ABSTRACT

Title of Document: THE CONTROLLED DELIVERY OF HYDROGEN SULFIDE FOR THE PRESERVATION OF HEART TISSUE

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There are over 100,000 patients on organ transplant waiting lists, creating a significant need to expand the donor pool. The heart is the most difficult organ to preserve *ex vivo*, with a short viable storage time of 4-6 hours, because damage to mitochondria during preservation can impair the heart's contractile function. By extending the viability time, the geographical range of donors can be extended. Hydrogen sulfide (H_2S) has been shown to reduce metabolism, increase preservation times, and enhance graft viability. We have developed gelatin microspheres under 10 microns able to slowly release H_2S and investigated different crosslinking concentrations to understand the time release profiles. These microspheres were then used to maintain H_2S levels in cardiomyocyte cell cultures without decreasing cell viability. Histological samples from 20 cold-stored rat hearts in various experimental treatments show H_2S-releasing microspheres offer protection against preservation injury comparable to the current clinical standard, University of Wisconsin solution.

THE CONTROLLED DELIVERY OF HYDROGEN SULFIDE FOR THE PRESERVATION OF HEART TISSUE

By

Team Organ Storage and Hibernation

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

I/R	Ischemia-Reperfusion (injury)
UW	University of Wisconsin
ROS	Radical oxygen species
ETC	Electron Transport Chain
MTP	Membrane Transition Pore
ATP	Adenosine Triphosphate
K-ATP	Potassium ATP Channels
K^+	Potassium ion
Na^+	Sodium ion
Ca^{2+}	Calcium ion
NaHS	Sodium Hydrogen Sulfide, an H_2S donor
H_2S	Hydrogen Sulfide
HIF-α	Hypoxia inducing factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
DMEM	Dulbeco's Modified Eagle Medium
IgG	Immunoglobulin G
rcf	Relative centrifugal force
µL	Microliter
mL	Milliliter
mg	Milligram
M	Molar
KOH	Potassium Hydroxide
$KHCO_3$	Potassium Bicarbonate
H_2O_2	Hydrogen Peroxide
PBS	Phosphate Buffered Saline
DAB	Diaminobenzidine
OCT	Optimal Cutting Temperature compound
bFGF	Basic Fibroblast Growth Factor
GHM	Gelatin hydrogel microsphere
GA	Glutaraldehyde solution
Live/Dead	Also known as a Viability Assay
MTT assay	Also known as the Metabolic Activity Assay

I. Introduction

Nearly 110,000 people in the United States are on the organ transplant waiting list, yet only 77 actually receive organ transplants daily. Organ transplantation today is hampered not only by the shortage of available organs, but also by current methods of organ storage, which provide a limited timeframe of organ viability. Currently, the viability of hearts is limited to a mere four to six hours, creating significant restrictions and logistical problems with regard to the timing of organ transportation. The duration of hypothermic storage and the perfusion techniques utilized to protect organs from ischemia-reperfusion (I/R) injury are important. Preservation-induced injury is a major contributing factor to early graft dysfunction in recipients. By extending the limits of organ storage, it would be possible to broaden the geographical radius to which a donated organ could be delivered, and ultimately widen the pool of potential organ transplantation recipients.

The most common method used for organ storage today is cold storage. Standard methods for static cold storage involve the use of low temperatures and the University of Wisconsin (UW) solution. The components of the UW solution help reduce swelling of the organ, while hypothermic preservation reduces metabolism and therefore harmful metabolic waste. Additionally, the solution provides rapid cooling as well as a sterile environment. However, while the UW solution lowers aerobic metabolism, anaerobic metabolism persists. The oxygen free radicals subsequently generated lead to I/R injury, inflammation, tissue damage, and cell death. Additives to UW solution, such as perfluorocarbons, improve the preservative capabilities of the solution through a variety of mechanisms. However, despite nearly two decades of research into potential additives

to static cold storage solutions, heart storage time has yet to clinically exceed 8 hours. Methods other than static cold storage include hypothermic machine perfusion, used mainly for kidneys, and normothermic perfusion, which is performed at 37°C. Both are promising, but limited in their applicability because they require relatively large pieces of equipment to maintain storage conditions. In short, existing techniques for organ storage are inadequate, and have limited viability time and high risks of injury and inflammation. These obstacles create the need for a better means of organ storage.

This research study focuses on utilizing a promising chemical, hydrogen sulfide (H_2S), to extend and improve upon the current organ storage methods. Recent research has shown that H_2S can induce a state of hibernation, which has been proven to protect hearts in storage from I/R injury. However, as will be discussed in the sections to follow, H_2S does have some inherent problems. For example, H_2S cannot be used as a simple additive to the original storage solutions, as an estimated one-third of the molecules are completely unused and escape in the form of a deadly gas. In addition, H_2S may be consumed by the heart cells themselves, which also compromises its effectiveness for storage.

In order to address these issues, we propose the use of gelatin microspheres that will allow for continuous delivery of H_2S to the heart and potentially improve both the practicality of using H_2S for organ preservation and the protective effects requiring the presence of H_2S. This hydrogel delivery system utilizes polymer networks that can provide a protective haven for many drugs that are normally degraded in circulation. Gelatin polymers in particular are advantageous for their biocompatibility,

biodegradability, and biologically recognizable moieties, and have previously been used in cardiac drug delivery applications with no side effects.

Objectives

The goal of this research study was to explore a novel preservation method utilizing H_2S to induce a protective state of hibernation against I/R injury. Although H_2S has been shown to reduce metabolic rates, ATP consumption, and the production of reactive oxidative species (ROS), research has not adequately addressed how long hearts should be exposed to H_2S for optimal protection. It was hypothesized that continuous release of H_2S in the preservation solution will result in better protection of the heart during cold storage. Such continuous release may be achieved by gelatin hydrogel microspheres loaded with sodium hydrogen sulfide (NaHS), an H_2S donor.

The global hypothesis was that H_2S-loaded gelatin hydrogel microspheres will deliver H_2S throughout the heart in a continuous, controlled manner to enhance protective effects associated with H_2S, including K-ATP channel opening, ROS scavenging, and hibernation, which will prolong heart viability, reduce I/R injury, and be practical for clinical implementation.

The overall goal was to develop a novel method for heart preservation that would not only extend heart viability in storage, but would also be applicable to today's organ transport methods. We hypothesized that compared to existing methods of storage, controlled delivery of H_2S will improve heart viability as determined by metabolic activity, viability assays, and ATP assay and caspase-3 assays.

The specific aims of the proposed project were:

1. To evaluate the relationship between NaHS concentration and its effect on tissue viability. To characterize cell metabolism of H_2S, cardiomyocytes were exposed to NaHS over 1 hour and the subsequent levels of H_2S in the cell culture were assessed. The cardiomyocytes were then assessed using metabolic activity and viability assays. Therefore, we investigated the effect of varying NaHS concentrations on cell viability.

2. Hydrogels are biocompatible polymers with a wide variety of applications. By controlling the degree of crosslinkage and gelatin acidity, absorption and release rates of NaHS were varied until a desired time-release profile of NaHS is achieved. We investigated crosslinkage in the fabrication of gelatin microspheres in order to control the release of H_2S.

3. In order to analyze the clinical applicability of the previously developed concentration and release profiles, the biological efficacy of NaHS treatment was analyzed in comparison to existing methods of organ storage. The most commonly used method today is hypothermic storage in UW solution. We analyzed three possible outcomes of applying microsphere delivery of NaHS to isolated hearts. First, we determined whether H_2S enhances, hinders, or has no effect on preservation with UW solution. Second, the viability effects of a fixed initial concentration of H_2S were compared to that of constant replenishment of H_2S, which was accomplished using gelatin microspheres. Lastly, an experiment was conducted to verify whether gelatin microspheres have an effect on heart viability. The extension of tissue survival during storage was measured by ATP and caspase-3 assays.

With the completion of these specific aims, we have developed a new, promising method for heart storage that has the potential to reduce I/R injury and ROS and can be incorporated into current organ transport methods.

II. Background

Organ Storage

Introduction

Organ transplantation today is limited by the time an organ can remain viable outside the body. This time range influences several key decisions (e.g. where the heart can be transported and thus where the surgery can be conducted) that ultimately determine the number of patients who can successfully receive a transplant. For the human heart, clinical storage time is limited to four to six hours under current storage methods as the extent of ischemia-reperfusion (I/R) injury is proportional to storage time (Jamieson and Friend 2008). The standard method of organ transplantation today is static cold storage of the organ in solution, such as Viaspan^{TM} solution. Widely known as the UW solution, this solution was the first to be thoughtfully designed for organ preservation and is widely used clinically. The various components of the UW solution have specific functions in maintaining the viability of the organ during storage. There have been many additions and alterations made to the UW solution ever since it was first designed in an attempt to improve organ preservation.

Heart Transplantation Milestones

Heart transplantation research began in 1956 in the United States when Watts R. Webb and James Hardy investigated both heart and lung transplantations in dogs. By 1957, Webb was able to demonstrate limited survival in dogs undergoing heart transplantations; with the success of the lung transplant in 1963, research moved on to human subjects. On January 23, 1964, the first transplant patient received a chimpanzee

heart. Retrograde gravity flow of cold, oxygenated blood through the coronary sinus (Figure II-1) was used as the preservation technique (Lower, Stofer et al. 1961; Hardy and Chavez 1968; Hardy 1999). After the heart was transplanted, it was warmed back to 37°C and defibrillated, restoring function immediately. Since the heart is a highly innervated organ, transplantations bring about complete extrinsic autonomic denervation, which results in almost total loss of the myocardial stores of catecholamines (Daggett, Willman et al. 1967). The inability of a trans-species heart to reinnervate contributed to the failure of the above study (Daggett, Willman et al. 1967). In addition, the chimpanzee heart was too small to sustain human functions. Other factors that led to the death of the recipient included the metabolic deterioration of the donor heart as well as the donor's state of intermittent shock.

The first successful human heart transplantation was conducted in Britain in 1967. However, the recipient died 18 days later of pneumonia. The first heart transplantation in which the recipient did not die shortly after the procedure occurred a year later and was the tenth heart transplantation ever conducted (Proctor and Parker 1968).

