Histological analysis of Static Cold Storage vs Hypothermic Machine Perfusion in porcine hearts

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Abstract

Heart failure is a growing health issue and a frequent cause of mortality worldwide. Heart transplantation is the current gold standard to treat people that suffer from end-stage heart disease. Even though there has been significant progress in the field of heart transplantation over the last 45 years, there are still important challenges and obstacles facing the field, which limit the application of heart transplantation, including donor organ shortage. In this sense, the optimization of the process is crucial for medical practice. Currently, static cold storage (SCS) is the clinical standard for heart preservation. However, there is a number of factors that can complicate the procedure, including myocardial ischemia injury. On the other hand, hypothermic machine perfusion (HMP) is considered an ideal approach to extend the donor pool and increase the utilization rate. In the present study, we sought to investigate the effects of hypothermic machine perfusion vs static cold storage in slaughterhouse porcine hearts, in order to compare the two preservation strategies and therefore enhance the existing knowledge, regarding the histological changes that happen in these hearts during preservation. In this context, we used apical biopsies from porcine hearts that were preserved for 4 hours with SCS or HMP, and subsequent 4 hours of normothermic reperfusion to evaluate each method. H&E and PTAH staining were performed to assess the myocardial injury and mitochondrial damage. Altogether, this approach could not give us a comprehensive overview of the effect of HMP vs SCS on tissue integrity. Future studies are essential to further elucidate the effects of machine perfusion on porcine hearts.

Plain language summary

Heart failure is a serious medical problem and a major cause of death globally. Heart transplantation is currently the most effective treatment for people with end-stage heart disease. However, there are many challenges facing the field, including a shortage of donor organs. To address this issue, researchers are exploring different methods to improve heart preservation. The current standard method for preserving hearts for transplantation is called static cold storage (SCS). However, this technique has limitations, including the risk of myocardial ischemia injury. An alternative approach is hypothermic machine perfusion (HMP), which can help extend the donor pool and increase the utilization rate. In a recent study, researchers investigated the effects of HMP versus SCS on pig hearts. The goal was to compare the two methods and gain a better understanding of how they affect tissue integrity. In the present study, we took biopsies from pig hearts that had been preserved using either SCS or HMP for four hours and then reperfused the hearts at normal temperature for another four hours. We used special stains to evaluate the extent of myocardial injury and mitochondrial damage. Although this study did not provide a comprehensive overview of the effects of HMP versus SCS on tissue integrity, it is an important step in understanding the potential benefits of machine perfusion. By comparing the two methods, we hope to improve heart preservation techniques and increase the number of donor organs available for transplantation. Future studies are necessary to further explore the effects of HMP on pig hearts, as well as its potential benefits for human heart transplantation. Despite the challenges and limitations in heart transplantation, researchers are making important trials to improve heart preservation and increase the success rate of this life-saving procedure.

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Chapter 1: Introduction

1.1 Heart transplantation: A therapeutic approach towards heart failure

Despite the progress of medical research, heart failure (HF) still represents a severe clinical syndrome. 17.9 million people die from cardiovascular disease each year, with an estimated 32% of all global deaths (Şahin & İlgün, 2022). Hence, there is a high correlation between the age of the patients and the prevalence of HF: In developed countries, the prevalence of HF has been recorded in 1-2% of the adults, yet that number rises to over 10%, among people aged over 70 years. From a clinical perspective, the diagnosis of patients is arduous since many effects of the disease are unspecific (Hessel, 2021).

The defining moment for cardiovascular research came from the first human-to-human heart transplantation in 1967 by Christiaan Barnard and his team (Javier et al., 2021). At present, heart transplantation describes the most effective treatment for people that suffer from end-stage heart diseases, with more than 5.000 transplants being conducted annually. In agreement with the International Society for Heart and Lung Transplantation (ISHLT) and International Thoracic Transplant Registry, more than 73.000 heart transplants have been performed worldwide, of which more than 800 have been recorded in the Netherlands (Taylor et al., 2007). Though the percentage of people that currently wait for transplants is estimated at approximately 50.000 per year, unfortunately, this number keeps increasing.

Therefore, it is essential to discover methods that could optimize the process of transplantation. Essentially, a dominant regulator of this process is the preservation of the heart, prior to transplantation. Interestingly, James H. Southard et al. have defined organ preservation as *"the supply line for organ transplantation"*, to describe its role in myocardial protection (Southard & Belzer, 1995). In this sense, the preservation of the donors' hearts is one of the limiting factors for expanding the donor pool. Therefore, a **successful** preservation strategy aims to ensure the maintenance of organ viability, extend the time of the operation and ensure the safety of the process, in order to achieve the best clinical outcome.

1.2 Organ Preservation: The standard practice

Since the first trial of heart transplantation, static cold storage (SCS) was the gold standard method. Under these circumstances, the heart is supplemented with specifically designed cardioplegic solutions, that lead to cardiac arrest, followed by storage on ice. By SCS, the organ can be safely stored for up to 4 hours, without increasing the risk of primary graft dysfunction (PGD). Primary graft failure is a devastating complication that has been described as the leading cause of early death after heart transplantation, which can be related to ischemia-reperfusion injury (Struck et al., 1985; Tonsho et al., 2014).

1.3 Myocardial ischemia injury/Reperfusion injury

Myocardial ischemia is commonly termed as the condition where a deficiency in blood flow supply is incapable to provide the oxygen and the components that are essential for the metabolic requirements of the myocardium. Myocardial ischemia causes a cascade of detrimental events, that affect the function of the heart. Even though the mechanisms behind myocardial ischemia injury are not yet fully understood, it has been suggested that accumulation of Ca^+, induction of ROS, and accumulation of metabolic end products are the main effects that trigger the pathophysiology of ischemia-reperfusion injury (Hausenloy & Yellon, 2013).

