FACULTY OF MEDICINE AND HEALTH SCIENCES

Cracking a hard egg: exploring the use of the chicken chorioallantoic membrane assay in tissue engineering research

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List of abbreviations

3D	Three-dimensional
ASC	Adipose-derived stem cell
BM	Basement membrane
CAM	Chorioallantoic membrane
DAB	Diaminobenzidine
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EDD	Embryonic developmental day
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
FBS	Fetal bovine serum
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
PBS	Phosphate buffered saline
PLA	Polyactic acid
qRT-PCR	Quantitative real-time polymerase chain reaction
STL CAD	Standard Tessellation Language Computed-Aided Design
SVF	Stromal vascular fraction
TE	Tissue engineering
VEGF-A	Vascular endothelial growth factor A
VEGF-R2	Vascular endothelial growth factor receptor 2
X^2	Chi-square
ZSF	Zinc salts-based fixative

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Abstract

Introduction: The field of tissue engineering has gained great interest with a variety of applications, such as adipose tissue engineering; using hydrogels, such as Gel-MA and RCPhC1-MA. However, a major challenge is to assure the viability of the newly formed tissue. An important factor hereby is the blood supply. Currently, mostly animal research is performed for this purpose; however, this is quite costly and time-consuming and thus research should be done to find attractive alternatives. This is where the chorioallantoic membrane assay (CAM) comes into the picture.

Materials and methods: Both *in ovo* and *ex ovo* methods were performed and compared. Scaffolds printed from Gel-MA and RCPhC1-MA hydrogels were placed onto the CAM, either in a silicone ring or on a double-layered mesh. Half of each group of scaffolds were seeded with HT1080s. These scaffolds tended to dry out and so 3 types of closed constructs were used: a plastic drinking straw, a 3mm 3D-printed construct, and a 5mm 3D-printed construct; all attached to a mesh. Only the *ex ovo* method was performed for these closed constructs, with only Gel-MA scaffolds. Again, half of the scaffolds of each group were seeded with HT1080s. Visualization was done after clearing, ink injection, and DAB-whole mount.

Results: *In ovo* and *ex ovo* gave the same results regarding tissue surface, surface gel, and number of blood vessels per surface area. Gel-MA and RCPhC1-MA were equal to each other as well. The silicone ring made it easier to perform histology when compared to the double-layered mesh as well as visualisation. Presence of cells had no noticeable effect on number of blood vessel.
The closed constructs did indeed tend to dry out less than the scaffolds as such. A small, non-significant difference was seen in number of blood vessels between the scaffolds with and without HT1080s. The embryos showed higher mortality with the larger and heavier constructs. The smaller constructs however tended to crook more, but this did not influence embryo mortality.

Discussion: *In ovo* and *ex ovo* are equally performant, as well as Gel-MA and RCPhC1-MA. Scaffolds that are placed on the CAM as such, tend to dry out. A possible solution to this problem could be the use of the closed constructs. In the constructs, the presence of cells gave a non-significant result of effect on the number of blood vessels; however, a slightly higher number of vessels could be noticed in presence of HT1080s. This confirms our theory that, with some optimization, this model could be fit to investigate what factors influence new vessel formation in scaffolds in tissue engineering.

Samenvatting

Inleiding: Weefseltechnologie heeft aan grote belangstelling gewonnen met een verscheidenheid aan toepassingen, onder andere door gebruik te maken van hydrogels zoals Gel-MA en RCPhC1-MA. Een majeure moeilijkheid is het verzekeren van viabiliteit van nieuw weefsel. Een belangrijke factor hierbij is bloedtoevoer verzekeren. Momenteel wordt het meeste onderzoek hieromtrent uitgevoerd door middel van proefdierexperimenten; dit is kostelijk en tijdrovend en daarom zou er onderzoek naar aantrekkelijke alternatieven gedaan moeten worden. Hierbij komt de chorioallantoïsche membraan (CAM) assay in beeld.

Materialen en methodes: Zowel een *in ovo* als *ex ovo* methode werd uitgevoerd en met elkaar vergeleken. Scaffolds geprint van Gel-MA en RCPhC1-MA hydrogels werden op de CAM geplaatst, ofwel in een silicone ring ofwel op een dubbellaag van nylon mesh. De helft van elke groep scaffolds werd bezaaid met HT1080-cellen. Deze scaffolds hadden de neiging om uit te drogen en dus werden 3 types gesloten constructen gebruikt; een rietje, een 3 mm 3D-geprint construct en een 5 mm 3D-geprint construct; alle bevestigd aan een mesh. Enkel de *ex ovo* methode werd hierbij uitgevoerd, met enkel Gel-MA scaffolds. De helft van de scaffolds in elke groep werd bezaaid met HT1080-cellen. Visualisatie werd uitgevoerd na klaring, inkt injectie en DAB-onderdompeling.

Resultaten: *In ovo* en *ex ovo* gaven dezelfde resultaten wat betreft oppervlakte nieuw weefsel, oppervlakte gel en het aantal bloedvaten per oppervlakte weefsel. Gel-MA en RCPhC1-MA waren eveneens gelijkwaardig aan elkaar. De silicone ring maakte het makkelijker om histologie uit te voeren in vergelijking met de dubbellaag mesh; de ring vergemakkelijkte visualisatie. De aanwezigheid van cellen had geen merkbaar effect op het aantal bloedvaten. De gesloten constructen hadden inderdaad minder de neiging om uit te drogen dan de scaffolds op zich. Een klein, niet-significant verschil in aantal bloedvaten tussen de scaffolds met en zonder HT1080-cellen kon bemerkt worden. De embryo's hadden een hogere mortaliteit bij de grotere, zwaardere constructen. De kleinere constructen daarentegen vielen vaker om, maar dit beïnvloedde de mortaliteit niet.

Discussie: *In ovo* en *ex ovo* zijn even performant, net als Gel-MA en RCPhC1-MA. Scaffolds die zonder meer op de CAM worden geplaatst, hebben de neiging om uit te drogen. Een mogelijke oplossing voor dit probleem zou het gebruik van gesloten constructen kunnen zijn. In de constructen gaf de aanwezigheid van cellen een niet-significant effect op het aantal bloedvaten, een enigszins groter aantal bloedvaten kan worden opgemerkt in aanwezigheid van HT1080-cellen. Dit bevestigt onze theorie dat mits optimalisatie dit model gepast kan zijn om te onderzoeken welke factoren in scaffolds een invloed hebben op bloedvatvorming in weefseltechnologie.

1 Introduction

Autologous fat grafting, which is the transfer of fat from one body part to another, is a widely used technique in the field of reconstructive plastic surgery, for instance in breast augmentation and reconstruction, which still has some limitations. The biggest disadvantage is the high and unpredictable resorption rate, ranging from 20 to 90%. As a result, re-surgery has to be done, often multiple times, which is distressing for patients. (1-3)

1.1 Tissue engineering

One of the major limitations of fat grafting is the viability of the graft. Tissue engineering (TE) can be seen as an attractive alternative for fat grafting because it could overcome some of the problems of fat grafts. Tissue engineering creates a new tissue which can be used as an implant and focuses mainly on supporting scaffolds, bioactive molecules, and manipulation of cells, in particular stem cells. (4, 5) It is used in a variety of medical fields for regeneration, such as adipose tissue engineering, and replacement of tissue and has gained great interest in the past years. An important principle in tissue engineering is the replace-like-with-like principle, that states that it is always best to try to mimic native tissue as closely as possible. (6)

1.2 Vasculogenesis and angiogenesis

An important factor in the viability of tissue engineered constructs is the adequate supply of nutrients through blood vessels. New blood vessels can be formed with endothelial progenitor cells (EPCs), thus forming an early vascular plexus, a process called vasculogenesis. The EPCs switch to endothelial cells (ECs), which can be stimulated by binding of vascular endothelial growth factor A (VEGF-A) onto the vascular endothelial growth factor receptor 2 (VEGF-R2).

Afterwards, the plexus undergoes angiogenesis, a process in which new blood vessels develop from existing vessels. Angiogenesis takes place in physiological as well as in pathological conditions, for example in tumours or in chronic inflammation. There are several types of angiogenesis: sprouting angiogenesis, intussusceptive angiogenesis, co-option, and vascular mimicry. The 2 most common types of angiogenesis (*fig. 1*) are sprouting angiogenesis and intussusceptive angiogenesis.

Sprouting angiogenesis (*fig. 1*) is the most studied type of angiogenesis; this is the formation of new blood vessels from pre-existing vessels through a tip cell and stalk cells. These cells are endothelial cells with a certain behaviour, expression of certain receptors, and secretion of specific molecules. The first step in sprouting angiogenesis is degradation of the basement membrane (BM) of the vessels and detachment of the pericytes by angiogenic signalling. One of the endothelial cells becomes a tip cell whereas the other cells develop into stalk cells. The

tip cell has filopodia which function as a sensor for fibroblast growth factor in hypoxic regions. It passes the information onto the stalk cells, which proliferate in answer to the angiogenic factors, such as VEGF-A, thus forming a vessel with a lumen and basement membrane. (7, 8)

Intussusceptive angiogenesis (*fig. 1)* on the other hand has a less clear mechanism but essentially forms new blood vessels from a pre-existing one by 'splitting' the vessel through a 'pillar'. Co-option and mimicry are less important forms of angiogenesis. Angiogenesis can be upregulated under hypoxic conditions. (8)