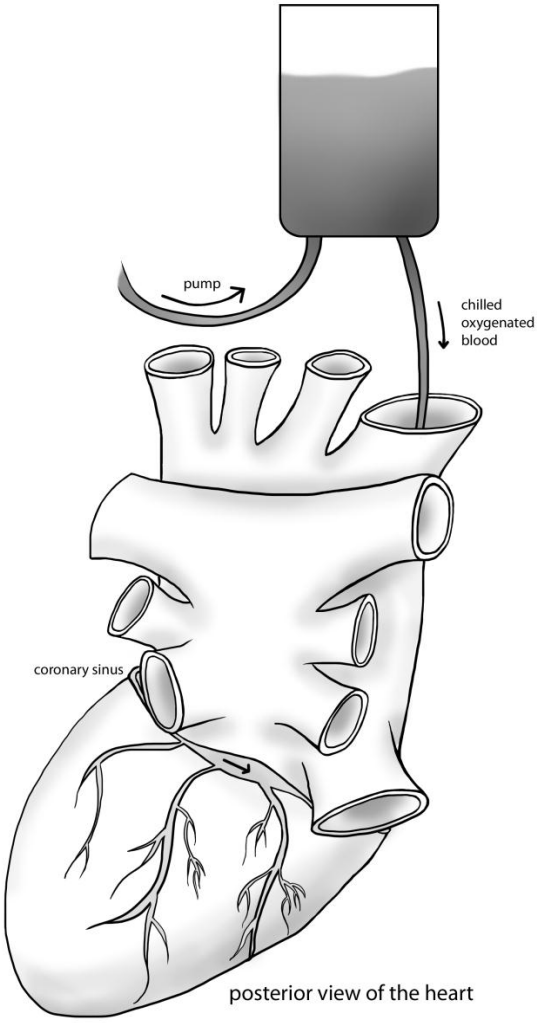

Figure II-1: Heart preservation in first transplant. Retrograde gravity flow of chilled oxygenated blood through the coronary sinus. (Adapted from Hardy, 1999)

Donor Management and Heart Extraction Today:

Today, organ harvesting in the United States undergoes many regulatory steps meant to standardize and maintain the quality of transplantation procedures. Physicians identify potential donors using patient history and immunization records. Before harvesting can occur, the donor must be identified, declared brain-dead, and have given consent. Next, donors undergo tests for HIV, Hepatitis B, Hepatitis C, Epstein-Barr virus, and the toxoplasma antibody. Negative results must be obtained for each of these tests to ensure that the donating individual's organs are in healthy condition for transplantation. A blood cell count and blood typing are also performed, followed by a detailed analysis of organ function, in order to ensure the viability and quality of organs before they are transplanted into the recipient. To assess the viability of donor organ health for the heart, a standard test includes electrocardiograms. Additionally, changes in troponin and creatine phosphokinase are monitored. If all tests prove negative, an echocardiogram is then ordered to assess morphology (Tatarenko 2006).

Removal of the heart is only conducted on brain-dead, heart-beating donors. In the procedure, donor bodies must be maintained at 90 mmHg systolic blood pressure and a heart rate less than 100 bpm. After a stand-off period has occurred to ensure the death of the donor, surgeons cross-clamp the aorta and perform *in situ* perfusion through a single cannula. Organs are rapidly cooled because the core body temperature is reduced by the perfusion. Moreover, subsequent graft function is significantly improved by a flush with streptokinase (an anticoagulant) at a flow pressure of 150 mmHg; it has been shown to lead to the highest recipient survival rates (Brockmann, Vaidya et al. 2006).

The heart is then stored in UW solution at a reduced temperature of 4^{o}C (Tatarenko 2006).

Preservation injury

When organs are removed, they unavoidably undergo a period of warm ischemia, typically followed by a period of cold ischemia in a low oxygen preservation solution. Ischemic-reperfusion (I/R) injury is the biphasic damage to tissue during periods of metabolic stress due to low blood flow and oxygen, followed by oxidative stress accompanying re-oxygenation. It is a paradoxical situation in that the restoration of blood flow rescues the ischemic tissue and reduces the infarct size, or area of localized dead tissue, but reperfusion itself contributes to tissue death. I/R injury manifests itself in a variety of organs and tissues, ranging from the brain, to skeletal muscle. A familiar example is injury after blood flow is restored following a myocardial infarction; I/R injury contributes to up to half of the infarct size. This damage to the heart leads to a 25% likelihood of cardiac failure (Yellon and Hausenloy 2007), and an array of dysfunctions like stunning (mechanical weakness), no-flow (areas of impeded perfusion), and arrhythmias (abnormal heart rhythm).

The mechanisms driving I/R injury are complex and still not fully understood (Henderson, Singh et al. 2010). The general progression is postulated to occur in two stages: an ischemic stage, and a reperfusion stage. During ischemia, oxidative phosphorylation is compromised because there is limited oxygen. The depletion of ATP impacts essential cellular self-maintenance processes and anabolic pathways, compromising the production of essential components needed to keep the organ alive. Conversely, catabolic pathways continue to break down these components (Ijichi,

Taketomi et al. 2006). Lactate and other metabolic by-products like hypoxanthine (a byproduct of ATP) build-up, as the organ uses anaerobic metabolism to support basic homeostatic processes (like the sodium potassium pump) in the low-oxygen environment. These become fuel for ROS generation upon the re-introduction of oxygen.

During ischemia, much calcium is accumulated in the cell, particularly in the mitochondria (Nishida, Sato et al. 2009). This calcium overloading is a critical event in the progression of I/R injury. Calcium accumulates inside the cell through various mechanisms related to ionic imbalance due to energy depletion. With less ATP to power the membrane Na^+/K^+ pump, sodium enters the cell down its concentration gradient. Sodium is exchanged for calcium via a membrane-exchange pump (Dorweiler, Pruefer et al. 2007), and indirectly opens voltage-gated calcium channels (Storey 2004). The mitochondria uptakes calcium, which is driven by the ionic potential across the inner-mitochondrial membrane. The high calcium concentration inside the mitochondria has an important role in reperfusion, as it contributes to the opening of the mitochondria transition pore (MPT). Proteases and phospholipases leak out of lysosome in high-calcium environments. These and other calcium-activated enzymes destroy proteins or lipid membranes, making the cell more susceptible to rupture (Murphy and Steenbergen 2008), and conduct apoptotic signaling cascades (Dorweiler, Pruefer et al. 2007).

The low-oxygen environment also reduces the cell's antioxidant defenses and promotes the generation of ROS. The mitochondria, under normal conditions, uses oxygen quite efficiently, converting 95% of its oxygen to ATP without production of ROS (Dorweiler, Pruefer et al. 2007), highly reactive species that can directly damage the cellular membrane and organelles. This efficiency drops during ischemia. Much more

ROS is generated due to inefficient electron transfers to the limited oxygen present (Becker 2004), and these can further damage the electron transport chain (ETC), leading to more ROS generation in a dangerous, self-propagating cycle (Roth and Nystul 2005). ROS generation is possible because catalase, dismutase, and the glutathione peroxidase system, the enzymes responsible for safely neutralizing superoxide to peroxide to water, do not function well in ischemia (Becker 2004), leading to a build-up of hydrogen peroxide, which in turn, generates the hydroxyl radical, a particularly dangerous ROS.

Upon reperfusion, there is a "burst" of ROS in a short amount of time. This ROS generation is fueled by the metabolic byproducts that accumulated during ischemia, and these ROS directly damage cellular structures and lead to tissue necrosis (Jamieson and Friend 2008), as well as signal inflammatory response (Dorweiler, Pruefer et al. 2007). The mitochondria are sites of particular interest as it plays a critical role in mediating I/R injury. A substantial portion of damage to the electron transport chain occurs during ischemia. The MTP is a non-specific channel in the inner mitochondrial membrane that opens under conditions of high matrix calcium and ROS, conditions that occur during the start of reperfusion (Murphy and Steenbergen 2008). The opening of the MTP leads to the dissipation of proton gradient and the inability of mitochondria to make ATP, which leads to cell death. Due to membrane disruption in the mitochondria by ROS, cytochrome c is released, which leads to the activation of caspases and apoptotic pathways (Chen, Camara et al. 2007). Thus, the extent to which MTP remains open largely determines the extent of reperfusion injury. The most effective therapeutic measures, like ischemic preconditioning, thus target the events during ischemia that set the stage for MTP

opening, including high calcium loading and ROS production (Halestrap, Clarke et al. 2007).

Preservation Methods

For the human heart, clinical *ex vivo* storage time is limited to four to six hours. Additionally, there is an organ donor shortage; as of February 2011, 3,215 patients were registered on the waiting list for a heart transplant. However, in 2010 there were only 2,135 donors, all deceased, leaving at least 1,000 still in need (Organ Procurement and Transplant Network 2011). Since quality organs are limited in supply and always high in demand, prolonging storage time can expand supply by expanding the geographic range of potential donors.

The quality of a donor organ is determined by a variety of factors, including donor age and donor management prior to procurement of the organ. The duration of hypothermic storage and the perfusion techniques utilized to protect organs from ischemia-reperfusion injury is also extremely important.

Static cold storage involves the use of low temperatures and UW solution, a commonly used storage solution with its own advantages and disadvantages. The additives of UW solution greatly reduce swelling over previous methods, while hypothermic preservation reduces metabolism and therefore metabolic waste. Additionally, the solution provides rapid cooling and a sterile environment. Since UW solution lowers aerobic metabolism, it induces anaerobic metabolism, whose end-products generate oxygen free radicals, leading to I/R injury. Inflammation, tissue damage, and cell death are also prevalent when using UW solution.

Other methods of organ storage are also available, such as the use of perfluorocarbons, which is an additive that induces the cells to absorb oxygen more efficiently. However, despite nearly two decades of research into potential additives to cold static storage solutions, heart storage time has yet to exceed 8 hours (Skrzypiec-Spring, Grotthus et al. 2007). More existing methods include hypothermic machine perfusion, used mainly for kidneys, and normothermic perfusion, which is performed at 37^{O}C (Jamieson and Friend 2008). Both are promising, but are limited in their applicability due to the requirement of relatively large pieces of equipment to maintain storage conditions.

