# HUMAN AMNIOTIC MATERIAL USE IN POST-INFARCT SURGERY TO MITIGATE PATHOLOGICAL CARDIAC OUTCOMES

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by **Emily Gudvangen**, titled ***Human Amniotic Material Use in Post-Infarct Surgery to Mitigate Pathological Cardiac Outcomes*** and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

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# Abstract:

Many different factors contribute to the development and progression of heart disease. Although substantial advancements have recently been established in the treatment and prevention of heart disease, it continues to produce one in every four deaths in the United States[3]. This indicates a growing need for treatment options for both cardiovascular disease and its related complications. Human Amniotic Material (HAM) presents a treatment option for the preservation of myocardial functionality as well as prevention of post-operative pericardial inflammation and development of altered electrical activity in the heart. Our research has shown that the application of HAM led to a significant reduction in the development of post-operative atrial fibrillation in humans undergoing cardiac surgery. Furthermore, HAM injection in an ischemic mouse model led to reduced pathological remodeling of the left ventricle, demonstrating its ability to improve the retention of cardiac function in ischemic tissues.

# Chapter 1: Introduction

**General Background:**

Cardiovascular disease is currently the leading cause of death in the United States, outnumbering both cancer and unintentional injuries, the second and third leading causes combined. It is estimated that 45.1% of the United States' population will have cardiovascular disease by the year 2035, with the subsequent healthcare cost increasing from approximately $351 billion to $1.1 trillion[4]. Although incidence rates have declined in the last few decades due to advances in treatment and prevention[5], it is apparent that cardiovascular disease will persist throughout the United States population, and a need for the development of novel treatments will continue to be a top-priority research area.

Myocardial infarction (MI) is produced by the occlusion of a blood vessel limiting the oxygen exchange in the myocardium, creating an area of ischemia where the tissue does not receive the blood flow necessary to survive and/or function normally. This leads to the loss of cardiomyocytes and inability of the ventricular wall to contract and function at the degree required to pump a sufficient amount of blood to the rest of the body; thereby contributing to the progression of heart failure. Early revascularization is proven to be crucial to effectively salvaging myocardium from necrosis and reducing infarct size. However, when blood flow is restored to the area of infarct, further damage and necrosis occurs, categorized as ischemia reperfusion (IR) injury. Although revascularization is vital in limiting the infarct size and preventing long term heart failure, the process also leads to further loss of cardiomyocytes as illustrated in

**Figure 1**. The mechanism of IR injury is a rise in oxygen-derived free radicals and calcium overloading, which then further damage the already at-risk tissue resulting in the IR injury[1].

Reperfusion injury is also in part due to the inflammation triggered by the return of blood flow to the damaged myocytes, resulting in accumulation of neutrophils in numbers directly correlated to the duration of the ischemic event[6].

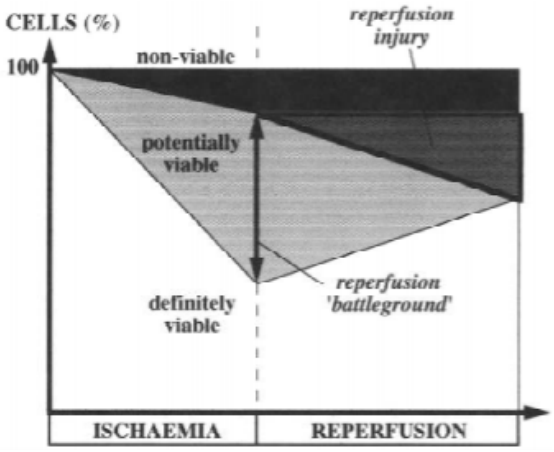



**Figure 1:** (Maxwell SRJ) A representation of the progression of myocyte death during ischemia and after reperfusion.

Inflammation plays a crucial role in the progression of the infarct and the remodeling that occurs in the affected tissue. The infiltration of neutrophils then leads to a further increase in reactive oxygen species, as well as release of inflammatory mediators and the no-reflow phenomenon, in which distal blood vessels experience additional blockages[7]. Inflammation may also instigate the alteration of electrical activity of the heart, triggering new onset post-operative atrial fibrillation (POAF). Although the cause of POAF is relatively unknown, studies suggest that inflammation is a strong contributing factor[8]. During cardiac surgery it is necessary to rupture the pericardial sac that surrounds and protects the heart from friction and foreign debris. Disruption of this barrier leads to the infiltration of cells and debris into the fluid within the pericardial sac. This results in local inflammation surrounding the heart, which then promotes cardiomyocyte death and the alteration of electrical activity that then generates arrhythmias[9].

Animal models have displayed a direct correlation between the extent of inflammation and the prevalence of POAF.

POAF is known to occur in approximately 35% of cardiac surgery patients[9], although recent studies have estimated that the occurrence rate is up to 50% due to underestimation and lack of diagnosis[10,11]. POAF is correlated with several undesirable outcomes, such as a two-fold increase in the risk of cardiovascular mortality, a 2.3-fold increase in the risk of stroke, and a five-fold increase in risk for congestive heart failure[12]. It poses a higher risk for renal failure and sudden cardiac death[12], along with significantly longer hospital stays, hospital costs, and hospital readmission rates[13]. Efforts to reduce the incidence rates of POAF after cardiac surgery have proven to be ineffective, with rates seeing little to zero decline. Given its strong correlation with poor patient outcomes and higher overall costs, additional research into the prevention and management of POAF is essential to improving patient outcomes.

Current treatment of POAF include the use of beta-blockers, calcium channel blockers, positive inotropes (i.e. digoxin), and anti-arrhythmic (i.e. amiodarone). Electrical cardioversion is also used in patients that are not hemodynamically stable but presents undesirable risks to the patients such as stroke and additional medication, so it is usually a last-resort method. Beta-blockers and calcium channel blockers are normally the first-line-drugs used to treat POAF, however it is not uncommon for the drugs to be ineffective or poorly tolerated in patients with systolic heart failure[14]. Anti-arrhythmic drugs such as amiodarone can cause bradycardia and hypotension and is therefore not suitable

for use in all patients[15]. Digoxin is used to slow the heart rate and control the heart rhythm but is not very effective in instances with higher sympathetic tone such as after invasive surgery[16]. Anticoagulation therapy is also a treatment option if POAF persists longer than 48 hours, but it is necessary to take into consideration the risk of bleeding after a major cardiac surgery. Corticosteroids are used to combat inflammation if it is detected and severe enough to warrant treatment, but their use is limited as they are associated with infection and hyperglycemia[17]. Cardiac ablation is only used in severe and chronic cases of atrial fibrillation and therefore is not used for POAF treatment. All current and commonly used treatment therapies for POAF come with severe risks and undesirable side effects. HAM application during cardiac surgery could offer a solution for prevention of post-operative inflammation and atrial fibrillation with minimal risks associated with its uses.

**Human Amniotic Material:**

Human Amniotic Material (HAM) presents a possible solution for the need of novel treatments post cardiac surgery, particularly following myocardial infarct intervention surgery. HAM has a very low immunogenicity while offering anti-inflammatory, antibacterial, anti-adhesion and anti-fibrotic capabilities that may limit pathological remodeling pathways[18]. HAM has a long history of use for wound dressings as it offers an allograft filled with growth factors and components to help speed healing while also providing a barrier that is biocompatible and combats infection.

The innermost layer of the placenta contains the amnion which is in direct contact with the amniotic fluid and fetus during gestation. The chorion is located at a more superficial level, loosely connected to the amnion[19]. The amnion layer comprises live amnion cells, cytokines, proteins, growth factors, extracellular matrix, fibronectin, elastin, laminin, nidogen and multipotent cells. These components are essential for cell scaffolds, as well as the promotion of regrowth and healing. The amnion contains epithelial and mesenchymal amniotic cells that secrete anti-inflammatory proteins such as Activin A, and IL-10, along with a notable suppression of proinflammatory cytokines, and expression of IL-1α and IL-1β, all contributing to its anti-inflammatory properties[20]. Amniotic tissue also produces β-defensins, a major group of antimicrobial peptides leading to the anti-microbial properties of the material[21]. Amniotic material limits the activation of fibroblasts through the suppression of TGF-β and the receptor expression on fibroblasts, which activate fibroblasts and lead to fibrosis, resulting in the material's anti-scarring property[22]. Although cells in the amnion have been shown to produce an extremely mild immune response, this response is counteracted by the presence of immunoregulatory factors listed above. Because the amnion and chorion layers separate the fetus from the immune system of the mother, it is necessary for the material to have low immunogenicity in order to prevent mother and fetus from producing an immune response[23]. Amniotic epithelial cells, for example, express HLA-G antigens which do not elicit an immune response instead of HLA-A, HLA-B, or HLA-DR antigens that are immunogenic and would be typical expressions of the cell type[24,25].

The chorion layer consists mostly of a dense collagen matrix; it contains a collection of growth factors and cytokines very similar to that of the amnion layer, but because the chorion is much thicker, it provides a larger amount of those desired components[26]. The addition of the chorion layer thereby offers significantly more growth factors and cytokines effective in boosting healing processes, while the amnion provides more of a vast array of contributing features to the suppression of inflammation and scarring[22,27]. However, the chorion has been known to cause an antigen response, and although it is mild, many processing methods isolate the amnion so that immune reactions are kept to a minimum. When the material is dehydrated it reduces the antigen response, due to the processing methods stripping and decellularizing the material, which is why many of the dehydrated versions include the chorion layer.

HAM tissue grafts contain the amnion layer, or a combination of the amnion and chorion layers of the placenta. HAM is commercially available and produced from donated placental material in three general formats implemented in our studies. Two of these classify as a membrane allograft that is adhered to the tissue surface upon treatment; the dehydrated version which includes derivatives from the amnion and chorion layers of the placenta, and the viable version which includes amnion material only, with the addition of a live stem cell layer adhered to the scaffold of extracellular matrix. The third format is an injectable solution that is a liquid allograft suspension of the material derived from the amnion, the injection serves the purpose of increasing infiltration of the material into the tissue

and therefore enhancing the delivery of the anti-scarring and anti-inflammatory material deeper into the tissue.