Upon SCS, anaerobic metabolism occurs. In the absence of oxygen, mitochondrial oxidative phosphorylation rapidly ceases, resulting in the depletion of ATP production. On a cellular basis, this imbalance contributes to the high secretion of metabolites, including the accumulation of lactate and hypoxanthine. Initially, the high concentration of lactate causes a decreased cellular pH. Metabolism is affected progressively in a variety of different stages and pathways, including ion transport systems. Subsequently, to restore the low levels of pH, activation of the Na^+-H^+ ion exchanger promotes the escape of protons outside the cells and the entrance of Na^+. Cell swelling occurs, respectively. Next, in response to Na^+ overload, the reverse Na^+-Ca^+ exchanger enhances the intracellular Ca^+ concentration. During the initial stage of ischemia injury, the elevation of Ca^+ levels is the key element that stimulates specific enzymes, that will contribute to the generation of ROS. Accordingly, the accumulation of ROS initiates oxidative deterioration that will end in cardiomyocyte necrosis (Carden & Granger, 2000; Hausenloy & Yellon, 2013; Jennings, 213).

Notably, there is a spectrum of responses associated with myocardial ischemia-reperfusion injury, and it is essential to declare that these alterations act synergistically to affect cardiomyocytes. Reperfusion ischemia injury provokes, a variety of unfavorable effects that have an impact on the myocardium (Basso & Thiene, 2006). Upon the initiation of reperfusion and oxygen restoration, aerobic metabolism takes place. The reperfusion injury is associated with stimulation of inflammatory responses, arrhythmia, and severe damage in regions of cardiomyocytes that are already been severely impaired (Carden & Granger, 2000). Moreover, enzymes that were overexpressed during ischemia, such as NADPH oxidases and xanthine oxidases, are capable of generating ROS, when oxygen is present. Subsequently, ROS, as the most dominant outcome during reperfusion injury, lead to oxidative stress and membrane damage. As a result, Ca^+ overloading takes place, which stimulates the opening of the Mitochondrial Permeability Transition Pore (Carden & Granger, 2000). Due to this, the selectivity of the mitochondrial membrane decreases, which results in mitochondrial swelling and loss of cristae. Under these mechanisms, ROS accumulation contributes to mitochondrial damage, cell death, and distributed blood vessels, which will lead to severe hemorrhage. Accordingly, these parameters lead to activation of the immune system and, initially, the outcome is cell death by apoptosis and necrosis (Carden & Granger, 2000; Halestrap, 2004).

1.4 Hypothermic Machine Perfusion and Normothermic Machine Perfusion: A Potential Therapeutic Approach

Since 2009, the Registry of the International Society for Heart and Lung Transplantation has recorded that extending the time of ischemia, causes a higher risk of early graft dysfunction and accordingly, an increased mortality rate (Taylor et al., 2009). Hence, the time that the heart can be preserved by cold storage is limited. To this end, it is crucial to discover alternative approaches, to maintain heart function, thus, preventing the detrimental effects of prolonged ischemia during preservation.

Hypothermic machine perfusion (HMP) describes a new, sufficient, and safe technology that aims to set up a proper environment for hypothermic storage by utilizing machine perfusion (Michel et al., 2014). During preservation, the heart is connected to an ex-situ perfusion system, which supplements the organ with oxygen and nutrients that are required for the metabolic pathways. The hypothermic machine perfusion system is capable of maintaining the temperature from 4°C to 8°C (Madsen, 2014). In contrast to SCS, the continuous oxygenized crystalloid-based solution enables the restoration of ATP levels, via oxidative metabolism. Simultaneously, lactate and end products of metabolism are eliminated and washed out, while cellular procedures continue to occur. Therefore, by decreasing the risk of myocardial ischemic injury, the preservation time can be extended (Ou et al., 2014; Qin et al., 2022). Importantly, earlier studies have demonstrated that 12 hours of hypothermic crystalloid perfusion provided myocardial preservation superior to cold storage, with functional and aerobic metabolic recovery approaching normal levels (Ou et al., 2014).

Pioneering work using the hypothermic machine perfusion system came by Nilsson et al, in which they performed the first human cohort. In this study, SCS and HMP were compared in brain-dead donors. The outcome indicates differences between those two groups, as all patients in the HMP trial had an event-free survival in contrast to those who were subjected to SCS. However, this statement is not yet established, and more research is needed (Nilsson et al., 2020).

In current clinical practice, two systems of machine perfusion have been identified: Hypothermic machine perfusion, and normothermic machine perfusion. Both approaches provide better preservation compared to SCS since they reduce oxidative stress and DNA damage. Moreover, the techniques have the advantage of aerobic metabolism and at the same time the removal of toxic byproducts, such as lactate (Ou et al., 2014). However, in HMP the heart is continuously perfused with a cold oxygenated solution while in NMP, warm blood supplements the heart with oxygen and energy demands. During NMP, the heart is kept at 37^{o}C, and therefore the organ is closer to its standard, physiological condition. In this sense, the heart can be preserved and evaluated in a normal state (Jing et al., 2018).

1.5 Aim of this study

Nowadays, there is increasing knowledge indicating that machine perfusion can prevent the negative effects of ischemia-reperfusion injury during heart preservation. Notably, a recent study performed by John M. Trahanas and colleagues aims to investigate the effects of 12 hours of NMP in myocardial structure. Histological analysis revealed no evidence of tissue damage throughout perfusion. Hence, the structure of the tissue was preserved, with no impact on the endothelial cells or the myocardial tissue (Trahanas et al., 2016).

As mentioned above, mitochondria play a key role in ischemia/reperfusion injury. In respect of mitochondrial injury, studies reported differences between perfused and cold-storage hearts. In fact, 12 hours of cold storage led to severe damage in mitochondrial phenotype, in contrast to perfused hearts, in which this pattern was not observed (Michel et al., 2015). Therefore, targeting the restoration of mitochondrial function may provide a potential therapeutic approach for heart failure.

Despite all these well-demonstrated findings, more evidence is still needed to prove and investigate the beneficial effects of the HMP, even at a shorter time of perfusion. Therefore, this focus study aims to investigate and compare the effects of hypothermic machine perfusion and initial normothermic reperfusion in porcine ex-situ perfused hearts, as to standard cold storage. *Why is there a healthier pattern in the hearts that are perfused by HMP technology, and how does this translate on a histological level?* This question is the main purpose of this research, to provide a clearer perception of tissue damage during heart storage. Within this context, we aim to enhance the existing knowledge, regarding the histological changes that happen in these hearts during preservation.

