

Immunohistochemical analysis to assess the effects of static cold storage versus hypothermic machine perfusion on porcine hearts

Pepijn van der Putten

Supervisor: Selma Kaffka genaamd Dengler

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Abstract

Background

Because of population ageing and a high prevalence of overweight, heart failure is a growing global health issue. Currently heart transplantation (HTx) is the gold standard treatment, but this treatment is paired with a high waiting-list mortality, partially caused by a shortage of donor hearts. One way to increase the donor pool would be to change the preservation of the donor hearts. A novel way to preserve donor hearts is hypothermic machine perfusion. In this study, the effects of the currently used preservation method static, cold storage and hypothermic machine perfusion on slaughterhouse porcine hearts have been compared using immunohistochemical analyses.

Methods

Slaughterhouse porcine hearts were removed and preserved for 4 hours using either static cold storage (SCS) or hypothermic machine perfusion (HMP) followed by 4 hours of normothermic machine perfusion (NMP). At the end of the experiment, tissue samples were collected and phosphotungstic acid-haematoxylin (PTAH) staining was used to score contraction band necrosis and erythrocyte extravasation. An active caspase-3 staining was performed to score apoptosis.

Results

After normalising the data for the survival time of the hearts, The SCS hearts showed significantly higher contraction band necrosis compared to HMP hearts.

Conclusion

HMP shows great potential to improve the donor pool. In this study we discovered that more contraction band necrosis is present in hearts that are preserved using SCS compared to HMP. Further studies should include a higher sample size and more stainings could be performed to further elucidate the effects of SCS and HMP on porcine hearts.

Plain language summary

Heart failure is a growing health issue. Currently most treatment is focused on slowing down the progression of the disease and there is only one curative treatment at the moment, namely heart transplantation. Due to a low number of donor hearts, there is a high waiting-list mortality. A way to improve the donor pool would be to change the way the heart is preserved. Currently hearts are preserved using a method called static cold storage (SCS). During this preservation, the hearts are stored on ice. In this study we compared this preservation to a novel method of preservation named hypothermic machine perfusion (HMP) using slaughterhouse porcine hearts. Using HMP, there is a continuous flow through the heart, which leads to a decrease in harmful substances. Using microscopy analysis we discovered that more damage occurred to the hearts that were preserved using SCS than to the hearts that were preserved using HMP.

Introduction

Heart failure (HF) is a growing global health issue, with a current estimate of over 64 million people diagnosed. The incidence of HF still continues to rise due to population ageing and a high prevalence of overweight (1). Treatment is focused on decelerating the disease using various pharmacological agents for instance with angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB), beta blockers and spironolactone (2).

For patients with end-stage HF, heart transplantation (HTx) remains the gold standard treatment. However, the demand for donor hearts exceeds the number of hearts suitable for transplantation (3). The donor heart shortage makes stringent regulations on eligible patients necessary and the shortage results in a waiting-list mortality up to 15% over a period of five years (4). A desirable way to reduce the donor shortage, would be to expand the donor pool.

The preservation of donor hearts is one of the limiting factors for expanding the donor pool. Currently, static cold storage (SCS) is used to preserve donor hearts. During SCS, the heart is perfused with a preservation solution, which leads to cardiac arrest, after which the heart is stored on ice. During SCS metabolism is converted to anaerobic respiration, which leads to the production of lactate and a decreased intracellular pH (5). This decrease in pH disregulates the Na^+/H^+ exchanger and the Na^+K^+ exchanger and the $2\text{Na}^+\text{H}^+$ exchanger with as final result a intracellular Ca^{2+} overload leading to cell swelling. When the heart is reperfused after transplantation, reactive oxygen species (ROS) are generated due to xanthine oxidase, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide synthase (NOS) induction as well as the reactivation of the electron transport chain (6). The generated ROS can cause cell damage and even cell death by activating metalloproteinases and calpains and by the opening of the mitochondrial permeability transition pore (MPTP). This event is known as ischemic reperfusion injury (IRI) and causes more damage to the heart following ischemia (5).

A novel way to preserve a donor heart is hypothermic machine perfusion (HMP). Using HMP, the donor heart is continuously being held in a cardioplegic state, thereby limiting the oxygen demand of the cardiomyocytes, similar to SCS. In addition, the affluent as a result of continuous flow leads to a decrease in harmful substances. This decreases the ischemia time and reduces IRI. HMP may also be used to resuscitate hearts after circulatory death (DCD), thereby increasing the donor pool (7). The aim of this study is to compare the effect of 4 hour SCS to HMP on the heart of slaughterhouse pigs using an *in vivo* porcine model.

Methods

Slaughterhouse animals

Pig hearts were harvested within five minutes after being sacrificed by means of electrical stunning. A parasternal incision was made in the thorax and the heart and lungs were removed en-bloc. After harvesting, the heart was immediately topologically cooled. Next, the pericardial sac was opened, the pulmonary artery was transected under the bifurcation and the aorta was transected under the first supra-aortic vessel (8). The heart was then isolated and prepared as described in a study performed by Hannon et al (9). Immediately after removal, the aorta was cannulated and 2 L of heparinized modified St. Thomas 2 crystalloid cardioplegic solution (Table 1) was administered through the coronary arteries at a mean pressure of 80-100 mmHg and a temperature of 4 °C (10).

Preservation

After the cardioplegic solution was added, the hearts were preserved for 4 hours either using SCS or HMP. In total 12 hearts were included in this study. 6 hearts were preserved with SCS and the other 6 with HMP.

