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DEVELOPMENT AND TESTING OF *IN VITRO* MEASUREMENT SYSTEM FOR EPITHELIAL BARRIER DYNAMICS

Bachelor of Science (Tech) Thesis
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ABSTRACT

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Epithelial barrier is an interface between tissue compartments formed by epithelial cells. It plays a central role in regulating the transport of fluids, ions, and other molecules in physiological processes. Research on the dynamics of the epithelial barrier is important because disruptions in its functions, particularly related to the tightness of the barrier, are a common cause of many diseases. More profound understanding of the function of the epithelial barrier helps to elucidate the mechanisms of many diseases and develop new treatment methods.

Transepithelial resistance (TER) measurements are a traditional method to assess epithelial barrier dynamics. As a structure that regulates the passage of substances, the epithelial barrier resists the movement of charged molecules through it, and thus, transepithelial resistance can be considered as a measure of ion permeability. TER measurements are a common way to assess integrity of epithelia non-invasively, but they also provide more precise information on the permeability of the epithelial barrier and the underlying mechanisms.

In this bachelor's thesis, an existing measurement system was developed and tested to examine epithelial barrier dynamics, specifically transepithelial resistance and changes of it due to delivering pharmaceutics during cell culture, i.e., *in vitro*. Current methods are lacking a system that would allow administering drugs and studying changes in the epithelial barrier in real-time with sufficient accuracy. The study tested an injection device that can administer drugs to cells monitored by an automated TER measurement device while the device is inside an incubator. The focus was on the functionality, reliability, and compatibility to in vitro environment. The device was tested by injecting substances with known responses into Madin-Darby Canine Kidney (MDCK) cell line cells. The cells were cultured on semi-permeable membrane, and the transepithelial resistance of the epithelia formed by cells was monitored during the tests.

The results indicate that the injection device functions as expected and does not seem to cause disturbance in TER measurements. Some of the results were unexpected, indicating that the device is not yet entirely reliable and requires some adjustments and additional testing. Additionally, solutions to improve user-friendliness should be considered if the injection system's capacity is to be increased. Overall, based on the results, the measurements system is a promising tool for epithelial barrier dynamics research after a few adjustments.

Keywords: Transepithelial resistance, epithelial barrier, in vitro, epithelial permeability, impedance spectroscopy, injection device

TIIVISTELMÄ

Julia Harju: Epiteeliesteen dynamiikan *in vitro* -mittausjärjestelmän kehittäminen ja testaus
Kandidaatintyö
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Epiteelieste on epiteelisolujen muodostama rajapinta kudosten ja elinten eri osa-alueiden välillä. Sillä on keskeinen rooli nesteiden, ionien ja muiden molekyylien kuljetuksen säätelyssä fysiologisissa prosesseissa. Epiteeliesteen dynamiikkaan liittyvä tutkimus on tärkeää, koska epiteeliesteen toimintaan ja erityisesti sen tiiviyteen liittyvät häiriöt ovat yleinen syy monien sairauksien taustalla. Tarkempi ymmärrys epiteelin toiminnasta auttaa selvittämään monien sairauksien mekanismeja, sekä kehittämään uusia hoitomuotoja.

Transepiteliaalisen resistanssin (TER) mittaukset ovat perinteinen metodi epiteeliesteen dynamiikan tutkimiseen. Aineiden kulkua säätelevänä rakenteena epiteelieste vastustaa sen läpi tapahtuvaa varauksellisten molekyylien liikettä, ja transepiteliaalista resistanssia voidaan näin ollen pitää ionien läpäisevyyden mittarina. TER-mittaukset ovat yleinen keino tarkastella epiteelin eheyttä ei-invasiivisesti, mutta TER-mittaukset antavat myös tarkempaa tietoa epiteeliesteiden läpäisevyyden ja sen taustalla olevien mekanismien tutkimiseen.

Tässä kandidaatintyössä kehitettiin ja testattiin olemassa olevaa mittausjärjestelmää, jolla epiteelikudoksen dynamiikkaa, tarkemmin transepiteliaalista resistanssia, ja siinä eri lääkeaineiden vaikutuksesta tapahtuvia muutoksia, voitaisiin tarkastella soluviljelyn aikana eli *in vitro*. Nykyisissä menetelmissä on aukko järjestelmälle, jolla voitaisiin annostella lääkeaineita ja tutkia muutoksia epiteeliesteessä reaaliaikaisesti tarpeeksi tarkasti. Työssä testattiin injektiolaitetta, jolla voidaan annostella lääkeaineita soluja monitoroivaan automatisoituun TER-mittauslaitteeseen laitteen ollessa inkubaattorin sisällä. Tarkastelun kohteena oli injektiolaitteen toimivuus ja luotettavuus, sekä yhteensopivuus *in vitro* -ympäristöön. Laitetta testattiin injektoimalla aineita, joiden vaste on tiedossa, Madin-Darby Canine Kidney (MDCK) -solulinjan soluihin. Solut viljeltiin puoliläpäisevälle kalvolle ja niiden muodostaman epiteelin transepiteliaalista resistanssia seurattiin testauksen aikana.

Tulokset osoittavat, että injektiolaite toimii odotetulla tavalla, eikä näytä aiheuttavan häiriöitä TER-mittauksissa. Osa tuloksista on myös odottamattomia, ja ne osoittavat, että laite ei ole vielä täysin luotettava, vaan kaipaa muutamia korjauksia ja lisätestausta. Lisäksi on syytä harkita keinoja käyttömukavuuden parantamiseksi, mikäli injektiosysteemin kapasiteettia halutaan kasvattaa. Kokonaisuudessaan mittaussysteemi on tulosten perusteella potentiaalinen väline epiteeliesteen dynamiikan tutkimukseen muutamien korjausten jälkeen.

Avainsanat: Transepiteliaalinen resistanssi, epiteelieste, in vitro, epiteelin läpäisevyys, impedanssi spektroskopia, injektiolaite

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

PREFACE

I am deeply grateful to Soile Nymark and Teemu Ihalainen for trusting this thesis subject for me. It has been a delight to be able to contribute to the research of the Biophysics of the eye group under their guidance. I would also like to express my sincere thanks to Nemanja Milicevic for his invaluable support and guidance in the laboratory work and for always being available to answer my questions. Despite the twists and turns along the way, their collective support allowed me to easily adapt and refine the project. All of this resulted in a truly authentic research experience that has shaped my academic growth.

