Endothelial cell viability and histology as parameters for *ex-vivo* mouse heart perfusion system optimization

Abstract

Heart failure is a main cause of morbidity and mortality worldwide. To reduce the disparity between the waiting list and available donor hearts, extended criteria donor hearts are an alternative, because the development of *ex-vivo* heart perfusion in many countries has led to utilisation of extended criteria donor organs recently. Compared to SCS and hypothermic machine perfusion, normothermic machine perfusion provides a higher protective capacity, a superior prediction of the early graft function, and more accurate assessment of donor heart functionality. Importantly, the endothelium should also be considered specifically when optimising preservation solutions. In this work, mouse hearts were perfused in KHB solution at normothermic temperature. Histology and microscopy analysis revealed no statistically significant differences in SWT, AWT, and LVWA when comparing different perfusion times and switching from Langendorff to working perfusion mode. In addition, the degree of ischemia was lowest after 15-20 minutes of Langendorff perfusion and increased with 2-hour Langendorff perfusion, but fluctuated afterwards, suggesting that ischemia is a dynamic process. Furthermore, no statistically significant differences were found in AWT and LVWA for mouse hearts exposed to low oxygen levels in the KHB solution compared to high oxygen levels. However, a statistically significant higher SWT was observed in mouse hearts exposed to a low oxygen level compared to the hearts exposed to a high oxygen level. A higher oxygen level seems to preserve mouse hearts better. Moreover, the average cardiomyocyte size showed no statistically significant difference between mouse hearts exposed to either high or low oxygen levels. After performing flow cytometry and data-analysis, no statistically significant difference in HMEC-1 cell viability was found when exposed to cold preservation solution St Thomas cardioplegia, although an indication might be found for the cells to be not affected during the different incubation periods. Using the RTCA xCELLigence system (Roche) to determine the viability of HMEC-1 cells over time when exposed to different colloids and different perfusion fluids, showed that albumin might be the preferred colloid and KHB seem to preserve the viability better than Steen solution based on the experimental data. To conclude, the results suggest that better preservation could be achieved with normothermic perfusion of mouse hearts in an *ex-vivo* heart perfusion system at high oxygen level compared to a low oxygen level. Moreover, ischemia for perfusion system optimisation may be important to consider. Furthermore, from the *in-vitro* experiments can be concluded that the viability of endothelial cells might not be affected over different incubation periods with St Thomas cardioplegia. Also, albumin may be preferred over the artificial colloids when only considering the experimental results, although for determining whether KHB or Steen solution is more optimal as perfusate on the system, additional experiments are necessary.

Front page source: hands with heart from (CCEB Research)[1]

Layman summary

Heart failure is a main cause of disease and death in the world. Heart failure is the condition of the heart where it is not able to pump enough blood, including the amount of oxygen, needed for the body to function properly. In The Netherlands 1% of all adults is suffering from the chronic form of heart failure and around 20% of all people will get a diagnosis with this disease during their life. The number of patients advancing to the end stage of heart failure is increasing because of the improved medical care for heart failure patients as well as due to the aging of the population. The heart transplant remains a golden standard treatment for end stage heart failure patients. Although the number of patients on a transplant waiting list is increasing, the amount of donor hearts available is relatively fixed. Currently the prognosis for patients with end-stage heart failure is poor, because besides heart transplantation no alternative treatment is available. Storing hearts on ice in a cold solution used to preserve the heart during transportation from donor to recipient has been the gold standard method. However, machines to perfuse the heart outside of the body are developed. Compared to the preservation of hearts on ice in a cold preservation solution and machine perfusion of hearts with a cold solution, machine perfusion of hearts with a warm solution for preserving hearts provides a better protection and allows to make a more accurate assessment of the donor heart functionality. This makes it possible to consider using a group of hearts normally not used, the hearts that need more care. Although, heart machine perfusion needs to be optimised. In this work, different cold and warm preservation solutions are tested on blood vessel cells and mouse hearts as well as the different compounds added to the solutions to preserve the heart better and different amounts of oxygen. An indication has been found for better preservation of mouse hearts when a high amount of oxygen was added to the solution. Moreover, the survival of blood vessel cells might not be affected when stored in a cold preservation solution over time, a certain compound added to the preservation solutions may be preferred over other compounds tested, although more tests are needed to determine which preservation solution is more optimal. By testing a new machine for the perfusion of mouse hearts with a warm preservation solution, and optimising the preservation solution, taking into account the effects on blood vessel cells, this study provides information that contributes to closing the knowledge gap in how donor hearts can be preserved in a more optimal way. As a result, decreasing the difference between the number of patients on the list and the availability of donor hearts.

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Glossary

AWT = anterior wall thickness
DBD = hearts donated after brain death
DCD = hearts donated after circulatory death
H&E = hematoxylin and eosin
HMEC-1 = human dermal microvascular endothelial cells
KHB = Krebs-Henseleit buffer
LVWA = left ventricular wall area
PTAH = phosphotungstic acid hematoxylin
RTCA = real time cell analyser
SCS = static cold storage
SWT = septal wall thickness
UMCU = University Medical Center Utrecht
WGA = wheat germ agglutinin-FITC conjugated antibody

Introduction

Heart failure is a main cause of morbidity and mortality in the world[2]. Heart failure is the condition of the heart where it is not able to pump enough blood to meet the requirements of bodies demands[3]. Due to inefficient pump function, the oxygen demand of the body is not met[4]. 64.3 million people worldwide are affected by this disease and the prevalence of this disease in the adult population of developed countries is estimated to be around 1.5%[5,6]. In The Netherlands 1% of the adult population is suffering from the chronic form of heart failure and around 20% of the entire population will get a diagnosis with this disease during their life[7]. The incidence of heart failure is increasing, which also results in more patients advancing to the end stage of heart failure[8]. This is caused by the increase of cases with heart failure due improved medical care for heart failure patients, the aging of the population. A left ventricular assist device is used in such patients with advanced heart failure as a bridge to transplantation, but the heart transplant remains a golden standard treatment for end stage heart failure patients. Although the number of patients on a transplant waiting list is increasing, the amount of donor hearts available is relatively fixed[8–10]. The scarcity of donor hearts is due to multiple reasons such as 60% of potential donor hearts are not eligible to be transplanted, due to the old age of the donor, female gender, the existing comorbidities such as hypertension and diabetes mellitus, logistical problems, and the presence of donor left-ventricular dysfunction and/or hypertrophy[9,11]. Currently the prognosis for patients with end-stage heart failure is poor, because besides heart transplantation no alternative treatment is available[8]. In The Netherlands around 40 heart transplants are performed each year since 1987, whereas around 130 patients are on a waiting list.

The disparity between the number of patients on this list and the availability of donor hearts keeps growing further, leading to longer waiting times[8]. The mortality rates for patients on the waiting list for heart transplantation are high and 23% of the patients on the waiting list die or get removed[12,13]. Moreover, patients on the waitlist for paediatric heart transplantation must wait on the list for a transplant the longest and are facing the highest mortality for the waitlist compared to every other solid organ waiting list[14]. However, in the last ten years, a lot of research has been done to try to expand the donor pool as well as to improve the allocation of heart allografts to a suitable recipient[9]. To reduce the disparity between the waiting list and donor hearts available, extended criteria donor hearts are considered as an option for heart transplantation. These hearts would otherwise be rejected due to comorbidities, age, and increased ischemic time. All these parameters have been associated with increased morbidity and mortality compared to the normally used donor hearts. However, the use of extended criteria donor hearts increases the donor pool and improves the waiting list mortality[14–16].

Since the 1960s, static cold storage (SCS) has been used as the gold standard method heart preservation. The preservation method involves flushing the procured organ with preservation solution at 0-4 degrees Celsius, then immersing it into preservation solution at the same temperature until transplantation[17–19]. The hypothermic environment is responsible for decreasing cellular metabolism, and the preservation solution reduces cellular metabolism and provides cytoprotection[17,20].

However, preservation time with SCS is limited as prolonged cold storage increases the risk of early graft dysfunction that contributes to chronic complications. This is due to tissue damage upon prolonged hypothermic preservation and the occurrence of ischemia-reperfusion injury. Besides, the assessment of functionality as well as viability of the donated organ is difficult and the possibilities for organ repair are limited[17]. Also SCS can only be used for preservation and storage of hearts donated after brain death (DBD) as the functional assessment of the DBD hearts before procurement is possible and the hearts are not exposed to warm ischemia, which makes the simple way of cold storage a possibility[19]. Using hearts donated after circulatory death (DCD) is needed to increase the donor pool, but DCD hearts are exposed to different degrees of damage due to warm ischemia and injury upon reperfusion, and as a consequence of the difficulties in assessing their functionality and because these hearts are not able to undergo resuscitation and preservation by SCS, the transplantation of hearts has been relying solely on DBD[13,15,21,22]. In addition, SCS only preserves standard DBD hearts for

approximately 4 hours[13]. Despite improving the preservation solutions used for SCS, the development of machine perfusion lead to results showing a higher protective capacity compared to what can be achieved by the optimised preservation solutions and hypothermia[23,24].

The development and extended use of *ex-vivo* heart perfusion in many countries has led to utilisation of extended criteria donor organs in recent years. Recently, *ex-vivo* machine perfusion trials were started up in The Netherlands and resulted in successfully transplanting a DCD heart for the first time in this country[25]. Although the overall beneficial effect of *ex-situ* heart perfusion when compared to SCS are regardless of perfusion temperature and the beneficial effect is seen across in all temperature groups, most studies are performed in hypothermic (<15 degrees Celsius) or (near-) normothermic range (>32 degrees Celsius) in *ex-vivo* machine perfusion. Hypothermic machine perfusion can supply cyroprotective substrates while inducing a metabolic depression of the heart. The intravascular pressure in the heart upon perfusing the heart on an *ex-vivo* perfusion system can induce edema formation as well as result in damage of the microvasculature, accounting for both the hypothermic machine perfusion as well as the normothermic machine perfusion. Although the incidence of edema formation in the myocardium was greater in case of hypothermic machine perfusion, SCS was associated with a worse early graft function compared to hypothermic machine perfusion. The latter is an very important finding, because a variety of studies showed that edema formation and graft failure are correlated, which for recipients of transplants is the main predictor for mortality after 1 year[13,24,26]. To decrease the amount of edema formation colloids, large molecular weight particles, were introduced to the crystalloid solutions, which are water-based solutions containing small molecules like glucose as well as electrolytes. Colloids include natural colloids (products consisting of proteins), such as albumin, and synthetic colloids, for example dextrans and polysaccharides. Colloids elevate the oncotic pressure in the vessels. As a result, less fluid extravasates from the vessels into the surrounding tissue[23,24,27]. Comparing the normothermic machine perfusion of isolated guinea pig hearts with Krebs-Henseleit buffer (KHB) containing colloids, albumin or hydroxyethyl starch, with the perfusion of the same buffer containing crystalloid (saline), showed that the extravasation of fluid was lower when the perfusate consisted of colloids[28]. Normothermic machine perfusion shares several benefits of the hypothermic machine perfusion. For example, the delivery of nutrients, elimination of waste products as well as carbon dioxide, and the performance of biochemical and blood gas analysis of the perfusion solution. Additionally, normothermic machine perfusion can measure coronary blood flow and blood pressure, enables assessment of donor hearts (metabolism and functionality) and the option for the delivery of therapeutic drugs, while being under physiologic conditions, which in comparison with hypothermic machine perfusion results in a superior prediction of the early graft function and a more accurate assessment of the functionality of the donor heart. This is a very attractive feature to expand the number of eligible donor hearts[24,26]. Additionally, the identification of biomarkers for the prediction of organ viability is an important barrier for normothermic machine perfusion to reach its full potential[24,26]. Currently, the only available system approved to be used in the clinic is the Organ Care System (TransMedics, Andover, USA). Comparing this system to SCS showed promising results. However, at the moment assessment of heart functionality is not possible because the system only supports Langendorff mode, in which the heart is perfused at a non-working state. Besides, the Organ Care System is very expensive, €50000.00 for each time the system is used[24,26,29].

To be able to perform assessments, the metabolic demands of the heart on a normothermic perfusion system at working mode should be met[30]. Previous research showed that oxygenated reperfusion of the donor heart immediately after warm ischemia was necessary for obtaining a viable heart function[29]. This can be achieved by adding an oxygen carrier to the perfusion fluid[30]. The amount of data regarding hypothermic machine perfusion is scarce. However, early results of studies showed that modest oxygen demands can be maintained by hypothermic machine perfusion, resulting in a low concentration of accumulating lactate and an adequate functionality in canines as well as pigs. However, knowledge about normothermic heart machine perfusion and the required oxygenation is lacking[24].

To increase the preservation time, preservation solutions are being optimised. A variety of studies optimising the perfusion solution, using either a cold crystalloid solution or arm blood showed their superiority compared to SCS in preserving DBD and DCD donor hearts[13]. A study observed that using rabbit hearts that underwent ischemia for a short time, followed by normothermic machine perfusion on working mode with KHB solution containing erythrocytes provided superior results in hemodynamic parameters as well as metabolic parameters compared to the hearts that were perfused with KHB. They concluded that the usage of perfusates based on blood results in a superior preservation of heart functionality[31]. Moreover, perfusion with blood-based perfusates resulted in longest times in perfusion[32–34]. However, perfusion with blood-perfusates comes with a risk of for example unwanted responses of the immune system, thrombosis, transmitting infectious diseases by blood, and erythrocyte hemolysis, which reduces the oxygenation of the tissues[17,26,35]. Furthermore, the regular use of warm blood on the perfusion system is limited as a consequence of complexity, high costs, and ethical concerns. Therefore, developing acellular perfusion solutions is an important direction. The same accounts for the way to oxygenate the hearts. More interest occurred for de development of acellular oxygen carriers that have a capacity to transport oxygen similarly to capacity of hemoglobin[13,17]. More research is needed about the best way to oxygenate the perfusion solution to meet the metabolic demands of the normothermic machine perfused heart.

