A Thesis
For the Degree of Doctor of Philosophy

Kidney on a Chip Development and Drug Efficacy Testing for Renal Hypoxic Reperfusion Injury

GRADUATE SCHOOL
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Department of Mechatronics Engineering

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(Supervisor: Prof. Dr. Kyung Hyun Choi)

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Preamble

This thesis is submitted for partial fulfillment of the requirements for the degreeof Doctor of Philosophy in the Department of Mechatronics Engineering at Jeju National University, Republic of South. All the research work presented here was established inAdvanced Micro-Mechatronics Lab at Jeju National University. No content or segmentof this thesis has been submitted for any degree at any other institution or university. This thesis work is original according to the best of the author's knowledge unless referenceis taken from related work published. Content from this thesis has already been published in 4 research articles in international peer-reviewed journals listed below.

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My thesis is dedicated to my beloved parents, daughter, and siblings who have sacrificed a lot giving me what they couldn't have in their lives, And Dr. Ansuja, Dr.Nilesh Kumar Meghani and Dr.Afaque Manzoor Soomro for helping me to fulfill my PhD dream

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During my school days, teachers usually ask the students, what is your ambition in your life? I always gave just one answer, which was to become a scientist. All my fellow mates made fun of me for what I said, but I had my teachers as support, strength, motivation and confidence. Thanks to them, I have reached so far with their help. Now that I am completing my PhD degree at Jeju National University and being a part of a scientific community group, my childhood dream is about to come true. I firmly believe that in this world, "everything is possible, nothing is impossible", but you need to believe, be passionate, have confidence, and work hard to make your dreams come true. My family has always been very supportive and helpful to me, and now that I am finishing my PhD, it is all thanks to their prayers.

After finishing my Master, I kept looking for PhD positions. Still, due to my family's financial situation, I had to take on odd occupations as a shopkeeper, butcher, labourer, driver, microbiologist, teacher, and farmer. I got different experiences with different people and job areas; these jobs made me strong. Even though I was doing other jobs at those times, my ambition was still alive, and I didn't lose hope. I am grateful that this PhD degree has driven me directly to my career. I want to thank Allah for always protecting me and helping me unconditionally. Dr. Ansuja was the person who held my hand to begin this journey of my research career in South Korea. Not to be forgotten, Dr. Nilesh, you opened the gates so that I can achieve my dream. I'd be forever grateful to you. Thank you for believing in me and my potential and also for your assistance and guidance.

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Abstract

The microphysiological system (MPS), dynamic complex cell culture technology has the ability to mimic the human pathophysiological system. The main goal of MPS is to recreate physiologically relevant human diseases to improve drug efficacy and unknown mechanism of disease conditions. The significance of MPS is to mimic the human pathophysiological system to replace animals, patient-relevant data, high precision-low toxicity, and save money and time. Moreover, animal models have limitations to mimic human physiology and human disease conditions. The MPS has the capacity to perform conventional bioassays including western blot, ELISA, qRT-PCR, flow cytometry, biochemical assays and embedded sensors assays. Human kidneys are responsible for filtration, filtering blood and removing waste materials, the kidney is the vital organ for drug screening, efficacy, and toxicity studies. The hypoxic condition can affect renal function, predominantly homeostasis, blood pressure, the balance of electrolytes, body fluids and pH. Finally, the hypoxic condition leads to chronic kidney disease (CKD) and other organ diseases as well. The renal hypoxic condition is challenging to perform in an animal model due to the lack of a human pathophysiological system, ethical concerns, intraspecies genetic variations, lack of robustness, and time and money. This thesis work encloses the advancement of the microphysiological system for renal hypoxic reperfusion injury model and drug efficacy test to overcome the current limitations of an animal model. Renal MPS was incorporated with impedance-based transepithelial-endothelial electrochemical resistance (TEER) for real-time monitoring of renal normoxic conditions, hypoxic conditions, and drug efficacy. Furthermore, TEER data was incorporated with conventional assays for further validation. This thesis can serve as a replacement for animals to improve drug discovery and disease models.

1. Objectives of Thesis

The key objectives in the present thesis emphasize the advancement in the microphysiological system for the renal hypoxic reperfusion injury model development using primary human renal proximal tubule epithelial cell (RPTEC) and primary human umbilical vein endothelial cell (HUVEC) were cultured on the apical and basal side of the porous membrane equivalent like human pathophysiology system embedded with TEER sensor. The TEER gold electrode was employed in a microfluidic glass chip to accelerate the continuous analysis of hypoxic and treatment conditions. The renal hypoxic reperfusion injury model was established using deoxygenated media and oxygenated media at specific intervals of time and the drug was administered before the reperfusion injury to check the protective effect of drug efficacy. Moreover, continuous TEER measurement collected both apical and basal areas and correlated with molecular assays. The advancement in MPS platform may support to develop different disease models and conditions, drug efficacy, drug mechanism of actions, and toxicity evaluation better ,quicker and cost effective manner compared to current 2D and animal model. The MPS platform will be the best suited for an animal replacement for ADME-Tox (absorption, distribution, metabolism, excretion and toxicity) and pharmacokinetic (PK) and pharmacodynamics (PD) for drugs evaluation.

2. Introduction

To better understand human physiology for disease and drug assessment, it is imperative to replicate the complexity of human tissues and organs in a lab setting. Beginning in the late 19th century, scientific cell culture was first practiced, and the "Petri dish" became the accepted medium for two-dimensional (2D) cell culture[2]. The current in vitro two-dimensional (2D) cell culture and in vivo animal models remain inadequate for an effective and precise preclinical assessment of drug toxicity and efficacy prior to the approval of clinical trials for testing in human subjects [3]. Recently, advanced three-dimensional (3D) culture tools have been developed using cell culture techniques to mimic human tissues, organs, and organ functions at the nanoscale and microscale[4-7]. Tumor microenvironment (TME), Cancer, rare diseases and viral infected diseases continue to be major obstacles for humanity to overcome, but this can be done by creating the best possible progress in our ability to replicate human pathophysiology[7]. In microphysiological systems (MPS), human pathophysiology can mimic on a microscale and nanoscale form. The ultimate objectives of the study's purpose and target tissues or organs determine the creation and design principle of MPS. The platforms for the 3D cell culture are Transwell ™, spheroids plates, organoids dishes or chips, Organ-on-a-Chip (OoC), organotypic culture, air-liquid interphase methods, microfluidic culture systems, and cell sheets for the purpose of demonstrating various microphysiological applications and satisfying various use contexts[8].

OoC has an emerging tool for mimicking human pathophysiology at the micro level among the most recent 3D cell culture techniques. The main obstacle to drug development is the lack of relevance between human pathophysiological outcomes and animal models and 2D cell culture outcomes[9]. When OoC was first being developed, the goal was to replace animal models with an

alternative platform that would mimic human pathophysiology including human-specific cells, organs functions, and cross-communication in the most pertinent way, if not perfectly but outcomes should be similar to humans. Drug research and development required more accurate simulation of human biology to be more successful, and cost-effective in a short time[10, 11]. OoCs have been effectively expanding their ability to mimic human pathophysiology using biologically formulated human organs that have been reverse-engineered. Better in vitro research is made possible by our good understanding of the cellular environments and responses to many external inputs, which was not attainable in 2D culture or animal models. The main objective of 3D cell culture is to engineer human pathophysiology for disease modeling to enhance drug screening analytics. The use of laboratory animals for drug testing can be replaced by complex OoC models that replicate human pathophysiology without any ethical issues compared to animal models.

As human excretion is predominantly carried by the kidney, filtration, reabsorption, and fluid excretion are key functions of the kidney that keep the body's fluid balance. Drug excretion depends on active and passive tubular absorption and glomerular filtration, these functions are critical for drug screening studies [12]. In order to predict drug-induced nephrotoxicity, pre-clinical drug testing may be made easier by simulating kidney function in a microfluidic device. Acute kidney injury (AKI) is a serious medical condition that often results in death. Around 1.7 million people worldwide pass away from AKI each year [13]. The kidney encounters large amounts of xenobiotics in removing waste materials from the blood, making it susceptible to (drug-induced) toxicity [14]. Kidney renal proximal tubule epithelial cells (RPTEC) express wide variety of ATP-dependent transporters and are crucial in the reabsorption of vital nutrients. Renal blood flow provides the necessary oxygen, and RPTEC have a high energy need to run active transporters [15]. AKI is brought on by renal ischemia/reperfusion injury (rIRI), which results in the loss of function

and cell destruction of the proximal tubule structure [16-18]. Additionally, the formation of reactive oxygen species is caused by an intracellular ionic imbalance (ROS). However, the cells create very modest levels of ROS at this stage of rIRI damage [19]. As a symptom following severe acute respiratory syndrome coronavirus2 infection, AKI has recently been the subject of substantial research [20].

In vitro drug testing, disease modeling, and reverse bioengineering have all been transformed by organ-on-a-chip technology. Additionally, the ability to mimic human pathophysiological processes, researchers have made significant progress in understanding kidney disorders and drug-induced renal impairment. Organ-on-chip technology has so far been used to represent the renal tubule, collecting duct, proximal tubule, glomerulus, and nephron. These models have been used to research a variety of topics, including renal fibrosis, medication toxicity, pharmacokinetics, and pharmacodynamics [21]. Furthermore, modern OoC-based MPS systems make extensive use of conventional end-point bioassays like western blots, PCR, and biochemical testing. Traditional bioassays are challenging to execute to study MPS systems because to their complexity and the restricted amount of cells and culture media available for sample collection. Such analytical constraints in MPS highlight the possibility of integrating real-time or continuous sensors for effective monitoring of cell conditions and responses. Moreover, organ-on-chip technology is incorporating biochemical and optical sensors to support real-time monitoring [22]. Transepithelial/transendothelial electrical resistance (TEER) sensors, for instance, can be utilized for a various purpose, and they have been utilized to assess the integrity of tight junctions or barriers in cells, cell survival, drug toxicity, and fibrosis [4, 6, 23].

The goal of this thesis to create a rIR injury-on-chip model utilizing primary human cells to investigate the impact of antioxidant vitamins. The rIR injury-on chip model consist of proximal

tubular portion and endothelial part were made using primary human renal proximal tubular epithelial cells (RPTECs) and human umbilical vein endothelial cells, which were grown on apical and basal side of the biocompatible porous membrane. In order to cause hypoxia and reperfusion damage, normoxic and hypoxic cell culture mediums were perfused simultaneously. A therapeutic dosage for treating rIR injury was established using different concentration of ascorbic acid and retinol concentrations. The molecular underpinnings and interactions of these cytokines during the course and prognosis of rIR damage were further investigated using kidney injury molecule 1 (KIM-1), ROS, endothelin-1 (ET-1), heat-shock protein 70 (HSP70), interleukin 6 (IL-6), and cell viability assays.