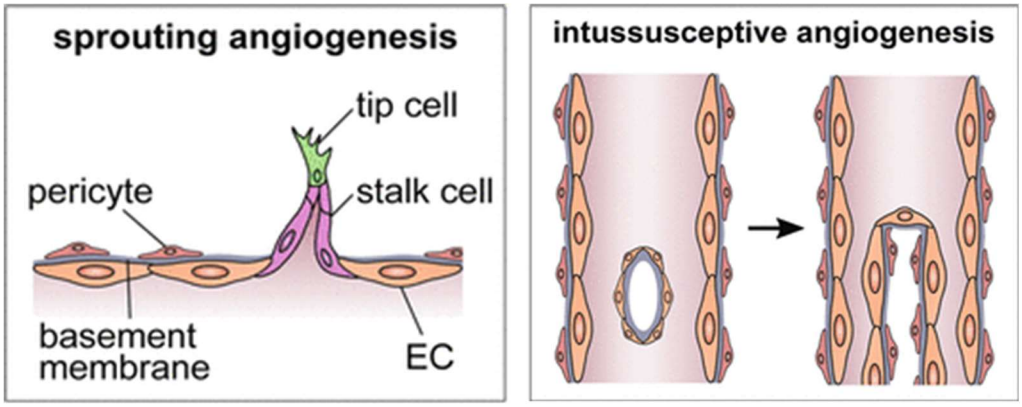

Figure 1. Two most important types of angiogenesis. Adapted from: Basic and Therapeutic Aspects of Angiogenesis Updated, 2020. (3)

1.3 Blood vessels

Mature blood vessels generally consist of 3 layers: the tunica adventitia, tunica media and tunica intima. The tunica intima is the inner layer which consists of ECs with a basement membrane and a subendothelial layer, whereas the tunica media consist of smooth muscle cells and an extracellular matrix (ECM). The tunica adventitia consists mostly of connective tissue and fibroblasts. However, these layers are not the exact same for all blood vessels: arteries generally have a thicker wall compared to veins to withstand the higher pressures. In capillaries, there is only one layer of endothelial cells with fenestrations which enable them to exchange oxygen and take up carbon dioxide and other waste products from the tissues. (8)

1.4 Adipose-derived stem cells

Another factor that has an influence on graft survival, are adipose-derived stem cells (ASCs). Adipose-derived stem cells are a specific type of mesenchymal stem cells (MSCs) harvested from adipose tissue (*fig.2)*. MSCs are readily available both in bone marrow and in adipose tissue. They have already shown to have great potential in tissue engineering and wound repair. They play a role in fat graft survival by supplying precursor cells but also because of their role in angiogenesis. (9) Besides their role in viability in fat grafts, stem cells are of great interest for tissue engineering given their self-renewable and inducible character. (5)

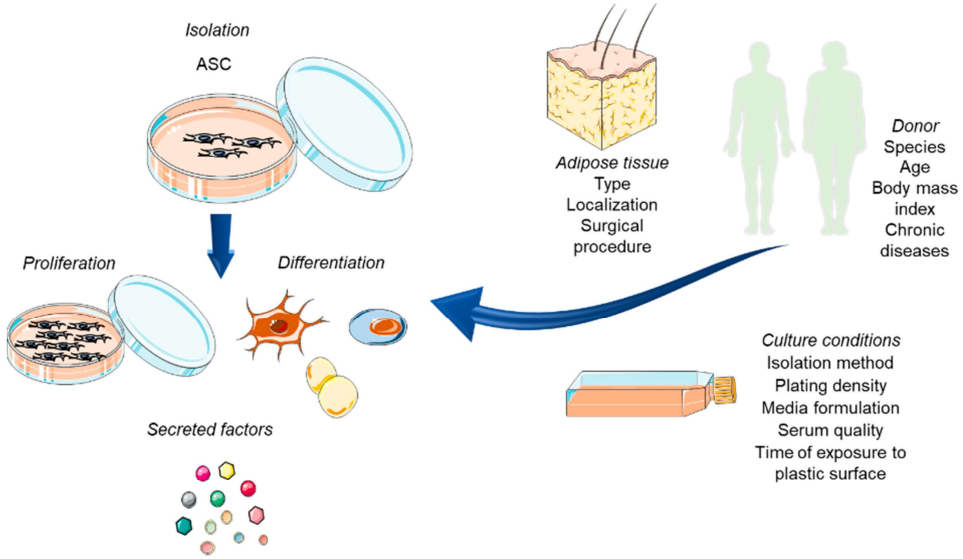

Figure 2. Isolation procedure and factors influencing properties of ASCs. From: Adipose Tissue-Derived Stem Cells: The Biologic Basis and Future Directions for Tissue Engineering, 2020. (1)

In general, there are criteria for a cell to be considered an ASC. First, it has certain surface markers. Second, as stem cells, they have the capacity for self-renewal and due to their telomerase activity, their DNA can remain stable during the different numerous cell divisions. Last, they have the ability to differentiate into 3 cell lineages: a chondrogenic lineage, an osteogenic lineage and an adipogenic lineage; differentiation occurs when the right conditions are applied. Adipose-derived stem cells are readily available in adipose tissue through harvesting during surgery. After enzymatic digestion, for example with collagenase, and centrifugation, a collection of cells is obtained which is called the stromal vascular fraction (SVF). The ASCs can be cultured from SVF by simply putting them in the right medium on a plastic adherence plate. (10, 11)

Adipose-derived stem cells can influence angiogenesis: they have both a paracrine effect to stimulate angiogenesis and can differentiate into endothelial cells, supporting vessel formation. They secrete growth factors, angiogenic factors, adipokines and neurotrophic factors and cytokines; VEGF for example is secreted under hypoxic conditions and stimulates the formation of new blood vessels. (12) ASCs have a light immunogenicity. (1, 9, 13)

Furthermore, the microenvironment is of great importance for the ASCs. This microenvironment can be influenced, a process called biomodulation, by for example lowering the O_2 concentration or genetic manipulation of the ASCs. (12) ASCs have shown to have a bigger angiogenic potential when used in co-culture with ECs compared to ASCs alone.(14) However, is still unclear how ASCs and ECs influence angiogenesis in tissue engineering with use of Gel-MA and RCPhC1-MA scaffolds. (10) Only one article investigates the effect of co-

culture of ASCs and ECs in a hydrogel; however, this is a collagen hydrogel and not Gel-MA hydrogel. (15) Before investigating the effect of ASCs, further optimization of a model has to be performed; this can be done with HT1080s, human tumor cells which are known for their high angiogenic potential. If these cells give a clear effect on vessel formation, it is clear that the model is fit to investigate the effect of cells and other factors on angiogenesis.

1.5 The extracellular matrix

The extracellular matrix is of great importance for the microenvironment. The extracellular matrix (ECM) consists of macromolecules, mostly fibrous-forming proteins such as collagen, elastin, laminins, and minerals. ECM functions as a physical support for cells as well as creating the right microenvironment for these cells to grow, differentiate, survive, and migrate. Collagen, one of the major components of the ECM, can be degraded into gelatin, a material which is much easier to handle given the lower viscosity when compared to collagen. There are RGD sequences that enable cells to adhere to gelatin. In addition to the RGD sequences, there are matrix metalloproteinases (MMP) sequences which make the gelatin easily biodegradable. (16, 17)

Gelatin can be used to form hydrogels. Hydrogels are three-dimensional (3D) networks of hydrophilic polymers that are able to absorb and retain large amounts of water. They are used in a variety of applications, such as tissue engineering; grafts should mimic the ECM as closely as possible. (18)

1.6 Hydrogels

Hydrogels can be modified with methacrylic anhydride which can result in a stable construct at body temperature following crosslinking with an appropriate photo-initiator. The crosslinking can be either physical by cooling the gel, chemical by UV irradiation after using a photo-initiator, or dual by combining both physical and chemical crosslinking. The dual crosslinking ensures that the gel stays stable at 37°C instead of becoming fluid again and thus being resorbed into the blood vessels quite quickly. Gel-MA is formed through a chain-reaction of polymerisation and is widely used in literature, assuring that the hydrogel mimics the ECM of adipose tissue as closely as possible. However, the gelatin originates from an animal source which leads to batch-to-batch variation and risk of pathogenic vectors, such as prions, and for immunogenicity, for instance anaphylactic reactions to vaccines with gelatin as a stabilizer. (19) This means that it is not fit fot clinical use.

Both problems could be solved by using a recombinant peptide based on collagen I (RCPhC1) instead of gelatin. (20) RCPhC1 is made by a yeast or by a bacterial system. The yeasts can generate recombinant gelatin by expressing collagen gene fragments of specific composition and length, thus overcoming the problem of heterogeneity. This technology makes it possible

to produce low-cost gelatin which is highly reproducible. Use of a bacterial system, such as *Escherichia Coli*, is less efficient for this purpose. (21) In research, mostly Gel-MA is investigated, while RCPhC1-MA is less investigated; however, this will be the form under which clinical use is possible. Comparisons between both gels are scarce. (20, 22)

1.7 Scaffolds

Scaffolds can be made from these hydrogels with all types of cells, for example by seeding. Scaffolds are three-dimensional constructs that help deliver cells or substances to the adipose tissue. Scaffolds can be formed from hydrogels by indirect three-dimensional printing, a technique in which a negative mould is obtained of the desired dimensions. This sacrificial mould gets filled with the material of interest, for example a hydrogel, followed by dissolving the mould. This technique has the advantage that it is possible to make more complex scaffolds in comparison with direct printing, for example for vessel formation. Furthermore, it is not necessary to bring the characteristics into account which have an effect for direct printing. The radicals formed during UV-crosslinking will not negatively affect the cells since crosslinking will be performed prior to seeding. (23)

The scaffolds should mimic the extracellular matrix of fatty tissue and give cells the opportunity to differentiate and proliferate. There is a variety of materials which can be used to form the scaffolds. Aside from the materials, other characteristics, such as the 3D structure, have to be considered. (1, 9, 13)

Currently, tissue engineered graft testing is mainly performed on nude mice, besides the *in vitro* testing. Mouse models however have some disadvantages; it is quite time-consuming and labour-intensive. Besides, some ethical arguments should be taken into consideration, such as the animals ability to feel pain. In addition, it is a costly method for testing tissue engineered grafts. (24-26)