The underlying concept behind most preservation methods is the suppression of metabolism via hypothermia. Despite its limitations, the UW solution in conjunction with hypothermia is the most common method of preservation today. The UW solution can preserve the liver, pancreas, and kidney for up to two days, and its effectiveness can be attributed to various cell impermeant agents that prevent cells from swelling during cold ischemic storage. In addition, it contains agents that stimulate recovery of normal metabolism after reperfusion by amplifying the antioxidant capacity of the organs or by stimulating high-energy phosphate generation (Southard and Belzer 1995). When used with machine reperfusion, the perfusion fluid is modified by utilizing gluconate instead of lactobionate, which is ineffective in continuous machine perfusion for unknown reasons. Machine perfusion has yielded the best-quality and longest-term preservation as it allows for continual delivery of oxygen and substrates to the organ for the synthesis of ATP and other metabolites, but has yet to see widespread use (Southard and Belzer 1995).

Before the UW solution was formulated, general organ preservation was limited to four to six hours in Collins' solution, and heart preservation times were far worse. Collins' solution was first created when a perfusate derived from human plasma preserved a kidney for three days with continuous machine perfusion at 6-8^{o}C. The final perfusate, cryoprecipitated plasma, became the standard perfusion fluid for human kidneys. However, because it was derived from human plasma, there were concerns regarding its potential for spreading disease. Subsequent research focused on synthetic perfusates. Despite the success of the subsequent perfusates, none were applicable to general organ preservation. Furthermore, in the 1970s, organ rejection and immunosuppressive drugs became the forefront of research. Once immunosuppressive drugs such as cyclosporine were developed, the focus shifted to better methods of organ preservation. Thus, the development of UW solution became the focus of research in the early 1980s.

Overall, UW solution is highly efficient in preserving the liver, pancreas, and kidneys. UW solution organ preservation at 0-5^{o}C meets most clinical needs, with the transplant survival rate for these organs at nearly 90%. However, it remains difficult to preserve the heart and lungs for more than 4-6 hours, and this time cap also limits the geographical range from which a donor heart can be procured. The difficulty of heart preservation is twofold: heart function is vital to the heart's own survival, and the high content of contractile protein means that energy stores are used rapidly - during cold storage, organs lose 95% of their ATP within 2-4 hours. This loss does not compromise heart viability as it quickly regenerates the ATP a few days post-transplantation. Although the heart loses ATP slower compared to other organs, it undergoes ischemic

contracture when ATP concentration falls below a certain point (Stringham, Southard et al. 1992; Southard and Belzer 1995). Unlike any of the other organs, the heart must regain near-optimal functional conditions immediately upon transplantation to support the circulation of the recipient. Therefore, it is vital that the heart suffers minimal preservation injury. The primary goals of cardiothoracic transplantation are to achieve successful preservation of the heart and lung for 24 hours or more (Southard and Belzer 1995).

The UW Solution

While many of UW solution's components (Table II-1) were originally included for their proven ability to decrease cellular edema, others were included simply because they were common to other organ preservation solutions and had not been shown to have detrimental effects (Askenasy, Vivi et al. 1996). Research has clarified how these other components also protect the heart by combating three obstacles of preservation: impaired energy metabolism, acidosis, and radical oxidative species (Jeevanandam, Barr et al. 1991). Further research has attributed additional myocardial benefits to the calcium-chelating properties of UW solution.

Substance	Amount
K^+-lactobionate	100 mM
KH_2PO_4	25 mM
$MgSO_4$	5 mM
Raffinose	30 mM
Adenosine	5 mM
Glutathione	3 mM
Insulin	100 U/L
Bactrin	0.5 mL/L
Dexamethasone	8 mg/L

Allopurinol	1 mM
Hydroxyethyl starch	50 g/L

Table II-1: Components of the original UW solution. This version was originally used to preserve the pancreas. (Askenasy et al., 1996)

Benefits of Cold Storage

Most enzymes exhibit a 1½- to 2-fold decrease in activity for every 10°C decrease in temperature. Therefore, cooling organs from 37°C to 0°C can decrease metabolic rate 12-13 fold and extend preservation time by 12-13 hours because most organs can tolerate 30-60 minutes of warm ischemia without complete loss of function (Sumimoto, Dohi et al. 1992). The problem with this theory is that hypothermic storage also activates processes that will ultimately be deleterious to the preserved organ, including cellular swelling, extracellular edema, acidosis, depletion of metabolic substrates, reperfusion injury, calcium overload, and endothelial injury (Askenasy, Vivi et al. 1996). Although normothermic preservations are possible with continuous perfusion (Askenasy, Vivi et al. 1999), previous studies have shown that UW solution is limited to hypothermic storage (Rosenkranz 1995).

Cellular Edema

Preventing cellular edema is often considered one of the most significant goals of organ preservation (Karck, Vivi et al. 1992), as deficient volume regulation is an early indicator of cell injury (Jahania, Sanchez et al. 1999). During ischemia and hypothermia, an increase in a cell's cytosolic osmolarity leads to cellular swelling (Belzer and Southard 1988). Therefore, many preservative solutions are composed of impermeable molecules that create hyperosmotic conditions. Three particular components of the original UW

solution have been shown to decrease transcapillary and osmotic fluid transport, which reduces cellular swelling (Maurer, Swanson et al. 1990): lactobionate, an impermeant anion (358 Daltons); raffinose, a saccharide with a relatively large molecular mass (594 Daltons); and hydroxyethyl starch, a stable, non-toxic colloid that prevents expansion of the extracellular space and interstitial edema (Schubert, Vetter et al. 1989). Hydroxyethyl starch is sometimes excluded in experimental modifications to UW solution. Its exclusion seems to improve cardioprotection by reducing the viscosity of the solution, thereby permitting faster organ perfusion (Jovanovic, Lopez et al. 1998). Using dextran-40 in its place may benefit cardioprotection (Snabaitis, Shattock et al. 1997).

Cellular swelling can also be caused by an influx of sodium ions in both normothermic and hypothermic conditions (Jovanovic, Lopez et al. 1998). Consequently, regulation of factors affecting sodium transmembranal transport, such as $Na/K/Cl_2$ cotransport, NaH antiport, and Na^+ voltage-gated channels, can influence cardioprotection. This effect is demonstrated by the experimental correlation between inhibition of sodium transmembranal transport and reduced water accumulation during ischemia (Maurer, Swanson et al. 1990).

Although UW solution and several of its constituents have been repeatedly correlated with a reduction in cellular swelling, some research challenges the idea that such reduction or prevention of edema is a causative factor in cardiac preservation (Jahania, Sanchez et al. 1999). Data showing that membrane deterioration precedes cellular swelling suggests that the integrity of the sarcolemmal membrane is an alternative and more accurate indicator of ischemia injuries (Maurer, Swanson et al. 1990). Cellular stress events, such as intracellular Ca^{2+} overload and the excess

production of reactive oxidative species, may weaken the sarcolemmal membrane and cause necrosis during reperfusion (Rosenkranz 1995).

Metabolic Suppression and ATP Recovery

Myocardial metabolism produces ATP that is used to maintain intracellular homeostasis and a functional contractile apparatus (Pacher, Nivorozhkin et al. 2006). Decreased metabolic activity prevents the production and accumulation of toxic waste products, such as free radicals (Burgmann, Reckendorfer et al. 1992). However, a myocardial preservation solution must maintain a certain level of ATP for functional recovery because the heart, unlike other organs, can develop an irreversible contracture during preservation or reperfusion, known as ischemic contracture (Jahania, Sanchez et al. 1999). In UW solution, adenosine helps stimulate the synthesis of ATP to be used in regenerating Na^+/K^+ ATPase pump activity and other energy-requiring steps of metabolism, which in turn facilitates rapid defibrillation and recovery of myocardial function (Burgmann, Reckendorfer et al. 1992).

As an intracellular hyperkalemic solution, UW solution also contains a high potassium concentration that mimics intracellular ionic conditions (Burgmann, Reckendorfer et al. 1992). The high extracellular potassium content equilibrates extracellular and intracellular ionic concentrations; this minimizes the ionic gradient and subsequent ion transfer across membranes, which allows more rapid spontaneous defibrillation upon reperfusion (Jovanovic, Lopez et al. 1998). Although potassium's importance in UW solution is due to its depolarizing effect, depolarization in cardiac preservation has been associated with arrhythmias and the activation of enzyme systems that use up ATP, cause I/R injury, and cause myocyte swelling. A hyperpolarizing

solution may be a more effective cardioplegic agent, inducing cardiac arrest without these side effects (Maurer, Swanson et al. 1990; Jahania, Sanchez et al. 1999). However, other data also suggests that adenosine protects against such effects by inhibiting hyperkalemia-induced Ca^{2+} loading, a mechanism that could contribute significantly to ventricular dysfunction (Jahania, Sanchez et al. 1999). Additionally, the mere presence of only potassium in St. Thomas' solution did not confer the same protective effects as potassium in UW solution, suggesting a complex mechanism of potassium action in UW solution (Maurer, Swanson et al. 1990).

Acidosis

Although hypothermic storage slows reaction rates and delays cell death, UW solution and ischemic storage induce reduced oxygen consumption that promotes the use of anaerobic glycolysis for ATP production (Rosenkranz 1995). Anaerobic glycolysis produces lactic acid and hydrogen ions during ischemia, which can injure cellular organelles and activate macrophages that initiate cytokine production and an inflammatory response (Pacher, Nivorozhkin et al. 2006). In UW solution, potassium phosphate and magnesium sulfate, along with other buffers, minimize pH changes caused by lactate production (Burgmann, Reckendorfer et al. 1992).

Oxygen Radical Scavenging

In addition to radical oxygen species (ROS) being formed as waste products of metabolic activity during organ storage, the reintroduction of blood flow and oxygen during reperfusion can lead to the creation of ROS (Murphy and Steenbergen 2008). The hydroxyl radical is one of the most damaging species formed during ischemia and reperfusion (Maurer, Swanson et al. 1990). Glutathione is an endogenous oxidative

radical scavenger that reduces hydroxyl radical levels as well as other cytotoxic agents, such as H_2O_2, lipid peroxides, disulfide, and ascorbate (Rosenkranz 1995). Because glutathione is depleted during ischemia, UW solution also contains glutathione (Belzer and Southard 1988). However, it has been suggested that the glutathione is oxidized to a less effective form, and has little effect on preservation (Pacher, Nivorozhkin et al. 2006). Allopurinol is another ROS scavenger in UW, mainly targeting xanthine oxidase, but it also acts on other ROS (Pacher, Nivorozhkin et al. 2006). The protective effects against myocardial I/R injury have been demonstrated in both animal and human models (Amir, Rubinsky et al. 2003; Amir, Rubinsky et al. 2004). Hypothermic conditions also contribute to the reduction of free-radical generation in isolated hearts (Scheule, Jost et al. 2003).