Chapter 2: Methods

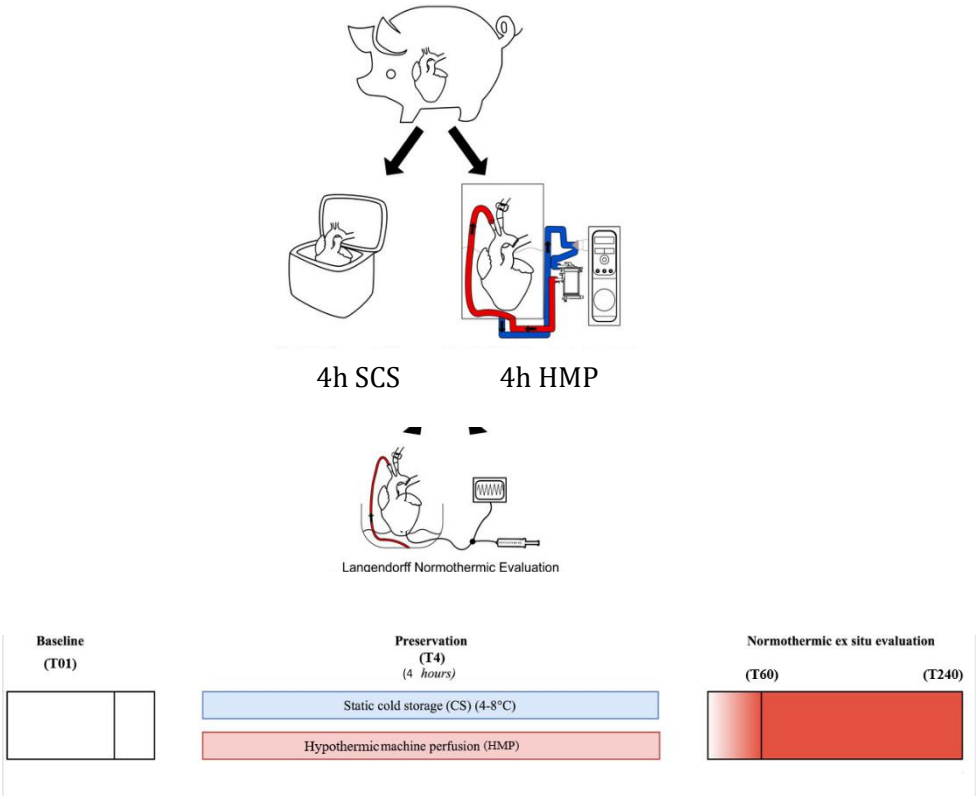

Figure 2.1: Overview of the study design and timing for endpoints. *Main figure adapted by* van Suylen et al., 2022

In this study, we used apical paraffin-embedded biopsies of ex-situ perfused porcine hearts, that were already been taken after harvesting, four hours post-preservation (Hypothermic machine perfusion/ Static cold storage), 60 minutes, and finally 4 hours after normothermic reperfusion. We will use the following two methods: Machine perfusion and static cold storage.

2.1 Slaughterhouse animals

Nineteen pigs were used for this study. After being sacrificed by means of electrical stunning, a parasternal incision was made in the thorax and then the heart was removed en-bloque. After harvesting, the heart was immediately cooled by submersion in cold saline. After opening the pericardial sac, the aorta was cannulated, and a crystalloid cardioplegic solution was administrated to the heart.

2.2 Preservation

When the cardioplegic solution was added, the hearts were preserved for 4 hours either using SCS or HMP. 10 hearts were preserved with SCS and 6 with HMP. The hearts in the SCS

group were retrospectively divided into 2 separate groups depending on their function. 4 hearts that maintained their function, and survived, formed the SCS survival group. Survival was defined by a CO >3 L with a MAP >60 mmHg and a LAP <15 mmHg. The rest 9 hearts that did not, form the SCS non-survival group.

2.3 Hypothermic machine perfusion

For the HMP group, the hearts were perfused with a homemade modified Steen heart solution at 4ºC. After harvesting, the hearts were placed in the perfusion box system (XVIVO Perfusion, modified Kidney Assist transport box). The heart box system consists of a reservoir, a pulsatile-driven rotary pump (60BPM), and a control unit. The hearts were perfused with a pulsatile aortic pressure to aim for a coronary flow of 100-200 mL/min. The temperature was regulated and maintained at around 8°C by placing ice around the reservoir. The hearts were preserved on HMP for 4 hours.

2.4 Static cold storage

Hearts were submerged in the crystalloid cardioplegic solution and preserved on ice for 4 hours.

2.5 Normothermic evaluation

After preservation, all hearts were reperfused ex-vivo using the PhysioHeartTM platform. For the reperfusion, approximately 3000 mL of normothermic, heparinized oxygenated whole blood was used, which was supplemented with glucose and insulin, and mixed with 1500 mL crystalloid prime (Krebs-Henseleit buffer). During normothermic reperfusion, the hearts were first perfused for 60 minutes in Langendorff mode, followed by 3 hours of working heart mode perfusion to access heart function. Atrial pressure and aortic pressure were constantly measured. A glucose and insulin mixture was supplemented to keep the blood glucose level at sufficient levels. Calcium was also supplemented when needed. Furthermore, during the conduction of the experiment, cardiac output and coronary flow were continuously monitored.

2.6 Sample Collection

Biopsies have been collected from the apex of each heart at 4 time points: at the baseline, after 4 hours of preservation (SCS/HMP), after 60 minutes, and after 4 hours of Normothermic Reperfusion ***(Figure 2.1)***. At the end of the experiment, biopsies were preserved and subsequently embedded in paraffin. Next, a microtome cutting instrument (ADAMAS INSTUMENTEN BV Microm HM 355S) was used to cut the paraffin-embedded tissue blocks into slices with a thickness of 3,5 μm. Afterward, two types of staining were performed to assess the functionality of the hearts.

2.7 Stainings

Prior to staining, all slides were deparaffinized in xylene 3 times, ethanol 100% 2 times, and alcohol 70%. Finally, the samples were washed in demineralized water.

