ENDOMETRIUM-ON-A-CHIP: a more Biologically Accurate model of the Endometrium with the Addition of a Vascular Compartment and a Collagen Type I Membrane

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Abstract

The endometrium is a dynamic organ, undergoing major structural, cellular and vascular changes during the 28-day menstrual cycle. The endometrium is the inner layer of the uterus wall, responsible for the implantation of an embryo during pregnancy. The endometrium, and the rest of the female reproductive tract, are difficult to study *in vivo* because of ethical considerations. So, there is a high need for a healthy model of the endometrium. Previously used animal models do not suffice, because they differ greatly from humans regarding reproduction. The AMBER research group made a vertical oviduct-on-a-chip containing an epithelium in the top chamber. The bottom vascular chamber, containing no cells, was separated by a synthetic polycarbonate membrane. A similar architecture was used for the endometrium-on-a-chip, however it was improved with the addition of an endothelium in the vascular compartment and a collagen type I membrane. HUVECs were used to create endothelium in the vascular compartment. In this work, three different substrates were compared for the culture of endothelial cells, namely a collagen coated polycarbonate membrane, a collagen type I membrane and gelatin coated polystyrene. HUVECs were successfully cultured on all three substrates until day 2. The collagen type I membrane was characterised after hydration: it had a constant thickness and diameter. The rigidness of the membrane significantly decreases after hydration. So, for its implementation into a microfluidic chip, the focus was on creating a strong attachment between the microfluidic device and the membrane. The so-far explored attachment methods lead to significant leakage, so the method of attaching the membrane to the chip needs to be researched and developed further. The effect of the membrane on the HUVECs and the attachment method to the microfluidic device needs to be studied further, however they are very promising for improving the endometrium-on-a-chip. The endometrium-on-a-chip will be a useful tool for studying diseases related to the endometrium, the effect of microplastics on reproductive health and studying pregnancy and early maternal-embryo interactions.

1 Introduction

1.1 The female reproductive tract: complexity, purpose and complications

The human female reproductive tract (FRT), a complex system of multiple interconnected organs, is in need of more research in order to better understand reproduction and the many conditions related to it. The cervix, uterus, fallopian tubes and the ovaries are the main organs of the FRT, see figure 1a. They undergo constant changes because of the 28 day menstrual cycle. The most significant purpose of the FRT is reproduction; it guides ovulated oocytes, it prepares for the implantation of an embryo and it nurtures the foetus so that it is able to develop as an independent organism [2]. Steroid hormones, such as estrogen and progesterone, direct the menstrual cycle which remodels the FRT to the shape needed for that moment in the cycle. The uterine wall, consisting of the endometrium, myometrium and perimetrium, is also prone to structural, cellular and vascular changes during the 28 day cycle. The endometrium, see figure 1b, thickens and secretes nutrients during decidualisation, so that an embryo can be implanted. Part of the endometrium is also shed during men-

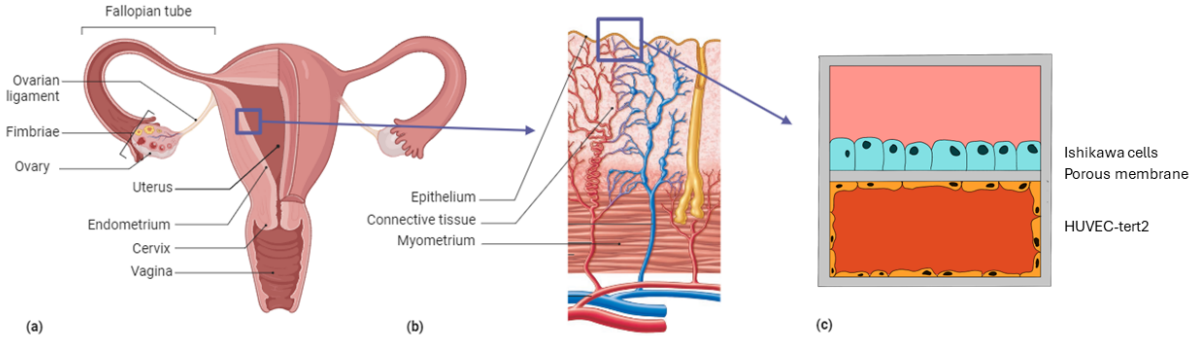

Figure 1: Schematic visualisation of the female reproductive tract created with Biorender (a), the endometrium [1](b) and the crosssection of the endometrium-on-a-chip (c).

struation [1].

There exist numerous significant medical conditions associated with the female reproductive system, such as ovarian, cervical or uterine cancer, infertility, endometriosis, preterm birth or other complications of pregnancy [2]. Pregnancy, and diseases and conditions related to it, are difficult to study in humans, because of ethical considerations of the mother and child. Most of these conditions have been studied in animal models, but their similarity to the human situation has been debated. Menstrual cycles of many animals differ significantly to those of humans or are simply not present. Also, animal models with female reproductive tracts similar to those of humans, such as nonhuman primates, are difficult to obtain because of high costs and ethical considerations [3]. So, health issues associated with the FRT have been difficult to study, despite their high prevalence.

Endometriosis, for instance, is a chronic disease affecting around 170 million women worldwide. Endometriosis is the condition where tissue containing stroma, epithelial and endometrial cells is located outside of the uterus. It was first discovered about a hundred years ago. Yet currently there is no known permanent cure and knowledge about its pathophysiology is unclear. Also, most current treatments, for example hormonal treatment, pain medications or laparoscopy, are very invasive or not suitable for long term use [4]. Also, recent studies have highlighted the effect of micro- and nanoplastics (MPs and NPS) on female health and reproductive fitness. These plastic particles are very pervasive in the environment and are able to enter the human body via inhalation, ingestion or skin contact [5].
Microplastics have recently been detected in the human endometrium in a study by Sun et al. These MPs and NPs have been reported to cause cytotoxicity, leading to fibrosis, endometrium thinning and dysfunction [6]. The study of the effect of MPs and NPs on reproduction has only been conducted in mice. This highlights the need to study the effect of NPs and MPs on reproductive health in a more human-like model.