Importantly, the endothelium should also be taken into account specifically when optimising preservation solutions. Endothelial cell injury was found when the cells were incubated with either a cardioplegic solutions as well as solutions used for organ preservation, which can be caused by the fact that during the optimisation of the preservation solution only the preservation of cardiomyocytes are taken into account. Moreover, it is necessary to distinguish the effect of the solutions on the endothelial cells from the damage induced by other factors that are part of the procedure, such as ischemic-reperfusion injury as well as hypothermia, to examine how specifically the solutions affect the endothelial cells[36–38]. Preservation of endothelial cell barrier is not only important to prevent edema formation, but because endothelial cell damage can result in hypoperfusion and immunological complications as well. Consequently, leading to graft failure[39–43]. Colloids were found to be better at preserving the endothelial barrier function compared to crystalloid solutions[44]. Albumin and dextran are colloids commonly used in the clinic[45]. Albumin was found to have anti-inflammatory as well as antioxidant properties and showed to have beneficial effects on the integrity of the endothelial cell layer. Interestingly, the prevention of edema formation by albumin may not only be caused by providing colloid osmotic pressure, but by the special connection of albumin towards the endothelial glycocalyx[28,45,46]. Dextran is used for improving the local flow of the microcirculation during microsurgery, because of its positive effect on the blood viscosity, the prevention of aggregation by erythrocytes, and his antiplatelet effect[45,47]. PEG was shown to reduce the erythrocyte destruction by phagocytes by preventing binding of antibodies to ABO blood group antigens. Moreover, attachment of PEG to the vessel wall decreased the amount of blood cells that bind to the endothelium because of the masking effect on antigens[48,49].

By testing a new *ex-vivo* mouse heart perfusion system using normothermic machine perfusion, and optimising the perfusion solution, taking into account the effects on endothelial cells, this study provides information that contributes to closing the knowledge gap in how donor hearts can be preserved in a more optimal way. As a result, decreasing the disparity between the number of patients on the list and the availability of donor hearts[8].

Materials and Methods

Animals used for the *ex-vivo* heart perfusion system and exclusion criteria mouse hearts

C57BL/6 mice were used to perform the *ex-vivo* heart perfusion system in both experiments (general pilot experiment and pilot experiment oxygen level). Characteristics of the mice used in the experiments are shown in table 1. The hearts were excluded when they did not restart on the perfusion system, when the hearts stopped while performing the experiment, and when fibrillation occurred that was not solved after pacing. Moreover, the exclusion criteria 'a flow rate lower than 1 ml/min during Langendorff mode' and 'an afterload pressure lower than 25 mmHg during working mode' were determined during the general pilot experiment and applied during the pilot experiment oxygen level. Experiments were approved by the Animal Experiments Committee of the University Medical Centre Utrecht (Utrecht, Netherlands).

	Sex (% male)	Age (wk)	Housing (% of total)	Weight (g)	Origin (% of total)
General pilot experiment	77	9 - 25	Individual: 26.7 Group: 73.3	18 – 35.1	Utrecht University: 10 Charles River: 33.3 RMI Utrecht: 50 GDL Utrecht: 6.7
Pilot experiment oxygen level	100	16 - 30	Individual: 100	27.3 – 34.6	Charles River: 100

TABLE 1 – CHARACTERISTICS MICE USED FOR THE EX-VIVO HEART PERFUSION SYSTEM EXPERIMENTS.

Cells used for the *in-vitro* experiments

For the *in-vitro* experiments human dermal microvascular endothelial cells (HMEC-1)(gift CDL UMC Utrecht)[50] were used. This cell-type is derived from the first immortalized cell line from human dermal microvascular endothelial cell origin. The morphology, phenotype, as well as functionality are highly similar to the endothelial cells that are part of the microvasculature. Moreover, this is a very stable cell line, and high passages can be reached with these cells[51].

Ex-vivo machine perfusion experiments

With these experiments the perfusion system (Radnoti, 130101EZ) will be setup and optimised. 30 mouse hearts were perfused with KHB, a commonly used perfusion fluid on this system[52]. The hearts were perfused in Langendorff mode and working heart mode. In the Langendorff mode the heart is unloaded, and the perfusion solution flows into the aorta retrogradely (the opposite way to the physiologic flow direction). The resulting pressure closes the aortic valves that cause the perfusion solution to flow into the coronary arterial vasculature of the heart and is flushed out of the heart via the coronary veins, which come together in the right atrium[52]. During working mode, the heart is loaded and is performing mechanical work which mimics physiological situation. The perfusion solution flows via the left atrium to the left ventricle and after filling and contracting, the solution is ejected via the aorta[53]. To optimise the system, it is necessary to look at the functionality of the mouse hearts over time by removing the hearts from the perfusion system at different timepoints: TP1, TP2, TP3, TP4, and TP5. The experiment was blinded for which mouse heart belongs to which timepoint.

To meet the metabolic demands of the normothermic machine perfused heart, a way of and the required oxygenation was tested[24]. 7 mouse hearts were exposed to either low (20% O_2 + 5% CO_2) or high oxygen levels (carbogen, 95% O_2 + 5% CO_2) at the perfusion system with KHB, which were both optimised in the previous experiment. The experiment was blinded for which mouse heart belongs to which oxygen level. To measure the functionality of the mouse hearts over time at different oxygen

levels, the hearts were removed from the perfusion system at different timepoints: TP1, TP2, TP3, TP4, and TP5.

Additional information on the used materials	
10x Krebs-Henseleit Buffer	Homemade (UMCU); $CaCl_2$*$2H_2O$ 2.2 mM, KCl 3.5 mM, NaCl 118 mM, $MgSO_4.7H_2O$ 1.1 mM, KH_2PO_4 1.2 mM dissolved in MiliQ water
10x Krebs-Henseleit Buffer (optimised)	Homemade (UMCU), optimised; $CaCl_2$*$2H_2O$ 2.0 mM, KCl 3.0 mM, NaCl 118 mM, MgSO4.7 H_2O 1.2 mM, KH_2PO_4 1.2 mM dissolved in MiliQ water
Pentobarbital	Apotheek Faculteit Diergeneeskunde; Pento barbitalNa 60 mg/ml (20 ml) + ethanol 8% (g/v) en propyleenglycol, Ref: 20002824
Heparin	Leo Pharma BV; Heparine LEO 5000 U.I./ml, Lot: C47478
Sodium chloride	B Braun; Sodium chloride 0.9% solution for infusion 100 ml, Lot: 194158131

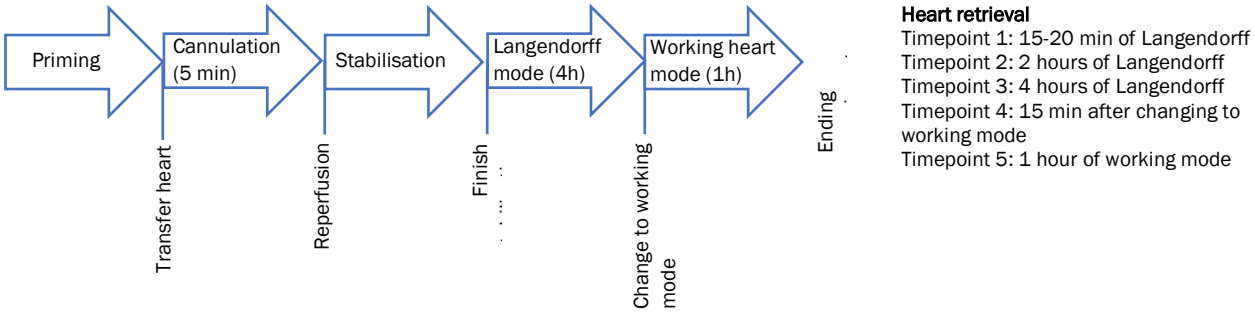


FIGURE 1 – EXPERIMENTAL PLAN EX-VIVO MACHINE PERFUSION EXPERIMENTS AND OVERVIEW TIMEPOINTS FOR HEART RETRIEVAL.

Priming perfusion system

An overview of the experimental plan for the *ex-vivo* machine perfusion experiments can be found in figure 1. On the day of the experiment, the perfusion fluid for the system was prepared. 10x KHB was diluted with MiliQ water to 1x KHB. Moreover, $NaHCO_3$ 31 mM, Glucose.H_2O 15 mM, and HEPES 10 mM was added to the perfusion fluid and the pH was adjusted to 7.4. Subsequently, the water jacket and perfusion system were primed. The temperature of the water was set at 37 degrees Celsius. Subsequently, the reservoir of the perfusion system was filled with 500 mL perfusion fluid (KHB) and oxygenated with carbogen (95% O_2 and 5% CO_2) with a pressure of 0.65 Bar from the gas supply. Then, the atrial and aortic line, the bubble trap, the afterload bubble trap (for working mode), the compliance chamber (for working mode), the reservoir of the post-heart chamber, as well as the heart chamber were filled with the perfusion fluid. Afterwards, the perfusion system was prepared to receive the heart and the pressure sensors for measuring the pressure of the aorta and atrium were calibrated using LabChart 8 software (AD Instruments).

Excising and cannulating the mouse hearts

Bench and the material needed for dissection of the mouse was cleaned with 70% Ethanol. Mouse was anesthetized by an intraperitoneal injection of 0.2 mL of 100 mg/kg pentobarbital (diluted with sodium chloride). After confirmation of sedation by toe-pinch 0.1 mL of 200 IU/animal heparin (diluted with sodium chloride) was injected into the orbital plexus. The heart was flushed using a 21G needle, connected to a 10 mL syringe containing oxygenated warmed perfusion fluid. After which, the chest cavity of the mouse was opened for harvesting the heart-lung bloc, which was immediately transferred to cardioplegia (ice cold) in a petri dish. Then, the heart was transferred to another petri dish, where the lungs as well as other tissue were removed under a microscope (Olympus SZX7). Hereafter, a left atrial cannula was inserted into the pulmonary vein and an aortic cannula was connected. After the cannulation the heart was transferred and attached to the perfusion system. After reperfusion when the heart started beating (in Langendorff mode), perfusate samples were taken (blood gas analysis, perfusate, and dfDNA/RNA perfusate), ECG electrodes were attached to the heart, and the perfusion system was switched to constant pressure mode with a pressure of 75-80 mmHg. The pressure was not constant in 60% of the TP1 perfused mouse hearts, 67% of the TP2 hearts, 83 % of the TP3 hearts, 17% of the TP4 hearts, and 33% of the TP5 hearts due to complications. After 15 minutes of stabilisation, and after 2 hours and 4 hours of heart perfusion the perfusate samples were taken again as well as the flow was measured. Then, the perfusion system was adjusted (flow rate changed to keep atrial pressure at 15-20 mmHg), enabling heart perfusion in working mode. Perfusate samples were taken after 15 minutes of stabilization and after 1 hour. At the end of the experiment a cfDNA/RNA perfusate sample was taken. Hearts were stopped (by injecting KCl in the cannula attached to the aorta), cut (obtaining the part with the atria together with upper part of the ventricles, the down part was cut into the front and back ventricle), and stored at different timepoints (see experimental plan), six mouse hearts for each timepoint: TP1, TP2, TP3, TP4, and TP5. The atria with upper part ventricles were stored overnight in 4% formalin at 4 degrees Celsius and the next day, after washing with PBS, in 70% ethanol at -20 degrees Celsius. The front and back ventricles were separately stored in cryotubes at dry ice. Measuring with the LabChart 8 software (AD Instruments) and oxygenation with carbogen was stopped afterwards.

Setting up the perfusion system and perfusing the mouse hearts to test the difference between oxygen levels was done with the same procedures used during the first experiment for testing ischemia and wall thickness of the mouse hearts over time, only during this experiment all the hearts were taken off the system after T5 and the procedures were optimised. During the harvesting step of the mouse heart, cold KHB was used instead of cold cardioplegia and during this step the 'vena cava' was decided to be punctured, whereafter the mouse was flushed with 5-10 mL of cold cardioplegia instead warm oxygenated perfusion fluid. Also, the time needed to perform certain procedures was determined. Moreover, instead of oxygenating all the hearts with carbogen (containing 95% O_2 and 5% CO_2), a high oxygen level, hearts were exposed to either low oxygen level (20% O_2 + 5% CO_2) or high oxygen level. Besides, the perfusion fluid, the 10x KHB, was optimised (see 'additional information on the used materials') as well as the salts added after diluting the stock: $NaHCO_3$ 25 mM, Glucose.H_2O 11 mM, and HEPES 10 mM. Furthermore, 80 µL/L insulin was added to the perfusion fluid (KHB) when the heart was on working mode.

Histology

The heart functionality was examined by embedding, performing histology using histochemical stains as well as immunohistochemistry, and analysing the microscopic pictures of the stained hearts, while being blinded for which heart belonged to which timepoint. Histochemical stains 'hematoxylin and eosin' (H&E) and 'phosphotungstic acid hematoxylin' (PTAH), as well as immunohistochemistry with wheat germ agglutinin-FITC conjugated antibody (WGA) were used for histology. The H&E staining was used to look at the wall thickness, because edema formation upon ischemia-reperfusion increases the thickness of the cardiac wall. Moreover, this staining was performed to examine whether different oxygen levels affect the wall thickness over time as a response to ischemia-reperfusion injury[54,55]. Furthermore, the PTAH staining was performed examine the hearts samples for ischemia to look at ischemia-reperfusion injury[56–58]. Can a difference be observed in wall thickness and amount of ischemia the longer the heart is on the perfusion system and after switching to working mode? Immunohistochemistry with WGA, which binds to cell membranes, was performed to determine the size of the cardiomyocytes[59]. As a result of a variety of pathophysiologic signals, for example hypertension, exercise, and ischemia, the size of the cardiomyocytes can increase (hypertrophy) and the heart enlarges[60,61]. Therefore, it would be interesting to determine how perfusing mouse hearts on the perfusion system with different oxygen levels affects the cardiomyocyte size. Thus, can a difference be observed in wall thickness and cardiomyocyte size between high and low oxygen levels?