1.8 CAM

The chorioallantoic membrane (CAM) assay is an assay to study angiogenesis that utilizes the CAM of a chick or other avian embryo. The CAM is a highly vascularized membrane and consists of endoderm, ectoderm, and mesoderm (*fig. 3*). Histologically, the upper epithelium is made of ectoderm called the chorionic membrane, the lower epithelium is made of endoderm called the allantoic membrane and the middle part is made of mesoderm, in which the capillary plexus can be found as well as the lymphatic system of the embryo, draining its waste products. The CAM grows from embryonic developmental day 3 (EDD 3) until EDD 13 and attaches to the shell from EDD 4 on. (27)

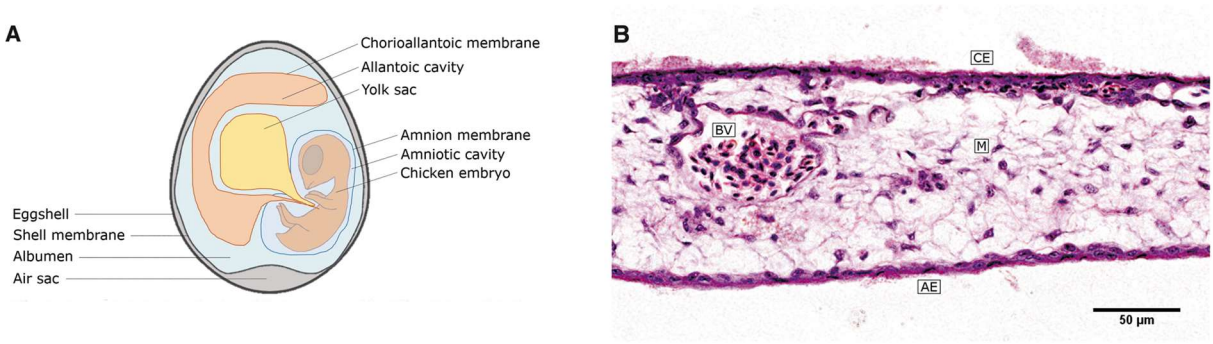

Figure 3. (A) General anatomy of fertilized chicken egg. (B) Histological view of the CAM. (CE: chorionic ectoderm, M: mesenchymal layer, AE: allantoic endoderm, BV: blood vessel). From: Chorioallantoic Membrane Assay as Model for Angiogenesis in Tissue Engineering: Focus on Stem Cells, 2020. (2)

The CAM essentially has the function of a lung for the embryo: it ensures the diffusion of oxygen, carbon dioxide, and nutrients that the embryo needs to survive. It transports calcium from the eggshell to the embryo, maintains the acid-base balance, homeostasis of water and electrolytes and the body temperature. (27)

CAM assays can be used for a variety of applications such as drug delivery systems, toxicity tests, tumour growth, and tissue engineering, which is particularly of interest. Results of the CAM assay have been compared to results of mouse models, suggesting that it is a good replacement. Given the 3R principle to replace, reduce and refine the use of laboratory animals, the CAM assay could be a suitable alternative. The main advantage when using the CAM assay instead of mouse models is the speed in which results can be obtained: it is possible to get results in 1 to 3 months with the CAM assay instead of about 3 to 6 months in mice models. (24, 25)

The formation of new blood vessels in the CAM mostly takes place between embryonic days 5 and 15. Angiogenesis usually starts on EDD 5, creating mature blood vessels with a capillary network on day 13-14. In the beginning, mainly sprouting angiogenesis takes place, later on followed by intussusceptive angiogenesis. (2, 28)

The embryo has no mature immune system of its own until EDD 15, making it very suitable to place biomaterials or human cells on the CAM. The embryo is in fact completely immunodeficient until EDD 10, thereby human cells can be implanted before the 10th embryonic day. Another advantage of the CAM model is the lower cost, simplicity and time needed to get results compared to the much more widespread mouse model. In addition, the embryos are unable to feel pain until EDD 13, strongly reducing the ethical concerns in comparison to other animal models. Moreover, this model is not considered as animal testing, strongly reducing the ethical concerns and regulation that should be taken into account. (24)

Despite these many advantages, there are still some limitations to this model. First, the CAM is not a mammalian model and so the plasma contains more water and fewer proteins as well as lipoproteins when compared to human plasma. Second, the red blood cells are nucleated. Third, since the embryos hatch at EDD 21, only limited sampling is possible, and the implantation time remains short. Last, since the membrane is already highly vascularized of its own and a high amount of angiogenesis takes place, it is hard to make a difference between a reaction to a test substance and the normal development of the membrane. (29)

Generally, there are two ways in which the CAM assay can be performed (*fig. 4*): *in ovo* and *ex ovo*. With the *in ovo* assay, the embryo stays inside the eggshell with only a small hole through which the embryo or CAM can be manipulated. The biggest advantage of *in ovo* is that there may be a higher survival rate since the embryo lives in more physiological conditions. This could be due to the calcium and phosphor the embryo obtains from its eggshell. Moreover, it has a lower risk of contamination from the outside as there is only a very small window that could possibly come into contact with dust or other contaminants. It is however harder to manipulate and visualize the CAM. Since it has such a small surface, placement of multiple testing substances on one embryo is not possible, which may lead to less reliable test results due to inter-egg variability when compared to the *ex ovo* CAM assay. This makes the *in ovo* considerably more time-consuming compared to the *ex ovo* model.

On the other hand, the *ex ovo* CAM assay, where the egg is cracked and transferred to a square weighing boat, is much easier to manipulate, allowing to test multiple substances on one embryo thus minimizing the effect of inter-egg variability. Furthermore, it is easier to visualize and quantify the ongoing angiogenesis. Since the entire CAM and embryo are exposed to the outside world, the risk of contamination is higher than for the *in ovo* assay. In addition, the conditions are less physiological compared to the *in ovo* setup, which could result in a higher mortality rate. (30, 31)

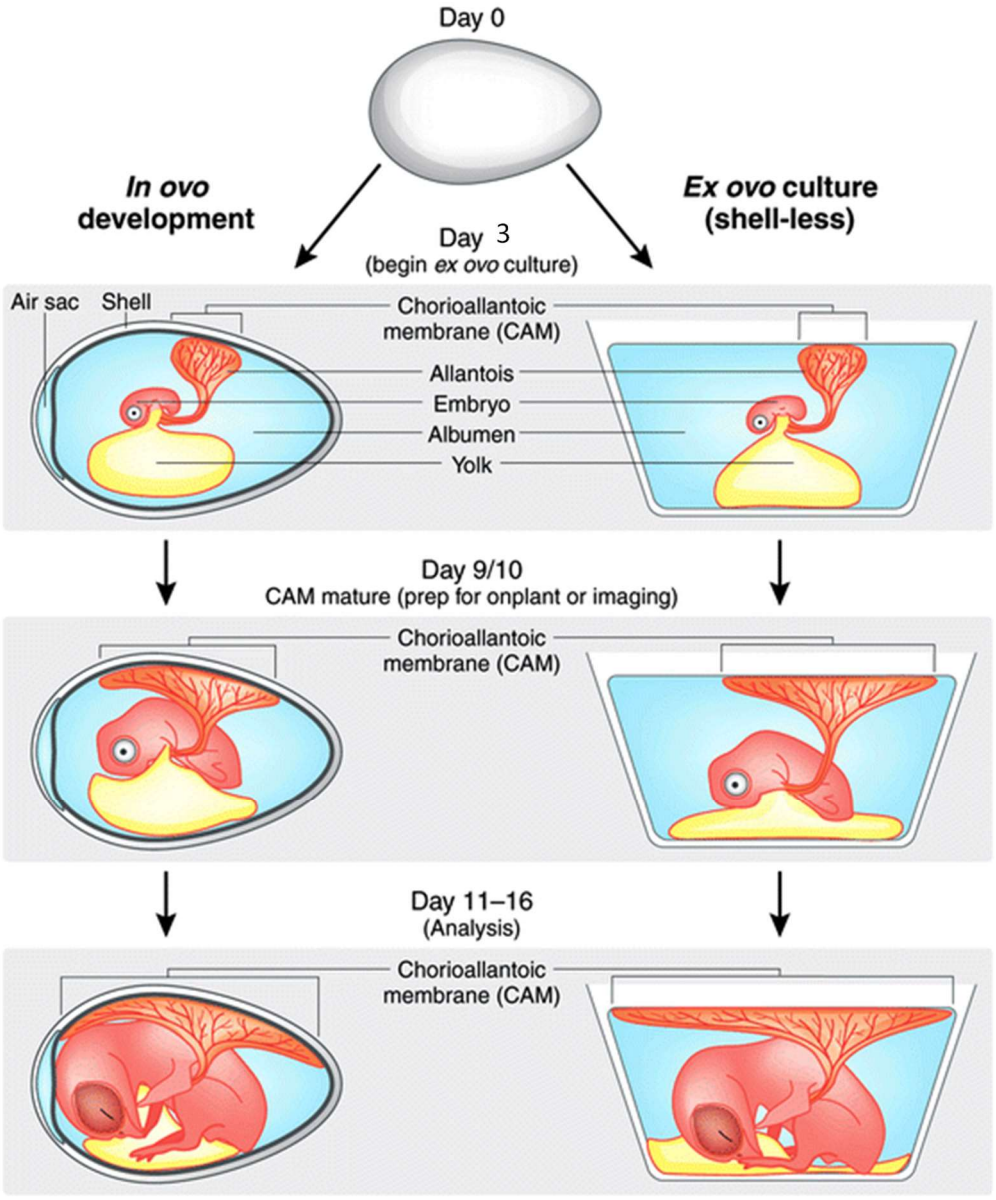

Figure 4. In ovo and ex ovo application of the chorioallantoic membrane assay. Adapted from: Visualization and Quantification of De Novo Angiogenesis in Ex Ovo Chicken Embryos, 2012. (32)