Tampere, 9.4.2024

Julia Harju

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

AC	Alternating current
AJ	Adherence junction
C_a	Capacitance of apical cell membrane
C_b	Capacitance of basolateral cell membrane
DC	Direct current
E-cad KO	E-cadherin knockout
EDTA	Ethylenediamine tetra acetic acid
PBS	Phosphate buffered saline
R_a	Resistance of apical membrane
R_b	Resistance of basolateral membrane
R_{med}	Resistance of culture media
R_p	Paracellular resistance
TJ	Tight junction
TER	Transepithelial resistance
TEP	Transepithelial potential
WT	Wild type

The abbreviations and symbols used in the thesis are collected into a list in alphabetical order. In addition, they must be explained upon first usage in the text.

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1. INTRODUCTION

Epithelium is a single cell layer present at the interfaces of different tissue compartments. Epithelial tissue is found in the gut where its most important function is the absorption of nutrients, whereas in the skin epidermis its mechanical protection properties are highlighted. Epithelial barriers vary depending on the needs of surrounding tissue and organs. [1] This thesis focuses on the role of epithelial barriers, particularly as the regulators of solute and ion passage between physiological compartments, and current methods to assess the permeability of the barrier tissue. The correct functioning of the epithelium is especially emphasized in retinal pigment epithelium (RPE), epithelium locating in the eye underneath the neural retina, and serving as the primary metabolic support for the photoreceptor cells in charge of image formation. RPE is found to be crucial for retaining sight, and dysfunction in RPE is the origin for several clinical disorders such as age-related macular degeneration and diabetic retinopathy. [2,3]

This thesis is made in collaboration with the Biophysics of the eye research group whose interest is specifically in RPE physiology. As a tissue absorbing stray light that has passed through photoreceptor layer, RPE is under heavy stress. Likewise other barrier tissues facing similar stress, it is prone to dysfunctions and diseases. [2] To treat and prevent the diseases related to barrier tissue dysfunctions, it is necessary to gain understanding of the structure and function of barrier tissues, which have great physiological roles in various organs. As the regulators of solution passage, the knowledge on barrier tissues is also crucial for the development of pharmaceutical approaches as the drugs must be able to penetrate the barrier to reach the target tissue.

The movement across barrier tissues is highly dynamic, governed by electrical and chemical differences across it. Substances move according to their electrical and chemical gradients and induce various electrophysiological phenomena in the epithelium. Transepithelial transport is widely studied to gain better understanding on barrier tissues, and common techniques exploit electrophysiological measurements and permeability assays. Transepithelial resistance (TER) measurements are commonly used to gain understanding on permeability and selectivity of barrier tissue. Differences in ion concentrations between internal and external environments of the epithelial cells and between the different sides of the epithelium result in electrical potential differences across the epithelial barrier. The measurement of TER quantifies the resistance across the epithelium thus reflecting the epithelial tightness and the ability of the barrier to counteract ion movement through it. [1]

This thesis centres in transepithelial resistance measurements as a method to observe barrier dynamics and the permeability of the epithelial barrier. Abundance of methods are available to assess the tissue integrity and electrical characteristics. However, real time monitoring of TER before and after administration of pharmaceutics or other substances to evaluate their effects in TER is still a problem to be solved and is lacking measurement systems allowing this. The current methods are either prone to errors or closed systems that do not allow changes. Measurements with living cells are highly sensitive to disturbances including changes in the in vitro environment such as temperature, pH, and the amount of physiological solution. The aim of this thesis was to develop and test an injection system that would allow administration of pharmaceutics into cellZscope, an automated cell monitoring system for TER measurements, while observing changes in TER during culture without changing the other factors that could potentially affect TER.

2. BACKGROUND

2.1 Epithelial barrier

Epithelial barrier is formed by epithelial cells attached together to generate a physical barrier that separates two different tissue compartments. It plays a vital role in regulating the environment of tissues in varying physiological conditions. The most important function of the barrier tissue is to regulate the passage of ions and solutes. Other key functions include waste secretion and the protection against toxins. Epithelial tissue barrier can be found for example in the bowel, retinal pigment epithelium in the eye and in capillary walls. [1] Dysfunctions in epithelial barrier are associated with numerous human diseases such as age related macular degeneration, [4] Crohn's disease and viral infections such as hepatitis C [1,5].

Adherent cell monolayer of the epithelia regulates ionic homeostasis of two biological compartments by vectorial transport of ions and solutes facilitated by transport proteins and ion channels [6]. The passage of solutes across the barrier can happen via two routes; active transcellular route, where passage of solutes is controlled by transmembrane proteins of both sides, or in passive paracellular route, through selective gates between the cells. As a passive route, paracellular transport requires a driving force such as an electrical potential difference, hydrostatic pressure, or a concentration gradient. The control of the passage across the epithelial barrier is very dynamic consisting of complex cascade of biochemical signalling and crosstalk between the two pathways. [1]

The cells composing the epithelial barrier exhibit polarization, meaning that the components of the cell membranes differ on either side of the barrier. The surface facing the outside compartment of organism is called apical surface, and the surfaces facing the inside compartment of the organ (basal surface) and neighbouring cells (lateral surface) is often referred to as basolateral surface. [6] The polarization is a precondition to proper functioning of the barrier tissue and is possible due to complex intercellular junctions, most essential of which being tight junctions (TJ). Tight junctions work as a fence that restrict the diffusion of membrane lipids and proteins forming a continuous intercellular barrier that separates apical and basolateral sides of epithelia. [5] Another intercellular junction working closely together with TJ's is adherens junction (AJ) that establishes strong adhesive interface between the cells [7]. Other intercellular junctions include gap junctions exchanging ions and small molecules between adjacent cells via cytoplasmic connection, and physically anchoring desmosomes [1].