3. Research Background

3.1 Impact of Serum Concentration in Cell Culture Media

Clinical practice is increasingly utilizing microphysiological systems (MPSs)[24]. These microfluidic models are effective for drug testing and toxicological studies for the reason that to pathophysiological mimicking and biological reverse engineering. Subsequent advancements in relative accuracy and relevance to human physiology, drug regulatory bodies are now considering the promise of MPSs seriously[25, 26]. Additionally, Due to the rise in the number of drugs failing in clinical trials, the pharmaceutical industry has recently seen a major decline in investigation and innovation efficiency[27]. Typically, less than 12% of all treatments proposed in the last 10 years have been developed successfully, resulting in enormous financial losses for the pharmaceutical industry[28]. Due to the increasing failure of prospective pharmacological targets, the cost of drug discovery and development for a single drug up until the time of its FDA approval has increased

to more than 2 billion US dollars. The field of OoCs anticipates the strategic objectives for the earliest practicable stage elimination of ineffective lead candidates to reduce the financial burden and time constraints.

MPSs lack standardization, nevertheless, as a result of the biological assays and cell culture techniques' additional complexity[29]. For the preservation, expansion, and differentiation of human and animal cell lines in cell culture, serum is added to the growth medium[30]. Growth factors, trace minerals, hormones, and transport proteins are just a few of the many diverse substances that make up the complex mixture known as serum, which is essential for cellular differentiation and proliferation[31]. Due to its strong growth-promoting effect and low gamma globulin concentration, fetal bovine serum (FBS) is the serum that is employed in cell culture media the most frequently. Serum's primary purposes in cell culture medium are to provide : (i) hormones stimulating cell division and expansion (ii) transport proteins that carry hormones, such as transcortin, as well as lipoproteins, trace minerals, such as ceruloplasmin, and minerals; (iii) Aspects of the extracellular matrix, such as cellular attachment and expansion factors (iv) cellular defenses and detoxifying mechanisms essential for pH maintenance and protease inhibition[32].

One of the several transmembrane molecular structures that make up cell-to-cell tight junctions is made up of tight junction proteins (TJPs). All epithelia and endothelia depend on TJPs because they connect neighboring cells to create a permanent barrier that prevents the invasion of extracellular substances[33]. TJP complexes, which are composed of the junctional adhesion molecule families occludin, cadherin, and their transmembrane counterparts, bind to the actin cytoskeleton through ZO-1, ZO-2, and ZO-3[34, 35].

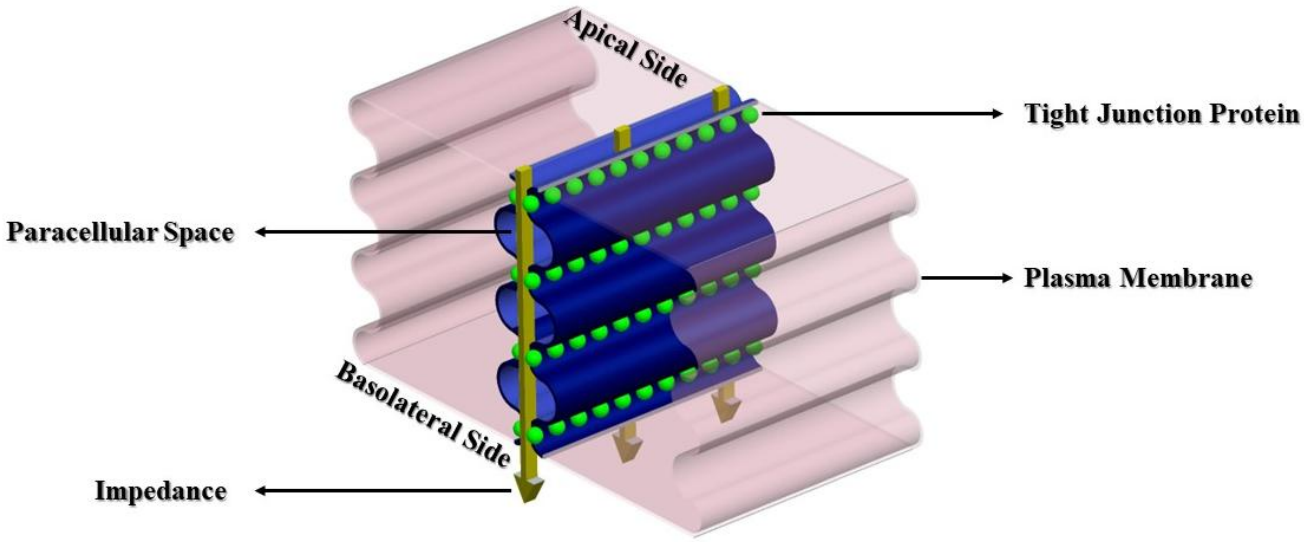


Figure 1. Schematic representation of cellular tight junctions

3.2 Extracellular Matrix Optimization for Microphysiological System

The cellular scaffolds in MPS are supported by biocompatible materials and porous membranes. A supportive extracellular matrix holds cells to a surface or suspends them in hydrogels (ECM). Cell adhesion, cell differentiation, cell-cell communication, tissue healing, tissue regeneration, and tumor growth processes are all influenced by ECM interactions[36, 37]. The loss of cellular polarity and significant molecular features results after cell separation from the native ECM[37]. Commonly used as cell culture substrata are ECM components that are readily available in the market. Exclusive ECM elements, including fibronectins, collagens, and laminins, have long been used in cell culture and have been shown to have a significant impact on the survival and attachment of cells grown in vitro as well as the homeostasis of many physiological functions[38]. It has become essential to standardize the MPS in order to receive regulatory authorities' approval. For the purpose of simulating human physiology, it is necessary to standardize issues relating to cell

culture in MPSs, such as cell number, cell type, tissue-specific ECM, and consistent biomarker testing procedures. By employing a TEER sensor to study the impact of serum concentration on tight junction protein within MPSs, Salih et al. were able to demonstrate the direct impact on tight junction proteins (TJPs), which are necessary for attachment and the formation of biomarkers[6]. A growing body of research has shown that MPS surface alteration by ECM is beneficial for a particular tissue type. ECM also affects the preservation of pluripotent stem cells (PSCs) and is crucial for PSC development. It is necessary to identify the attachment proteins needed for each organ in order for tissues to adhere to a particular ECM[39].

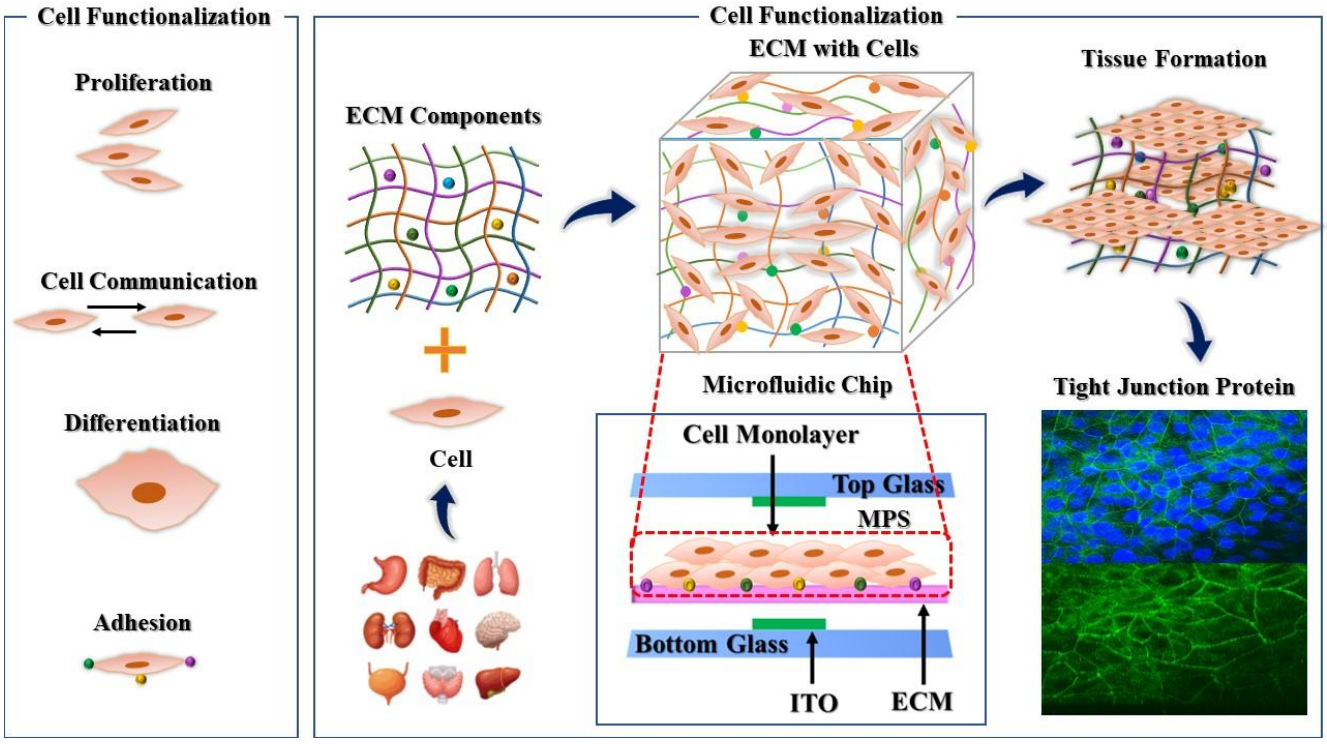


Figure 2. Role of Extracellular matrix in organ on a chip. A supportive extracellular matrix holds cells to a surface or suspends them in hydrogels (ECM). Cell adhesion, cell differentiation, cell-cell communication, tissue healing, tissue regeneration, and tumor growth processes are all influenced by ECM interactions.