There is a variety of methods to measure the formation of blood vessels in the CAM. The first is microscopy *in vivo*, where vessels, branching points, length of the vessels, vessel density or a combination of these factors can be measured. The quality of the blood vessels can be assessed on a scale from 0 to 4 or simply quantified by counting the vessels. To make a difference between new vessels and pre-existing vessels, it is useful to compare pictures before application to pictures after application. There are several options to test the angiogenic potential of biomaterials. The first is to place the biomaterial on top of a double mesh on the CAM. The vessels growing on top of the mesh are new vessels. This can make it easier to quantify the number of vessels by comparing the number of squares containing vessels to the total number of squares. Additionally, it is possible to simply place the biomaterial on top of the

CAM inside a silicone ring. This facilitates locating the biomaterial since it can move along with the movements of the embryo. The counting can be done manually or with the help of a software program, which can make the process more time-efficient and accurate. Another widely used method to measure angiogenesis is histology, where a simple haematoxylin/eosin staining as well as immunohistochemistry can be used. Furthermore, it is possible to use more advanced techniques, such as X-rays, MRI with gadolinium-contrast, dual staining, FITC, 2-photon-microscopy and RNA analysis. (2, 33, 34)

The CAM assay has already been used widely in tissue engineering research for a variety of biomaterials, such as sponges and gels. However, the use of a gelatin and recombinant gelatin hydrogel on the CAM has not yet been studied sufficiently and so the CAM assay has not been optimized for this use yet. (25)

Former research has shown that the gelatin gels cannot be placed onto the CAM as such, as can be done for collagen gels. The gelatin hydrogels degraded and could not be found anymore after a couple of days on the CAM. This is why scaffolds were printed from the hydrogels. The scaffolds contain pores to ensure that interaction with the CAM was possible, and given the importance of 3D-structure in scaffolds and tissue engineering. (22)

In conclusion, there is a need for a cost- and time-efficient, ethical alternative to the classically used mouse model to investigate angiogenesis and viability in adipose tissue engineering. The CAM assay has already been used in numerous research papers investigating solid biomaterials. However, it has not yet been used to investigate angiogenesis in scaffolds from hydrogels. In this paper, the optimal setup of the CAM assay for this application is investigated.

2 Materials and methods

2.1 CAM

Broiler eggs from a local hatchery were disinfected with povidone iodine and placed horizontally for 15 min to place the embryo on top. They were then opened at EDD 3 with a Dremel tool and either a small hole was made at the top of the egg for the *in ovo* method or the contents were transferred into square weighing boats for the *ex ovo* method *(fig. 5).* Weighing boats were covered with a square petri dish as lid and small holes were covered with tape to avoid drying. They were placed in the incubator at 37.8°C and 76% humidity in a cardboard holder in a horizontal position for the *in ovo* method or in boxes per 16 weigh boats for the *ex ovo* method. After an additional 6 days of incubation, at EDD 9, the scaffolds were implanted onto the CAM. (22)

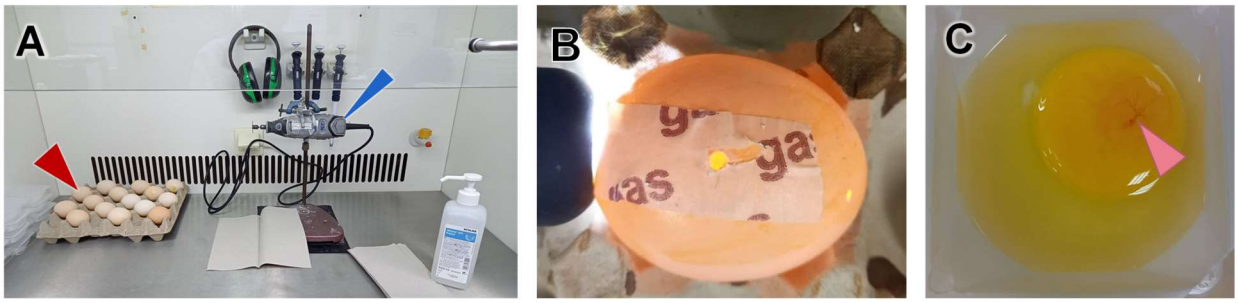

Figure 5. (A) Setup for opening of the eggs. (Red arrow: broiler eggs, blue arrow: Dremel tool). (B) In ovo EDD3 opening of the egg: small hole on top of the egg. (C) Ex ovo EDD 3 after opening of the egg. (Pink arrow: embryo with surrounding CAM).

2.1.1 Scaffolds

Both RCPhC1-MA and Gel-MA were made according to the protocol by Van Den Bulcke et al (35). In brief, 1g of RCPhC1 (Cellnest™, Fujifilm) or gelatin type B was dissolved in 10 mL of a 0.1 M phosphate buffer (pH 7.8) at 40°C. Methacrylic anhydride (2.5 equivalents) was added relative to the amount of amines in RCPhC1 (66.4 mmol amines/100g) and gelatin (38.5 mmol amines/100g). This solution was diluted with 10 mL of double distilled water and dialyzed against double distilled water for 24h to remove any methacrylic acid or unreacted methacrylic anhydride. Subsequently, the obtained RCPhC1-MA and Gel-MA were frozen (-20°C) and lyophilized (-80°C, 0.37 mbar, Christ freeze-dryer alpha I-5). The degree of substitution was determined using ^{1}H-NMR spectroscopy at 40°C with D_2O in a Bruker WH 500 MHz NMR spectrometer.

Moulds were printed with a three-dimensional printer in polylactic acid (PLA). They were designed with a Standard Tessellation Language Computed-Aided Design (STL CAD) based on a negative mould of the targeted scaffolds, printed with the Cura 13.06.4 software at 210°C with a speed of 11 m/s. These moulds were printed with the Ultimaker 3 (Ultimaker, Gerldermalsen, The Netherlands).

The scaffolds were obtained by dissolving one gram of either RCPhC1-MA or Gel-MA in 10 mL of double distilled water at 40°C to create a 10% (w/V) mixture. Next, Li-TPO-L, a photoinitiator (2 mol% respective to amount of methacrylic amide) was added. The moulds were submersed in the mixture under vacuum conditions for 1h to enable sufficient intrusion inside the pores. Afterwards, they were placed in the fridge for 15 min prior to exposure to UV-A light for 1.5h allowing both physical and chemical crosslinking respectively. The scaffolds were then transferred to a flask with chloroform to dissolve the PLA moulds. Sterilization was done with submersion in ethanol 70% for 24h. Ethanol was refreshed after 12h and the scaffolds were placed under UV-C light for 30 minutes. The scaffolds were rinsed with PBS prior to seeding the cells. Half of these scaffolds were seeded with 100.000 HT1080s/scaffold. Cells were cultured in basic culture medium, containing Dulbecco's modified eagle medium (DMEM), 10% FBS, 1% penicillin/streptomycin.

2.1.2 Setup first experiment

Two experiments were done; in the first experiment, the scaffolds as such were placed onto the CAM, whereas in the second experiment a closed construct with scaffolds was used.

For the *ex ovo* CAMs, 4 scaffolds were placed on top of the CAM, and for the *in ovo* CAMs, only one scaffold per CAM was implanted on embryonic developmental day 9 *(fig. 6)*. There were 8 groups in total for both *in ovo* and *ex ovo* experiments: RCPhC1-MA or Gel-MA scaffolds, each with or without HT1080 cells, either on top of a nylon mesh or inside a silicone ring respectively.

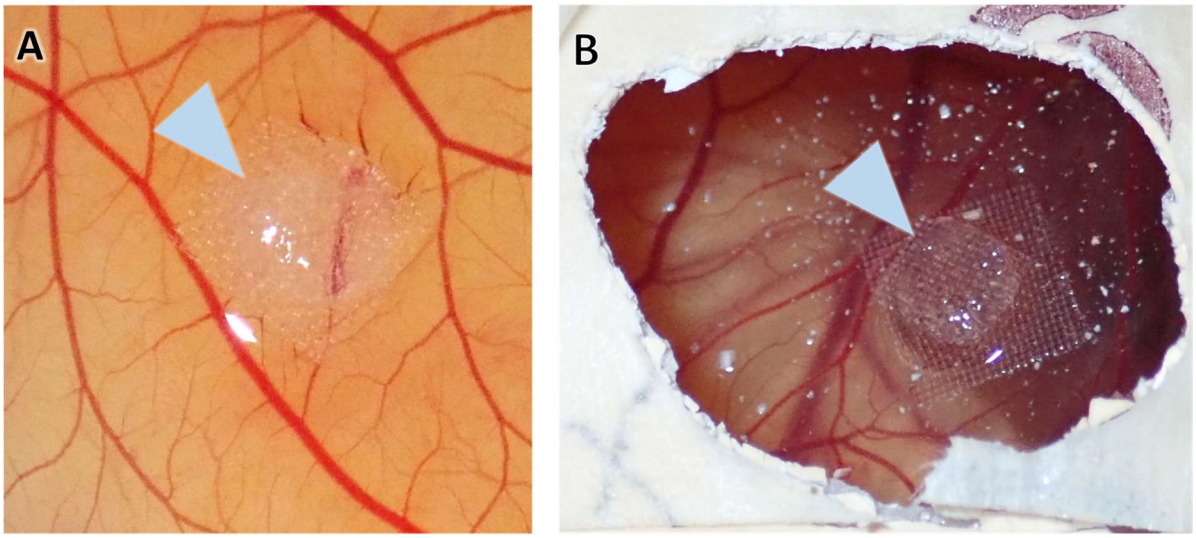

Figure 6. (A) Implant of a scaffold (blue arrow) ex ovo, on top of a double-layered mesh. (B) Implant of scaffold (blue arrow) in ovo, on top of a double-layered mesh.

Every day from EDD 12 to 15, visualization was performed with a stereomicroscope *in vivo* (Axio.zoom V16, Zeiss) in the bright field and DAPI channel (excitation 358nm, emission 463nm) for proper assessment of blood vessels. Bright field images showed information about

the structure of the scaffolds. Pictures in the DAPI channel visualized the blood vessels clearly, making them darker than the background. On EDD15, the embryos were decapitated, and the implants were collected for histology.