Additional information on the used materials	
Trisodium citrate dihydrate, ACS, 99.0% min, crystalblue ($Na_3C_6H_5O_7$. $2H_2O$)	Thermofisher (Kandel) GmbH; Alfa Aesar, Lot: R04G032
Wheat germ agglutinin- FITC conjugated antibody	Sigma-Aldrich; L4895-2MG-lectin from Triticum vulgaris (wheat), FITC conjugate lyophilized powder, Lot: #036M4119V
Mounting medium	Vectashield^{R}; Antifade Mounting Medium, Lot: ZG1028, Ref: H-1000
Glass slides	Leica Biosystems; Surgipath X-tra Adhesive Precleaned Micro Slides, 26x76x1.0mm (72 pcs) 3800203AE green, Lot: 4900058509

The embedding of the mouse heart samples was performed at the Hubrecht. Mouse heart information (timepoint and specific mouse number, initials, date experiment) was printed on a cassette. The atria with the upper part of the ventricles were used from the perfusion system experiment. Each mouse heart sample was placed in a cassette and brought to a tissue processor for a standard overnight program. The next day, after 18 hours, the program was stopped, the heart samples were removed from the tissue processor, and were taken to an embedding machine. The embedding machine was used to embed the heart samples in liquid paraffin. A metal cup was filled with liquid paraffin, each mouse heart sample was transferred from the cassette to a liquid paraffin filled metal cup, a cold plate was used to immobilise the heart sample in the paraffin, liquid paraffin was added to the cassette, and the metal cup with the cassette was transferred to a cold plate (minimal 30 minutes).

Afterwards, the embedded hearts were taken to the pathology department of the University Medical Center Utrecht (UMCU) for slicing. The heart samples were embedded in such a way that the ventricles were sliced before the atria. The embedded hearts were cooled down to approximately -9 degrees Celsius with a cold plate (Adamas CP1500 Koelplaat) and cut in 3 µm sections with a microtome

(Adamas Instrumenten BV, microm HM 3555). The heart sections were transferred to a warm water bath of approximately 42 degrees Celsius (Störk-Tronic) and added to glass slides in pairs of consecutive sections afterwards. The sections dried for at least 15 minutes, subsequently these were taken to a warm plate of 60 degrees Celsius to melt the paraffin and were stored. The embedding and slicing of the mouse heart samples to test different oxygen levels were done in the same way for testing ischemia and wall thickness over time.

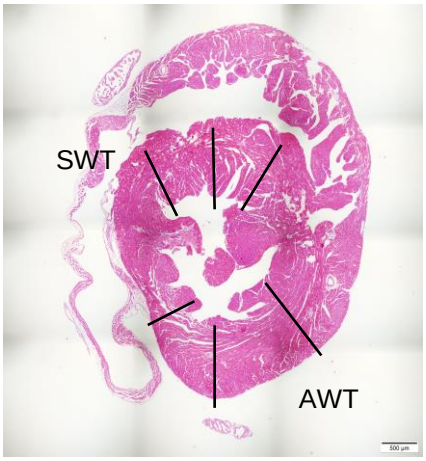


Figure 2 – Locations measurements wall thickness of hematoxylin and eosin pictures. *Septal wall thickness (SWT) and anterior wall thickness (AWT) were measured from microscopic pictures (Olympus BX53, 4x magnification) of hematoxylin and eosin stained mouse heart sections with ImageJ[62], each at three different locations as shown in this figure.*

The heart sections used to test ischemia and wall thickness over time were stained with histochemical stains H&E and PTAH using an automated H&E stainer (pathology department UMCU) and performed by employees of the pathology department (UMCU), respectively. The heart sections used to test different oxygen levels were stained with a histochemical stain 'H&E' in the same way and immunohistochemistry with WGA was performed on the other heart sections as followed: a citrate buffer was made (2.94 g/L trisodium citrate dihydrate dissolved in Aqua Dest, adjusted to pH 6) and cooked for 20 minutes (500 degrees Celsius). Subsequently, one glass slide (with a pair of heart sections) of each heart was deparaffinized with xylene for 10 minutes, followed by ethanol (moving slides from 99.5%, 96% to 70%), and washed with Aqua Dest. Afterwards, the glass slides were transferred to the hot citrate buffer to cook for 20 minutes at 250 degrees Celsius and were cooled in cold water for 15 minutes. Then the sections were washed with PBS sequenza for 5 minutes and each heart section was incubated with 50µL 2 mg/ml WGA 1:40 in PBS for 30 minutes in the dark at room temperature. The sections were washed with PBS sequenza and incubated with DAPI for 5 minutes in the dark at room temperature. After incubation with DAPI, the sections were washed with PBS sequenza and Aqua Dest, and the glass slided were dried. At the end, mounting medium was added to the sections with cover slips (glued to the glass slides with nail polish) for long term preservation of the heart sections and were stored for analysis.

A microscope (Olympus BX53) was used with CellSens Dimension Imaging Software (Olympus) to make microscopic pictures. Microscopic pictures (4x magnification) of the H&E stained mouse heart sections of either the timepoints and different oxygen levels were used to measure the left ventricular wall area (LVWA), the thickness of the walls septal wall thickness (SWT), and anterior wall thickness (AWT) with ImageJ[62]. An average of the measured wall thickness at three different locations in the wall was calculated for the SWT and the AWT, see figure 2. Furthermore, microscopic pictures (4x magnification) of the PTAH-stained mouse heart sections were made. These pictures were used to score the amount of ischemia based on the percentages of 4 categories. A pathologist of the UMCU explained how to characterise which areas can be depicted as ischemic (brown colour). The changes in colour of the staining towards brown (ischemia) are scored as well. A brown colour was scored as 'severe ischemia', amount brown > blue scored as 'moderate ischemia', amount brown < blue scored as 'mild ischemia', and a blue colour was scored as 'no ischemia'. The scoring was separately done by a second examiner as well (blinded for timepoints) and averages were taken for the end score (in percentage) for each category at each timepoint. The microscope (Olympus BX53) was also used with CellSens Dimension Imaging Software (Olympus) together with a fluorescence illumination system (X-CiteR series 120, EXFO) to perform fluorescence microscopy with the WGA stained heart sections with settings: ISO1600, greyscale off, and exposure times 6 ms (green), 1 ms (blue), and 80 ms (red). The pictures were taken of different parts in the heart sections and were equally divided over the heart: 5 pictures of the right ventricle and 5 pictures of the left ventricle (including 2 pictures of the septal wall)

were made. The pictures were analysed for the cardiomyocytes size with the image analysis software Imaris (Oxford Instruments Imaris). Afterwards, only the measured areas between 150 and 800 μm^2 were included, because of previous research that was done for cardiomyocyte size in mice[63,64]. Besides, an average was taken for the sizes of all analysed areas for each mouse heart.

Flow cytometry and data-analysis

Because of the importance to test preservation solutions on endothelial cells, the effect of cold St Thomas cardioplegia, one of the preservation solutions used in the clinic for cold preservation, on the viability of endothelial cells was tested[65]. First, cell culture was performed with HMEC-1 cells, followed by the exposure of the cells to cold St Thomas cardioplegia for different incubation periods. Finally, cells were obtained and flow cytometry was performed to determine the viability of the cells.

Additional information on the used materials	
St Thomas cardioplegia	Homemade (UMCU); NaCl 92.1 mM, KCl 14.9 mM, $MgSO_4.7H_2O$ 1.2 mM, $MgCl_26H_2O$ 15.1 mM, KH_2PO_4 1.2 mM, $CaCl_2*2H_2O$ 1.2 mM, $C_{13}H_{21}ClN_2O_2$ 1.0 mM dissolved in MiliQ water
DPBS (1x)	Gibco™; Ref: 14040-091
PBS (1x)	Gibco™; without $CaCl_2$ and $MgCl_2$, pH 7.4, Ref: 10010-056
Medium	Gibco™; MCDB 131 Medium (1x) without L-glutamine, Ref: 10372-019. Added: 10% FBS, 1% P/S, 0.1% HEGF, 0.1% Hydrocortisone, 1% L-glutamine
Accutase	Innovative Cell Technologies; without Ca^{2+} and Mg^{2+} (dissolved in DPBS)
Trypan Blue solution	Sigma-Aldrich; Ref: T8154-100 mL
Trypsin-EDTA solution	Sigma-Aldrich; Ref: T4049-100 mL
FACS buffer	Home-made (UMCU); 5% heat-inactivated FBS and 0.2% EDTA dissolved in PBS
Zombie NIR™ Viability kit	Biolegend; Ref: 423105

Before culturing the cells, medium had to be prepared and T75 flasks were coated. The flasks were filled with 10 mL DPBS (1x) + 0.1% gelatine and incubated in an incubator (5% CO2, 37 degrees Celsius) for at least 15 minutes. The DPBS (1x) + 0.1% gelatine was removed after and the flasks were washed with 10 mL PBS (1x) for three times. During the last time washing the flasks, PBS stayed in the flasks till the moment HMEC-1 cells were added. The medium was removed from a T75 flask containing cultured HMEC-1 cells and washed three times with 10 mL PBS (1x). Subsequently, the PBS (1x) was removed and 1 mL of pre-warmed (warm water bath, 37 degrees Celsius) accutase was added to the flask and incubated in the incubator for 4 minutes. The flask was shaken afterwards and 10 mL of pre-warmed (warm water bath, 37 degrees Celsius) medium was added to inactivate the accutase. Another 10 mL of pre-warmed medium was added to the flask, cells were homogenised, divided over the coated flasks, and the flasks were transferred to an incubator. Every two days the medium of the cells was changed. Two days before the experiment, the HMEC-1 cells were added to 3 wells of five 6-well plates and 6 wells of one 6-well plate using the latter cell culture protocol. However, after inactivating accutase with medium, the cells were transferred to a 50 mL tube and centrifuged for 5 minutes (RCF 350, brake 9). Afterwards, the supernatant was removed, 1 mL of medium was added, 10 µL of Trypan

Blue solution was mixed with 10 µL of the medium/cell solution on parafilm, 10 µL of this mixture was transferred to a counting slide, and the number of cells was counted with an Automated Cell Counter (Beckman). After calculations, 130000 cells in 2 mL medium were added to each well.

On the day of the experiment, the medium was removed from each of the six 6-well plates, wells were washed three times with PBS (1x), and St Thomas cardioplegia was added at different times in the morning. In this way creating different incubation periods (0 min, 10 min, 30 min, 1 hour, 2

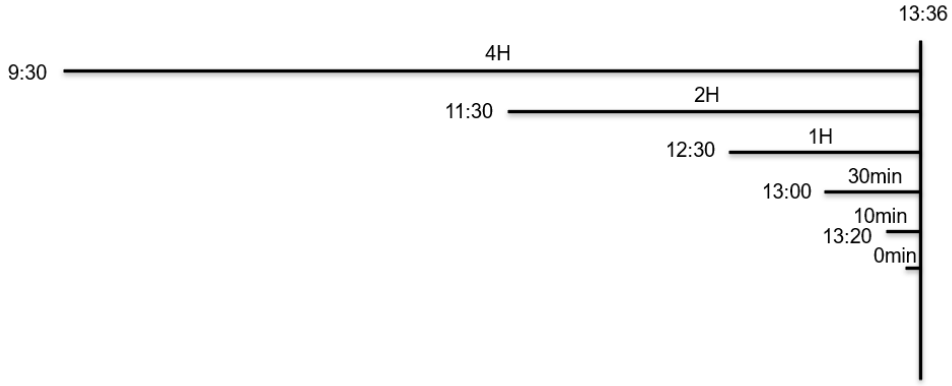


FIGURE 3 – EXPERIMENTAL PLAN ST THOMAS CARDIOPLEGIA INCUBATION.

hours, and 4 hours), each consisting of three wells with cells except for the 0 min incubation period, see figure 3. At the end of all the incubation periods, St Thomas cardioplegia was removed, and the cells were incubated in the incubator with pre-warmed medium (warm water bath, 37 degrees Celsius) for 15 minutes. The '0 min' incubation period represents the 6-well plate with the positive (3 wells) and the negative control (3 wells), which was not exposed to St Thomas cardioplegia. The medium was removed and new medium was added at the same time the St Thomas cardioplegia was removed and medium added for the other incubation periods. Afterwards, the wells were washed three times with PBS (1x), 1 mL of trypsin was added to detach the cells, 2 mL of medium was added, and the cells of each well of every incubation period and control was added to a separate 15 mL tube. The tubes were centrifuged for five minutes (RCF 330, brake 9). Then, the supernatant was removed, and the cells of each incubation period were dissolved in 1 mL FACS buffer, each in a separate 15 mL tube. The cells of the positive control were exposed to dry ice for 30 seconds followed by hot water for 30 seconds (92 degrees Celsius), which was repeated two times to induce cell death. Subsequently, the cells of all incubation periods were centrifuged for five minutes (RCF 330, brake 9), the supernatant was removed, the cells were dissolved in 1 mL PBS (1x) and incubated with 1 µL Zombie NIR™ in a fridge (4 degrees Celsius) for 30 minutes. During the incubation, the CytoFLEX (Beckman Coulter) was started up. After incubation, 2 mL FACS buffer was added to the cells, the cells were centrifuged (RCF 330, brake 9), and were dissolved in 250 µL FACS buffer. Then, flow cytometry was performed, data was obtained from the system. This experiment was done three times, and a positive control was included in the last two experiments.

Data-analysis was performed with the software Kaluza (Beckman Coulter). After gating for the cells of interest for each sample (incubation period, control), information was provided about the percentage of alive and death cells over the total number of cells. An average was taken from the percentage of alive cells for all the samples for each incubation period and control of each experiment.

