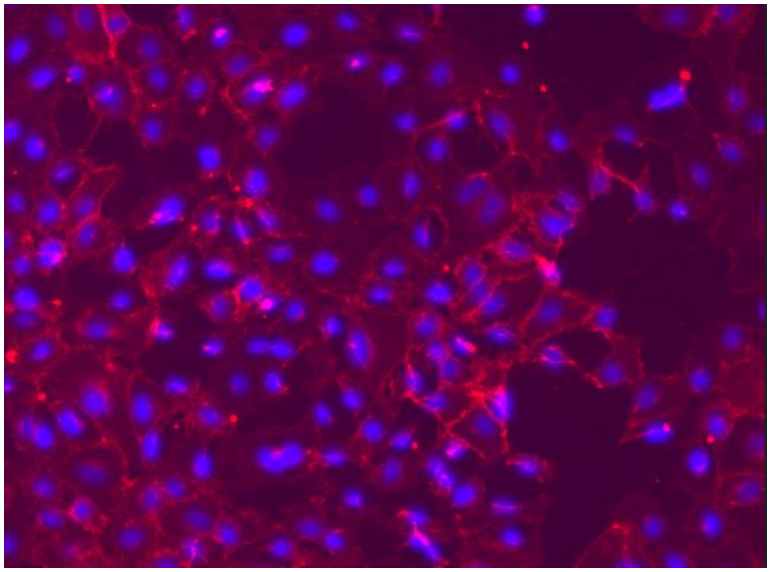

THE EFFECT OF HISTAMINE AND CETIRIZINE ON ENDOTHELIAL CELLS

The effect of histamine and its antagonist cetirizine on the permeability of a monolayer of Human Umbilical Vein Endothelial Cells (HUVECs)

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Bachelorthesis BMT M12

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Abstract

Age-related Macular Degeneration is a progressive eye disease that affects millions of people worldwide. It is distinguishable between wet and dry AMD but are both initiated by an inflammatory cascade reaction. There are a couple of known treatments but they are primarily for late stage AMD. To simulate the inflammatory state of the endothelium in the disease, Human Umbilical Vein Endothelial Cells (HUVEC) were incubated with an antihistamine drug cetirizine, an aspired treatment drug for early stage AMD, and its antagonist histamine, a potent inflammatory mediator. The HUVECs were incubated until confluent and after incubation of either histamine, cetirizine or histamine and cetirizine, were stained with an immunostaining for VE cadherin, a molecule that composes the tight junctions between cells. A transwell permeability assay was also performed under similar conditions. There was a noticeable difference in cell morphology upon a concentration of 100 µM histamine and 100 µg/mL cetirizine incubation, the 100 µg/mL cetirizine also seemed toxic for the HUVECs monolayer. The 10 µM histamine and 10 µg/mL cetirizine concentration did not alter the morphology conclusively. Further on, the 10 µg/mL cetirizine concentration did not influence the monolayer permeability induced by a 100 µM histamine concentration. Future experiments should focus on permeability assays like the transwell experiment or a trans-endothelial electrical resistance (TER) measurement.

Nederlands:

Macula degeneratie (AMD) is een progressieve oog ziekte die miljoenen mensen aantast wereldwijd. Er zijn twee verschillende soorten AMD 'natte' en 'droge' AMD. Deze worden beide geïnitieerd door hetzelfde complexe inflammatoire proces. Er zijn een aantal behandelingen bekend, maar deze zijn voornamelijk bedoeld voor AMD in een laat stadium. Om de ontstekingssituatie van endotheel weefsel van deze ziekte te simuleren, werden menselijke navelstreng-endotheelcellen (HUVEC) geïncubeerd met de antihistamine cetirizine, een beoogd behandelingsmedicijn voor het vroege stadium van AMD, en zijn antagonist histamine, een krachtige ontstekingsmediator. De HUVECs zijn geïncubeerd totdat ze confluent waren en na incubatie van histamine, cetirizine of zowel histamine als cetirizine werden de cellen gefixeerd en werd immunostaining toegepast. Een transwell-permeabiliteitstest werd ook uitgevoerd onder vergelijkbare concentraties. Er was een duidelijk verschil in cel morfologie na incubatie van een concentratie van 100 µM histamine en 100 µg/mL cetirizine, de 100 µg/mL cetirizine leek ook toxisch effect te hebben op de HUVEC's-monolaag. De concentraties van 10 µM histamine en 10 µg/mL cetirizine veranderde de morfologie niet overtuigend. Verder had de 10 µg/mL cetirizine concentratie geen invloed op de permeabiliteit van de monolaag die werd geïnduceerd door een 100 µM histamine concentratie. Toekomstige experimenten zullen zich moeten richten op permeabiliteitstests zoals het transwell-experiment of een Trans-endothelium electrical resistance (TER)-meting.

Inhoudsopgave

1. Introduction

Age-related Macular Degeneration (AMD) is the leading cause of irreversible blindness globally and affects around 200 million people worldwide[1]. It is the progressiveness of the disease which makes it a particular hard condition to live with. There are a couple of treatment options possible, but mainly for late stage AMD, this will be discussed later on.