**Previous Research:**

For over a century, amniotic material has been used to accelerate wound healing and serves as a contributing factor to the regenerative processes, leading to improved patient outcomes[28]. Interestingly, HAM is considered an allograft, but it has been documented to be equally effective as autologous skin grafts, with superior engraftment in comparison to other allografts[29]. Furthermore, the injection of amniotic cells and membrane matrix into chronic fibrotic scar tissue showed significant tissue remodeling and total elimination of pain relating to the scar tissue[30]. This indicates that injection of the amnion material may contribute to a reduction in scar tissue even in late-stage fibrosis. Applying these properties to cardiac repair has only recently become a topic of investigation and may offer a solution to lessen the damaging effects of remodeling after infarct.

In a recent three-month-long study performed on ischemic rats, the animals treated with amniotic cells and membrane showed improved cardiac function, both systolic and diastolic, and reduced scar formation at the 30-day evaluation[31]. It was hypothesized that the amnion membrane serving as a scaffold would enhance cell engraftment and keep scar formation to a minimum on a more long-term timeline. However, the 90-day evaluation revealed that the scar formation and ejection fraction was no longer statistically significant from the non-treated group and no cell engraftment was observed[31].

More recently, it has been observed that the application of amniotic membrane to the epicardial surface during cardiac surgery has reduced the

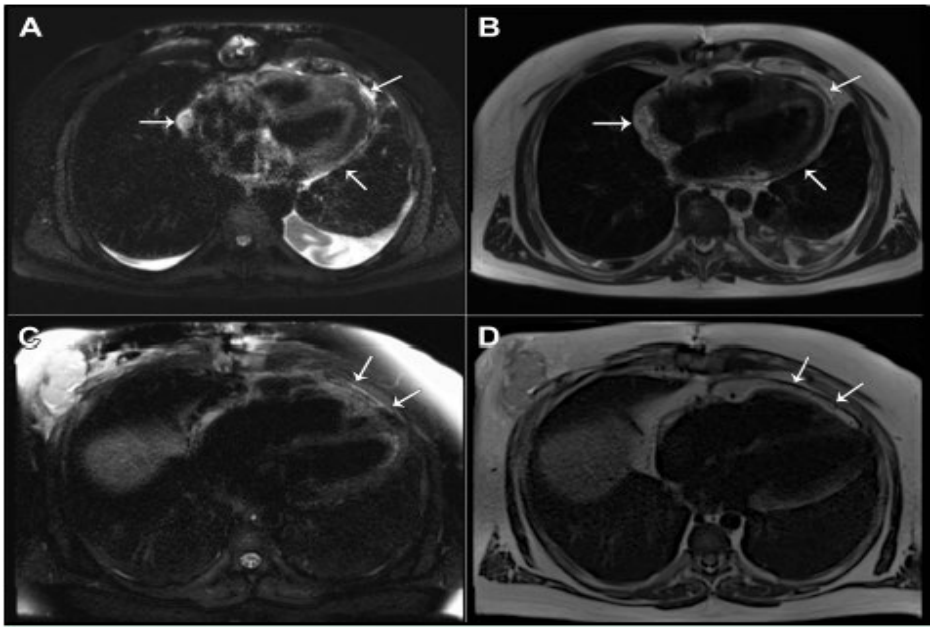



**Figure 2:** (Khalpey 2015) T2-weighted MRI obtained on post-operative day 6 of 56-year-old patient who did not receive amniotic membrane (A, B) and the 24-year-old patient who received an amniotic patch perioperatively (C, D). A, C with fat suppression B, D without fat suppression to localize edema.

amount of post-operative inflammation. This is shown in **Figure 2** with arrows indicating the area of inflammation and reduced inflammation in the area surrounding the heart in the patient that received an amniotic membrane during cardiac surgery[2]. This case study was published as a proof-of-concept trial in which a 24-year-old patient suffering from pericarditis due to prior transplantation received pericardial stripping and adhesiolysis treatment with the addition of HAM placement. This patient showed reduced inflammation post-operatively and lack of development of POAF in comparison to a 56-year-old Coronary Artery Bypass Graft (CABG) recipient who went on to exhibit POAF. Although these patients are not closely comparable due to their differences, the data suggest that HAM placement could reduce inflammation after cardiac surgery. Due to the

large contribution of inflammation in the suggested mechanism of post-operative atrial fibrillation motivated further study of POAF occurrence rates in patients receiving amniotic membrane placement during cardiac surgery.

Ninety-three eligible patients undergoing CABG or valve surgery (n=57 and n=36 respectively) were included in a single-surgeon study to further explore the observed reduction in inflammation and absence of POAF manifestation due to amniotic allograft placement. As predicted by previous research, patients receiving valve surgery experienced a higher rate of POAF occurrence in the control group than that of the CABG group. The application of the amniotic allograft led to elimination of POAF occurrence in valve patients and a large reduction in the incidence rate of CABG patients, as shown in **Figure 3** and **Figure 4** below (unpublished data). This data shows the incidence rate of POAF can be significantly reduced by the application of HAM on the cardiac surface at the end of operation. However, it cannot be determined if inclusion of the chorion layer of the placenta reduces this incidence rate further, which would require further exploration. This is the first indication that POAF could possibly be treated by the application of HAM. The observed outcomes warranted additional research into the resulting mechanism due to the addition of amniotic membrane allografts. Research revealing the mechanism for improved outcomes would allow for the optimization of those processes in order to best fit the need of the patients. Although HAM application has recently been explored in cardiac diseased animal models, further analysis into the benefits and possible risks of its treatment is needed to be able to apply HAM in not only POAF treatments, but other cardiac

complications as well. The mechanisms provided by the introduction of HAM to the myocardial tissue are necessary to determine the full range of benefits provided by HAM. To explore this mechanism, however, it is necessary to turn to animal models to be able to perform in-depth studies of the material's regenerative processes and integration into myocardial treatment areas.

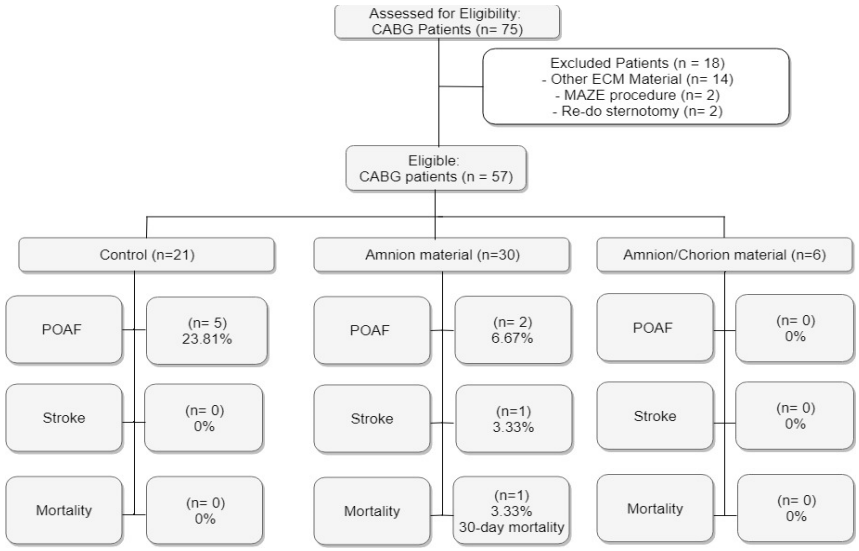



**Figure 3:** Schematic of the occurrence rates of POAF and its correlated conditions including stroke and death in CABG procedures with 21 control patients, 30 patients that received amniotic membrane allografts, and 6 recipients of amnion/chorion allografts.

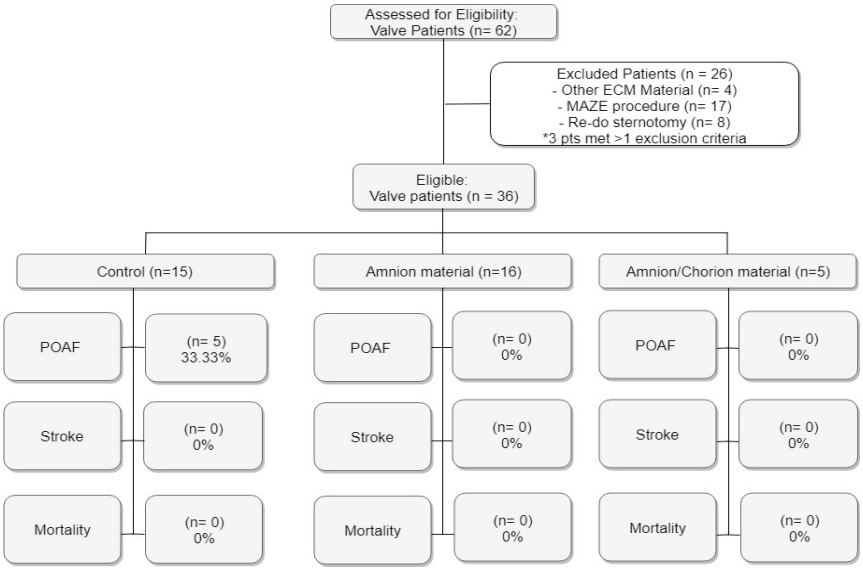



**Figure 4:** Representation of POAF rates in valve patients including stroke and death rates. Valve patient numbers consisted of 15 control without treatment, 16 amniotic membrane allograft recipients, and 5 amnion/chorion membrane allograft recipients.

