FACULTY OF MEDICINE AND HEALTH SCIENCES

The use of a CO_2 independent medium for vascular bioprinting

Caro Brys
Student number: 01805598

Supervisor(s): Prof. Dr. Phillip Blondeel, Dr. Bernard Depypere

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Medicine in Medicine

Academic year: 2021 – 2023

GHENT UNIVERSITY

"The author and the promotor give the permission to use this thesis for consultation and to copy parts of it for personal use. Every other use is subject to the copyright laws, more specifically the source must be extensively specified when using results from this thesis."

Date

10/11/2022

(Signature)

Name (student)

CARO BRYS

(promotor)

Prof. Dr. Ph. BLONDEEL
Kliniek voor Plastische Heelkunde
UNIVERSITAIR ZIEKENHUIS GENT
RIZIV nr. 1-06972-19-210

Dr. B. DEPYPERE
Kliniek voor Plastische Heelkunde
UNIVERSITAIR ZIEKENHUIS GENT
RIZIVnr. 1-48710-88-210

Preface

This thesis was written to complete my degree in Medicine at Ghent University. This thesis investigated the use of a CO_2 independent medium for vascular bioprinting.

In my third year, I had chosen this topic because I thought this was a very innovative concept. Making a functioning blood vessel from separate components seemed very exciting to me. 3D bioprinting offers many possibilities for reconstructive medicine and this is where I wanted to contribute. In terms of transplant medicine, it also seemed like a step in the right direction to solve the donor shortage. In my social circle, I have already seen people waiting a long time for a donor organ. This was an additional motivation to choose this topic.

With this thesis, I gained a lot of techniques, insights and experiences that were otherwise not covered in my education. For this, I would particularly like to thank my supervisor Florian Vanlauwe. As part of his PhD, I was given the opportunity to do this research. He patiently taught me all the techniques and guided me in every step of the process.

I would also like to thank my promotor Prof. Dr. Blondeel and co-promotor Dr. Depypere. As a result of the monthly research meetings they organized, I learned how to approach and present scientific articles. They were a great support and took the time to provide feedback or new insights on this thesis.

To end, I would like to thank my parents and closest friends. They have supported me throughout my education and especially with this thesis.

Caro Brys

Table of contents

Abstract

There is a need for other tissue transplantation techniques. In recent years, tissue engineering has been trying to find appropriate techniques for this. Three-dimensional (3D) printing is an emerging strategy within the field of vascular reconstruction. The printing process can take a long time for large organs. Microscopy takes a long time and sophisticated devices are needed for this purpose. To ensure that the cells do not die during this process, there is a need for medium that allows the cells to survive outside the incubator. This thesis aimed to make such a CO_2 independent medium. Using a PrestoBlue viability assay, a CO2 independent medium provided by GIBCO® and a HEPES buffered DMEM were examined. In terms of supplements, FBS and ESM were compared. Commonly used EGM-2 in an incubator was used as a control. The ideal proliferation concentration was also examined as well as which molar concentration of HEPES gave the best results. This thesis showed that a HEPES buffered DMEM provided sufficient survival outside of an incubator. However, no significant difference was found between different molar HEPES concentrations. Further research is needed on the effect of HEPES on seeding and attachment of the cells in the printed blood vessel.

Samenvatting

In het gebied van reconstructieve chirurgie is er nood aan nieuwe weefseltransplantatie technieken. De laatste jaren wordt er in de tissue engineering gezocht naar geschikte technieken hiervoor. Driedimensionaal (3D) printen is een opkomende strategie op het gebied van vasculaire reconstructie. Het printproces kan echter lang duren voor grote organen. Microscopie kost veel tijd en voor het printen zijn geavanceerde apparaten nodig. Om ervoor te zorgen dat de cellen tijdens dit proces niet afsterven, is er behoefte aan een medium dat de cellen buiten de incubator laat overleven. Deze thesis had tot doel een dergelijk CO_2-onafhankelijk medium te maken. Met behulp van een PrestoBlue viability assay werden een CO_2-onafhankelijk medium van GIBCO® en een HEPES gebufferd DMEM onderzocht. Qua supplementen werden FBS en ESM vergeleken. Als controle werd EGM-2 in een incubator gebruikt. Ook werd onderzocht wat de ideale proliferatieconcentratie was en welke molaire HEPES concentratie de beste resultaten opleverde. Uit deze thesis bleek dat een met HEPES gebufferd DMEM een goede celoverleving gaf buiten een incubator. Er werd echter geen significant verschil gevonden tussen verschillende molaire HEPES-concentraties. Verder onderzoek is nodig naar het effect van HEPES op het seeden en hechten van de cellen in het geprinte bloedvat.

1. Introduction

A growing world population is causing the total number of cardiovascular problems to rise (1, 2). These problems can be traumatic, congenital of disease induced (3-5). Some of these problems can only be solved by using autologous grafts or by allogenic transplantation (2, 3, 6-8). There are issues with both options. A disadvantage in using autologous grafts is that if vascular disease is present, the graft will also be affected. Thus, it is not possible to obtain a completely healthy donor graft, and this leads to faster graft failure (9). Another disadvantage is that replacement of narrower vessels (< 6mm) causes problems because of poor patency (2, 5). Also, there is also no infinite usable reservoir of autologous blood vessels (3, 5, 7). In addition, the prelevation of such a vessel creates donor site morbidity (3, 5, 10).

Disadvantages of using allogeneic blood vessels involve: not matching in size with the native blood vessel, difficulties with children (small vessels; they still need to grow and the graft is unable to evolve) and complications such as infection, immune reactions, … (5, 11-13). In addition, there is a long waiting list for donor organs and it gets longer every year (currently 1514 in Belgium and 13 462 in Europe) (1, 14-16). Due to a greater demand for donor organs and a shortage of donors, there is a major organ shortage crisis (14). This is the reason that the number of people on the waiting list increases as well as the number of people who die while waiting on the list (13% - 14% each year in Europe) (16).

In recent years, tissue engineering has been trying to solve these problems. Walgenbach et al defines tissue engineering as “the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain or improve tissue function.” (17). Three-dimensional (3D) printing is an emerging strategy within this field for vascular reconstruction (5, 6, 18). Biomaterials and biochemical components are precisely controlled by a preset computer program to continuously print living cells (19). This can create a blood vessel adapted to the patient based on medical imaging (5, 20). Currently, 3D-printing is used for many things such as drug screening, cancer research, transplant and tissue engineering and regenerative medicine (19, 21, 22). The following is a further discussion of bioprinting and its application in the vascular field.

a) Bioprinting

Bioprinting is defined by Turnbull et al as “the production of complex living and non-living cells, molecules, extracellular matrices and biomaterials.” This involves using 3D-printing technology to arrange the cells and materials in a particular pattern (3). Gao et al defines 3D-bioprinting as “procedure of synchronous printing of biomaterials and living cells for biological applications” (18).

To 3D-bioprint, several components are needed: a computer aided design (CAD) to control the micro- and macro-architecture. This design can be based on medical imaging. A bioprinter based on a particular technique (see section ‘1. Bioprinting techniques’). A container for distributing biomaterials, live cells or cell aggregates. A bio-ink, this is a processable hydrogel with or without self-assembling cells. These cells can be stem cells or differentiated cells (7, 23). The utilization of the patient's cells ensures that there is no potential for rejection and thus no need for immunosuppression (6, 15).

Printing consists of three phases (figure 1): pre-printing, printing and post-printing. Pre-printing is the phase where imaging is collected, the design is created on the computer and the bio-ink is assembled. This is where the different cell types and materials are chosen. The second phase is printing. Here, the target cells and biomaterial are molded into a 3D-structure (15, 23). In case of printing macrovascular channels, the scaffolds are rotated so that the cells cover the entire luminal wall when printing is complete (8, 24). The printed tissue is checked under the microscope during this phase (25). The final stage is post-printing. This is where the construct undergoes a step of cell growth and maturation (15, 23). The object is placed in a culture medium in an incubator. Daily, this culture medium should be refreshed as well as growth factors should be added regularly (23).

For printing larger organs or tissues, different types of cells, hydrogels and bio-inks are needed. This makes it very complex and gives more chances of error occurring. While smaller tissues take a few minutes or hours to print, larger tissues and entire organs can take days of printing. This can cause cell death and necrosis to occur in tissues that are even still on the printer. This is a problem that must be considered and addressed. Some solutions to this are: multiple parallel bioprinters, faster printers with high resolution, better ways to preserve the part already printed, ... (25).

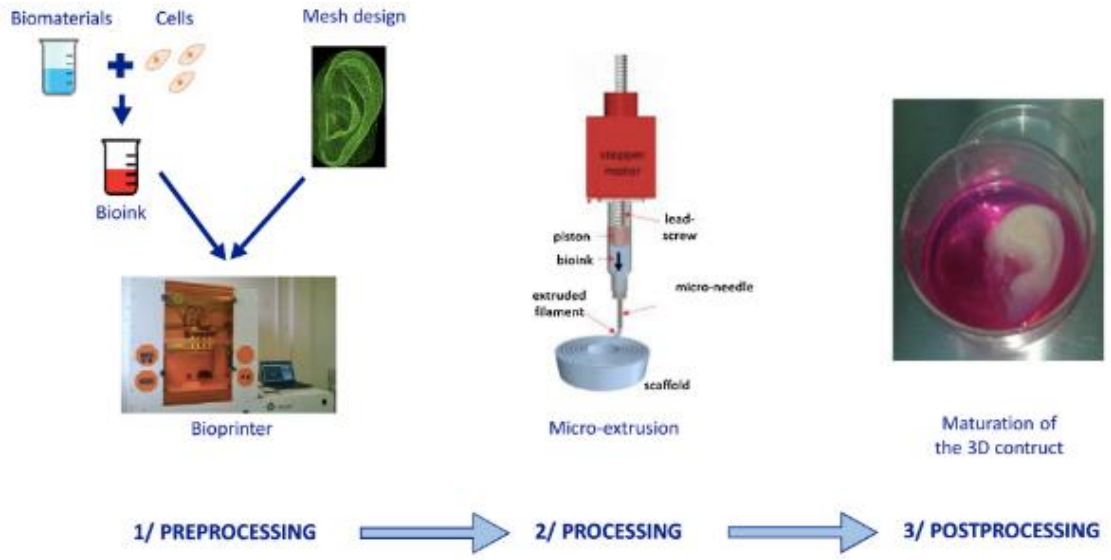


***Figure 1**: The process of bioprinting in three phases: 1/pre-printing, 2/ printing and 3/ post-printing. Figure adapted from Sigaux et al (23).*

1. Bioprinting techniques

There are three printing techniques: inkjet-based (IBB, also called droplet-based), laser-assisted (LAB) and extrusion-based bioprinting (EBB) (15, 21, 22, 26).

Inkjet-based bioprinting (IBB) involves serial placement of droplets of biomaterials and/or cells (bio-ink) in very precisely defined 3D-designs (26, 27). The resolution of this technique is around 50-300µm, this does not allow capillaries to be printed. Therefore, the formation of a capillary network depends on the self-assembly of endothelial cells (26). It is important that the bio-ink has low viscosity, otherwise it is difficult to obtain droplets and the nozzle may become clogged (18, 26). A disadvantage of this technique is that making larger tissues or organs is challenging, given that the printed droplets are small. This also causes a longer printing time. Therefore, this method is especially good for 2D-structures (26). An advantage is that it is low-cost (3) and gives low damage to the cells (27).

Extrusion-based bioprinting (EBB) is the most used technique. Unlike IBB, high viscosity bio-inks can be used here. Multiple layers can be printed, it is cheaper and faster (26, 28). The ink is printed in continuous cylindrical filaments, layer by layer. This allows larger 3D structures to be printed. A disadvantage of this technique is high shear stress resulting in cell death (26, 27). Additionally, this technique has the lowest resolution (> 100µm) and is therefore less suitable for micro-vasculature (26). This low resolution is caused by the need to use a larger nozzle, as a diameter < 150µm can cause clogging (18).

Laser-assisted bioprinting (LAB) manipulates cells and photoreactive biomaterials with light energy, based on a digital model. The laser is focused on an energy-absorbing substrate. This provides the generation of energy, heat and pressure. This causes a drop of bio-ink to be injected onto the collector substrate (26, 27). This method has a very high resolution (< 10µm) and is the only technique without the use of a nozzle. That makes this technique the most suitable for microvasculature (18, 26). The disadvantage of this technique is that the bio-inks must have a low viscosity and low material concentration. In addition, it is an expensive technique (18, 26, 27).

2. Bio-inks

Barrs et al define bio-inks as "formulations of biomaterials and/or cells that serve as the writing material for bioprinting." (26). There are several types of bio-inks: polymer-based microcarriers, polymer-based hydrogels, cell aggregates, tissue spheroids and decellularized matrix (dECM) (21, 27). Hydrogels are the most commonly used type of bio-ink. These are high-molecular-weight cross-linked structures. They determine the physical-mechanical properties of the bio-ink. Hydrogels are widely used because they have a great water holding capacity and are biocompatible in vivo (3, 21). There are three types of hydrogels: natural, synthetic and the combination of these two. Natural hydrogels include gelatin, cellulose, collagen and alginate. The disadvantages of these hydrogels are uncontrolled degradation and weaker mechanical properties (3). Polyethylene glycol (PEG) and poloxamers (e.g., Pluronic F-127) are examples of synthetic hydrogels (21, 26). These gels are chemically formulated so the biomechanical properties are an improvement on natural ones. A disadvantage is that they do not support cell adhesion (26).

b) Vascular bioprinting

1. Anatomy of blood vessels

The cardiovascular system consists of several vessels. The larger vessels, such as the veins and arteries, transport large volumes of blood. The smaller vessels such as the arterioles, venules and capillaries provide perfusion of the various organs, exchange of substances and control of blood pressure (26).