Together with adherens junctions, tight junctions form complex network of strands that are connected to the cytoskeleton of the cells and form close contact points with the strands of neighbouring cells [1,7]. These contact points result in so-called kissing points appearing in the membrane interface [1]. As a barrier separating different compartments, tight junctions function as size and charge selective gates for paracellular diffusion of solutes. [5] The paracellular pathway can be further distinguished into charge selective pathway and size selective “leaky” pathway that differ in mechanisms and regulation. The charge selective pathway is mediated by junctional intra membrane strands forming ion-selective channels that are regulated to open and close. However, the precise mechanisms of the size selective pathway are still under debate, but it has been suggested that it may occur in stepwise manner, as the macromolecular diffusion is slow. [5] TJs have a rather complex protein composition compared with other intercellular junctions, as many as 40 proteins have been found in the protein composition of junctional strands. However, the claudin protein family is highly expressed in tight junctions and studies have shown that claudins have central role in controlling the paracellular, specifically, the size selective pathway. [5,8]

2.2 Epithelial barrier dynamics

Understanding of the tissue dynamics is vital for grasping the complexity and purpose of collective behaviour of cells within tissues. As highly dynamic structures, epithelial barriers undergo constant chemical and mechanical interaction, cell renewal and differentiation, and maintain organization. [9] The dynamics of epithelial barriers is orchestrated by junctional complexes, biochemical and mechanical cues, and cytoskeleton connected to the extracellular matrix [1]. The highly dynamic barrier tissues are constantly studied, and barrier models are developed to understand the key developmental and physiological processes within tissues [9]. The dynamical nature of the epithelia is also reflected in the diverse range of functions carried out by junctional complexes. Tight junctions have been shown to possess a range of different functions including contributing to cell polarization, performing as a signalling platform, and enabling paracellular passage [5]. Similarly, adherens junctions, in addition to adhesion, have been shown to have a role in regulating morphology and cell division, while also exhibiting mechanosensitivity [10].

As active systems, cells can sense mechanical stimuli, demonstrating their capability of mechanosensing. [10] The ability of cells to detect and react to mechanical cues is essential for tissue remodelling, as mechanosensing has been shown to play a crucial role in cell migration and the promotion of junctional remodelling and polarization. [10,12] Applied shear stress has also been shown to affect the permeability of barrier tissue, indicating the significance of mechanosensing in tissue homeostasis. [1] These mechanical dynamics are carried out by junctional complexes, primarily AJs, TJs, and desmosomes, composed of proteins that are connected to the cytoskeleton. Key proteins, which have been the focus of numerous studies, include zonula occludens, occluding, tricellulin, claudins, and cadherins. [12,13]

Years of studies have also shown organisms to possess circadian clock, regulating properties, such as transepithelial resistance, and the function of tissues, as well as many physiological processes. [11] The 24-hour circadian rhythm is established by genes whose expression cycles approximately every 24 hours [14]. While light signals have shown to be the primary environmental cue affecting the circadian clock, a study by Balsalobre et. al. (1998) revealed that so-called serum shock induces the circadian expression of various genes [11,14]. However, these are only few dynamic processes briefly highlighted here that are relevant to this thesis, and there is much more to cover.

2.3 Transepithelial resistance (TER)

A common method to assess epithelial barrier dynamics is transepithelial electrical measurements. Between the two sides of the epithelial barrier is electrochemical gradient, the combined influence of charge and concentration difference between the apical and basolateral side. The electrochemical gradient drives the movement of ions across the cellular membrane. This movement results in generation of transepithelial potential (TEP). [2] The barrier monolayer resists this ion movement reflecting its capacity to slow down the flow of ions and to allow it only selectively. This resistive property is known as transepithelial electrical resistance (TER), the electrical resistance across the cell layer and a sum of paracellular and transcellular resistances. [1] Transepithelial resistance serves as a measure of ion permeability providing information of the electrical characteristics of the epithelial tissues. It is a common method to non-invasively assess tissue integrity and barrier function. [1,15]

The tightness of epithelial barrier tissues, which is determined by the quality and quantity of their tight junctions, plays a crucial role in TER. There is a direct correlation between the tightness of the epithelial cell layer and its electric resistance, and epithelial tissues can be categorized as 'leaky' or 'tight' based on their TER values. Tight epithelia have high TER values, indicating low permeability and high selectivity for solutes. [1,8] An example of tight epithelium is Retinal Pigment Epithelium (RPE) that has TER value among the largest found in the human body [16]. On the contrary, epithelia responsible for the swift transport of ions and water typically have a lower paracellular resistance classifying them as leaky barriers. An example of a leaky barrier can be found in the kidney, in the proximal tubule of the nephron in charge of reabsorption. [1] In addition to TJs, TER value is affected by multiple factors that can result in wide range of TER values for the same cell type and it is crucial to acknowledge and address this variability. These factors include physical factors such as temperature and shear stress, as well as factors related to cell culture such as passage number, used culture inserts, medium formulation, and culture period. [8,15]

2.4 TER measurement techniques

To measure TER, epithelial cells are usually grown on semipermeable inserts mimicking the interface between tissue compartments and two electrodes are placed on the different sides of the cellular sheet so that one is in the basal and one in the apical extracellular space [17,18]. Commercial TER measurement systems are based on two mechanisms relying in different physical principles to measure TER: Ohm's law and impedance spectroscopy. To obtain the resistance across the cell layer from the read values of the device, electronic characters of epithelia can be approximated to equivalent circuit and the corresponding mathematical models allow extracting parameters mirroring barrier properties. [1,18] The unit in which TER is represented is $\Omega \cdot cm^2$ [19].