**Specific Aims and Objectives:**

The goals of this research focused on exploring the mechanistic basis for the observation that placement of human amniotic membrane allografts on the epicardial surface during cardiac surgery led to a reduction in post-operative inflammation and POAF. This was examined through its use in two animal models with cardiac disease: permanently ligated ischemic mice, and ischemia reperfusion models in swine to represent coronary artery disease. These models were used to elucidate cellular and molecular mechanisms of HAM allografts as well as the contribution of HAM allografts to the preservation of cardiac function. These mechanisms were studied using echocardiography for functional assessment and histology for examination of material integration and its associated enhancement and suppression of specified cellular pathways. The overarching hypothesis of this research is that the use of HAM in ischemic cardiac tissue would reduce inflammation and scar formation, leading to a greater retention in cardiac function. In the next chapters I will discuss two animal models that were used to explore the more long-term remodeling pathways through a mouse model (Chapter 2) and the acute processes in a swine model (Chapter 3). Chapter 4 then includes a general discussion of the research we performed and the future directions of HAM research in cardiac applications.

# Chapter 2: *In vivo* Mouse Model

## Materials and Methods

All experiments followed guidelines for the use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona. The goals of this experiment included the examination of long-term implications of HAM use in both membrane and injection formats with ischemic mice models. Due to the reduction in POAF occurrence seen in the human applications, we hypothesized that the material will reduce immune responses initiated by the ischemia and surgery. Therefore, when applied to the infarction, the immune response is reduced, myocardial tissues are preserved, and scar formation will be reduced long-term. To test this, we used a permanent ligation mouse model over a timeline of one month to allow for remodeling to occur. To examine the scar formation, we used histological picrosirius red staining of collagen in contrast to cardiac tissue. For a closer look at the immune response, we used IL-6 (a tissue injury or infection related cytokine[32]) and CD68 (a marker for macrophages) histology staining. Finally, for an indication of myocardial preservation, we used echocardiography for functional assessments and cardiac measurements.

**Experimental Design:**

An adult male mouse model was used to explore the contribution of the Human Amniotic Material to the preservation of cardiac function and the overall

remodeling of the myocardium at risk due to the occlusion of the Left Anterior Descending (LAD) artery. This model suggests a primarily long-term process of remodeling and the progression to heart failure seen in many cardiac disease patients.

Three-month-old male mice (N=25) were anesthetized with an IP injection of 250mg/kg tribromoethanol, intubated and ventilated with 0.5-2% Isoflurane in combination with tribromoethanol. Mice were then provided with subcutaneous analgesia (buprenorphine SR at 0.5mg/kg) before undergoing a left thoracotomy performed by a left lateral incision and transection of the third rib. The left coronary artery was ligated with an 8-0 polyethylene suture tied perpendicular to the long axis of the heart to produce the ischemia. Mice were split into; 1) control MI (N=7); and treatment groups of 2) MI plus HAM Membrane (N=8) and 3) MI plus HAM injection (N=10). Control MI mice received no treatment while treatment groups were split as follows: Amnion Membrane (PalinGen Membrane by AmnioTechnology) application to epicardial surface and Amnion injection (PalinGen Kardia-Flow by AmnioTechnology) into the myocardium.

*Implantation of Human Amnion Material:*

Amnion Membrane: One square centimeter of the membrane was placed onto the myocardium over the area of ischemia and secured with two single mattress sutures.

Intramyocardial Injection: A 30-gauge sterile beveled needle was inserted near the base of the heart and advanced into the ischemic myocardium to the right of the

left main coronary artery. Fifty microliters of solution were injected into the myocardium and held in place until the solution dissipated and the needle was withdrawn.

**Echocardiography:**

Echocardiography was recorded at baseline (before surgery), 10-day post-surgery, and 28-day post-surgery using LogicE Ultrasound to document the changes in cardiac functional performance[33]. B-mode echocardiography was used to produce 2-D views of the long axis of the heart and measure ejection fraction (EF), fractional shortening (FS) and left mid-ventricular posterior wall thickness (LVPW). Two-dimensional M-mode echocardiographic images from parasternal short-axis views at basal, mid-ventricular and apical locations were used to calculate the left ventricular internal diameter (LVID) and left ventricular volume. Measurements were obtained in triplicate and averaged in both systolic and diastolic phases.

**Immunohistochemistry and Histological Staining:**

*Tissue harvest and preservation:*

Two mice, one control and one HAM membrane treatment were euthanized at day 10 post-op to examine the progression of the membrane integration. Four weeks post-operation, the rest of the mice (N=23) were anesthetized and sacrificed through cervical dislocation and vital organ harvest. After removing the heart, it was sliced down the center on the long axis and

incubated in 10% formalin for fixation before being placed in methanol to dehydrate. Tissue was then cleared with xylene and embedded in a wax block for sectioning. All tissues were cut at 5 μm and placed on charged Superfrost slides for staining. Sections were imaged on a Zeiss microscope (M1 model) through Axion Vision Software (version 4.72) and photos were merged using Adobe Photoshop to visualize the entire heart.

*Hematoxylin and Eosin (H&E):*

H&E was used for visualization of overall tissue morphology and cellular infiltration. Tissues were rehydrated in an ethanol gradient before staining with Richard Allen Hematoxylin and Eosin incubation for 30 seconds. Heart sections were then imaged in 5x light microscopy.

*Picrosirius Red (PSR):*

PSR was used for visualization of collagen as a marker for scar development. Nuclei were stained with Weigert's hematoxylin before tissues were placed in picrosirius red for 1 hour. Hearts were visualized and imaged in fluorescent light microscopy at 5x under Alexa 488 and Texas red and overlaid to show both live tissue and PSR stained collagenous tissue. Quantification was performed using ImageJ Software to determine the pixel area of the differing colors. The left ventricular area was used as a normalization method. The Color Threshold function was used to calculate the area of the left ventricle and the area of

collagen in the left ventricle to give a percentage of collagen which was then presented as an average across each group.

*Anti-Interleukin-6 (IL-6) Antibody:*

IL-6 is a cytokine released due to tissue injury or infection[32]. IL-6 staining was used to visualize the amount of IL-6 cytokine present in the tissue in relation to these processes in order to assess the amount of immune response that occurs in each treatment group. Antigen retrieval was performed using sodium citrate buffer (Dako 1x Solution) for 40 minutes at 95℃. For blocking, tissue is incubated in 0.3% hydrogen peroxide ($H_2O_2$) in PBS for to block endogenous peroxidase activity and then incubated with 10% goat serum and 2% BSA in PBS for 60 minutes to reduce nonspecific background staining. For the primary antibody, a 1:300 dilution of rabbit anti-IL-6 was used overnight at 4℃. Secondary antibody was incubated for 1 hour at 1:500 dilution of goat anti-rabbit IgG HRP. To allow for visualization, slides were incubated with DAB chromogen for 5 minutes. The chromogen interacts with the HRP enzyme conjugated to the secondary antibody, converting it to a brown product to be visualized. Tissue was counterstained with hematoxylin for 45 seconds to allow for visualization of nuclei. Once mounted, slides were imaged under light microscopy.

*Anti-CD68 Antibody:*

CD68 is an antigen expressed by macrophages, which play a vital role in the progression of tissue necrosis due to inflammatory responses. Macrophages also release factors that activate fibroblasts and lead to scar formation. To evaluate the relative amount of macrophages present in the myocardium after MI and treatments, tissues were stained with anti-CD68 primary antibody. Antigen retrieval was performed using sodium citrate buffer (Dako 1x Solution) for 40 minutes at 95℃. Blocking was carried out using 0.3% $H_2O_2$ in PBS to block endogenous peroxidase activity and then incubated with 10% goat serum, 2% BSA in PBS for 60 minutes to reduce nonspecific background staining. The rat anti-CD68 primary antibody was diluted at 1:100 and incubated overnight at 4℃. A 1:500 dilution of goat anti-rat IgG HRP was used for the secondary antibody and incubated for 1 hour. Six minutes of DAB chromogen was then used before counterstaining nuclei with hematoxylin. Slides were imaged under light microscopy at 10x-40x.

*Statistical Analysis:*

All data was analyzed through a 2-Way ANOVA with multiple comparisons or mixed model analysis for significance. Significance was determined by a P-value $< 0.05$. Graphical error bars represent the standard error of the mean (±SEM).