Calcium Regulating Properties

In addition to the previously identified protective effects of UW solution, calcium regulation is an important factor in UW solution's success as a preservation solution. Several components of UW solution prevent the accumulation of intracellular calcium, which can lead to cellular dysfunction and death. Lactobionic acid, known for regulating intracellular water accumulation, also effectively binds calcium. Increasing concentrations of lactobionic acid have been correlated with decreasing concentrations of free calcium, especially at higher pH values when tested at a pH range of 5 to 8 (Burgmann, Reckendorfer et al. 1992). Furthermore, potassium phosphate and magnesium sulfate may indirectly regulate calcium concentrations by contributing to the hydrogen ion gradient (Murphy and Steenbergen 2008).

The UW solution contains various components designed to effectively preserve hearts in storage. Research has demonstrated which components are responsible for which protective effects, including the reduction of metabolic activity through cold storage, the prevention of cellular edema, and ATP recovery. Furthermore, UW solution has been shown to prevent acidosis and the accumulation of ROS, and to also have calcium-chelating properties. Although these characteristics make UW an effective preservation solution, the advantages of some components, such as potassium, are still in question. In addition, some studies have investigated other substances with great potential for maximizing the cardioprotective effects of the UW solution.

Additives to UW Solution

While UW solution has been a major step in preserving hearts, it is not optimal and can be improved. In order to combat I/R injury, there have been continuous developments of additives that increase heart viability (Table II-2).

Additive	Proposed Benefits	Experimental Findings
2,3-Butanediol	By adding 2,3-butanediol (a cryoprotectant agent that prevents ice crystal formation) and preserving hearts at subzero temperatures, rat hearts can be preserved for longer periods of time and retain viability.	In rat hearts with 2% 2,3-butanediol preserved at -1ºC, the left ventricle had significantly better recovery with significantly higher amounts of creatine kinase and lactate when compared to the control group (Scheule, Jost et al. 2003).

Additive	Proposed Benefits	Experimental Findings
Antifreeze Proteins (AFP)	Antifreeze proteins are able to bind ice crystals and inhibit recrystallization, which allows subzero preservation.	Hearts with UW solution and AFP were preserved at -1.3ºC, and the viability of the hearts was significantly better after reperfusion. There was also better myocyte and mitochondrial structural integrity (Fischer and Jeschkeit 1996).
Cariporide	Cariporide is an NaH antiport inhibitor that can improve cellular integrity after ischemia and reperfusion.	Addition of cariporide in UW solution along with constant perfusion resulted in increased stroke-work index and decreased levels of troponin I in pig hearts (Nishida, Morita et al. 1996).
Endothelin-A receptor Receptor Antagonist	By adding a selective endothelin-A receptor antagonist (to block receptors for blood-constricting proteins) to oxygenated UW solution, edema and vasoconstriction can be minimized.	After preservation for 24 hours using perfusion with oxygenated UW solution and endothelin-A receptor antagonist, the percent recovery rate of cardiac output and coronary flow was increased (Okada et al., 1996).
Hyaluronidase	Hyaluronidases are a family of enzymes that degrade hyaluronic acid.	In hearts treated with hyaluronidase, edema formation was reduced; coronary flow, metabolic recovery, and stroke volume were improved as well (Xing, Gopalrao Rajesh et al. 2005).

Additive	Proposed Benefits	Experimental Findings
Lazaroid	Lazaroid is an inhibitor of iron-mediated lipid peroxidation. This compound has been previously shown to reduce ischemia-reperfusion injury by reducing free radicals.	Rabbit hearts pre-treated with lazaroid had less ischemia-reperfusion injury, better ventricular function, and lower lipid peroxide levels (Kuroda, Kawamura et al. 1995; Matsumoto and Kuroda 2002).
Nucleoside-Nucleotide Mixture	A nucleoside-nucleotide mixture consisting of varying concentrations of inosine, guanosine monophosphate, cytidine, uridine, and thymidine has a positive effect on protein and energy metabolism. This combination helps restore myocardial ATP concentrations and is used in forming high-energy phosphate.	Recovery of heart rate, aortic pressure, rate pressure, stroke volume, and stroke work was increased. Coronary flow and aortic flow also increased. Coronary vascular resistance was decreased (Kobayashi, Tanoue et al. 2008).
Perfluorocarbons (PFCs)	Perfluorocarbons are compounds derived from hydrocarbons with fluorine replacing hydrogen. The compound has a high capacity to dissolve oxygen and a low oxygen-binding constant, which allows better oxygen transfer.	In a heterotropic rat heart transplant model, preservation time was extended up to 48 hours (Fremes, Zhang et al. 1995).

Additive	Proposed Benefits	Experimental Findings
Rho-Kinase Inhibitor	By adding a Rho-kinase inhibitor to UW solution, the detrimental effects of the Rho-kinase transduction pathway can be prevented and provide better myocardial preservation and reduce ischemia-reperfusion injury.	In rabbit hearts where Rho-kinase inhibitor was added to UW solution, coronary blood-flow and heart rate increased. Endothelial nitric oxide synthase mRNA levels increased 4-fold, which reflects a positive effect on endothelial function (Lee, Drinkwater et al. 1996).
Varying Calcium and Magnesium Concentrations	Modifying the cation concentrations has been shown to be beneficial and may enhance cardiac recovery after extended preservation.	Developed pressure was increased with 0.1 mmol/L calcium and 20 mmol/L magnesium. Coronary flow was recovered best with 15 mmol/L magnesium. Diastolic function was reduced. Addition of calcium in concentrations of 2.5 mmol/L was harmful (Rosenfeldt, Conyers et al. 1996).
Varying Potassium Concentration	High potassium concentrations are suggested to be related to coronary artery endothelial damage.	In one study, lowering potassium concentration resulted in better protection of the endothelium, which correlated to increased nitric oxide release (Hardy 1999). In another study, lowering potassium concentration in a non-starch UW solution decreased the protective effect by 30%.

Table II-2: Additives to UW solution that have improved heart viability or extended ex vivo storage time.

Antifreeze proteins (AFP) allow fish and insects to survive freezing temperatures by lowering the freezing point in their bodies without changing osmolarity. AFP I from

winter flounder and AFP III from ocean pout have been successfully utilized to improve heart preservation. Rat hearts have been preserved in UW solution containing AFP at concentrations of 15-20 mg/cc for 2-6 hours at -1.1ºC to -1.3ºC (Kuroda, Kawamura et al. 1995; Matsumoto and Kuroda 2002). While three of the four hearts in the control group froze and died, electron microscopy showed that the myocyte structure and mitochondrial integrity were preserved in the AFP-preserved hearts. The control hearts had disrupted Z-lines, mitochondrial swelling, and destruction of myocyte structure. It was concluded that AFPs prevent the freezing of hearts in subzero preservation, and that using subzero preservation can increase storage time and viability of preserved hearts (Kuroda, Kawamura et al. 1995; Matsumoto and Kuroda 2002).

Cariporide, a NaH antiport inhibitor (HOE642) has also been shown to have beneficial effects when used with UW solution. In the experimental group with cariporide, the stroke-work index was higher and malondialdehyde levels were lower, as revealed by light microscopy. This study implies that cariporide has potential to improve recovery and decrease myocardial damage (Kobayashi, Tanoue et al. 2008).

Perfluorocarbons (PFCs) are additives that maintain a constant supply of oxygen. PFCs have small oxygen binding constants, which allow oxygen to be released more easily in solution, thereby making it an excellent carrier. In experiments conducted with a two-layer method, which utilizes oxygenated UW solution and PFCs, ATP production continued during storage, leading to longer preservation times (Rosenfeldt, Conyers et al. 1996).

Signaling pathways related to I/R injury involving Rho and Rho-kinase have also been investigated. The Rho-kinase pathway directly affects actin cytoskeleton

organization, cell adhesion, cell migration, and endothelial nitric oxide synthase levels. Inhibiting Rho-kinase would preclude those events and could consequently increase heart viability. Japanese white rabbit hearts were immersed in UW solution with or without inhibitor for 24 hours. The performance of the left ventricle was measured using the Frank-Starling curve, indicating that the inhibitor group produced higher aortic flow with less left atrial pressure. Coronary blood flow and heart rate was measured using a Langendorff apparatus. Phosphorylated myosin light chain and nitric oxide synthase mRNA levels were measured as indicators of effectiveness of the inhibitor and endothelial function, respectively. Low levels of myosin light chain indicated that the inhibitor had worked and there was increased blood flow, heart rate, and endothelial function. Overall, the addition of the inhibitor could be used to prevent myocardial damage during preservation (Lee, Drinkwater et al. 1996).

Varying the concentrations of the potassium, magnesium, and calcium ions has produced different effects. Evidence suggests that the high concentration of potassium currently in UW solution is responsible for endothelial damage. One study in Australia compared the effect of using a starch-free UW solution with a lowered potassium concentration to St. Thomas' solution. When the potassium concentration was lowered, the protective effect was compromised (Fremes, Zhang et al. 1995). However, in another study conducted by the UCLA Medical Center, there were differing results. When potassium concentrations were lowered to 25 mEq/L from 129 mEq/L, there was better protection of the endothelium, which was measured by an increase of nitric oxide release (Buckberg, Brazier et al. 1977). Due to conflicting results, it is necessary for more

research to be conducted on the effect of potassium concentration on endothelial function and viability.

Calcium concentrations from 0.025 mmol/L to 10 mmol/L have also been researched. At the maximum concentration, the calcium was detrimental and decreased the developed pressure. The concentration that resulted in the highest developed pressure was 0.1 mmol/L. Coronary flow was significantly better than in UW solution without additives at 15 mmol/L. When the effect of the cations is compared cumulatively, the best developed pressure was achieved by 0.1 mmol/L calcium and 20 mmol/L magnesium. Consequently, the addition of calcium and magnesium could enhance recovery from preservation (Oshima, Morishita et al. 1999; Tsutsumi, Oshima et al. 2001).