Arteries consist of three circular layers (figure 2) (22). The innermost layer is the tunica intima. This consists of a single-layered squamous epithelium called: the endothelium. The function of the endothelium is to protect against infections and to provide a non-thrombotic environment. In addition to the endothelium, the intima also contains the lamina basalis. This

consists of laminin and type IV collagen. This cell layer is surrounded by a narrow layer of elastin, which is the lamina elastica interna. The second layer is the tunica media (8, 22). This layer contains type I and type III collagen, proteoglycans, as well as smooth muscle cells (SMCs) (22, 29). These SMCs provide vasoconstriction or vasodilation. This layer is also surrounded by elastin and is called the lamina elastica externa. The outer layer is the tunica adventitia. This tunica consists of a loose collagen matrix produced by fibroblasts (8, 22, 26). This outer layer causes the blood vessel to adhere to the surrounding tissue (29).

These arteries reduce to arterioles with a diameter of 10-200µm. These vessels consist of the same anatomy as described above, but the layers are thinner. The adventitia here is richly innervated by sympathetic nerves. In turn, the arterioles reduce to capillaries. Capillaries have a diameter of 5-10µm and belong to the microscopic vessels (22). These vessels have only a tunica intima surrounded by a few pericytes for support (26, 30). Thus, they do not contain smooth muscle cells or a tunica adventitia. This is to allow good permeability and exchange (26).

These capillaries then empty into venules. These small vessels, 8-100µm in diameter, have approximately the same anatomy as arterioles. Several venules then come together to form the veins (22). The anatomy of these veins can be compared to that of arterioles, but with less muscle and elastic tissue given that the blood pressure in the veins is lower (26, 29).

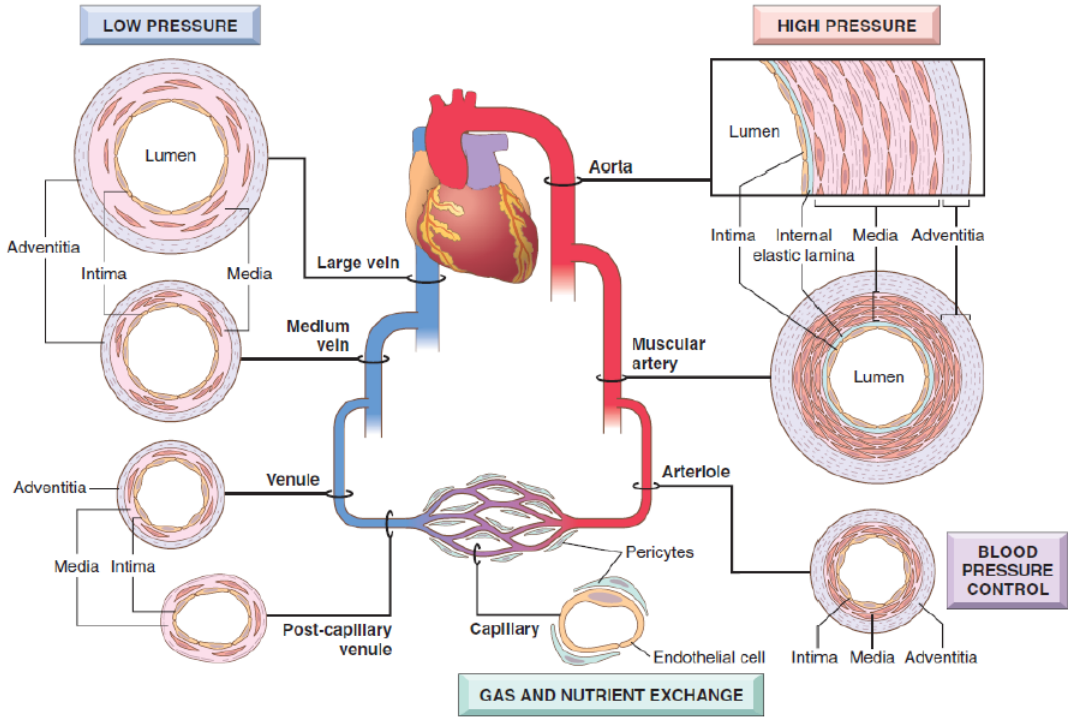


***Figure 2**: Anatomy of the different vessels of the vascular system. Figure adapted from Prof. Dr. Ferdinande, Ghent University (31).*

2. Human umbilical vein endothelial cells

As mentioned in '1. anatomy of blood vessels', the inner layer of a blood vessel is delineated by endothelial cells. To form this inner layer, human umbilical vein endothelial cells are commonly used in research. Human umbilical vein endothelial cells (HUVECs) are derived from the endothelium lining the veins of the umbilical cord (26). These endothelial cells are obtained by perfusing the umbilical cord with collagenase (32). HUVECs are most often used to study vascular pathology and function and therefore also in tissue engineering. It is an inexpensive and abundant source of endothelial cells given the umbilical cord is otherwise disposed of (26).

3. Vascular bioprinting techniques

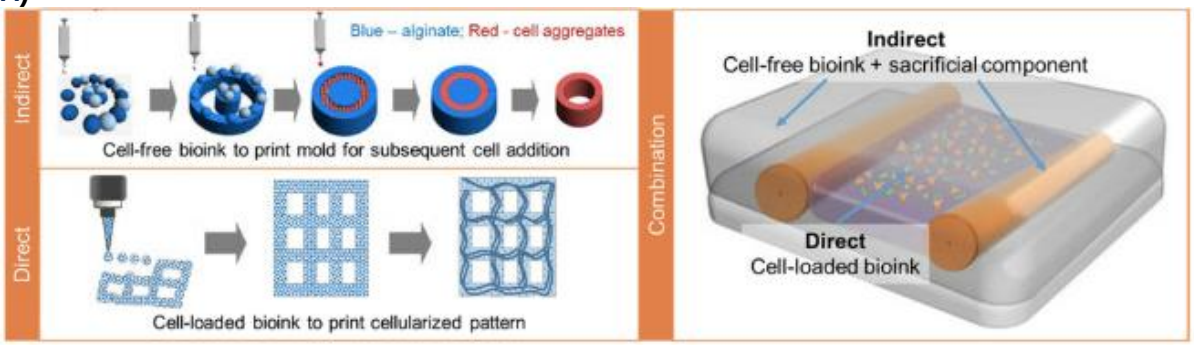
There are three approaches to bioprinting vasculature: Indirect, direct and scaffold-free bioprinting (figure 3) (22, 26, 33).

Indirect: soluble, cell-free bio-inks are used to print hollow tubes that can transport fluids within a tissue construct (26, 33). These bio-inks can be printed as solid pieces and removed after printing to leave hollow channels that can be perfused and endothelialised. The resolution of indirect printing is not good enough for capillaries (> 100µm vs. 10µm) (26).

The direct approach does not use soluble materials, but vascular stimulating bio-inks containing endothelial cells. These inks engage cell-cell and cell-matrix interactions, helping the cells to form capillary networks. This strategy is more suitable for smaller blood vessels than indirect bioprinting because they stimulate endogenous vasculature (26, 33).

In the scaffold-free approach, cells are not encapsulated in external biomaterials (27). They are first seeded in non-adherent well plates to stimulate self-assembly into tissue spheroids. These spheroids are then printed into the desired shape without scaffold support (22, 27). This technique mimics natural embryogenic development (27).

A)



B)

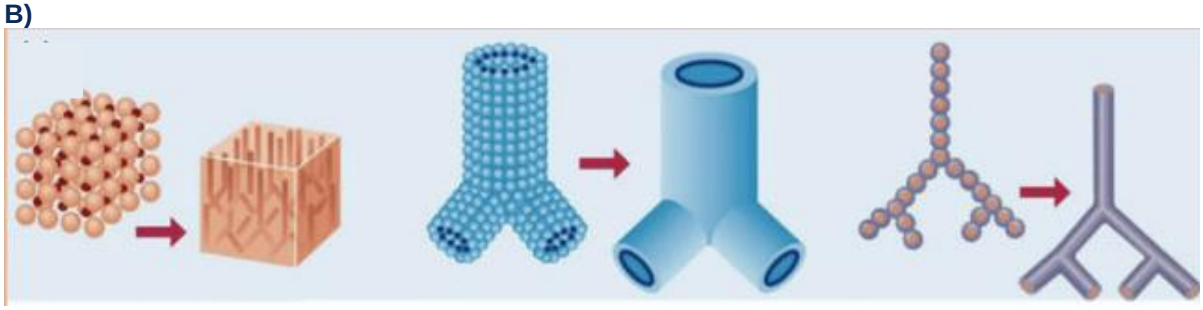


***Figure 3**: The different approaches for vascular bioprinting. A) Indirect en direct bioprinting. B) scaffold free bioprinting. Figure adapted from Richards et al (33).*

4. Seeding of the scaffolds

As mentioned in ‘a) bioprinting’, the scaffolds must be rotated. When the construct is fully printed and the cells are present (direct/indirect approach), the cells are in the center or at the bottom of the vessel. To cover the entire luminal surface, the vessels are rotated. For an average of 4-6h, the scaffolds are rotated every 15-30 min (8, 24, 34-37). There are two options for this. This can be done manually; this requires a lot of effort from the operator and it must be done in a sterile chamber in a standard tissue incubator. However, this can also be done automatically. This allows for ease of technique as well as scale-up of seeding. There are sophisticated devices for this, in which this rotation is done in an incubator. Since this may not be available everywhere, Kinstlinger et al provided an open-source gel flipping system (photo 1). This system has a lower cost and consists of more basic materials. This system must be placed outside an incubator, as it cannot withstand the humidity of an incubator (24).

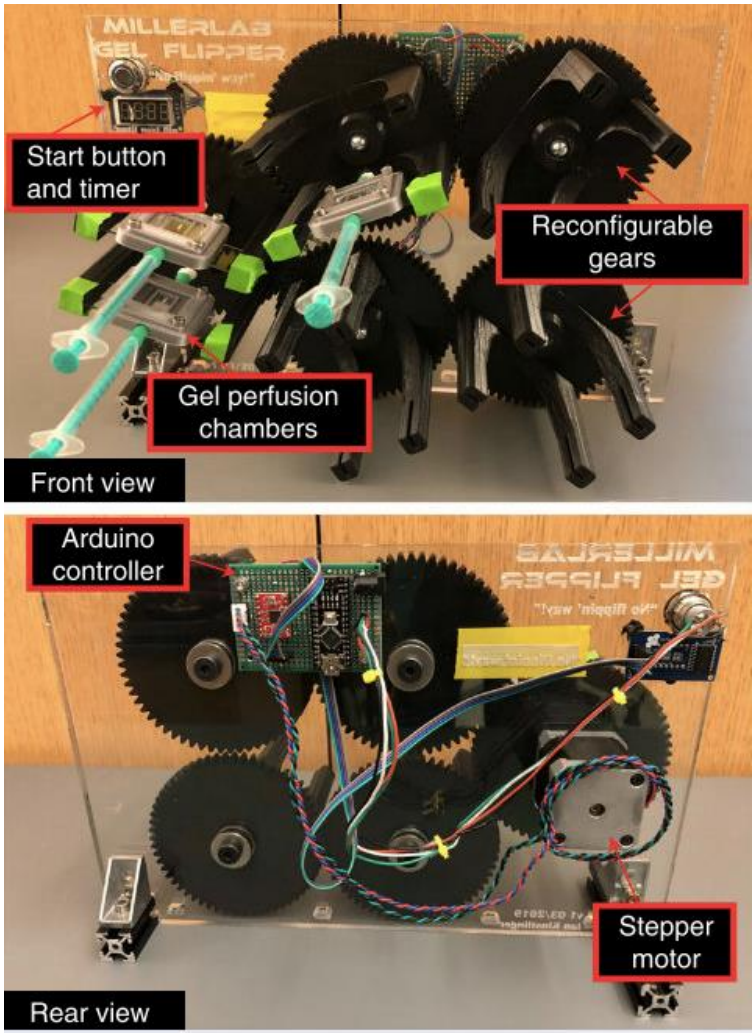


***Photo 1**: The open-source multiplexed gel flipping system (MillerLab) with gears that hold the constructs and rotate synchronously. Photo adapted from Kinstlinger et al (24).*

c) Culture media

A culture medium serves for the transport, storage and growth of cells. There are many different types (38). In the field of bioprinting, a culture medium is used for the proliferation of cells in the pre-printing phase, the preservation of cells and printed scaffolds and in the post-printing phase for maturation and cell proliferation (39, 40). The media relevant to this master's dissertation are discussed in this section.