# Results

**Immunohistochemistry and Histological Staining:**

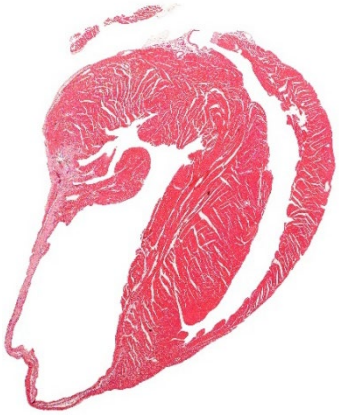
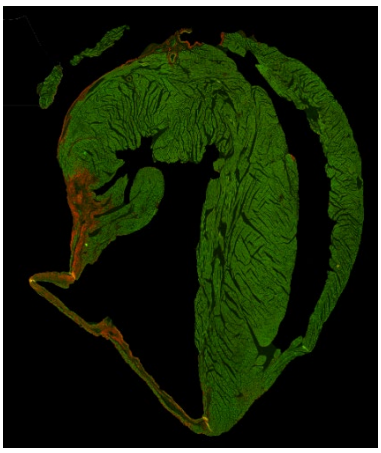
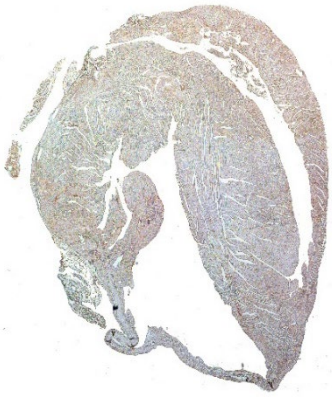
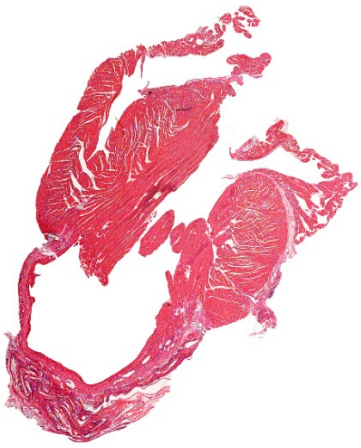
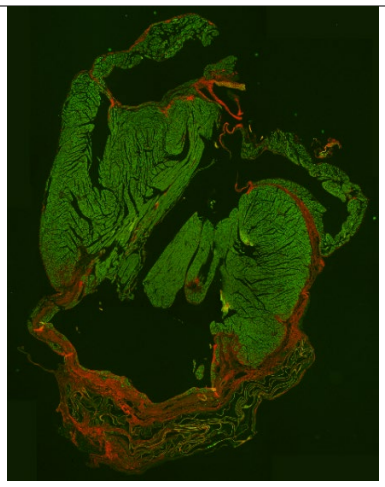
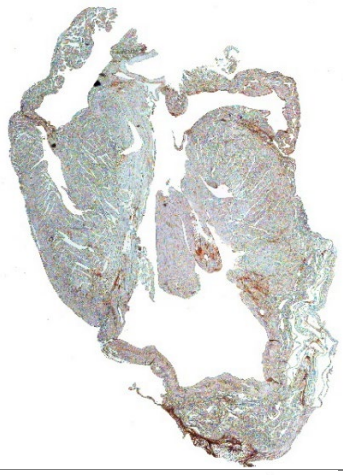
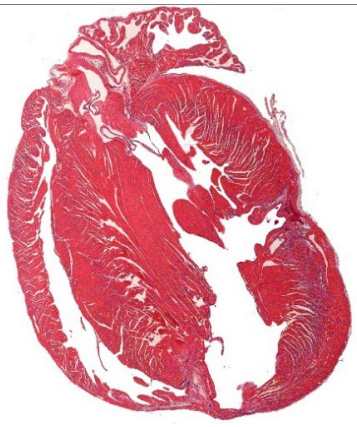
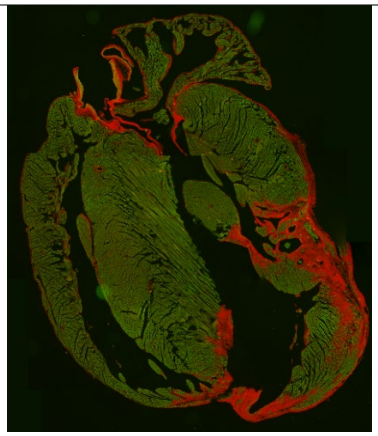
| | H&E | PSR | IL-6 |
|---|---|---|---|
| **Control: (Animal number 519569-4)** **N=6** |  |  |  |
| **HAM Membrane (Animal number 519570-3)** **N=7** |  |  |  |
| **HAM Injection (Animal number 519572-1)** **N=8** |  |  |  |

**Figure 5:** Histological comparisons between groups with H&E, PSR and IL-6 staining.

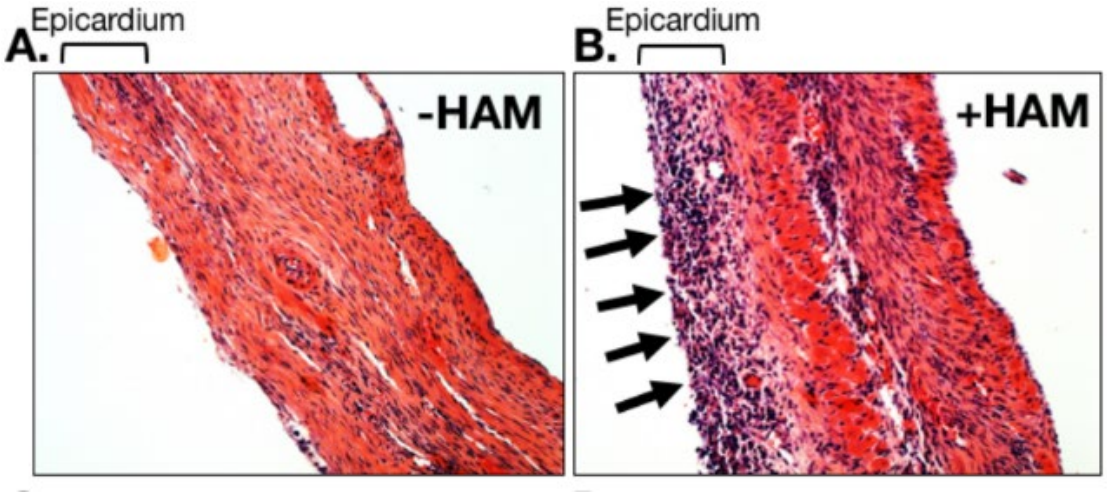



**Figure 6:** H&E staining showed cellular accumulation at the epicardial surface at day 10 with the application of the HAM membrane to an infarcted heart (**A**) in comparison to the control MI (**B**).

Histological staining presented interesting results in both the membrane and injected HAM treated groups. With the HAM membrane group, we noticed that in multiple mice there was an appearance of tissue adhered to the ventricular wall. This tissue displayed an abundance of nuclei but did not display the typical morphology of collagen or cardiac muscle. In some mice, as displayed in **Figure 5** above, the injection of HAM led to a retention in myocardial tissue in hearts that clearly suffered a large ischemia due to the presence of the collagen and remodeling from near the top of the left ventricle to the apex of the heart. However, between the two areas of collagen development remained a visible patch of live myocardium as displayed by the PSR staining. IL-6 staining did not show any notable differences in amount of cytokine across the groups, or in comparison to the control MI mice.

**Figure 6** shows that the application of the membrane led to a significant accumulation of cells at the surface of the heart during a 10-day period. Since the dehydrated version of the HAM membrane was used which does not possess live cells, we know that this accumulation is due to cellular recruitment and/or growth.

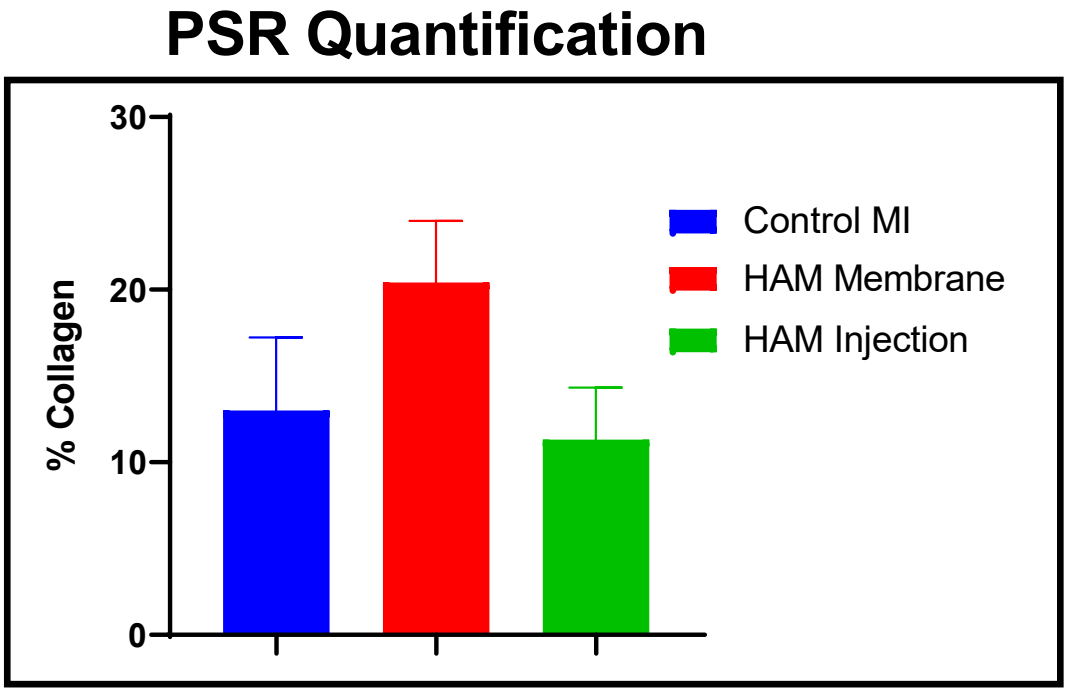



**Figure 7:** Picrosirius red staining quantification shows the percent collagen in the left ventricle across treatment groups. Data is represented as mean values with error bars ±SEM.

Although the quantification of collagen did not show significant differences across groups, it should be noted that HAM treatment introduces a large quantity of collagen to the tissue which could counteract the reduction in scar formation and lead to the similar collagen quantities of the left ventricles in each group. Even with that in mind, the data shows a trend for the HAM membrane to lead to an increase in collagen in the left ventricle, while the injection of the material led to a decrease in the mean percent collagen (**Figure 7**).