3.3 Renal Microphysiological System

Approximately one million nephrons make up a human kidney. The nephron, which is the kidney's functional unit, provides a chance to microphysiologically mimic the kidney. The recent development of physiologically appropriate human tissue models has significantly expanded the potential for in vitro mimicry of organ biology and multi-organ systems. Because the nephron is one of the body's most anatomically intricate and sequentially connected microfluidic components, tiny microfluidic technologies are great candidates for in vitro capture of kidney biology. Although these models are interesting, there are a few things to keep in mind before really putting them into practice in a drug development paradigm. Drug safety assessment is frequently the first step in new MPS model implementations in the pharmaceutical business[40]. Models of the kidney present a chance to enhance drug-induced toxicity prediction because they are a common target organ. Animal models are frequently used to simulate human risk for evaluating preclinical safety, although their accuracy in predicting clinical outcomes is frequently questioned. In a data set described by Monticello et al. (2017), animal predictions of unfavorable human renal effects in phase 1 studies have a high specificity and negative predictive value but a low sensitivity[41]. Therefore, dissemination of human-based preclinical data may aim to reduce false positives while enhancing translational confidence from animal models. To assess a kidney MPS model's appropriateness for pharmaceutical applications, it is generally recommended to balance its kidney phenotype, kidney function, and responsiveness to xenobiotics. These traits work as a unit to define the context-of-use (CoU) for the model system in safety evaluation[40].

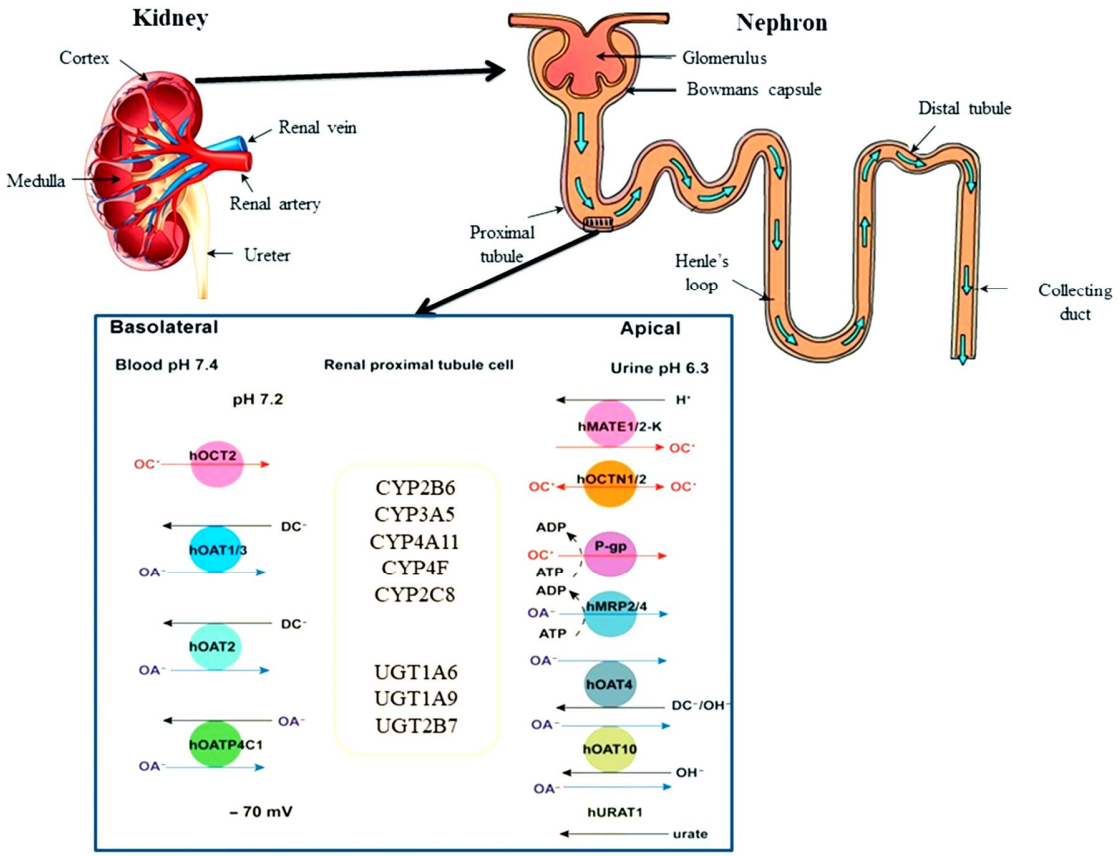


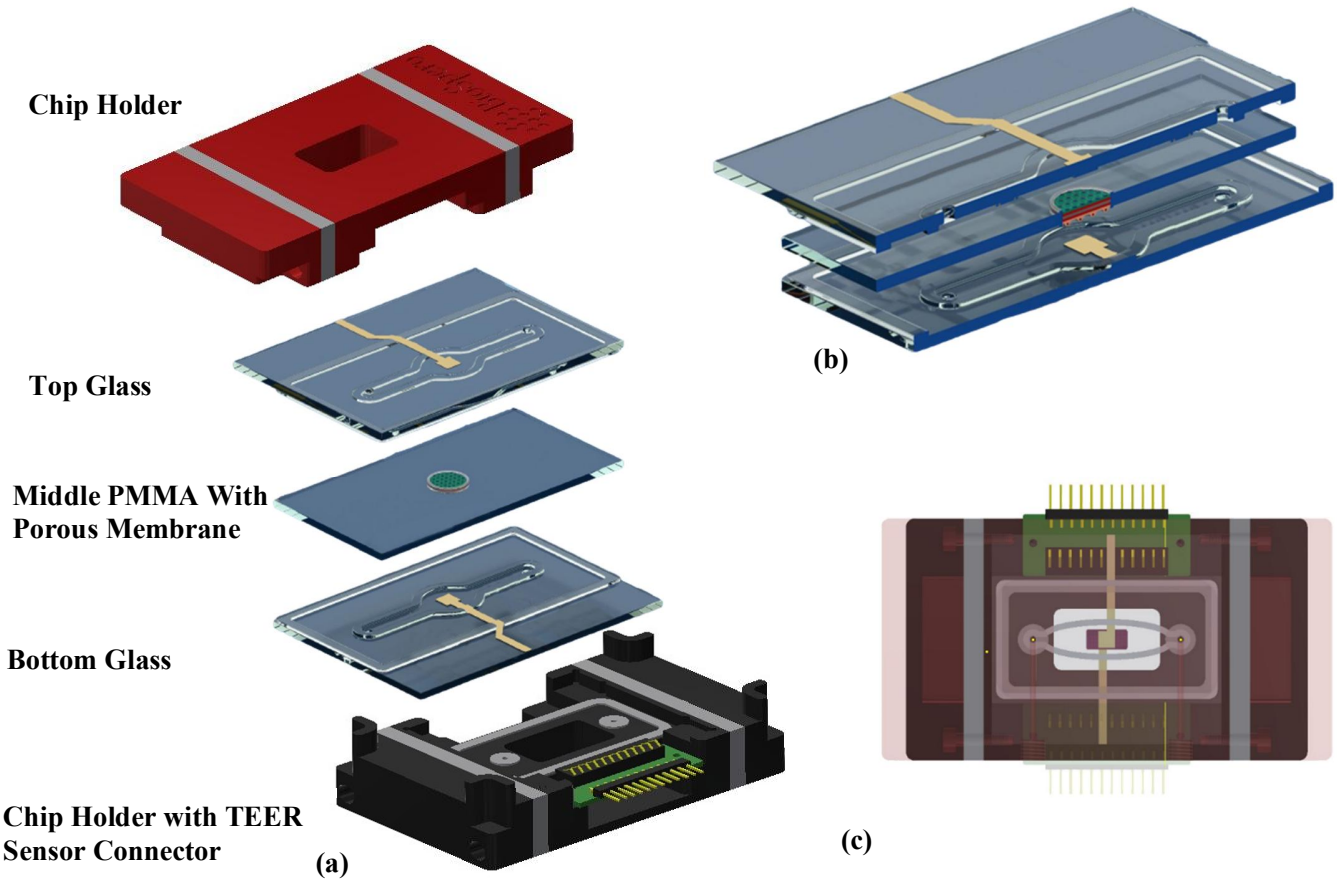
Figure 3. Overview of renal proximal tubule. Expression of metabolic enzymes and transporters in the renal proximal tubule (adapted from Yin et al.,[1] and Yeung et al)

4. Methodology

4.1 MPS Design and Development

The bilayer microfluidic chip comprises two (top and bottom) soda lime glass layers (with printed microfluidic channels and printed electrochemical sensors) and one polymethyl methacrylate (PMMA) middle layer. A circle was cut in the center of the middle layer with a diameter of 7 mm, and a microporous polycarbonate (PCTE) (complete information shown in (Figure 4) was fixed on top for bilayer culture using biocompatible silicone glue. After fabricating the sensor patterns at appropriate locations, the microfluidic channels were printed on the top, and bottom glass layers using the same dispenser printing system tweaked using Nusil medical grade silicone (MED-6033) elastomer as the printing material.14 The nozzle diameter was selected to be 600 μm. The standoff distance was set at 400 μm, and the print speed was set at 4 mm min^{-1} at a discharge pressure of 70–90 kPa. After printing the channel, it was sintered at 150 °C for 30 min. Glass was chosen as the material for the top and bottom layers in chip assembly as it is gas impermeable, which is essential for creating a controlled oxygen environment. The top and bottom glass chips had PVD printed Au patterns for trans epithelial and endothelial electrical impedance (TEER) measurement with a 16 mm^2 active overlap area. The renal hypoxic reperfusion injury-on-chip model was developed using a glass-based microfluidic chip comprising a top, middle, and bottom layers. A porous membrane was attached to a cut-out in the middle layer for culturing renal proximal tubule epithelial cells RPTEC and HUVECs on either side of it. The top and bottom layers had inkjet-printed microfluidic channels, which covered the bilayer tissue on the middle layer from both sides and routed media to the respective compartments. The inlet and outlets were bored at the extreme ends of these microfluidic channels on top and bottom chips to connect cell culture media via external fluidic routings. The chip was assembled and placed in a chip holder,

as shown in Figure.4. The chip holder could provide fluidic connections through holes from external microfluidic tubing to the glass chip. Leak-tight fluid flow was ensured using polydimethylsiloxane (PDMS) gaskets at fluidic connections on these chip holder holes and bilayer glass chip holes. Two peristaltic pumps for media circulation in each channel (for epithelium and endothelium) and washing purposes were used the same way, 70% Isopropyl alcohol and 1X PBS were used for washing the microfluidic channels and microfluidic tubings. The cell culture media reservoirs were changed with the containers of 70% Isopropyl alcohol and the alcohol was passed once through the whole microfluidics for 30min at the speed of 100 µL per min. After that, the containers of 70% Isopropyl alcohol were switched with the containers of 1X PBS, and the microfluidics was washed for 30min at the speed of 100 µL per min. The process of washing the microfluidics with the 1X PBS was repeated thrice (Figure 5).



