate. This whole set- up was placed in an incubator for maintaining optimal growth conditions. Staining of corneal tissues after induced pressure revealed disrupted ZO-1 expression (tight-junction marker), which points towards the barrier integrity of the endothelium being compromised. Similarly, we observed a disruption of the actin cytoskeleton (phalloidin staining) and enlargement of the cells in the treated tissues when compared to that of the control tissues.

From the previous literature it was noted that an increase in intraocular pressure, disrupted the tight and adherens junctions that caused the disengagement of cadherins. This led to the increase in Rho A-GTP, that caused the activation of Rho kinase, that resulted increase in phosphorylation of the myosin light chain, increase in actomyosin contraction that ultimately resulted in loss of barrier integrity. To test this theory, we looked for the doubly phosphorylated myosin light chain and observed an increase in ppMLC expression in treated tissues when compared to control.

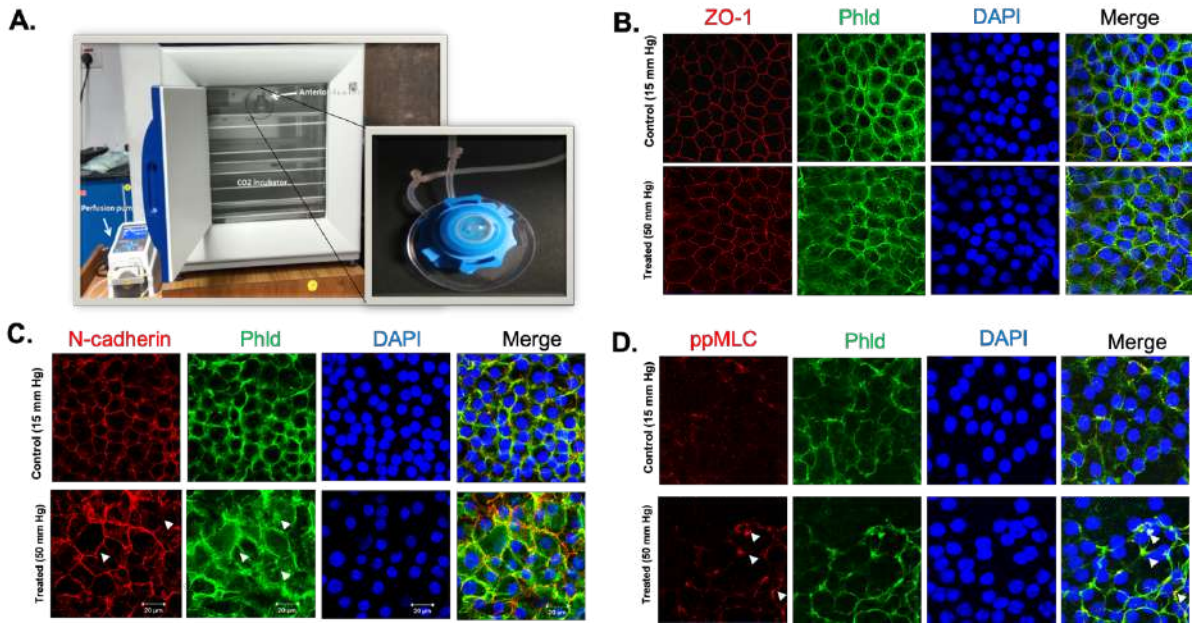

Figure 6: Representative image of ex-vivo perfusion system (panel A). Representative image of human CE cells after 6 hours of perfusion showing immunofluorescence of Control (15mmHg) and Treated (50mmHg) tissues expressing - ZO-1 (tight junction marker; red staining) and phalloidin (actin cytoskeleton marker; green staining) (panel B - Scalebar-20µm. Representative image of human CE cells after 6 hours of perfusion showing immunofluorescence of Control (15mmHg) and Treated (50mmHg) tissues expressing – N-cadherin (cell-cell junction marker; red staining) and phalloidin (actin cytoskeleton marker; green staining). Images were taken at 40x (panel C- Scalebar-20µm). Representative image of human CE cells after 6 hours of perfusion showing immunofluorescence of Control (15mmHg) and Treated (50mmHg) tissues expressing – pp-MLC (Myosin light chain marker; red staining) and phalloidin (actin cytoskeleton marker; green staining). DAPI was used to stain the nucleus blue. Images were taken at 40x by using Zeiss Confocal Inverted Microscope (Scalebar-10µm).

7. Statistical analyses

Data is represented as Mean ± SD for Pearson's correlation.

8. Discussion

Various stimuli activate the TRPV channels such as pressure, pH, osmolarity, membrane stretch etc. In this scenario, the TRPV channels could get activated through the pressure itself or through a membrane stretch as seen in some cases of the increased intraocular pressure where a change in the radius of the cornea was observed. Therefore, Acute increase in IOP, causes disruption of the junction markers such as ZO-1 and N-cadherin; and contraction of the

actin cytoskeleton as seen by the positive expression of doubly phosphorylated MLC, which could be driven through the Rho-kinase pathway. TRPV channels are expressed in the corneal endothelial cells and are functional as seen with the help of calcium live cell imaging. As TRP channels colocalize with membrane markers such as ZO-1 and N-cadherin and the actin cytoskeleton, and an increase in IOP caused visible disruption of the membrane proteins and the actin cytoskeleton, the TRP channels could be contributing to regulating the cells response to increased IOP. The overall change in cell morphology could be driven through 2 pathways – Rho or the TRPV regulated pathway or both. Previous literature has stated that an increase in pressure increased the calcium influx through TRPV channels that led to an increase in cytoplasmic calcium which resulted in calcium binding to calmodulin and calmodulin being the ultimate signaling molecule by binding to the TRPV channels or phosphorylate myosin resulting in the remodeling of the actin cytoskeleton and therefore, the regulation of the cytoskeleton could be driven through these two major pathways or both.

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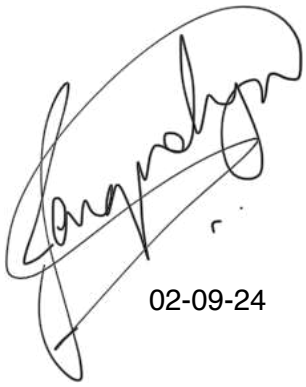

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