**Echocardiography:**

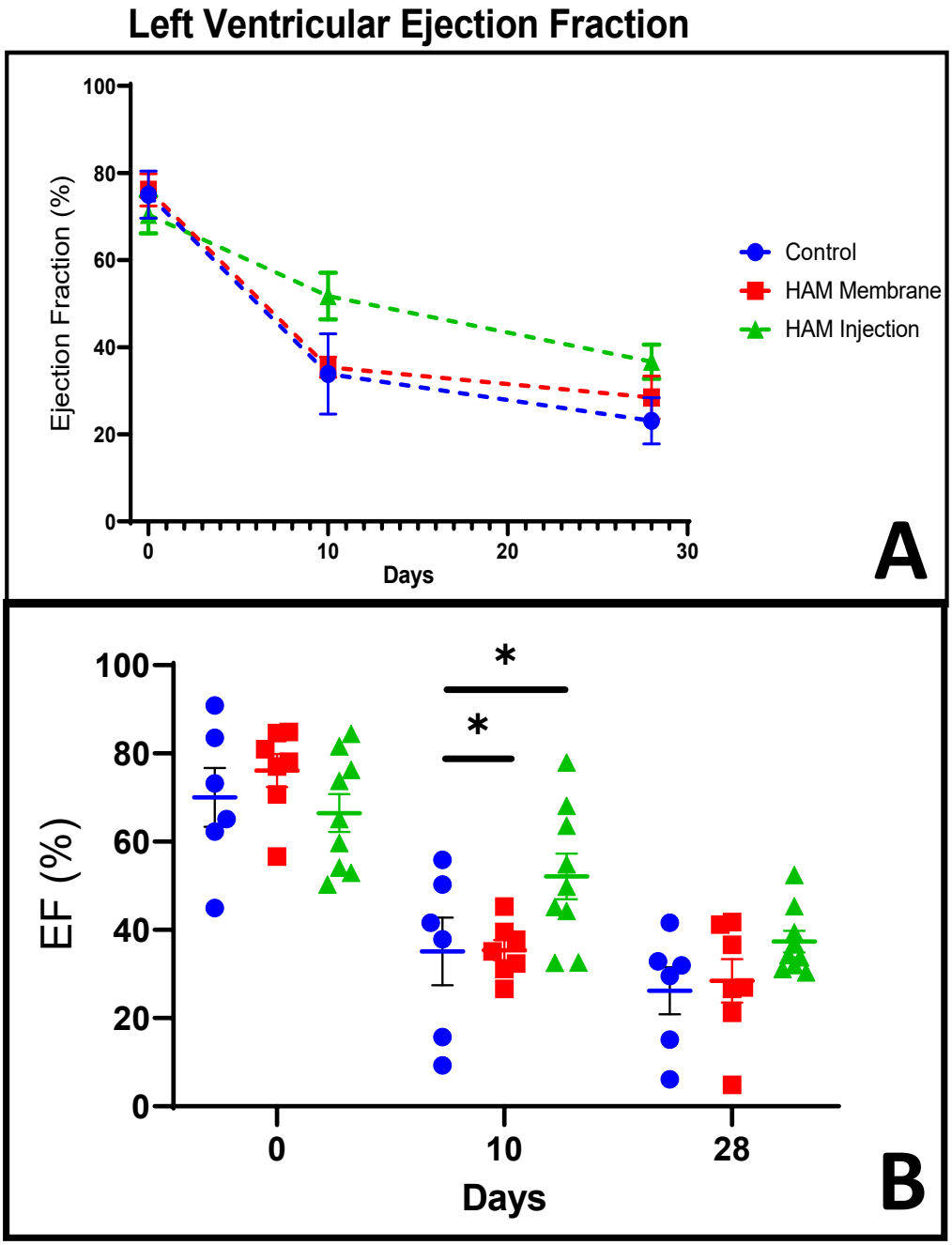



**Figure 8:** A graphical representation of the changes in ejection fraction over time (**A**). **B** shows the scatter of data points for ejection fraction to emphasize the outliers and general trends. Error bars are representative of ±SEM.

Ejection fraction is commonly used as a measure for ventricular function. Ejection fraction is measured as the amount of blood that is contracted out of the ventricle in relation to the amount of blood that the ventricle contains at the end of relaxation. Ejection fraction is determined by measuring the volume of blood at end-systole and end-diastole, it is then expressed as a percentage of the blood at end-diastole that leaves the ventricle during contraction.

A mixed-effects analysis with Tukey's multiple comparisons showed significant improvement in ejection fraction between the HAM injection group and control at day 10 ($p = 0.0298$). Large variation in the control group prevented significant comparison to the HAM injection at day 28. Interestingly, the injection of HAM led to an increase in ejection fraction for several mice at the 10-day period and a single mouse at the 28-day measurement.

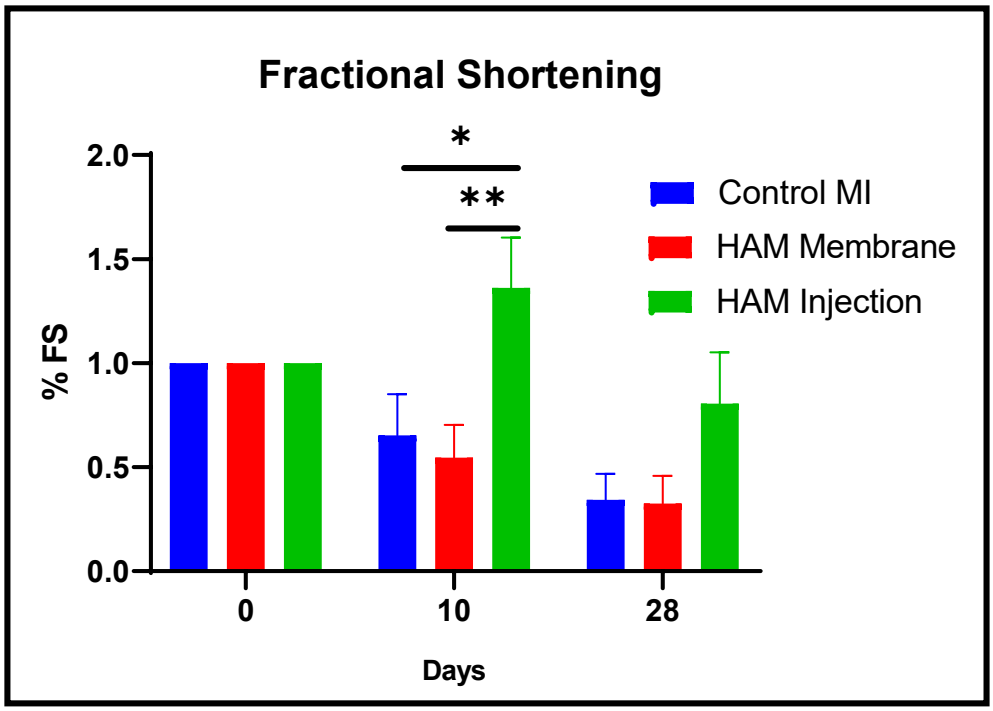



**Figure 9:** Fractional shortening was measured at the posterior mid-ventricular level using echocardiography. Data is represented as mean values normalized to baseline values with error bars ±SEM.

Fractional shortening (FS) represents a second method for determining systolic function of the ventricle. Fractional shortening recorded through two-dimensional B-mode echocardiography is represented in **Figure 9** through normalization of each data set to the baseline measurements. Statistical analysis was performed through a 2-way ANOVA with Tukey's multiple comparisons test. This showed significant differences between the control and HAM injection ($P = 0.0179$) as well as HAM membrane and HAM injection groups ($P = 0.0034$) at day 10. Although there is not a statistical significance between these groups at day

28, there is a visible trend of an increase in FS in the group treated with HAM injection.

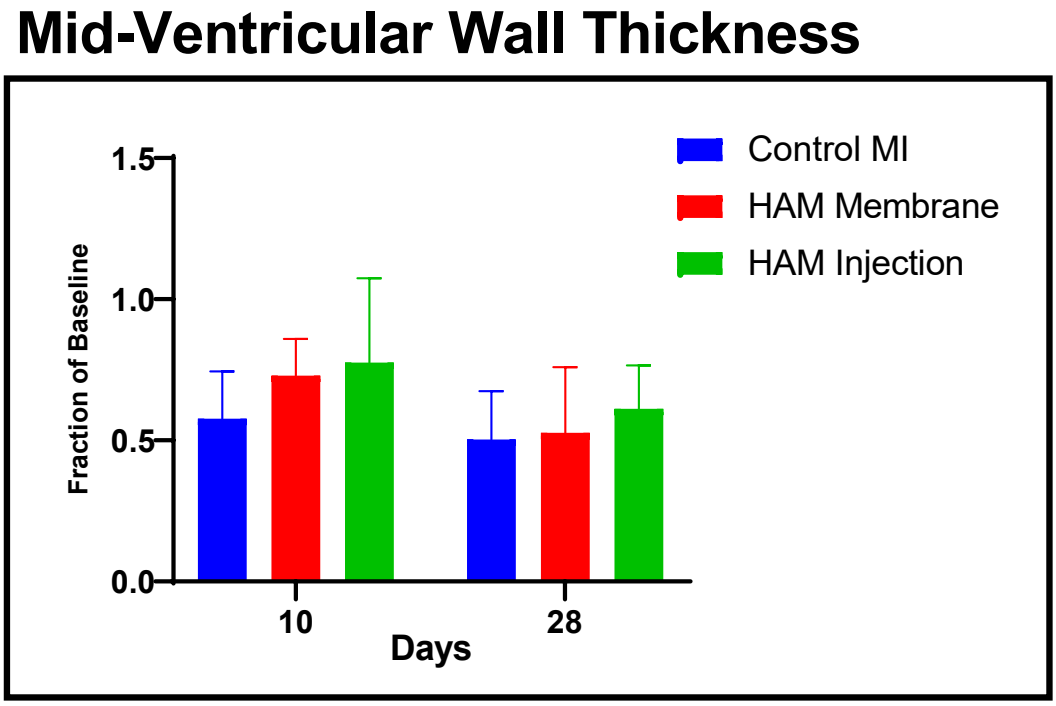



**Figure 10:** Wall thickness was measured at the posterior mid-ventricular level during systole using echocardiographic imaging. Data is represented as mean values normalized to baseline values with error bars ±SEM.

Mid-ventricular wall thickness is a measure of determining the functional capabilities of the left ventricle, the retention of myocardial tissue, and dilation of the ventricular wall. When a permanently ligated MI is given to mice the ventricle dilates to severe degrees and the muscle loss leads to ventricular wall thinning. The reduction in wall thickness is shown in **Figure 10** where values are normalized as a fraction of the baseline data. This wall thinning was observed across all groups, though the mean wall thickness is slightly higher in HAM treatment groups.

With this introduction of an ischemia, it is expected to lead to severe ventricular dilation as seen in the histological representations in **Figure 5**. This dilation was also measured as the internal diameter of the left ventricle through short-axis M-mode echocardiography. Internal diameter during systole represents a method of analyzing the contractile function in combination with the degree of dilation. Collected data is graphically represented in **Figure 11** below as mean values across the groups. A Tukey's multiple comparisons 2-Way ANOVA showed significant reduction in ventricular diameter during systole between the HAM injection group and the control (P = 0.0341) in addition to the HAM injection group and the HAM membrane group (P = 0.0264) after four weeks.