TER can be measured by applying Ohm's law and calculating ohmic resistance as the ratio of the voltage and current. In this method, direct current (DC) or single frequency alternating current (AC) is directed to electrodes to measure the resulting voltage. [15] Common measurement system using Ohm's law method is handheld chopstick electrodes that are placed in the apical and basal compartments and current is passed via a volt/ohm meter. To obtain the resistance across the cell layer, two measures are needed: one from an empty insert and the other from an insert with cultured cells. Resistance measured from the empty insert is then extracted from the resistance of the cell culture insert yielding the remaining TER. [8,15]

More accurate representation of TER can be acquired with impedance spectroscopy. In the impedance spectroscopy a small amplitude alternating current with wide spectrum of frequencies is applied to the barrier and the electrical response is measured in terms of impedance. [15] The concept of impedance can be simplified as the resistance encountered by the alternating current [20]. The obtained impedance data can be analyzed and interpreted using equivalent circuit models containing resistances and capacitances as described in Figure 1. In the equivalent circuit model, the apical and basolateral membranes are represented in proximity to capacitors (C_a, C_b), due to the insulating nature of the lipid bilayer, and the ion permeabilities of the apical and basolateral membranes, constituting the transcellular route, are associated with resistances (R_a, R_b). The ion permeability of the paracellular route can be equated with a resistance (R_p) in a parallel coupled pathway. In addition, the resistance (R_m) of the media is usually added in the circuit to acknowledge external factors between the electrode and the cell layer. [1] In contrast to the Ohm's law method, impedance spectroscopy recognizes that capacitors become increasingly conductive with increasing frequency. In principle, under AC conditions at high frequencies the capacitator's impedance becomes very low short circuiting the membrane resistor. Capacitator then causes a lag phase in voltage and current. [20] With impedance spectroscopy, the transcellular capacitive pathway can even be distinguished from the paracellular pathway by using two-path method [19].

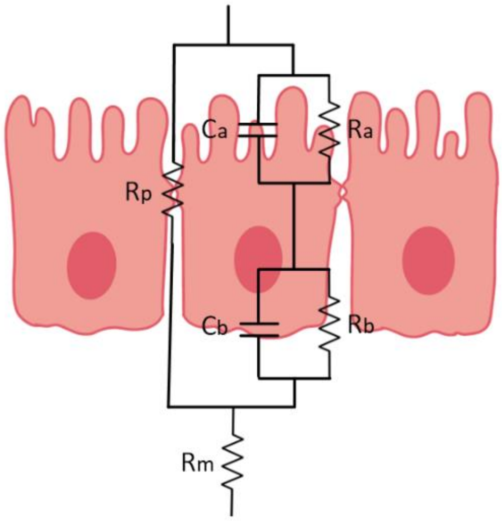

Figure 1. *Equivalent electrical circuit model for an epithelial cell layer. R_p, paracellular resistance; R_a, apical resistance; R_b, basal resistance; R_m, Resistance of culture media; C_a, apical capacitance; C_b, basal capacitance. Adapted and redrawn from [1]*

TER measurement is a widely used and approved method, as it serves as a valuable tool to monitor live cells through different stages of growth and differentiation non-invasively [15]. It is also a label-free method to track tight junction functionality [18]. Depending on the aim of the study there are multiple methods to measure TER. For assessing tissue integrity, a slightly less accurate representation of TER is sufficient whereas assessing tissue specific TJ strength requires more accuracy from the measurement technique. It is crucial to understand the measurement setup and technical parameters of the system to correctly interpret and compare TER values [19]. Measurement systems vary from handheld chopstick electrodes coated with Ag/AgCl to automated TER monitoring systems with microelectrodes [18]. There are several commercial devices from different manufacturers to fulfil different needs, some of them enabling quick measurements manually and some offering high throughput data. In Table 1 some commercial TER measurement systems are compared based on their physical principle, advantages, and disadvantages.

Probably the best-known measurement systems utilizing ohm's law as a physics principle are products by World Precision Instruments using epithelial volt/ohm meter EVOM™ series complemented with either chopstick electrodes or EndOhm chamber. With chopsticks, TER can be measured directly from the cell culture. In EndOhm the cell culture insert is placed into the chamber that includes circular disc electrodes in the top and bottom compartments. [8,18] Another popular technique for TER measurements is a Ussing chamber, an apparatus developed in 1950s by the Danish biologist Hans H. Ussing. It is a high accuracy device and it vastly increased knowledge on epithelial transport mechanisms in its time. Many manufacturers offer Ussing Chambers with different features and personalization while maintaining the main features of the original device. [1]

Electrical impedance spectroscopy is a common physics principle in so-called second- and upper-generation devices [18]. The device used in this thesis project, CellZscope system by NanoAnalytics, is based on this principle and it measures electrical parameters from cell monolayers cultured on inserts. The inserts are placed in titanium wells and the lid with circular disc electrodes is placed on top. The impedance spectra are automatically measured and mathematically analyzed. [19] A further application to electrical impedance spectroscopy is electrical cell-substrate impedance sensing (ECIS®) by Applied BioPhysics. Cells are cultured on the integrated gold-film electrodes resulting in high sensitivity measurements. [18]

Table 1. *Comparison of commercial TER measurement systems*

TER measurement system	Physics principle	Advantages	Disadvantages	Ref
Ussing Chamber	Ohm's law	+ Uniform current distribution + Enables other types of measurements, such as monitoring of diffusion + Allows a great degree of flexibility and customization	- Challenging setup, methodology and interpretation of results - Limited number of samples that can be studied simultaneously	[1,21]
EndOhm + EVOM™ (World Precision Instrument)	Ohm's law	+ Creates a uniform current density and thus provides more reliable data and better reproducibility of TER values compared to handheld chopstick from the same manufacturer + Closed system; minimizing environmental effects on measurements	- Inserts need to be moved into the chamber which may cause disturbances to cells and require stabilization time - Does not allow continuous long-term monitoring	[8,18]
STX 2 Handheld chopstick electrodes + EVOM™ (World Precision Instrument)	Ohm's law	+ Allows quick measurement manually without removing inserts from the culture wells + Simple and readily accessible	- Current is not uniform - Allows measurement only across a small section of the monolayer - Poor reproducibility - Elevated risk of contaminations or disturbances to the cells	[8,18]
CellZscope (NanoAnalytics™)	Impedance spectroscopy	+ Acquires capacitance in addition to TER + Allows continuous long-term monitoring + Easy to use and interpret results, mathematical analysis is done by the software	- Closed system doesn't allow modifications during the measurement - Takes a while to stabilize when starting measurements	[18,19]
ECIS® (Applied BioPhysics)	Impedance spectroscopy	+ Can be used to study single cell's electrical behavior + High sensitivity, reproducible measurements	- Inability to study transport phenomena due to absence of basolateral fluid compartment - Complex theory, setup, and data interpretation - Requires cells to be grown on special inserts provided by the manufacturer.	[1,18, 22]