It can thus be concluded that there is an urgent need for more extensive research into the FRT, and specifically the development of better models of the endometrium. This research will thus focus on a model of the endometrium, as seen in figure 1. This model of the endometrium will have applications for the study of maternal-embryo interactions, female toxicity studies but also for the study of microplastics on female health.

1.2 Organs-on-a-chip

Organs-on-a-chip (OOCs) offer an advanced way of modelling tissues or organs in vitro, which will be used in this research to model the endometrium. OOCs are devices made out of polydimethylsyloxane (PDMS), silicon, glass or certain polymers and have microchannels in which miniature tissues can be formed and grown [7]. There are different possible layouts for the organs-on-a-chip, namely a single channel OOCs, vertical OOCs and planar OOCs. Planar OOCs have multiple channels located next to each other and are separated by so-called phase guides; micro structured barriers which establish certain flow patterns. The vertical OOC has channels located on top of each other which are separated by a porous membrane [8]. The vertical OOC will also be used in this research. The microchannels in these chips allow for a 3D-like cell environment, which is a big improvement in comparison to the old approach of culturing cells in polystyrene well plates [9]. Also, the microchannels provide a high level of control

of the micro environment of the cells and an easier way to observe the behaviour of cells and tissue than *in vitro* [7].

There are multiple ways to produce OOCs, for example PDMS-based soft lithography, thermoplastic-based injection moulding or UV curable resin-based 3D printing. PDMS based soft lithography is a very popular choice for OOC production, because the production steps are relatively simple and the microchannels can be made with high resolution and very precise details [7]. PDMS is currently also the most widely used material for OOCs. PDMS is optically transparent, has excellent gas permeability and a good biocompatibility with cells and blood. An important drawback of PDMS is its hydrophobicity which leads to the absorption of small hydrophobic molecules, potentially causing a misbalance of molecules in the culture medium. The hydrophobicity and bioinertness of PDMS prevent cell adhesion, but this problem can be overcome with protein coating [10].

OOCs have proven successful for the modelling of parts of the FRT, for example an endometrium-on-a-chip by Gnecco et al. [11]. The chip consists of two channels separated by a porous synthetic membrane. The top chamber contains endometrial stromal fibroblasts and the bottom vascular chamber contains HUVECs. They also integrated the menstrual cycle into the chip, by supplementing media with different hormone concentrations over 28 days. With the menstrual cycle mimicked in the chip, the stromal cells differentiated into decidual cells similar to the *in vivo* situation. Another OOC was made by Ferraz et al. which modelled the oviduct in order to mimic early reprogramming of the (epi)genome of the embryo and improve the quality of in vitro fertilised (IVF) zygotes. The OOC also consisted of two channels separated by a porous synthetic membrane, with a confluent layer of bovine oviduct epithelial cells (BOECs) cultured in the top compartment, see figure 2A. Media with different concentrations of progesterone and estrogen was circulated through the vascular compartment to mimic the human hormonal cycle. The research showed that the BOEC differentiation and growth was similar to the *in vivo* epithelium, which made the oviduct-on-a-chip a useful tool for the production of *in vivo*-like zygotes and the study of early embryo development [12]. The oviduct-on-a-chip was partly developed by the research group Applied Microfluidics for BioEngineering Research (AMBER), and the endometrium-on-a-chip will be very similar to this model. The model could be improved with the addition of an endothelium in the vascular compartment and a more biological and *in vivo*-like membrane.

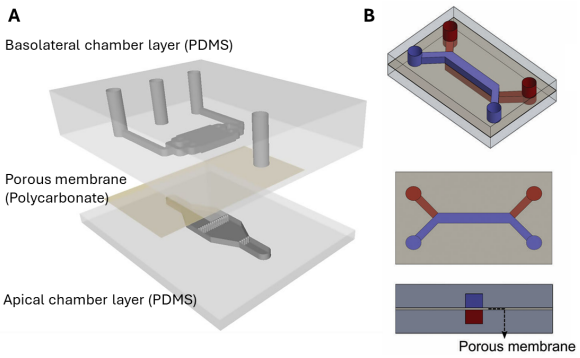

Figure 2: Two OOC designs: (A) oviduct-on-a-chip by the AMBER research group [12] and (B) layout of the endometrium-on-a-chip (not up to scale) [8]

1.3 The Addition of a Vascular Compartment

In this research, an endometrium-on-a-chip will be used consisting of two channels located on top of each other which are separated by a horizontal porous membrane, see figure 2B. This design is based on earlier models developed by the AMBER research group, see figure 2A. In the top compartment, Ishikawa cells (human endometrial carcinoma cells) will be cultured in order to model the epithelial layer of the endometrium. In the bottom compartment, a blood vessel will be modelled. For this research, the focus will be on the addition of this bottom vascular compartment. The integration of vasculature in OOCs is necessary, because it replicates the physiological complexity of organs and tissue and makes the models more biologically acurate [13]. Human umbilical vein endothelial cells (HUVECs) are used to add endothelium to the vascular compartment. These cells have been proven effective to mimic endothelium. They are robust and have well defined protocols for cell culture [11].

1.4 The Addition of a Collagen Type I Membrane

Membranes in OOCs serve multiple purposes, the most important purpose is acting as a scaffold for the cells and supporting cell adhesion. There are multiple membrane characteristics that influence cell adhesion, such as the material, topography and the chemistry of the surface. Many organ-on-a-chip models use a synthetic membrane in the chip, for example membranes made out of polycarbonate (PC), PDMS or polyethylene terephthalate (PET) [14].

Synthetic polymer membranes are often robust, have a wide availability and they have very precise features. However, these membranes are bioinert and need to be coated before cells are able to adhere to the membrane. It has been reported that these surface coatings are able to compensate for some of the missing properties of the natural extracellular matrix (ECM). Their thickness is also usually fixed at 10 μm, which hinders cell-cell contact across the membrane. Uninted morphology of cells grown on synthetic membranes has also been reported [9]. In contrast, biological OOC membranes are made out of natural ECM proteins, such as collagen, fibronectin or laminin. These membranes are very bioactive, by providing familiar mechanical and chemical cues to the cells and providing a large surface contact area [14]. The stiffness of the biological OOC membranes is also more similar to the stiffness of biological basement membranes from human tissue [15].