Hypothermic machine perfusion

For the HMP group, the heart was kept in a modified Kidney Assist perfusion box. For the perfusion, a homemade Steen heart solution was used and the temperature was kept at 8 degrees Celsius. After harvesting, the hearts were placed in the perfusion box and perfused with a pulsatile aortic pressure with a mean pressure of 20-25mmHg with a resulting flow > 100 ml/min. The hearts were maintained on HMP for 4 hours.

Na+	136 mmol/L
K+	23 mmol/L
Ca ²⁺	1.3 mmol/L
Mg ²⁺	8.0 mmol/L
Cl-	142 mmol/L
HCO ₃ ⁻	25 mmol/L
PO ₄ ²⁻	1.3 mmol/L
D-Glucose	6.3 mmol/L
Dextran	70 g/L
Noradrenaline	6 pmol/L
Adrenaline	6 pmol/L
T3	3 pmol/L
T4	2 pmol/L
Cortisol	420 pmol/L
Insulin	8 U/L

Figure 1: Steen solution composition (10)

Static cold storage

Hearts were submerged in 1L of St. Thomas 2 crystalloid cardioplegic solution and stored on ice for 4 hours.

Normothermic testing

After preservation, all hearts were reperfused ex-vivo using the PhysioHeart™ platform. For the reperfusion, 4500 mL diluted whole blood of normothermic, heparinized oxygenated blood was used, which was supplemented with glucose and insulin. First, the hearts were perfused for 60 minutes in Langendorff mode, followed by 3 hours of working heart mode perfusion. Atrial pressure and aortic pressure were kept at respectively 10-20 mmHg and 60-100 mmHg. A glucose and insulin mixture was added to keep the blood glucose level between 5-7 mmol/L and a pH of 7.40 was maintained by adding sodium bicarbonate. In order to measure the cardiac output, an ultrasound flow probe (SonoTT™ Clamp-On Transducer, em-tec GmbH, Finning, Germany) was placed after the afterload, and another ultrasound probe was placed after the pulmonary artery to measure the coronary flow (8).

Sample Collection

At the end of the experiment, perfusion was terminated and left and right ventricle samples were collected and preserved in 4% formaldehyde and subsequently embedded in paraffin. A microtome cutting instrument was used to cut slices with a thickness of 3 μ m from the formalin-fixed, paraffin-embedded tissue samples. Several stainings were performed to assess the functionality of the hearts.

Stainings

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

Haematoxylin and Eosin

To assess general tissue morphology, a haematoxylin and eosin (H&E) staining was performed. The haematoxylin stains the nuclei blue and the cytoplasm and ECM pink. First the slides were deparaffinized using xylene. After deparaffination, the slides were incubated for 5 minutes in haematoxylin. Hereafter, the slides were washed in tap water for 10 minutes and subsequently incubated in eosin for 1 minute. Finally, the slides were washed using demineralized water for 15 seconds and dried on a heater at 60 degrees Celsius.

Activate Caspase-3

In order to evaluate apoptosis, an activate caspase-3 staining was performed.

First the slides were deparaffinized in xylene and subsequently washed in ethanol. Hereafter, the slides were washed in demineralized water and boiled for 20 minutes in 30 mL EDTA + 1500 mL demineralized water (pH 9).

When the slides were cooled down, they were again washed in demineralized water and subsequently washed in PBS 3 times for 1 minute. After this, 150 microliter Active Caspase-3 antibody diluted 1:200 in PBS/BSA Azide was added to the slides and the slides were incubated overnight at 5 degrees Celsius. Next, the slides were incubated for 30 minutes at room temperature and washed with PBS 3x for 15 minutes, after which the secondary antibody Bright Vision Rabbit AP was added to the slides. Hereafter, the slides were incubated for 30 minutes at room temperature and subsequently washed with PBS 3 times for 1 minute. Subsequently, Dako Liquid Permanent Red Chromogen was added to the slides and after a 10 minute incubation the slides were washed with

demineralized. Hereafter, the slides were incubated in 1:3 diluted hematoxylin for 15 seconds and washed in tap water for 10 minutes and finally dried on a heating plate at 60 degrees Celsius.

Phosphotungstic Acid Haematoxylin

A Phosphotungstic Acid Haematoxylin (PTAH) stain was performed to assess contraction band necrosis and erythrocyte extravasation. First, the slides were deparaffinized in xylene 3 times, ethanol 100% 2 times and alcohol 70% and finally washed in demineralized water. Subsequently, ammonium iron (III) sulphate was added to the slides, followed by 30 minutes of incubation. Hereafter, the slides were washed in demineralized water for 1 minute, after which oxalic acid (5%) was added to the slides. After 10 minutes of incubation, the slides were again washed for 1 minute using demineralized water after which phosphotungstic acid was added to the slides. Hereafter the slides were incubated at 60 degrees in a laboratory oven for 3 hours. Finally, the slides were dehydrated using 70% alcohol and 100% alcohol 2 times, after which they were washed in xylene 3 times.