Machine Perfusion

Cold storage is limited on a conceptual basis. The goal is to limit metabolism in order to induce a state of suspended animation, which would theoretically allow the heart to remain outside of the body longer for transportation. However, the heart never truly hibernates; it uses anaerobic metabolism, which leads to the build-up of dangerous ROS (Ozeki, Kwon et al. 2007) that subsequently react with nearly every molecule in the cell, impacting protein functions and damaging the cell. Although UW solution's radical oxygen scavengers help alleviate these effects, storage time is limited, and I/R injury persists. Additionally, this leads to a buildup of metabolic waste and severe depletion of ATP energy stores (Opie 2004).

Machine perfusion (MP) is advantageous because it removes metabolic waste as it is generated, which prevents acidosis (Collins, Moainie et al. 2008). In addition, MP provides oxygen to the myocardial cells by virtue of its continuous delivery system.

Without a continually replenishing oxygen supply, the heart must work 20 times harder to produce ATP (Conte and Baumgartner 2000). MP has already been well established for renal allografts and transplants, and the Organ Care System, a proprietary perfusion system, has already received FDA approval for kidney transplantations. MP can even use the UW solution as a perfusate during the *ex vivo* period.

Despite its benefits, MP has raised several concerns that have prevented its adoption in clinical practice. Myocardial edema is one strong concern, but it has been proven to have no effect on function after the *ex vivo* storage period (Tsutsumi, Oshima et al. 2001; Poston, Gu et al. 2004; Ozeki, Kwon et al. 2007). The two other main concerns are cost and technical complexity. Cold storage cannot be surpassed in these terms because of its simplicity. Additionally, the UW solution is extremely cost-effective, available commercially, and readily made from a simple recipe. In contrast, MP requires maintenance: it is currently a bulky machine and not easily transportable. However, these concerns can be easily addressed with time and research.

The two main types of MP are normothermic and hypothermic. As their names imply, normothermic perfusion is conducted by heating the perfusate to body temperature, 37°C, and is generally conducted using a perfusate that is based on whole blood, often with some additives like antibiotics. Hypothermic MP is conducted by cooling the perfusate to 4°C and generally uses a synthetic perfusate.

Normothermic MP has been one of the most successful techniques in extending *ex vivo* storage time for cardiac transplants. Although the technique was advantageous because it was used for heart and lung preservation simultaneously, it was abandoned due to the technical complexity and cost of these systems. Thus, researchers believed it would

not be applicable for distant thoracic organ procurement (Collins, Moainie et al. 2008). However, the Organ Care System™ has recently shown promise for distant heart procurement although its cost and size are still issues. It has been approved in Europe for marketing, and is currently undergoing FDA testing.

Hypothermic MP has also been demonstrated to sustain aerobic metabolism (Collins, Moainie et al. 2008), and should maintain cell integrity and vital functions much better than anaerobic metabolism. However, MP does have its challenges. While successful perfusate and pump parameters are known, the lack of unified research has created confusion in the field. Myocardial edema has been a significant concern, but does not appear to affect heart function once blood flow has been restored (Sakaguchi, Taniguchi et al. 1998). One of the concerns with hypothermic MP has been that metabolism cannot be observed. Although the heartbeat can be observed in normothermic MP, this is not seen in hypothermic MP. Additionally, hypothermic MP has been observed to revive only 50% of hearts; this is theoretically due to the already sustained I/R injury (Amir, Rubinsky et al. 2003; Amir, Rubinsky et al. 2004).

Other Alternatives

The purpose of cold storage at $4^{O}C$ is to reduce metabolic rate and biochemical reactions, to reserve energy reserves, and to avoid waste build-up. However, low temperature and ischemic conditions limit ATP synthesis, thus limiting vital cellular functions. New techniques have been derived from finding optimal oxygenation and temperature conditions for ATP-synthesis (Jamieson and Friend 2008). Oxygenation of storage solution helps maintain mitochondrial function for ATP synthesis and antioxidant mechanisms. Oxygen persufflation and perfluorochemicals with UW solution (two-layer

method) have improved ATP recovery and maintained antioxidants better than cold storage (Matsumoto, Kuroda et al. 1996). The mild two-layer method, a variation of the two-layer method at $20^{O}C$, has led to faster ATP recovery and resuscitation of pancreases (Stubenitsky, Booster et al. 2000). Studies have shown the potential of normothermic preservation, which involves the perfusion of cell-culture media or blood-based perfusate near physiological temperatures, to lengthen storage times and improve graft survival (McAnulty, Reid et al. 2002). Another development in the research of additives to cold storage solution is trophic factor supplementation. Adding various growth factors prolonged storage time of kidneys, possibly by accelerating regeneration of tissue or activating protective signaling cascades (Nakao, Kaczorowski et al. 2008). Together, these new generation methods represent an opposite approach to preservation, namely creating conditions that support rather than inhibit the metabolism of the organ during storage.

One potential organ preservation improvement lays in unlocking cytoprotective genes inherent in organs. HO-1 is a particularly important gene responsible for breaking down heme (which converts H_2O_2 into more reactive free radicals) into cytoprotective molecules CO and biliverdin. HO-1 is a heat shock protein that is expressed in various stress situations, such as oxidative stress, hot temperature, ischemic preconditioning, and chemicals (Jamieson and Friend 2008). The advancement of gene manipulation has made it possible to transfer protective genes to the preserved organ through an engineered virus vector. Successful experiments have transferred various genes including HO-1, Bcl-xl, and Interleukin-10 (Jamieson and Friend 2008).

It is recognized that improvement of the cold-storage method has met an impasse due to the inherent problems related to its mechanism. Temperature reduction does indeed lower metabolism by a factor of 10-12, but anaerobic metabolism continues (Jamieson and Friend 2008; Nakao, Kaczorowski et al. 2008). With ischemia: 1) end-products of metabolism such as protons, lactate, and hypoxanthine build-up (Cotter 2003), 2) ATP levels drop to 50% of normoxic levels within 4 hours of ischemia (Hale, Dai et al. 2008), 3) Impairment of mitochondrial antioxidant defenses occurs, 4) Calcium influx and leakage from mitochondria are prevalent. For these reasons, the time in cold storage is proportional to the extent of ischemia-reperfusion (I/R) injury. While perfusion techniques appear to attenuate these problems, their use is restricted due to the cost and relative complexity of machinery. Similarly, the setbacks of technology-intensive gene therapy include issues of practicalities making it inadequate for organ preservation.

Conclusions

While there has been significant money spent and research conducted in improving the UW solution, these findings have not translated into clinical practice. Factors such as ion concentrations, mainly potassium and calcium, have yet to be optimized. The current configuration is considered an intracellular type of preservation solution because its high potassium content mimics the intracellular environment. However, varying the potassium and calcium concentrations within UW solution has been reported to significantly improve both the viability of the heart after preservation, and extend the time the heart can be preserved. In addition, there has been no research conducted into unifying various additives into a new solution.

There are also entire classes of compounds that have not been investigated for heart preservation. One such possibility is the usage of compounds that have been shown to induce a hibernative state. A new class of molecules called gasotransmitters has been of interest in this application. Gasotransmitters are a group of molecules that are toxic in higher concentrations, but induce extremely low metabolism with no permanent side-effects at lower concentrations. Some research into gasotransmitters has been conducted, but there is not enough information to make any conclusions regarding their potential benefits to cardiac preservation (Staples & Brown, 2008). It may be that some of these components will interfere with the mechanisms of action of other components, but once the correct configuration is identified, this may lead to a solution that is a very significant improvement over the current UW solution.

Hydrogen Sulfide

Introduction

Hydrogen sulfide (H_2S) is a small, gaseous molecule primarily known for its toxicity and the danger it poses (See Table II-3for chemical properties). It is produced by biological decomposition, crude petroleum, and industrial activities. Commercially H_2S is mainly used to manufacture elemental sulfur and sulfuric acid, an important industrial product (Strickland, Cummings et al. 2003). Even in low concentrations, prolonged H_2S exposure can have detrimental effects, and exposure to high concentrations can be fatal.

Recently H_2S has been discovered to be an endogenously produced gaseous signaling molecule and, as a result, is beginning to receive much attention as a possible therapeutic drug. A growing body of literature has increasingly recognized that H_2S plays a critical physiological role, particularly in vascular tone regulation. In the span of a

decade, H_2S has been transformed from a poisonous gas into a marketable product with growing therapeutic potential for protecting the heart from ischemic disease.

Property	Information
Molecular weight	34.08
Color	Colorless
Taste	Sweetish taste
Physical state	Gas (under normal ambient conditions)
Melting point	-85.49°C
Boiling point	-60.33°C
Density in air	1.19 (air=1.00)
Density at 0 °C, 760mmHg	1.5392g/L
Odor	Rotten eggs
Odor threshold: Water Air	 0.000029 ppm 0.005-0.3 ppm
Solubility: Water Other solvents	 5.3 g/L at 10 °C; 4.1g/L at 20°C; 3.2 g/L at 30 °C Soluble in glycerol, gasoline, kerosene, carbon disulfide, crude oil
Partition coefficients	Not applicable
Vapor pressure at 25 °C	15,600 mmHg
Acid dissociation pk_{a1} pk_{a2}	$\rightleftharpoons H^+(aq) + HS^-(aq)$ (1); $HS^-(aq) \rightleftharpoons H^+(aq) + S^{2-}(aq)$ (2) 7.04[b] $Ka_1 = \frac{[HS^-(aq)][H^+(aq)]}{[H_2S(aq)]}$ 11.96[b] $Ka_2 = \frac{[S^{2-}(aq)][H^+(aq)]}{[HS^-(aq)]}$
Henry's law constsnt at 20 °C at 30 °C at 40 °C	 468 atm/mole fraction 600 atm/mole fraction 729 atm/mole fraction
Autoignition temperature	260 °C
Conversion factors	1ppm = 1.40 mg/m^3
Explosive limits	Upper, 45.5%; lower, 4.3% (by volume in air)

Table II-3: The physical and chemical properties of hydrogen sulfide (Strickland, Cummings et al. 2003).