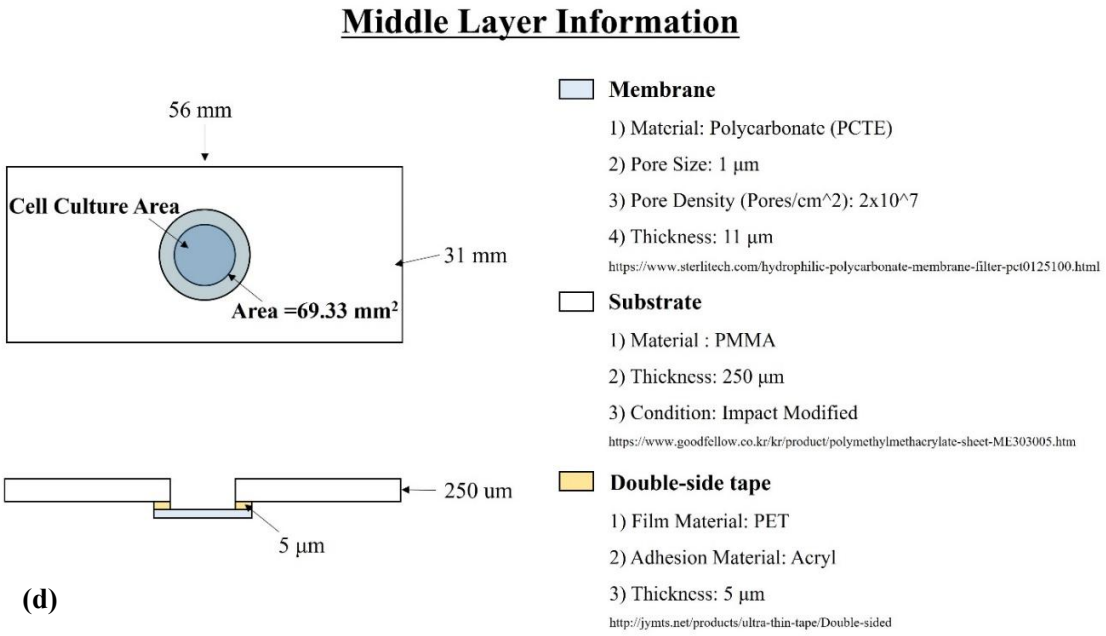


Figure 4. Renal Hypoxic Injury on a Chip Components. (a) The exploded view of the microfluidic glass chip with the chip holder. (b) The cross-sectional view of the microfluidic glass chip. (c) Top view of the microfluidic glass chip within the chip holder (d) Middle layer information.

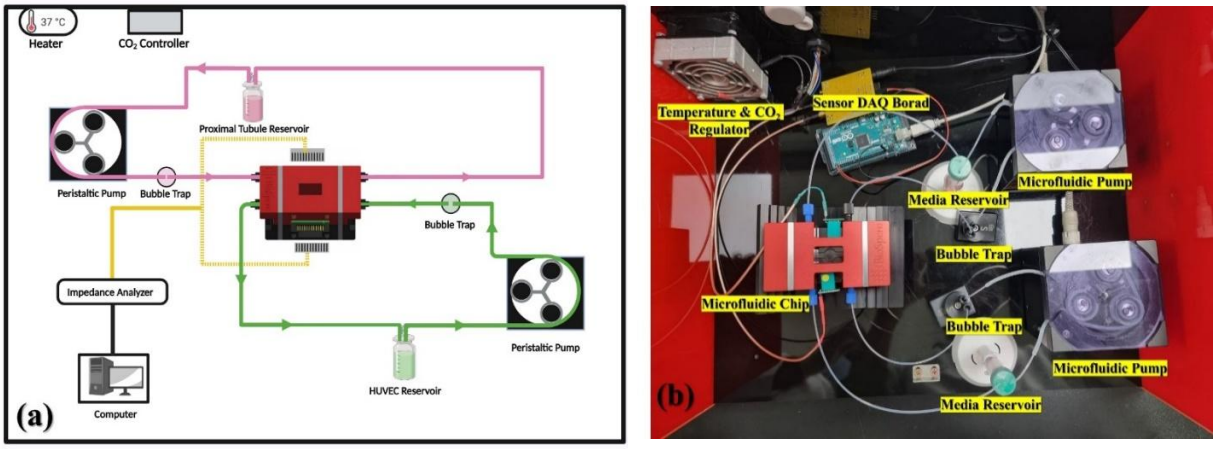


Figure 5. (a) Schematic of the renal proximal tubule microfluidic chip platform setup. Two peristaltic pumps for media circulation in each channel (for epithelium and endothelium) and washing purposes were used the same way, 1X PBS was used for washing channels and tubings. TEER sensor was connected to PC through a custom-developed impedance measuring unit (b) Real picture of the renal proximal tubule microfluidic chip platform.

4.2 Renal MPS Cell Culture

The primary human RPTEC were donated by Seoul National University Bundang Hospital, Seoul, South Korea, and grown in a complete Renal Epithelial Cell Growth Medium BulletKit (REGM, cat# CC-3190, Lonza). While, primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (cat# C2519A, pooled human umbilical vein endothelial cells) and maintained in complete Endothelial Cell Growth Medium-2 BulletKit (EGM-2, cat# CC-3162) for culture. As recommended by the cell providers, both types of cells were passaged once before being seeded on the polycarbonate porous membrane. Briefly, To eliminate cell metabolites and dead cells, the cells were washed with 1X phosphate-buffered saline (PBS) after cells achieved 80 % confluence on the petri dish. The cells were then trypsinized at 37 % with a 0.05 % trypsin–EDTA solution until entirely detached from the petri dish. To normalise the trypsin effect, the media were added. RPTEC cell pellets were suspended in REGM medium, whereas HUVECs were suspended in endothelial cell growth medium-2 (EGM-2) bullet kit TM. Following that, a BioSpero seeding kit was used to seed on a collagen-coated membrane. As an extracellular matrix for cell adhesion to the surface, a collagen type I solution (250 µg/mL diluted in 1X phosphate buffer saline) was applied to both sides of the membrane. Chip assembly was done using a three-layer arrangement (Printed TEER sensors on the bottom glass, printed TEER sensors on the top glass, and a porous PCTE membrane in the middle PMMA). The layers were sterilized overnight using UV light and 70% alcohol, then air-dried. The PMMA layer was placed on the BioSpero seeding kit with an attached PCTE membrane with a pore size of 1 µm. The PCTE membrane was pre-coated with a 250 µg mL-1 collagen solution in cold 1x phosphate buffer saline. The seeding kit was incubated for 1 hour at room temperature after 470 µL of collagen solution was added to the apical side of the membrane. The apical side was rinsed with 1 % cold PBS after incubation,

and the excess collagen was removed. The middle layer (PMMA with PCTE membrane) was then flipped over, and 470 µL of collagen solution was added to the membrane's basal section. The seeding kit lid was closed, and the cells were incubated for 1 hour at room temperature before being washed in cold 1 x phosphate buffer saline and having the excess collagen removed from the basal side. Another collagen-coating stage was performed on the PMMA with PCTE membrane in the seeding kit by using a flipping mechanism to apply 470 µL of 250 µg mL-1 collagen solution to both sides, i.e., the apical and basal regions of the membrane, and then closing the lid to ensure no bubble formation. After another overnight incubation at 4 °C, the ECM solutions were pipette withdrawn from both the apical and basal sections, and the excess ECM was washed away with 1 x cold PBS. Seeding the cells on the middle layer was the next stage. For this, 470 µL of REGM medium was initially put to the BioSpero seeding kit's basal side and covered, ensuring that no bubbles formed inside the seeding kit. The seeding kit was then inverted, and no media movement across the membrane was confirmed. Then, at a density of 2×10^5 cells per 500 µL, 470 µL of RPTEC cell suspension was poured to the apical route, and the lid was carefully put without touching the cell suspension media, and incubated overnight at 37 °C, 5% CO2, and 18% oxygen. The medium and cell debris from the apical region were removed after incubation and replaced with EGM media. The seeding kit was switched over, and the EGM medium was taken from the basal side, then washed with 1 % PBS to remove any remaining medium. The basal side was then supplemented with 470 µL of HUVEC suspension at a density of 2×10^5 per 500 µL. It was covered with a lid and incubated overnight at 37 °C with 5% CO2 and 18% oxygen, making sure the lid did not come into contact with the cell suspension media. Both the apical and basal media were removed after incubation, and the PMMA intermediate layer with the PCTE membrane was removed. 1x PBS was used to wash the seeded bilayer tissue. On the basal side, 10

μL of HUVECs media was supplied to the cell culture space. A printed microfluidic channel was inserted on the bottom glass. After that, the chip was flipped over and a top glass layer with a printed microfluidic channel was put to cover the apical side, completing the complete chip assembly. The chip was then placed in the bilayer chip holder once it had been built.

4.3 Renal Hypoxic reperfusion Injury on a Chip Model Development

The standard cell culture media contained 18% oxygen for culturing renal proximal tubular cells. In this experiment, we chose nitrogen (N_2) bubbling to remove oxygen from the media and obtain hypoxic cell culture media. The media of renal proximal tubular epithelial cells were collected in a 50 mL falcon tube, and nitrogen was bubbled into the media after 20 min. Then, the dissolved oxygen concentration was verified using a commercial dissolved oxygen sensor (PreSens). Nitrogen bubbling provided a dissolved oxygen level of 1.21 mg/L, and the obtained media were used in the hypoxic renal reperfusion injury experiments

The primary human RPTEC were donated by Seoul National University Bundang Hospital, Seoul, South Korea, and grown in a complete Renal Epithelial Cell Growth Medium BulletKit (REGM, cat# CC-3190, Lonza). In addition, primary human umbilical vein endothelial cells were purchased from Lonza (cat# C2519A, pooled human umbilical vein endothelial cells) and maintained in complete Endothelial Cell Growth Medium-2 BulletKit (EGM-2, cat# CC-3162) for culture. As recommended by the cell provider, both types of cells were passaged once before being seeded on the polycarbonate porous membrane. Collagen type I solution (250 μg/mL dissolved in 1× phosphate buffer saline) was applied on both sides of the membrane as an extracellular matrix for cell attachment to the surface. RPTEC (2×10^5 cells/500 μL) were seeded on the apical side of the membrane, while human umbilical vein endothelial cells (2×10^5 cells/500 μL) were cultured on

the basal side of the membrane. First, the dissolved oxygen content in the cell culture media was reduced to 1.21 mg/L by nitrogen bubbling for preparing the hypoxic media. Then, the media were used for inducing the hypoxic injury in the RHR injury-on-chip model. A commercial sensor (Microx 4, PreSens) was used to validate the dissolved oxygen content in the hypoxic cell culture media. The RHR injury-on-chip model was developed by using the M-Physio™ Platform (BioSpero, Inc, Republic of Korea). The microfluidic chip containing the middle layer was assembled and placed in the RHR injury-on-chip platform. Cell culture media were supplied through a media reservoir, while a bubble trap was set up to avoid bubble formation in the microfluidic channels (Figure 5). Additionally, a chip-embedded TEER sensor was employed to examine the barrier integrity of the RHR injury-on-chip model.