Degenerative changes below the retina (figure 2) of the macula because of a para-inflammatory reaction is what pathologically categorizes AMD [2]. The macula is the part of the eye where the concentration of rods and cones is the highest, this part is mostly responsible for sight. There are factors which heighten the risk of the macular changes like aging, family history, cigarette smoking and sunlight exposure, yet the underlying initiator of it all remains uncertain [3]. Active components of the para-inflammatory state however are known.

There are two different types of AMD, distinguished by the reason for the vision loss. There is 'dry-AMD', where the vision loss is caused by photoreceptor dysfunction and degeneration and dysfunction of the retinal pigment epithelium (RPE), which plays an essential role in maintaining homeostasis in photoreceptor cells. The other type is 'Wet-AMD', where vision loss occurs because of choroidal neovascularization which penetrates past the RPE and can cause fluids to leak in the retina which can cause vision loss. This is the main cause for approximately 80% of severe vision loss in AMD[1][2][4].

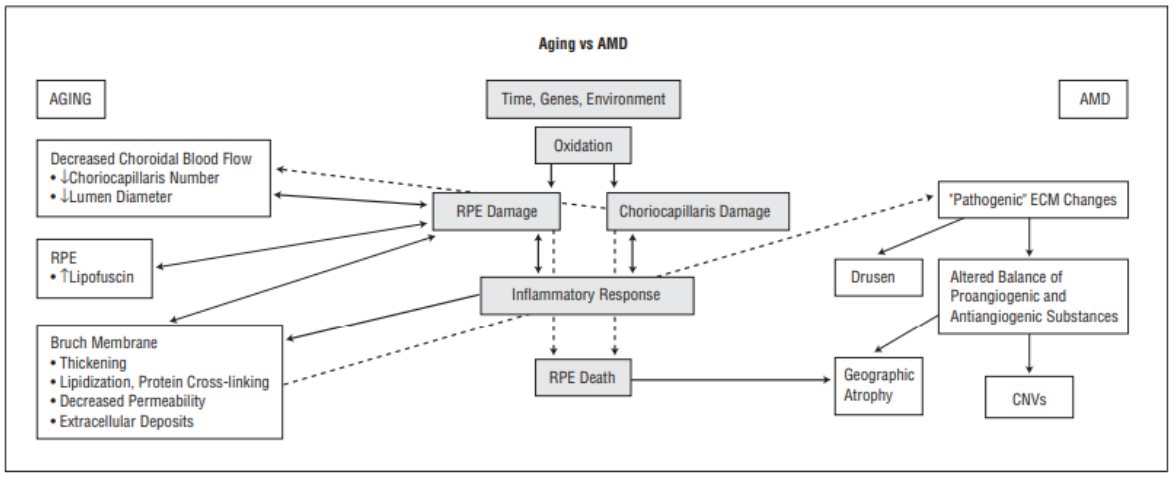


Figure 1: Aging vs age-related macular degeneration[4]. *Retinal Pigment Epithelium (RPE), Extra Cellular Matrix (ECM) Choroidal New Vessels (CNVs)*

As depicted in figure 1 above, the inflammatory response is the main initiator of the cascade that eventually results in the pathological changes that give rise to the different types of AMD and its symptoms. In dry AMD, the pathogenic changes result in subretinal deposits called drusen[5]. These deposits are localized between the RPE and Buch's Membrane (BM) (figure 2) and cause imperfections in the RPE which causes localized photoreceptor disruption. Vascular endothelial growth factor (VEGF) is one of the main culprits for 'wet-AMD'. The VEGF protein is a very potent promotor for angiogenesis of Choroidal New Vessels (CNVs) into the RPE and again promotes other inflammatory cytokines[6].

There are a three lines of treatment options for AMD patients. The first line of treatment for AMD is to prevent progression by eliminating risk factors. In late stage dry AMD, where there is only accumulation of subretinal drusen, the drusen can be laser photocoagulated to eliminate the deposits[4][5]. Based on the role in VEGF in wet-AMD, one of the main treatments for late stage wet

AMD are anti-VEGF intravitreal injections. This prevents further CNV formation and exudation of fluids in the RPE[1][4]. There is no definitive treatment for early stage AMD.

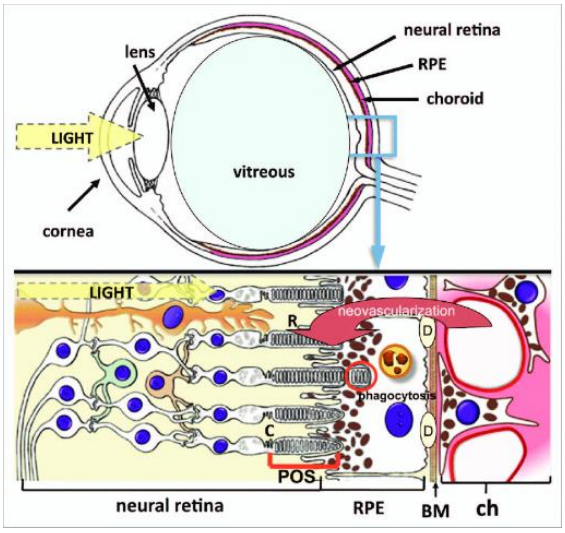


Figure 2: Depiction of cross section of the human eye for anatomical context[7].