Dangers of H_2S

H_2S exists primarily in a gaseous form, in which it is colorless, flammable, and has a pungent odor of rotten eggs. With a molecular weight of 34.08 grams, H_2S is heavier than air and as such collects near the ground, often resulting in dangerous pockets where H_2S concentrations reach high levels. H_2S poisoning comes mainly through inhalation into the lungs, from which it spreads to the rest of the body. Oral administration of the solid sulfide salts has been used in studies, but no case of toxic effects in people ingesting H_2S has been recorded (Beauchamp, Bus et al. 1984; Agency for Toxic Substances and Disease Registry 2006).

H_2S acts as an irritant at lower concentrations, burning the eyes, nose, and lungs; at higher concentrations, studies show that H_2S affects the respiratory, nervous, and cardiovascular systems. Specifically, H_2S was demonstrated to cause vacuolization, ciliocytophthoria, and nasal sloughing and lesions in the respiratory system at a concentration of 300 ppm (Lopez, Prior et al. 1987; Brenneman, James et al. 2000). In the brain, a multitude of effects were observed at a range of concentrations from 10 ppm to 500 ppm, including inhibition of enzymes, such as carbonic anhydrase, to alterations in brain waves in the hippocampus. In the heart, rabbits exposed to 72 ppm H_2S for 0.5 hours for five days demonstrated arrhythmias for several days, while those exposed for 1.5 hours for only one day demonstrated no negative effect.

Biological Functions

Despite its toxic effects, H_2S is actually produced in mammals as a signal molecule. H_2S in the blood and tissues comes from the metabolic breakdown of the amino acid L-cysteine from two enzymes (cystathionine B-synthase and cystathionine Gam-lyase) found in virtually every organ of the body. Cystathionine β-synthase and cystathionine γ-synthase, which are responsible for the metabolism of L-cysteine, produce H_2S in extremely low concentrations, on the order of picomoles per milligram of protein (Szabo 2007). Other H_2S sources include microflora of intestinal epithelium and the inorganic reduction of elemental sulfur in erythrocytes (Szabo 2007). One of the fundamental questions that remains disputed is exactly how much H_2S exists. Plasma concentrations of H_2S have been reported in the range of 30 to 50 μM, though it varies significantly depending on experimental procedure (Szabo 2007; Furne, Saeed et al. 2008). This issue is important in understanding the therapeutic mechanisms of H_2S and their relationship to its physiological effects. At such low concentrations, H_2S loses its cytotoxic properties and becomes cytoprotective instead, playing a role in regulating vasodilation, blood pressure, and smooth muscle contraction (Lefer 2007; Szabo 2007; Zhang, Huang et al. 2007). In micromolar concentrations, H_2S also induces upregulation of anti-inflammatory genes. However, it appears that H_2S levels must fall in a very specific concentration range for the molecule to retain its protective effects. If H_2S concentration is too high, H_2S regains its toxicity and becomes proinflammatory, while concentrations that are too low have been associated with heart disease (Szabo 2007). The discovery of these regulatory properties of H_2S has led to increasing interest in the therapeutic potential of H_2S and its cardioprotective abilities.

H_2S and Ischemia-Reperfusion Injury

Among the therapeutic applications of H_2S of interest is reducing I/R injury, which represents a serious issue in organ transplantation. In several studies, H_2S has been shown to reduce the damage from I/R when applied both pre-ischemia and post-ischemia. Pretreatment with administration of NaHS, a H_2S donor, 15 minutes before myocardial ischemia has been shown to significantly reduce myocardial infarct size by approximately 26% (Sivarajah, McDonald et al. 2006). A later study confirmed an even greater cytoprotective effect when treatment with an H_2S donor at the time of reperfusion, instead of before ischemia, significantly reduced myocardial infarct size by as much as 72%, as revealed by triphenyltetrazolium chloride staining (Elrod, Calvert et al. 2007). The infarction-reducing effects in this study varied depending on the concentration of H_2S; the maximum reduction of 72% was achieved with 50 μg/kg (0.9 μM) of the H_2S donor (Elrod, Calvert et al. 2007). Other studies also support the idea of concentration-dependent limitation of infarct size, but quote different optimal concentrations. For example, one study found the smallest percentage of infarction with a 1 μM concentration of NaHS (Ji, Pang et al. 2008). In addition, this study also suggests an upper boundary to infarction limitation, as the 100 μM concentration of NaHS did not significantly reduce infarction compared to the control (Sivarajah, McDonald et al. 2006). Furthermore, the cytoprotective effects of H_2S are not limited to the aforementioned myocardial tissue. H_2S has been shown to confer similar protection to intestinal, muscle, and cutaneous tissue (Weinstein, Henderson et al. 2009; Jimenez, Henderson et al. 2010).

Research has suggested that H_2S may play a role in regulating various cardiovascular functions, including vasodilation, blood pressure, and, in isolated tissues,

the contraction of smooth muscle (Szabo 2007). However, many of H_2S's cytoprotective effects may occur only in low concentrations. In situations of heart disease, H_2S levels are lower than in healthy hearts (Szabo 2007). Furthermore, micromolar concentrations of H_2S help induce expression of anti-inflammatory or cytoprotective genes. However,H_2S at higher concentrations has pro-inflammatory effects (Szabo 2007). H_2S also regulates the gene for subunit V of cytochrome c oxidase, the terminal enzyme complex of the electron transport chain (Blackstone, Morrison et al. 2005). The proposed mechanism through which low concentrations of H_2S reduce metabolic rates is inhibition of cytochrome c oxidase, which blocks oxygen uptake and prevents ATP production, effectively inducing a suspended-animation-like state in a mouse model (MacDonald and Storey 1999).

The literature provides various experimental models and methods that have been developed to study H_2S cardioprotection. Studies have measured the extent of myocyte injury to determine the effects of H_2S. One way to measure myocyte injury is by using myocardial protein markers. Significantly reduced levels of a cardiac-specific isoform of troponin-I in hearts treated with 50 μg/kg of the H_2S donor confirmed the protective effect of H_2S (Sivarajah, McDonald et al. 2006). Measurements showing a concentration-dependent limitation of the release of creatine kinase, another protein marker, from coronary effluent also indicate the protective role of H_2S (Szabo 2007). Another method of measuring myocyte injury is through the trypan blue exclusion assay (Bian, Yong et al. 2006). One study found that the percentage of viable cells increased in a concentration-dependent manner, with the greatest percentage of viable cells seen after ischemia in a solution of 100 μM NaHS (Hu, Li et al. 2007).

ATP content is an important measurement of the protective effects of H_2S since it is one of the best indicators of myocyte recovery (Hu, Li et al. 2007). Hearts treated with NaHS, an H_2S donor, showed significantly higher levels of myocardial ATP following reperfusion (Hu, Li et al. 2007). As stated previously, H_2S has been proven to lower the metabolic rate (Blackstone, Morrison et al. 2005). In return, this lowers the levels of production of ATP and induces a "hibernation" state in which H_2S reduces injury by reducing the need for high levels of O_2.

Isolated hearts treated with an H_2S donor also tend to exhibit lower levels of apoptosis. TUNEL staining identified apoptotic cells with brown nuclear staining (Hu, Li et al. 2007). Although there is some amount of apoptosis in H_2S-treated hearts, it is significantly reduced (Elrod, Calvert et al. 2007). In some cases, H_2S-treated hearts were found to have a 59% reduction in the number of apoptotic cells (Hu, Li et al. 2007; Zhang, Huang et al. 2007).

Although many studies have shown that H_2S is able to reduce I/R injury, the mechanism by which H_2S acts is not well understood. However, researchers have pointed to the mitochondria, and in particular, relationships between ROS, calcium, and metabolism, to elucidate the mechanisms of H_2S cardioprotection. As described previously, ischemia-reperfusion (I/R) injury is the biphasic damage that tissues undergo during periods of metabolic stress due to low blood flow and oxygen, followed by oxidative stress accompanying re-oxygenation. Important to the progression of injury during ischemia is the role of calcium. Calcium loading occurs as the acidic environment leads to high Na^+ concentration, which in turn is exchanged for calcium via a membrane exchange pump. Proteases and phospholipases leak out of lysosomes in high-calcium

environments; these and other calcium-activated enzymes destroy proteins or lipid membranes, making the cell more susceptible to rupture (Murphy and Steenbergen 2008). Lactate and other metabolic byproducts build up as the organ uses anaerobic metabolism to support basic homeostatic processes (like the sodium-potassium pump) in the low-oxygen preservation solution. Upon reperfusion, these byproducts fuel the generation of ROS, which directly damage cellular structures (Jamieson and Friend 2008).

The mitochondrion is a site of particular interest as it plays a critical role in mediating I/R injury. A substantial portion of damage to the electron transport chain (ETC) occurs during ischemia. ROS are generated due to inefficient electron transfers, and these can further damage the ETC, leading to further ROS generation in a dangerous feedback cycle (Roth and Nystul 2005). Due to membrane disruption in the mitochondria by ROS, cytochrome c is also released, which leads to the activation of caspases and apoptotic pathways (Chen, Camara et al. 2007). The membrane transition pore (MTP) is a non-specific channel in the inner mitochondrial membrane that opens under conditions of high matrix calcium and ROS, conditions that occur during the start of reperfusion (Murphy and Steenbergen 2008). The opening of the MTP leads to the dissipation of the proton gradient and the inability of mitochondria to make ATP, which leads to cell death. Thus, the extent to which the MTP opens determines the extent of reperfusion injury. The most effective therapeutic measures, such as ischemic preconditioning, thus target the events during ischemia that set the stage for MTP opening, including high Ca^{2+} loading and ROS production (Halestrap, Clarke et al. 2007).

In this context, H_2S cardioprotection can be explained by mechanisms that inhibit mitochondrial calcium overload, ROS production, and metabolism. In general, research

on H_2S in hearts focuses on ATP-activated potassium channels as the main mechanism of the cytoprotective influence of H_2S. Several studies show that the introduction of glibenclamide, a selective K-ATP channel blocker, blocks the protective effects of H_2S (Hu, Li et al. 2007; Zhang, Huang et al. 2007). For example, mitochondrial K-ATP channels were implicated in this mechanism by blocking the channels with 5-hydroxydecanoate and observing the subsequent protective effects of H_2S (Sivarajah, McDonald et al. 2006; Ji, Pang et al. 2008). The activation of mitochondrial K-ATP channels in particular is believed to confer protection during ischemia, and so would be beneficial for scenarios like organ storage. The opening of these channels increases the potassium influx to the mitochondria, which lowers the membrane potential. As calcium uptake depends on this membrane potential, mitochondrial K-ATP channels contribute to preventing calcium overload (Halestrap, Clarke et al. 2007). In addition, H_2S itself is a potent antioxidant, and either directly scavenges free radicals or signals intracellular antioxidant defenses (Szabo 2007). H_2S has been shown to trigger multiple anti-apoptotic and antioxidant pathways (Jha, Calvert et al. 2008). The targets for these pathways include: GSH, HO-1 (intracellular antioxidants), HSP-90, and Bcl-2 (anti-apoptotic agents) (Szabo 2007; Bliksoen, Kaljusto et al. 2008; Jha, Calvert et al. 2008). Finally, and perhaps most interestingly, H_2S is capable of suppressing metabolic activity.