Numerous studies have demonstrated that using a biological membrane for an OOC significantly enhances cell behavior. A research by Wang et al. developed an OOC of the colon, consisting of two channels separated by a collagen membrane. Human caco 2 cells were cultured in the top compartment. The research showed that using collagen membranes in OOCs leads to a significantly higher expression of adhesion and cell function proteins (F-actin, ZO-1 and ezrin), suggesting that a collagen membrane creates an improved micro environment in comparison to synthetic polymers. Higher cell viability was also reported [9]. Another research by Mondrinos et al. cultured HUVECs, murine pericytes, human lung adenocarcinoma cells, human bronchial epithelial cells and human lung fibroblasts on different types of ECM membranes. They reported increased FAK signalling in cells cultured on a collagen membrane. FAK is a crucial component in the focal adhesion complex for integrin engagement with the ECM. The assembly of thick bundles of stress fibres along the periphery of the cell were also noted. The research concluded that the cells had a stronger interaction with their surroundings, contributing to an enhanced structural integrity [15].

There are numerous strategies to attach a membrane to an OOC. One of the most common methods used is plasma oxidation, which activates the surface of PDMS or glass so that they are able to bond together [11, 16, 17]. Another frequently used method to attach membranes to chips is by using a PDMS prepolymer or a PDMS prepolymer and toluene mixture as a mortar, so that the membranes and chip are fixed after curing [12, 15, 18]. Other researchers used double sided pressure sensitive adhesive (PSA) tape to fix the membrane to the chip. [19].

For OOCs with a collagen membrane, the membrane can also be synthesised directly on top of one of the of the PDMS slabs to facilitate attachment [20]. In this research, a membrane made out of collagen type I will be used. This protein is one of the most common in the ECM and has been proven effective for good cell adhesion and cell culture. Most coatings of bioinert materials also rely upon collagen type I [9] . Many conventional membrane attachment methods are unsuitable for the attachment of a collagen membrane. Methods using a mortar often rely on high curing temperatures, which causes denaturation of the collagen [21]. Collagen membranes are also relatively fragile and sensitive for chemicals and unavailable for plasma activation. These factors makes the attachment to microfluidic devices difficult [14]. It is thus necessary to find an efficient and non-harmful way to incorporate a collagen membrane into an OOC.

1.5 Goal of this Research

So, there is a knowledge gap in the research into health of the female reproduction tract, reproduction and the effect of microplastics on the FRT. More accurate models of the FRT are needed to fill this knowledge gap. The AMBER research group has been working on these kind of models, namely the oviduct-on-a-chip and the endometrium-on-a-chip. These current models however, lack a vascular compartment and rely on a synthetic membrane. The goal of this research is to improve the endometrium-on-a-chip with the addition of a vascular compartment and a collagen membrane, which will make the chip more *in vivo* like. The final design of the chip can be seen in figure 1c. For the collagen membrane, the amount of cell adhesion, cellular morphology and changes in membrane thickness will be characterised. It is hypothesized that a collagen membrane will contribute to better cell adhesion and a more in vivo like morphology. Two versions of the endometrium-on-a-chip with a collagen will be tested, to find out what is the best collagen attachment method for the chip. The collagen membrane will either be attached to the microfluidic device with an PDMS mortar cured at 37 °C or with pressure sensitive adhesive tape. The attachment strength will be characterised with the burst pressure test. This improved model of the endometrium-on-a-chip will be a vital addition to the research of female health and microplastics, conditions and diseases associated to the FRT and

it will provide a healthy model to study pregnancy and maternal-embryo interactions.

2 Materials and methods

2.1 Interactions between HUVEC-tert2 and Cell Culture Substrate

HUVEC culture and maintenance All materials in this section were bought from Gibco, Thermo Fisher Scientific (Waltham, MA, USA) unless specified otherwise. The human umbilical vein endothelial (HUVEC/TERT2) cell line (Evercyte, Vienna, Austria) was cultured in maintenance medium (endothelial cell growth basal medium (EBM) with 0.2% bovine brain extract, 5 ng/ml rh EGF,10 mM L-glutamine, 0.75 Units/ml heparin sulfate, 1 μg/ml hydrocortisone, 50 μg/ml and 2% fetal bovine serum) using 75 ml tissue culture treated flasks with a 0.1% gelatin coating (2 mL added and incubated for at least 30 minutes at 37 °C and 5% CO_2). The medium was refreshed every 2-3 days and the cells were passaged every 3-4 days. The HUVECs were grown at 37 °C and 5% CO_2. Passages 17 to 21 were used for experiments.

Preparation of the polycarbonate membranes for cell culture A 10 μm thick porous polycarbonate (PC) membrane (TRAKETCH PC10, pore size: 0.4 μm, pore density: $100 \cdot 10^6$ cm^{-2}, SABEU GmbH & Co. KG) was used. PC membranes were prepared for cell culture. All chemicals were bought from Sigma Aldrich (Zwijndrecht, the Netherlands) unless otherwise specified. First, the membranes were plasma oxidised (CUTE, Femto Science) and afterwards immediately transferred to a silanization solution (400 μL (3-Aminopropyl)triethoxysilane (APTES) in 20 mL 20% ethanol) and were incubated for 20 minutes at RT. The PC membranes were sterilised in a cell suspension culture 48 well plate using UV light for an hour in a laminar flow cabinet. A polydopamine (PDA) solution (10 mg PDA and 5 mL Tris-HCl buffer) was made and filtered through a 0.20 μm filter. 300 μL PDA solution was added to all wells, and was incubated for one hour at RT. After incubation, all wells were washed twice with phosphate buffered saline (PBS). A collagen type I solution (177 μL collagen and 4.8 mL PBS) was prepared on ice and 300 μL was added to each well. It was incubated for half an hour at 37 °C and 5% CO_2. The wells were washed once with PBS.

Preparation of the collagen membranes for cell culture Collagen membranes (Viscofan bioengineering, thickness: 20 μm) were prepared according to manufacturer's instructions. 150 μl of PBS was pipetted in wells of a 48 well suspension culture plate. In every well, a collagen membrane was added carefully without submerging it. The membranes were incubated for 30 minutes at RT. After incubation, PBS was carefully removed and the membranes dried without lid over the weekend at RT. Then, 300 μL of cell culture media was added to each well, then it was incubated for four hours at 37 °C and 5% CO_2.