1. EGM-2

Endothelial cell growth medium is an endothelial basal medium that is growth factor and serum free. To this medium is added: fetal bovine serum (FBS), epidermal growth factor (EGF), fibroblast growth factor, long R3 insulin-like growth factor (IGF), vascular endothelial growth factor, ascorbic acid, heparin and hydrocortisone (41, 42). These factors ensure differentiation,

increased integrity and proliferation (43). Many studies have shown that EGM-2 has a very good effect on the proliferation of HUVECs. Therefore, this medium is often used for this cell type (43, 44).

2. HEPES buffered DMEM

Cells get their energy from glucose. When processing this glucose, lactic acid is formed. This acid lowers the pH of the culture medium. An acidic environment is bad for cell viability (45). Normally, the pH is maintained by the $NaHCO_3$ in the culture medium and the CO_2 in the incubator, but sometimes lactate formation can exceed this buffering capacity (46, 47). The pH changes by transporting the media between rooms with different CO_2 partial pressures (e.g. in and out the incubator) (47). Therefore, a buffer is often added to the medium to avoid this pH change. This can be either bicarbonate or, for example, a HEPES buffer (45, 47).

HEPES stands for 4-(2-hydroxyethyl)-1-piperazineethanesulfonic ($C_8H_{18}N_2O_4S$). It is a zwitterionic sulphonic acid buffering agent commonly used in culture media. The buffering capacity is CO_2 independent. It is better at preserving pH (7,2 – 7,4) despite changes in the CO_2 concentration than a commonly used bicarbonate buffer (45, 48)**.** Of this buffer, 10-25mM is usually added to the medium (49, 50).

Dulbecco's Modified Eagle's Medium (DMEM) is a commonly used product to assemble the culture medium. DMEM simulates the human blood plasma (51). It contains inorganic ions and organic substances such as glucose, vitamins, amino acids. There are different types where the glucose concentration can be chosen according to nutritional need (46). Since DMEM does not contain growth factors, FBS is often added to this medium (51, 52). FBS is a very popular supplement and is used worldwide. It contains many growth factors, hormones, vitamins and proteins. It is obtained by drawing blood from calf fetuses (46, 52, 53).

d) Viability assay

To see if the cells survive and proliferate before, during or after printing, a viability assay is used. PrestoBlue® Cell Viability Reagent (Invitrogen™) is a reagent that has been on the market for about ten years (54). PrestoBlue uses resazurin as a cell viability indicator (55). This is a water-soluble dye. By taking over electrons from NADPH, FADH, FMNH, NADH and cytochromes, resazurin is reduced to resorufin. This occurs only in viable cells. Thanks to this reduction, PrestoBlue changes color and changes to a highly fluorescent form (54). This conversion is proportional to the number of active cells and can be measured quantitatively by

a fluorescence spectrophotometer. PrestoBlue has a high sensitivity and can measure cell viability as early as ten minutes of incubation (54-56).

e) Goal of this research

As mentioned above, the cells should be rotated during the post-printing phase so that they cover the entire lumen. This requires expensive machinery. As this is not available in Ghent, a less expensive, external rotator (photo 1) would be used here. But as this cannot withstand the humidity of the incubator, a solution must be found to use this rotator outside the incubator (24).

A CO_2 independent medium can offer this solution. The usefulness of such a medium is that the scaffolds can be seeded in such an external rotator (as above). Also, there can be printed for hours without the cells dying. Further, long-term microscopy would then no longer be a problem. Additionally, the printed vessels need to be transported afterwards (from lab to lab or later the operating room). This may take some time but with such a medium, this should not be an issue.

The goal of this research is to see if we can make such a medium, in which the cells survive outside the CO_2 incubator. This will be investigated in three different experiments:

- Experiment 1: Can cells survive outside the CO_2 incubator? Which medium gives the best results for this? A new medium based on a HEPES buffer, and a medium provided by GIBCO, are made once with FBS and once with Endothelial SupplementMix (ESM). These four are then compared with the normal EGM-2 in the incubator.

- Experiment 2: What amount of cells is best used to start the protocol? At which concentration are the cells still in the proliferative phase? Since the cells seeded in the printed blood vessel have yet to proliferate, is it ideal to observe the cells in the proliferative phase during the experiment. The number of cells started with can affect the further survival of these cells/blood vessels. If the cells are with too many, they detach and die. If they are with too few, they do not proliferate enough. This experiment compares 500, 1000 and 5000 cells in both a 48 well plate and a 96 well plate.

- Experiment 3: Does the molar HEPES concentration affect cell survival? This question was created based on the results of experiment 1 (which showed that a HEPES + ESM

medium could be a good alternative to EGM-2 + incubator). 5mM, 10mM and 20mM are compared with the EGM-2 medium in the CO_2 incubator. Here, we also examine whether the use of a controlled temperature chamber can affect cell proliferation/survival.

f) Contribution of the student

This thesis was written as within the framework of Florian Vanlauwe's PhD research. Each part of this thesis was performed by the author under the supervision of Dr. Vanlauwe. The author isolated, thawed, counted and seeded the cells; made the culture media; performed the PrestoBlue; ran the statistics; made the graphs.

2. Materials and methods

a) Experiment 1: CO_2 independent medium

To see in which culture medium, the cells survive and multiply best, a test was performed between five different mediums (GIBCO + ESM, GIBCO + FBS, HEPES + ESM, HEPES + FBS and EGM-2). EGM-2 was as control medium placed in a CO_2 incubator. The others were placed in a warm room (34°C).

In preparation for this experiment, HUVEC's were thawed and stored in a T75 flask with an EGM-2 medium (Promocell®). This medium consists of endothelial basal medium and endothelial SupplementMix (ESM). The culture medium was changed twice a week. After ten days, cells were isolated by trypsinization (TrypLE™). A T75 flask required 2ml of trypsin. After isolation, cells were manually counted under a microscope (Olympus IX 81) using a Bürker chamber. A 1:5 dilution was made: 20µl of cell suspension and 80µl of Trypan Blue (Gibco™) *(step-by-step plan in appendix 1 a-d.*).

1. Seeding cells

Seeding of the cells occurred the day before the start of the PrestoBlue™ analysis. This day the cells were still placed in the normal culture medium to allow them to adhere to the well plate. In this experiment, 96 well plates were used. The remaining cells, not used in this experiment, were refrozen (HUVEC P5). 5000 cells per 200µl of EGM-2 were seeded in each well. Six wells were provided for each media. This gave a total of 36 wells, which were divided among three 96 well plates: plate 1 with six wells, plate 2 with eighteen wells and plate 3 with twelve wells. These plates are further clarified in section 3 of this experiment (*calculations and step-by-step plan in appendix 1 e.).*

2. Making the culture media

This preparation has also been done the day before the start of the PrestoBlue™ analysis. Five different cultural media were created as described below. 20ml of each medium was made, except EGM-2 100ml. This was enough to reach the end of the experiment. All the media were stored in the fridge at 4°C.

1. **GIBCO + ESM**

A non-HEPES CO_2 independent medium, provided by GIBCO™ Life Technologies, was used. To this solution 560µl ESM was added, as well as 200µl of Penicillin-Streptomycin and 400µl of glutamine to reach 20ml.

ESM:

- 400µl of fetal bovine serum (FBS)
- 20µl of gentamicin sulfate – amphotericin (GA-1000)
- 20µl of ascorbic acid
- 20µl of hydrocortisone
- 80µl of bovine brain extract (BBE)
- 20µl of human epidermal growth factor (hEGF)
- => A total of 560µl

The Penicillin-Streptomycin (Pen-Strep) was added to the culture media to prevent bacterial contamination (1ml Pen-Strep/100ml culture media).

2. **GIBCO + FBS**

17,4ml of the GIBCO solution was supplemented with 2ml FBS; 0,4ml glutamine and 0,2ml Pen-Strep to reach 20ml.

3. **HEPES + ESM**

1M HEPES (Sigma-Aldrich®, CAS 7365-45-9) and DMEM (10g/dl glucose) powder (Sigma-Aldrich®, D5523-10L) were used. In this experiment, a 10mM solution was required. HEPES (10mM) was added to a 10g/dl DMEM solution in distilled water. The final pH was 7,36. The same amount of ESM and Pen-Strep was used in this medium as in GIBCO + ESM (respectively 0,56ml and 0,2ml). This means that 19,24 ml of the HEPES/DMEM solution was supplemented with the ESM and Pen-Strep to reach 20ml (*calculations and step-by-step plan in appendix 2).*

4. **HEPES + FBS**

This medium was made in the same way as mentioned in appendix 2 in the step-by-step plan. This medium had a final pH of 7,33. Again, the same amounts of FBS and Pen-Strep are used as in GIBCO + FBS. (2ml and 0,2ml, respectively). Thus, to obtain 20ml of culture medium, 17,8ml of the HEPES solution was needed.

5. **EGM-2**

An EGM-2 medium provided by Promocell® was used. As mentioned above, this medium consists of endothelial basal medium and endothelial SupplementMix (ESM). To this 100ml, 1ml of Pen-Strep was added. This media will serve as a control against the four others.

3. PrestoBlue™ analysis

This analysis is performed on day zero, one, three and seven of the experiment.

3.1 Day zero

Given that the cells were seeded in normal EGM-2 culture medium, on day zero, only that medium was used. After the PrestoBlue™ analysis, the cells were transferred to the correct medium. The four media were tested CO_2 independent, in a warm chamber at 34°C. To maintain proper humidity, they were placed in a dome with a cup of water. They each counted six wells. EGM-2 media was used as control medium: as a positive control, six wells were placed in the CO_2 incubator, as a negative control, six wells were placed in the warm chamber (34°C).

- **Preparation of the PrestoBlue™ solution**

In total there were 36 wells. An additional three wells were filled with the medium without the cells. This way, the reactivity of the medium itself could be subtracted from the reactivity of the cells. That made a total of 39 wells. PrestoBlue requires a 1:10 solution (PrestoBlue/culture medium). Each well should contain 100µl. It was sealed off from light with aluminum foil.

- **Preparation of the well plate**

See appendix 3 for the step-by-step plan.

After two hours, were the solutions transferred to a new well plate to make a read out.

- **Replacing culture medium**

Before replacing the media, the five media were heated to a temperature of 37°C. As mentioned in item ‘1 Seeding cells’, three well plates were used:

- Well plate 1 contained the positive control (EGM-2). In the six wells of this plate, 200µl of EGM-2 culture medium was added. After this, it was placed in the CO_2 incubator.

- Well plate 2 contained the negative control (EGM-2), GIBCO + ESM and GIBCO + FBS. 200µl of each medium was added to the respective wells. Then it was placed in the dome in the warm room.
- Well plate 3 contained HEPES + ESM and HEPES + FBS. 200µl of each medium was added to the respective wells. This plate was also placed in the dome in the warm room.

A) **B)**

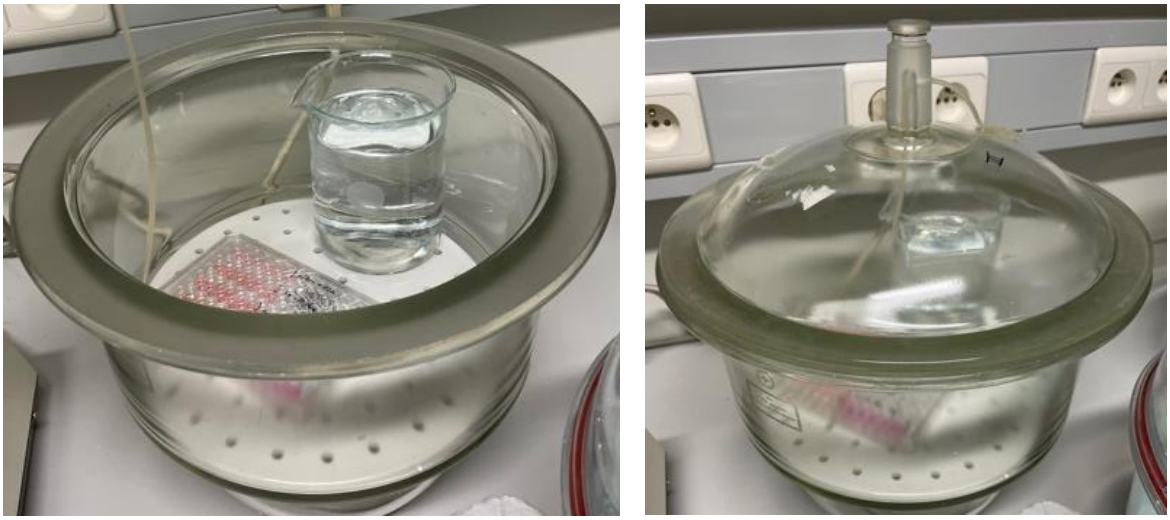


***Photo 2A and B**: Setup of the well plates in the warm chamber. Placed in a dome with a cup of water*

3.2 Day one – seven

The preparations for day one to day seven were the same and are therefore discussed together here.

- **Preparation of the PrestoBlue™ solution**

As mentioned in 3.1, a 1:10 solution of the PrestoBlue™ was required. This had to be done separately for each medium. For each medium, an extra three wells were used as a background check for the analysis. As on day zero, the wells were filled with 100µl of solution.

- **Preparation of the well plate**

Same as in 3.1.

The solution was transferred to a new well plate to make a read out *(see appendix 4)*.

- **Replacing culture medium**

Same as in 3.1.