- **Haematoxylin and Eosin (H&E) staining**

To assess general tissue morphology, a haematoxylin and eosin (H&E) staining was performed. The haematoxylin stains the nuclei blue and the cytoplasm and the extracellular matrix (ECM), pink. The H&E staining was performed at the *Department of Pathology, at UMC Utrecht.*

❖ **Phosphotungstic acid-haematoxylin (PTAH) staining**

PTAH stain was performed in order to assess and compare mitochondrial injury. The haematoxylin binds to mitochondria, erythrocytes, and glial fibers nuclei and stains blue. On the other hand, phosphotungstic acid binds to collagen, fibrin, and connective tissue, and stains brown/red color. Firstly, the samples were deparaffinized in xylene 3 times, ethanol 100% 2 times, and alcohol 70%, and finally washed in demineralized water. Then, ammonium iron (III) sulphate was added and the slides were incubated for 30 minutes. Subsequently, slides were rinsed with demineralized water and after oxalic acid (5%) was added. After 10 minutes of incubation, samples were washed one more time with demineralized water, and then phosphotungstic acid was added. Next, the slides were incubated at 56 degrees for approximately 3 hours. Finally, the slides were rapidly dehydrated using 70% alcohol and 100% alcohol 2 times, after which they were washed in xylene 3 times. The slides were then covered and they were ready to be evaluated under the OLYMPUS BX53 Fluorescence Microscope.

2.8 Histological analysis

❖ **Myocardial injury scoring**

For the analysis of the H&E staining, the myocardial injury was scored based on the scoring system adapted by the group of *J.M. Trahanas et al*. Myocardial injury scoring was performed based on the presence and severity of myofiber degeneration, myocardial hemorrhage, interstitial edema, and endothelial alterations. A myocardial injury grading scale was used to score and evaluate each heart (***Table 1***). The average score was compared for each group between the SCS survival, SCS non-survival, and HMP group. Slides were evaluated and scored under the confocal microscope.

Tissue degradation can be described as the accumulation of muscle damage and cell death driven by multiple factors including inflammation, abnormal mechanical forces, and altered vascularization. On this basis, muscle damage could be visualized under the microscope with a pale pink formation. The scoring for myofiber degeneration was performed according to how extended the region of tissue damage was on each sample (***Figure 2.2.A.***). As regards myocardial hemorrhage, the assessment was performed according to the accumulation of erythrocytes in each tissue sample (***Figure 2.2.B.***). Edema, which is one of the greatest concerns of myocardial injury, was visualized as the excess accumulation of fluid in the myocardial interstitium (***Figure 2.2.C.)***. Finally, endothelial changes were the most triggering part to score, because of the large variation between the size of the tissue samples. Nevertheless, abnormal structures in cardiac vessels have been reported in all of the groups (***Figure 2.2.D.)***.

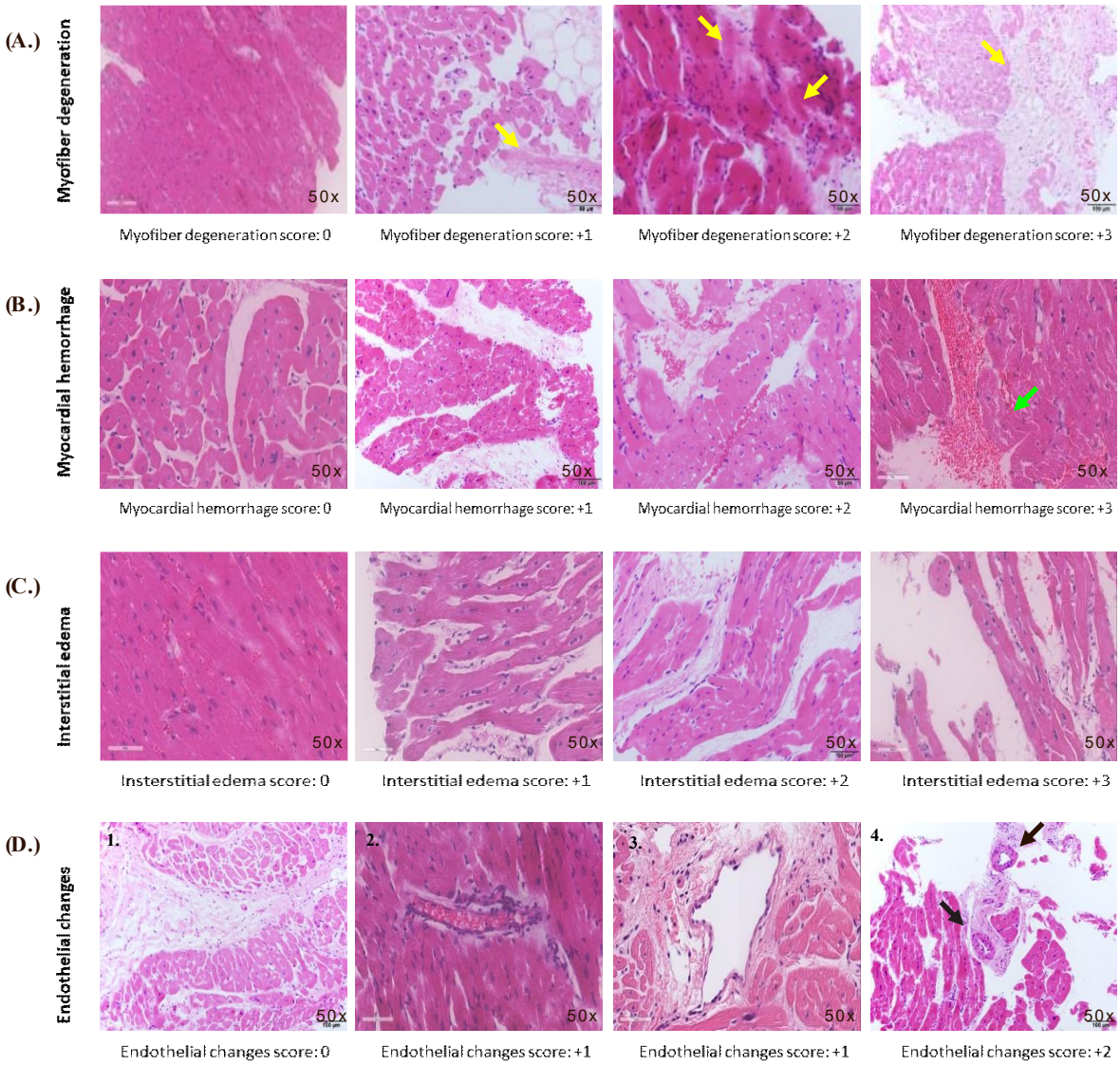

Figure 2.2: Myocroscopy images after H&E staining. *Myocardial injury was graded based on the presence and severity of* ***(A.) myofiber degeneration****: The scoring was performed according to how extensive the region of the damaged tissue was (yellow arrows),* ***(B.) myocardial hemorrhage:*** *The assessment was based on the accumulation of erythrocytes in the tissue(green arrow),* ***(C.) interstitial edema:*** *Edema was evaluated according to the fluid that separates the cardiomyocytes, and* ***(D.) endothelial alterations: D.2,*** *Endothelial cells in the vessel have a pump shape instead of the normal round/* ***D.3,*** *Abnormal structure of the vessel was scored as +1****, D.4*** *Two arterioles with different structures (black arrows).*