HUVEC and membrane interactions A cell culture treated well plate was coated with 0.1 % gelatin (2 mL added and was incubated for at least 30 minutes at 37 °C and 5% CO_2). For the well plate with the coated PC membranes, the well plate with the collagen membranes and the gelatin coated well plate: 300 μL of cell suspension was added to the wells with either a concentration of $3.3{\cdot}10^5$ cells/ml or $1.67{\cdot}10^5$ cells/ml. The plates were incubated at 37 °C and 5% CO_2.

Cell fixation For these well plates, medium was refreshed every 2-3 days. On days 2, 4 and 7, cells were fixated. For fixation, cell culture media was removed and washed once with PBS. Subsequently, 100 μl of formaldehyde (Sigma Aldrich (Zwijndrecht, the Netherlands)) was added to each well and was incubated for 20 min at 37 °C and 5% CO_2. After incubation, the wells were washed three times with PBS.

Staining of HUVECs and membrane samples All stainings and antibodies used in this section were bought from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). The other chemicals were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands) After fixation, PBS was removed from the samples and 100 μl of 0.1% Triton X-100 in PBS was added. The samples were incubated for 30 min at RT. Then, the cells were washed twice with PBS and 150 μl blocking buffer (1% Bovine serum albumin (BSA) in 1x PBS) was added. The samples were incubated for 60 min at RT. The blocking buffer was removed and 100 μl 1:200 dilution VE-cadherin rabbit in blocking buffer was added. The samples were incubated overnight at 4 °C. Afterwards, the cells were washed twice with PBS. 100 μl 1:200 dilution goat antirabbit Alexafluor 488 in blocking buffer was added and was incubated for an hour at RT. Cells were washed twice with PBS. A dilution of 1:40 phalloidin and 1:100 DAPI in blocking buffer was added and the cells incubated for 30 min at RT. Then, the cells were washed twice with PBS. The membranes were removed from the wells and placed on a glass plate and imaged with a confocal microscope (Zeiss LSM 880).

Cell viability To test the effect of cell seeding densities on the cell viability, HUVECs were

seeded at $1.67 \cdot 10^5$ cells/ml and $0.33 \cdot 10^5$ cells/ml in a gelatin coated cell culture treated 48 well plate. Cell viability was assessed with a LIVE/DEAD assay (Sigma Aldrich (Zwijndrecht, the Netherlands)) on day 2 and day 4. Cells were washed twice with PBS. Then, 300 μl of LIVE/DEAD staining was added (1.5 mL EBM, 3 μl 2 mM EthD-1, 0.75 μl 4mM Calcein AM) to the cells and incubated for 15 min at 37 °C and 5% CO_2. Pictures were taken with a fluorescence microscope (EVOS FL).

Collagen membrane characterisation Collagen type 1 membranes were soaked in PBS and incubated at 37 °C and 5% CO_2. Thickness and diameter measurements were taken at day 1 until 9.

2.2 Integration of the Collagen Type I Membrane into the Microfluidic Chip

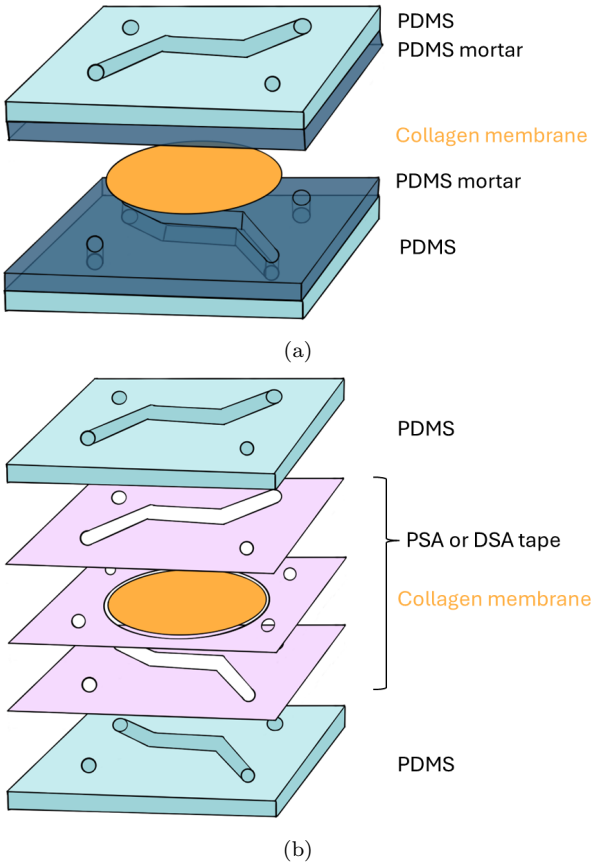

Figure 3: Schematic drawing of the microfluidic chip with attachment of the collagen membrane facilitated by a PDMS mortar (a) and pressure sensitive adhesive (PSA) or double sided adhesive (DSA) tape (b). Drawings are not up to scale.

Microfluidic chip fabrication The mold for the microchannels was fabricated with stereolithography (SLA). The design was printed using the Form 3B+ 3D-printer and FL clear resin V4 (Formlabs, Sommerville, MA, USA) with a layer thickness of 50 μm. The mold was post-processed with a UV treatment (λ=405 nm) and a post-bake at 120 °C. Microchannels were fabricated with soft lithography. Per device, two polydimethylsiloxane (PDMS) slabs were fabricated, PDMS prepolymer and a curing agent (SYLGARD 184, Sigma Aldrich (Zwijndrecht, the Netherlands) were mixed at a weight ratio of 10:1, after which it was placed in a vacuum chamber of -0.08 bar to degas. Then, the PDMS was poured into the mold and returned to the vacuum chamber to degas. After all air bubbles were removed, the mold cured at 65 °C overnight in an oven. After curing, the PDMS slab was removed from the mold and cut into the desired shape. Scotch tape was placed on the top of the slabs to prevent dust from accumulating on the chips.

Collagen type I Membrane and Microfluidic Chip Attachment Methods In this research, the Collagen Cell Carrier membrane from Viscofan bioengineering, see figure 4. The membrane is made from pure collagen type I fibres from bovine skin. It has a thickness of 20 μm and a diameter of 10 mm [22].