Since the main initiator of the symptoms in both types of AMD is a complex inflammatory reaction, an option for early stage AMD treatment is taking anti-inflammatory medicine[3][4]. This is exactly what a professor at the University of Twente realised after some research after being diagnosed with AMD. Under the supervision of his general physician he purchased two medicines at the local drugstore: aescin, an anti-inflammatory agent, and cetirizine, an anti-histamic medicine. He used both of these medicines and recommended them to friends. Interestingly, his friends reported that the disease has not progressed since then. This anecdotal evidence is the initiator to research the effect of aescin and cetirizine on the choroidal endothelial cells during AMD progression. This research focusses on the effect of cetirizine.

Cetirizine is the antagonist of Histamine, which is a potent mediator in inflammatory responses[8][9]. Histamine can be produced by a wide variety of different cell types like mast cells and histaminergic neurons[10]. It mostly plays a large roll in allergic reactions but there is also increasing evidence that it modulates the response to chronic arterial injuries[11]. Histamine binds to the four histamine receptors (H1-H4) which start a cascade that results in vasodilation and elevated microvascular permeability. Histamine phosphorylates VE cadherin adherens junctions between cells[12]. The permeability is increased because the phosphorylation of VE cadherin causes VE cadherin displacement and creates small gaps between adjacent endothelial cells at sites of a VE cadherin junction [13].

Cetirizine is a very potent H-receptor blocker[14]. It is used primarily as antihistamine for people with hay fever, per example. Since cetirizine has a higher affinity to the H-receptors than histamine, it is hypothesized that the permeability of endothelial cells will be not increase when incubated with both histamine and cetirizine.

A similar experiment was performed on Human Umbilical Vein Endothelial Cells (HUVECs) by a member of the Applied Stem Cell Technology research group of the University of Twente. The conclusions from that research was that histamine incubation did not induce any morphological changes in the HUVECs[15] and therefore the healing abilities of cetirizine were not observed. It was hypothesized that the lack of morphological changes was because the treatment of histamine was not stable enough to observe a homologic change throughout the wells. Another hypothesis is that

the monolayer was too mature. In a more developed monolayer the VE cadherin adherens junctions are more stable and less susceptible for phosphorylation[10][11]. This thesis continues on this previous experiment. Previous studies by Yong et al. indicate that histamine generates a heightened permeability in a HUVECs [9], while Andriopoulou et al. showed that cetirizine significantly inhibits the response of HUVECs to histamine[8].

In this study the effect of histamine and its antagonist cetirizine on the permeability of a monolayer of Human Umbilical Vein Endothelial Cells (HUVECs) is analysed. This is done in two ways. Firstly an immunostaining is done for VE cadherin, to assess the tight junctins, and F-actin, to assess the morphology of the cell. Next a permeability assay will be performed, this gives an insight of the integrity of the and the permeability of the monolayer.

2. Method

2.1 Cell culture

To be able to perform the two experiments on the HUVECs, they needed to be prepared. The Human Umbilical Vein Endothelial Cells (HUVEC, Lonza) were cultured in ECGM-2 (PromoCell GmbH, Germany) supplemented with 1 v/v penicillin/streptomycin (P/S) (Thermofisher) in a T-75 flask (Cellstar, Greiner bio-one, Germany). The flask was coated with 0,1 mg/mL collagen I (Rat tail collagen I, Corning, USA) in dPBS (Gibco, Thermofisher) and incubated for at least 30 minutes and prior to seeding washed with dPBS(Gibco, Thermofisher). HUVECs were incubated in the T-75 flask at 37 ◦C, 5% CO_2 until a confluence of approximately 80%. When confluent, the HUVECs were obtained from the flask using 0,05% Trypsin/EDTA (Gibco, Thermofisher). They were centrifuged at 1200 rpm for 5 minutes (5810R, Eppendorf) at room temperature (RT).The cells were seeded in ECGM-2 at 5000 cells/well (15000 cells/cm^2) in a flat bottom, tissue culture treated (TCT) 96-well plate (VWR, USA) which was first incubated for 30 minutes with 0,1 mg/mL collagen I and then flushed with dPBS. After 2 days of incubation in ECGM-2 the medium was changed to the starvation medium ECBM-2 (PromoCell GmbH, Germany) supplemented with 1% P/S and 2% FBS for 24 hours. After 24 hours the medium was changed to ECBM-2 with 10 µg/mL or 100 µg/mL cetirizine (Sigma Aldrich) , 1% P/S, 2% FBS. The next day the wells were incubated with 10 µM or 100 µM histamine and/or 10 µg/mL or 100 µg/mL cetirizine ECBM-2. The wells were incubated with histamine for 30 minutes prior to fixating at 37 ◦C, 5% CO_2.