2.5 The aim of the study

The aim of the study is to further develop and test an injection system developed to minimize the environmental factors such as temperature changes, gas composition changes resulting in pH changes, and the effects of moving the system and causing disturbance to cells. The injection system allows the chemical treatment of cells while maintaining relatively unchanged conditions. The aim is to test the device with living cells and to see the effects in TER when injecting biochemicals whose expected effects on TER are known. The cells are treated with EDTA that is known to disturb TJ proteins by reducing the levels of Ca^{2+} and Mg^{2+} ions, as well as causing oxidative stress i.e. disturbance in the balance of free radicals [23,24]. To see if the injection system itself affects the cells or TER, the cells are also treated with PBS i.e. saline which should not cause any effect, and the possible effects would be caused by the injection system.

The used cell line is MDCK (Madin-Darby Canine Kidney), that is an epithelial cell line originally derived from normal dog's kidney. MDCK is a common cell line used for studying epithelial development and function since the cells form a monolayer of closely packed cells and retain epithelial characteristics such as transepithelial transportation. [25] There are multiple strains of MDCK, and the strain used in the experiments is MDCK II strain, a strain isolated from high passage MDCK cells. The strains vary in their tight junction composition and demonstrate different tightness of the barrier with MDCK II having low TER values (< 300 $\Omega \cdot cm^2$) compared to MDCK I strain, for example, that demonstrates tighter barrier with TER values over 4000 $\Omega \cdot cm^2$. [26]

In this study, two different genotypes of MDCK IIs are observed; MDCK II wild type cells (WT) with no changes in genotype and MDCK II E-cadherin knockout cells (E-cad KO) with E-cadherin gene removed. E-cadherin forms adherens junctions in epithelial cell layers, thus knocking the gene out results in weaker adhesion strength. Ability to form tight junctions remains, but the E-cadherin knockout cells were observed in this study out of the interest to see if there are any differences in the effects of treatments. [12]

3. MATERIALS AND METHODS

3.1 Cell line

The cell line used in the study was epithelial MDCK (Madin-Darby canine Kidney) cell line originally derived from normal dog's kidney. The cell line was type II MDCK line with two genotypes: wild type and E-cadherin knockout. Cells were cultured with Gibco™ MEM with GlutaMAX™ medium supplemented with 10 % FBS and 1% Pen Strep. For the experiment, both cell lines were seeded separately on the apical side of the Sarstedt™ 24 well PET transparent inserts with 1µm holes, at a density of 1 x 10^5 cells/ml. Prior to seeding, the membranes were coated with rat tail collagen type I. The coated well plate was left to sterilize for 30 minutes in UV light. To achieve the wanted density of cells the cells were counted with haemocytometer and the cell suspension diluted to wanted cell density with culture media so that the density of 1 x 10^5 cells/ml was in the 300 µl volume of media on the apical side of the insert. Total of 24 inserts, 12 inserts of each cell types were prepared and incubated (37˚C and 5% CO_2) on Costar™ 24 well culture plate with 300 µl of culture media on the apical side and 700 µl on the basal side.

3.2 Measurement setup

Transepithelial resistance measurements were carried out using cellZscope2 (NanoAnalytics, Münster, Germany), automated, computer-controlled cell monitoring system that includes 24-well cell module, controller and computer run software. CellZscope allows undisturbed, long-term monitoring of various barrier forming cells cultured on semipermeable inserts. The device measures real time values of the transepithelial resistance (TER) and capacitance (C_{cl}) of the cell layer by recording the frequency-dependent impedance (Z) when applying a small alternating current (AC) voltage (V) through the electrodes in the upper and lower compartment. [27]

The aim of the experimental work was to test and modify an injection system developed to be compatible with the cellZscope. The injection system allows the chemical treatment of cells while maintaining unchanged conditions. This way drugs, for example, can be injected into the cellZscope during ongoing measurement without the need of opening the lid of the device or moving it to laminar hood, thus avoiding stress to the cells as well as modifications to the system's conditions such as temperature changes that are known to affect TER.

The basic principle of the function of the device is to pressurise the preloaded system with air so that the loaded liquid is released into the desired wells of the device. The system consists of small tubings for delivering the drugs and bigger 'airway tubings' to pressurise the system. The system is built so that the small delivery tubings are secured to the magnetically attached electrode bars on the lid of the cellZscope cell module using zip tides. The open end of the tubing is fixed close to the electrode of the desired well and curved to 90 degrees angle to precisely deliver the liquid inside the insert. The way tubings are fixed into the cellZscope is better depicted in the Figure 2. There is total of six delivery tubes that allow drug administration to six wells.