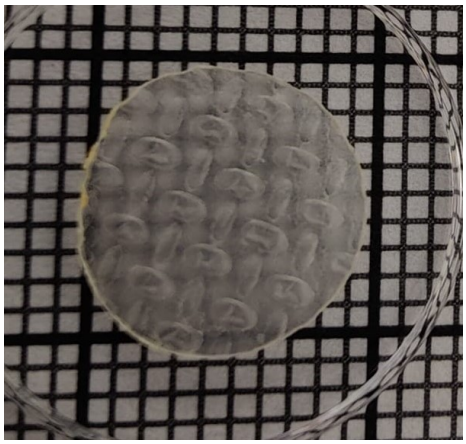

Figure 4: The Collagen Cell Carrier membrane from Viscofan bioengineering (dehydrated).

Two main methods were considered to attach the collagen membrane to the microfluidic device: a PDMS mortar and pressure sensitive adhesive (PSA) tape. Each attachment method had two variations. The designs of the microfluidic device are shown in figure 3.

PDMS and curing agent were mixed at a 10:3 ratio. The PDMS mortar was spincoated on a petridish at 2000 rpm for 3 minutes (Spin150). The

bottom PDMS slab was gently dipped in the PDMS mortar and a collagen membrane was placed on top of the mortar. For one of the chips, the collagen membrane was firmly pushed to secure adhesion. The slabs were incubated at 37 °C and 5% CO_2 overnight. The next day, the petridish with PDMS mortar was prepared again and the top PDMS slab was gently dipped in the mortar. For one of the chips, the top and bottom slabs were pushed firmly against each other so that the collagen membrane was properly secured in between. This makes two variations of the chip, one chip which was extra secured with firm pressure and one chip which was fabricated without extra pressure.

Double sided adhesive tape (DSA) was laser-cut to accommodate for the channels, the collagen membrane housing and the holes. Layers were assembled according to figure 3B. One chip variation had all three layers of DSA tape and the other chip was secured with only the middle layer of DSA tape. Both chips were gently pushed together for extra adhesion.

Microfluidic chip attachment evaluation Microfluidic chip attachment was evaluated with a burst resistance test and a leakage test. For the burst resistance test, three in- and outlets of the chip were closed off with scotch tape. the Flow EZ (Fluigent) was attached to the inlet of the top channel. The pressure was increased from 0 to 2000 mbar. Airflow was measured to find out at what pressure leakage occurred. For the leakage test, 10 μl of diluted food dye was slowly pipetted into the inlet of the bottom channel. The outlet of the bottom channel was covered with scotch tape.

Analysis Results Images of the HUVECs were enhanced digitally with Fiji ImageJ for higher visual quality and easier morphological analysis. The cellular surface area, maximum and minimum feret diameter were also measured with Fiji. 10 random cells per condition were selected and measured, see supplementary figure S1. The ratio between the maximum and minimum feret diameter was calculated by dividing the minimum feret diameter by the maximum feret diameter. A ratio close to zero signifies a thin and long cell, whereas a ratio close to one signifies a round cell.

VE-cadherin expression was also quantified with Fiji ImageJ. Images were taken with the same settings and not enhanced digitally, so that the fluorescence intensity could be compared. Images were converted to 8-bit for better comparison. A straight line was drawn through some cells, see supplementary figure S1. A profile plot was made and the ten highest peaks were selected, and their grey value was measured. The average grey value of these peaks was used as the fluorescence intensity of a condition.

For the cell viability, dead and living cells were counted to calculate the viability percentage. Collagen membrane thickness was evaluated using the confocal microscope. Start and endpoints of a z-stack were selected to measure the thickness of the membrane. The diameter of the collagen membrane over time was calculated by measuring the area of the membrane in Fiji ImageJ. The area was converted to the diameter.

3 Results and discussion

3.1 Interactions between HUVECs and Cell Culture Substrate

3.1.1 The Effect of Substrates on Cell Morphology and Monolayer Formation

HUVECs were seeded on three different substrates: (1) a polycarbonate (PC) membrane with a collagen type I coating, (2) a collagen type I membrane and (3) a polystyrene cell culture treated well plate with a gelatin coating. The cells were stained with phalloidin, DAPI and VE-cadherin. The results of the cells on day 2 are presented in figure 5A-F. Images of the HUVECs stained on days 4 and 7 can be found in the supplementary information, see figure S2. The average cellular surface area and cell shape were also quantified, see figure 5G and table 1. The expression of VE-cadherin in the HUVECs is presented in figure 5H. The results of VE-cadherin of the HUVECs cultured in the gelatin coated well plate are not displayed because the pictures were taken with different settings, so these results could not be compared.

As can be seen in figure 5, the different substrates cause different cell morphology. The HUVECs on the collagen coated PC membrane had an oval and spindle-like morphology, similar to the cells seeded on the gelatin coated well plate. The HUVECs on the collagen membrane appear more round or star-shaped. The ratio between the Feret diameter and the minimum Feret diameter also shows that the HUVECS seeded on the collagen membrane are in general more round than the cells on other substrates. As for the surface area, the HUVECs cultured on the collagen membrane are smaller than the HUVECs cultured on the other two substrates, see figure 5G. A lower seeding density seems to result in a bigger cellular surface area for the PC membrane and the well plate. However, the error bars do overlap and it was not possible to perform