❖ Mitochondrial assessment

For the analysis of the PTAH staining, reperfusion injury was evaluated by focusing on mitochondrial damage. On this basis, all stained slides were analyzed using QuPath software 0.4.2. QuPath software is a tool that enables to quantify and measure the area of the tissue which was stained blue (***Figure 2.3.)***. . Measurements of the whole tissue and the blue stained area of interest were obtained by defining two different thresholds, respectively. These measurements corresponded to the presence of the blue stain in each heart biopsy. However, since there was a large variation in the size of the samples among each group, each heart was evaluated by taking four individual snapshots of each biopsy: one snapshot from the upper edge of the tissue, one from the bottom edge, and two from the middle. Next, the blue positively stained area was analyzed in the QuPath software, for each individual image. Finally, the pool of the four images per sample was used for the statistical analysis. This principle was followed for each heart among the groups. Images of each biopsy were taken using OLYMPUS BX53 Fluorescence Microscope.

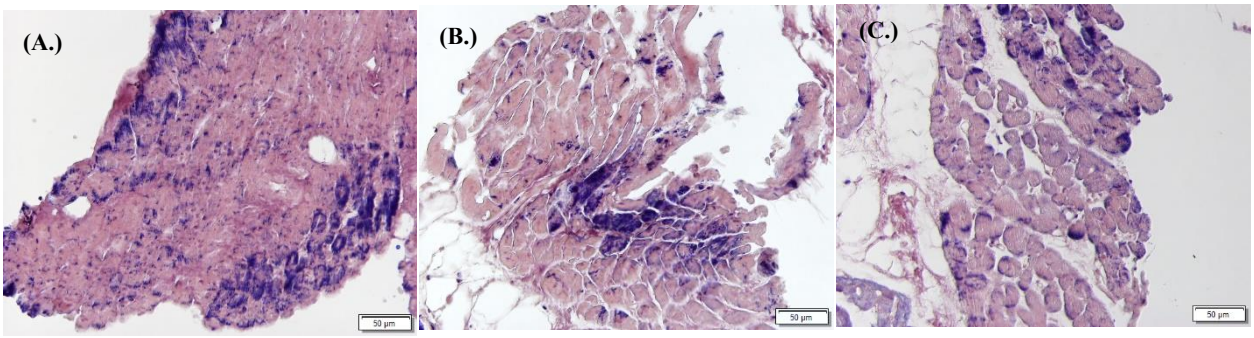

Figure 2.3: Myocroscopy images after PTAH staining. *Examples of images after PTAH staining for* ***(A.)*** *HMP group,* ***(B.)*** *SCS survival group,* ***(C.)*** *SCS non-survival group.*

2.9 Statistical analysis

By calculating median scores for each group, a statistical comparison was made using two-way mixed ANOVA analysis to assess differences between groups. The obtained data were interpreted in graphs by using GraphPad software 8.0.1.

Chapter 3: Results

3.1 Myocardial injury scoring

Assessment of the slides under the microscope revealed that myofiber degeneration, myocardial hemorrhage, interstitial edema, and endothelial alterations were present in both preservation techniques: hearts that were stored by SCS and hearts by HMP. No significant difference was observed (***Figure 3.1).***