Constructs

Cylindrical constructs *(fig. 7)* were made based on a protocol by Kilarski et al. (36) Plastic drinking straws (diameter 3 mm) and three-dimensional hollow PLA cylinders (diameters 3 mm and 5 mm) were attached to a mesh of 15x15 mm with cyanoacrylate. The mesh made it possible to differentiate between pre-existing and new vessels and divided the weight of the construct evenly. Then, the scaffolds were gently inserted into the cylinders onto the mesh. A few drops of PBS buffer with antibiotics were added and sunflower oil was added on top of this to create a sterile and closed environment. This was then implanted onto the CAMs on EDD 9. On EDD 14 or 15, whole eggs were fixed with Zn-fixer (ZSF) for 1h after live-imaging with FITC-dextran and injection with Indian ink. The constructs were implanted on EDD 9 and visualized on either EDD 14 or EDD 15.

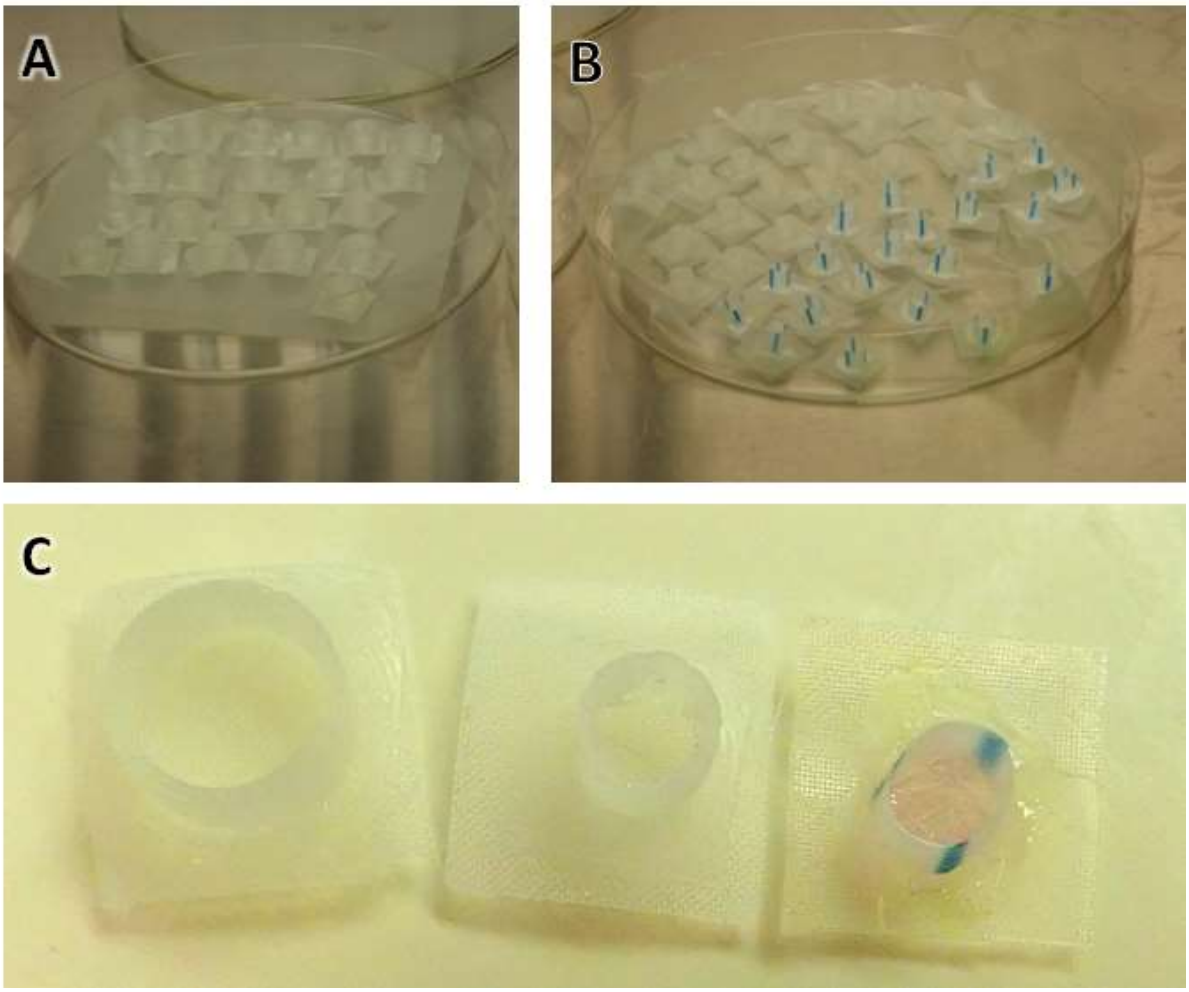

Figure 7. 3D-printed constructs. (A) Large 3D printed constructs. (B) Small 3D-printed constructs (top) and drinking straw constructs (bottom). (C) Top view of the constructs. From left to right: large 3D printed construct, small 3D printed construct, and plastic drinking straw construct.

2.1.3 Setup second experiment

For the second experiment, constructs were placed on the *ex ovo* CAMs, with 1 construct per CAM, on EDD 9 *(fig. 8)*.

There were 6 groups (for each group: n=10), in which the following conditions were tested: a plastic drinking straw (3 mm diameter) and two 3D printed hollow cylindrical constructs with each a different diameter (3 mm and 5 mm diameter). Only Gel-MA scaffolds were used in these constructs. These constructs contained either scaffolds without cells or scaffolds with 100.000 angiogenic HT1080s/scaffolds.

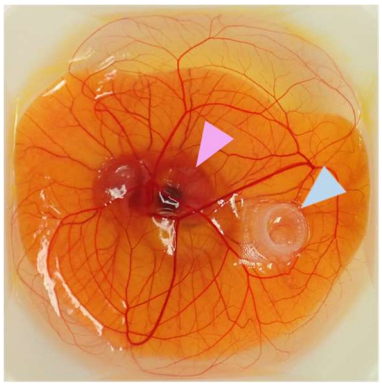

Figure 8. Placement of a large 3D construct on the CAM. (Pink arrow: embryo, blue arrow: 5 mm 3D-printed construct).

On EDD 15, a combination of DAB stain (diaminobenzidine; PBS, diaminobenzidine, 0.3% H_2O_2) and intravenous injection of Indian ink (0.5 mL, colloidal carbon) was performed; ink was injected before Zn-fixation. DAB stained for peroxidase, colouring blood-containing structures, while the ink coloured the functional blood vessels. By comparing ink-staining with DAB-staining, it was possible to differentiate between actively perfused vessels (ink) and vessels with no or low perfusion (DAB). Subsequently, the constructs were treated with cedarwood oil to clear the implant tissues.

2.2 Histology

The implants were collected for histology by cutting out the scaffolds and attached CAM.

This was briefly rinsed with PBS to remove residual blood, fixated in paraformaldehyde 3.5% and dehydrated in degraded alcohol baths (alcohol 70% for 3h, 80% for 1.5h, alcohol 90% for 4.5h and isopropyl for 4.5h). After clearing with xylene for 4.5h, they were embedded in paraffin. Samples were systematically cut and tissue sections of 8 µm were made with an interval of 500 µm. Pictures were taken with and analysed with imageJ focusing on following characteristics: surface scaffold, surface CAM, number of vessels, number of vessels/surface CAM.

2.3 Statistics

SPSS Statistics 27 was used to conduct statistic tests. The Man-Whitney-U test was used to compare means between groups. The Chi-square (χ^2) test was used to compare groups for categorical variables. When requirements (<20% of the cells have a value <5) for the chi-square test were not met, Fisher's exact test was performed. For the effect on vessel formation, deceased embryos were excluded to rule mortality out as a confounding factor.

3 Results

3.1 Optimization of the model

First, the *in ovo* method was compared to the *ex ovo* method using histological pictures. Both total surface area, consisting of remaining gel and newly formed tissue, and tissue surface area had non-significant results ($p>0.05$). Number of vessels as well as surface tissue were slightly higher for the *ex ovo* method. Number of vessels per surface tissue was at the same ratio, as well as ratio of large vessels per surface tissue. A cut-off of 50 µm diameter was used to define 'large' vessels, based on visible differences in vessels. Surface scaffolds was slightly higher for the *in ovo* method. All of these were statistically non-significant results ($p>0.05$). Mortality both *in ovo* and *ex ovo* was at 20%.

The use of a silicone ring was compared to a double-layered mesh for locating the scaffolds, which were able to move on the CAM. Quantitative analysis of the double-layered mesh was not possible due to the scaffolds slipping off the meshes and moving on the CAM, which made it impossible to locate them and count the vessels and surface accurately; additionally, the meshes interfered with counting vessels and surface. Furthermore, the scaffolds are not transparent making *in vivo* counting of the vessels impossible as well.

Histology showed that there was only little interaction of the CAM with the scaffolds and could not confirm whether the cells did really grow into the scaffolds or that they migrated into the pores. The scaffold was more encapsulated as such rather than real interaction taking place *(fig. 9).* The surface of some scaffolds tended to dry out at the side exposed to the air in the humid incubator (*fig. 10*), while other scaffolds seemed to liquefy, making it harder to locate the scaffolds. The drying out made performing histology more difficult. A closed system was designed in another experiment.