2.2 Immunostaining

For changes in morphology, the cells were stained for actin filaments, cell nuclei and VE cadherin. To fixate, the cells were incubated for 15 minutes at RT with a 4% formaldehyde (37%, Sigma Aldrich) solution in PBS++ (Gibco). Afterwards the cells were washed 3 times with PBS ++ before incubating the wells in permeabilization and blocking buffer (PBB). The PBB consists of 0,1% v/v Triton x-100 (Sigma Aldrich) and 1% w/v BSA (Sigma Aldrich). After 60 minutes at RT, the PBB is replaced with the primary antibody (AB) solution, which contains 2ug/ml VE-cad GaH (0,2 mg/mL, R&D Systems) in PBB, this is incubated at 4 ◦C overnight. The AB solution is aspirated and the cells are washed with PBS++ 3 times, with waiting steps of 20 minutes in between where the plate is put on a plate shaker. The secondary antibody solution, containing 5ug/mL Donkey-anti-Goat AB (Invitrogen by Thermofisher), 2 actingreen (Invitrogen by Thermofisher) droplets/mL and 6,25 ug/mL DAPI (Invitrogen by Thermofisher), was added after these washing steps. The cells were wrapped in aluminium foil and incubated with this second AB solution for 2 hours at RT. After incubation, the AB solution was aspirated and washed 3 times with PBS++ with wait steps of 20 minutes in between washing rounds. The cells are now ready to be analysed.

2.3 Quantification

After the staining, the cells were able to be analysed for quantification. The fluorescence staining of the cells was processed using an automatic microscope, the EVOS FL2 (Invitrogen by Thermofisher). These images were then downloaded into FIJI ImageJ and Cell Profiler to display the images and quantify the intensity of the immunostaining. In FIJI ImageJ the intensity of the VE cadherin per cell was measured and plotted.

2.4 Transwell permeability assay

Since the morphology reading does not necessarily conclude anything about the permeability of the monolayer, a different proof of principle experiment was done. The HUVECs were seeded at 15000 cells/cm^2 in 0,1% collagen coated transwell inserts (3µm transparent 24 wells insert, Cellquart) in ECGM-2. After 2 days the medium was substituted by starvation medium (ECBM-2) for 24 hours. After which the cells incubated with cetirizine for 24 hours, before adding histamine and/or

cetirizine to the transwells, which they were incubated with for 30 minutes. After 30 minutes 100 µg/mL FITC-dextran (Sigma Aldrich) was added to the top compartment and 100 µL was removed and afterwards added to the lower compartment every 5 minutes for a total time of 15 minutes. The extracts were placed in a black 96 wells plate (Greiner-bio one) and analysed using the fluorescence plate reader (VICTOR3, Perkin Elmer). To calculate the permeability the following formula can be used:

$$P_{membrane} = \frac{dC}{dt} * \frac{Volume_{chamber}}{C_i * Area_{chamber}} \qquad (1)$$

Where the $P_{membrane}$ is the permeability of the membrane, $V_{chamber}$ and $Area_{chamber}$ are the volume of the bottom well and area of the transwell insert respectively. $\frac{dC}{dt}$ is the difference in concentration over time of the bottom well.

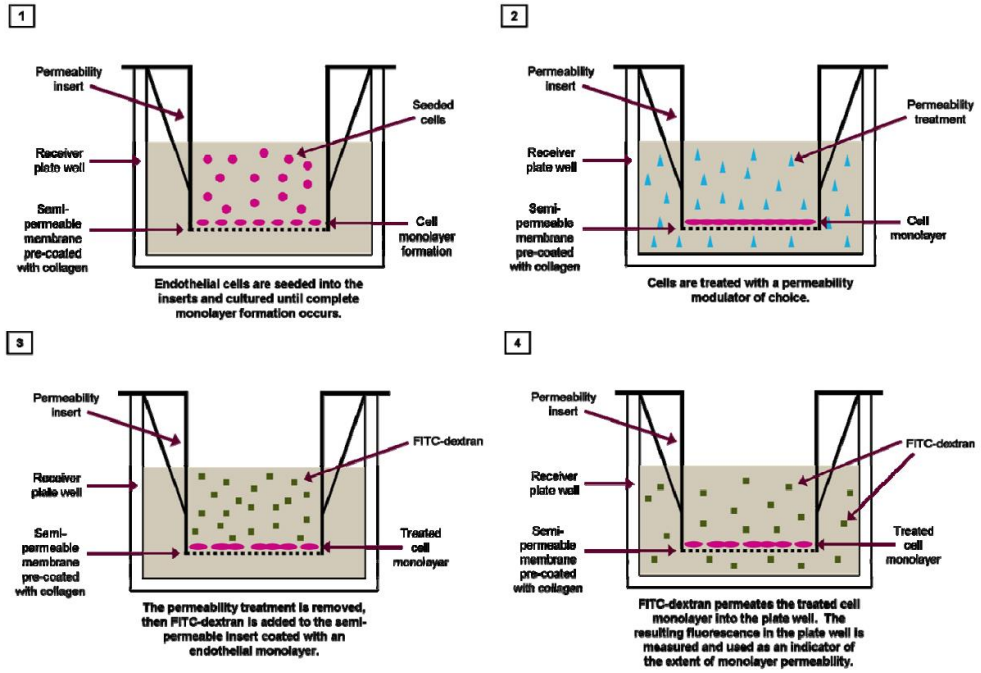


Figure 3: Schematic view of the transwell permeability assay [16]

3. Results

The results of the experiments consist of three groups: pictures of morphological changes in the HUVECs showed by VE cadherin, DAPI and F-actin immunostaining, VE cadherin intensities per cell and the transwell experiment.