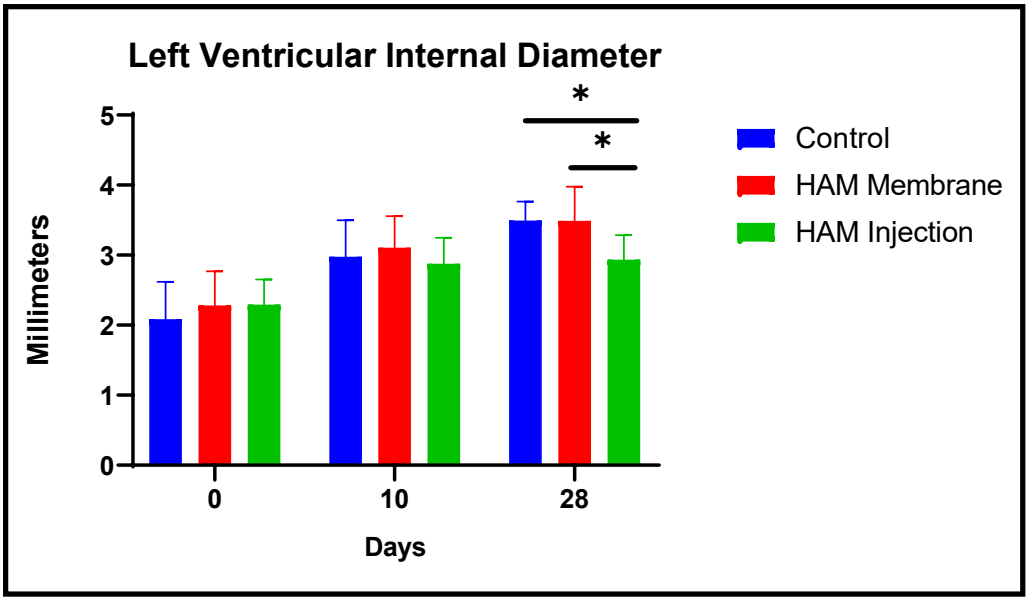



**Figure 11:** The internal diameter of the left ventricle of each treatment group measured during systole. Data is shown as mean values with ±SEM error bars.

The reduction in dilation and conservation of contractile function is further supported by the ventricular volumes. The volume of the left ventricle is a second

method of measuring contractile function of the entire ventricle as well as its pathological dilation. Data is shown in Figure 12 below as mean values across treatment groups. A Tukey's multiple comparisons 2-Way ANOVA showed significantly less ventricular volume between the HAM injection group and the control (P = 0.0196) in addition to the HAM injection group and the HAM membrane group (P = 0.0094) at day 28.

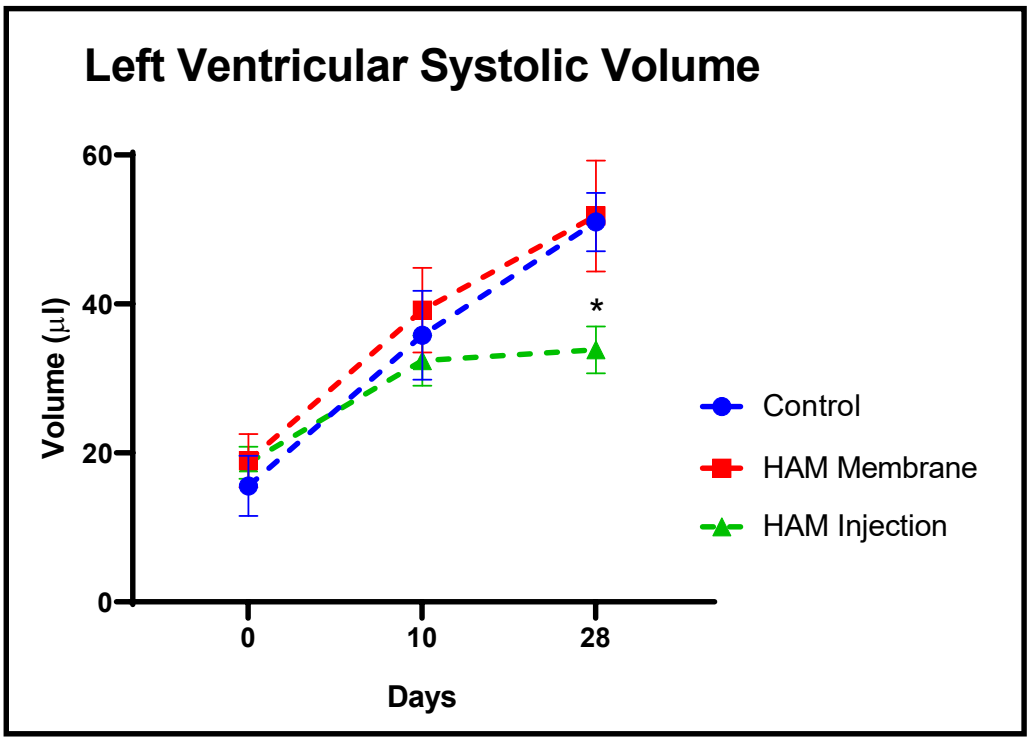



**Figure 12:** Volume of the left ventricle during systole. Data is shown as mean values with ±SEM error bars.

**Discussion:**

This study showed that HAM injection into ischemic myocardium may lead to improvement in cardiac function in comparison to non-treated tissue. We noted a decrease in dilation of the left ventricle and retention of contractile function as shown by the reduced ventricular internal diameter and volume during systole. These results warrant further study to see if data trends will become significant changes across groups if given a larger N. Histological staining also showed a change in morphology of the tissue when treated with HAM as well as an accumulation of cells in the HAM treated areas. This led us to believe that the HAM membrane was engrafting into the tissue and that HAM was promoting cellular recruitment and growth. We believe that integration of the HAM membrane occurred which led to a development of nuclei-rich section of the left ventricle which could possibly lead to myocardial growth over a longer time period.

CD68 staining did not show any significant changes in the presence of macrophages; the number of macrophages in the mice hearts was relatively low across all groups, but the activation of these monocytes is unknown. Picrosirius red fluorescently stains collagen and presents a solution for determining the extent of scar formation due to ischemia, however, when HAM is introduced to the tissue, whether it be through integration at the epicardial surface or injection, it introduces a large amount of collagen to the tissue. A large percentage of HAM is collagen, so by introducing it to the tissue it is likely that it then brings up the percent collagen in the ventricular wall, even though that collagen is not related to the scar formation. Therefore, it is reasonable to see that the percent of collagen

would not differ between groups, even if the scar formation is lessened in HAM treated tissues.

Injection into beating myocardial tissue only a couple millimeters in width is a difficult and not always successful task. Although measures were taken to promote the injection staying in the tissue, there is not a way of knowing the amount of injected material that remained in the tissue, dispersed, or leaked out of the injection site. These limitations provide room for improvement in the experimental design and reason for expansion to a larger N and larger species.

# Chapter 3: *In vivo* Swine Model

**Introduction:**

In this chapter I will review the experimental application of HAM in an *in vivo* ischemic swine model. First, it is important to note the additional treatments that were applied in this study. In addition to HAM, Platelet-Rich Plasma (PRP) and Trans-myocardial laser Revascularization (TMR) were used as supplemental treatments to HAM injection. PRP is derived from the centrifugation of whole blood and contains a large number of platelets in a small amount of plasma, as indicated by its name. Platelets provide a source of growth factors, so PRP is a dense accumulation of growth factors such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and platelet derived angiogenic factor (PDAF)[34]. These growth factors have a vital role in healing processes, most notably proliferation and angiogenesis[35]. Platelets also contribute fibronectin, vitronectin and other substances that are key initiators of wound healing[34]. It has also been reported that PRP may provide a potential fibrin and platelet regenerative matrix to advance healing pathways[36]. The benefits of PRP remain controversial as studies have not provided strong evidence of its efficacy and introducing growth factors can promote tumor growth. However, we hypothesized that using PRP as a suspension solution for HAM injection would provide additional growth factors and substances beneficial to wound healing, and any risks would be counteracted by anti-tumorigenic properties of HAM[37].

We also hypothesized that TMR would add to the benefits of HAM injection when combined as a treatment. TMR has been used since the early 90's to initiate revascularization and improve perfusion[38]. The general concept of TMR is to create an artificial pathway for blood to reach areas of ischemia[39]. Myocardial perfusion has been reported to increase by 20 percent in patients treated with TMR. The idea of combining TMR treatment with HAM injection was that increased perfusion could enhance myocardial preservation and provide vessel formation for increased HAM integration into the tissues. A porcine animal model offers advantages such as similarities to humans in terms of organ sizes, coronary anatomy and immunologic responses[40]. A large surface area and ease of multiple, separated injections was necessary for this experiment, making the swine the most translatable and readily available animal model that could be used.

# Materials and Methods

**Experimental Design:**

Experiments were approved by IACUC at the University of Arizona (protocol number: 17-217) and adhered to guidelines for care and use of laboratory animals. The main purpose of this study was to be a pilot study to serve only as a proof-of-concept and establishment of protocols for further investigation. Therefore, the study included a small sample size with a large number of treatments per animal. Tissues were separated into treatment sites, meaning the non-treatment areas served as control tissue.

*Surgical Procedure:*

Six-month-old domestic farm pigs (n=3) ranging from 54.7 – 58.75 Kg, received bupivicain at 2mg/Kg and lidocaine at 4-6 mg/Kg for local and regional analgesia. The animals then underwent 45-minute percutaneous ischemia reperfusion via balloon occlusion distal to the second diagonal of the Left Anterior Descending artery (LAD) under fluoroscopic guidance.