Scoring and statistical analysis

For the analysis of the PTAH staining, the contraction band necrosis was scored based on the percentage of contraction band necrosis over the whole slide. For this, a 4 class scoring system was used. I 0-10% positive staining, II 10-20% positive staining, III 20-50% positive staining and IV >50% positive staining. The extravasation of erythrocytes was scored based on a 3 class system: I: none to minor extravasation, II: reasonable extravasation, III: severe extravasation. The scoring of the active caspase-3 stained slides was performed by counting all positive stained cells on the whole slide and normalized to the size of the slides by first multiplying the amount of cells with the size of the slide and subsequently dividing it with the average slide size. Scoring was done blindly and the PTAH slides were also scored by a second observer. Images of the slides were taken using cellSens Dimension.

The obtained data underwent statistical analysis using the Mann-Whitney U test using IBM SPSS Statistics.

Results

Microscopy images of contraction band necrosis and erythrocyte extravasation

Assessment of the slides with the microscope revealed that contraction band necrosis and erythrocyte extravasation were present in both the SCS hearts (figure 2 a,c) and the HMP hearts (figure 2 b,d).

Overall however, notably more contraction band necrosis and erythrocyte extravasation were seen in the SCS hearts.

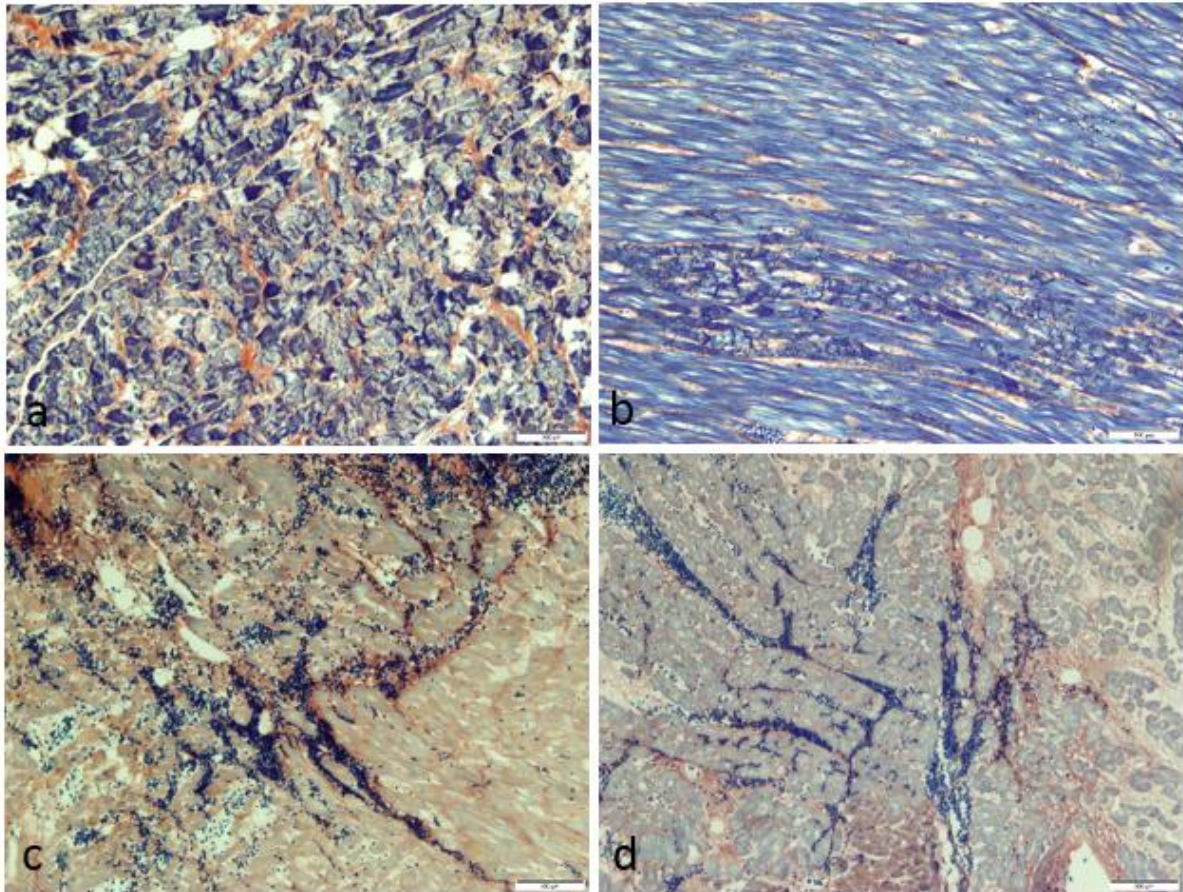


Figure 2: Comparison of contraction band necrosis and erythrocyte extravasation using PTAH staining. More contraction band necrosis was generally seen in the SCS hearts (a) compared to the HMP hearts (b). Erythrocyte extravasation was also mostly in the SCS hearts (c) than in the HMP hearts (d).

Contraction band necrosis

In both groups contraction band necrosis could be detected. The SCS hearts showed increased contraction band necrosis compared to the HMP hearts for both the left ventricle samples as well as the right ventricle samples. However, no significant difference could be seen.

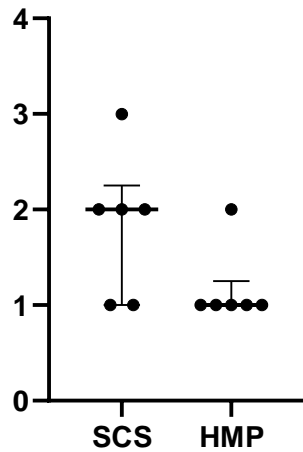


Figure 3: Contraction band necrosis scoring of the left ventricle. The SCS group has a higher median (2,00(1,00-2,25)) compared to the HMP group (1,00(1,00-1,25)), but this difference is not significant.