4.4 Proximal Tubule Permeability Assay

The microvascular lumen permeability was measured using a fluorescein isothiocyanate-dextran transporter with and without microvascular endothelial cells on a polycarbonate membrane (pore size of 3 μm). A fluorescein isothiocyanate-dextran concentration of 0.2 mg/mL (0.005 mol/m^3) was introduced into the basal compartment of the bioreactor. After 2 h of cell culture media circulation, 10 μL of media were collected from the apical part mixed with 100 μL phosphate-buffered saline and then transferred into 96 well plates. The fluorescence intensity was measured according to the manufacturer's protocol.

4.5 TEER apparatus and assessment method

A Trans-epithelial/endothelial Electrical Resistance (TEER) sensor was applied to measure the barrier integrity of the tissue formed by the renal proximal tubule cells (RPTEC). The TEER sensor

was characterized by employing the impedance spectroscopy (IS) technique. IS was carried out with a commercial impedance analyzing system from 1 Hz to 10 kHz with an excitation signal of 0.1 V AC. The influence of different fluid flow rates (23.37 μL min^{-1}, 46.67 μL min^{-1}, 70.11 μL min^{-1}, 83.33 μL min^{-1}) was measured. Figure 6 is representing that different flow rates have not affected the electrical impedance responses and any flow rate can be chosen for the real-time impedance monitoring during the experiments. The IS response for the empty channel (without any flow) was also given to highlight the performance comparison of the TEER sensor. Then the IS response of different concentrations of the collagen was measured, as the viable cells require a biocompatible coating for their attachment to the membrane. The chip was evaluated with different concentrations of the collagen solution (0.4 mg mL^{-1}, 0.8 mg mL^{-1}, 1.2 mg mL^{-1}, 1.6 mg mL^{-1}) and an impedance spectrum was measured, as presented in Figure S6. It can be found that it has no significant effect as a nearly identical spectrum was observed. Hence, any concentration of the collagen solution (0.4 mg mL^{-1}, 0.8 mg mL^{-1}, 1.2 mg mL^{-1}, 1.6 mg mL^{-1}) can be utilized for experimentation. The model measurement method for the bilayer chip was the measurements of the separate monolayer impedances of RPTEC and HUVEC cells. An additive technique was utilized to describe the total bilayer tissue impedance. The measurements were collected for a total of 79 hours while the measurement interval selected was 1 hour. An in-house developed measurement interface was designed with an onboard AD5933 impedance analyzer. The frequency and the amplitude of the excitation signals were selected to be 60Hz and 0.2 V AC respectively. The bilayer tissue impedance was recorded for both normoxic and hypoxic reperfusion conditions. The TEER values collected can be evaluated as the sum of the individual contributions of the epithelium (Z_{epi}), endothelium (Z_{endo}), and membrane-solution ($Z_{mem+sol}$) impedance values ignoring other variables such as temperature, etc. A mathematical equation is given below to

explain the relationship of the TEER values

$Z \cong Z_{epi} + Z_{endo} + Z_{mem+sol}$

The individual contributions of the epithelium or endothelium were also recorded in a previously describe monolayer chip as presented in Figures 6 (c) and (d). The membrane and collagen solution impedance have not tended to change with time, and these could not be considered constant mainly the contribution to TEER change is by the virtue of the bilayer tissue itself. As it is evident from Figure 6 (d) that the Z_{endo} is not changing over time, and it can be assumed that Z_{epi} is the major contributor to the change in the TEER values.

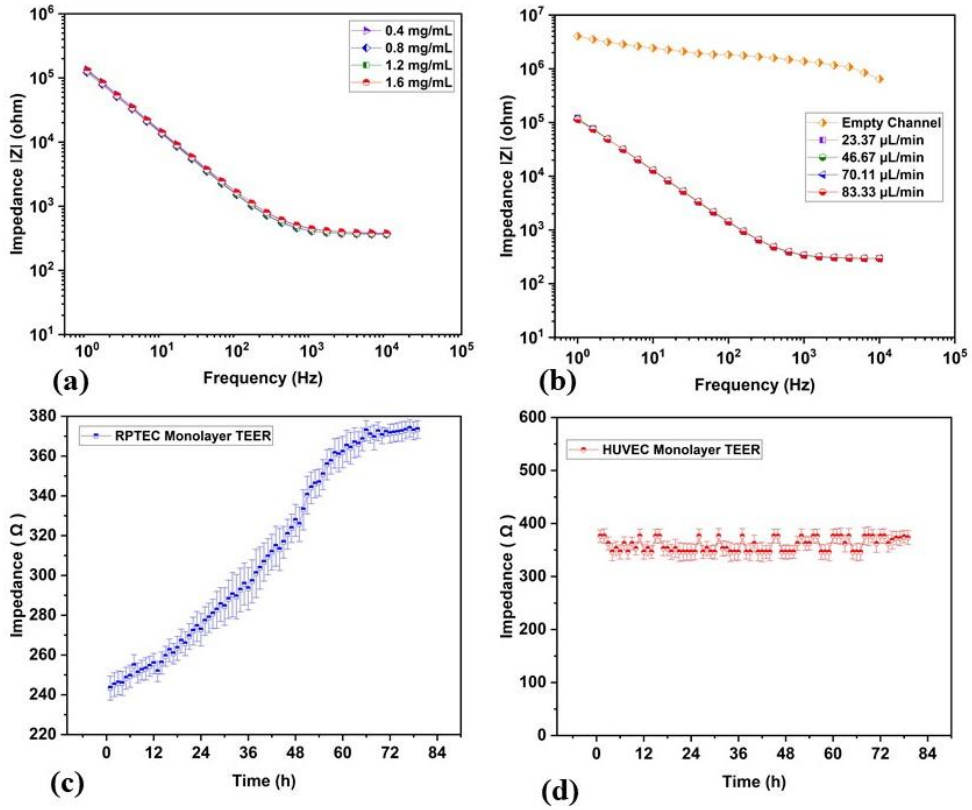


Figure 6. TEER sensor characterization. (a) TEER sensor impedance spectroscopic response without media flow and media flow with different flow rates values. (b) TEER sensor impedance spectroscopic response with different collagen concentrations for the same incubation time. (c) TEER value of monolayer tissues of RPTEC (d) HUVEC measurement. TEER measuring chip the RPTEC cell impedance is an almost constant and negligible value.

4.6 Drug Efficacy Testing on a Chip

The impact of vitamins on the developed RHR injury-on-chip model was evaluated by supplementation of vitamin C, retinol, and their combination at the time of reperfusion of the bioreactor. Vitamin C (L-Ascorbic acid, catalog no. A92902-25G, Sigma Aldrich), retinol (Retinol, catalog no. R7632, Sigma-Aldrich), and a combination of vitamin C and retinol were used at concentrations of 30 µg/L, 50 nM/mL, and their combined concentration, respectively. The stock solutions were prepared in DPBS, while the working concentrations were prepared in Renal Epithelial Cell Growth.

4.7 Biochemical Assays

4.7.1 Cell Viability Assay, Dicholorodihydroflourescein Diacetate Staining and Image Processing.

The live/dead assay was executed according to the corresponding instructions using the LIVE/DEAD Viability/ Cytotoxicity Kit for mammalian cells (catalog no. L3224, ThermoFisher Scientific). The bilayers were washed three times with Dulbecco's phosphate-buffered saline (DPBS), and 200 µL of live/dead assay reagent was applied. The bilayers were incubated in a cell culture incubator at 37 °C for 30 min. Then, the bilayers were rinsed with DPBS, and mounting media were used to apply the coverslip. Dichlorodihydrofluorescein diacetate (DCFDA—ab113851) staining was performed by washing the monolayers twice with DPBS, and 200 µL of DCFDA solution was applied. Then, the monolayers were incubated in a cell culture incubator at 37 °C for 45 min in the dark. The monolayers were washed twice with DPBS after incubation, and mounting media were used to apply the coverslip. A confocal laser scanning microscope (FV123, Olympus) was used for taking micrographs, which were processed using the open-source ImageJ software (version 1.52p, NIH).

4.7.2 Elisa Assay for Renal Biomarkers

KIM-1, HSP70, ET-1, and IL-6 assays were performed to evaluate the impact of injury in the RHR injury-on-chip model. KIM-1 ELISA Kit (catalog no. DSN199, R&D Systems), HSP70 ELISA Kit (catalog no. ab133060, Abcam), and IL-6 ELISA Kit (catalog no. BMS213-2, Invitrogen) were used for measuring the corresponding biomarkers collected from the renal proximal tubular lumen. In addition, ET-1 ELISA Kit (catalog no. ab133060, Abcam) was used for the estimation of ET-1 from the vascular lumen of the RHR injury-on-chip model. All the biomarkers were measured following the manufacturer's instructions. A multipurpose microplate reader (SpectraMax i5 Multimode Reader, Molecular Devices) was used for taking ELISA readings.

5. Results and Discussion

5.1 Renal hypoxic Reperfusion Injury on a Chip Model

The RHR injury-on-chip model based on glass microfluidic chips were constructed by fusing three chips. Apical and basal compartments represented the proximal renal tubule and microvascular lumens of the RHR injury-on-chip model, respectively (Figure 7). An embossed biocompatible membrane divided the proximal renal tubule and microvascular portions of the RHR injury-on-chip model (Figure 7). The media flow within the microfluidic channels (apical, 25 μL/min; basal, 45 μL/min) applied the in vivo shear stress of 0.2 dyn/cm^2 for the optimal growth of cell–cell tight junctions and the formation of the cell monolayer. Culturing RPTEC and vascular endothelial cells on each side of a porous membrane can further increase the cell viability proliferation and gain of the phenotype of the RPTEC by increasing mitochondrial activity[42]. On the other hand, the lack of physiological shear stress results in poor development of cellular phenotypic characteristics and a low yield of physiological biomarkers[43]. The proximal renal

tubular lumen and microvascular lumen were confluent with respective monolayers on day 3 (Figure 7). Hypoxic media containing the dissolved concentration of 1.21 mg/L were perfused in the proximal renal tubule for inducing hypoxia in the RHR injury-on-chip model for 2 h. Then, the proximal renal tubular lumen was treated with normoxic cell culture media for 4 h to induce RHR injury, which severely decreased the cellular viability in both lumens within the model (Figure 8 and 9).