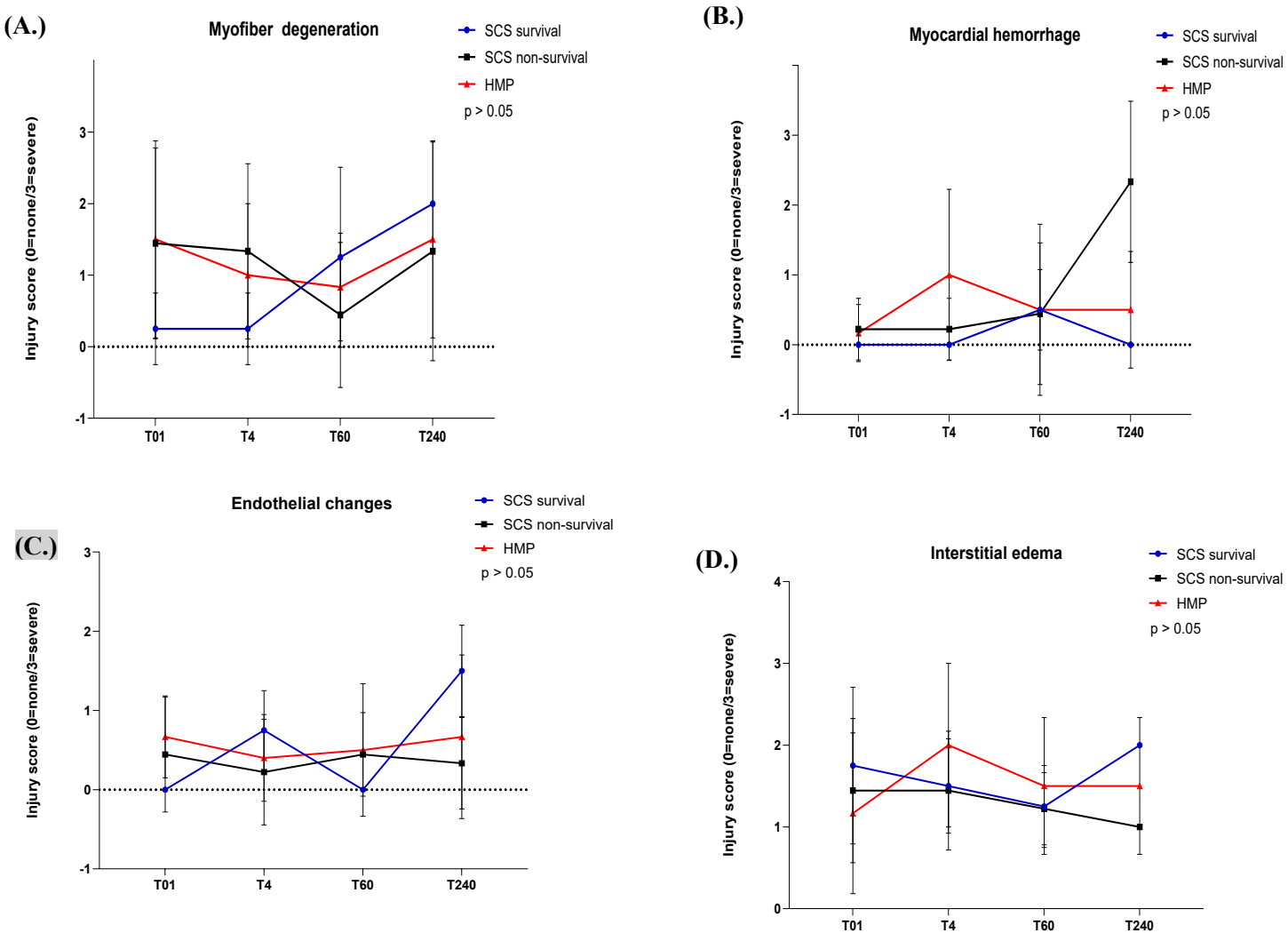

Figure 3.1: Myocardial injury scoring. *For* ***(A.)*** *Myofiber degeneration,* ***(B.)*** *Myocardial hemorrhage,* ***(C.)*** *Endothelial changes,* ***(D.)*** *Interstitial edema*

3.2 Detection and quantification of mitochondria

Mitochondrial injury can be one of the main causes of cardiac tissue injury. In this context, we sought to investigate whether we could find a correlation between the condition of mitochondria for each preservation method. Therefore, we performed the PTAH staining followed by QuPath software analysis, as described in Chapter 2.8. To this aim, we aim to detect and quantify the positively stained blue region in each tissue sample.

Regards mitochondrial destruction, no statistical significance was observed. However, upon preservation, the PTAH analysis showed a trend, in which mitochondrial damage was detected less in the HMP group, following the SCS survival and the SCS non-survival group (***Figure 3.2***). However, during normothermia reperfusion, this trend changes.

During the first 60 minutes of reperfusion, there is an overall increase in the positively stained area in all groups. Hence, the SCS non-survival group was evaluated with the highest levels. At the end of the experiment and the normothermic assessment, all groups were approximately at the same level of positively stained area detection. Nonetheless, no significant difference could be seen in this analysis (***Figure 3.2***).

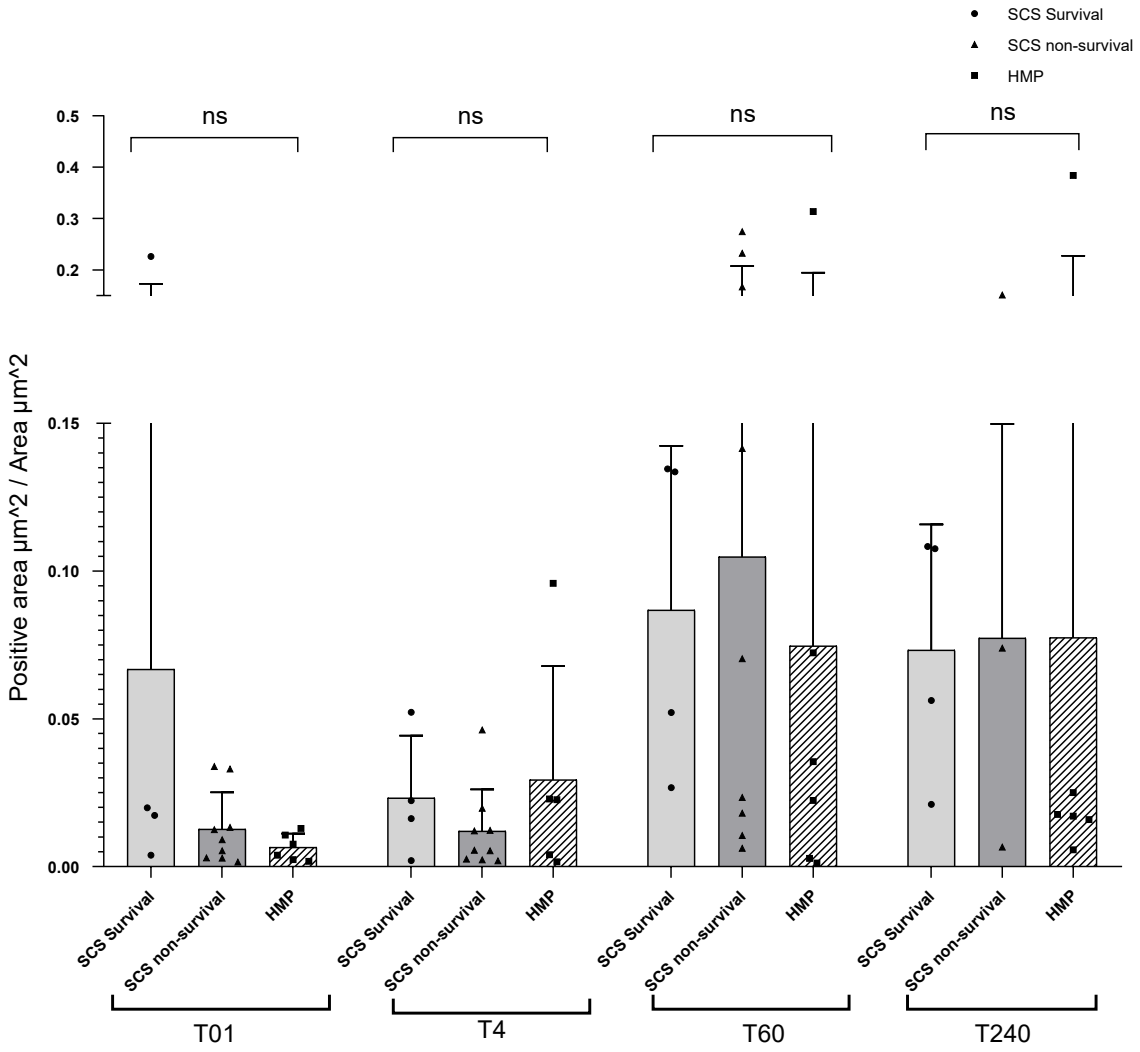

Figure 3.2: Comparison of mitochondrial damage using PTAH staining. *The assessment was performed at baseline (T01), after 4 hours of preservation SCS/HMP, after 60 minutes of reperfusion (T60), and after 4 hours of reperfusion (T240).*