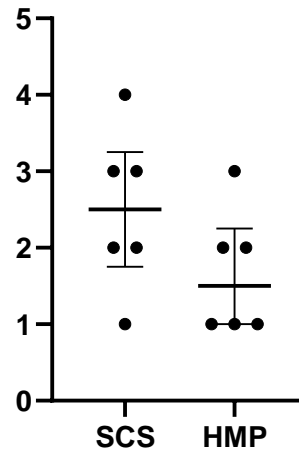


Figure 4: Contraction band necrosis scoring of the right ventricle. A higher median can be observed in the SCS group (2,5(1,75-3,25)) compared to the HMP group (1,50(1,00-2,25)), but this difference is not significant.

Erythrocyte extravasation

The medians of the erythrocyte extravasation in the left ventricle samples were the same, but for the right ventricle samples more erythrocyte extravasation could be seen in the SCS group. The difference was not significant however.

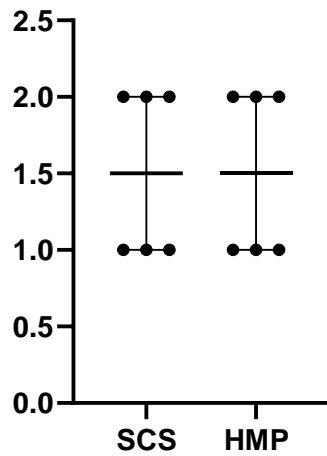


Figure 5: Erythrocyte extravasation scoring of the left ventricle. The medians of the SCS group and the HMP group are the same (1,5(1,00-2,00)).

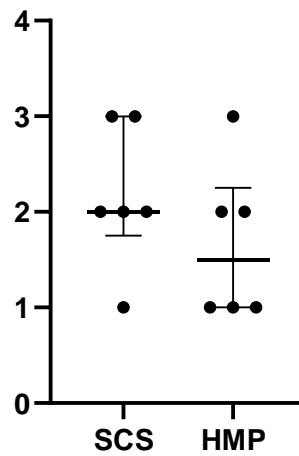


Figure 6: Erythrocyte extravasation scoring of the right ventricle. The SCS group has a higher median (2(1,75-3,00)) compared to the HMP group (1,50(1,00-2,25)), but this difference is not significant.

Active caspase-3

In the left ventricle samples, more active caspase-3 positive stained cells were present in the SCS group compared to the HMP group, but in the right ventricle samples the opposite was seen. For both the left and right ventricle however, the differences were not significant.

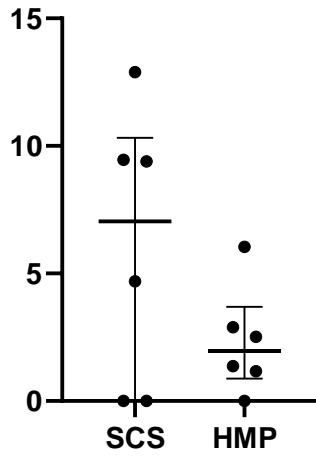


Figure 7: Scoring of active caspase-3 positive cells of the left ventricle. The SCS group has a higher median (7,04 (0,00-10,32)) compared to the HMP group (1,95(0,88-3,69)), but this difference is not significant.

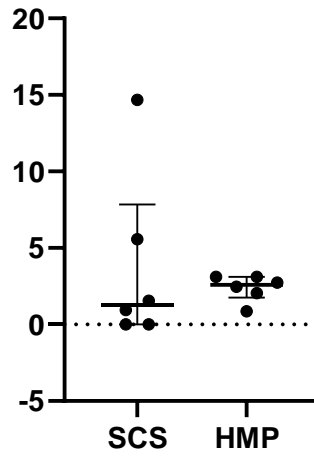


Figure 8: Scoring of active caspase-3 positive cells of the right ventricle. The HMP group has a higher median (2,60(1,76-3,12)) compared to the SCS group (1,25(0,0-7,85)), but this difference is not significant.

Overall survival

Since not all hearts made it to the end of the experiment, a Kaplan-Meier survival plot was performed to assess the survival between the two groups. This revealed a significant difference between the SCS and HMP group. It is likely that more damage would have occurred to the hearts that failed early, which could impact the results.