3.1 The effect of histamine and cetirizine on cell morphology

To examine whether there is any visual difference between the expression of morphological cell markers between cells treated with histamine, cetirizine, both or neither, the cells were treated with immunostaining for VE cadherin, DAPI and F-actin. The VE cadherin is the marker for cell-cell adhesion which can be an indicator for layer permeability, the cell-cell borders are shown by VE cadherin in the figure 4 below.

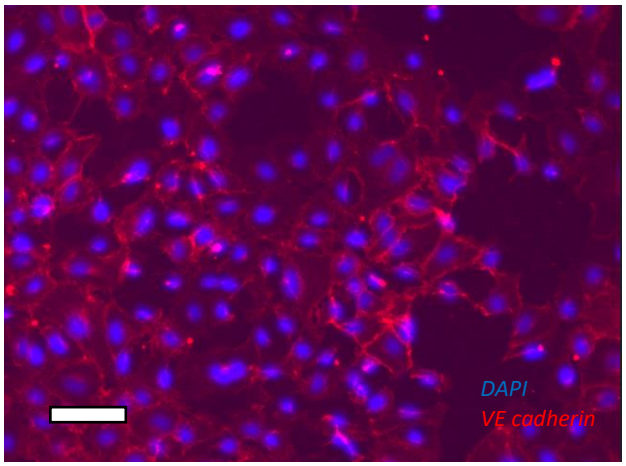
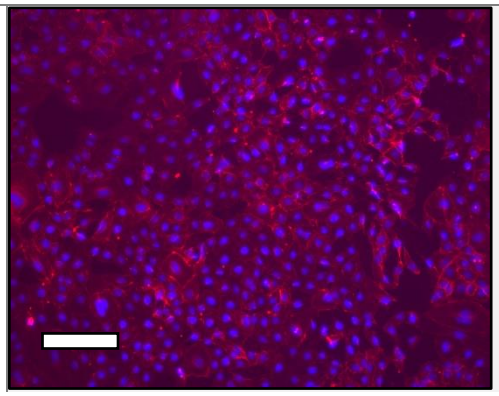
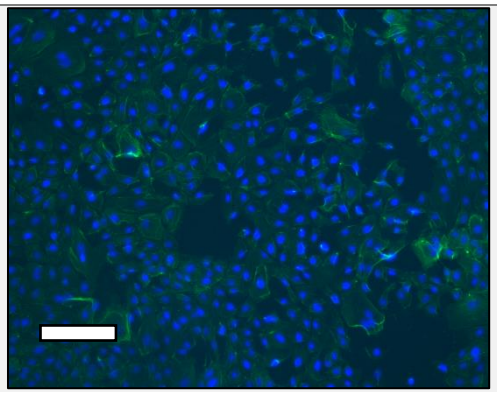
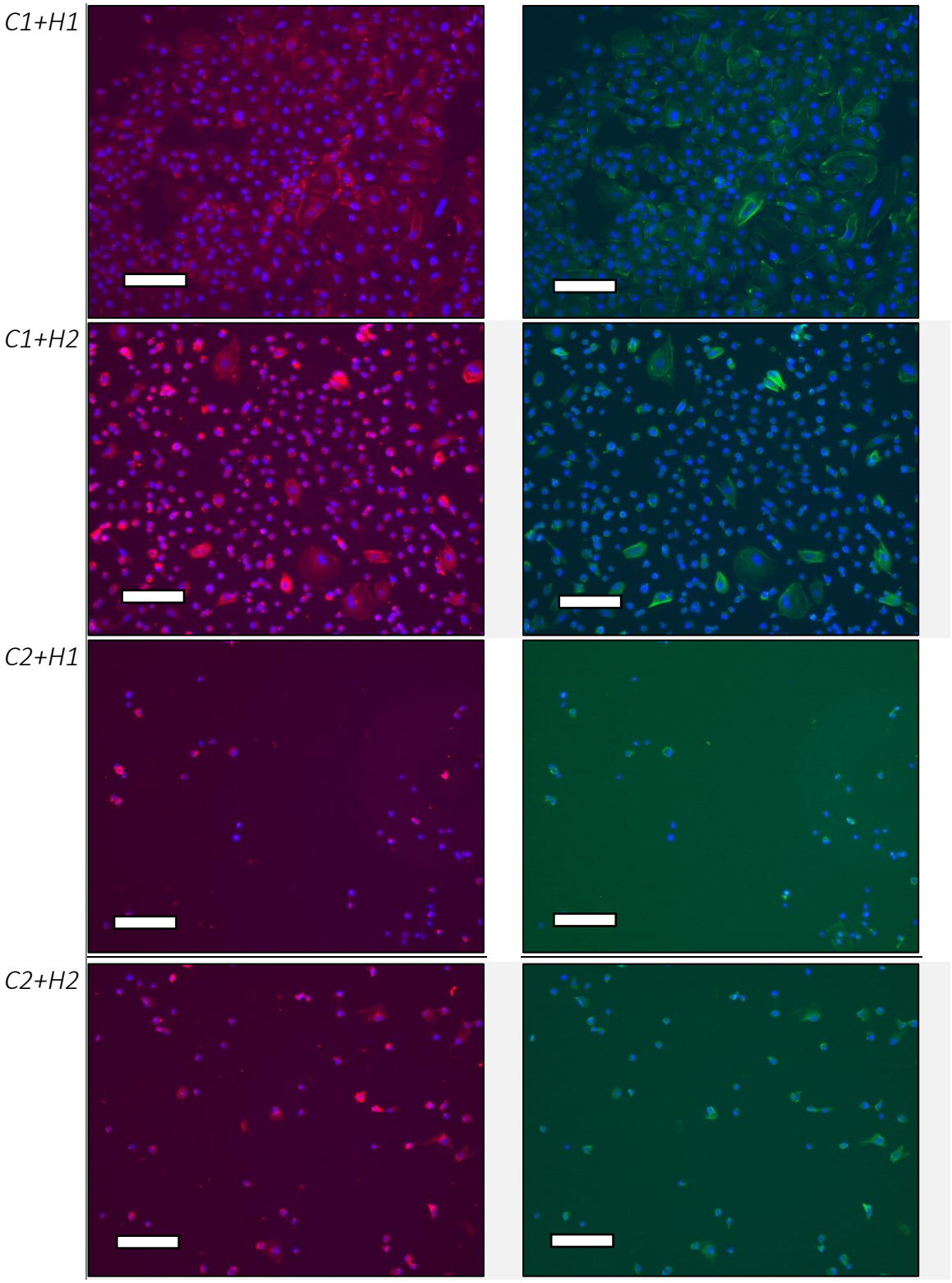