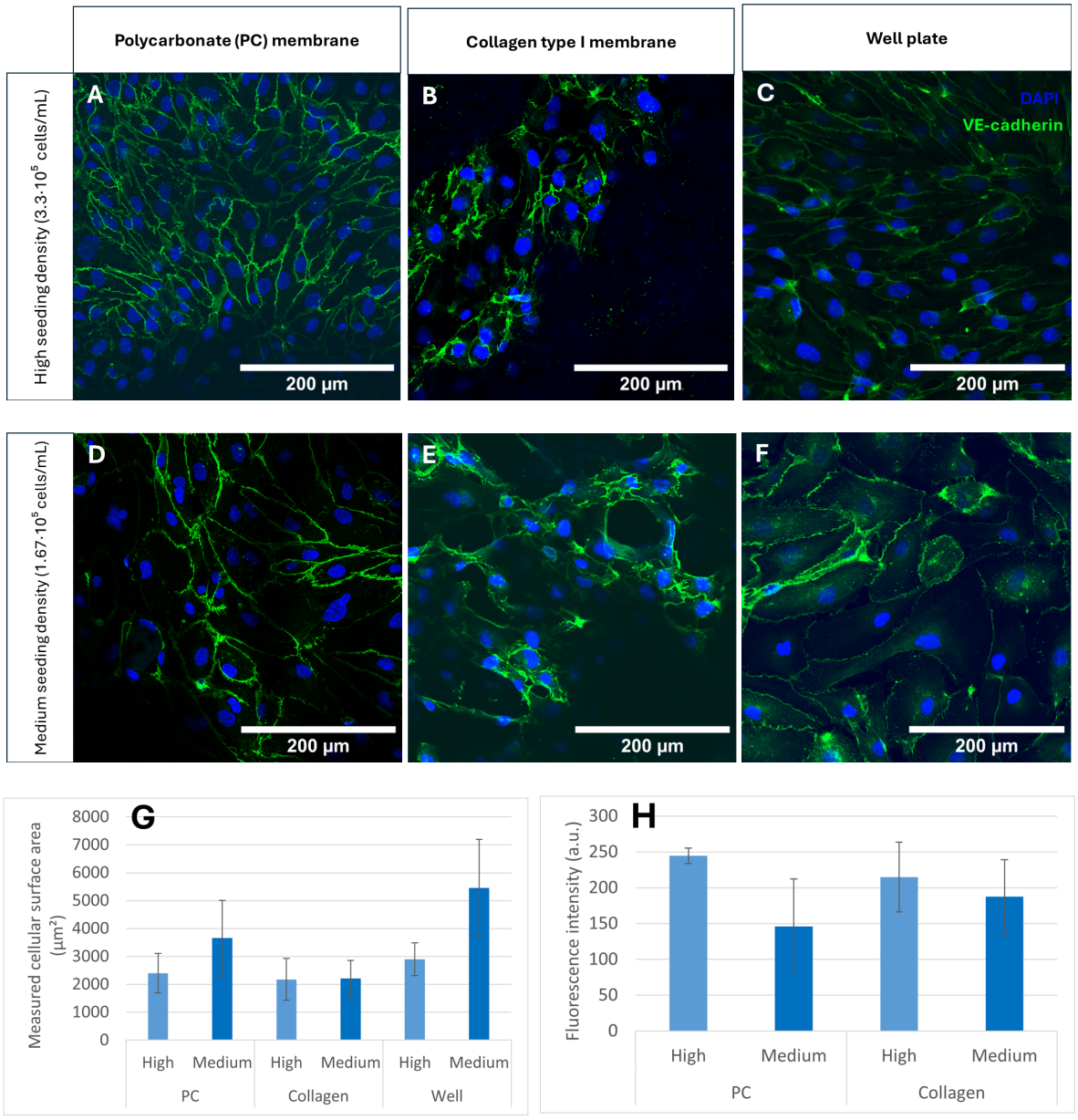

Figure 5: Morphological results of HUVECs seeded on different substrates. Panel A-F: fluorescence images were taken with a confocal microscope. The substrates used are a collagen coated PC membrane, a collagen membrane or a gelatin coated well plate. The cells were seeded at two densities: high ($3.3{\cdot}10^5$ cells/ml) and medium ($1.67{\cdot}10^5$ cells/ml). Cells are stained with DAPI (blue) and VE-cadherin (green). The contrast of the images was enhanced for better visual quality. Panel G: The average cellular surface area of HUVECs seeded at high and medium density on different substrates. Panel H: the expression of VE-cadherin quantified with the peak fluorescence intensity (arbitrary units). Error bars were calculated with the standard deviation. n=10 cells

Table 1: Morphological characteristics of the HUVECs seeded at different densities and on different substrates. A high ($3.3{\cdot}10^5$ cells/ml) and medium ($1.67{\cdot}10^5$ cells/ml) cell density were used. The ratio between the minimum and maximum Feret diameter gives an indication of the cell shape. A ratio close to zero signifies a long and thin cell, and a ratio close to one signifies a round cell. The range is calculated with the standard deviation. n=10 cells

	Seeding density	Feret diameter (μm)	Minimum Feret : maximum feret
PC membrane	High	79.3 ± 18	0.41
	Medium	100 ± 20	0.37
Collagen membrane	High	60.1 ± 8.8	0.55
	Medium	67 ± 12	0.57
Well plate	High	130 ± 27	0.25
	Medium	129 ± 45	0.40

a test on the data for statistical significance. More data and a statistical test is required to draw definite conclusions.

Figure 5H shows that the expression of VE-cadherin was highest for the HUVECs seeded at a high density on the PC membrane. A higher seeding density also leads to more VE-cadherin expression. High VE-cadherin expression signifies the formation of a good cell monolayer and more cell-cell adhesion. For similar reasons as the quantification of cellular surface area, more data and a statistical test are needed for a more definite conclusion.

It has also been reported that some cell lines such as endothelial cells are able to integrate themselves into the collagen membrane [9]. Orthogonal views of z-stack images were evaluated to see whether a similar effect took place for the HUVECs. The collagen membrane exhibited a low autofluorescence, so it could be imaged with confocal microscopy. Z-stack images were made with 10 stacks, see supplementary figure S3. The resolution was too low to determine if the HUVECs integrated themselves into the collagen membrane.

After day 2 of cell culture, a high amount of cell death occurred. The cells shrunk and expressed barely any VE-cadherin, signifying the disappearance of the cell monolayer, see supplementary figure S2. There was also a high amount of detached cells because of cell death. There are multiple possible causes of the cell death. The medium in the wells was refreshed every 2-3 days and no severe discoloration of the medium was observed. So, the cell death was probably not caused by this. It was hypothesized that the HUVECs might have been seeded at a too high density. A LIVE/DEAD staining was performed to evaluate the effect of the seeding density on the viability. The viability of the cells seeded at medium ($1.67{\cdot}10^5$ cells/ml) and low ($0.33{\cdot}10^5$ cells/ml) density can be seen in supplementary figure S4. After day 2, the viability of the HUVECs seeded at medium density seemed lower than the viability of the HUVECs seeded at low density. However, there were only two measurements available per condition, so the relevance of these results is highly debatable. It can thus not be concluded whether the used seeding densities were related to the cell death.
Also, other articles used higher seeding densities than those used in this research, and no complications were mentioned. For example, Rayner et al. fabricated a kidney-on-a-chip where HUVECs were seeded with $6 \cdot 10^6$ cells/ml [23]. The cells were seeded at a higher density and on a smaller surface than in this research. No problems with this high seeding density were mentioned, so it is unlikely that the high seeding density used in this experiment was the cause of cell death.