- **Refreshing culture medium**

Every two days the culture medium was changed. Given that days zero, one and three followed each other shortly, the medium was already refreshed there. So, the media was only refreshed between day three and seven. Refreshment was done by removing the old medium and adding 200µl of the right medium again.

b) Experiment 2: proliferation test

In this experiment, different starting concentrations and two different sizes of well plates were used, to see in which condition the cells multiplied and survived best. By cell isolation and cell counting (as described in appendix 1), a 48 well plate and a 96 well plate were seeded (HUVEC P5). On each plate, three wells received 500 cells, three wells received 1000 cells, and three wells received 5000 cells as the starting count. EGM-2 culture medium was used for this experiment.

1. PrestoBlue™ analysis

Three wells were used per condition. An additional three wells were provided as a background check for the analysis. As in experiment 1, this was done to subtract the reactivity of the medium itself from the cell reactivity. This analysis was done on day zero, one, three, seven and fourteen.

- **Preparation of the PrestoBlue™ solution**

As described above, for the 96 well plate, 100µl of PrestoBlue solution was used. For the 48 well plate this was 200µl. 4ml of an 1:10 solution was made (PrestoBlue/culture medium).

- **Preparation of the well plate**

96 well plate: same method as experiment 1

48 well plate: 200µl of the solution was needed. And, after the two hours of incubation, 160µl of the solution was transferred to a new 48 well plate (this is to account for the fact that max 20% may have been evaporated). The other steps were the same as for the 96 well plate.

- **Replacing and refreshing culture medium**

96 well plate: 200µl of warm (37°C) culture medium + CO_2 incubator

48 well plate: 400µl of warm (37°C) culture medium + CO_2 incubator

Refreshment of the culture medium was done every two days.

c) Experiment 3: HEPES culture medium

As described in the results section, HEPES + ESM gave the best outcome in experiment 1. A controlled temperature chamber, different HEPES concentrations, GIBCO + ESM and EGM-2 medium (positive and negative control) were used. The cells used were HUVECs (P5) that were thawed, isolated, and refreshed before the start of the research as described in experiment 1.

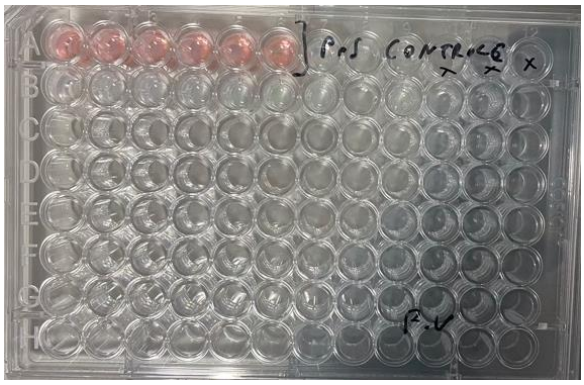
1. Seeding cells

As discussed in the results section, the starting concentration of 500 cells gave the best result in experiment 2. Therefore, after cell counting, 500 cells were inserted per well. Three 96 well plates were used (photo 3):

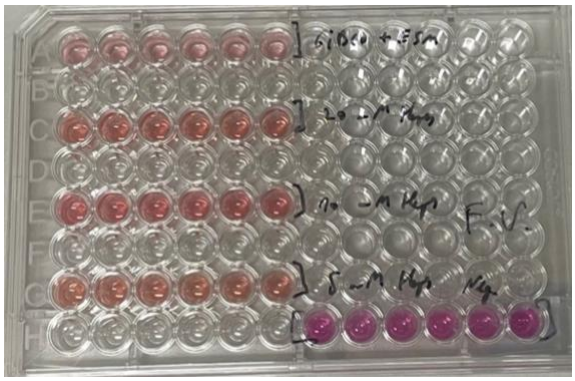
- Plate 1 contained six wells for the positive control in the CO_2 incubator.
- Plate 2 contained 30 wells for negative control, GIBCO + ESM, HEPES 20mM, 10mM and 5mM.
- Plate 3 contained six wells for 10mM HEPES in the controlled temperature chamber.

That made a total of 42 wells. At seeding, all cells were still placed in EGM-2 culture medium and in the CO_2 incubator.

A)



B)



C)

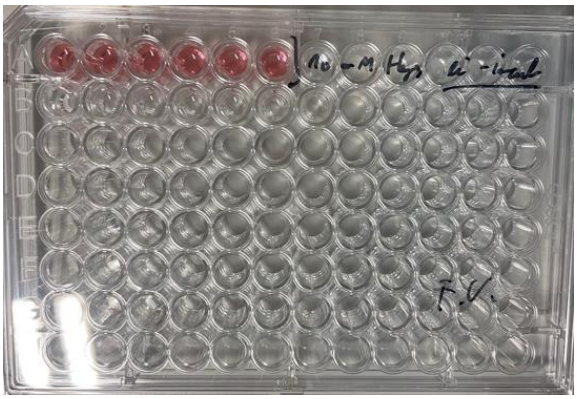


***Photo 3A, B and C**: The layout of the three well plates used in experiment 3. A) Plate 1 B) Plate 2 C) Plate 3*

2. The culture media

Both a 5mM, a 10mM and a 20mM HEPES concentration were used. 200µl was added per well.

1. HEPES

1M HEPES (Sigma-Aldrich®, CAS 7365-45-9) and DMEM (10g/dl glucose) powder (Sigma-Aldrich®, D5523-10L) were used again. The calculations and making of the media were almost the same as for 10mM in experiment 1. In this experiment a 5mM, a 10mM and a 20mM solution was required. HEPES (respectively 5, 10 and 20mM) was added to a 10g/dl DMEM solution in distilled water. Optimal pH's (7,2 – 7,4) were titrated to 7,25 (5mM); 7,30 (10mM) and 7,23 (20mM).

After this, the culture media were filtered. 19,24ml of each culture medium was added in a new bottle. To this, 0,56µl growth factors and 0,2µl Penicillin-Streptomycin were added to each medium.

2. EGM-2

As described in experiment 1 section 2.5.

3. PrestoBlue™ analysis

This analysis was performed on days zero, one, three, seven, and fourteen.

3.1 Day zero

Considered that the wells were seeded in EGM-2 medium on day zero, only this medium was used.

- **Preparation of the PrestoBlue™ solution**

We had 42 wells and three more were being used as background control, what made a total of 45 wells. Each well required 100µl of PrestoBlue solution. A 1:10 solution was made (PrestoBlue/culture medium)

- **Preparation of the well plate**

For the distribution on the well plate see appendix 5. The method was the same as in experiment 1.

- **Replacing culture medium**

The culture media was heated to a temperature of 37°C. For each culture media, six wells were filled with 200µl.

As mentioned in section one there were three well plates:

- Plate 1: 200µl EGM-2 in each well + CO_2 incubator
- Plate 2: warm room (34°) in a dome with a cup of water (see photo 2).
  - 200µl of GIBCO + ESM in well A1-6
  - 200µl of 20mM HEPES in well C1-6
  - 200µl of 10 mM HEPES in well E1-6
  - 200µl of 5mM HEPES in well G1-6
  - 200µl of EGM-2 in well H7-12
- Plate 3: 200µl 10mM HEPES + controlled temperature chamber (photo 4). A cup of water was put on the side of the incubator and the bottom was also filled with water. The desired temperature was set at 36°C-37°C. The temperature and humidity needed to be checked daily!

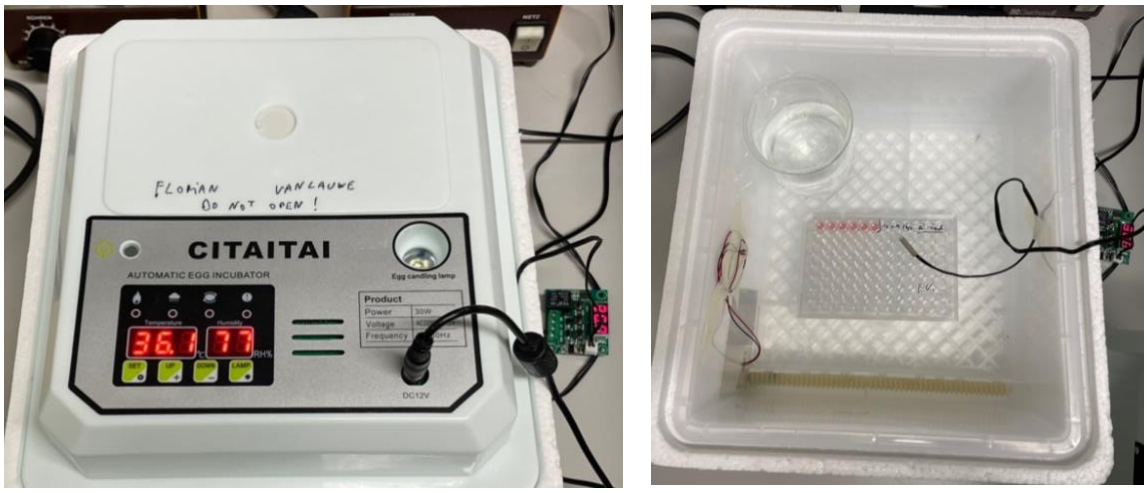


***Photo 4A and B**: setup of the well in the controlled temperature chamber.*

3.2 Day one – fourteen

The preparations for day one to day fourteen were the same and are therefore discussed together here.

- **Preparation of the PrestoBlue™ solution**

The PrestoBlue™ solution had to be made separately for each medium. For each medium, the positive and negative controls; an extra three wells were used as controls. As on day zero, the

wells were filled with 100µl of solution. An 1:10 solution for each media was made (PrestoBlue/culture media).

- **Preparation of the well plate**

For the distribution on the well plates see appendix 5. The method was the same as in experiment 1.

- **Replacing culture medium**

Same as in 3.1.

- **Refreshing culture medium**

Every two days the culture medium was changed. Refreshment was done by removing the old medium and adding 200µl of the right medium again.

d) Analyzing the results

Through the fluorescence spectrophotometer (Perkin Elmer Wallac 1420 Victor2 Microplate Reader), the PrestoBlue™ reaction was recorded. This provided an excel file. This file contains fluorescence measurements per well. Via Excel, the controls were subtracted from the results so that the true reactivity of the cells were measured and not that of the medium. The viability of each well was then calculated by this formula:

$$Viability\ \% = \frac{Fluorescence\ on\ day\ 1, day\ 3, day\ 7\ or\ day\ 14\ after\ using\ the\ different\ culture\ media}{Fluorescence\ on\ day\ 0\ before\ using\ the\ different\ culture\ media} * 100$$

These results were then further analyzed via GraphPad Prism. The analytical assay used for each experiment was the one-way ANOVA. Tukey's multiple comparisons test was used as post hoc test. PAULA Cell imager was used to view the cells under the microscope.

3. Results

a) Experiment 1: CO_2 independent medium

The purpose of this experiment was to see if the cells can proliferate and survive in a CO_2 independent medium. Small differences were already seen on day one. The HEPES medium and the positive control had a higher percentage of proliferation than the other media. On day three, cells in each media increased in number. On the last day, day seven, the difference in proliferation was more pronounced. HEPES + ESM and the positive control are almost equal and well above the other media. Only these two continued to proliferate. In the other media, there was a decline in terms of growth and survival. GIBCO + FBS performed as poorly as the negative control. GIBCO + ESM finished around the middle (figure 4).

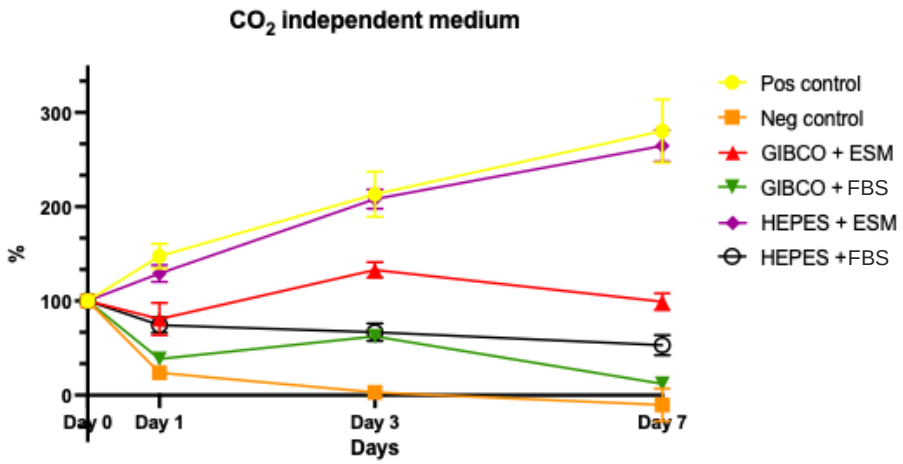


***Figure 4**: Survival and proliferation expressed as percentage of proliferation on day 1, 3 and 7 in the different media.*

After performing a one-way ANOVA analysis, these results were obtained. In terms of percentage growth, every comparison between conditions was statistically significant except between: positive control and HEPES + ESM ($p = 0.9064$); negative control and GIBCO + FBS ($p = 0.1225$); GIBCO + FBS and HEPES + FBS ($p = 0.2725$). This means that HEPES + ESM without the CO_2 incubator performs as well as the control in the incubator, but GIBCO + FBS performs as poorly as the negative control (figure 5).