Cell culture experiments

To determine which colloid would be the best one to introduce in the perfusion solution, it is important to compare the effect of different colloids on endothelial cells. During this experiment, HMEC-1 cells were incubated with KHB consisting of albumin, dextran-70 or PEG, to compare the effect of each colloid on the viability of endothelial cells over time. The real time cell analyser (RTCA) xCELLigence system (Roche) was used to measure the viability of the endothelial cells over time. Is there a difference in endothelial cell viability when exposing the cells to different colloids? Next to testing different colloids, it is important to test the effect of different perfusion fluids on specifically endothelial cells. Steen solution is a frequently used solution for *ex-vivo* lung perfusion as well as for the perfusion of different organs with machine perfusion. The solution includes colloids for maintaining the oncotic pressure. Examples are albumin and dextran. The glucose in the Steen solution is the source of energy, the dissolved buffers keep the pH around the normal pH, and the osmolarity is regulated by physiological concentrations of ions, which are added to the solution[17]. Previous research about Steen solution as a perfusion fluid showed that normothermic ex-vivo heart perfusion of porcine hearts resulted in an improved preservation of the contractile function, which can be caused by the increased oncotic pressure of the solution that counteracts the continuous positive hydrostatic pressure where the heart is exposed to during ex-vivo heart perfusion as well as diminishes the swelling of the myocardium[66]. During this experiment, Steen solution and KHB (used during the previous in vivo experiments) were compared for their effect on the endothelial cell viability by using the RTCA xCELLigence system[52]. Is there a difference in endothelial cell viability when exposing the cells to different perfusion fluids?

Additional information on the used materials	
St Thomas cardioplegia	Homemade (UMCU); NaCl 92.1 mM, KCl 14.9 mM, $MgSO_4.7H_2O$ 1.2 mM, $MgCl_26H_2O$ 15.1 mM, KH_2PO_4 1.2 mM, $CaCl_2*2H_2O$ 1.2 mM, $C_{13}H_{21}ClN_2O_2$ 1.0 mM dissolved in MiliQ water
10x Krebs-Henseleit Buffer	Homemade (UMCU); $CaCl_2*2H_2O$ 2.2 mM, KCl 3.5 mM, NaCl 118 mM, $MgSO_4.7H_2O$ 1.1 mM, KH_2PO_4 1.2 mM dissolved in MiliQ water
10x Krebs-Henseleit Buffer (optimised)	Homemade (UMCU), optimised; $CaCl_2*2H_2O$ 2.0 mM, KCl 3.0 mM, NaCl 118 mM, $MgSO_4.7H_2O$ 1.2 mM, KH_2PO_4 1.2 mM dissolved in MiliQ water
DPBS (1x)	Gibco™; Ref: 14040-091
PBS (1x)	Gibco™; without $CaCl_2$ and $MgCl_2$, pH 7.4, Ref: 10010-056
Medium	Gibco™; MCDB 131 Medium (1x) without L-glutamine, Ref: 10372-019. Added: 10% FBS, 1% P/S, 0.1% HEGF, 0.1% Hydrocortisone, 1% L-glutamine
Accutase	Innovative Cell Technologies; without Ca^{2+} and Mg^{2+} (dissolved in DPBS)
Trypan Blue solution	Sigma-Aldrich; Ref: T8154-100 mL
Trypsin-EDTA solution	Sigma-Aldrich; Ref: T4049-100 mL
70% ethanol	Klinipath BV; Ref: 40709010
Dextran-70	Carl Roth; Ref: 9228.2
Albumin	Roche; -Bovine Serum Albumin Fraction V, Ref: 10735086001
PEG	Sigma-Aldrich; PEG 35 kDa, Lot: #BCCD7740, Ref: 81310-1KG
Noradrenaline	Centrafarm; Ref: 14211.43011
Adrenaline	Centrafarm; Ref: 17125.43011
Liothyronin sodium	ZGT Apotheek; Ref: 16796462
Levothyroxine sodium	Apotheek der Haarlemse Ziekenhuizen; Ref: S142
Hydrocortisone	Pfizer
Sodium chloride	Merck; 1.370.171.000
Potassium chloride	Sigma-Aldrich; Ref: P9333-1KG
D-glucose	Fresenius Kabi
Sodium phosphate	Sigma-Aldrich; Ref: 342483-500G
Magnesium chloride hexahydrate	Sigma-Aldrich; Ref: M2393-500G
Calcium/Magnesium	Apotheek A15; Ref: EP00264
Sodium bicarbonate	Sigma-Aldrich; Ref: S5761-500G
E-plate	ACEA Biosciences, Inc; E-Plate 16, Ref: 5469830001
18G needle 1,2 x 50 mm	BD Microlance™; 3 needle 18G 1.2 x 50 mm pink, Ref: 301900
Syringe	BD Discardit™; 2 piece eccentric luer 20 mL, Ref: 300296
0.45 µm filter	Corning®; syringe Filter 0.45 Micron SFCA Membrane, Ref: 431220

Different colloids were dissolved in 1x KHB, which is the perfusion solution used on the *ex-vivo* mouse heart perfusion system. 1x KHB was prepared from the 10x KHB stock by dilution with MiliQ water. The RTCA xCELLigence system as well as the software on the computer were started, and the schedule of the experiment was saved in the software, see table 2.

Step	Information step	Interval	Total time
1	Seeding/incubation HMEC-1 in medium	Measurement every hour for 19 hours	19 hours
2	Incubation in St Thomas cardioplegia	Measurement every 10 minutes for 1 hour	20 hours
3	Incubation with different colloids, only 1x KHB (negative control), and medium (positive control)	Measurement every 15 minutes for 4 hours	24 hours
4	Incubation with different colloids, only 1x KHB (negative control), and medium (positive control)	Measurement every hour for 20 hours	44 hours
5	Incubation with different colloids, only 1x KHB (negative control), and medium (positive control)	Measurement every two hours for 12 hours	68 hours

TABLE 2 – SCHEDULE REAL TIME CELL ANALYSER XCELLIGENCE EXPERIMENT TO TEST DIFFERENT COLLOIDS.

After starting up the RTCA xCELLigence system (Roche) and the software, HMEC-1 cells were cultured. 200 µL DPBS (1x) + 0.1% gelatine was added to all the wells of an E-plate and incubated in an incubator (5% CO_2, 37 degrees Celsius) for fifteen minutes. The wells were washed with PBS (1x) for three times and were transferred to an incubator, with a layer of PBS (1x) in the wells from the third wash. The cells were cultured and counted as done before during the previous experiment (flow cytometry experiment: cell culture HMEC-1 cells). This time, a mixture of 200000 cells/mL HMEC-1 medium was made, resulting in 40000 cells/200 µL HMEC-1 medium in each well of the E-plate. This cell/HMEC-1 medium mixture was added to the coated E-plate from the incubator. The E-plate was inserted in the the RTCA xCELLigence system (Roche)located in an incubator (5% CO_2, 37 degrees Celsius), and in the software the experiment was started with step 1, see table 2. The cells were incubated in medium for 19 hours and every hour the system measured the cell index, the change in impedance, which is a parameter for the viability of the endothelial cells. The cell index increased when the cells grow/proliferate and decreased when cells die[67]. 1x KHB was mixed with Dextran-70 (60 g/L Dextran-70 in 1x KHB solution) and Albumin (55 g/L Albumin in 1x KHB solution). The negative control for this experiment was 1x KHB and the positive control was pre-warmed medium (warm water bath, 37 degrees Celsius). The 1x KHB solutions (with colloids and the negative control) were filtered using a separate 0.45 µm filter, a 18G 1,2 x 50 mm needle, and 20 mL syringe for each solution. After 19 hours of incubation with medium, the E-plate was taken out of the RTCA xCELLigence system (Roche), and the wells were washed three times with 200 µL 1x PBS. Afterwards, the E-plate was put in the incubator with 200 µL of St Thomas cardioplegia in each well and incubated for one hour, see step 2 of table 2. After one hour of incubation, the E-plate was removed from the system, the wells were washed three times with 200 µL 1x PBS, and 200 µL of each 1x KHB solution (dextran-70, albumin, and negative control) as well as the positive control were added to 4 wells of the E-plate (in total 16 wells). The E-

plate was brought back into the RTCA xCELLigence system (Roche) and the cells incubated in the solutions for 48 hours while the system was measuring the cell index over time. The system measured the cell index every 15 minutes for the first four hours, then every hour for another 20 hours, and finally the system measured the cell index every 2 hours for 24 hours, see step 3, 4, and 5 of table 2. After the 48 hours of incubation with the different solutions, the experiment ended. The data was obtained, and the E-plate was removed from the system and cleaned. This experiment was repeated four times. However, during the second, third, and fourth time, 30 g/L PEG in 1x KHB solution was added as a solution. This resulted in 4 wells of the E-plate for the positive control and 3 wells of the plate for the negative control and the 1x KHB with colloid solutions.

To test the different perfusates, the same setup (including the colloids Dextran-70, PEG, and Albumin) was used as the experiment for testing the different colloids. This time two E-plates were used, one plate with the 1x KHB solutions (with different colloids and negative control) and positive control (medium). The other plate with Steen solution (with the same colloids and a negative control, MiliQ water), and positive control. Besides, similar to the experiment to test the effect of high and low oxygen level, the optimised KHB solution was used (see 'additional information on the used materials') as well as the salts added after diluting the stock: $NaHCO_3$ 25 mM, Glucose.H_2O 11 mM, and HEPES 10 mM. Moreover, the optimised 1x KHB was mixed with an optimised number of colloids: Dextran-70 (57 g/L Dextran-70 in 1x KHB solution), Albumin (60 g/L Albumin in 1x KHB solution) and PEG (30 g/L PEG in 1x KHB). Furthermore, during this RTCA xCELLigence experiment, the exposure to St Thomas cardioplegia was 10 minutes instead of 1 hour, the cells were exposed to the different solutions for 24 hours instead of 48 hours (step five was excluded), see step 2 of table 2. Moreover, 1x PBS as well as the positive control (medium) were pre-warmed (warm water bath, 37 degrees Celsius) before exposure to the cells, to not expose the cells to too many fluctuations in temperature as well as cold shock. This experiment was performed one time. Averages of the measured cell index for all the wells for each solution were calculated for each experiment.

The Steen solution was home-made. 11.4 grams of Dextran-70 (end concentration 57 g/L), 12 grams of albumin (end concentration 60 g/L), and 5.7 grams of PEG (end concentration 30 g/L) were added to 60 mL MiliQ water (was later filled up to 75 mL). Afterwards, 100 µL of noradrenaline (5.91 µM) as well as 110 µL of adrenalin (5.46 µM) was added to 100 mL of MiliQ water. This solution was called 'solution 1'. Mixing 1 mL of solution 1 and 99 mL of MiliQ water resulted in solution 2. This was stored in the -20 degrees Celsius freezer. After solution 2 was made, solution 3 was prepared. 1 mL liothyronine sodium (37.15 µM), 0.2 mL levothyroxine sodium (125.2 µM), and subsequently 75 µL hydrocortisone (68.975 µM) was put together with 249 mL MiliQ water. This was followed by adding 1 mL solution 3 to 99 mL MiliQ water to make solution 4. Then, 25 mL NaCl (3.8 M), 25 mL KCl (0.8 M), 1.95 mL D-glucose 50% (2.78 M), and 0.4 mL $NaPO_4$ (3 M) were dissolved in 419 mL MiliQ water. In addition, 25 mL MgCl (0.256 M), 1.75 mL of solution 4, and 2.1 mL CaMg (0.54 M Ca^{2+} and 0.24 M Mg^{+}) were added to latter solution to make solution 5. Afterwards, 115 mL of solution 5, 20 µL of solution 2, 16 µL of human insulin for injection (100 IU/mL), and 10 µL of $NaHCO_3$ (0.5 M) were put together to form the final solution. This final solution was made four times and 75 mL of dextran-70, albumin, PEG, and MiliQ water were each dissolved separately to one of the four solutions. The pH was checked and adjusted to 7.4.

Statistics

GraphPad Prism8 (GraphPad Inc, San Diego, California USA) was used for the statistical analysis and for making graphs. To test the normality of the data a Shapiro-Wilk test was performed with SPSS Statistics 28. A one-way analysis of variance and Tukey's multiple comparisons test were performed for the averages at each timepoint of SWT, AWT, and the LVWA for the heart samples tested for ischemia and wall thickness over time. An unpaired t-test with Welch's correction was performed for the averages of SWT, AWT, the LVWA, and the averages of the cardiomyocyte size for the heart samples tested for the different oxygen levels. For the flow cytometry experiment, an average was taken from the percentage of alive cells for all the samples for each incubation period and control of each experiment and plotted into a graph. A Kruskal Wallis with Dunn's multiple comparisons test was performed on the data to test for statistically significant differences in percentage of alive cells of all cells between the averages of the negative control (not exposed to St Thomas cardioplegia, 0 min), the positive control (pre-warmed medium), and the different incubation periods (exposure to St Thomas cardioplegia for 10 min, 30 min, 1 hour, 2 hours, and 4 hours). The averages of each experiment from the cell culture experiments for each solution were used to plot graphs (first four hours and full incubation period). Moreover, for the cell culture experiment, at four hours of incubation with the 1x KHB solutions (negative control and different colloids) and positive control to test the different colloids, a one-way analysis of variance and Tukey's multiple comparisons test were performed. For the cell culture experiment with the 1x KHB solutions (negative control and different colloids) and positive control to test different perfusates a Kruskal Wallis with Dunn's multiple comparisons test was used, whereas for the Steen solutions (negative control and different colloids) and positive control to test different perfusates, a one-way analysis of variance and Tukey's multiple comparisons test were performed on the data to test for statistically significant differences in cell index between the different solutions at that timepoint. A p-value smaller than 0.05 was considered statistically significant.

Results

Histology *ex-vivo* machine perfusion experiments

The H&E histochemical staining was performed and followed by measurements of the SWT and AWT (in µM), as well as the LVWA (in μM^2) by ImageJ[62] of microscopical pictures (Olympus BX53, 4x magnitude) of the H&E stained mouse heart sections to determine whether a change occurs in the thickness of the heart walls and the size of the heart the longer the mouse heart is on the perfusion system and after switching to working mode. Timepoint 1 represents the mouse hearts that were exposed to 15-20 minutes of Langendorff, timepoint 2 includes the hearts exposed to 2 hours of Langendorff, timepoint 3 includes the hearts exposed to 4 hours of Langendorff, timepoint 4 includes the hearts exposed to 4 hours of Langendorff as well as 15 minutes of working mode, and timepoint 5 represents the hearts exposed to 4 hours of Langendorff and 1 hour of working mode. A one-way analysis of variance and Tukey's multiple comparisons test were performed ($p < 0.05$ was considered statistically significant) to compare the difference in the means of the SWT, AWT, and LVWA between the mouse hearts of the different timepoints. No statistically significant differences were found for the hearts between the different timepoints and after switching to working mode for SWT ($p > 0.9695$) (figure 4A), AWT ($p > 0.9836$) (figure 4B), as well as the LVWA ($p > 0.8035$) (figure 4C).