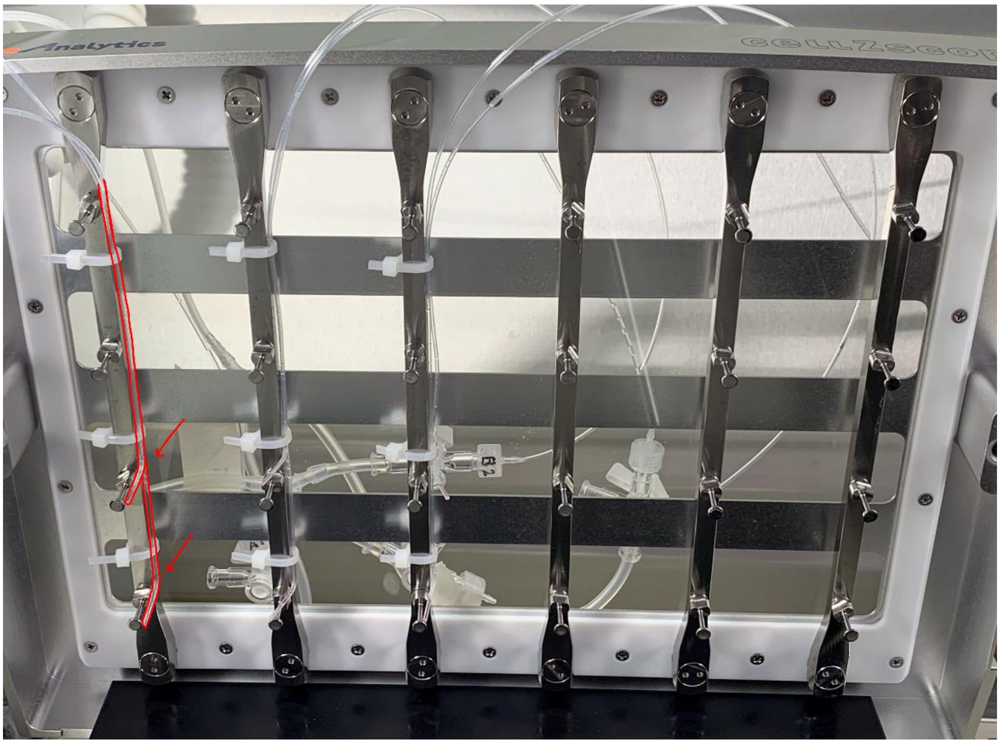

Figure 2. *Photo of the system's delivery tubes.*

The other end of the delivery tubing is connected to three-way tap that allows switching routes in between loading and releasing the liquid. One of the routes of the tap is open and the entry is used as the site to place pipette tip and inject the desired liquid into the small delivery tube while keeping the airway closed. The third route is connected to air-injecting tubes, and this allows switching routes between loading and releasing the liquid. These air injecting tubes are connected to 25 ml syringe, which can be loaded with air and left outside the incubator. The piston can then be pushed down at the time of injection. When preparing for the injection, the syringe should be loaded with air and the tap in the tip of the syringe connecting it to the airway tubing should be closed. When loading the injection system with a drug the three-way tap should be turned so that the airway is closed, once the administration is done and the liquid is in the injection tube the tap can be turned so that the airway is open. With the tap turned this way, the cellZscope can be placed in the incubator to wait the time of the injection, which can be now done any time by opening the tap of the syringe and by pushing down the piston. This air pressure in the whole tubing system leads to the release of the liquid in the each well's delivery tubes into the wells.

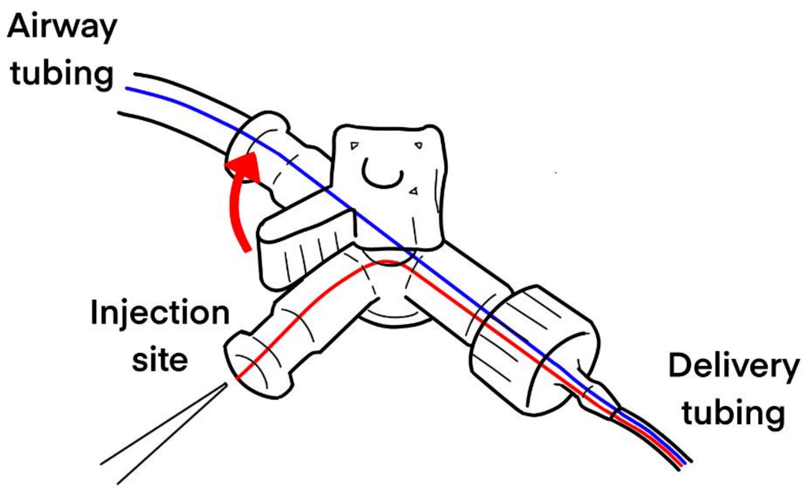

Figure 3. *Illustration of the three-way tap for altering between two routes. When administering the loaded liquid into the wells the tap should be in the position shown in the illustration and the route marked with blue is open. When loading the delivery tubing the red route should be open, and the tap turned the way shown by the red arrow.*

For the experiments, the original prototype of the injection system was modified. The original system had three delivery tubes and now three additional tubes were added. Originally, there was one tubing attached to each electrode bar, but after the alterations, two tubes were fixed into one electrode bar allowing injection to total of six wells. The addition of injection channels also required additional airlines and some modifications ensuring easier handling of the device. The delivery tubes were marked to avoid mixing up the tubes and ensuring that the administration is done to the right well.

Before the experiments, the cellZscope was properly sterilized by autoclaving the autoclavable parts and by washing other surfaces with washing liquid and then wiping with 70 % EtOH. The tubing system was disassembled, and the parts were rinsed first with soap water, then multiple times with water and finally with 70 % EtOH. The parts also received 30 minutes of UV-light and were left to dry in the laminar hood.

3.3 Measurement procedure

The experiment was divided into two measurement periods with genotypically different test groups. Since the injection system is limited to six wells at the same time, the cell lines had to be monitored in two batches. The idea was to get data on the effect of the injection system to the cells. To test this, the TER values of three different setups with three replicates were observed. Out of the six wells equipped with injection tubing, three were loaded with PBS and another three with EDTA. These were compared to measurements from three inserts containing no tubing and thus, remaining untreated. The tubes were loaded with EDTA solution that was prepared so that the final concentration would be 5mM, known to sufficiently cause effects in TER.

The wild type MDCK II cells were observed first. Nine wild type inserts were placed into a 24-well cell module of the cellZscope system five days after seeding the cells on the inserts. 700 µl of culture media was added on basal side and 300 µl on apical side of the inserts in the wells. Additional empty insert without cells but the same amount of media was added as a reference for the cellZscope measurements. Six of the WT inserts were placed in the wells containing injection tubing. Three of the wells containing tubing were loaded with 50 µl of PBS and the other three with 50 µl of EDTA solution. The system was then placed in the incubator and TER measurements started with 15-minutes set as the wait time in between the measurements. The development of TER was observed for 23 hours to make sure the system was stabilized before the injection experiments. The injection was done approximately at 23-hour mark, and WT cells were then observed with cellZscope for additional 44 hours. Measurement was stopped, and after disassembling cellZscope, the inserts that had received treatment, were fixed with 4 % PFA (paraformaldehyde) for future staining and further observations.