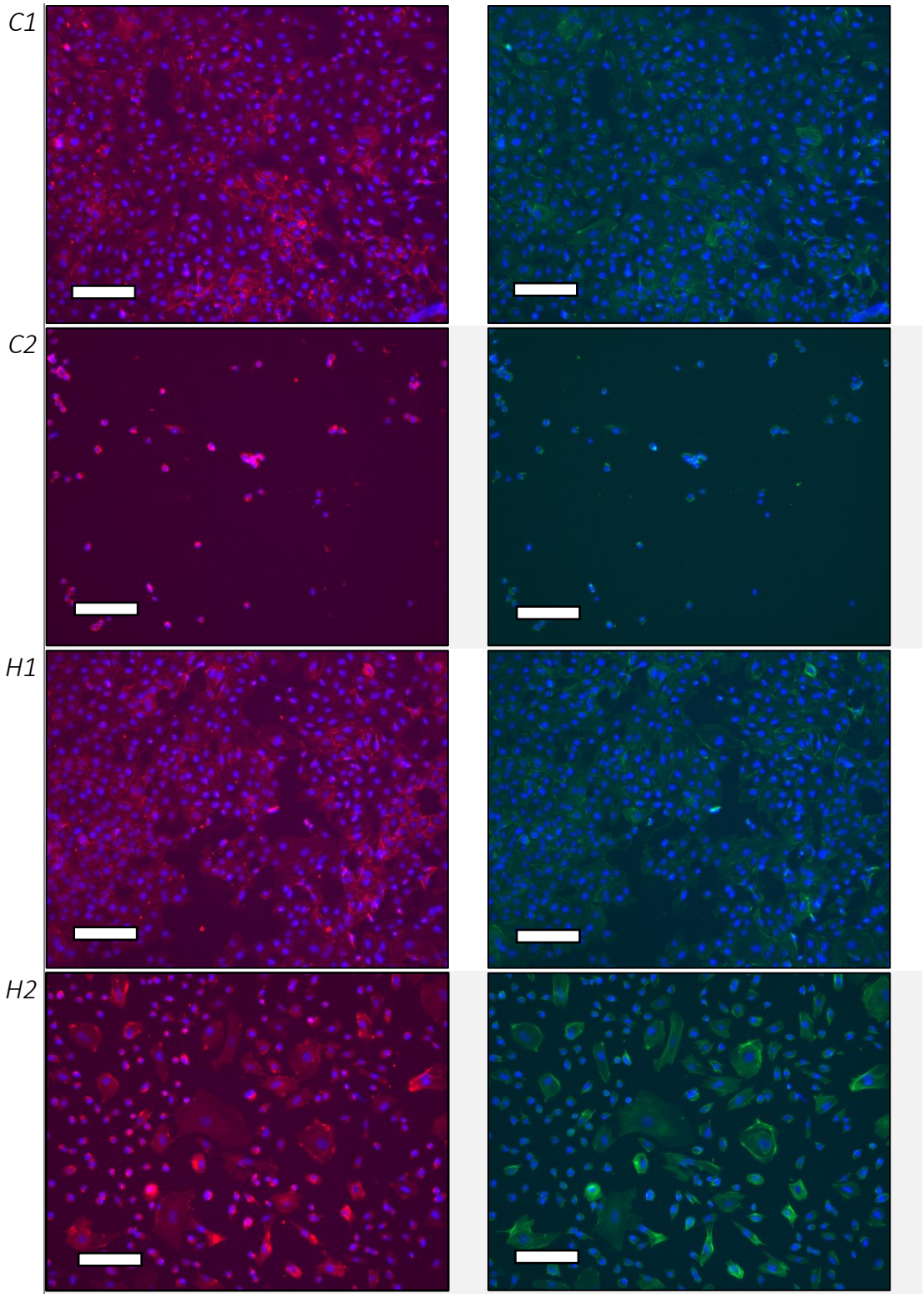
Figure 4: Control VE cadherin staining. Example of well illuminated VE cadherin borders. Scalebar: 250 µm

As seen in table 1 below, the wells with either a high cetirizine concentration (C2, 100 µg/mL) or the wells with a 100 µM histamine concentration (H2) had a very different cell morphologies than the other conditions. The cells had shrunk compared to the control and took up less of the surface area, as seen in both the F-actin and VE cadherin immunostaining. The conditions of 10 µg/mL cetirizine or histamine concentrations (C1+H1, C1, H1) had a similar morphology to the control, except that the H1 and C1+H1 seemed to have less clear VE cadherin cell boarders.