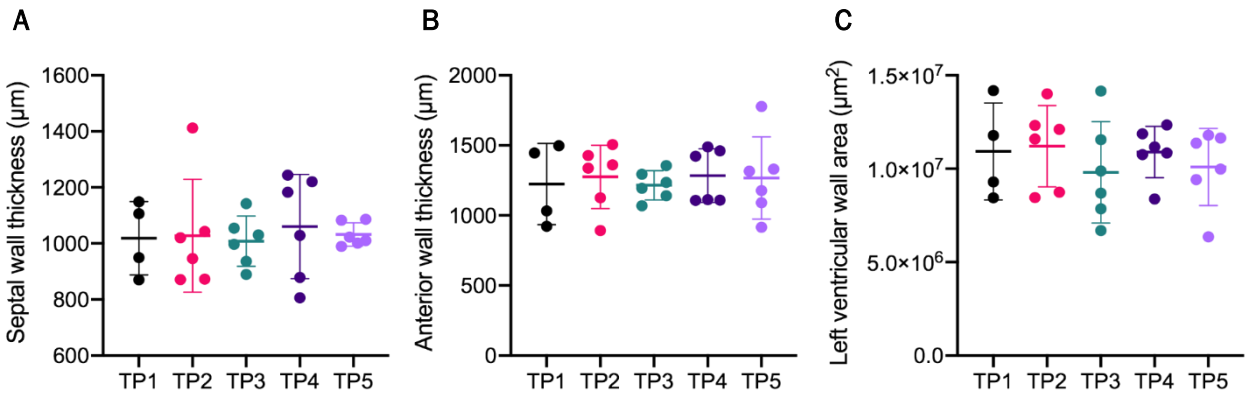


Figure 4 -Overview hematoxylin and eosin staining results for mouse hearts at different timepoints on the perfusion system. *The averages of the septal wall thickness (in µM)(4A), of the anterior wall thickness (in µM)(4B), and of the left ventricular wall area (in μM^2)(4C) for all the mouse hearts for each timepoint based on measurements of microscopical pictures (Olympus BX53, 4x magnitude) of the hematoxylin and eosin stained mouse sections with software ImageJ[62] are shown. Timepoint 1 represents the mouse hearts that were exposed to 15-20 minutes of Langendorff, timepoint 2 includes the hearts exposed to 2 hours of Langendorff, timepoint 3 includes the hearts exposed to 4 hours of Langendorff, timepoint 4 includes the hearts exposed to 4 hours of Langendorff as well as 15 minutes of working mode, and timepoint 5 represents the hearts exposed to 4 hours of Langendorff and 1 hour of working mode. A one-way analysis of variance and Tukey's multiple comparisons test were performed ($p < 0.05$ is statistically significant) with GraphPad (GraphPad Inc, San Diego, California USA). The differences in septal wall thickness ($p > 0.9695$), anterior wall thickness ($p > 0.9836$), and left ventricular wall thickness ($p > 0.8035$) between timepoints was shown to be not statistically significant.*

The PTAH histochemical staining was performed and followed by scoring the microscopical pictures (Olympus BX53, 4x magnitude) of the PTAH-stained mouse heart sections to determine whether a change occurs in the amount of ischemia the longer the mouse heart is on the perfusion system and after switching to working mode. The scoring was done by looking at the amount of ischemia based on the percentages of 4 categories. A brown colour was scored as 'severe ischemia', amount brown > blue scored as 'moderate ischemia', amount brown < blue scored as 'mild ischemia', and a blue colour was scored as 'no ischemia'. At timepoint 2, the average scoring of mouse hearts for 'severe ischemia' and 'no ischemia' was relatively the same and lower (0% and 44.2%) compared to timepoint 1 (0.3% and 77.2%). However, the percentage 'mild ischemia' as well as 'moderate ischemia', was higher for timepoint 2 (42.3% and 12.9% respectively) compared to timepoint 1 (20% and 2.5% respectively).

Comparing the mouse hearts of timepoint 2 to timepoint 3, the amount of 'moderate ischemia' is lower again from 12.9% to 5.8%, the amount of mild ischemia became higher for timepoint 3 (42.3% to 50.4%), and the amount of 'no ischemia' relatively stayed the same (44.2% to 43.8%). Then after 15 minutes of working mode (timepoint 4) the amount of 'no ischemia' was higher (55.4%) than timepoint 3 (43.8%). The amount of 'mild ischemia' was lower for timepoint 4 (36.6%) compared to timepoint 3 (50.4%) and the amount of 'moderate ischemia' relatively stayed the same for timepoint 4 (8.3%) and timepoint 3 (5.8%). However, at timepoint 5, after 1 hour of working mode, the amount of 'moderate ischemia' was higher (19%) compared to timepoint 4 (8.3%), the amount of 'mild ischemia' was lower (36.3% to 29.3%), and the amount of 'no ischemia' relatively did not change that much (55.4% to 51.7%). Taken it all together, the amount of ischemia was shown to be the lowest in the beginning (15-20 minutes of Langendorff), increased when comparing 15-20 minutes to two hours of Langendorff, but fluctuated afterwards, see figure 5, 6, and table 3.

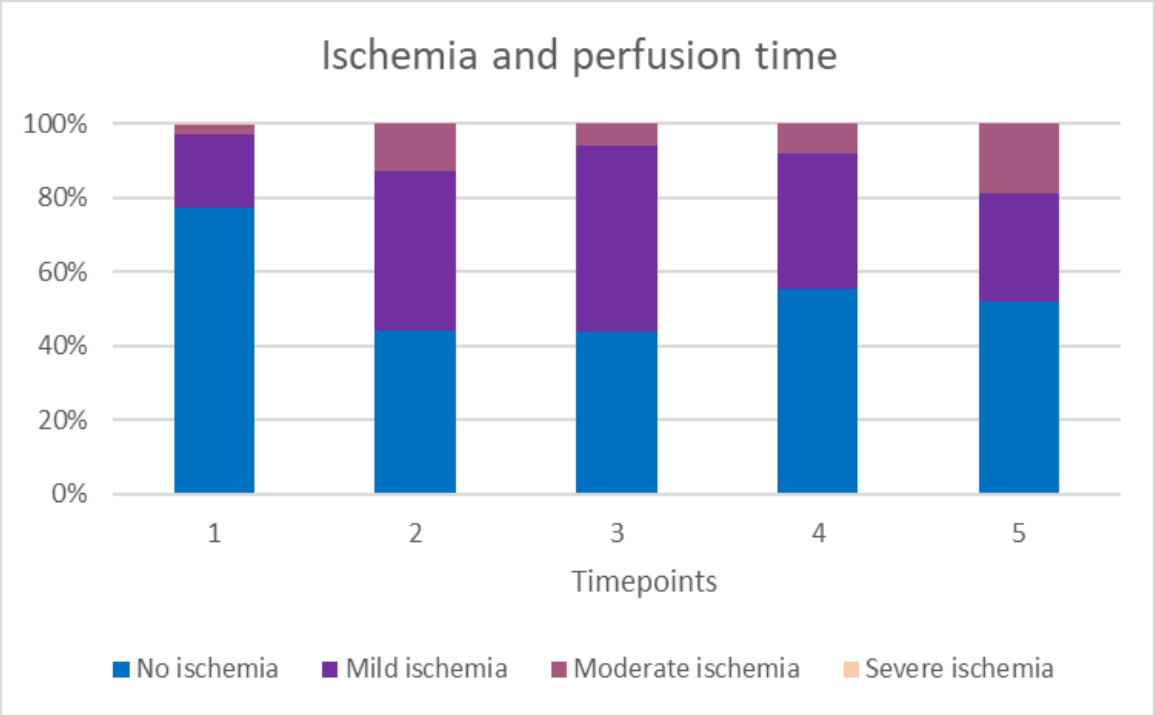


Figure 5 – Overview scoring results phosphotungstic acid hematoxylin stained mouse heart samples. *Averages scoring results of phosphotungstic acid hematoxylin stained mouse heart samples (based on microscopy, Olympus BX53, 4x magnitude) at different timepoints of perfusion. The scoring is based on the percentages of four different categories: a brown colour was scored as 'severe ischemia', amount brown > blue scored as 'moderate ischemia', amount brown < blue scored as 'mild ischemia', and a blue colour was scored as 'no ischemia'. Timepoint 1 represents the mouse hearts that were exposed to 15-20 minutes of Langendorff, timepoint 2 included the mouse hearts exposed to 2 hours of Langendorff, timepoint 3 includes the mouse hearts exposed to 4 hours of Langendorff, timepoint 4 includes the mouse hearts exposed to 4 hours of Langendorff as well as 15 minutes of working mode, and timepoint 5 represents the mouse hearts exposed to 4 hours of Langendorff and 1 hour of working mode.*

Timepoints	No ischemia (%)	Mild ischemia (%)	Moderate ischemia (%)	Severe ischemia (%)
Timepoint 1	77.2	20	2.5	0.3
Timepoint 2	44.2	42.3	12.9	0
Timepoint 3	43.8	50.4	5.8	0
Timepoint 4	55.4	36.3	8.3	0
Timepoint 5	51.7	29.3	19	0

Table 3 – Overview scoring results phosphotungstic acid hematoxylin stained mouse heart samples, in numbers. *Averages scoring results (in numbers) of phosphotungstic acid hematoxylin stained mouse heart samples (based on microscopy Olympus BX53, 4x magnitude) at different timepoints of perfusion. The scoring is based on the percentages of four different categories: A brown colour was scored as 'severe ischemia', amount brown > blue scored as 'moderate ischemia', amount brown < blue scored as 'mild ischemia', and a blue colour was scored as 'no ischemia'. Timepoint 1 represents the mouse hearts that were exposed to 15-20 minutes of Langendorff, timepoint 2 included the mouse hearts exposed to 2 hours of Langendorff, timepoint 3 includes the mouse hearts exposed to 4 hours of Langendorff, timepoint 4 includes the mouse hearts exposed to 4 hours of Langendorff as well as 15 minutes of working mode, and timepoint 5 represents the mouse hearts exposed to 4 hours of Langendorff and 1 hour of working mode.*

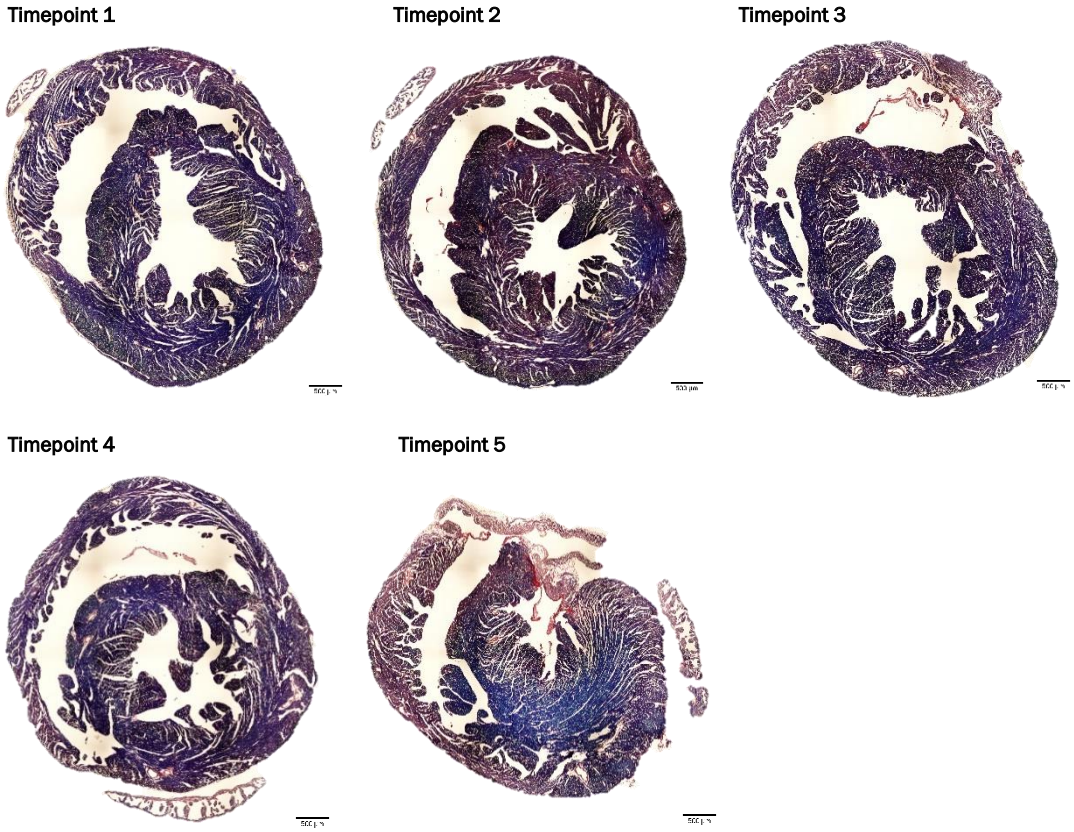


Figure 6 – Phosphotungstic acid hematoxylin stained heart samples. *Phosphotungstic acid hematoxylin stained mouse heart samples (microscopy, Olympus BX53, 4x magnitude) at different timepoints of perfusion. Timepoint 1 represents the mouse hearts that were exposed to 15-20 minutes of Langendorff, timepoint 2 included the mouse hearts exposed to 2 hours of Langendorff, timepoint 3 includes the mouse hearts exposed to 4 hours of Langendorff, timepoint 4 includes the mouse hearts exposed to 4 hours of Langendorff as well as 15 minutes of working mode, and timepoint 5 represents the mouse hearts exposed to 4 hours of Langendorff and 1 hour of working mode.*

The H&E histochemical staining was performed and followed by measurements of the SWT and AWT (in µM), as well as the LVWA (in μM^2) by ImageJ[62] of microscopical pictures (Olympus BX53, 4x magnitude) of the H&E stained mouse heart samples, to determine whether a difference can be found in the average thickness of the heart walls and the size of the heart between mouse hearts exposed to high (95% O_2 + 5% CO_2) and low (20% O_2 + 5% CO_2) oxygen levels. An unpaired t-test with Welch's correction was performed for the averages of SWT, AWT, and LVWA between high and low oxygen levels ($p < 0.05$ was considered statistically significant). No statistically significant differences were observed in AWT ($p = 0.7034$) (figure 7B) and LVWA ($p = 0.7478$) (figure 7C) for the mouse hearts exposed to low oxygen levels compared to high oxygen levels. However, SWT ($p = 0.0477$) (figure 7A) was found to be statistically significant.