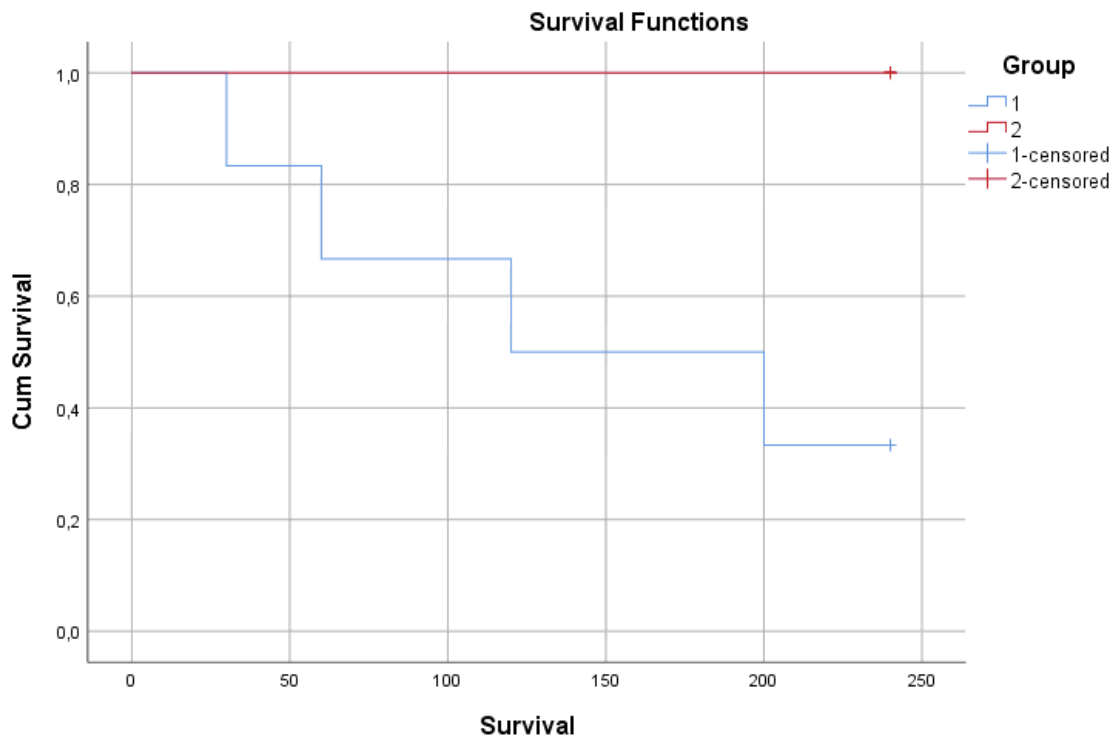


Figure 9: Kaplan-Meier Survival plot. In blue the cumulative survival of the SCS hearts and in red the cumulative survival of the HMP hearts.

Normalised data for survival

Because of the difference in survival between groups, time could have an impact on the results. In order to overcome this confounding factor, the data was normalized for the survival time. The data was normalized by dividing the scored value of a heart by the survival time of that heart and subsequently multiplying it with the average survival time. After normalization, there was a significant difference in contraction band necrosis between the SCS and HMP group, indicating increased contraction band necrosis in the hearts that were preserved using SCS. This difference was visible in both the left as well as right ventricle. For the extravasation of the erythrocytes as well as the active caspase-3 positive cells, again no significant difference was found.

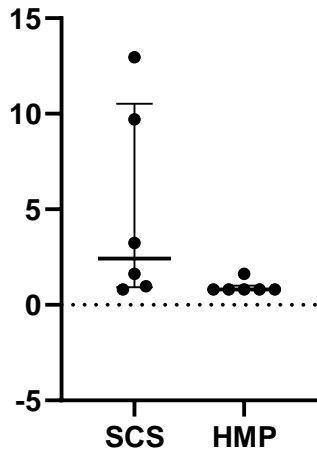


Figure 10: Contraction band necrosis scoring of the left ventricle after normalization for survival time. The SCS group has a significantly higher median (2,43(0,93-10,52)) than the HMP group (0,81(0,81-1,01)).

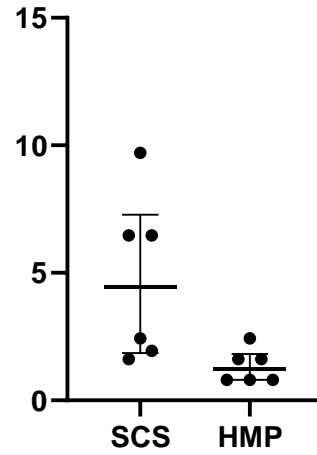


Figure 11: Contraction band necrosis scoring of the right ventricle after normalization for the survival time. The SCS group has a significantly higher median (2,59((0,81-6,47)) than the HMP group (0,81(0,81-1,62)).

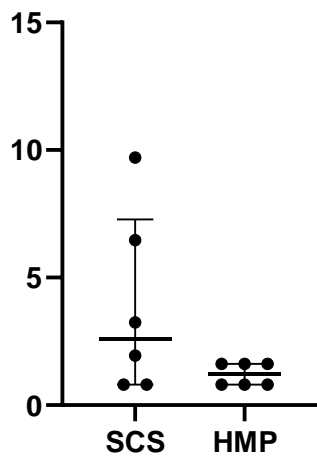


Figure 12: Erythrocyte extravasation scoring of the left ventricle after normalization for the survival time. The SCS group has a higher median (2,59(0,81-6,47)) compared to the HMP group (1,22(0,81-1,62)), but this difference is not significant.

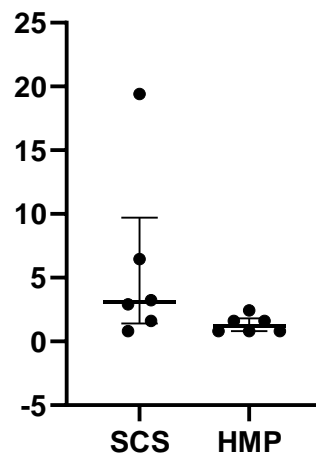


Figure 13: Erythrocyte extravasation scoring of the right ventricle after normalization for the survival time. The SCS group has a higher median (3,08(1,42-9,71)) compared to the HMP group (1,22(0,81-1,82)), but this difference is not significant.