Image processing of the live/dead assay results showed the intensity percentage and decreased cell viability of the proximal renal tubular lumen and microvascular lumen, respectively. Reperfusion of an oxygen-deprived tissue with physiological normoxic dissolved gases leads to the abrupt release of ROS by mammalian cells, constituting an oxidative burst (Figure 8 and 9). The oxidative burst results in the mass production and release of free radicals such as superoxide, hydroxyl radical, and hydrogen peroxide, leading to caspase activation and eventually apoptosis in cells[44]. The RHR injury-on-chip model showed an increase in the production of ROS compared with the control experiments (Figure 8b). However, there is a need and a possibility to increase the overall length of the experiment (from 4 hours up to 7 days) and incorporate immune cells and other part of the nephron for the RHR injury-on-chip models to reproduce in vivo environments more realistically.

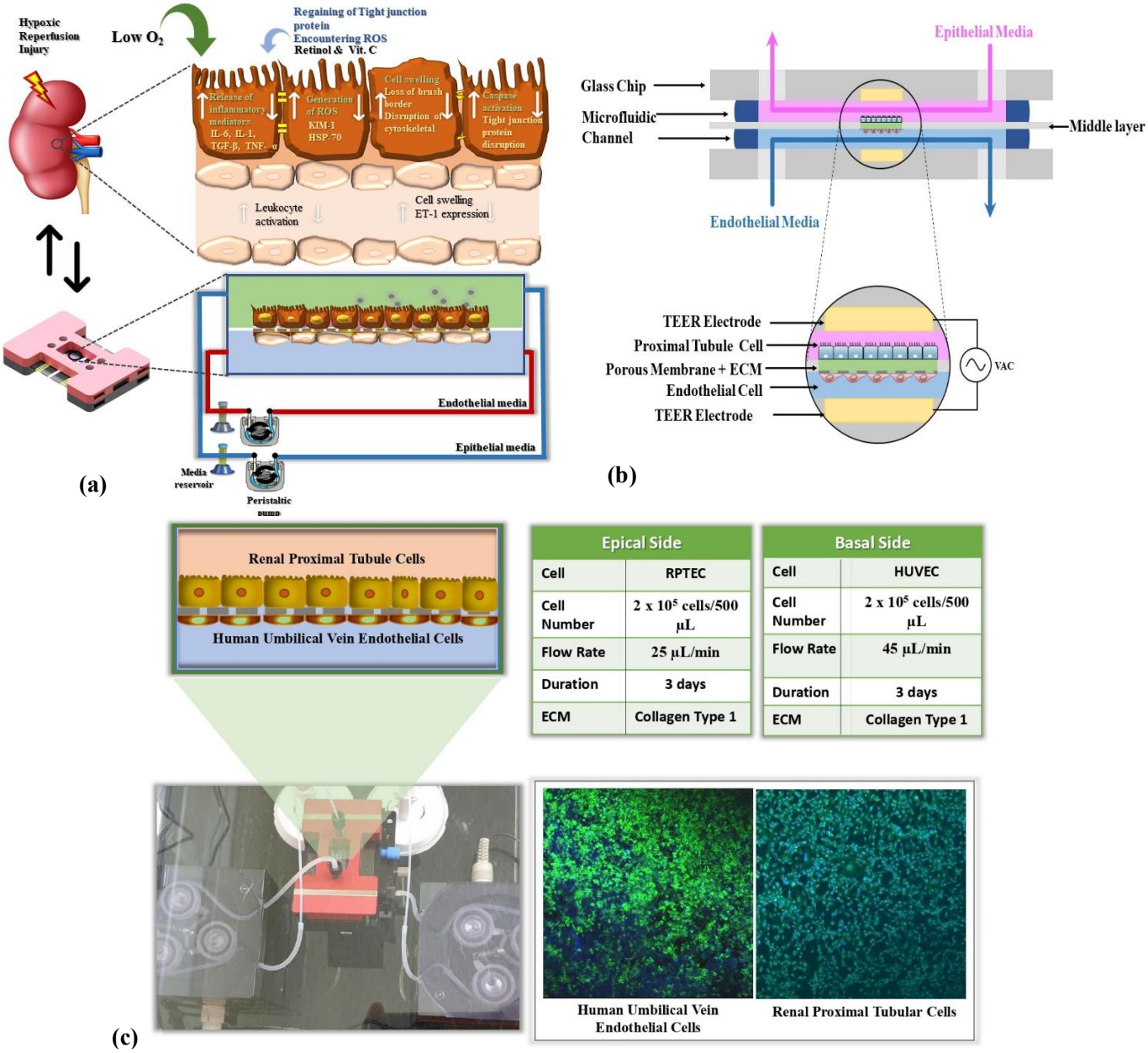


Figure 7. (a) Schematic of RHR injury-on-chip model; (b) The illustration of the RHR injury-on-chip model representing the arrangement of different components of the microfluidic chip. (c) Three-day experiments in the proximal renal tubular lumen and microvascular lumen for cell confluency confirmation. (ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells; RPTEC, renal proximal tubular epithelial cells)

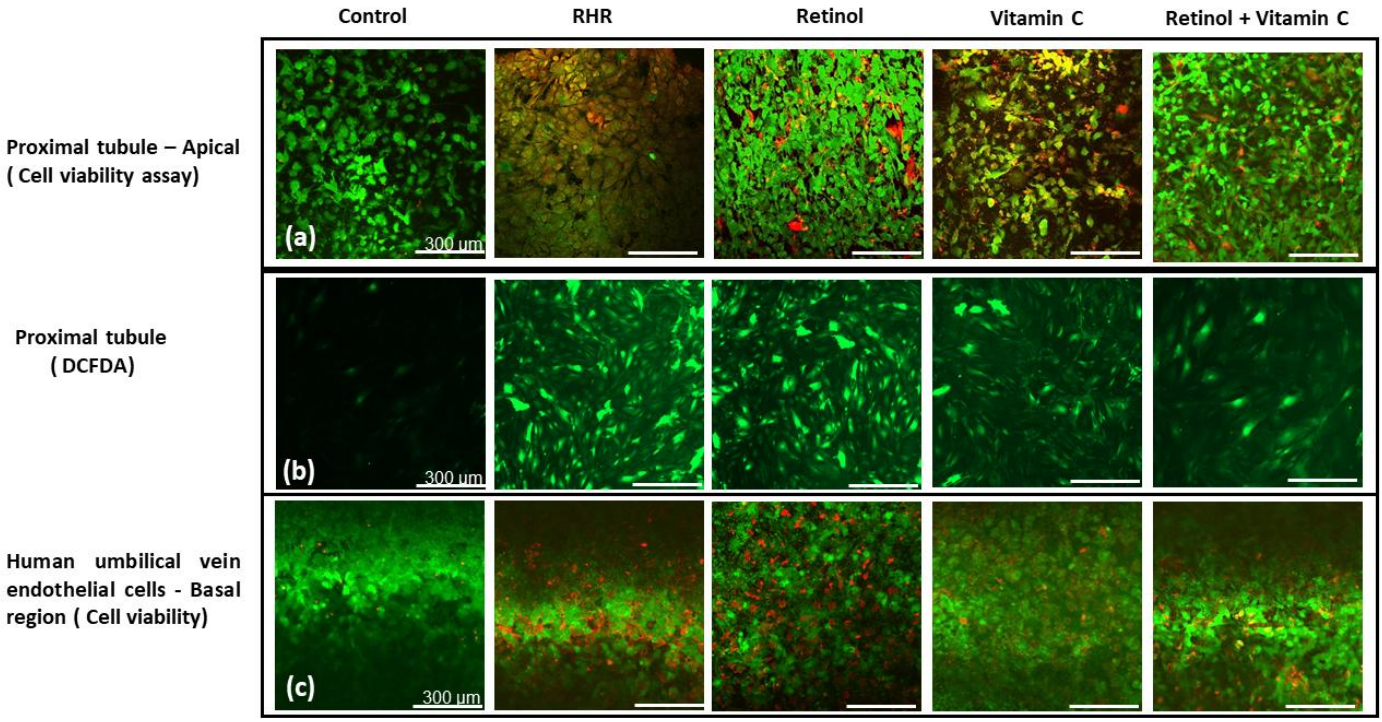


Figure 8. (a) Live/dead assay results of cell viability for RHR injury-on-chip model with reperfusion injury and under the effect of vitamin C, retinol, and combinational vitamin doses. The reperfusion injury in the RHR injury-on-chip model decreased the cell viability to 51% compared with the control (89.9%). In contrast, the RHR injury-on-chip model treatment with vitamin C, retinol, and combinational vitamin dose provided cell viability rates of 61%, 66%, and 74.2%, respectively. (b) Results of DCFDA staining for RHR injury-on-chip model with reperfusion injury and under the effect of vitamin C, retinol, and combinational vitamin doses. The control experiment showed a fluorescent intensity of 9.89%, while reperfusion injury increased the intensity to 81.4%. In contrast, the RHR injury-on-chip model treatment with vitamin C, retinol, and combinational vitamin dose decreased the DCFDA fluorescent intensity to 55.41%, 72.58%, and 32.5%, respectively. (c) Live/dead assay results of cell viability for basal compartment (endothelial cell).