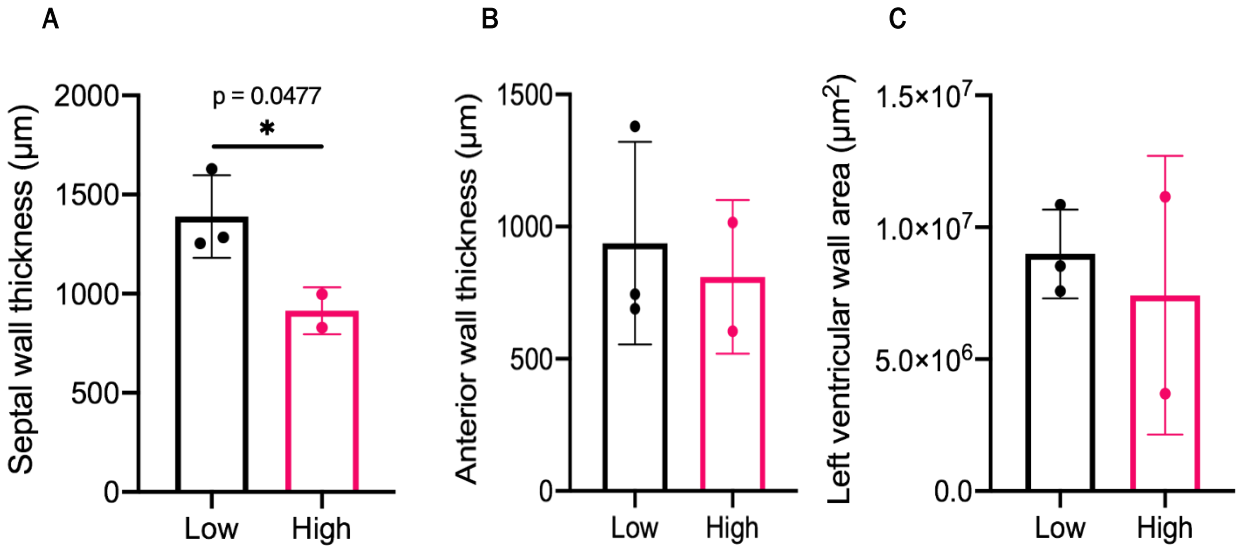


Figure 7 - Overview hematoxylin and eosin staining results for mouse hearts exposed to high and low oxygen levels on the perfusion system. *The averages of the septal wall thickness (in µM)(7A), of the anterior wall thickness (in µM)(7B), and of the left ventricular wall area (in μM^2)(7C) for all the mouse hearts exposed to high (95% O_2 + 5% CO_2) and low (20% O_2 + 5% CO_2) oxygen levels based on measurements of microscopical pictures (Olympus BX53, 4x magnitude) of the hematoxylin and eosin stained mouse hearts with software ImageJ[62], are shown. An unpaired t-test with Welch's correction was performed ($p < 0.05$ is statistically significant) with GraphPad (GraphPad Inc, San Diego, California USA) showing no significant differences in anterior wall thickness ($p = 0.7034$) and left ventricular wall area ($p = 0.7478$) of the mouse hearts exposed to a low oxygen level compared to the hearts exposed to a high oxygen level. However, the septal wall thickness ($p = 0.0477$), was found to be statistically significant.*

To determine whether a difference can be observed in the average cardiomyocyte size (in µM) between mouse hearts exposed to high (95% O_2 + 5% CO_2) and low (20% O_2 + 5% CO_2) oxygen levels, immunohistochemistry with WGA was performed. This was followed by fluorescence microscopy (Olympus BX53 20x magnitude together with a fluorescence illumination system X-Cite^R series 120 EXFO) of the WGA stained mouse heart sections followed by analysis of the microscopy images with the image analysis software Imaris (Oxford Instruments Imaris). Statistical analysis with GraphPad (GraphPad Inc, San Diego, California USA), using an unpaired t-test with Welch's correction ($p < 0.05$ is statistically significant) for the averages of the cardiomyocyte for all the mouse hearts showed that no statistically significant difference can be observed in the average cardiomyocyte size between mouse hearts exposed to high and low oxygen levels ($p = 0.7707$) (figure 8).

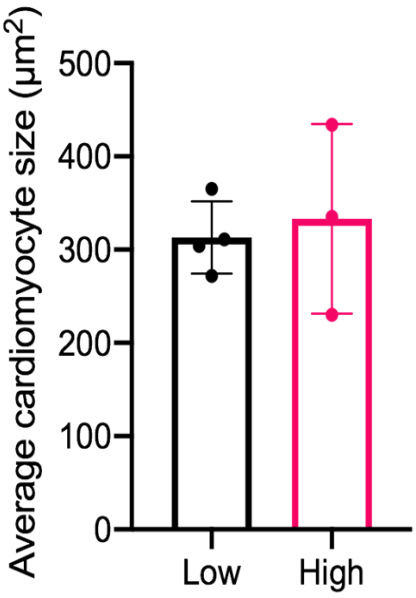
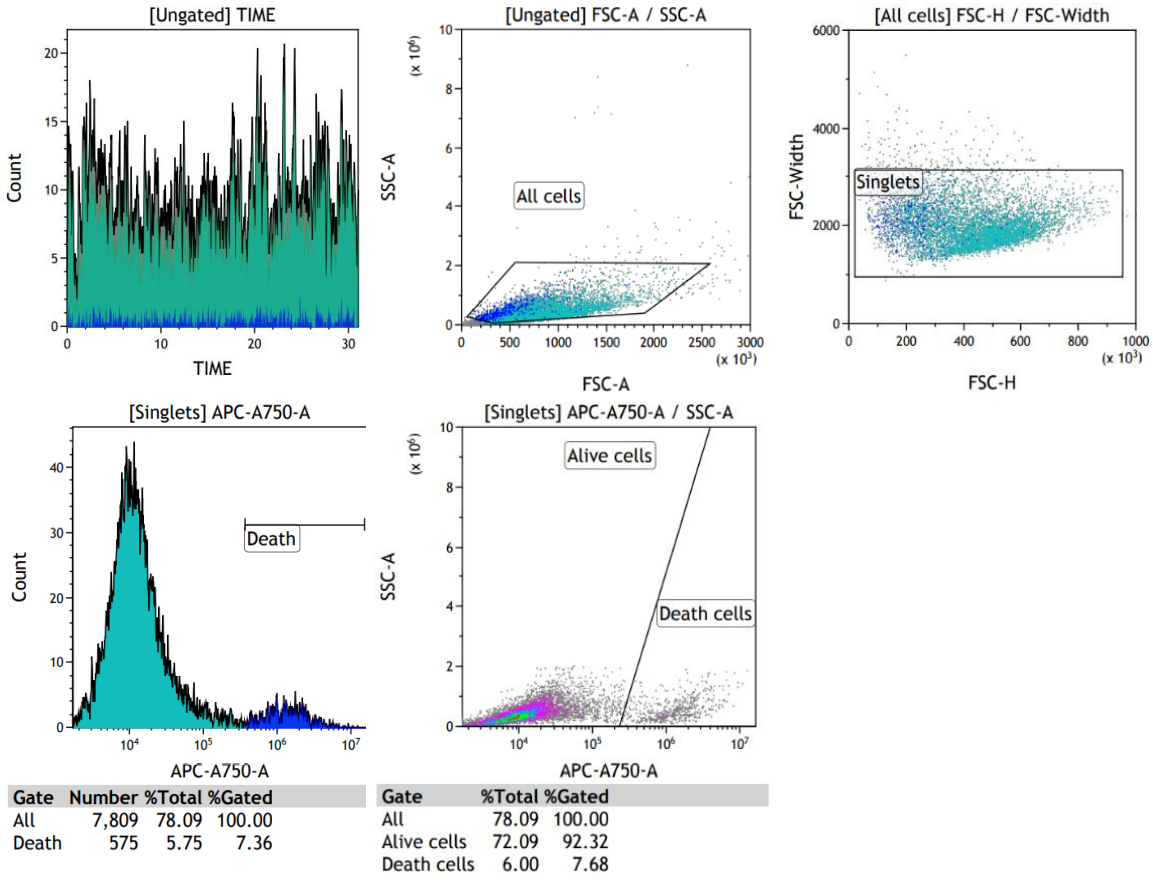


Figure 8 – Immunohistochemistry with Wheat germ agglutinin-FITC conjugated antibody results for mouse hearts exposed to high and low oxygen levels on the perfusion system. *In this graph the averages of the cardiomyocyte size (in µM²) of all the mouse hearts exposed to high (95% O_2 + 5% CO_2) and low (20% O_2 + 5% CO_2) oxygen levels were shown based on measurements of microscopical pictures (Olympus BX53 20x magnitude together with a fluorescence illumination system X-Cite® series 120 EXFO) of the Wheat germ agglutinin-FITC conjugated antibody stained mouse hearts sections followed by analysis with image analysis software Imaris (Oxford Instruments Imaris). A unpaired t-test with Welch's correction ($P < 0.05$ is statistically significant) with the averages of the cardiomyocyte size for all the mouse hearts (high and low oxygen levels) with GraphPad (GraphPad Inc, San Diego, California USA). no statistically significant difference can be observed in the average cardiomyocyte size between mouse hearts exposed to high and low oxygen levels ($P = 0.7707$).*

Flow cytometry

To test whether the viability of endothelial cells is affected during different incubation periods with St Thomas cardioplegia, HMEC-1 cells were exposed to St Thomas cardioplegia for different time periods (10 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours) and flow cytometry (CytoFLEX, Beckman Coulter) was performed afterwards with viability marker Zombie NIR™. The negative control represents HMEC-1 cells that were not incubated with cardioplegia, and the positive control are HMEC-1 cells not exposed to cardioplegia and received a heat shock/cold shock treatment. The data obtained after using the CytoFLEX (Beckman Coulter) were analysed with the software Kaluza (Beckman Coulter). The gating strategy used, is shown in figure 9. The gating for alive and death cells is based on a fluorescent intensity of death cells that is higher than 10^5 and the two separate groups (alive and death cells) shown in the results of SSC-A/APC-A750-A.



Gate	Number	%Total	%Gated
All	7,809	78.09	100.00
Death	575	5.75	7.36

Gate	%Total	%Gated
All	78.09	100.00
Alive cells	72.09	92.32
Death cells	6.00	7.68

Figure 9 – Results obtained by flow-cytometry of human dermal microvascular endothelial cells exposed to St Thomas cardioplegia and the gating strategy applied to the results of all incubation periods. *Flow cytometry results are shown for human dermal microvascular endothelial cells exposed to St Thomas cardioplegia for 10 minutes. Flow cytometry (CytoFLEX, Beckman Coulter) was performed with viability marker Zombie NIR™ and the obtained data was analysed with the software Kaluza (Beckman Coulter). The gating strategy used in Kaluza to measure the viability of the human dermal microvascular endothelial cells, is included in the results. The gating for alive and death cells is based on a fluorescent intensity of death cells that is higher than 10^5 and the two separate groups (alive and death cells) shown in SSC-A/APC-A750-A.*

An average was taken of the percentage alive cells of all cells (figure 9, SSC-A/APC-A750-A results) for all wells of each incubation period and controls. This experiment was performed three times and the averages were calculated for each incubation period over all the experiments, results are shown in figure 10. Statistical analysis of the data with a Kruskal Wallis with Dunn's multiple comparisons test ($p < 0.05$ is considered statistically significant) performed with GraphPad (GraphPad Inc, San Diego, California USA) showed that the percentage of alive cells of all cells was not statistically significant different for the averages of the positive control, negative control, and the different incubation periods (exposure to St Thomas cardioplegia for 10 min, 30 min, 1 hour, 2 hours, and 4 hours).