The next test group observed was E-cadherin knockouts (E-cad KO). 8 days after seeding all twelve prepared KO inserts were placed into the cell module of the cellZscope. Similar to the first experiment series, 700 µl of culture media was added on basal side and 300 µl on apical side of the inserts in the wells. Additional empty insert with the same amount of media was again added. The inserts were placed into the wells so that E-cad KO cells in observation received the injection tubing and the treatment. The injection system was again loaded so that three of the inserts would receive 50 µl of PBS and other three 50 µl of EDTA solution. The system was then placed in the incubator and TER measurement started with 15-minutes set as the wait time in between the measurements. Measurements were running over 2 days, and the injection was done approximately at 70-hour mark. The E-Cad KO cells were then observed for 47 hours before stopping the measurement. The inserts that had received treatment were fixed with 4 % PFA similarly to the first experiment.

4. RESULTS AND DISCUSSION

The tests showed that the injection device functions correctly, but there were some unexpected results. The results are presented in Table 2. and unexpected results highlighted with red. CellZscope analyses the data automatically and the data was exported to Excel. For the comparison of the effect of the treatments average TER values before and after injection time were inspected. In Table 2. the TER values from the wells containing injection tubing loaded with PBS (wells A1-A3) and the wells containing injection tubing loaded with EDTA (wells B1-B3) are compared with a well with untreated inserts which did not contain tubing. The two different genotypes, WT and E-cad KO were not compared due to differing observation periods and injection times; furthermore, it was not relevant for the purposes of this study.

Unexpectedly, in both test groups, the well A3 loaded with PBS caused a drop in TER. Injection of PBS was expected to not have effects. The cause of the drop in TER is unknown, but possible causes could be that the tubing was accidentally loaded with EDTA instead of PBS, or that there are some toxic residuals inside the tube. However, the reason behind the drop in TER has most likely something to do with the tubing connected to A3 well. The reason remains unknown, but one possible explanation could be residuals of cytotoxic substance inside the tube. Another unexpected result occurred in the test group with E-cad KO cells. In the well B1 loaded with EDTA the drop in TER was not observed. This was most likely due to unsuccessful injection. There were problems with the stability of the device and the tubes are easily detached. If the tube is detached the air pressure will not push the drug into the system and it stays loaded in the tubing. Apart from these outliers the device seems to function correctly, and injections are successful with expected results. It can also be noted that there are differences in the TER values before the injection inside each group. These differences can be explained by individual inserts' different reactions to media exchange and by the stabilization of the system.

Table 2. *Calculated averages of TER before and after injection time in both experiments. Unexpected results are highlighted in red.*

Test group	Well	Treatment	Mean TER ($\Omega \cdot cm^2$)	
			Pre-treatment	Post-treatment
WT MDCK II cells	A1	PBS	51.00	40.55
	A2	PBS	49.53	39.51
	A3	PBS	37.73	21.82
	B1	EDTA	42.94	11.49
	B2	EDTA	42.04	15.29
	B3	EDTA	72.62	12.88
	A4	Control, no tubing	49.03	38.17
E-cad KO MDCK II cells	A1	PBS	48.31	36.90
	A2	PBS	41.85	35.87
	A3	PBS	34.71	17.71
	B1	EDTA	48.57	34.03
	B2	EDTA	42.58	14.12
	B3	EDTA	40.51	20.86
	A4	Control, no tubing	41.70	35.58

The results of the first experiment series, carried out using the WT cell line, are depicted in Figure 4. Mean values, as shown in Table 2 were analysed with repeated measures by two-way ANOVA using GraphPad Prism 10 software. Figure 4a illustrates that treatment with EDTA results in an anticipated drop in TER, with no significant difference observed between untreated cells (No injection) and cells treated with PBS. Holm-Šídák's multiple comparisons test revealed a significant difference in EDTA treatment when comparing values before and after treatment (p=0.0015). In contrast, the difference in PBS treatment was not statistically significant (p=0.2448), and similarly, no injection demonstrated no significant difference as well (p=0.1695). The Figure 4b illustrates changes in TER as time progresses, showing changes in TER in different treatment groups. In the beginning of the measurement TER is elevated, which is a typical phenomenon after media exchange and application of fresh serum causing a so-called serum shock. This phenomenon can also be seen in Table 2, in the test series with WT cells, where in the well B3 average TER before treatment was significantly higher. There were variations in the response to the serum shock observed among individual samples. Figure 4b reveals a similarity in the behaviour of PBS-treated and untreated cells, while EDTA-treated cells exhibit a drastic drop in TER instantly after the injection is performed.

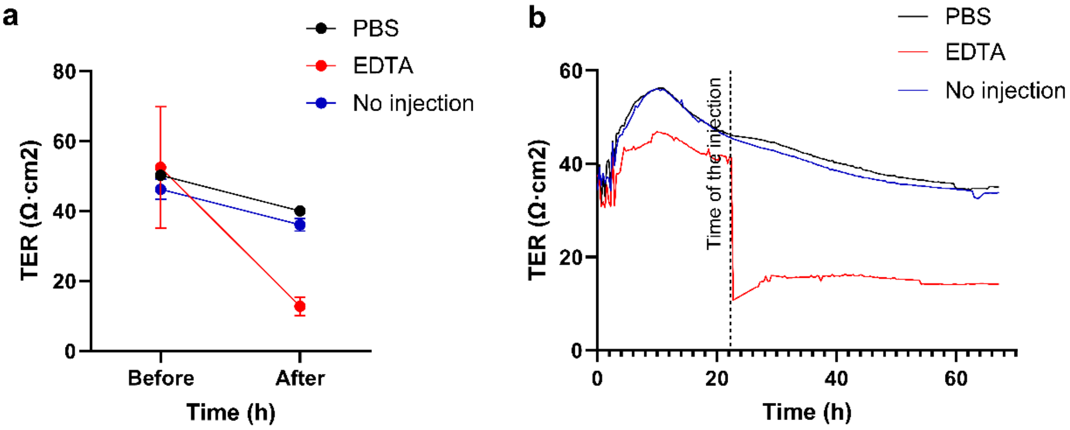

Figure 4. *Responses to treatments in Wild type MDCK II cells. (a) Statistical analysis was performed with two to three biological replicates using means of TER of before and after treatment. The effect of time and treatment on TER were assessed by repeated measures two-way ANOVA. The test showed that there was a statistically significant interaction between the effects of time and treatment (p=0.0377) as well as statistical significance in the effects of time (p=0.0055) and treatment (p=0.0152) to TER. Graph shows the mean and error ± SD, TER values of each group are shown as dots. (b) Graph shows TER measurement data from three individual replicates and the timepoint of injection is marked.*