Table 1: DAPI (blue), VE cadherin (red) and F-actin (green) staining on HUVECs treated with 10 µg/mL cetirizine (C1) or 100 µg/mL cetirizine (C2) for 24 hours and/or 10 µM histamine (H1) or 100 µM histamine (H2) for 30 minutes. Scalebar: 1 mm

	VE cadherin and DAPI	*F-actin and DAPI*
Control		


C1+H1
C1+H2
C2+H1
C2+H2


C1
C2
H1
H2

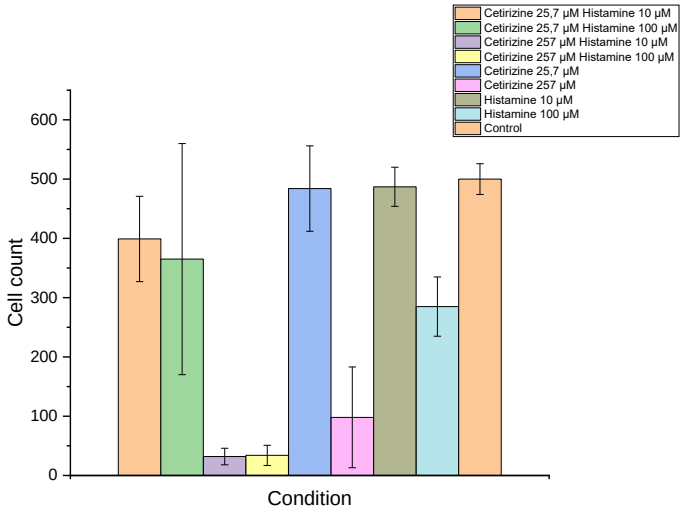


Figure 5: Average cell count per EVOS picture in the different conditions 25,7 µM and 257 µM equals 10 and 100 µg/mL respectively.

As is seen in the morphology of the cells, the cell count also differs. The cell count for C2, C2+H1, C2+H2 and H2 are noticeably less than the cells counts in the other conditions. The very low values of cell counts in the conditions with both cetirizine in high concentration in combination with histamine in low or high concentration stand out.

3.2 Fluorescence intensity reading

To quantify the VE cadherin differences in the different conditions, the intensity was measured for every EVOS picture using ImageJ. Figure 6 shows that the differences in VE cadherin intensity are not consistent with the divergent morphology conditions described above. The high histamine concentration has the highest intensity and the high cetirizine, low histamine well has the lowest raw integrated density per cell. Overall the difference in VE cadherin intensity per cell per condition is relatively small.

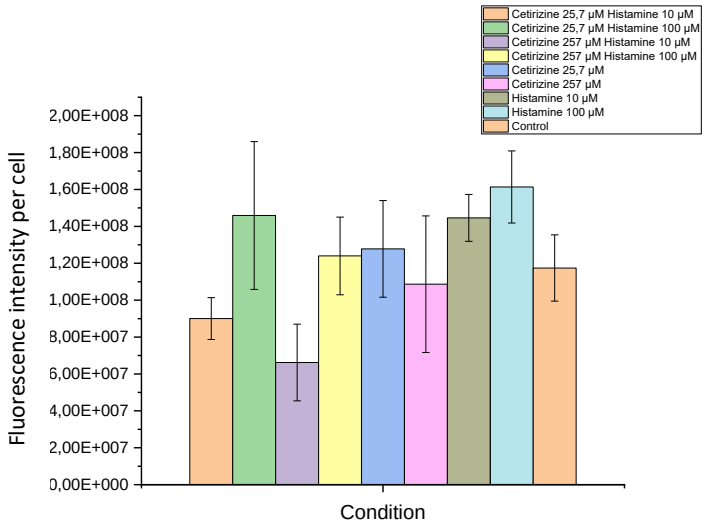


Figure 6: Raw integrated density of the intensity of VE cadherin relative to the amount of cells per condition 25,7 µM and 257 µM equals 10 and 100 µg/mL respectively.

3.3 Effect of histamine and cetirizine on the monolayer permeability

The permeability of a membrane is determined by the tangent of the concentration curve. It is very noticeable that the cetirizine + histamine and histamine curve have the steepest slope. However, the histamine measurement is less reliable because of the remarkable shape and large error bar. The high permeability of cetirizine + histamine and histamine is consistent with the morphology changes, because in those results it is visible that the cells are not in direct contact with one another anymore. The control and the cetirizine wells are very similar in slope and are within the error margins of one another.