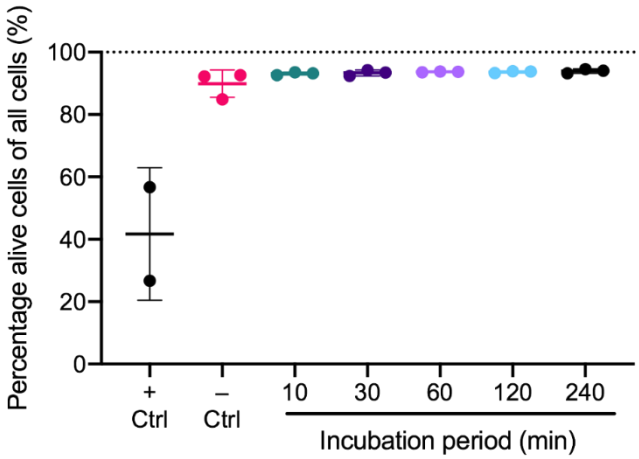


Figure 10 – Overview flow cytometry results of human dermal microvascular endothelial cells exposed to St Thomas cardioplegia for different time periods. *The averages (experiment performed three times) of the with Kaluza (Beckman Coulter) analysed flow cytometry (CytoFLEX Beckman Coulter, viability marker Zombie NIR™) results (in percentage alive cells of all gated cells) are shown for human dermal microvascular endothelial cells incubated with St Thomas cardioplegia for 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and controls. The negative control represents human dermal microvascular endothelial cells that were not incubated with cardioplegia and the positive control are human dermal microvascular endothelial cells not exposed to cardioplegia and received a heat shock/cold shock treatment. Performing a Kruskal Wallis with Dunn's multiple comparisons test ($p < 0.05$ is considered statistically significant) with GraphPad (GraphPad Inc, San Diego, California USA) showed no statistically significant difference in percentage of alive cells to all cells for the positive control, negative control, and the different incubation conditions.*

Cell culture experiments

To determine whether the viability of endothelial cells is affected differently by the exposure to different colloids, HMEC-1 cells were exposed to St Thomas cardioplegia for 1 hour followed by the exposure by either dextran-70, albumin, or PEG (all dissolved in perfusion solution 1x KHB). Incubation of HMEC-1 cells with 1x KHB without colloids represented the negative control and the incubation of the cells with pre-warmed HMEC medium (warm water bath, 37 degrees Celsius) is the positive control. The cell index (impedance, parameter for endothelial cell viability) of the cells was followed over time with a RTCA xCELLigence system (Roche)[67], see figure 11. This experiment was done four times. For all the wells of each solution the averages of the cell index were calculated for every experiment. Afterwards, the averages were calculated for the averages for each solution of all the experiments combined. The cell index of HMEC-1 cells exposed to 1x KHB with albumin remained relatively high for a longer time compared to the other incubation conditions (figures 11A and 11B). Although, performing a one-way analysis of variance and a Tukey's multiple comparisons test ($p < 0.05$ is statistically significant) with GraphPad Prism8 (GraphPad Inc, San Diego, California USA) on the averages of the cell index after 4 hours of incubation with the 1x KHB solutions (negative control and different colloids) and positive control, showed that no statistically significant differences in the cell index can be found between the different solutions at 4 hours of incubation (figure 11C).

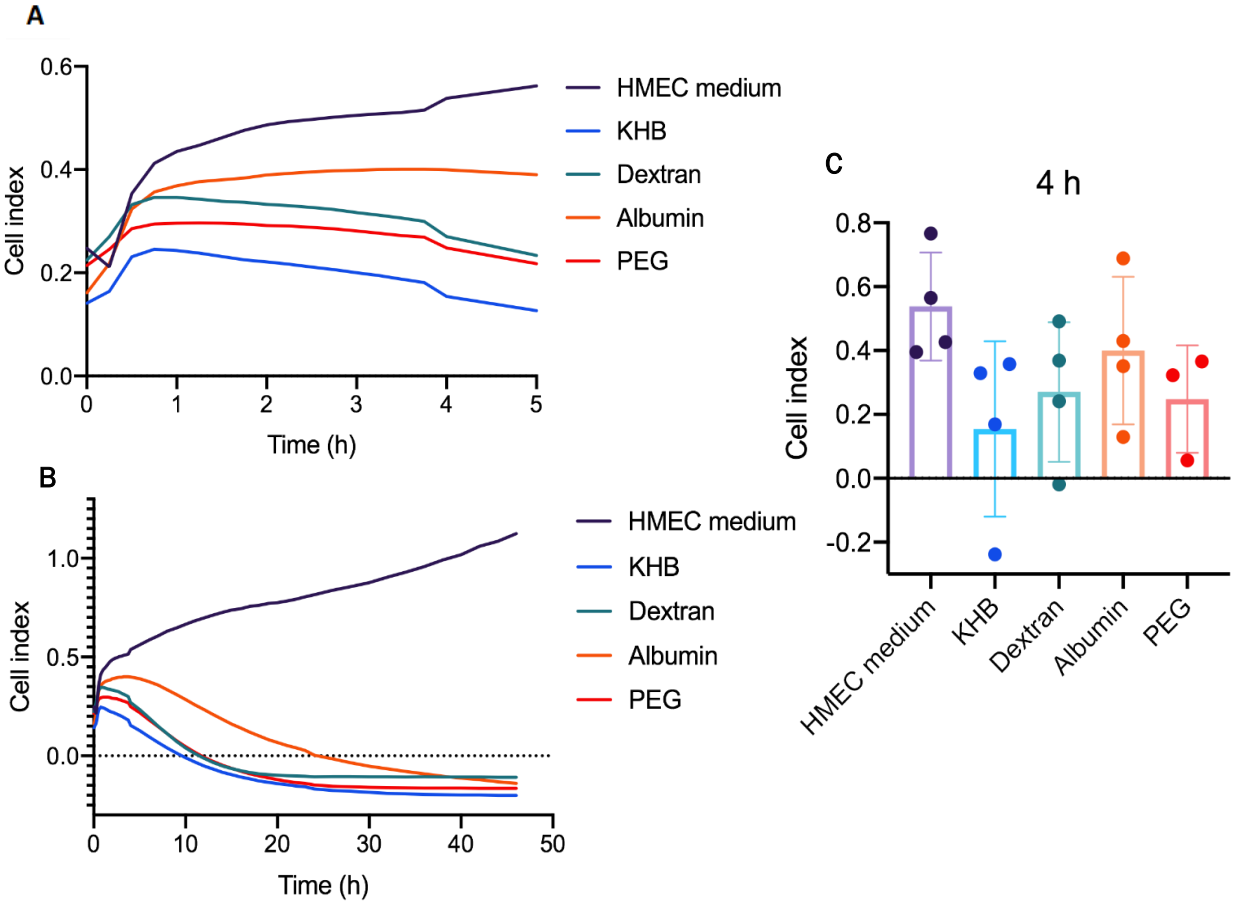


Figure 11 - Overview real time cell analyser xCELLigence system results of human dermal microvascular endothelial cells exposed to St Thomas cardioplegia and different colloids over time. The averages were measured (experiment performed four times) of the cell index measured by a real time cell analyser xCELLigence system (Roche) of human dermal microvascular endothelial cells exposed to St Thomas cardioplegia for 1 hour followed by the exposure by either dextran-70, albumin, PEG (all dissolved in perfusion solution 1x Krebs-Henseleit buffer, negative (only 1x Krebs-Henseleit buffer) or positive (pre-warmed HMEC medium, 37 degrees Celsius warm water bath) control for 2 days (figure 11A and 11B). A one-way analysis of variance and a Tukey's multiple comparisons test ($p < 0.05$ is statistically significant) with GraphPad Prism8 (GraphPad Inc, San Diego, California USA) showed no statistically significant difference in the averages between the different incubation conditions at 4 hours of incubation (figure 11C).

To determine whether the viability of endothelial cells is affected differently by the exposure to different perfusates, HMEC-1 cells were exposed to St Thomas cardioplegia for 10 minutes followed by the exposure by either dextran-70, albumin, or PEG (all dissolved in perfusion solution 1x KBH (figure 12A, 12C, and 12E) or Steen solution (figure 12B, 12D, and 12F)). Incubation of HMEC-1 cells with 1x KBH without colloids represented the negative control for the 1x KBH based solutions and incubation with Steen solution without colloids represented the negative control for the Steen solution based solutions. Besides, the incubation of the cells with pre-warmed HMEC medium (warm water bath, 37 degrees Celsius) is the positive control for both perfusates based solutions. The cell index (impedance, parameter for endothelial cell viability) of the cells was followed over time with a RTCA xCELLigence system (Roche)[67], see figure 12. This experiment was done one time. For all the wells of each solution the averages of the cell index were calculated. Comparing the results of the 1x KBH HMEC-1 exposure during previous experiment (in vitro experiment: effect of different colloids on endothelial cells) (figure 11) with the 1x KBH HMEC-1 exposure results of this experiment (figure 12), the cell index for the different incubation conditions seems to be closer to each other in this experiment. Besides, the cell index of albumin was found to be lower than the cell index of the other incubation conditions compared to the higher cell index found in previous experiment. Furthermore, performing Kruskal

Wallis with Dunn's multiple comparisons test ($p < 0.05$ is statistically significant) with GraphPad Prism8 (GraphPad Inc, San Diego, California USA) on the averages of the cell index after 4 hours of incubation with the 1x KBH solutions (negative control and different colloids), and positive control, showed that no statistically significant differences in the cell index can be found between the different solutions (figure 12E). However, after performing a one-way analysis of variance and a Tukey's multiple comparisons test ($p < 0.05$ is statistically significant) with GraphPad Prism8 (GraphPad Inc, San Diego, California USA), a statistically significant higher cell index was found for the positive control compared to the different Steen solution solutions (negative control $p < 0.0001$, dextran-70 $p = 0.0002$, albumin $p = 0.0014$, and PEG $p = 0.0002$) after 4 hours of incubation (figure 12F). In addition, over time the cell index of the HMEC-1 cells exposed to the Steen solution solutions seems to be lower than the cell index of the HMEC-1 cells exposed to the 1x KBH solutions.

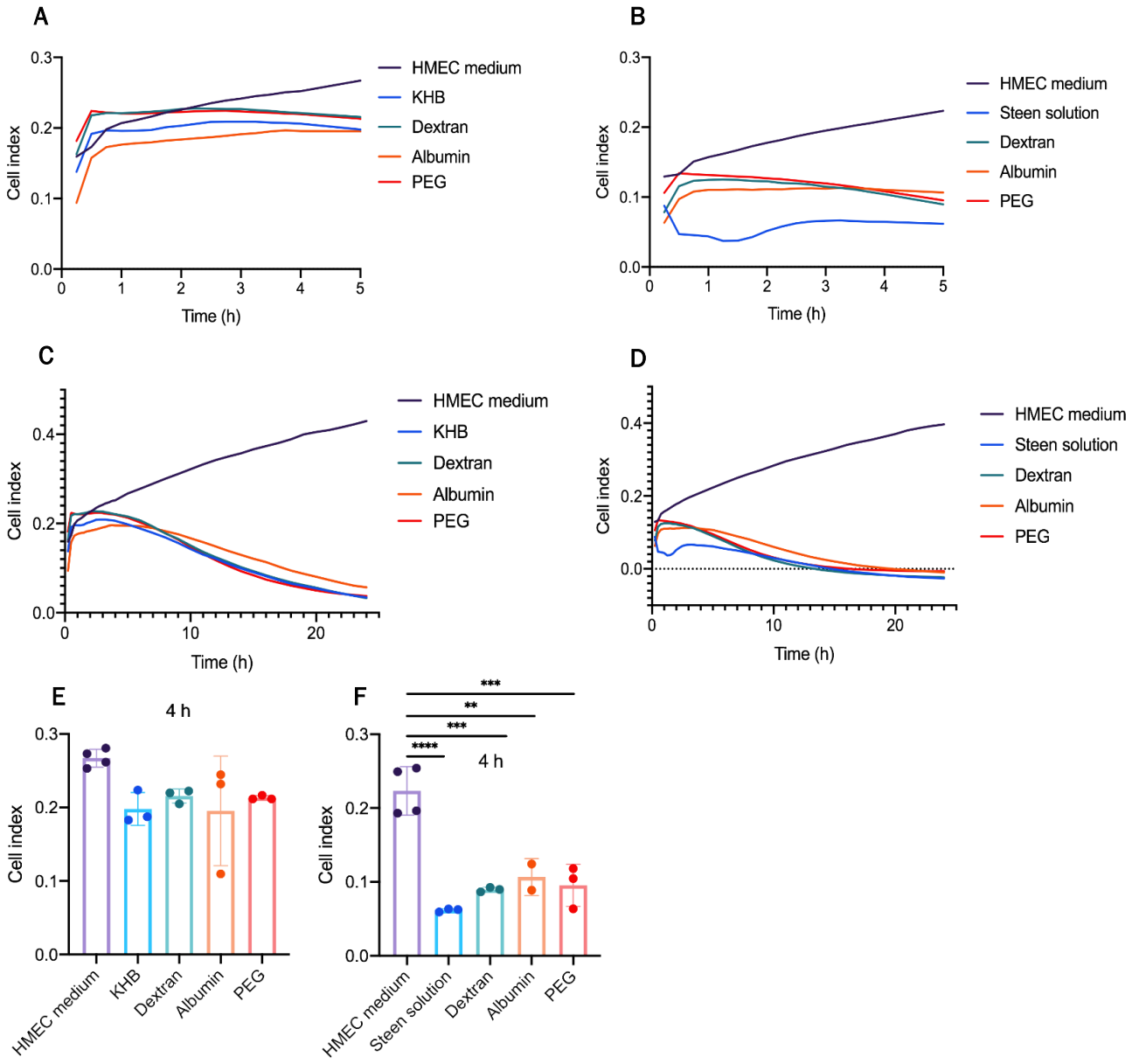


Figure 12 - Overview real time cell analyser xCELLigence system results of human dermal microvascular endothelial cells exposed to St Thomas cardioplegia and different perfusates as well as colloids over time. The averages were measured (experiment performed one time) of the cell index measured by a real time cell analyser xCELLigence system (Roche) of human dermal microvascular endothelial cells exposed to St Thomas cardioplegia for 10 minutes followed by the exposure by either dextran-70, albumin, PEG (all dissolved in perfusion solution 1x Krebs-Henseleit buffer or Steen solution), negative (only 1x Krebs-Henseleit buffer or only Steen solution) or positive control (pre-warmed HMEC medium, 37 degrees Celsius warm water bath) for 1 day (figure 12A, 12C, and 12E for results perfusate 1x Krebs-Henseleit buffer and figure 12B, 12D, and 12F for results perfusate Steen solution). A Kruskal Wallis with Dunn's comparisons test ($p < 0.05$ is statistically significant) with GraphPad Prism8 (GraphPad Inc, San Diego, California USA) showed no statistically significant difference in the averages between the different incubation conditions with 1x Krebs-Henseleit buffer at 4 hours of incubation (figure 12E), whereas after performing a one-way analysis of variance and a Tukey's multiple comparisons test, a statistically significant higher cell index can be found for all the different incubation conditions with Steen solution (negative control $p < 0.0001$, dextran-70 $p = 0.0002$, albumin $p = 0.0014$, and PEG $p = 0.0002$) compared to the positive control (figure 12F).