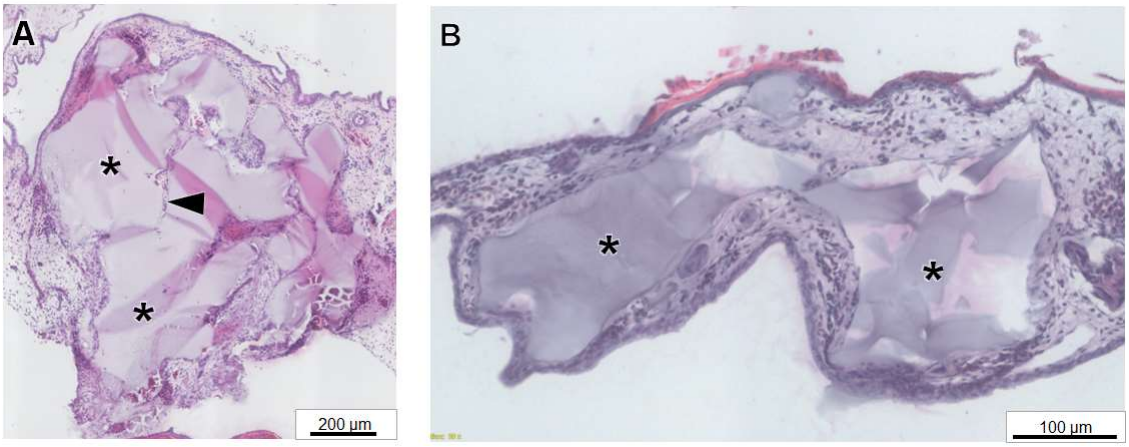

Figure 9. (A) Some cells interact with the scaffold. It is unclear whether these cells are HT1080s or interaction with CAM. (B) Gel stays intact and does not really interact with the CAM. (Black arrow: cells. Asterisk (*): scaffold).

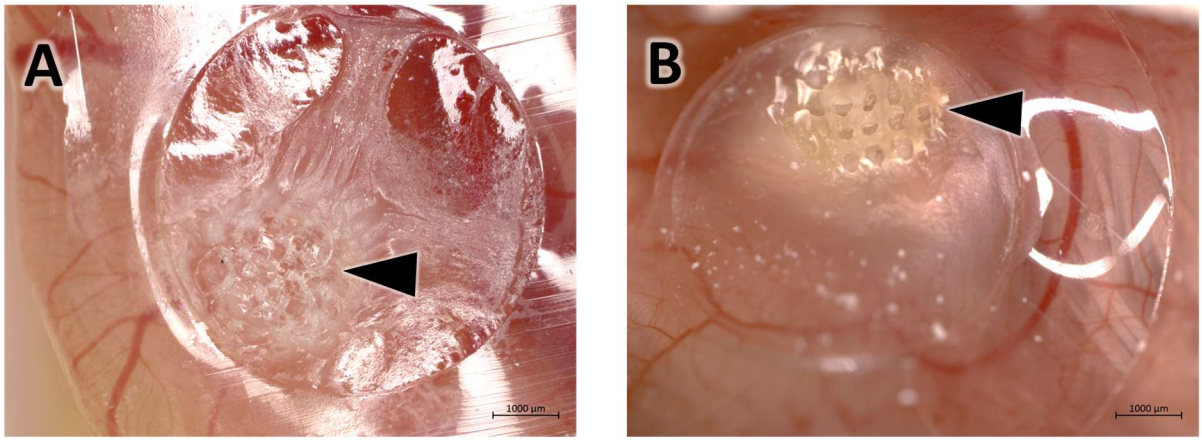

Figure 10. Drying out of the scaffolds. (A) Scaffold (black arrow) without drying out. (B) Scaffold (black arrow) drying out.

The variables in the closed construct were evaluated. The influence of the type of construct on viability was investigated. For the link between the distinct types of constructs and embryo viability, p=0.035 was found, which indicates that there is a statistically significant ($p<0.05$; χ^2) difference in viability between the types, showing highest mortality in the drinking straw, followed by the large 3D-printed construct *(fig. 11)*.

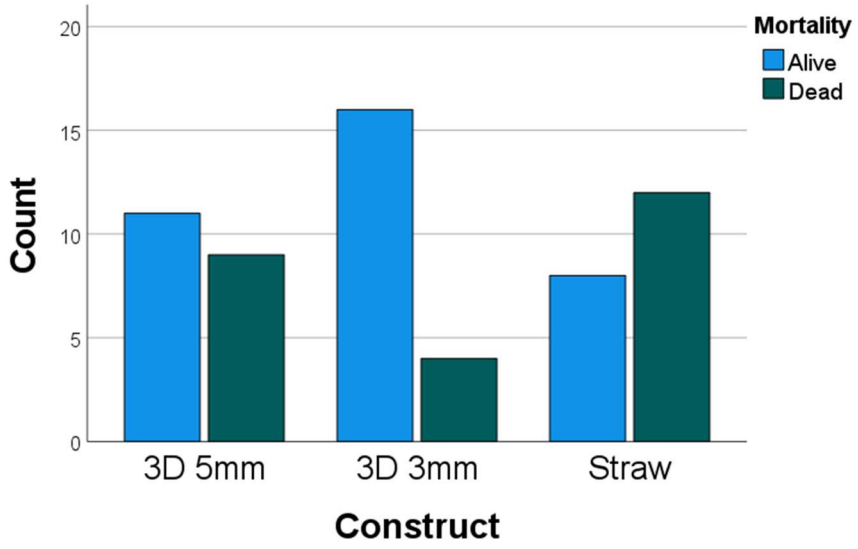

Figure 11. Mortality compared between the distinct types of constructs.

Next, crooking of the scaffolds could be noticed in some constructs. Moreover, the crooking of the constructs took place more frequently for one of the types of constructs. This gave a statistically significant result with $p<0.05$ (χ^2). Crooking was most frequent with the straw construct. The 3D-printed smaller construct crooked more frequently than the larger 3D-printed construct. Moreover, some sinking into the CAM of the constructs could be noticed.

Blood vessels could be identified as black structures on the pictures of the scaffolds, taken out of the constructs after ink injection *(fig. 12)*. DAB gave mainly artifacts and was not used to

identify vessels. Histology could not confirm the presence of blood vessels. No cell growth inside the gels could be identified with certainty. Some interaction can be seen with the scaffolds, mainly on one side of the scaffold. The scaffold did conserve its shape with pores quite closely. Moreover, some artifacts can be seen on histology.

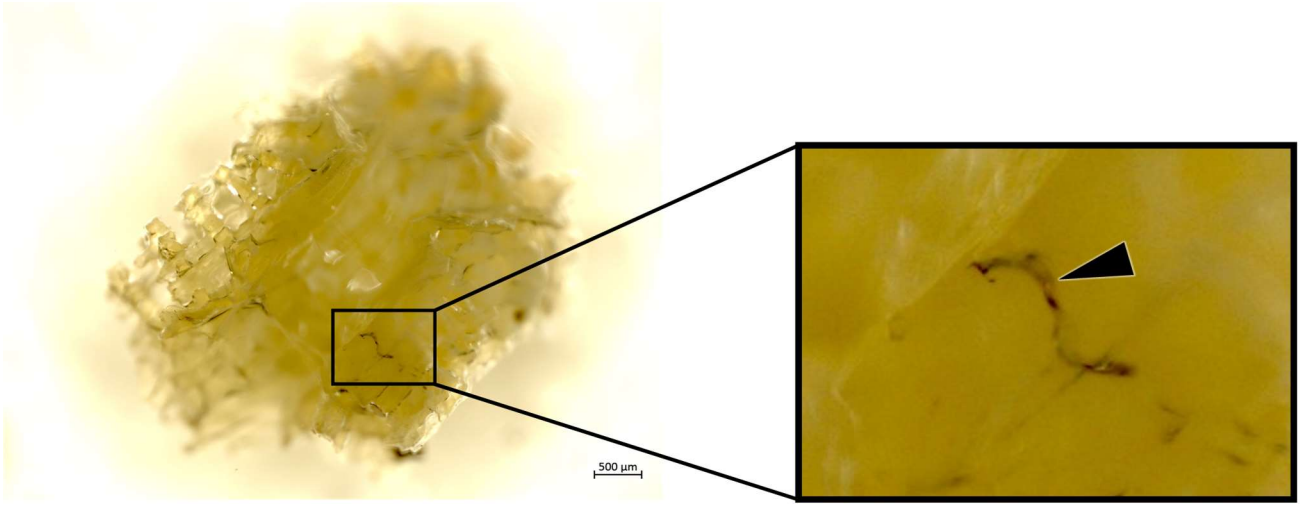

Figure 12. Blood vessels can be identified due to ink injection. Left: overview of scaffold with blood vessels. Right: same scaffold, zoomed in on a blood vessel (black arrow).

The deceased embryos were excluded from the statistics of the second experiment regarding vessel formation to rule mortality out as a confounding factor; only 35 embryos were included for the statistics regarding blood vessels.

3.2 Experimental section

Additionally, a comparison between histological sections of Gel-MA and RCPhC1-MA was made in the first experiment. The tissue surface was slightly higher for Gel-MA compared to RCPhC1-MA, but no statistically significant difference was found. For the scaffold surface, no significant difference was found ($p>0.05$, *fig. 13*). Likewise, no significant result was found for the total surface area. No difference was observed when the percentage of vessels per surface tissue nor when the percentage of large vessels per surface tissue were compared between Gel-MA and RCPhC1-MA. Both gave a statistically non-significant result with $p>0.05$.