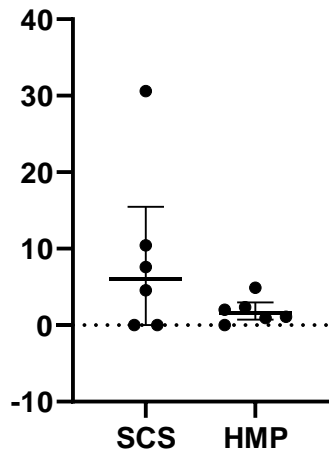


Figure 14: Scoring of active caspase-3 positive cells of the left ventricle after normalization for the survival time. The SCS group has a higher median (6,08 (0,00-15,48)) compared to the HMP group (1,58(0,71-2,99)), but this difference is not significant

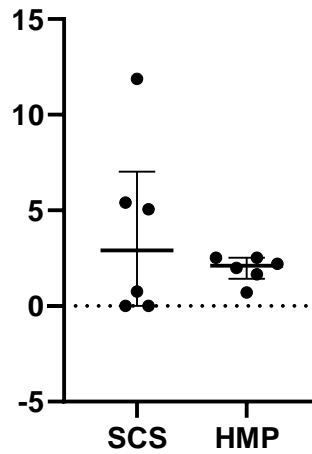


Figure 15 : Scoring of active caspase-3 positive cells of the right ventricle after normalization for the survival time. The SCS group has a higher median (2,91(0,00-7,03)) compared to the HMP group (2,10(1,42-2,52)), but this difference is not significant

Damage markers between failing and non-failing hearts

We also compared the damage markers between the hearts that didn't meet the inclusion criterium of >3L CO until the end of the experiment and the hearts that did meet it. Figure 16 shows the difference between the groups before normalization for survival time and figure 17 after normalization for survival time. Both figures show that more severe contraction band necrosis and erythrocyte extravasation can be seen in hearts that had a failure, but a lower number of apoptotic cells compared to the hearts that did not fail.

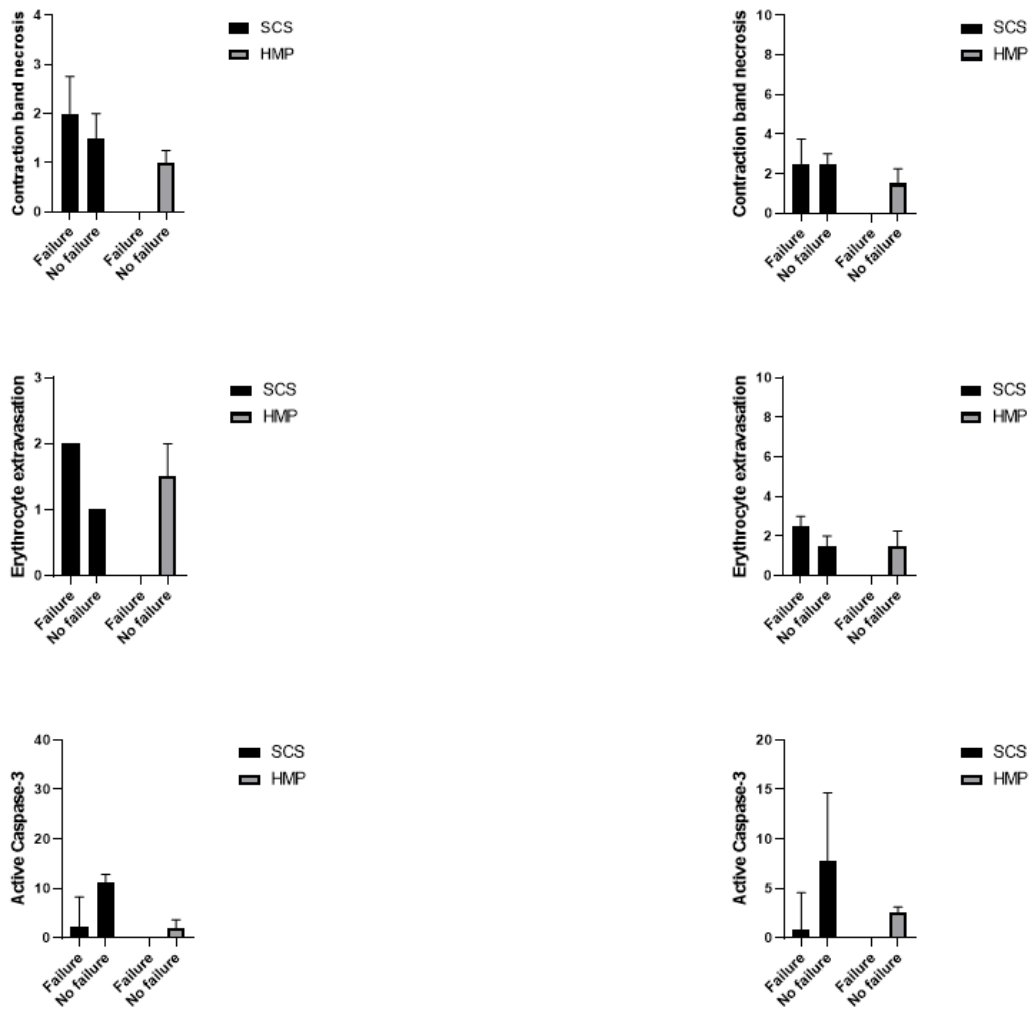


Figure 16: Comparison of damage markers between the hearts that did not fail compared to the hearts that did not fail. On the left hand side are the left ventricle samples and on the right side the right ventricle samples. These graphs indicate more severe contraction band necrosis and erythrocyte extravasation in the hearts that had a failure, but a lower number of apoptotic cells compared to the hearts that did not fail.