These results show that all substrates successfully lead to healthy HUVECs on cell culture day 2. The PC membrane, collagen membrane and well plate (negative control) facilitate cell attachment and VE-cadherin was expressed, which signifies the formation of a confluent monolayer. However, data of HUVEC cell culture is only available from cell culture day 2. The limited amount of data makes it difficult to draw conclusions on the effect of the different substrates on HUVECs. In order to draw conclusions about the actual effect of the substrates on HUVECs, data from at least two weeks of cell culture with healthy HUVECs is needed.

3.2 Characterisation of the Collagen Type I Membrane

The thickness and diameter of the collagen type I cell carrier membrane in phosphate buffered saline (PBS) were measured at different points in time. The change in thickness is displayed in figure 6. The diameter of the collagen membrane slightly increased by approximately 0.5 mm after hydration. The membrane also lost its rigidness after hydration.

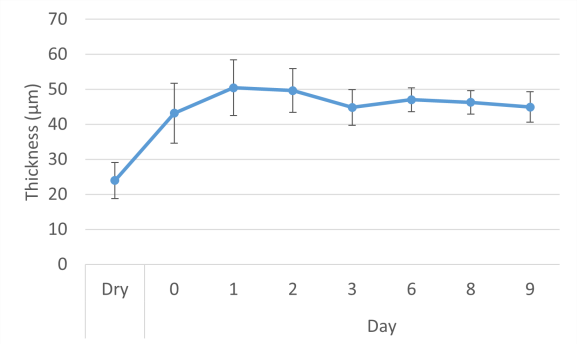

Figure 6: The thickness of the collagen membrane incubated phosphate buffered saline (PBS) at 37 °C and 5% CO_2 over time. Error bars were calculated with the standard deviation. n=6 locations on one collage membrane.

In figure 6, it can be seen that the membrane thickness increases with approximately 20 μm after hydration in PBS. After an hour of incubating in PBS at 37 °C and 5% CO_2 over time (day 0), the thickness of the collagen membrane is almost constant over time with some small fluctuations. A constant collagen membrane thickness is consistent with other reports [24].

Another important factor to consider is the effect of cell culture on the thickness of the collagen membrane. Many cells also have the ability to remodel the collagen membrane. A decrease in collagen membrane thickness due to this cause has been reported in other research. Rayner et al. cultured HUVECs and human kidney cells in channels in a collagen type I gel, separated by a collagen type I membrane for 14 days. The thickness of the collagen membrane had a decrease in thickness of 90% [23]. Because of the collagen gel, the study is not fully similar, but it does show that these cells are able to remodel the collagen membrane. Another study by Lo et al. cultured HUVECs and H441 cells on a collagen type I membrane for 14 days and compared it with a collagen membrane in culture media. The thickness of the membrane with cells cultured on it had a decrease in thickness of 30% whereas the thickness of the membrane in only culture medium stayed the same [24].

So, after the collagen membrane has been fully hydrated, the thickness stays relatively stable over time, which has also been reported in other research. However, other articles have reported a decrease in collagen membrane thickness due to remodelling performed by cells. These articles used HUVECs, among other cells, so it is highly likely that HUVECs will influence the collagen membrane thickness over time. This effect needs to be quantified and studied further in order to know how it will influence the attachment of the membrane to the microfluidic chip.

3.3 Collagen Type I Membrane and Microfluidic Chip Attachment Methods

Multiple methods were used to attach the collagen membrane to the microfluidic chip. The collagen membrane was attached via a PDMS mortar where one device was firmly pressed together for extra attachment and one device was not. The used PDMS mortar was uncured PDMS with a 10:3 ratio. The collagen membrane was also attached with double sided adhesive tape (DSA), one design with 3 layers of tape and one design with only the middle layer of DSA, see figure 3B. The design with only the middle layer of DSA tape was also tested, because the fabrication process required less effort than the fabrication process with three layers. DSA tape was used as a replacement for pressure sensitive adhesive tape (PSA), because the PSA tape was not available at the time of the experiment. The results of the fabrication of the chips and a leakage test are presented in figure 7. The results of the burst pressure test can be found in the supplementary materials, figure S5.

The burst pressure test was performed to quantify the bonding strength of the microfluidic chip. Three in- and outlets of the chip were closed so that an airflow would only occur when the chip broke and created a leakage. However, when air pressure was applied on the chips, an airflow was already present. This phenomenon happened for all chip designs, meaning that all microfluidic chips had leaks and were not bonded sufficiently. The graphs

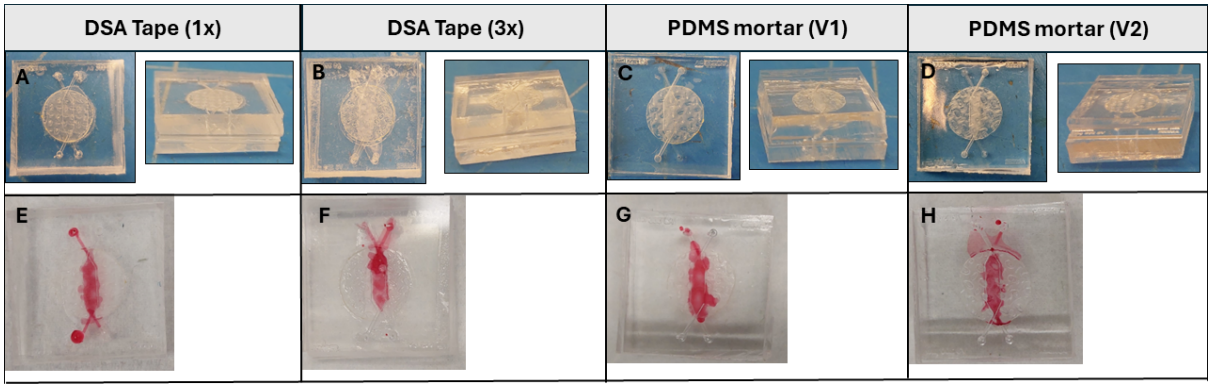

Figure 7: The four versions of the designed chips: (1) one middle layer of double sided adhesive (DSA) tape (2) three layers of DSA (3) firmly pressed PDMS mortar and (4) lightly pressed PDMS mortar. Subfigure A-D shows the top and side view of the chips. Subfigure E-H shows a leakage test, fluid was injected in the top left inlet.