Discussion

At present, information is lacking about the optimal conditions for donor heart storage. During this study, an *ex-vivo* mouse heart perfusion system was set up using normothermic machine perfusion. The system was optimised for working in Langendorff Mode and Working Mode while perfusing the hearts for varied amounts of time. Moreover, due to complications and limitations regarding the use of blood-based perfusates, an acellular perfusion solution, KHB, was used[17]. Furthermore, to meet the metabolic demands of the heart perfused at normothermic temperature, the required oxygenation was tested in absence of an oxygen carrier[24]. In order to test specifically the preservation solutions as well as the effect of SCS on endothelial cells, and to optimise the perfusion solutions to minimise the amount of edema formation when one of the solutions will be used on the system, cell culture experiments were performed to examine the effect of a cold preservation solution and different combinations of perfusion solutions and colloids on the viability of endothelial cells[23,24,27,36,37,39,42]. No statistically significant difference is observed in wall thickness of the hearts perfused at normothermic temperature over time, whereas ischemia might have been shown. After perfusion in two different levels of oxygen, a statistically significant higher SWT was seen following exposure to low oxygen levels. However, the average cardiomyocyte size is not statistically significantly different between high and low oxygen levels. The *in-vitro* experiments showed that the endothelial cell viability was not statistically significant affected over different incubation periods with St Thomas cardioplegia. The cell index of endothelial cells exposed to KHB with albumin remained relatively high for a longer time compared to the other incubation conditions, although no statistically significant difference can be found between the solutions with different colloids after exposure of 4 hours. Besides, over time the cell index of HMEC-1 cells exposed to the Steen solution seems to be lower than that of HMEC-1 cells exposed to the KHB solutions. Nevertheless, the cell index of the positive control compared to the different Steen solutions and the negative control was statistically significant higher after 4 hours, whereas no statistically significant differences in cell index were found between the different KHB solutions and the negative control compared to the positive control after 4 hours of incubation.

After performing histology and microscopy analysis, no statistically significant differences were found for SWT, AWT, and LVWA between the different timepoints and after switching to working mode. This result was not expected because edema formation upon ischemia-reperfusion was found frequently in previous studies in hypothermic and normothermic machine perfusion, which increases cardiac wall thickness[26,54,55]. Here, SWT and AWT as indicators of edema formation were quantified by taking an average of wall thickness at three locations for both walls; it is possible that this method is not accurate enough to show a difference between timepoints. Using cell swelling and interstitial edema formation separately, as done in a previous study, could be a better way to examine heart edema development, because both mechanisms play a role in the occurrence of myocardial edema[55,68]. Interestingly, a porcine study involving a closed-chest ischemia-reperfusion for 40 minutes, sacrificing the pigs after 2 hours, 1, 4, and 7 days, found that edema formation in the myocardium followed a bimodal pattern. Specifically, after reperfusion, a sudden formation of edema was induced, which decreased to a low level at 1 day. However, myocardial edema elevated after 4 and 7 days. The occurrence of this second increase in edema was linked to tissue healing mechanisms[69,70]. The question remains whether the mechanisms of ischemia-reperfusion injury *in vivo* can be translated to the ischemia-reperfusion injury after mounting a heart on an *ex-vivo* perfusion system, as well as whether this reaction differs between species, e.g. using a murine or porcine heart. Moreover, knowledge about the bimodal pattern of edema formation can possibly be used to optimise this *ex-vivo* mouse heart perfusion system to administer specific therapeutics that reduce edema at the right time.

In addition, ischemia was shown to be the lowest in the beginning (15-20 minutes of Langendorff), increased when comparing 15-20 minutes to 2 hours of Langendorff, but fluctuated afterwards. Accounting for the mouse hearts on Langendorff mode and after switching to working mode, this suggests that ischemia can also be part of a dynamic process. However, it should be verified that colour changes in the used staining towards brown (ischemia) can be characterized as moderate and mild ischemia before conclusions can be made. During heart resuscitation using machine

perfusion, ischemia-reperfusion injury induces intracellular and interstitial edema. Blood vessel compression due to increased interstitial pressure and cell swelling can lead to the collapse of the smallest vessels, inducing no-reflow[26,71]. Besides, endothelial activation in response to ischemia-reperfusion can cause thrombus formation, resulting in decreased microvasculature perfusion. Lower tissue perfusion can induce additional ischemia[72]. Mouse hearts exposed to either 4 hours of Langendorff mode as well as 4 hours of Langendorff and 15 minutes of working mode, were shown to have less ischemia compared to those exposed to 4 hours of Langendorff and 1 hour of working mode. This may be caused by changed locations of the perfusion tubes when switching to working mode, causing a difference in exposure between myocardium regions to ischemia or reperfusion. This suggests that ischemia and reperfusion might be a dynamic process. This possibility for a dynamic process should considered when optimising different parameters, such as the perfusion solution and pressure[73–75]. For example, pressure overload was shown to aggravate ischemia-reperfusion injury in the myocardium[76]. Due to complications during the experiments, a few mouse hearts were treated as different timepoints, and the pressure of the perfusion solution through several hearts changed, even though the pressure was planned to be constant. This possibly led to different pressures in different regions of the heart and may have contributed to the fluctuations in the amount of ischemia. It would be interesting to look at the ischemia locations of each heart to determine where the perfusion pressures were possibly not optimal. Reducing the infarct size and edema with ischaemic preconditioning and postconditioning has been found to be successful in patients with pre-infarction angina and myocardial infarction[77]. It would be interesting to see whether applying these cardioprotective methods in an adjusted way diminishes ischemia-reperfusion injury in *ex-vivo* machine perfusion. Furthermore, examining other important markers for mechanisms linked to ischemia-reperfusion injury, such as oxidative stress and apoptosis, could give more information about heart functionality during machine perfusion[68].

No statistically significant differences were found in AWT and LVWA for the mouse hearts exposed to low oxygen levels compared to high oxygen levels. Even though more mouse hearts should have been included and measuring cell swelling as well as interstitial edema formation would have been a more accurate method, a statistically significant higher SWT was observed in hearts exposed to low oxygen compared to those exposed to high oxygen[68]. This could be explained by the lower myocardial perfusion flow and perfusion in the septum compared to the anterior wall of the heart, which makes this region more vulnerable for hypoperfusion[78–80]. A higher oxygen level seems to work better at preserving the heart. Moreover, no statistically significant difference can be observed in the average cardiomyocyte size between hearts exposed to high and low oxygen levels. Together with the potential evidence for increased SWT in hearts exposed to low oxygen, this might indicate that interstitial edema formation developed earlier compared to cell swelling. However, edema formation in the form of cell swelling was suggested to appear in the initial stages of ischemia during myocardial infarction, whereas cell swelling together with interstitial edema formation occurred upon reperfusion[69,70,81,82]. How edema, including cell swelling and interstitial edema, develops upon ischemia-reperfusion injury has not been elucidated for *ex-vivo* heart machine perfusion. Optimising the methods to measure cell swelling and interstitial edema formation, as well as including more mice and optimising the analysis of cardiomyocyte size with Imaris software (Oxford Instruments Imaris), are necessary to give valid conclusions. Information in how edema formation occurs during machine perfusion can give insight about when and how to prevent edema during *ex-vivo* machine perfusion.

Furthermore, to test the effect of the cold preservation solution (St Thomas cardioplegia) specifically on endothelial cells, HMEC-1 cells were incubated in the solution for different periods[65]. The percentage of alive cells was not statistically significant different for the averages of the positive control, negative control, and the different incubation periods (exposure to St Thomas cardioplegia for 10 min, 30 min, 1 h, 2 h, and 4 h). The variability between the few positive control samples is much

larger than that of samples within the negative control and the different incubation periods, and the percentage of alive cells in negative control is similar to all the different incubation periods, which might indicate that HMEC-1 cell viability is not affected during the different incubation periods for the first 4 hours. However, a variety of studies showed that damage to endothelial cells occurs when exposed to a crystalloid cardioplegic solution[36,83,84]. Nevertheless, a lot of studies overlooked how endothelial cell behaviour is affected by factors other than the solution, for example, temperature and incubation period[36]. Although HMEC-1 cell viability may not be different during the different incubation periods with St Thomas cardioplegia, the endothelial cells can be affected by the exposure. Functional measurements for endothelial cell permeability, such as the trans-endothelial electrical resistance assay and a transwell permeability assay, are options to determine the response over time to St Thomas cardioplegia[65,85,86].

To test whether a difference in endothelial cell viability can be found when exposing the HMEC-1 cells to different colloids over time, the RTCA xCELLigence system (Roche) was used. The cell index of HMEC-1 cells exposed to KHB with albumin remained relatively high for a longer time compared to the other incubation conditions. This can be explained by the fact that besides the antioxidant and anti-inflammatory effects of albumin, as well as providing a colloid osmotic pressure, albumin was suggested to prevent edema formation better than artificial colloid, partly because of albumin interacting with the glycocalyx of the endothelium. Considering this, albumin would be the preferred colloid to use[28,45,46]. Nevertheless, the cell index averages after 4 hours of incubation with the KHB solutions (negative control and different colloids) and positive control, showed that no statistically significant differences in cell index can be found between the different solutions at 4 hours of incubation. However, this depends on the perfusion solution composition. For example, when blood is included in the preservation solution, dextran might be beneficial as a result of decreasing leukocyte activation and their interaction with the endothelium. More information is needed about the interaction between the glycocalyx, the integrity of the vascular wall, and the inflammatory response, along with how they react to different perfusion solution compositions as well as factors of the hemodynamic system[45].

To test whether a difference in endothelial cell viability can be found when exposing the cells to different perfusates over time, experiments with the RTCA xCELLigence system (Roche) were performed. A possible explanation for the cell index of the different incubation conditions being closer to each other compared to the previous experiment when comparing colloids, is the optimisation of the KHB solution. Furthermore, the lower cell index of albumin relatively to the other perfusion solutions compared to the relatively higher cell index in the previous experiment, can be caused by damaged electrodes in one of the wells with albumin observed as a much lower cell index for one of the wells compared to the other two wells. Similar to the previous experiment, no statistically significant differences in cell index were found between the KHB solutions and the positive control after 4 hours of incubation. The cell index of the positive control compared to the different Steen solutions (negative control and different colloids) was statistically significant higher after 4 hours. Unexpectedly, over time the cell index of the HMEC-1 cells exposed to the Steen solutions seems to be lower than that of cells exposed to the KHB solutions, which may be due to suboptimal preparation of the Steen solution. The colloid concentrations in the Steen solution were lower than calculated (51.5 g/L instead of 57 g/L Dextran-70, 53.4 g/L instead of 60 g/L albumin, and 26.4 g/L instead of 30 g/L). Rather than using the commercially available new Steen solution used in lungs, a homemade Steen solution developed for heart perfusion was prepared[87,88]. Moreover, the statistically non-significant differences between different compositions of the Steen solution (negative control and different colloids) can be a cause of the solution originally not being developed for the cells at 37 degrees Celsius, normothermic machine perfusion, but for hypothermic machine perfusion[89,90]. It is questionable that normothermia would be significantly worse for the endothelial cells[42]. Furthermore, an important

component of Steen solution are red blood cells, which were not included during these experiments[87,88]. This could be another reason for the Steen solution not performing better than KHB. Moreover, the sudden decrease in cell index shown for the negative control of the Steen solution solutions, could not be explained. Examining the proliferation rate of the remaining endothelial cells might provide some answers[91]. In addition, despite the fact that the negative control of the Steen solutions does not consist of colloids, the expectation was that the cell index would be more similar to the negative control of the KHB solutions, and not performing worse, because Steen solution is optimised for organ machine perfusion[87]. However, more experimental replicates are necessary before any conclusions can be drawn.

Conclusion and future perspectives

To conclude, the results suggest that better preservation could be achieved with normothermic perfusion of mouse hearts in an *ex-vivo* heart perfusion system at high oxygen level compared to a low oxygen level. Moreover, ischemia for perfusion system optimisation may be important to consider. Furthermore, from the *in-vitro* experiments can be concluded that endothelial cell viability might not be affected over different incubation periods with St Thomas cardioplegia. Also, albumin may be preferred over the artificial colloids when only considering the experimental results, although for determining whether KHB or Steen solution is more optimal as perfusate on the system, additional experiments are necessary.

Several aspects can be improved, including the method for measuring edema formation and the analysis of cardiomyocyte size with the Imaris software (Oxford Instruments Imaris), as well as performing additional experiments to compare the endothelial cell viability when exposed to KHB and Steen solution. Additionally, the *in-vitro* method to mimic the situation of the endothelial cells in the mouse heart on the perfusion system can be improved by exposing the endothelial cells to the perfusion solution under flow. For example, the ibidi pump system can be used, followed by functional assays, such as real-time polymerase chain reaction to look at markers for endothelial dysfunction upon exposure to the different perfusion solutions under flow[92,93]. Moreover, antioxidants, anti-inflammatory drugs, and nutrients, e.g. amino acids and vitamins, have to be considered to optimise the perfusion solution for prolonged normothermic machine perfusion. Knowledge is lacking about whether improving the antioxidant capacity of the perfusion solution can optimise the preservation of hearts during normothermic machine perfusion[24,30]. In addition, the only source of energy for Steen solution and the perfusion solution provided for the Organ Care System (TransMedics, Andover, USA) is glucose, which is not enough to meet the organ metabolic demands during normothermic perfusion[17]. Currently, several promising biomarkers have been developed to assess the functionality of donor hearts, which are biomarkers for metabolism, tissue damage, function of the vessels, and inflammation, for example, the consumption of oxygen by the heart, vascular leakage, and lactate release. Besides, new ways of assessment are being developed, such as omics, exosome profiling, and heart imaging such as cardiovascular magnetic resonance. Moreover, machine perfusion makes it possible to measure the change in the amount of biomarker that is released over a period by sampling the perfusion solution, resulting in more accurate assessment of the heart functionality. Nevertheless, for identifying predictive factors that are reliable enough, the conditions during machine perfusion, for example temperature and the composition of the perfusion solution, must be standardised[94].

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