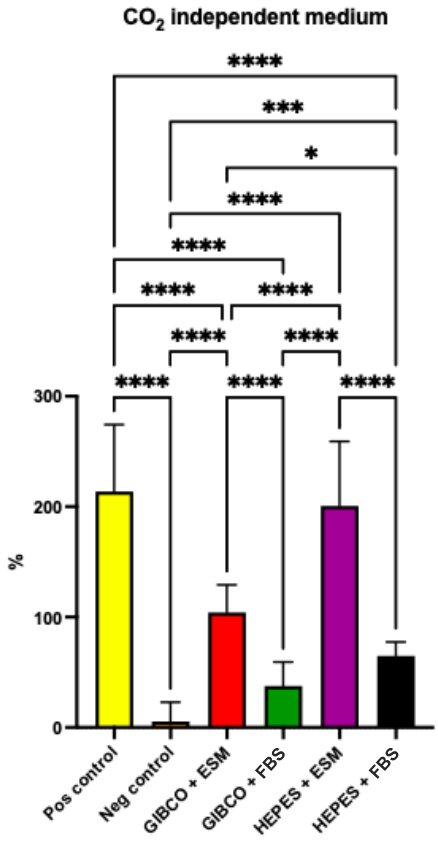


Figure 5*: Proliferation rate of cells throughout the experiment in positive/negative control, GIBCO + ESM/FBS and HEPES + ESM/FBS after day 14. The bar resembles the mean ± SD. * indicates statistical difference of p < 0.05; *** indicates p < 0.001 and **** indicates p < 0.0001.*

b) Experiment 2: proliferation test

This section looked at the right condition to analyze endothelial cells in a proliferative phase. First, the 96 well plate is discussed. On day one, no differences could be seen between the wells. Day three showed that the wells with 1000 as the starting number, multiplied the most. There is no real difference from 500 yet. On day seven, the cells which started with 500, surpassed those of 1000. For those at 5000, there was a limited increase. At the end of the experiment, day fourteen, 500 did a bit better than 1000 (1906,68% vs 1654,15%). Compared to the others, the wells of 5000 were not much expanded over the entire line (475,659%) (figure 6A).

The 48 well plate showed no difference between 5000 and 1000 on day one. However, the wells with 500 cells went to a negative number. On day three, all three conditions were around the same value (± 200%). On day seven, more differences were seen: the cells from the wells

of 500 were around 1500%, those of 1000 around 850% and those of 5000 around 280%. On the last day, the wells of 500 clearly multiplied the best (10 249%) (figure 6B).

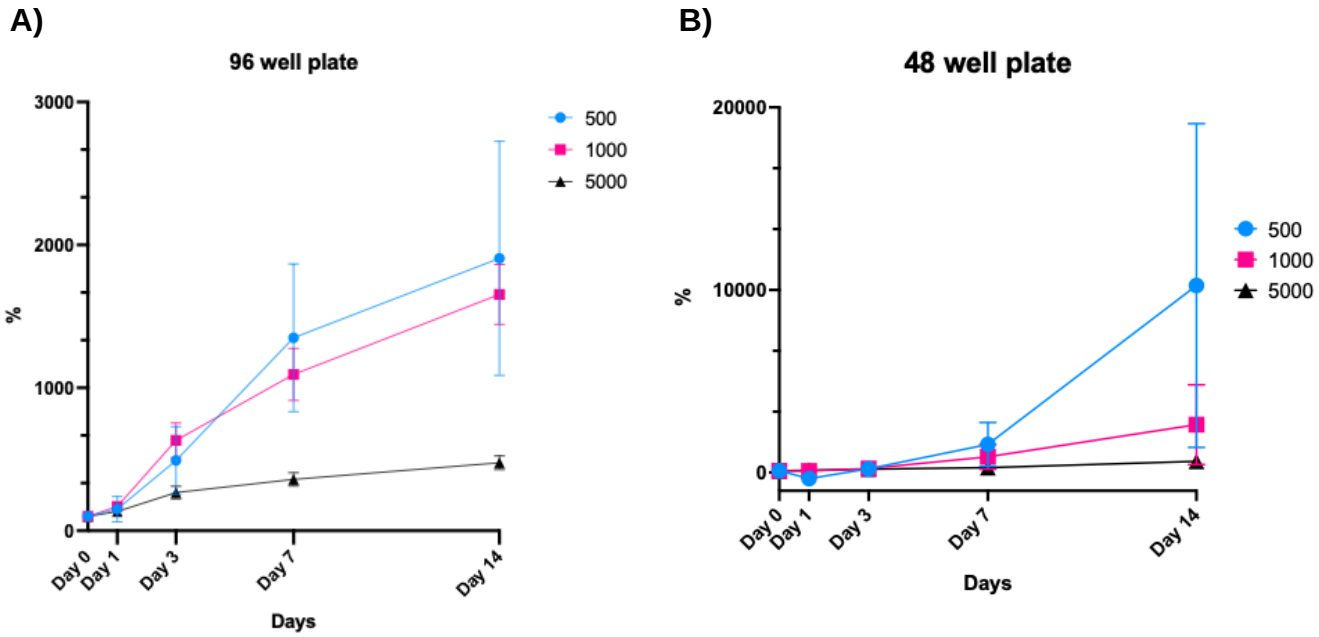


Figure 6: *Survival and proliferation expressed as percentage of growth on day 1, 3, 7 and 14 at different conditions in a 96 well plate (A) and a 48 well plate (B).*

After performing a one-way ANOVA analysis for both well plates, these results were obtained after day fourteen. For the 96 well plate, there is a statistically significant difference between 500 and 5000 ($p = 0{,}0267$). The other values are not statistically different from each other. For the 48 well plate, no significant differences were measured (figure 7).

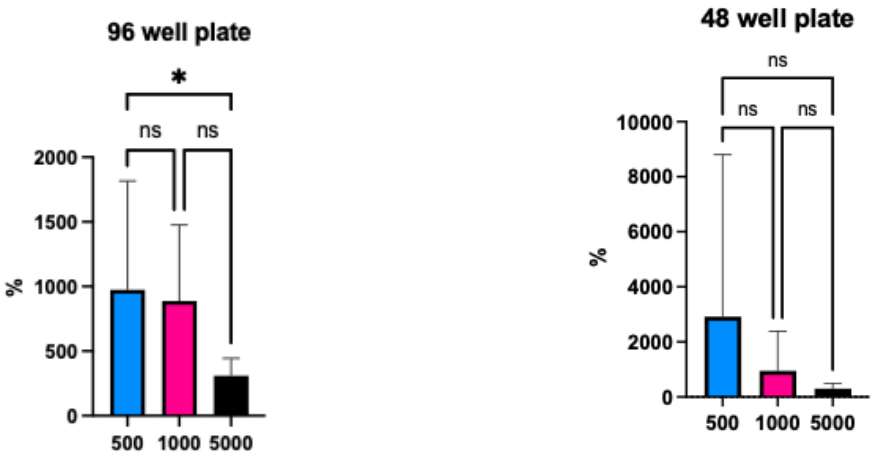


Figure 7*: Proliferation rate of cells throughout the experiment with a start amount of 500, 1000 and 5000 cells in a 96 well plate (A) and a 48 well plate (B) after day 14. The bar resembles the mean ± SD. * indicates statistical difference of $p < 0{,}05$.*

c) Experiment 3: HEPES culture medium

This section looked at which molar concentration of HEPES was best in a CO_2 independent environment. In this experiment, the results of the positive control were found to be much higher than the other media (3627,29% and 607 737 cells at day fourteen). The aim of the experiment was to check whether the cells could survive outside a CO_2 incubator. Therefore, the positive control was not included in this section. The graph showing the positive control can be found in appendix 6.

In terms of proliferation rate, there was not much difference on day one between the HEPES media (± 30% gain). The GIBCO + ESM culture medium already showed reduced viability (-22%). This trend was observed throughout the whole experiment, until almost all cells were dead (1% left on day fourteen). Day three showed higher proliferation in the HEPES medium of the controlled temperature chamber. After this, viability decreased for this medium. Between day three and fourteen, a growth was observed for 5 mM, 10mM and 20mM HEPES. On both day seven and fourteen, the best results were recorded for 20mM HEPES (final percentage of 403.26%) (figure 8).

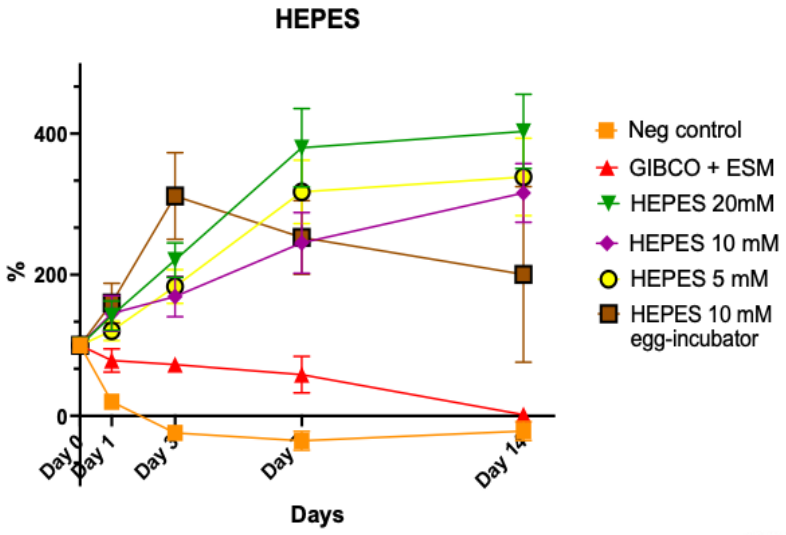


Figure 8: *Survival and proliferation expressed as percentage of proliferation on day 1, 3, 7 and 14 in the different media (without the positive control).*

As above, the positive control is not considered in this section. The figure showing the statistical results for this medium can be found in appendix 3. After performing a one-way ANOVA analysis, these results were obtained (figure 9): in terms of growth rate, the negative control gave significant results ($p < 0.0001$) with every medium except GIBCO + ESM. Thus, this medium gave equally poor results. The GIBCO + ESM medium also showed a statistical

significance of p < 0.001 with all HEPES media; with HEPES 20mM, there was even a very strong difference (p < 0.0001). No significance could be shown between the HEPES media. The graph showed that the 20mM did stand out slightly.

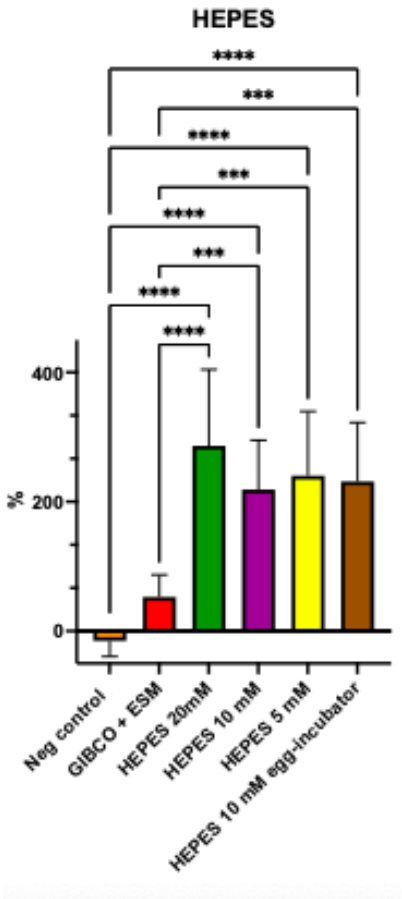


Figure 9**: Proliferation rate of cells throughout the experiment in negative control, GIBCO + ESM and HEPES 20mM/10mM/5mM/controlled temperature chamber after day 14. The bar resembles the mean ± SD. *** indicates statistical difference of p < 0,001 and * indicates p < 0,0001.*

4. Discussion

a) HEPES vs GIBCO

The purpose of this experiment was to see if the cells can proliferate and survive in a CO_2 independent medium. In conclusion, the cells were able to survive outside a CO_2 incubator. The HEPES + ESM medium gave the best results here.

Two clear differences could be noted. This between the ESM and FBS supplement as well as between the GIBCO and HEPES medium. Compared to ESM, FBS still lacks certain growth factors. It is difficult to state which exactly these are, considering that the composition of FBS is not yet fully elucidated, as well as that the composition of the FBS extract can vary greatly (53, 57, 58). Presumably the hydrocortisone, hEGF and BBE present in the ESM are nevertheless important for enhanced proliferation. All three of these have cell growth-promoting qualities.

A possible explanation for the poor result of the GIBCO medium could be that a gradual exposure of the cells to the GIBCO medium was required. It has been shown that, sudden exposure of some cell lines to this CO_2 independent medium, can lead to reduced cell survival (59). This may explain why there was a decrease in cells between day zero and one. The protocol always mentioned the use of a 0% CO_2 incubator and storage in 36-38°C. In this experiment, an incubator was not used, but a warm room instead. As a result, there is an atmospheric concentration of CO_2, which is not zero. The temperature in a warm chamber averages 34°C and does not remain constant. These suboptimal conditions may also have played a role in the results (59). The protocol does not mention how long the cells can be kept in the medium. In most studies, they use this medium for a few hours to a maximum of one or two days (60-63). Based on this experiment, a three-day storage in this medium is a maximum. For longer storage, a medium with a stronger buffer seems to be needed, e.g. HEPES buffer.