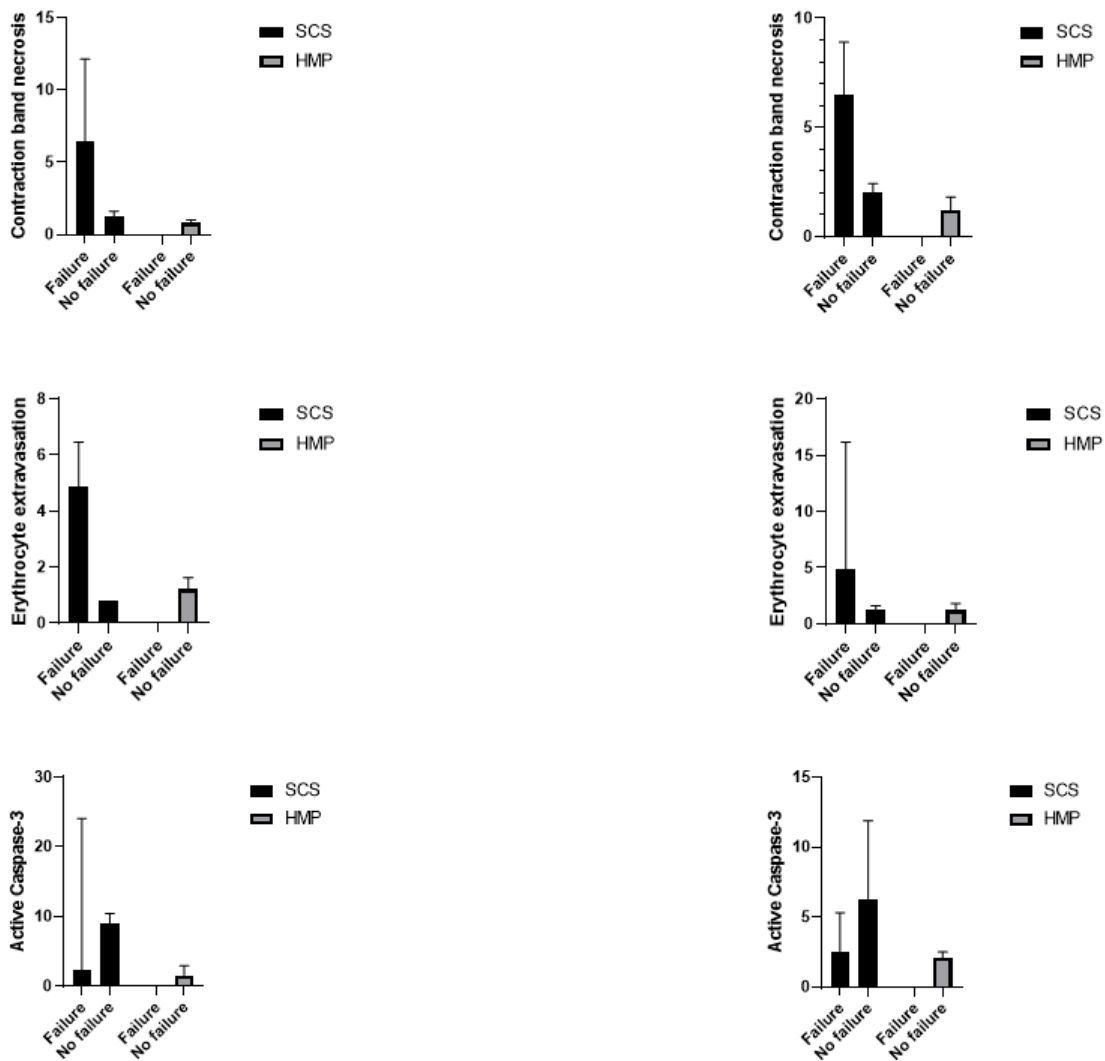


Figure 17: Comparison of damage markers between the hearts that did not fail compared to the hearts that did not fail after normalising the data for the survival time. On the left hand side are the left ventricle samples and on the right side the right ventricle samples. These graphs show more severe contraction band necrosis and erythrocyte extravasation in the hearts that had a failure, but a lower number of apoptotic cells compared to the hearts that did not fail.

Damage markers over time

Finally, we looked at the number of damage markers over the survival time. Since all of the HMP hearts made it until the end of the experiment, we only included the SCS hearts for this analysis. For the erythrocyte extravasation, a negative trend can be seen, while on the other hand the amount of caspase-3 positive cells increase over survival time.