Chapter 4: Discussion

According to World Health Organization (WHO) statistics, ischemic heart disease ranks as the greatest cause of mortality in humans (Şahin & İlgün, 2022). Heart transplantation describes the golden standard of treatment for people that suffer from end-stage heart diseases. Machine perfusion during preservation has been shown to reduce ischemic damage to the donor heart in various animal models. This investigation was performed as an effort to assess the effects of SCS and HMP on cardiac tissue integrity (Qin et al., 2022; van Suylen et al., 2022). Therefore, our ambition was to compare the two methods by finding a correlation between cellular architecture and cardiac function.

To this aim, we first focused on the cellular structure by looking at the overall picture of each tissue. Samples were embedded in paraffin, sectioned, and stained with H&E. The primary advantage of this technique is that the staining is relatively simple to perform and gives an overview of the tissue architecture. We used the H&E staining and then we scored each heart for myofiber degeneration, myocardial hemorrhage, interstitial edema, and endothelial alterations. Due to myocardial ischemia/reperfusion injury, myocardial reperfusion may itself induce further cardiomyocyte death. Therefore, reperfusion injury was expected to be seen in all groups. Previous findings have shown that machine perfusion maintains tissue viability with no observable tissue disruption or endothelial damage (Critchley et al., 2020). Moreover, in another study H&E analysis revealed that 4 hours of HMP lead to a better-preserved cell structure compared to the conventional cold storage method (Michel et al., 2014). In this context, we were expecting that more damage would be seen in the hearts that were preserved with cold storage, especially those that did not survive. Also, because of the damaging effects that occur during this period of time, we were expecting that in both preservation strategies, there will be an overall increase in the effects of myocardial injury.

H&E analysis verified that tissue damage was observed in all groups. With reference to myofiber degeneration, after 4 hours of preservation, less cell death was detected in the HMP group compared to the non-survival group. However, during reperfusion, this trend was reversed. After 4 hours of normothermic reperfusion, the SCS non-survival group was less compared to HMP, and SCS-survival group. Nevertheless, no significant difference could be seen.

Regarding myocardial hemorrhage, 4 hours of reperfusion led to higher levels of erythrocyte accumulation in the SCS non-survival group. Nevertheless, during the period of the experiments, no specific pattern could be seen among the 3 groups. Again, the difference was not significant.

After 4 hours of preservation, interstitial edema was found mostly severe in the HMP group. However, this can be explained because of the perfusion, since the development of myocardial edema is a consequence of ESHP (Hatami et al., 2020).

Furthermore, after 4 hours of preservation, interstitial edema was increased in the HMP group. However, this can be observed also due to the weight gain that occurs during perfusion. Notably, in a previous investigation researchers observed that 4 hours of HMP led to more severe myocardial edema compared to the cold storage group. However, in the aforementioned study, the edema that was observed under these conditions, although significant compared to the conventional cold storage method, did not lead to diastolic impairment of the ventricle and was resolved during the reperfusion period (Michel et al., 2014). On the other hand, in the present study, a strong decrease in edema was detected in the SCS non-survival group, compared to HMP, and the SCS-survival group during normothermia evaluation.

In the final part of the myocardial injury scoring, endothelial changes were analyzed. Similar to interstitial edema, SCS non-survival group was evaluated with the mildest change in the endothelium. Again, the difference was not significant, and essentially, no trend among the three groups could be seen.

Overall, the data of this project show that for each parameter of myocardial injury scoring, no specific pattern was followed. Most importantly, each parameter that was assessed, showed different trends for each group. Therefore, our final assumption was that the results of this type of macroscopic analysis were not corresponding to the cardiac function of the hearts. This assumption arrived because we found that this type of staining was not sufficient to give us a conclusion about the differences among the groups. The lack of correlation between the results of macroscopic analysis and cardiac function could be due to the fact that cellular architecture alone does not necessarily reflect the functional state of the heart. The heart is a complex organ, and its function is dependent on numerous factors, including electrical activity, blood flow, and the interaction between the cells that make up the heart tissue. The H&E staining provides a snapshot of the tissue architecture and does not necessarily capture the dynamic changes that occur in the heart during preservation and reperfusion. Therefore, we concluded that more comprehensive methods are necessary to gain a more complete understanding of the effects of preservation strategies on cardiac function.

Since the H&E staining was not sufficient to give us adequate conclusions about the differences among groups, we then decided to proceed with the PTAH staining. Our initial goal was to see whether we could compare the two preservation strategies by focusing on mitochondrial damage. For the quantification of the positively stained area, we decided to analyze 4 snapshots per sample, because the samples had varying sizes, which could have an impact on the results. In agreement with our expectations, after 4 hours of preservation, mitochondrial damage was detected higher in the SCS group compared to the HMP group. However, during reperfusion, this trend is reversed, with the SCS non-survival group being in the higher levels of the positive area (***Figure 3.2***). Because of the damaging effects that occur during reperfusion, we expect to see a response in mitochondrial function or/and integrity. Based on previous studies, electron microscopy showed that mitochondria were better preserved after the application of HMP compared to SCS in porcine hearts (Michel et al., 2014, 2015). Therefore, this interpretation did not correspond to our hypothesis.