That ESM is a better alternative to FBS is a promising finding. In this way, better growth and proliferation of cells can be obtained. However, ESM is more expensive then FBS alone, so it increases the price of the culture media. This is disadvantageous to use this method on a larger scale. In relation to reproductivity, it is also not necessarily better than working with FBS alone. ESM contains FBS which, as mentioned above, can vary from batch to batch. ESM also contains bovine brain extract (BBE), which is something similar to FBS and thus also different from batch to batch. This should be taken in consideration when repeating the method.

In the last few years, there have been many ethical discussions about the use of FBS (53, 57). FBS is obtained by drawing blood from calf fetuses. According to studies, fetuses experience pain with this puncture and it causes increased iatrogenic mortality (53). Given that the ESM also contains FBS, this ethical problem is not avoided. Less FBS is used here though (400µl instead of 2ml), so there is an improvement. Also remember that the ESM contains BBE. However, this is obtained after slaughter, so the calf did not feel pain and therefore does not contribute to the debate.

b) Proliferation start concentration

This section looked at the right condition to analyze endothelial cells in a proliferative phase. Here it can be concluded that, starting with 500 cells in a 96 well plate, is the best approach.

The statistics in this experiment only gave a statistical difference between the 500 cells and the 5000 cells in a 96 well plate. Yet, it can be clearly seen on the graph that there is almost equal difference between the 1000 and 5000 cells. The experiment only counted a small sample, and they had a large standard deviation. This may be a reason for not obtaining a statistical result. This also applies to the 48 well plate. There, 500-cells stood out strongly over 1000 and 5000, but 500 had a very large standard deviation. A large standard deviation and then also a small sample, reduce the likelihood of obtaining a statistically significant result. Therefore, the conclusion is mainly based on the graphs and microscopy.

For the 96 well plate, a difference could be seen as early as day three. The 500 and 1000 cells continued to proliferate until the last day. The wells with 5000 cells hardly showed any further growth. This can be explained by the principle of contact inhibition. If cells contact each other via the glycocalyx (complex of membrane-bound carbohydrates), they stop proliferating and moving. This is due to competition for mitogens and growth factors (64). Considering the 5000 cells soon reached confluence (around day three already), the cells stopped growing and they detached from the plate. This was confirmed via microscopy. This is therefore why the 500 cells were even more proliferating than the 1000 cells. The first had more room to keep growing.

For the 48 well plate, the 500 cells came out best in the end. At the beginning of the experiment, 500-cell values remained very low. This slow growth can be explained by the principle of paracrine stimulation. HUVECs secrete certain growth factors and soluble proteins. These secretions then go on to stimulate other HUVECs to grow (65-67). Microscopy showed that

the cells were widely spaced. As a result, the cells did not stimulate each other as much as with the 5000 cells. As the cells grew closer together, the growth rate increased and there was a large increase between day seven and fourteen.

Since a balance must be found between the possibility of paracrine stimulation and contact inhibition, the 500 cells in the 96 well plate were considered the best. This showed good proliferation from the beginning and by day fourteen there was no contact inhibition.

c) Molar concentration of HEPES

This section looked at which molar concentration of HEPES was best in a CO_2 independent environment. Here, it can be concluded that the molar concentration of the HEPES buffer does not affect cell survival/proliferation. It can also be stated that the controlled temperature chamber is not a positive influence on cell survival.

In experiment 1, cell growth was similar between EGM-2 and HEPES (10mM) + ESM. On the other hand, a big difference is seen between these two media in experiment 3. The EGM-2 gave a nine-fold higher proliferation rate than HEPES 10mM. The difference from experiment 1 could be explained by the number of cells started with. In experiment 1, a starting number of 5000 cells was used. Based on the proliferation test results, in experiment 3, 500 cells were used for starting. As mentioned in the section above, contact inhibition may explain the difference. Considering the cells here were started with less, they still have the most room to proliferate before competing with each other. Still, it is a finding that indicates that the HEPES-buffered DMEM medium still needs something else besides from growth factors (e.g. IGF, VEGF or heparin) to induce sufficient proliferation. It may be that the concentration of bicarbonate present in FBS is too low, and this while bicarbonate is also important for cell proliferation.

No statistically significant difference could be shown between the different molar HEPES concentrations. On the graph, the 20mM does stand out slightly above the others. It has already been described in the literature that this concentration gives the best results (49). The Gibco website then lists 25mM as the most commonly used concentration (68). 25mM was not included in this experiment as previous research showed poorer viability at this concentration (69). One explanation for not finding a difference could be that the sample was too small to obtain significance. It could also be that the pH did not fluctuate much and therefore always had enough buffer.

It is a curious finding that the controlled temperature chamber did not do much better. The first seven days did show better cell proliferation. From day seven, the cells started to die. This contrasted with the media placed in the warm chamber, here the cells did survive. As mentioned above, the chamber has an average temperature of 34°C. Also, stable humidity cannot be provided here. To mimic the condition of a CO_2 incubator, a controlled temperature chamber was used. Normally, the incubator should provide a more stable environment for the cells. The purpose of such an incubator is to keep the temperature (37°C) and humidity constant. It is also dark 100% of the time. In contrast, if someone enters the warm room, the media are briefly exposed to light. No light exposure is important to avoid hydrogen peroxide being formed by the HEPES buffer. Hydrogen peroxide can be cytotoxic to the cells (48, 70-73). So even though the incubator was a more stable cell environment than the warm chamber, towards the end of the experiment, it still did not give better results. However, it can be used for proliferation in the first seven days. This may indicate that the warm chamber temperature (34°C) appears to be a better temperature for the cells without bicarbonate after all.

In the first experiment, reduced survival between days three and seven was observed for the CO_2 independent medium provided by Gibco™. In that experiment, day seven was the last day so further observation was not possible. This decreasing survival trend was also observed in experiment 3. Given that this study lasted a week longer, the decreasing trend could be further established. At the end, as good as almost all cells were dead (1% survived). The same explanation as in section ‘a) HEPES vs GIBCO’ can be given for this (suboptimal conditions and need for sequential adaptation).

d) Future perspective

Several follow-up experiments can be suggested:

- Given that in this study, no significant difference could be demonstrated between the different molar HEPES concentrations, it would be better to repeat the experiment again with more wells. This will increase the sample and thus the chances of obtaining a statistically significant result.
- The controlled temperature chamber was only used for one medium and one experiment here. A subsequent experiment where different molar HEPES concentrations are put in the controlled temperature chamber is needed. This will allow to make a better conclusion towards the effect of this incubator.

- In the literature, hPL is proposed as a cheaper and more ethical alternative to FBS (74). A condition of HEPES buffered DMEM supplemented with ESM can be compared with a condition supplemented with hPL. In purchase, hPL is more expensive than ESM, but hPL can be obtained in the lab itself, however, this requires more time. It is a consideration to be made if hPL gives better proliferation results than ESM.
- The CO_2 independent medium provided by Gibco™ can be tested in the controlled temperature chamber. This provides more optimal conditions. Also, cells should be sequentially exposed to the medium as described in the protocol (59).
- The next step is that samples consisting of endothelial cells encapsulated in or seeded on top of the hydrogels should be tested for survival/proliferation in CO_2 independent medium. If these cells tend to survive, application in long lasting bioprinting and transport can be attempted. If cells tend to attach decently to the hydrogels while being suspended in CO_2 independent medium, the medium can be tested for seeding of bioprinted channel structures in a rotational device.

5. Conclusion

In this thesis, the use of a HEPES + ESM CO_2-independent medium was successful. The cells were still viable after fourteen days. This gives great prospects for the future where cells can be grown and rotated in a simple setup. This also gives the possibility of doing these experiments in less equipped or funded labs.

6. References

1. Wong VW, Rustad KC, Longaker MT, Gurtner GC. Tissue engineering in plastic surgery: a review. Plast Reconstr Surg. 2010;126(3):858-68.
2. Arrigoni C, Camozzi D, Remuzzi A. Vascular tissue engineering. Cell Transplantation. 2006;15(SUPPL. 1):S119-S25.
3. Turnbull G, Clarke J, Picard F, Zhang W, Riches P, Li B, et al. 3D biofabrication for soft tissue and cartilage engineering. Medical Engineering and Physics. 2020;82:13-39.
4. Machour M, Szklanny AA, Levenberg S. Fabrication of Engineered Vascular Flaps using 3D Printing Technologies. J Vis Exp. 2022(183).
5. Abdollahi S, Boktor J, Hibino N. Bioprinting of freestanding vascular grafts and the regulatory considerations for additively manufactured vascular prostheses. Transl Res. 2019;211:123-38.
6. De Freitas Soares BL, Golz K, Giri S, Bader A, Fellmer P. Point of care technology for standardized vascular graft engineering: Use of a novel bioreactor technique. Gefasschirurgie. 2013;18(5):488-9.
7. Da Graca B, Filardo G. Vascular bioprinting. American Journal of Cardiology. 2011;107(1):141-2.
8. Jin Q, Jin G, Ju J, Xu L, Tang L, Fu Y, et al. Bioprinting small-diameter vascular vessel with endothelium and smooth muscle by the approach of two-step crosslinking process. Biotechnology and bioengineering. 2022;119(6):1673-84.
9. Pavan Kalyan B, Kumar L. 3D Printing: Applications in Tissue Engineering, Medical Devices, and Drug Delivery. AAPS PharmSciTech. 2022;23(4).
10. Wang G, Zhang X, Bu X, An Y, Bi H, Zhao Z. The Application of Cartilage Tissue Engineering with Cell-Laden Hydrogel in Plastic Surgery: A Systematic Review. Tissue Eng Regen Med. 2022;19(1):1-9.
11. Hadlock TA, Vacanti JP, Cheney ML. Tissue engineering in facial plastic and reconstructive surgery. Facial Plast Surg. 1998;14(3):197-203.
12. Baltazar T, Merola J, Catarino C, Xie CB, Kirkiles-Smith NC, Lee V, et al. Three Dimensional Bioprinting of a Vascularized and Perfusable Skin Graft Using Human Keratinocytes, Fibroblasts, Pericytes, and Endothelial Cells. Tissue Engineering - Part A. 2020;26(5-6):227-38.
13. Gozuacik D, Irmak S, Karakas E, Koc B. Optimized 3D design and bioprinting of a blood vessel based on autophagy analyses. J Tissue Eng Regen Med. 2012;6:377.
14. Abouna GM. Organ shortage crisis: problems and possible solutions. Transplant Proc. 2008;40(1):34-8.
15. Ali M, P RA, Lee SJ, Jackson JD. Three-dimensional bioprinting for organ bioengineering: promise and pitfalls. Curr Opin Organ Transplant. 2018;23(6):649-56.
16. Eurotransplant. Annual raport 2021. Online 2022. Consulted at 24 october 2022. Available from: https://www.eurotransplant.org/wp-content/uploads/2022/06/Annual-Report-2021_LR.pdf.
17. Walgenbach KJ, Voigt M, Riabikhin AW, Andree C, Schaefer DJ, Galla TJ, et al. Tissue engineering in plastic reconstructive surgery. Anat Rec. 2001;263(4):372-8.
18. Gao G, Huang Y, Schilling AF, Hubbell K, Cui X. Organ Bioprinting: Are We There Yet? Adv Healthc Mater. 2018;7(1).
19. Abudupataer M, Chen N, Yan S, Alam F, Shi Y, Wang L, et al. Bioprinting a 3D vascular construct for engineering a vessel-on-a-chip. Biomed Microdevices. 2020;22(1).
20. Benmeridja L, De Moor L, De Maere E, Vanlauwe F, Ryx M, Tytgat L, et al. High-throughput fabrication of vascularized adipose microtissues for 3D bioprinting. J Tissue Eng Regen Med. 2020;14(6):840-54.
21. Aljohani W, Ullah MW, Zhang X, Yang G. Bioprinting and its applications in tissue engineering and regenerative medicine. Int J Biol Macromol. 2018;107(Pt A):261-75.
22. Datta P, Ayan B, Ozbolat IT. Bioprinting for vascular and vascularized tissue biofabrication. Acta biomaterialia. 2017;51:1-20.
23. Sigaux N, Pourchet L, Breton P, Brosset S, Louvrier A, Marquette CA. 3D Bioprinting:principles, fantasies and prospects. J Stomatol Oral Maxillofac Surg. 2019;120(2):128-32.
24. Kinstlinger IS, Calderon GA, Royse MK, Means AK, Grigoryan B, Miller JS. Perfusion and endothelialization of engineered tissues with patterned vascular networks. Nat Protoc. 2021;16(6):3089-113.