Figure 5 depicts the results of the second test group, similarly to the pattern seen in Figure 4. Notably, E-cad KO cells showcase behaviour comparable to WT cells. It is worth mentioning, that the observation period was longer in this experiment. In contrast to the first experiment, the effects of the serum shock observed in the first experiment was evident only in one insert. Holm-Šídák's multiple comparisons test performed on data from E-cad KO cells revealed a significant difference in EDTA treatment when comparing values before and after treatment (p=0.0008). No injection demonstrated no significant difference (p=0.0616), but PBS treatment was shown to be statistically significant (p=0.0293) in this instance. When looking at the Figure 5b TER seems to continuously decrease without reaching a clear plateau, indicating an ongoing evolution toward lower values which may explain the unexpected significance of PBS treatment in multiple comparison test. The single observation of serum shock mentioned earlier was seen in PBS treated cells. This elevation in TER significantly impacts the mean, as illustrated in Figure 5a, where the corresponding error is also evident. The similar occurrence can also be seen in Figure 4a where one of the EDTA treated inserts displayed the effects of the serum shock.

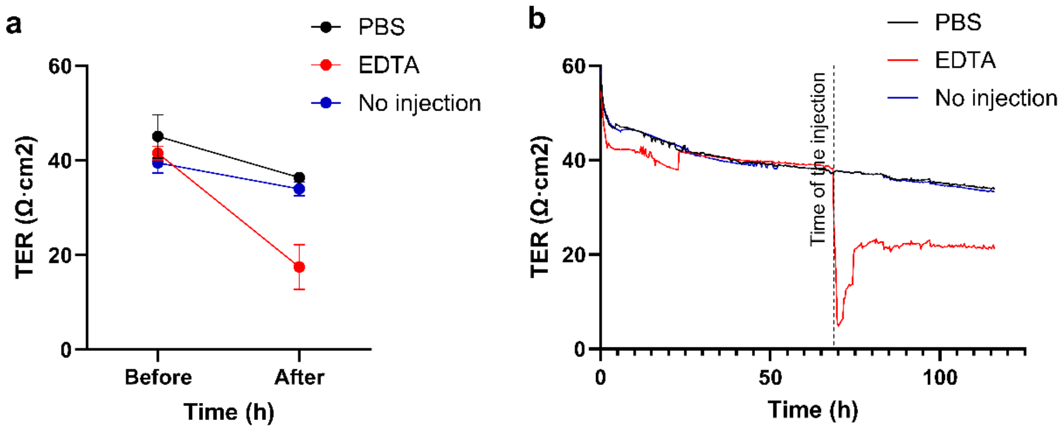

Figure 5. *Responses to treatments in E-cadherin knockout MDCK II cells. (a) Statistical analysis was performed with two to three biological replicates using means of TER of before and after treatment. The effect of time and treatment on TER were assessed by repeated measures two-way ANOVA. The test showed that there was a statistically significant interaction between the effects of time and treatment ($p=0.0123$) as well as statistical significance in the effects of time ($p=0.0009$) and treatment ($p=0.0124$) to TER. Graph shows the mean and error ± SD, TER values of each group are shown as dots. (b) Graph shows TER measurement data from three individual replicates and the timepoint of injection is marked.*

Based on the results, there are improvements to make to the device. The primary issue is the instability of the tube attachments. This is due to slight incompatibility of the parts resulting in easy detachment. A possible solution could be adding elastic silicon tubes as sealing parts at the connection points or match the tube sizing better. Secondly, the materials of the device present some challenges, as the airway of the device is currently assembled from scrap parts whose exact materials are unknown. This lack of knowledge presents a problem for cleaning the device, as the reaction of these materials to different chemicals is uncertain. It would be advisable to establish a cleaning protocol for the device to avoid any residuals or contaminations in the tubes when the injection device is re-used.

Furthermore, it would be desirable to upgrade the number of wells that could be injected in. However, achieving this would possibly require a bigger volume of air to pressurise the device, as well as coming up with solutions to make the device easier to handle, as it is already challenging to work with. Aligning multiple tubes inside the inserts when placing the lid on cellZscope device requires precision. Moreover, the crossing tubes and multiple taps serving as loading sites are prone to mix-ups. The taps are currently fixed on place with tape, but alternative methods should be considered. One suggestion for enhancing the user-friendliness of the device would be to incorporate a rack designed to be placed on the lid of the cellZscope, where the taps could be attached.

An even more significant update would be enabling the injection device to perform multiple injections in one run. By far reloading the device is only possible simultaneously with the medium exchange required by cells every 4 days. Achieving this would likely require more substantial alterations to the fundamental design of the device.

5. CONCLUSIONS

The goal of this thesis was to further develop and test a TER measurement setup for in vitro measurements with drug injections. A device that would make long term observations of TER while testing effects of pharmaceutics is sought after, as there is currently lack of measurement system that would allow administration of pharmaceutics while other environmental factors remain unchanged.

The injection device tailored for compatibility with cellZscope was modified and then tested in this work using two genotypically different epithelial cell lines. The correct function of the device was assessed based on changes in TER, confirming that injection device successfully performs injection while the presence of the device has no effect on TER. The results demonstrate correct functionality of the system, showing promising potential for further development. With the capabilities for drug testing during real-time observations, this device can be a valuable tool for advancing research. However, a few adjustments are required to improve the precision, functionality, and user-friendliness of the device.

Future directions for the device would be to fix the problems causing instability in the results and to continue testing the device. Improvements towards more user-friendly and functional device would be reinforcing the integrity of the tube connection points and organizing the injection sites into a more organised layout. Good act would be to conduct tests to assess the precision of the device, as well as to determine the minimum and maximum injection capacities. Should the device be reused, it is advisable to develop a method for adequately cleaning the device between experiments. By taking care of these points the device is promising asset for studying epithelial barrier dynamics.

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