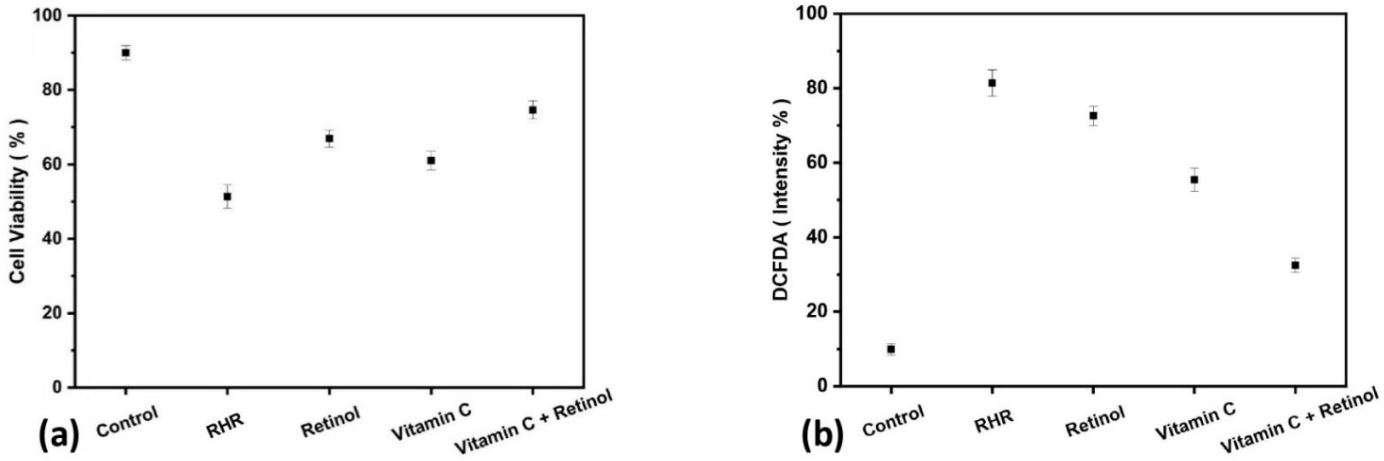


Figure 9. Flourescence intensity analysis (a) Live/dead assay results of cell viability and (b) DCFDA staining results of ROS in RHR injury-on-chip model with reperfusion injury and under the effect of vitamin C, retinol, and a combinational vitamin dose.

5.2 Impact of Renal hypoxic Injury on Proximal Tubule Barrier Integrity

Proximal tubular cells are the primary drivers of active tubular secretions that are vital for drug secretion and release and metabolic excretion. These secretions occur under membrane transporters and require cellular barrier integrity. The TEER of the RHR injury-on-chip model was evaluated as an indicator of cell–cell tight junction formation for examining the influence of RHR injury on the RPTEC monolayer. The TEER was monitored every hour from day 0 until the termination of the experiment using an in-house TEER monitoring system. The TEER values showed a consistent increase to reach the plateau (257 Ωcm^2) due to RPTEC growth, differentiation, and cell–cell tight junction formation. In addition, the TEER values showed a linear pattern on day 3, representing the formation of a compact monolayer (Figure 10). A TEER range of 167–257 Ωcm^2 may be considered a reference value for RPTEC monolayer formation within a microfluidic system.

The RHR injury was induced by perfusing the hypoxic and normoxic cell culture media, as described above. The RHR injury decreased the TEER value to 227 Ωcm^2 compared with the

control value of 257 Ωcm^2. A swift drop has been directly related to the pathophysiological condition of the cellular monolayer. The live/dead assay results validated the TEER values as the RHR injury substantially reduced the cellular viability and negatively disrupted the cell–cell tight junctions. The oxidative burst produced by the RHR injury also contributed to the lower TEER values compared with those from the control experiments. The correlation between the TEER and ROS release by epithelial cells has been previously reported, with increasing ROS production resulting in the loss of cell–cell tight junction formation and compromised TEER values[45].

Here, ROS release starts from the inflammation of the cells when reperfusion occurs leading to cell death. Cell death induces a decline in TEER measurements as compared to normal conditions, the main purpose of this study. Additionally, multivitamin therapy down-regulated inflammation and cell death due to reperfusion injury which further validates that the cell death was the main reason for TEER value decline. Overall, ROS and TEER results correlate only with ROS production due to inflammation. Also, TEER sensor cannot detect ROS directly as its main principle is to measure the resistance across the cellular barrier. This current study mainly focuses on the TEER values in correlation with inflammation, cell death, and cell viability. This study can be useful for ROS production due to inflammation, and cell death correlating to TEER.

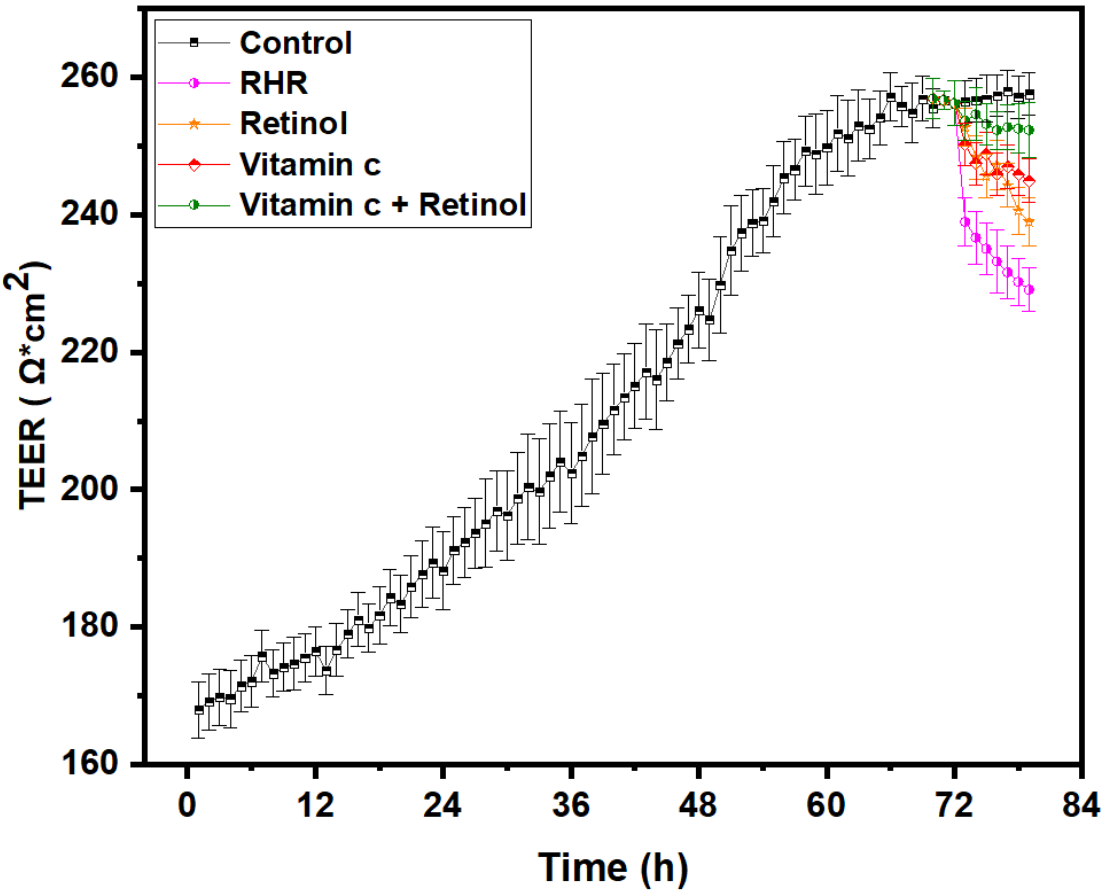


Figure 10. TEER measurements corresponding to RHR injury and vitamin treatment (impedance over time for n = 3). The TEER value was calculated in ohms per square centimeter square (Ωcm2). The TEER values over 79 h showed a consistent increase due to cellular expansion, differentiation, and cell–cell tight junction formation until the development of a compact monolayer. In addition, vitamin C, retinol, and their combinational dose decreased the reperfusion injury response in the RHR injury-on-chip model and helped to retain the reference TEER value.

5.3 Proximal Tubule Permeability Assay

Both the upper and lower channels' samples were taken after 2 hours. By comparing the fluorescence intensities to a 0.005 mol.m-3 intensity value, the relevant concentrations were extrapolated from the graph in Figure 11, where the fluorescence intensities were compared to that value. While the concentration discovered in the membrane alone was 0.12 mg.mL-1, it was greater than the membrane with cell value of 0.07 mg.mL-1. The concentration measured in the

upper channel was closer to the starting concentration supplied. The discrepancy between the membrane alone and membrane with cells responses can be explained by the disparity between the model parameters and the actual values of the species' transport properties, such as the diffusion constant and potentially variable cell properties[46]. The fact that the tight junction protein of endothelium was maintained for a sufficient amount of time under dynamic conditions during culture further contributes to this lower value's indication of the better barrier function.

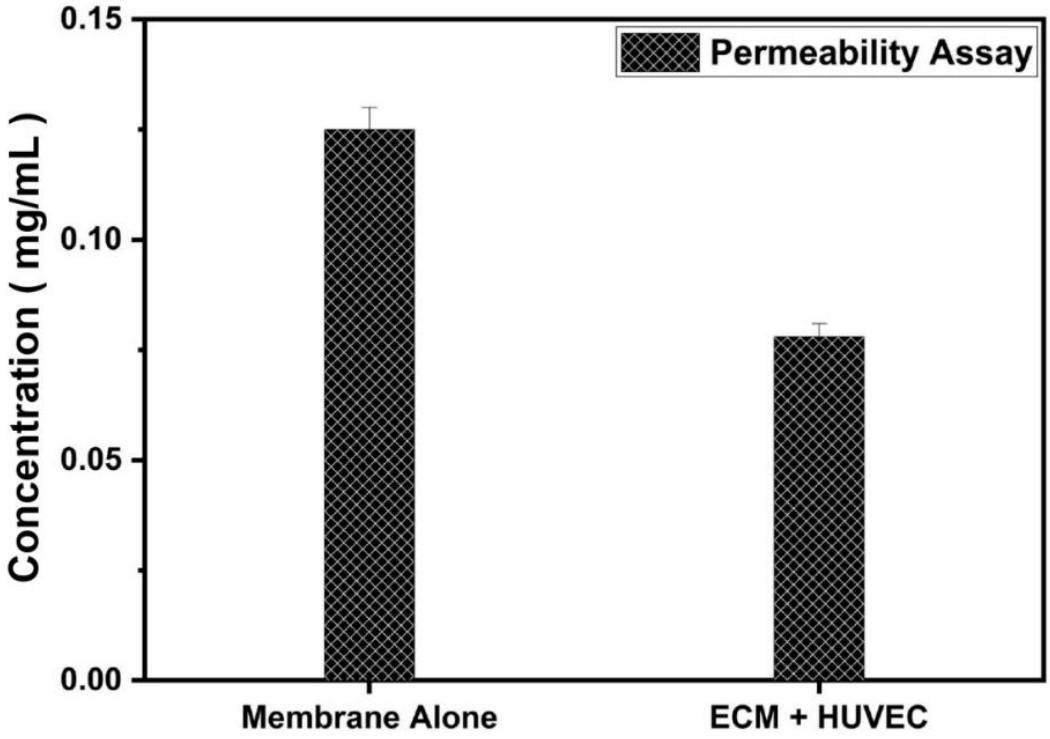


Figure 11. Permeability assay result. Comparison between membrane alone and ECM with cells

5.4 Renal Hypoxic Injury Biomarkers Analysis

Expression of KIM-1. KIM-1 is a precise and highly sensitive biomarker for RPTEC and is an early biological indicator of tubular pathophysiology. Primary and secondary renal stresses such as oxidative burst, infections, and endotoxins result in the cleavage of KIM-1 and its release into the tubular lumen. In addition, the upregulation of KIM-1 indicates various renal anomalies such

as CKD, fibrosis, and acute cellular injury. The RHR injury-on-chip model showed a higher KIM-1 compared with the control experiments. After reperfusion for 30 min with normoxic cell culture media, a fourfold increase in KIM-1 secretion was observed in the tubular lumen in the RHR injury-on-chip model. Elevated KIM-1 levels may indicate participation in RPTEC differentiation and division after acute cellular injury[47]. On the other hand, vitamins, and their combinational therapy substantially reduced the KIM-1 release within the RHR injury-on-chip model (Figure 12 a).