25. Mandrycky C, Wang Z, Kim K, Kim DH. 3D bioprinting for engineering complex tissues. Biotechnol Adv. 2016;34(4):422-34.
26. Barrs RW, Jia J, Silver SE, Yost M, Mei Y. Biomaterials for Bioprinting Microvasculature. Chem Rev. 2020;120(19):10887-949.
27. Quadri F, Soman SS, Vijayavenkataraman S. Progress in cardiovascular bioprinting. Artificial Organs. 2021;45(7):652-64.
28. Bova L, Billi F, Cimetta E. Mini-review: advances in 3D bioprinting of vascularized constructs. Biology direct. 2020;15(1):22.
29. Ng HY, Lee KA, Kuo CN, Shen YF. Bioprinting of artificial blood vessels. Int J Bioprint. 2018;4(2):140.
30. Caputo M, Saif J, Rajakaruna C, Brooks M, Angelini GD, Emanueli C. MicroRNAs in vascular tissue engineering and post-ischemic neovascularization. Adv Drug Deliv Rev. 2015;88:78-91.
31. Ferdinande L. Histologie van het cardiovasculair stelsel. 2019.
32. Park HJ, Zhang Y, Georgescu SP, Johnson KL, Kong D, Galper JB. Human umbilical vein endothelial cells and human dermal microvascular endothelial cells offer new insights into the relationship between lipid metabolism and angiogenesis. Stem Cell Rev. 2006;2(2):93-102.
33. Richards D, Jia J, Yost M, Markwald R, Mei Y. 3D Bioprinting for Vascularized Tissue Fabrication. Ann Biomed Eng. 2017;45(1):132-47.
34. Kolesky DB, Truby RL, Gladman AS, Busbee TA, Homan KA, Lewis JA. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. Adv Mater. 2014;26(19):3124-30.
35. Kolesky DB, Homan KA, Skylar-Scott MA, Lewis JA. Three-dimensional bioprinting of thick vascularized tissues. Proc Natl Acad Sci U S A. 2016;113(12):3179-84.
36. Lee VK, Kim DY, Ngo H, Lee Y, Seo L, Yoo SS, et al. Creating perfused functional vascular channels using 3D bio-printing technology. Biomaterials. 2014;35(28):8092-102.
37. Liu X, Wang X, Zhang L, Sun L, Wang H, Zhao H, et al. 3D Liver Tissue Model with Branched Vascular Networks by Multimaterial Bioprinting. Adv Healthc Mater. 2021;10(23):e2101405.
38. 2021. Biology Online.
39. Delkash Y, Gouin M, Rimbeault T, Mohabatpour F, Papagerakis P, Maw S, et al. Bioprinting and in vitro characterization of an eggwhite-based cell-laden patch for endothelialized tissue engineering applications. Journal of Functional Biomaterials. 2021;12(3).
40. De Moor L, Smet J, Plovyt M, Bekaert B, Vercruysse C, Asadian M, et al. Engineering microvasculature by 3D bioprinting of prevascularized spheroids in photo-crosslinkable gelatin. Biofabrication. 2021;13(4).
41. Chen Z, Htay A, Dos Santos W, Gillies GT, Fillmore HL, Sholley MM, et al. In vitro angiogenesis by human umbilical vein endothelial cells (HUVEC) induced by three-dimensional co-culture with glioblastoma cells. J Neurooncol. 2009;92(2):121-8.
42. PromoCell. Endothelial cell growth medium-2 2022.
43. Ma X, Yang C, Zhang J, Wang J, Li W, Xu C, et al. Culturing with modified EGM2 medium enhances porcine neonatal islet-like cell clusters resistance to apoptosis in islet xenotransplantation. Xenotransplantation. 2018;25(1).
44. Bala K, Ambwani K, Gohil NK. Effect of different mitogens and serum concentration on HUVEC morphology and characteristics: implication on use of higher passage cells. Tissue Cell. 2011;43(4):216-22.
45. Chen J, Zhang C, Feng YD, Zong C, Chen JR, Tang ZH, et al. Studies on Culture and Osteogenic Induction of Human Mesenchymal Stem Cells under CO2-Independent Conditions. Astrobiology. 2013;13(4):370-9.
46. Yao T, Asayama Y. Animal-cell culture media: History, characteristics, and current issues. Reprod Med Biol. 2017;16(2):99-117.
47. Michl J, Park KC, Swietach P. Evidence-based guidelines for controlling pH in mammalian live-cell culture systems. Commun Biol. 2019;2:144.
48. Bell-Young L. ReAgent: What is hepes buffer? Online 14/02/2021. Consulted at 24 october 2022. Available from: https://www.reagent.ie/what-is-hepes-buffer/.
49. ENCO. Protocol: HEPES Buffer Recipe. Consulted at 12 october 2022 Available from: https://www.enco.co.il/protocol.php?id=24.
50. Delmouly K, Belondrade M, Casanova D, Milhavet O, Lehmann S. HEPES inhibits the conversion of prion protein in cell culture. J Gen Virol. 2011;92(Pt 5):1244-50.

51. Rohanová D, Boccaccini AR, Horkavcová D, Bozděchová P, Bezdička P, Častorálová M. Is non-buffered DMEM solution a suitable medium for in vitro bioactivity tests? J Mater Chem B. 2014;2(31):5068-76.
52. Arora M. Cell Culture Media: A Review. Online 2013. Consulted at 24 12 october 2022. Available from: https://www.labome.com/method/Cell-Culture-Media-A-Review.html.
53. van der Valk J, Bieback K, Buta C, Cochrane B, Dirks WG, Fu J, et al. Fetal Bovine Serum (FBS): Past - Present - Future. Altex. 2018;35(1):99-118.
54. Boncler M, Różalski M, Krajewska U, Podsędek A, Watala C. Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells. Journal of Pharmacological and Toxicological Methods. 2014;69(1):9-16.
55. Xu M, McCanna DJ, Sivak JG. Use of the viability reagent PrestoBlue in comparison with alamarBlue and MTT to assess the viability of human corneal epithelial cells. Journal of pharmacological and toxicological methods. 2015;71:1-7.
56. Gaucher S, Jarraya M. Technical note: comparison of the PrestoBlue and LDH release assays with the MTT assay for skin viability assessment. Cell Tissue Bank. 2015;16(3):325-9.
57. Piletz JE, Drivon J, Eisenga J, Buck W, Yen S, McLin M, et al. Human Cells Grown With or Without Substitutes for Fetal Bovine Serum. Cell Med. 2018;10:2155179018755140.
58. Lee J, Kang M, Choi J, Chun Y, Jang J, Yang Y, et al. Comparative analysis of FBS containing media and serum free chemically defined media for adipose derived stem cells production. Cytotherapy. 2019;21(5):S82.
59. ThermoFisher. CO2 independent GIBCO medium PROTOCOL. 2014.
60. De Clerck LS, Bridts CH, Mertens AM, Moens MM, Stevens WJ. Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. J Immunol Methods. 1994;172(1):115-24.
61. Minet E, Arnould T, Michel G, Roland I, Mottet D, Raes M, et al. ERK activation upon hypoxia: involvement in HIF-1 activation. FEBS Lett. 2000;468(1):53-8.
62. Weltin A, Slotwinski K, Kieninger J, Moser I, Jobst G, Wego M, et al. Cell culture monitoring for drug screening and cancer research: a transparent, microfluidic, multi-sensor microsystem. Lab Chip. 2014;14(1):138-46.
63. Piret JP, Mottet D, Raes M, Michiels C. CoCl2, a chemical inducer of hypoxia-inducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2. Ann N Y Acad Sci. 2002;973:443-7.
64. Gu Q, Tomaskovic-Crook E, Wallace GG, Crook JM. 3D Bioprinting Human Induced Pluripotent Stem Cell Constructs for In Situ Cell Proliferation and Successive Multilineage Differentiation. Adv Healthc Mater. 2017;6(17).
65. Piard C, Jeyaram A, Liu Y, Caccamese J, Jay SM, Chen Y, et al. 3D printed HUVECs/MSCs cocultures impact cellular interactions and angiogenesis depending on cell-cell distance. Biomaterials. 2019;222:119423.
66. Qi H, Wang K, Li M, Zhang Y, Dong K, Heise S, et al. Co-culture of BMSCs and HUVECs with simvastatin-loaded gelatin nanosphere/chitosan coating on Mg alloy for osteogenic differentiation and vasculogenesis. Int J Biol Macromol. 2021;193:2021-8.
67. Axelrod R, Axelrod DE, Pienta KJ. Evolution of cooperation among tumor cells. Proc Natl Acad Sci U S A. 2006;103(36):13474-9.
68. ThermoFisher. Improve your cell culture with Gibco media supplements Online. Consulted at 24 october 2022. Available from: https://www.thermofisher.com/be/en/home/life-science/cell-culture/mammalian-cell-culture/media-supplements.html.
69. Vanlauwe F. The tissue engineering of adipose tissue and macrovasculature for breast reconstruction. 2020.
70. Zigler JS, Jr., Lepe-Zuniga JL, Vistica B, Gery I. Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. In Vitro Cell Dev Biol. 1985;21(5):282-7.
71. Lepe-Zuniga JL, Zigler JS, Jr., Gery I. Toxicity of light-exposed Hepes media. J Immunol Methods. 1987;103(1):145.
72. Bowers CW, Dahm LM. Buffers for cell culture [2]. Trends in Cell Biology. 1993;3(3):76-7.
73. Bowman CM, Berger EM, Butler EN, Toth KM, Repine JE. HEPES may stimulate cultured endothelial cells to make growth-retarding oxygen metabolites. In Vitro Cell Dev Biol. 1985;21(3 Pt 1):140-2.

74. Henschler R, Gabriel C, Schallmoser K, Burnouf T, Koh MBC. Human platelet lysate current standards and future developments. Transfusion. 2019;59(4):1407-13.

APPENDIX 1

a) Thawing of cells

Needed materials:

- Cup with sterile water
- Microwave
- Frozen vial with HUVECs
- PBS
- 1000µl pipette and tips
- Culture medium (EGM-2)
- Centrifuge
- 15ml CELLSTAR™ tube
- T75 CELLSTAR™ Cell culture flask
- CO_2 incubator

Step-by-step plan:

(Always work in the laminar flow cabinet and as sterile as possible)

1. Put ± 10ml aside of the cold culture medium. Warm the rest to 37°C.
2. Warm the cup with sterile water in the microwave.
3. Hold the vial with frozen cells in the cup with sterile water until 2/3 is thawed. *(The cap must NOT be submerged in water!!)*
4. Transfer the thawed cells with the pipette to the 15ml tube.
5. Rinse the tube TWICE with the cold culture medium and add it to the cells in the 15ml tube.
6. Fill the 15ml tube with cold culture medium until 10ml is reached.
7. Centrifugate the 15ml tube for 5 min at 1500 rpm.
8. Remove the supernatant. *(Be careful not to touch the cells!!)*
9. Suspend the cells with warm culture medium.
10. Put the cells in the T75 CELLSTAR™ Cell culture flask.
11. Rinse the tube TWICE with the warm culture medium.
12. Fill the flask with warm culture medium until 15ml is reached.

13. Move the bottle slowly from left to right, up and down. NOT in circles!
14. Place the flask in the CO_2 incubator at 37°C.
15. Refresh the medium twice a week and check the proliferation of the cells under the microscope

b) Refreshing cells

Needed materials:

- Warm culture medium (EGM-2) (37°C)
- 5000µl (5ml) pipette and tips
- Pasteur pipettes and an electric pipette
- PBS

Step-by-step plan:

(Always work in the laminar flow cabinet and as sterile as possible)

1. Remove the culture medium from the T75 flask with the pasteur pipettes.
2. Rinse the flask with 5ml PBS with the 5ml pipette. (*Do not touch the cells! Spray the PBS to the other side of the cells).* Move the flask slowly from left to right, up and down.
3. Remove the PBS with the Pasteur pipettes
4. Add the warm culture medium to the flask until 15ml is reached.
5. Place the flask in the CO_2 incubator at 37°C

c) Isolating cells

Needed materials:

- PBS
- Trypsine (TrypLE™)
- Warm culture medium (EGM-2) (37°C)
- 15ml CELLSTAR™ tube
- 5000µl (5ml) pipette and tips
- 1000µl pipette and tips
- Pasteur pipettes and an electric pipette

- Centrifuge
- PAULA Cell Imager

Step-by-step plan:

(Always work in the laminar flow cabinet and as sterile as possible)

1. Remove the culture medium from the T75 flask with the pasteur pipettes.
2. Rinse the flask TWICE with 5ml PBS. (*Do not touch the cells! Spray the PBS to the other side of the cells).* Move the flask slowly from left to right, up and down.
3. Remove the PBS with the pasteur pipettes.
4. Add 2ml trypsin with the 1000µl pipette. Move the flask slowly from left to right, up and down.
5. Place the flask in the CO_2 incubator at 37°C for 5min.
6. Tap 3 times with the flank on a surface and look with the PAULA microscope to see if the cells have become detached.
7. Add 3 times as much as warm culture medium as the volume of trypsin that you had added (6ml).
8. Resuspend the mix of trypsin and culture medium with the pasteur pipettes.
9. Collect the cells in the 15ml tube.
10. Rinse the flask with PBS and add it to the 15ml tube.
11. Centrifugate the cells for 5min at 1500 rpm
12. Remove the supernatant to obtain a pellet of cells. *(Be careful not to touch the cells!!)*

d) Counting cells

Needed materials:

- Bürkers counting chamber, already mounted
- Microcentrifuge tube 1,5ml
- Trypan Blue Solution (Gibco™)
- 200µl pipette and tips

- 5000µl (5ml) pipette and tips
- Warm culture medium (EGM-2) (37°C)
- Microscope (Olympus IX 81)
- Isolated cell pellet as in ‘c) Isolating Cells’

Step-by-step plan:

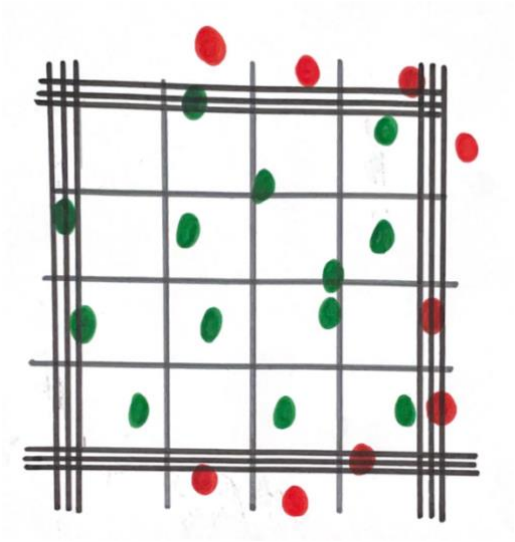
(Always work in the laminar flow cabinet and as sterile as possible)

1. Add 3ml of warm culture medium to the isolated cell pellet with the 5ml pipette and resuspend.
2. Make a 1:5 dilution of cells and trypan blue.
    a. Add 20µl of the cell suspension in a 1,5ml microcentrifuge tube with the 200µl pipette.
    b. Add 80µl of trypan blue to the microcentrifuge tube with the 200µl pipette.
3. Resuspend.
4. Bring this solution into the counting chamber. *(Do this slowly! The chamber will suck the solution itself)*
5. Count the cells under the microscope
    c. Count 1 large square delineated by 3 lines. This large square is divided into 16 smaller squares. Count the cells inside these squares, the cells on the left edge and on the top edge.
    d. This means that the cells outside this large square or the cells on the right edge or on the bottom edge do NOT count (Drawing 1).
    e. Count a total of 5 large squares.
6. Take the average of the cell count from these 5 large squares. *(e.g. (a + b + c + d + e + f)/5)*
7. Calculate the average number of cells per ml culture medium:

    Average amount from step 6 x dilution factor x 10^4

(In this case is the dilution factor 5 => see step 2)

8. Since 3ml of culture medium was used (see step 1), this number must be multiplied by 3.



Drawing 1: *Counting cells on a Bürker counting chamber: green dots are included; red dots are not.*

e) Seeding cells

Calculation of how many ml is needed:

1. 5000 cells were chosen as starting number. Since 5 mediums were tested and a positive control was also used, a total of 36 wells were in use.
2. Thus, a total number of 180 000 cells (5000 x 36) was required. Rounded off, this is about 200 000 cells.
3. After calculating the total number of cells per ml (see cell count), one can calculate how many ml are needed to obtain 200 000 cells.
4. The wells should be filled with 200µl containing 5000 cells. This means 25 000 cells per ml.
5. Since 200 000 cells are needed in total, a total of 8ml is required.
6. The number of ml calculated in step 3 should be subtracted from this 8ml. The number obtained is the amount of culture medium that still needs to be added.

Needed materials:

- 3 x 96 CELLSTAR® well plate
- Correct amount of culture medium with cells (see above)

- CO_2 incubator
- 200µl pipette and tips

Step-by-step plan:

(Always work in the laminar flow cabinet and as sterile as possible)

1. Cell isolation as in ‘c) Isolating cells.’
2. Cell counting as in ‘d). Counting cells.’
3. Calculation as above.
4. Resuspend
5. Add 200µl in 6 wells of plate 1*.
6. Add 200µl in 18 wells of plate 2*.
7. Add 200µl in 12 wells of plate 3*.
8. Place the well plates in the CO_2 incubator at 37°C.

*(*Further specification of the well plates in experiment 1 – item 3)*

APPENDIX 2 - HEPES medium

The calculation of how many ml of 1M HEPES GIBCO™ was needed:

1. To make 20ml, about 0,20g of this powder (= 0,020l) is needed. In this case 0,2612g was used (= 0,02612l)
2. 1M HEPES => 10mmol conc => 10 mmol/l x 0,02612l = 0,2612 mmol
3. 0,2612 mmol/1000 mmol/l = 0,2612 ml needed

Step-by-step plan:

1. Put 18ml of distilled water in an Erlenmeyer.
2. Place the flask on the magnetic stirrer and put a magnetic stir bar in it.
3. Weigh about 0,20g of DMEM powder. As mentioned above, in this case was it 0,2612g.
4. Put the DMEM powder carefully in the flask with distilled water.
5. Rinse the plate of the powder with 1ml of distilled water.
6. Add the amount of ml of 1M HEPES as calculated above.
7. Since 0,2612g was weighed, the total volume of this medium will be 26,12 ml.
8. Considering there was already 18ml of distilled water, 1 ml was used for rinsing and 0,261ml of HEPES was needed, we still need 6,859ml of water (26,12ml – 18ml – 1ml – 0,261ml = 6,859ml).
9. Use the pH meter to check the pH. The pH should be between 7,2 and 7,4. If it is not, adding HCl or NaOH can regulate the pH. In this experiment the pH was 7,36.
10. Filter out the culture medium.

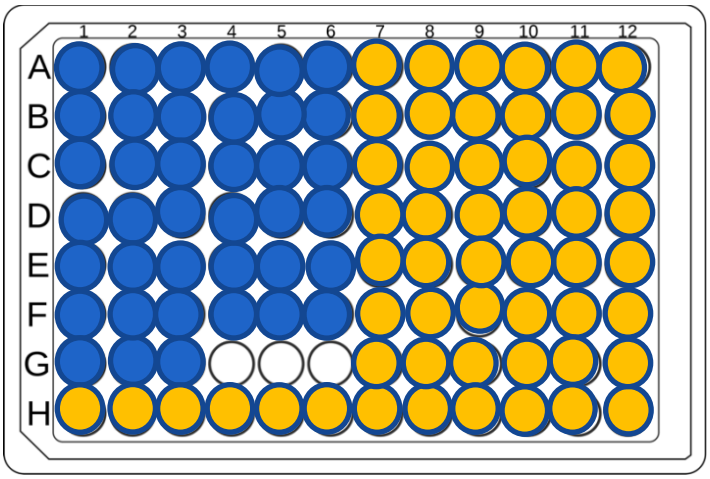
APPENDIX 3 – Preparation of the well plate experiment 1

Step-by-step plan:

(Always work in the laminar flow cabinet and as sterile as possible)

1. Remove the culture medium from your cell seeded welt plates
2. Add 100µl of the PrestoBlue solution into the 36 wells. Add 100µl to 3 additional wells as a control.
3. Wrap the 3 well plates in aluminum foil to protect from light.
4. Place the 3 well plates back in the incubator for 2 hours.
5. Take a new 96 well plate and add 80 microliters of each well to this new plate. The wells from well plate 1 will go on row 1, then the wells from plate 2 on row 2-4 and then the wells from plate 3 on row 5-6 (see appendix 3). We use 80µl instead of 100µl because there is always a small particle (max 20%) that evaporates during these 2 hours.
6. Wrap this new plate in aluminum foil.
7. It is ready to analyze.

APPENDIX 4 - Well plate experiment 1



Blue stands for the wells used on day 0:

A: positive control

B: negative control

C: GIBCO + ESM

D: GIBCO + FBS

E: HEPES + ESM

F: HEPES + FBS

G: background control of the medium

Yellow stands for the wells used on day 1:

A: positive control

B: negative control

C: GIBCO + ESM

D: GIBCO + FBS

E: HEPES + ESM

F: HEPES + FBS

G 7 – 9: background of GIBCO + ESM

G 10 – 12: background of GIBCO + FBS

H 1 – 3: background of the pos. control

H 4 – 6: background of the neg. control

H 7 – 9: background of HEPES + ESM

H 10 – 12: background of HEPES + FBS

APPENDIX 5 – Well plate experiment 3

Plate 1:

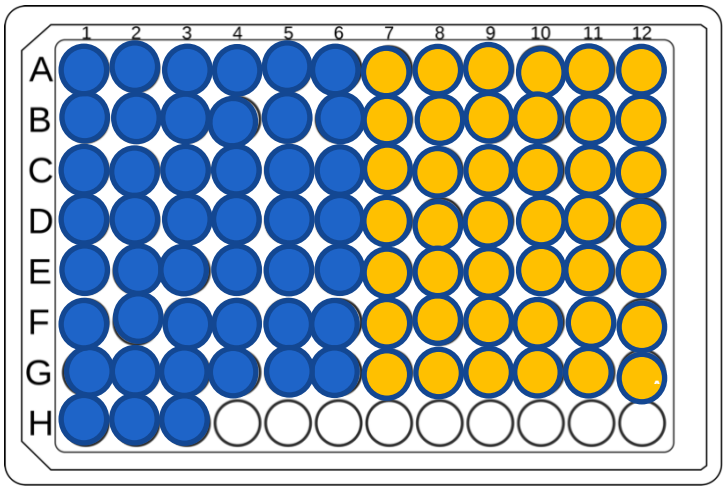
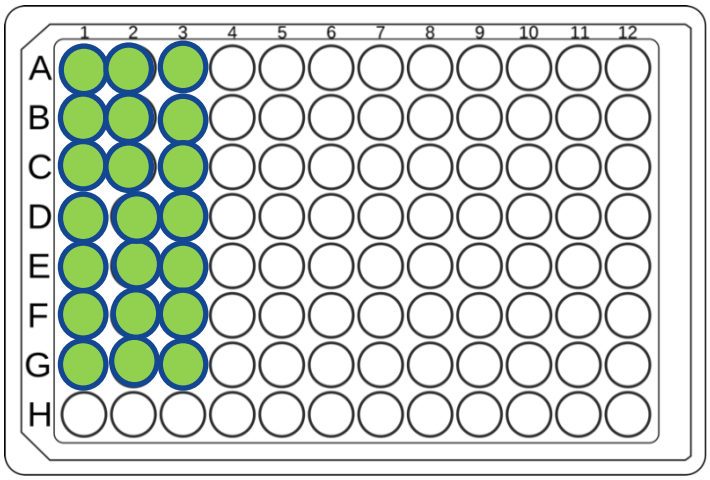


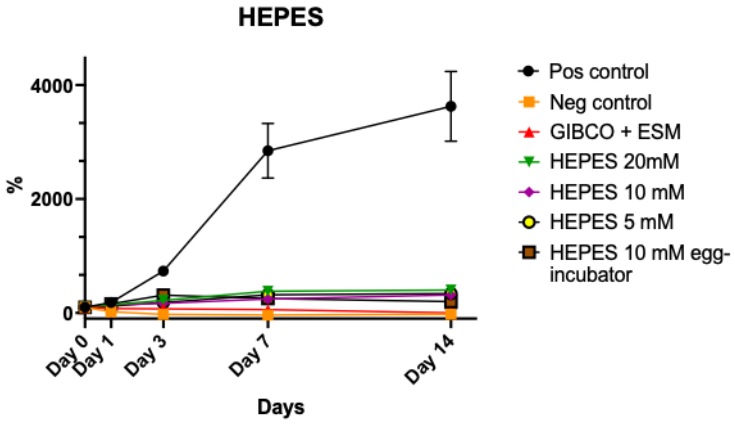
Plate 2:



Blue – plate 1 stands for the wells used **on day 0**:	Yellow – plate 1 stands for the wells used **on day 1**:	Green – plate 2 stands for the wells used **on day 1**:
A: positive control B: negative control C: GIBCO + ESM D: 20mM HEPES E: 10mM HEPES F: 5mM HEPES G: 10 mH HEPES controlled temperature chamber H: background control of the medium	A: positive control B: negative control C: GIBCO + ESM D: 20mM HEPES E: 10mM HEPES F: 5mM HEPES G: 10 mH HEPES controlled temperature chamber	A: background + contr. B: background - contr. C: background GIBCO +ESM D: background 20mM E: background 10mM F: background 5mM G: background 10mM controlled temperature chamber

APPENDIX 6 – Results experiment 3 with EGM-2

A)



B)

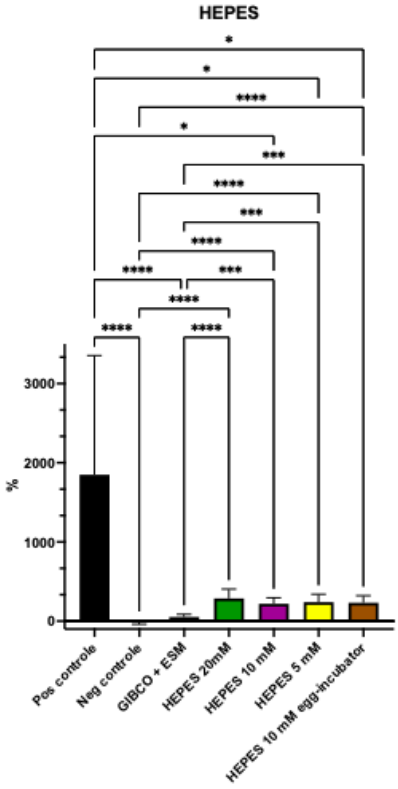


Figure A: *Survival and proliferation expressed as percentage of proliferation on day 1, 3, 7 and 14 in the different media.*
Figure B*: Proliferation of cells throughout the experiment in positive/negative control, GIBCO + ESM and HEPES 20mM/10mM/5mM/ controlled temperature chamber. The bar resembles the mean ± SD. * indicates statistical difference of p < 0,05; *** indicates p < 0,001 and **** indicates p < 0,0001.*