Chapter 5: Limitations and Future Directions

First of all, the use of isolated working slaughterhouse hearts for experimental purposes has been a common practice in various fields of research. One advantage of using isolated working slaughterhouse hearts is that they are readily available and relatively inexpensive compared to other animal models or human heart tissue. However, it is important to note that there are still some parameters that cannot be fully controlled, such as the age and health status of the animals. Despite the quick harvesting process, warm cardiac ischemia is still expected to occur and cause cardiac nutrient deficiency, hypoxia, acidosis, and necrosis. It is expected that these processes will continue to damage the tissue (Drewnowska, 1991). Nevertheless, this statement does not explain why not seeing differences between the groups, as this was the case for all hearts that were used in this experiment.

Therefore, the above-described adverse effects should be taken into account when comparing the results. These effects result in an increased chance of the loss of cardiac tissue integrity and function. In this sense, future studies should incorporate an experimental porcine model to obtain a better understanding of the process. This study was also limited by small numbers of pigs per group. Future studies should examine the two approaches with a higher number of samples. It is also noteworthy that apical biopsies are an important diagnostic tool in cardiovascular research (Cooper et al., 2007). However, there are concerns about the small circumscript lesions of tissue that we are able to investigate. Unfortunately, we could not avoid a large variation in the size and the orientation of the tissue specimens, which also hesitate the process of scoring and quantification. Furthermore, biopsies that are isolated from the apex are specifically localized. In this sense, they might not represent damage in the rest of the heart.

As mentioned above, future experiments should include a higher sample size to obtain more reliable results and more stainings could be carried out to assess the difference in damage between hearts stored with SCS compared to those preserved with HMP. Future steps could include Galectin-3 (GAL-3) staining. GAL-3 is a beta galactoside binding lectin and it has been shown in mice that GAL-3 levels are increased after ischemia-reperfusion injury (IRI). Previous studies have investigated the role of GAL-3 using porcine models (Mo et al., 2019; Navarro-Alvarez et al., 2018). Therefore, GAL-3 could be an interesting target to investigate differences between the two approaches (Al-Salam & Hashmi, 2018). Alternatively, more stainings could be done by using a TNF-α staining. Important studies have also demonstrated that the levels of Tumour necrosis factor-α (TNF- α) are elevated during ischemia-reperfusion injury (Formigli et al., 2001). In this context, this staining could be performed in order to compare the effects of IRI between the hearts that were preserved with SCS vs HMP.

Furthermore, while we did not see any effect of machine perfusion in cardiac histology, previous studies have demonstrated that electron microscopy is an excellent tool to investigate endothelial cell rupture, damaged muscle fibers, mitochondria swelling, and Z-band streaming and damage (Michel et al., 2015). Therefore, electron microscopy could be a sufficient technique that is capable of providing us with a high-degree resolution and visualization of subcellular structures

and the organization of the cardiac tissue. In this sense, perhaps we could get a better understanding of what is truly happening in these hearts during preservation and reperfusion.

Moreover, myocardial biopsies can also be used in order to determine levels of adenosine triphosphate, a marker of energy storage, and endothelin-1, a marker of endothelial dysfunction. Previous investigations have shown that SCS resulted in higher ET-1 levels compared to the perfused group (Madsen, 2014). Additionally, an important next step would be to test the effect of HMP by focusing on gene expression and genomic sequencing.

It is undoubtedly important to understand the molecular mechanisms of ischemia-reperfusion injury for the establishment of machine perfusion in medical practice. In this context, an important next step will be to test the effect of HMP on heart function by looking at gene expression patterns and comparing them to those observed in SCS. Several studies have shown that miRNAs are vital regulators of signaling pathways and that their role is associated with heart diseases, including heart failure, and ischemia-reperfusion injury (Ye et al., 2011). A key finding in the field of heart transplantation came from Liangyi Zhou and colleagues, who reported an expression profile of miRNA in I/R injured heart grafts in heart transplantation. In this study, researchers demonstrated that miR-711, miR-2137 miR-705, miR-5130, miR-346, miR-714, and miR-744 were significantly upregulated in I/R injured hearts, while miR-210, miR-490, miR-491, miR-425, miR-423-3p, and miR-532-3p were downregulated. Therefore, our future potential steps could be focused on the study of those genes (Zhou et al., 2013).

In conclusion, our study sought to investigate and compare the effects of hypothermic machine perfusion (HMP) to static cold storage (SCS) for ex-situ preservation of porcine hearts, to aid in understanding the underlying mechanisms for the difference in functional outcome on the histological level. In addition, the goal of this study was to provide insights into the histology involved in I/R injury and reveal a correlation between the functional outcome of HMP vs SCS. Altogether, the findings of our approach could not give us a comprehensive overview of the cellular architecture and the effects of ischemia injury that occur during HMP and SCS. Also, we did not find any significant changes in myocardial injury scoring and PTAH staining. Nevertheless , as described above, we initially knew that our approach has significant limitations and obstacles. Therefore, our conclusion is that as we move forward, more studies are clearly needed to enlighten the effects of HMP vs SCS on cardiac tissue integrity, and address whether HMP is the preferred approach to heart preservation.

Appendix

Myofiber Degeneration	
0	Absent
1+	Single to multiple foci of vacuolated, shrunken, or fragmented, hypereosinophilic myofibers
2+	More frequent multifocal foci to larger zones of vacuolated, shrunken, or fragmented hypereosinophilic myofibers
3+	Large coalescing to regionally extensive zones of vacuolated, shrunken, or fragmented hypereosinophilic myofibers
Myocardial hemorrhage	
0	Absent
1+	Focal to multifocal mild myocardial, epicardial, or endocardial hemorrhage
2+	Moderate multifocal to regionally extensive myocardial, epicardial, or endocardial hemorrhage
3+	Severe regionally extensive hemorrhage involving large portions of heart section
Interstitial edema	
0	Absent
1+	Mild and multifocal separation of myofiber bundles or expansion of perivascular spaces
2+	Moderate and multifocal separation of myofiber bundles or expansion of perivascular spaces
3+	Marked regionally extensive or multifocal separation of myofiber bundles and perivascular spaces
Endothelial changes	
0	Absent
1+	Plump endothelial cells, separation of endothelium from underlying basal lamina
2+	Cellular infiltration of vessel wall or disruption of layers of vessel wall
3+	Necrosis or severe vascular cellular infiltration

Table 1: Myocardial injury grading scale. *Adapted by* (Trahanas et al., 2016)

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