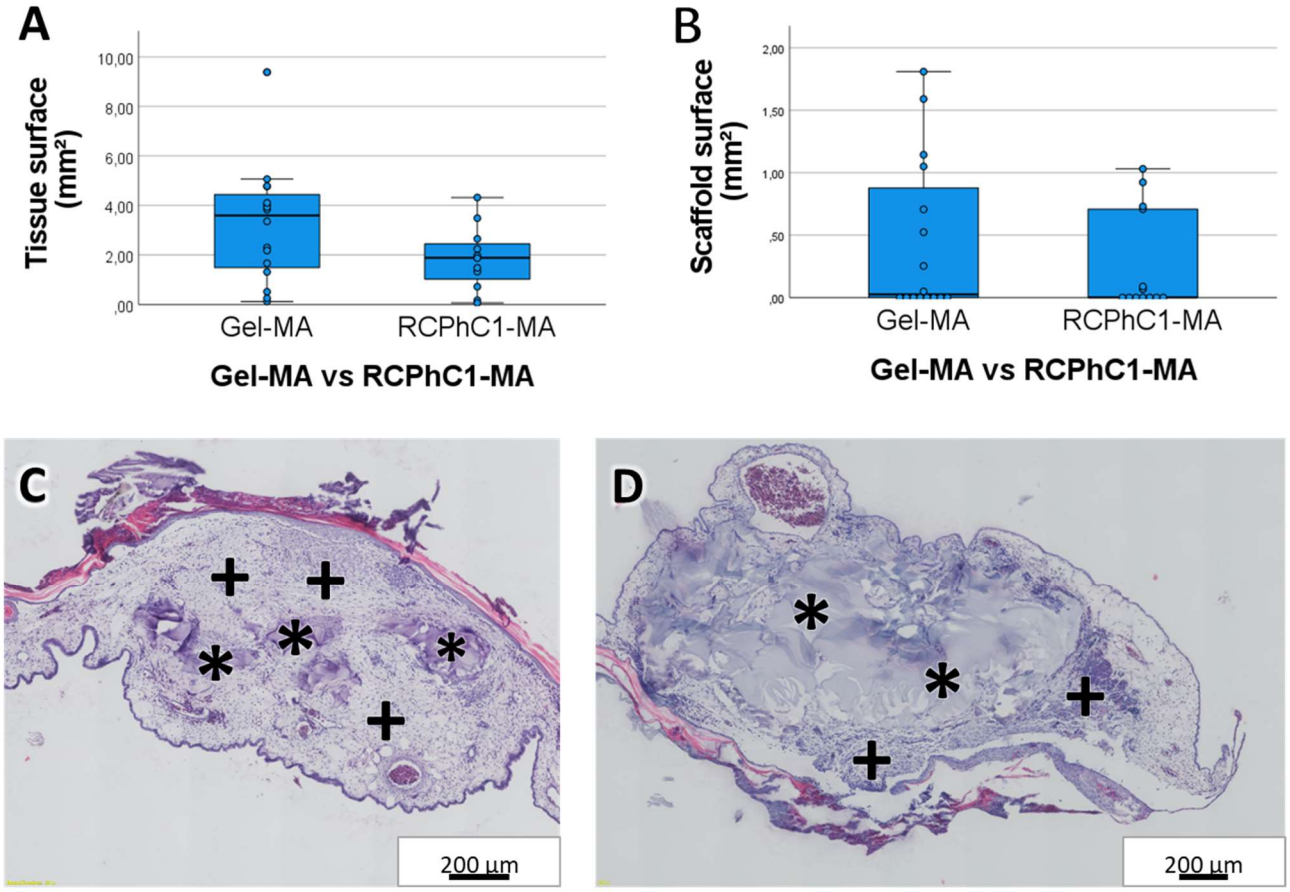

Figure 13. Comparison of Gel-MA with RCPhC1-MA. (A) Boxplot of comparison of tissue surface. (B) Boxplot of comparison of scaffold surface. (C) Histological picture of Gel-MA scaffold in CAM-tissue. (D) Histological picture of RCPhC1-MA scaffold in CAM tissue. (Plus (+): tissue, asterisk (*): scaffold).

Additionally, a comparison regarding the presence of cells was made in the first experiment. No statistically significant difference was found ($p>0.05$) when comparing total surface area, surface scaffold and surface tissue. Surface tissue seemed slightly higher in absence of cells than with the HT1080s (*fig. 14*). Number of vessels, number of vessels per surface tissue (*fig. 14*) and number of large vessels per surface tissue compared with and without cells was non-significant as well. Presence of cells did not influence the embryo mortality.

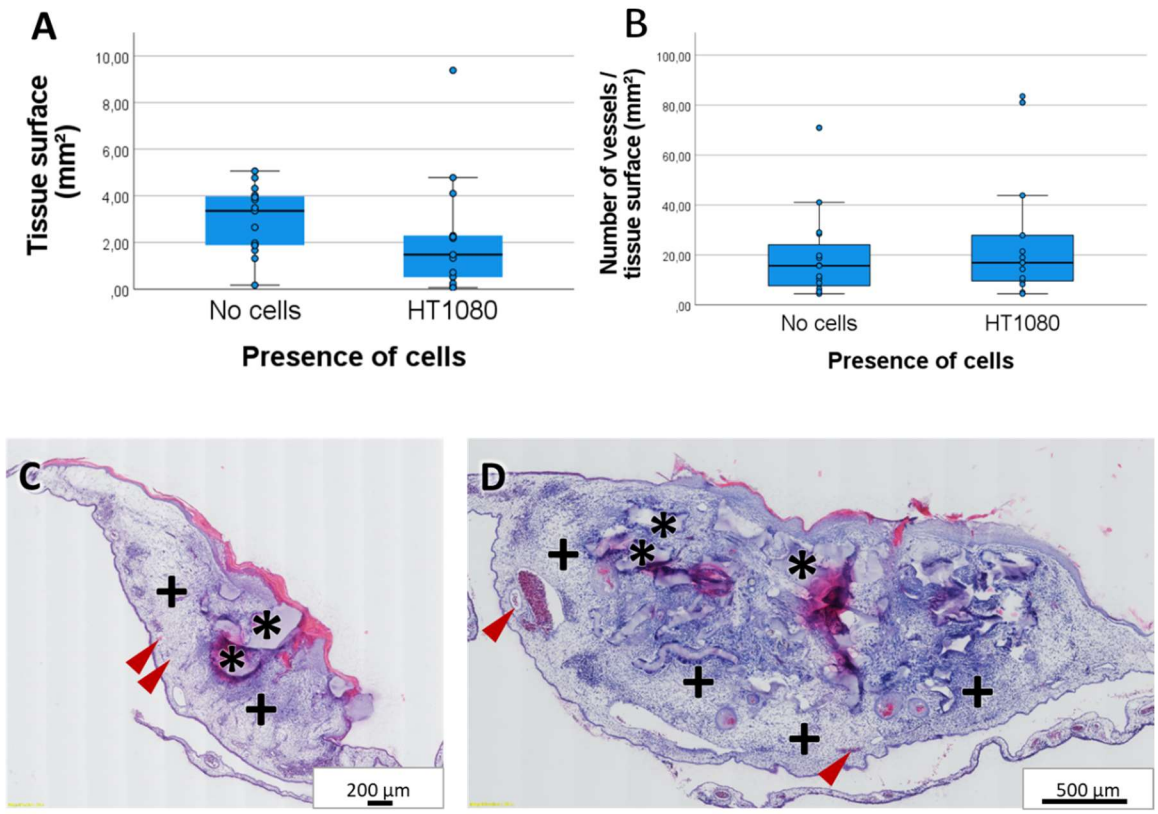

Figure 14. Comparison of scaffolds with and without cells. (A) Boxplot of comparison of tissue surface. (B) Boxplot of comparison of scaffold surface. (C) Histological picture of a scaffold in tissue without cells. (D) Histological picture of scaffold in tissue with HT1080s. (Red arrows: vessels, plus (+): tissue, asterisk (*): scaffold).

Moreover, the link between the presence of cells and the number of blood vessels was explored in the second experiment. This gave a non-significant statistic result with p>0.05 (Fisher's exact). Minor differences can be seen with a slightly higher number of blood vessels in presence of HT1080 when compared to absence of HT1080s in the bar chart *(fig. 15)*. Additionally, the link between the type of construct and the number of blood vessels was investigated. This gave a non-significant statistical result (p>0.05, Fisher's exact).

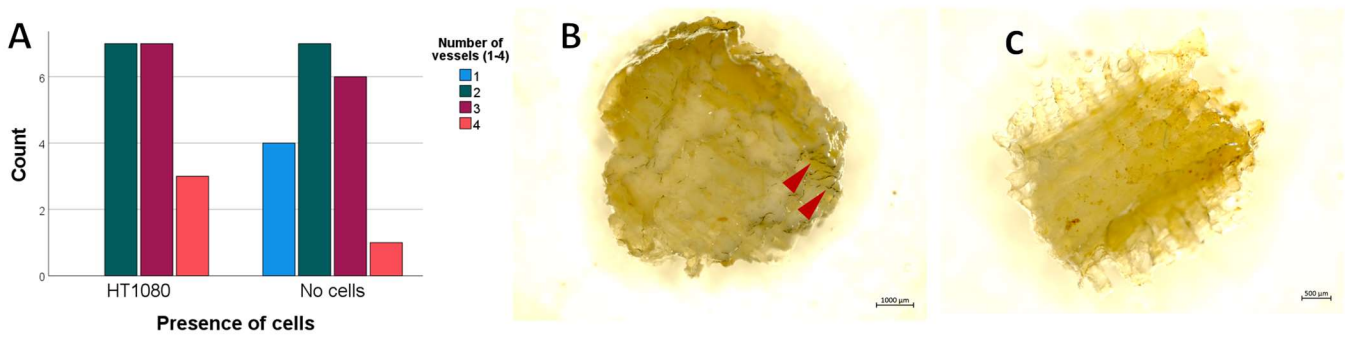

Figure 15. (A) Bar chart: number of vessels compared with and without HT1080s. A small difference can be noticed. (B) Picture of scaffold with HT1080s. Vessels (red arrows) can be identified. (C) Scaffold without HT1080s. No vessels can be identified.

4 Discussion

4.1 Optimization of the model

In the first experiment, no statistically significant difference was found between the *in ovo* and *ex ovo* method for the CAM assay regarding total surface area, percentage of new tissue, blood vessels, and large blood vessels. As a result, both methods of performing the CAM assay are equally valuable but given the ease of performing the *ex ovo* method regarding time efficiency and visualization, this method is preferred in further research.