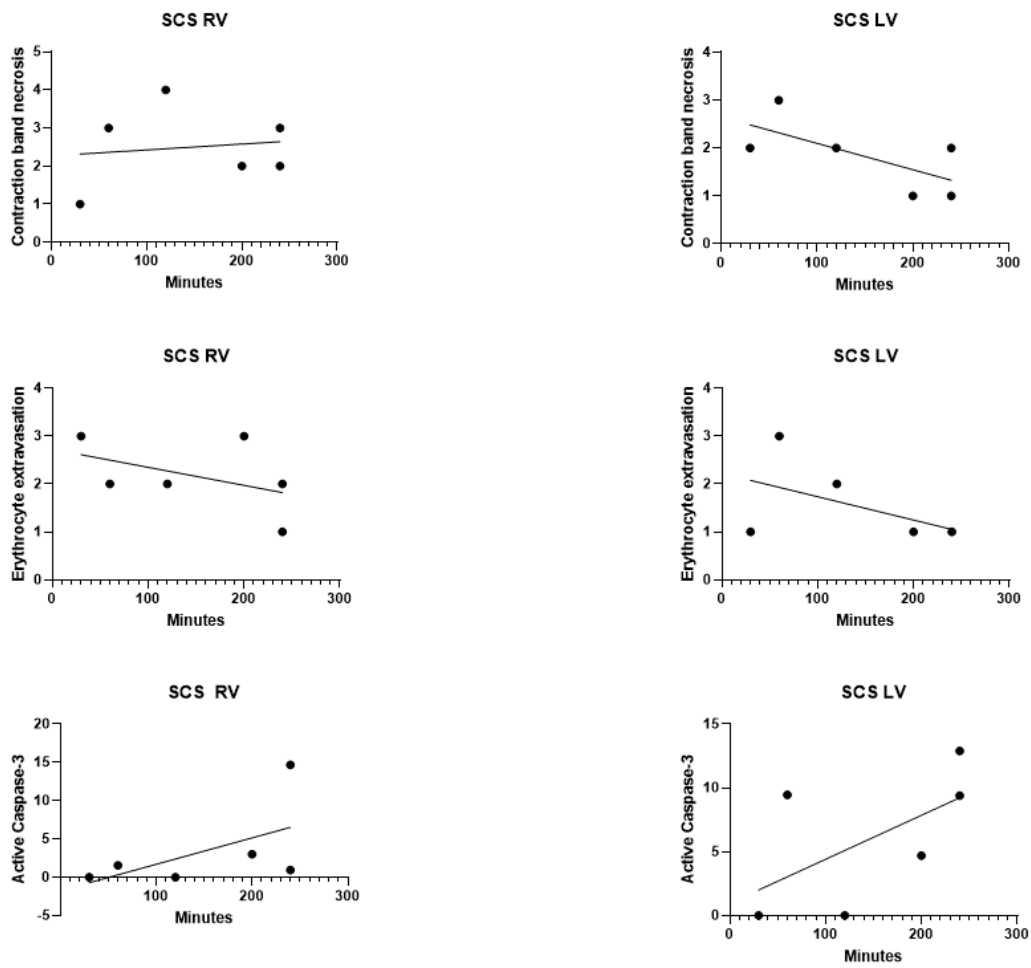


Figure 18: Incidence of damage markers over the survival time of the SCS hearts. A positive trend can be seen in the RV hearts and a negative trend can be seen for the LV hearts for the contraction band necrosis. The erythrocyte extravasation shows a negative trend in both the RV and LV hearts and more active caspase-3 positive cells can be seen in the hearts that had a higher survival time.

Discussion

This study was performed to assess the effects of SCS and HMP on cardiac function. While no significant difference can be seen between the two storage methods on apoptosis and erythrocyte extravasation, we did observe a significant difference in contraction band necrosis in both the left and right ventricle after normalizing the data for the overall survival time of the hearts.

In this study apoptosis was scored using an anti-active caspase-3 staining. The primary advantage of active caspase-3 staining is that once caspase-3 is cleaved, apoptosis is at a point of no return. This greatly reduces the number of false positives. Moreover, this staining is known to work well with formalin-fixed, paraffin-embedded tissues, and unlike TUNEL, which is heavily dependent on fixation conditions and pre-treatment procedures, activate caspase-3 staining does not require special pre-treatment procedures (11). Additionally, TUNEL stainings can provide false positives since it also detects DNA damage by other causes than apoptosis. However, we were informed by the pathologist that it is unlikely for apoptosis to occur in the timespan of our experiment, which is also supported by literature (12). This could explain the low number of apoptotic cells counted.

Initially we opted to score the contraction band necrosis and erythrocyte extravasation using the H&E staining, because the staining is relatively simple to perform and gives a comprehensive overview of the tissue. These were however difficult to notice with this staining, due to a lack of experience of the observers. Therefore, we decided to score contraction band necrosis and erythrocyte extravasation using the PTAH staining. For the scoring of apoptosis, we corrected the amount of positively stained cells for the size of the sample, because the samples had varying sizes, which could have an impact on the results. A different way to correct the scoring would be to count all the cells. Due to a lack of time, we were unable to perform this. Furthermore, we decided to normalize the data because the exclusion criterium of $>3.0L$ CO led to selection bias and this could have impacted the results.

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 and HMP. Possible stainings would first of all include Galectin-3 (GAL-3). GAL-3 is a beta galactoside binding lectin and it has been shown in mice that GAL-3 levels are increased after IRI (13) Secondly, Masson's Trichrome staining could be performed to assess the infarcted tissue(14). Finally, IRI has been shown to cause the production of tumour necrosis factor- α (TNF- α) by the injured tissue (15). A TNF- α staining could be performed to compare the amount of IRI between hearts preserved using SCS and hearts preserved using HMP.

Conclusion

The aim of this study was to compare and assess the effects of SCS and HMP on cardiac function. On apoptosis and erythrocyte extravasation we did not find any significant changes. However, HMP appeared to cause less heart band necrosis. Necrosis causes inflammation in its surroundings and is therefore regarded as highly damaging. In the case of cardiac tissue, this might lead to irreversible damage. Therefore, based on this study we conclude that HMP is the preferred choice of heart tissue preservation. Importantly, because this study has significant limitations as described above, it is important that more research is done regarding this subject.

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