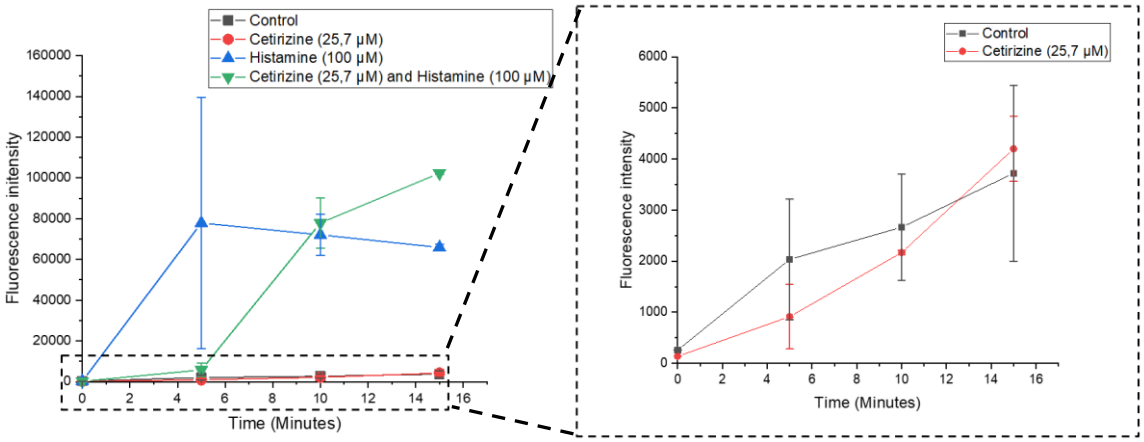


Figure 7: Fluorescence intensity of FITC dextran reading vs time with enhancement. 25,7 µM and 257 µM equals 10 and 100 µg/mL respectively

4. Discussion

In this study is assessed what the effect is of cetirizine and histamine on the permeability of HUVECs after growing to a confluent monolayer.

The most important results to take from the experiment are the knowledge of morphology, the transwell results and what concentrations are best to use for further experiments. The high concentration of histamine (100 µM) had a large effect on the morphology of the HUVEC monolayer, as the cells do not stand in direct contact with one another anymore and have shrunk. This is different than the conclusion of the previous research this thesis was based upon[15]. It can also be seen in figure 7 that the permeability of the HUVEC monolayer has increased a lot at this concentration in the, which is consistent with Yong, et al.[9]. This indicates an increase in permeability since there is now space between cells. The lower concentration of histamine (10 µM) does not show such a divergent cell morphology, but it does show that the VE cadherin seems to displace away from the cell-cell contact points, which is inconsistent with the control, which corresponds to known research[12]. This is however very hard to quantify, so in this experiment no conclusive difference in relocation of the VE cadherin molecules can be recognised. The displacement does not necessarily indicate a heightened permeability since there are still other junctions involved in cell-cell contact.

The experiments also showed usable results regarding the cetirizine concentrations. The morphology and cell count assay showed that the cetirizine concentration of 100 µg/mL was toxic for the HUVECs, which was inconsistent with literature which showed that 100 µg/mL was not toxic for HUVECs[17]. Figure 5 shows that the concentration of 10 µg/mL cetirizine had no toxic effect on the HUVECs nor does it produce any morphological changes. However it is seen in figure 7 that it does not seem to counter the permeability effect of the 100 µM histamine. This can be seen in morphology (table 1) and the transwell analysis (figure 7).

The results of the different concentrations show that the used concentrations are not yet optimal. The goal is to see if cetirizine has an effect on the permeability when histamine is incubated on HUVECs, and with these concentrations it is not yet distinguishable. In this experiment the concentration of histamine is either too high or the concentration of cetirizine too low to show a definitive result on the transwell and morphology experiment (figure 7 and table 1).

The transwell permeability assay was not optimized as a calibration curve to precisely calculate the permeability using formula 1 was not made. Figure 7 can still give an approximation of the difference in concentration over time but it is not as precise as it could be if a calibration curve was used. This is an important step to take in mind for further research.

Because the VE cadherin displacement is hard to quantify, the permeability assay of the transwell experiment has a large value in this experiment. In future experiments more concentrations should be tested, more biological duplicates should be used and more time points should be measured, because the last two give more measurements and therefor raises the credibility of the results.

Another way of accurately measuring the permeability of a membrane is a Trans endothelial Electrical Resistance (TER) measurement[8]. This test can also be a way to expand this research and give a better representation of the permeability changes when a HUVEC monolayer is exposed to cetirizine and histamine.

Both of these further research techniques should be performed be able to expand the knowledge of permeability and the mechanism behind it after exposure to histamine and cetirizine.

5. Conclusion

There was a noticeable difference in cell morphology upon 100 µM histamine and 100 µg/mL cetirizine incubation, the 100 µg/mL cetirizine also seemed toxic for the HUVECs monolayer. The 10 µM histamine and 10 µg/mL cetirizine concentration did not alter the morphology conclusively. Further on, the 10 µg/mL cetirizine concentration did not influence the permeability induced by a 100 µM histamine concentration. Future experiments should focus on permeability assays like the transwell experiment or a TER measurement.

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