When comparing the double-layered mesh with silicone rings, which are used to locate the scaffolds on the CAM in the first experiment, it appeared quite clearly that the double-layered meshes are less fit for this use, since the scaffolds are not transparent. This makes it impossible to see the vessels as can be done for other experimental set-ups, as described by Zijlstra. (32) In addition, the different layers of the nylon mesh tend to shift and do not stay in place as expected and interfered with counting of the blood vessels. This could be because the gel does not really permeate between the meshes. This is in contrast with the method of Zijlstra, where the meshes are embedded within a collagen gel, making them stick onto each other thus making it impossible to shift. (32) The silicone ring proved to be a valuable alternative to the double-layered nylon mesh for locating scaffolds, and non-transparent materials in general. However, when using a closed construct, it is not necessary to use an extra method to locate the scaffolds.

In the first experiment, we observed that the scaffolds seemed to dry out *(fig. 10)*, even in a humid incubator. This might be because they only touch the chorioallantoic membrane on one side leaving the other side exposed to the air in the incubator. They were also taken out of the incubator each day from EDD12-15 for microscopic examination, exposing both the embryos and the scaffolds to the outside air for several minutes.

To overcome the drawback of the scaffolds drying out and the difficulties locating the scaffolds after a few days on the CAM, a closed construct was used in another experiment so the scaffolds were not exposed to the outside air and could be found easily due to their placement inside the construct. In this experiment, only the *ex ovo* method was used given the ease of manipulation and visualization, the larger surface that is available for manipulation and since results are equally performant as the *in ovo* method, as seen in the first experiment. Since no difference could be found between both materials out of which the scaffolds were printed, only the Gel-MA scaffolds were used given their ease and lower costs of the material. These scaffolds did not dry out and seemed to liquefy less when compared to the open system. Furthermore, more interaction with the CAM was seen.

We also investigated the factors that could influence embryo mortality were investigated. Presence of cells showed no statistically significant difference in mortality of the embryos. This indicates that the cells had no adverse effect on the embryos. However, the type of construct did show an effect on the mortality; the plastic drinking straws gave the highest mortality, followed by the 3D-printed larger constructs. The slightly higher mortality in the larger constructs could be explained by the higher weight or larger size, possibly causing damage and bleeding of the chorioallantoic membrane and thus damaging the embryo. For the higher mortality in the drinking straws, the material of the straws could be a possible explanation, as it was not easy to get an even surface to place onto the CAM. The lowest mortality occurred in the smaller 3D-printed construct. This makes it a good method for investigating scaffolds in tissue engineering research. However, since the effects in fat tissue and tissue engineering are mainly of interest, a larger construct would be more feasible.

The constructs did seem quite heavy for the CAM, causing it to sink into it and damaging it. This was especially the case for the larger 3D-printed constructs. This problem could possibly be solved by using another material to print the constructs or by using a bigger size of mesh to divide the weight more evenly, or by a combination of both options. Furthermore, making smaller constructs could be a possibility but is for this research purpose less fit, since our major interest is to know what happens inside the scaffolds.

An additional disadvantage resulting from the weight and larger mesh is that placement of only one construct per embryo is possible. Moreover, the smaller scaffolds seemed to curl up and placement of smaller scaffolds into the plastic drinking straws and smaller 3D constructs were suboptimal, causing the scaffolds to crook. Hence, there should be investigated a way to make lighter constructs by using another material to print the construct. Another feasible solution for the damaging of the proportionally too high weight of the scaffolds, can be using another type of avian embryo, which is larger, to make it easier to manipulate and give a possibility to use larger constructs without damaging the embryo or to give the scaffolds more time on the CAM; for example duck or turkey embryos. Nevertheless, the chick embryo is more available in Belgium since only one hatchery for turkeys exists. (37)

A link between the type of construct and crooking of the scaffold could be found. This was highest in drinking straws, followed by the smaller 3D-printed construct which was then also higher than the larger 3D-printed constructs. We hypothesize that this is the effect of the smaller size of the constructs and scaffold, making it harder to place the scaffolds in the constructs in the correct way, which is the placement of the scaffold horizontally on top of the mesh.

The type of construct gave a non-statistical significant result regarding its effect on the number of blood vessels, and no difference could be seen across the groups. This indicates that, after exclusion of the deceased embryos, the distinct types of constructs did not differ. However, since the plastic drinking straws and the larger 3D-printed constructs gave a higher mortality, the smaller 3D-printed construct gave the best results.

On day 15, a histological analysis was performed. Cells could be identified but seemed to migrate into the pores and not really inside the scaffold itself. There is very little interaction between the scaffold and the CAM however this interaction can be mainly seen at the surface of the scaffolds. It might be better to do implantation of the materials earlier in the embryonic development so there is more time for the CAM to interact with the materials.

Histology was much easier to perform in the closed constructs when compared with the free scaffolds of the first experiment; since less degradation took place, the form of the scaffolds was conserved quite well, and the scaffolds were easier to locate. Histology was able to confirm that the structure of the scaffold with its pores was conserved quite closely. The general image of histology was one of the scaffolds with their pores, some artifacts, and some cells which showed some interaction with the scaffolds, mainly on one side, the bottom of the scaffold. Again, the cells did not invade into the scaffolds but only migrated into the pores. No blood vessels could be identified with certainty. Hence, we could not confirm that the black structures were in fact blood vessels and not artifacts or shadows in the pores. This could be due to the various manipulations of the tissue with ZSF, cedarwood oil clearing, Indian ink injection, and the histology techniques. It might be helpful to use another fixative instead of ZSF, as described in the original article (36), to make the cutting of the samples easier and give more reliable results. Additionally, it might be helpful to use another clearing, for example the one as described in the original article (36), instead of cedarwood oil. Besides, the whole-mount in DAB for 20 min seemed to give mainly artifacts on the scaffold and so optimization of the mounting time should be performed. These variables should still be judged for their effects on the scaffolds and on the tissue. Moreover, it could be a possibility that the blood vessels were damaged when removing them from the CAM, thus ripping them out of the scaffolds. Alternatively, the vessels could be stained using immunohistochemistry, for example staining against CD31; thus staining the endothelial cells of the blood vessels. This is however a more expensive alternative to ink injection and histology.

4.2 Experimental section

Our major focus was to investigate whether the presence of cells would affect the amount of blood vessels and which construct is best for investigating this effect. The deceased embryos were excluded, which means only 35 embryos could be included across the 6 groups. Both

variables gave a non-significant result. However, a small difference can be noticed when comparing the blood vessels in presence and absence of HT1080s *(fig. 14).* In the presence of HT1080s, a slightly higher number of vessels can be seen, which is in accordance to literature (22, 32). This could be due to the small sample sizes, and the experiment should be repeated with larger sample sizes to confirm whether this result is based on a coincidence or by a cause-and-effect relation. If the difference results from a cause-and-effect relation, this indicates that the model might be fit for testing effects of cells added to the scaffolds and other factors on the formation of new blood vessels, a major goal in tissue engineering research. This model could be a promising tool, as well as a feasible alternative to mouse experiments, following the 3R principle regarding animal experiments. It is however important to note that the presence of vessels inside the scaffolds could not be confirmed by histology. Visualization *in vivo* of the blood vessels in the embryo could be possible. This was attempted with TRITC-dextran injection; however, this is very time consuming and when performed by untrained researchers often unsuccessful, hence why this was not performed in this research. A possible solution for visualization could be immunohistochemistry, as already mentioned above.

Additionally, scaffolds printed from Gel-MA were compared with scaffolds printed from RCPhC1-MA and no statistically significant difference was found between the materials from which the scaffolds were printed regarding total surface area, tissue surface, blood vessels, and large blood vessels. As a result, it is possible to choose between both materials, given the fact that in reality RCPhC1 is more feasible to use in real life clinics. However, it may be a possibility to use Gel-MA in experiments since this is more cost-efficient. Moreover, it might be possible that the similarities result from the at the time unoptimized model.

Further research should look into the different variables, optimizing the clearing agent and clearing time, ink injection, DAB whole-mount time, and fixation. Therefore, comparing scaffolds without any clearing or ink to scaffolds with these manipulations could particularly be useful. Further optimization of fixation method could also be useful.

Another important variable that still needs optimization is the weight and size of the constructs, for example by using a lighter material than PLA, by using a larger mesh to divide the weight over a larger surface causing minimal damage and pressure to the CAM, or by using another avian type with a larger and stronger CAM.

This research shows that the CAM assay can thus be a valuable tool in tissue engineering research, and more specifically in testing blood vessel formation in scaffolds printed from hydrogels. It is a fast and cheap assay, which can help us to further replace, reduce, and refine the use of laboratory animals in research. Some disadvantages will have to be overcome, such as the visualization of the vessels *in vivo*, the differentiation between the already highly

vascularized CAM and the newly formed vessels, and the fact that there are some minor differences when compared to mammalian species. Moreover, the materials can be observed during only a couple of days to maximum one week, while the materials in tissue engineering should stay intact for much longer in the human body, and thus the assay is less fit to investigate important factors such as degradation and inflammation over a longer period of time.

Limitations to this research are mainly the small sample sizes and variety of variables tested in one experiment. These different variables should be investigated more closely to create an understanding of the effect they have on the outcome of the experiments.

4.3 Conclusion

This thesis investigates whether the chicken chorioallantoic membrane assay, an *in vivo* method to analyze distinct variables that affect blood vessel formation in scaffolds, is a suitable model for tissue engineering research, more specifically for the properties of hydrogel-scaffolds. Some difficulties were found when placing the scaffold as such on the CAM, which could be partially resolved using a closed construct, as found in literature, in which the scaffolds could be embedded. This enabled to locate the scaffolds much easier, and the scaffolds preserved their initial form better, making histology easier to perform. This closed construct can be used in further research for tissue engineering research, investigating different types of scaffolds and gels in the CAM assay.

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