Expression of HSP70. Heat-shock proteins are intracellular cytosolic proteins that mediate peptide assembly and folding mechanisms and sometimes participate in programmed cell death. A cytoprotective HSP70 is produced by stressed cells to counter acute reperfusion injury. A substantial increase in HSP70 occurs in the presence of acute tubular anomalies, and its level remains high in chronic renal conditions[48]. The control experiments showed a much lower concentration of HSP70 in the tubular lumen, whereas the RHR injury induced a fivefold increase in the HSP70 release (Figure 12c). The higher concentration of HSP70 in the RHR injury-on-chip model indicated a self-protective response of the tubular cells against the ROS. Similarly, the apoptosis controlling mechanism of HSP70 by the activity of cellular kinases to regulate Bax (pro-apoptotic proteins) has been reported[49].

Expression of IL-6. RHR injury is an inflammatory predecessor characterized by proinflammatory cytokines. Among them, IL-6 upregulates hypoxic reperfusion injury in several organs[50]. IL-6 measurements were performed to evaluate the impact of the injury on the RHR injury-on-chip model. The IL-6 values increased after RHR injury compared with those of the control experiment, where no hypoxic reperfusion injury was induced (Figure 12b). IL-6 is known to cause the degeneration of intracellular components of RPTEC[51]. Thus, a higher concentration of IL-6 can be

directly related to lower cell viability within the RHR injury-on-chip model after reperfusion.

Expression of ET-1. The role of endothelial cells in RHR injury is crucial as they produce several cytokines that modulate the microvasculature for protecting renal tissue from the oxidative burst. ET-1 is the most abundant biomarker released explicitly by endothelial cells to induce vasoconstriction and expand the destructive effect of RHR injury[52]. In addition, ET-1 performs upregulation in RHR injury. Hence, several therapeutic agents are being introduced to limit the localized release of ET-1. A drastic increase in ET-1 was observed in the vascular lumen of the RHR injury-on-chip model compared with the control. Urinary ET-1 and KIM-1 induce a synergetic effect in the progression of systemic lupus erythematosus and polycystic kidney disease. The ET-1 release may contribute to the progression of RHR injury in the renal proximal tubular lumen[53, 54]. However, the release of ET-1 was substantially reduced within the RHR injury-on-chip model after treatment with vitamins (Figure 12d).

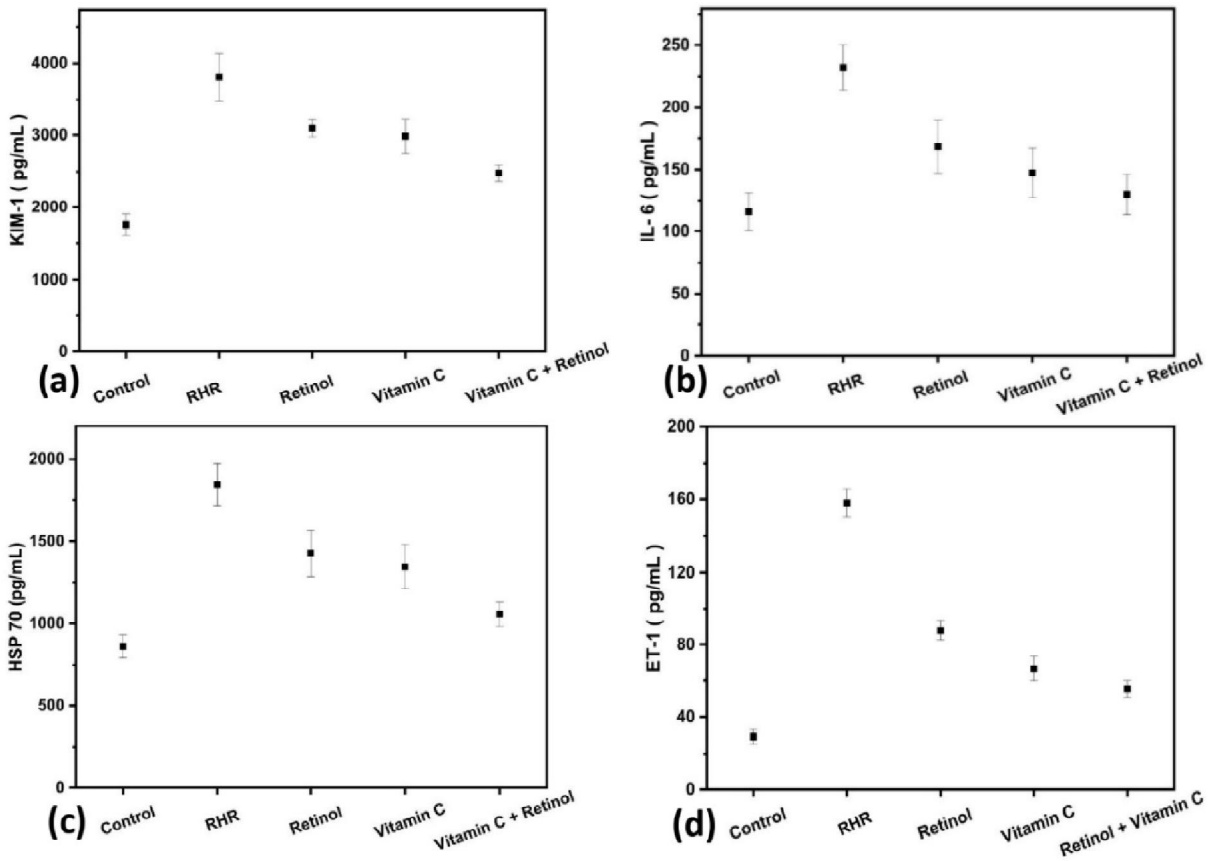


Figure 12. Biomarker concentrations in control experiment and RHR injury-on-chip model with reperfusion injury and after treatment with vitamin C, retinol, and combinational vitamin dose. (a) KIM-1 ELISA graph of KIM-1 concentration within the proximal renal lumen of RHR injury-on-chip model. A twofold decrease in KIM-1 concentration was observed after treating the RHR injury-on-chip model with a combinational vitamin dose compared with treatment with vitamin C or retinol alone. (b) IL-6 ELISA graph of IL-6 concentration within the proximal renal lumen of RHR injury-on-chip model. A substantial increase in IL-6 concentration was observed after reperfusion in the RHR injury-on-chip model. Vitamin and combinational vitamin doses considerably reduced the IL-6 release within the RHR injury-on-chip model. (c) HSP70 ELISA graph of HSP70 concentration within the proximal renal lumen of RHR injury-on-chip model. A substantial increase in HSP70 was observed after reperfusion in the RHR injury-on-chip model. Vitamin and combinational vitamin doses considerably reduced the HSP70 release within the RHR injury-on-chip model. (d) ET-1 ELISA graph of ET-1 concentration within the microvascular lumen of RHR injury-on-chip model. A substantial increase in ET-1 was observed after reperfusion of the proximal renal lumen in the RHR injury-on-chip model. Vitamin and combinational vitamin doses considerably reduced the ET-1 release within the microvascular lumen of the RHR injury-on-chip model.

5.5 Drug Efficacy and Biomarkers Analysis

Vitamins have been extensively evaluated for the treatment of RHR injury, showing protective characteristics. Antioxidant vitamins in high doses neutralize the negative effects of the oxidative bursts during reperfusion by either neutralizing the effect of ROS or by preventing the bursts from occurring[55]. Vitamin C has already been studied for limiting the effect of RHR injury in both animal models and in vitro studies[56]. In contrast, the role of retinol in countering RHR injury has not been studied to date. Therefore, this study aimed to evaluate the influence of both antioxidant vitamins and their combinational dose for preventing RHR injury. Vitamin C and retinol were perfused in the proximal tubule lumen and microvascular lumen with the cell culture media during reperfusion of the RHR injury-on-chip model. A substantial increase in cell viability and reduction in ROS generation was observed. In addition, both antioxidants considerably reduced the kidney-specific biomarker yield. Vitamin C was more effective than retinol in reducing the overall impact of reperfusion.

Combinational therapy using vitamin C and retinol is prevalent in dermatological therapy, but its effect is less known in RHR injury prevention. A combinational dose of vitamin C and retinol was studied in the developed RHR injury-on-chip model[57]. Interestingly, the combinational dose was more effective than the separate use of vitamin C or retinol to counter RHR injury. The cell viability of RPTEC and microvascular endothelial cells increased by 1-fold compared with the single-vitamin treatment. Similarly, the combinational dose of vitamin C and retinol was the most effective in reducing RPTEC injury biomarkers.

6. Conclusion and Future Perspectives

The kidney MPS used to develop an RHR injury-on-chip model for analyzing renal hypoxic reperfusion and studying the effect of antioxidant vitamins in RHR injury prevention. First, traditional glass chips were used with a PMMA sheet and a porous membrane to develop a two-lumen cell culture bioreactor. Then, normoxic and hypoxic cell culture media were perfused hierarchically to induce reperfusion injury in the renal proximal tubular lumen of the bioreactor. Various biomarkers were measured to confirm the RHR injury, and vitamin doses were used to mitigate the injury. Additionally, a chip-embedded non-invasive TEER sensor was used to determine the relation between RHR injury and barrier integrity of RPTEC. This study found that an injury-on-chip model is a viable alternative to an animal model and a conventional two-dimensional cell culture model for studying RHR injury. Additionally, vitamin combinational therapy likely outperforms single-vitamin therapy in the prevention of RHR injury. Overall, the use of animal models can be replaced by the use of microfluidic MPS platforms, which effectively imitate human organs and tissues. The pipeline for drug discovery and development will be accelerated by MPS platforms' clinical predictive capability. MPS performance is further improved by the embedded sensors' potential to continuously monitor the cellular microenvironment.

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