of the airflow during the burst pressure test can be found in supplementary figure S5. The leakage test was performed afterwards to test fluid leakage in the chip, see figure 7E-H. For all microfluidic chip designs, significant fluid leakage occurred. The leakage and burst pressure test showed that the current chip designs are not suitable for cell culture, and need to be developed further.

3.3.1 Evaluation PDMS mortar for Membrane to Microfluidic chip Attachment

The results of the burst pressure test are contradictory to results in other articles. Eddings et al. tested multiple PDMS-PDMS bonding methods, for example oxygen plasma treatment, corona treatment and uncured PDMS as a mortar. They reported that bonding with uncured PDMS resulted in an average burst resistance of 474 kPa, which was significantly higher than the other bonding methods [25]. In that article, a PDMS : curing agent ratio was used of 15:1, which might explain the different outcomes of that and this research. Also, the bonding of PDMS was tested without a membrane in between, which is different from the microfluidic chip in this research. Yet Mondrinos et al. managed to successfully attach a collagen membrane to a microfluidic device with a 10:3 PDMS mortar [15]. For future research, different ratios of PDMS mortar could be tested to attach the collagen membrane to the chip, because it has the potential to create a strong bond between the microfluidic device and the collagen membrane.

As far as fabrication difficulty, the method with the PDMS mortar was experienced as relatively easy. No mortar was observed in the channels during the fabrication of the chips. There was also a chance of PDMS mortar being absorbed into the collagen membrane. The assembled microfluidic devices were evaluated under the phase contrast microscope to find traces of PDMS mortar in the membrane, see supplementary figure S6. However, it was difficult to see whether PDMS mortar had leaked into the membrane. For future research, it needs to be tested whether the PDMS mortar leaks into the collagen membrane and what effect this has on cell culture in the microfluidic device.

The collagen membrane is not able to resist high temperatures which are usually used for PDMS mortar curing. The devices were cured overnight in an incubator at 37 °C, instead of 60 to 70 °C for a couple of hours. Incomplete curing of the PDMS might cause leakage of uncured oligomers into the cell culture media, which can be toxic to cells [26]. However, Mondrinos et al. cured their PDMS mortar at room temperature overnight and did not mention any adverse effects on the cell viability [15]. In future research, the effect of lower curing temperatures on the cell viability of the device needs to be studied.

3.3.2 Evaluation of DSA tape for Membrane to Microfluidic chip attachment

Assembly of the microfluidic devices with DSA tape also resulted in significant leakage. DSA tape was used as a substituent for pressure sensitive adhesive (PSA) tape, because PSA tape was unavailable at the moment of the experiment. The leakage might have been caused by the folding of the slices of

DSA tape during fabrication, causing the creation of ridges and crevices. DSA stuck to itself very easily, which made it difficult to properly align the multiple layers of DSA tape. This problem would probably be less present when using PSA tape. Using PSA tape, alignment and flattening the layers of tape will be easier, because adhesion will only occur when pressure is added. This method might prevent the formation of ridges and crevices, so that there will be less to no leakage. Both PSA and DSA tape have been used in the fabrication of other devices, and have not influenced the biocompatibility of the devices [19, 27]. However, in the devices of the previously mentioned articles, there was no close contact between the channels and the tape, whereas this design has the tape located very close to the cells. The effect of the tape on the viability of the cells in the device needs to be studied in future research.

3.4 Conclusions and Future Prospects

In conclusion, HUVECs were successfully cultured until day 2 for the three substrates: a collagen type I coated polycarbonate membrane, a collagen type I membrane and a gelatin coated well plate (polystyrene). Data from at least two weeks of cell culture is needed to determine the effect of the different substrates on the growth, viability and morphology of the HUVECs. The collagen type I membrane increased approximately 20 μm in thickness and 0.5 mm in diameter after hydration in PBS. After hydration, the dimensions of the collagen type I membrane stayed constant for at least 9 days. The rigidness of the collagen membrane decreased significantly after hydration. Taking into account the decrease in rigidness of the collagen membrane, two designs for embedding the membrane into the microfluidic chip were proposed. Both the design based on a PDMS mortar and the design based on the DSA tape resulted in significant air and fluid leakage. However, with further optimalisation they are very promising for a strong bonding between the collagen membrane and the microfluidic device.

Other models of the endometrium have also been developed, for example the endometrium-on-a-chip by Gnecco et al. as described in the introduction [11]. This chip also integrated the menstrual cycle into the microfluidic device, by supplementing media with different hormone concentrations over 28 days. A similar method was used in the oviduct-on-a-chip by Ferraz et al. [12]. Also, the vascular compartment of the endometrium-on-a-chip was dynamically perfused to mimic the shear stress that epithelial cells experience *in vivo*. The addition of these two elements resulted in the decidualisation of the stromal cells and the alignment of the HUVECS with the direction of the flow. These two elements could also be implemented in our endometrium-on-a-chip in order to make it more biologically accurate.

Once fully developed, the endometrium-on-a-chip will have a wide range of applications. It can be used as a healthy model for reproduction to study early maternal-embryo interactions. If patient-derived cells are implemented in the endometrium-on-a-chip, it could be used for the development of personalised medicine. Other applications include the study of endometrial carcinomas and other conditions related to the endometrium. Finally, the model can be used to provide various insights in the study of microplastics and other toxicants and their effect on reproductive health.

Disclaimer

During the preparation of this work the author used ChatGPT and Grammarly in order to evaluate grammar and writing style. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the work

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