Seven days post-ischemia, animals were sedated with ketamine 10-18mg/Kg and diazepam 1-2 mg/Kg, induced with 5% isoflurane in O2 via mask and anesthetized with 1-2.5% isoflurane in O2 via endotracheal tube before being placed in dorsal recumbency for median sternotomy to expose the heart. Lidocaine 2 mg/Kg IV bolus every 30 minutes along with fentanyl 0.003 mg/Kg IV bolus loading dose with constant rate infusion of 0.001-0.003 mg/Kg/hour was administered for pain management. In addition, buprenorphine HCl 0.01-0.05 mg/Kg intramuscular for analgesia at vet discretion.

Animals were scheduled for euthanasia after being sedated and anesthetized via KCl IV at 1-2 mmol/Kg in conjunction with isoflurane at post-operative day 1, 2 and 7 following second survival surgery.

*Treatment Groups:*

Treatments included an injection of two variations of HAM, which were referred to as particulate and viable. The particulate is a micronized and suspended form of the dehydrated amnion/chorion material. The viable is a suspension of the live amnion layer. One aim of this study was to test if

treatments could be further augmented through the addition of other methods known to induce healing and promote regenerative processes. Therefore, PRP and TMR were added to treatment sites to examine a local combined effect leading to further enhancement of tissue preservation and regeneration. PRP was used as an alternative solution for saline in suspension of HAM. TMR was added close to the injection site to investigate the combination of treatments. Treatments were grouped as follows: Control, PRP, TMR, PRP and TMR, Particulate in saline, Viable in saline, Particulate in PRP, Viable in PRP, Particulate in saline with TMR, Viable in saline with TMR, Particulate in PRP with TMR, and Viable in PRP with TMR. Each animal received all of the above treatments in the infarcted zone at different sites to create an N of 3 for each treatment. Each injection was 500 microliters of equal concentration of HAM. For Particulate suspension, dehydrated Amnion/Chorion matrix was micronized and thoroughly mixed with the suspension solution.

*Tissue collection and preservation:*

Following euthanasia, the sternum was re-opened, the heart was excised and washed with heparinized saline, and treatment sites were cored out of the heart and embedded in OCT, then frozen in liquid nitrogen and stored at -80℃. All tissues were sectioned at 5 micrometers in -20℃, placed onto charged slides, and stored overnight at -80℃ before proceeding with fixation and staining.

**Immunohistochemistry and Histological Staining:**

*Hematoxylin and Eosin (H&E):*

H&E was used to visualize the morphology of the tissues as well as neutrophil and cell accumulation in the tissue. Tissues were fixed in 100% methanol at -20℃ for 15 minutes before rehydration and Richard Allen Hematoxylin for 5 minutes for nuclei staining. Tissues were incubated in Eosin for 25 seconds to stain the cytoplasmic elements. Treatment sections were then imaged in 5x light microscopy and stitched using Photoshop.

*Picrosirius Red (PSR):*

PSR was used for collagen quantification as an indication for scar formation. Tissue sections were fixed in 95% methanol 5% acetic acid at -20℃ for 15 minutes. Nuclei were stained with Weigert's hematoxylin before tissues were stained with picrosirius red for 30 minutes. Tissues were then imaged in fluorescent light microscopy at 5x under Alexa 488 and Texas red and overlaid. Quantification of pixel area for collagen and live tissue was performed using ImageJ Software after Photoshop stitching. Quantification was performed by finding the pixel area for both green and red fluorescence using the Color Threshold function on ImageJ software. The sum of the live tissue (green) and collagen (red) was used as the total tissue area and the amount of collagen was then divided by total tissue area to give a percentage of collagen within the tissue section. This was presented as an average percentage for each group.

# Results

**Immunohistochemistry and Histological Staining:**

Histological staining did not show any noticeable trends across treatment groups. We did not notice any large areas of increased cellular accumulation as was expected in the HAM treated tissues. However, some tissues showed small pockets that did not have muscle tissue but were dense with nuclei, shown by H&E staining. This could indicate pockets of the injection that had yet to disperse. Further investigation is required to see if these were observed in control tissue. PSR staining did show collagen formation at TMR sites especially, however these sites can be difficult to distinguish from larger vessels. Histological comparisons across representative treatment groups are shown below in **Figure 13**.

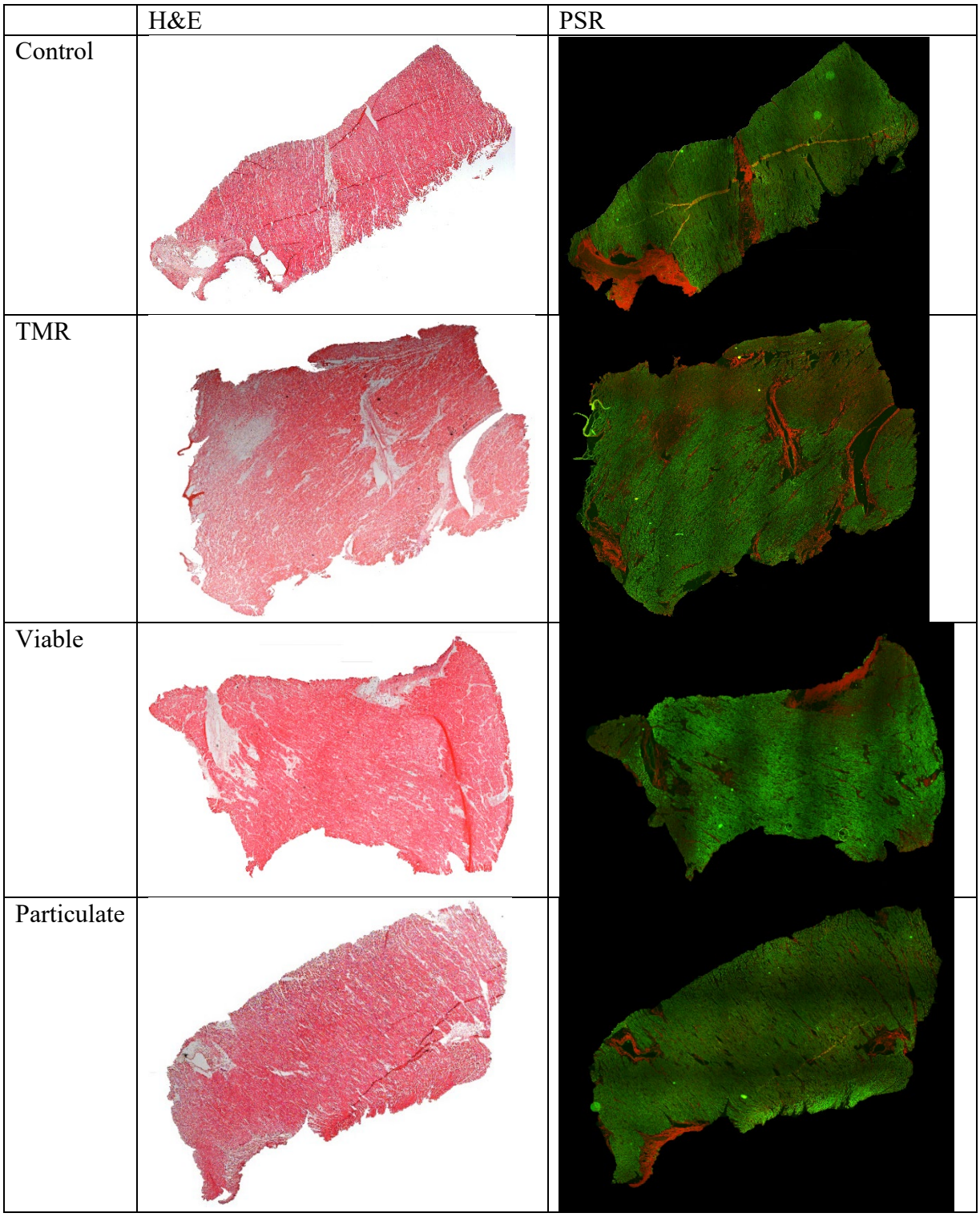



**Figure 13:** Histological comparisons between representative treatment groups with H&E and PSR.

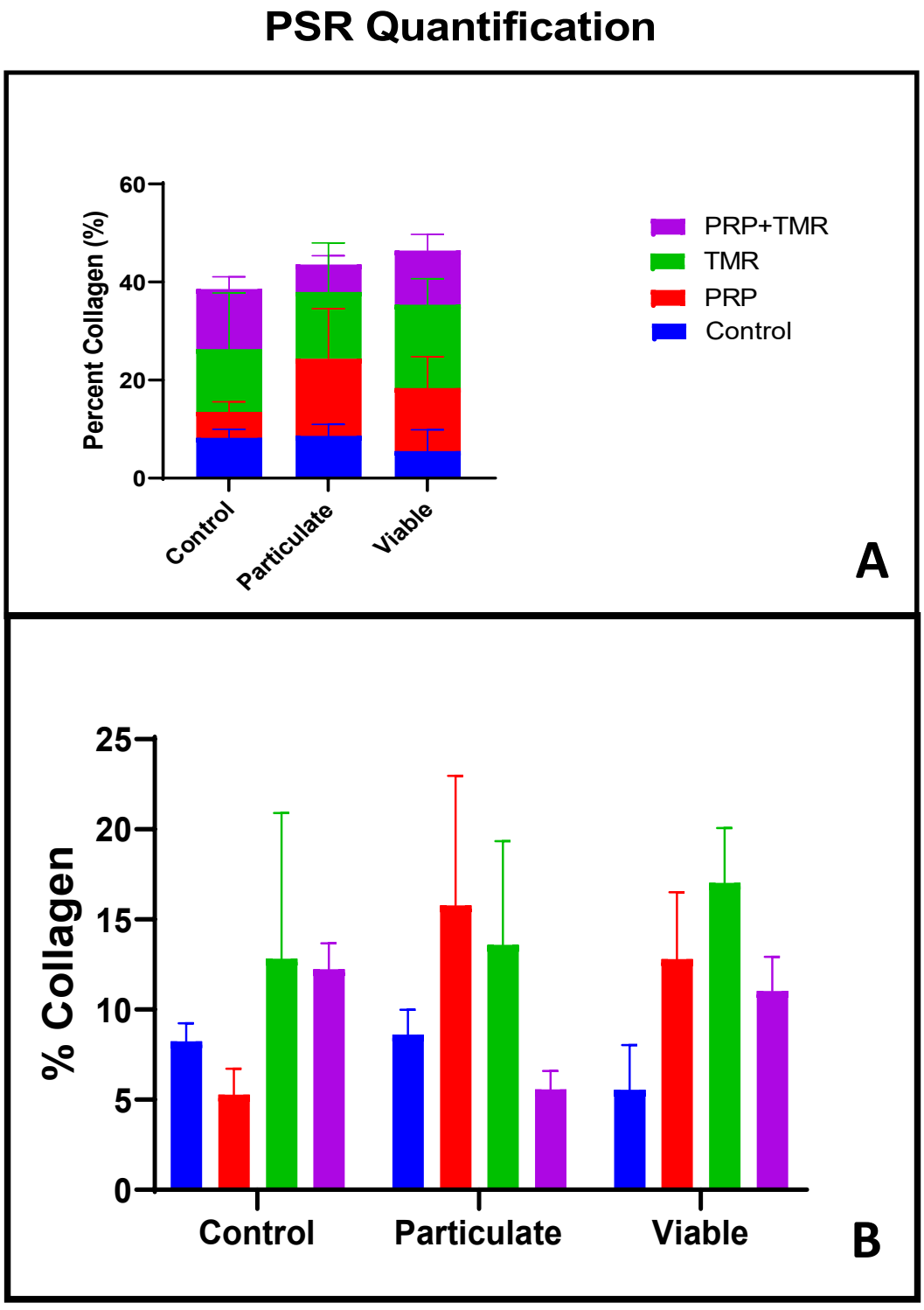



**Figure 14:** Percent collagen as determined my picrosirius red staining quantification in the tissue cores across treatment groups. Data is represented as mean values with error bars ±SEM.

PSR staining quantification showed no significant differences across treatment groups, however TMR groups tended to have a larger mean percentage of collagen as predicted by histological observations. **Figure 14, A** and **B** both represent percent collagen in the tissue, **A** shows the different HAM treatments across combinations of the additional treatments to emphasize the differences between control and HAM treatments.

**Discussion:**

This pilot study did not suggest any strong trends of data or observations. This study served the purpose of working out the experimental design and protocols for the combination of treatments in an ischemia reperfusion swine model to expand the study for our nest steps in swine models with HAM treated infarcted hearts. Limitations of this study included the small sample size as well as the timeline of the treatments. Inflammation and POAF reach their peak levels at post-operative days two and three, so a timeline stretched out around those days was chosen for comparison. Each animal was scheduled for a different timeline of euthanasia to assess the relatively acute effects of the treatments of up to one week. However, due to the strain on the animals, two of the pigs that were scheduled for euthanasia at days 2 and 7 died shortly after the second survival procedure. The first pig was euthanized at 24 hours post-op, the second and third pigs died approximately 3 hours after the median sternotomy and treatment application procedure. This led to an even shorter timeline than originally planned, and possibly not enough time for each treatment to take effect. Another

challenge that we faced was the preservation and fixation of the material. The first set of tissue that was collected suffered areas of freeze damage that was later corrected in the next tissue collection and preservation protocols for the last two animals.

# Chapter 4: General Discussion

Previous chapters reviewed literature pertaining to the current uses of Human Amniotic Membrane as well as its noted potential in cardiac application through animal models. Limited literature is available on the cardiac applications of HAM and the mechanism for its contribution to remodeling pathways are still unknown. I then discussed our application in human cardiac patients to prevent POAF and went over our use of HAM in MI animal models to demonstrate its abilities to preserve myocardial tissue and function. These experiments served as proof-of-concept pilot studies in which amniotic material was applied to ischemic hearts to examine response mechanisms triggered or suppressed by the presence of the material. This was done in order to gain a more thorough understanding of the observation that its use in cardiac surgery patients dramatically reduced the occurrence of POAF. This chapter will discuss the observed results of the studies performed, speculation into the mechanisms behind these observations and possible methods for enhancing outcomes.

Through the use of a four-week permanent ligation mouse model we came to the conclusion that HAM injection may offer a solution to improving the retention of myocardial function after ischemia. Our data suggests HAM injection leads to a preservation of the contractile function and a decrease in pathological remodeling as shown by a larger retention in fractional shortening and reduced dilation of the left ventricle. This was measured through the left ventricular internal diameter and volume during systole. These results are further supported by the trends of the ejection fraction, fractional shortening and wall thickness.

One unexpected observation was the increase in ejection fraction over the 10-day and 28-day period in some mice. We believe that this was due to the injection of HAM helping to preserve the myocardial function. However, it is important to point out that the functionality of the muscle was retained in these animals, and the group of mice that received the HAM injection showed a visible reduction in the mean percent decrease in ejection fraction. The control group was much more variable, we saw outliers in the control group that lost much more of their contractile function than was seen in any of the HAM injection animals.

A second notable observation was seen in the histology of the mice hearts. We saw a strong accumulation of cells at the epicardial surface when treated with HAM membrane. This was seen at 10-day post-operation, and even more so at the 28-day marker. At day-28 we noticed that in multiple mice there was an appearance of tissue at the location treated with HAM membrane on the ventricular wall. This tissue contained a dense accumulation of nuclei but not typical muscle or collagen morphology, so we believe it to be a result of HAM infiltration of the myocardium. This supported the idea that HAM would integrate into the myocardium and lead to regeneration, instead of simply being degraded by the body. This suggests that cellular recruitment and growth was an effect of HAM membrane integration into the myocardial tissue. The injection of HAM resulted in retention of cardiac muscle tissue in hearts that showed a large visible ischemic area through the scar formation that developed around the injection site. IL-6 staining did not show any notable differences in amount of cytokine across the groups, meaning that HAM did not elicit a larger immune response in HAM

treated tissues. This supports the claims of HAM's low immunogenic properties but did not illustrate further suppression of the immune response as expected.

**Speculations:**

Further speculation of the mechanism behind our data is that the primary contribution of HAM when applied to ischemic myocardium is its anti-fibrotic effects. The injection of HAM supported this claim through histological comparison to control MI hearts. Control MI mice showed ventricular wall remodeling into a thin section of collagen lacking contractile function. In the HAM injected MI model, we saw collagen surrounding the area of injection indicating the suppression of scar formation in the injected area. In addition, the anti-inflammatory properties may contribute to limiting the death of the cardiomyocytes in these injection sites. We also believe that if the model were extended to a longer timeline that the HAM membrane group may experience rebuilding of the myocardial wall due to the integration of the membrane and growth of cells that was observed. This growth is likely due to the abundance of growth factors available to the tissue through the application of HAM. Therefore, it is believed that the growth will lead to improvements in cardiac function long-term if the model was given a longer timeline.

A recent publication has shown that pericardial closure during surgery using an extracellular allograft scaffold has led to a reduction in POAF[41]. The proposed mechanism behind this research is that the closure of the pericardium provides a barrier that prevents infiltration of cellular debris and therefore

inflammation of the pericardium. However, pericardial closure has been strongly correlated to the occurrence of cardiac tamponade, so this method can be risky and the widely preferred method is to refrain from stitching the pericardium[42]. Our goal was to prove that the application of HAM served as more than a pericardial barrier, which supported by the injection of the material leading to significant retention of systolic contractile properties. The use of HAM is superior to that of the extracellular matrix barrier used in the study for several reasons. Primarily, in addition to a barrier, it provides anti-fibrotic and inflammatory suppressing factors which further the contributions to reducing scar formation and development of POAF. We saw this reduction in POAF occurrence without stitching it to the pericardium, meaning the risk of cardiac tamponade due to pericardial closure is eliminated with the use of HAM. In addition to this, HAM provides a variety of growth factors to support healing and angiogenesis which is predicted to improve cardiac function long-term. HAM has also been proven to be equally compatible as autologous skin grafts, superior to other allografts, so HAM is likely to elicit less of an immune response than the extracellular allograft[29]. These speculations can be proven by future study into HAM application in cardiac disease.

**Future Directions:**

The two animal studies that were performed were pilot studies that left substantial room for expansion and improvement. Although the data visually supports our hypotheses, but some do not show statistical significance, the

reduction in error through a larger sample size could possibly lead to a confirmation through statistical analysis. It is reasonable to believe that this larger sample size would lead to more reliable results in order to differentiate more clearly between groups. In addition to increasing the sample size, the inclusion of a second species of rodent would be beneficial to further supporting the trends of the initial data. Rats present a larger rodent model allowing for a larger surface area to work with and thicker ventricular wall for injection. It is also planned to further the swine study in both acute and chronic remodeling. Our next steps with the swine models will be to identify acute functional differences and inflammatory modulation seen in HAM treated ischemic tissues. Finally, we will study HAM implications in long term remodeling and functional outcomes in an infarcted swine model. In order to prove the material's superiority over an extracellular matrix use in pericardial closure, it will also be necessary to add in control groups provided with these barriers.

Eventually, it would be interesting to test the ability of HAM application in a wider range of cardiac procedures. For instance, Percutaneous Coronary Intervention (PCI) is an alternative method to CABG to increase blood flow to an ischemic area of the heart. PCI utilizes a catheter insertion of a stent into the blocked artery to expand the artery and restore blood flow[43]. Because this procedure is not an open-heart surgery, HAM application would be trickier. This is where it would be interesting to test the use of catheter injection of HAM during cardiac catheterization surgeries. Recent studies have opened up the possibility of doing this through the use of the PHOENIX ™ system[44]. Although

PCI procedures do not require puncturing the pericardial sac and therefore would not require treatment for inflammation or POAF caused by this, the results reported in this thesis indicate that HAM may contribute more to cardio-protection, retention of cardiac function and myocardial regeneration than originally hypothesized. Treatment of ischemic cardiac tissue with HAM presents an exciting possibility for improving cardiac disease treatment methods.

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