Suspended Animation

The inhibition of cytochrome c oxidase to block oxygen uptake and prevent ATP production is another proposed mechanism by which H_2S at low concentrations reduces metabolic rates and core body temperature, as low concentrations of H_2S effectively inducing a suspended-animation-like state in a mouse model (Blackstone, Morrison et al.

2005). When mice breathed 150 ppm H_2S for 20 minutes, they entered a hypometabolic state that allowed them to survive hypoxic conditions for more than six hours. Without H_2S pretreatment, mice could only survive for 15 minutes (Blackstone and Roth 2007). It is unclear how this adaptive response specifically operates (Cooper and Brown 2008). However, other researchers have explored blocking the ETC during ischemia as a means of protecting the mitochondria from ROS-mediated injury (Chen, Camara et al. 2007). By mild uncoupling of the ETC with pharmacological agents, the researchers observed lower mitochondrial potential, which is correlated with ROS-production during ischemia.

Metabolic reduction is expected to allow cells to survive low-oxygen conditions (Roth and Nystul 2005; Blackstone and Roth 2007). Hibernation, an example of a suspended animation state utilized by many animals, can protect organs through hypometabolism, by which 88% of energy is saved that would otherwise be expended in maintaining euthermic processes (MacDonald and Storey 1999). Hypometabolism is achieved through active controls that coordinate a strong suppression of both ATP-consuming and ATP-generating processes, prioritizing ATP consumption to sustain vital processes (Storey 1997). In addition, genes controlled by hypoxia-inducible factor alpha (HIF-α) are up-regulated in hibernation (Storey 1997). HIF-α helps control a range of cytoprotective responses, including red blood cell proliferation and activation of HO-1 (Bernhardt, Campean et al. 2006).

H_2S and Organ Preservation

As hibernation can increase resistance to hypoxic and ischemic insults, the protective effects of H_2S have been investigated in the context of transplantable organs. Studies examining H_2S in isolated hearts confirm several of its cytoprotective effects.

Although these studies differ in methodologies and experimental protocols, (such as variations in time of ischemia, time of reperfusion, and pre- or post-conditioning) they share several common elements. For example, most studies use a Langendorff perfusion column to perfuse the heart and measure overall function of the heart by monitoring various parameters such as coronary flow, coronary perfusion pressure, heart rate, temperature, and myocardial electrogram. Many studies also use NaHS in solution as an H_2S donor to deliver H_2S into the cardiac tissue (Ji, Pang et al. 2008). In addition, the research focuses on certain widely accepted indicators of myocardial injury or recovery, such as left ventricular function, infarct size, creatine kinase markers, ATP content, and apoptosis levels. Some studies have shown that NaHS treatments improve left ventricular developed pressure (LVDP) after reperfusion (Sivarajah, McDonald et al. 2006; Hu, Li et al. 2007; Zhang, Huang et al. 2007).

As previously mentioned, H_2S has many cardioprotective properties that should protect the organ from I/R injury in the context of organ preservation. One of the few studies that have ventured to apply H_2S to whole organ preservation was conducted by Hu et al. in 2007. This group added 1 μM NaHS to a cold Krebs-Henseleit (KH) buffer, and submerged hearts in this solution for six hours. For comparison, hearts were also submerged in a control group (KH buffer without NaHS) and St. Thomas solution (a cardioplegic solution). Afterwards, they tested ATP content, apoptosis index, and heart performance. The hearts submerged in NaHS had the highest ATP content, lowest apoptosis index, and performed equally well as hearts preserved in St. Thomas solution. Adding a K-ATP channel blocker (glibenclamide) to the NaHS solution erased the protective effects. Thus, the authors attributed H_2S protection mainly to its opening of K-

ATP channels and lowered Ca^{2+} accumulation. Other possible mechanism of H_2S protection include ROS scavenging, vasorelaxation at reperfusion, and lowering metabolism by promoting a state of suspended animation (Hu, Li et al. 2007).

Effect of Timing on Treatment

Hydrogen sulfide's effects may be divided into two categories: direct effects and signaling effects. Direct effects depend on the presence of the molecule as an effector for the protective mechanism. These include chemical modification of K-ATP channels and scavenging of ROS. In addition, H_2S is a trigger or mediator of multiple anti-apoptotic and antioxidant signaling pathways. Thus, the protective effects do not require the continued presence of the molecule, only an amount that begins and sustains the signaling cascade. The question of how best to administer H_2S (as a bolus injection or through continuous infusion) is closely related to the question of which type of effect is dominant in cardioprotection (Osipov, Robich et al. 2009).

There is convincing evidence for both types of effects. *In vitro* tissue studies adding NaHS to an ROS-generating system strongly support the notion of a direct ROS-scavenging effect (Geng, Chang et al. 2004). H_2S is thought also to directly open K-ATP channels, and there is evidence that it can do so by chemically reducing the cysteine residues on that protein. This theory is supported by the loss of H_2S stimulatory effect on K-ATP channels observed when point mutations are introduced at the cysteine residues (Jiang, Tang et al. 2010). In whole-cell patch clamp experiments, the hyperpolarization induced by H_2S was reversed by removing H_2S from the bathing solution (Tang, Wu et al. 2005). While K-ATP channels are generally thought to be an important signaling protein in the ischemic preconditioning pathway, it may act as an end-effector as well, by

raising mitochondrial membrane potential and lowering calcium uptake (Yellon and Downey 2003). If this were true, then sustained concentrations of H_2S would be important for preconditioning protection. Finally, H_2S inhibits mitochondria and slows O_2 reduction by out-competing oxygen at moderate concentrations (above 50-200 μM NaHS) and binding to cytochrome c on the ETC (Cooper and Brown 2008). As explained previously, this inhibitory effect on oxidative respiration is proposed to be cytoprotective by reducing ROS generation during the ischemic period (Roth and Nystul 2005; Blackstone and Roth 2007). The process can be reversed with sufficient oxygen (Collman, Ghosh et al. 2009). Interestingly, it has been found that a continuous infusion of NaHS solution is necessary to induce metabolic reduction in a porcine model, while bolus injection fails to do so (Simon, Giudici et al. 2008). On the other hand, it has been observed that mice breathing 150 ppm H_2S for 20 minutes entered a hypometabolic state that allowed them to survive in low-oxygen environments without additional H_2S for more than 6 hours after the pretreatment (Blackstone and Roth 2007). Clearly, there is still much research to be done to determine what effect the duration of exposure to H_2S has on the protection it confers (Osipov, Robich et al. 2009; Wagner, Asfar et al. 2009).

As previously explained, H_2S has been used as a preconditioning stimulus to protect the heart from simulated I/R injury, either through an initial bolus injection (Sivarajah, McDonald et al. 2006) or through brief periods of alternating perfusion with NaHS and buffer solution (Pan, Feng et al. 2006). H_2S has been shown to up-regulate several antioxidant genes including HO-1 and trigger anti-apoptotic pathways (Jha, Calvert et al. 2008; Calvert, Jha et al. 2009). However, similar to classical ischemic preconditioning, protection conferred by H_2S, preconditioning lies in a transient time

window. Using cardiomyocytes, one group observed this window of protection to be 1-3 hours after treatment (Pan, Feng et al. 2006). This window of protection is inadequate to protect the organ during preservation periods that ideally would greatly exceed four hours. The reason for the decay in the activation of these signaling pathways remains unclear. Interestingly, attempts to translate classical ischemic preconditioning to organ preservation have met limited success, and some have pointed to the time restrictions of the early window of protection as a possible explanation (Huang, Shan et al. 2009).

One of the complicating factors to understanding the mechanisms behind H_2S effects lies in its transient nature. As a small molecule, it is highly volatile and can easily escape from solution. For this reason, most researchers prefer to prepare the solution no more than 3 days ahead of the experiment (Dombkowski, Russell et al. 2004). The rate of H_2S release likely has a profound effect on the physiological response as shown by a study that investigated the vasorelaxation on rat aortic rings using a slow-release H_2S drug (GYY4137) and NaHS, a fast H_2S donor (Li, Whiteman et al. 2008). The slow-release drug gave a sustained relaxation effect while the fast-release drug induced rapid, transient relaxation (Li, Whiteman et al. 2008). Another possible mechanism for H_2S depletion could come from the fast metabolism of H_2S by mitochondria, despite the low oxygen concentrations and cold temperatures found in preservation solutions. The implications of this suggested mechanism on the therapeutic potential of H_2S are unknown.

Drug Delivery

Drug Delivery and its Effects on the Heart

There have been many developments in methods to treat diseased or damaged heart and cardiac tissues. Many of these recent advancements have focused on targeted drug delivery directly to these tissues, in contrast to oral or intravenous drug delivery. The efficacy of targeted drug delivery is dependent on the discovery of novel biomarkers that are up-regulated or uniquely expressed in such diseased tissues (Scott, Crabbe et al. 2008). Studies in recent years have demonstrated that bio-derived materials also improve cardiac function after implantation due to their angiogenic potential (Jiang, Wang et al. 2009).

One method of targeted drug delivery to the heart is through direct injection. Direct myocardial injection of therapeutic agents for the purpose of myocardial revascularization was explored nearly a decade ago as a feasible and safe alternative to surgical-based injection procedures, or open heart surgery (Kornowski, Fuchs et al. 1999). Direct injection can be administered to the peri-infarct myocardium or into the vasculature. For example, direct injection of an adenoviral vector that codes for heat shock protein 70 (HSP70) was able to restore post-infarct heart function in rats when administered as a pretreatment (Lau, Griffin et al. 2000). Other studies have shown that direct injection of a vector coding for human growth hormone can be effective in improving post-infarct cardiac function as well (Scott, Crabbe et al. 2008). Intramyocardial injection of skeletal myoblasts has also been shown to treat post-infarction chronic heart failures, particularly via the route of retrograde intracoronary injection (Fukushima, Coppen et al. 2008).

Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are both molecular signaling factors that also have cardioprotective properties. Direct injection of the gene for VEGF has been shown to stimulate angiogenesis, and is thought to induce the growth of functional vasculature after an infarction when delivered in conjunction with complementary compounds for angiogenesis such as angiopoietin-1, RhoA, and Rac1. Another study showed that injection of hydrogel microspheres containing bFGF into a peri-infarct region was able to improve left ventricular function and increase blood vessel density (Scott, Crabbe et al. 2008). Furthermore, studies show that bFGF-induced angiogenesis with cardiomyocyte transplantation is a promising procedure for myocardial infarctions (Freiberg and Zhu 2004).

Targeted Drug Delivery to the Heart

Targeted drug delivery can be accomplished with the aid of new developments in drug delivery vehicles, such as liposomes, polymeric micelles, and biodegradable nanoparticles. An ideal vehicle for drug delivery should be non-toxic, biocompatible, non-immunogenic and biodegradable (Scott, Crabbe et al. 2008).

Two main considerations in drug delivery to the heart tissues are the vehicles delivering drugs or treatment, and the identification of biomarkers to be targeted by these treatments. Other important considerations are the release rates afforded by these drug delivery vehicles and the specificity of the targeting. Drug release can be initiated by external energy sources, while specificity can be fine-tuned by modified drug delivery vehicles, such as ligand-coated drug carriers or the use of antibody targeting. These methods and similar approaches have advantages, but largely remain imperfect. For example, the levels of antibody expression do not usually differ greatly between normal

and diseased tissue (depending on the target chosen). In order for biomarkers to be effective, a drastic up-regulation in diseased tissue would be ideal. Appropriate biological targets, however, have yet to be discovered for many types of treatments (Scott, Crabbe et al. 2008).

One drug delivery method that has received significant attention has been hydrogels. Hydrogels are networks of crosslinked hydrophilic polymers. They have been found to be particularly successful in drug delivery due to their biocompatibility and ability for sustained release. The polymers that comprise hydrogels for biomedical applications are often specifically chosen because these base polymers already have little immunological reactions. For example, to enhance biocompatibility, many polymer microspheres are constructed from materials derived from natural polymers (such as algae and chitin) or by the employment of polymers made from naturally present monomers such as lactic and glycolic acids. Conversely, to enhance mechanical properties of the gel, these polymers may be co-polymerized with other polymers such as polyethylene. Also, as a result of its hydrophilic nature, hydrogels are largely water volume wise. This creates a microenvironment very similar to other soft tissue.

Hydrogels are also of great interest in biomedical applications because of their versatility. External stimuli, such as pH, temperature, antigens, electric fields and photofields, can cause a change in a hydrogel's shape, structure, or volume, depending upon the composition of the polymer. This can allow for the introduction of materials, drugs, or signaling factors into specific organs, tissues or body cavities with greater ease. For example, injection of α-cyclodextrin (α-CD)/MPEG-PCL-MPEG hydrogel into

infarcted myocardium has been shown to preserve left ventricular heart function by preventing systolic bulging (Jiang, Wang et al. 2009).

Polymer microspheres are drug delivery vehicles that can be sustainably rate-controlled and designed to be target specific, as opposed to exposing the whole body to a drug. Microsphere delivery of therapeutics also prevents the variance in drug levels that is inherent to oral drug delivery. Hydrogels accomplish this sustained delivery via two main mechanisms: either the diffusion of the drug through the crosslinked polymer, or via degradation of the polymer matrix, releasing the drug to the environment. Various properties of polymer microspheres can affect the desired release rate, including the molecular weight of the polymer used, the distribution of the drug to be delivered within the microsphere, polymer blending, crystallinity, microsphere size, porosity of the hydrogel, physical distribution of the microspheres in the target area, and the individual morphology of the spheres. Microspheres composed of more than one polymer can have a unique release profile based on the degradation rate and other properties of each polymer in the blend, as well the composition percentage of each of the polymers (Freiberg and Zhu 2004).

Although a common aim for a drug release profile is constant release rate over time, often times this desired release profile is not achieved. The most common drug release profiles exhibit an initial spike in concentration of expelled drug or medication from the sphere or a usually more constant drug delivery profile dependent on diffusion and degradation. There are additional strategies to trigger release upon encountering a changed pH of the system, such as intercellular pH levels (Freiberg and Zhu 2004). By

forming a biocompatible polymer that can be triggered through a change in pH, temperature, or antigens, localized and specific drug delivery can be accomplished.

Microspheres prepared from monomers can be polymerized by emulsion, suspension or dispersion techniques, and can be formed on the nanometer-size scale. Microspheres can also be prepared from the polymerization of linear polymers, many of which are available commercially (Freiberg and Zhu 2004).

Method of Delivery

Gelatin hydrogel microspheres (GHMs) have been used as a novel non-viral agent for gene delivery (Kushibiki and Tabata 2004). They can be delivered via catheter-based injection, a novel technique compared to the only previous option, delivery via open-chest surgical technique. One study examined three routes of injection: anterograde injection (AI) via coronary arteries, retrograde injection (RI) via coronary veins, and direct myocardial injection (DI) via the coronary sinus. A catheter-based strategy of drug delivery was used to reduce the procedure's invasiveness. The AI distributed microspheres homogeneously and broadly through the target area with a 73±11% retention. However, microinfarctions were detected, which most likely caused a lack of increase in coronary flow reserve. RI proved to have the lowest efficacy of the three delivery strategies but was still efficient nonetheless; only 22± 8% of the microspheres were retained and spread homogenously. The retention may have been affected in part by the timing of the injection. Lastly, DI was tested without any fatal events and resulted in a 47±14% retention rate in the heart, while the rest leaked to the lungs from the puncture site. Overall, the results suggest that RI and DI are effective delivery strategies while AI is not due to the occurrence of microinfarctions (Hoshino, Kimura et al. 2006).

Microspheres prove to be particularly versatile as drug delivery vehicles. GHMs are larger than viral vectors. As discussed in a previously mentioned study, the type of injection affected the spread of microspheres (Hoshino, Kimura et al. 2006). In addition, there are other factors that must be considered before GHMs are used as drug delivery vehicles. In particular, GHMs can aggregate after being suspended, with microspheres that average 10 μm in size being able to form clusters over 20 μm in size. Despite the effectiveness of AI when used for viral vector-based gene therapy, the occurrence of microinfarctions and micro-embolisms makes the use of GHMs with AI unsuitable for the heart. Furthermore, when microspheres were used in a clinical setting, the microspheres injected with RI or DI leaked out from the heart via the coronary sinus and likely became trapped in the lungs. (Hoshino, Kimura et al. 2006).

In particular, GHMs can carry genes, proteins, or therapeutic agents such as H_2S, which can be released gradually over an extended period of time. In addition to its ability to distribute, GHMs are very versatile due to their ability to be varied in diameters of 1 μm to greater than 100 μm, or even sheets. Additionally, gelatin has already been established as a compound safe for clinical use. GHMs containing bFGF were used with an ischemic cardiomyopathy model, along with cardiomyocyte transplantation. GHMs were able to effectively provide controlled release of bFGF. In doing so, the microspheres created an environment suitable for the survival and activity of transplanted cells and thus enhanced bFGF-induced angiogenesis (Yamamoto, Sakakibara et al. 2003).

Based on prior studies, it would appear that while many polymer fabrication methods may be available, a favorable biomaterial for the particular study of delivery of H_2S solution throughout cardiac tissue would be GHMs. Furthermore, due to a previous

study on different injections methods, the most favorable method of delivery would be RI of the fabricated GHMs.

III. Methods

Materials

The gelatin (Type A, from porcine skin) and glutaraldehyde (50% photographic grade) were obtained from Sigma-Aldrich. Tween 20 (enzyme grade) was purchased from Fisher-Scientific. Sodium hydrosulfide hydrate (NaHS) was purchased from Sigma-Aldrich. The ATP assay was purchased from Abcam, and a Caspase-3 Assay kit was obtained from BD Pharmingen. The cell line was obtained from ATCC and Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen. All reagents utilized were used as received. For immunohistochemistry, biotin-conjugated goat anti-rabbit IgG was provided by Jackson Immuno Research, and Cytoseal was provided by Fisher Scientific.

Microspheres

Microsphere Fabrication

Gelatin microspheres were fabricated with Type A gelatin from porcine skin. 0.26 g of gelatin was fully dissolved in 5.0 mL of distilled water, and heated to 50°C in a water bath. The dissolved gelatin was sprayed with an atomizer into 50mL of vegetable oil, previously heated to 50°C and stirred at 1150 rpm. The gelatin-oil solution was stored at 4°C and stirred at 1150 rpm for 30 minutes before 20mL of acetone was added and the solution was left to stir for another hour. Solution was transferred to 50 mL Falcon tubes and centrifuged for 5 minutes at 4000 rpm to isolate the microspheres after decanting the supernatant. Microspheres were transferred to microcentrifuge tubes. 1mL acetone was added and microspheres were centrifuged a second time for 5 min at 4000 rpm. The

supernatant was decanted and 0.5 mL isopropyl alcohol was added to each tube. After centrifuging for 5 min at 4000 rpm, the supernatant was decanted and the microspheres were stored at 4°C. All reagent volumes were scaled up linearly for fabricating larger quantities of microspheres.

The fabricated microspheres were imaged on an Axiovert 40CFL inverted light microscope (Zeiss). The sizes of the microspheres in the image were measured using SPOT (Diagnostic Instruments), a calibrated image analysis program. These sizes were recorded and reported as mean ± standard deviation.

Crosslinking Microspheres

Gelatin microspheres were crosslinked with glutaraldehyde after fabrication. 100 mg microspheres were mixed into a beaker with 100 mL of the desired glutaraldehyde solution (1M or 4.75M) and 100 mg Tween 20 and stirred at 1150 rpm at 4°C for 15 hours. The solution was transferred to 50 mL Falcon tubes and centrifuged for 5 minutes at 4000 rpm to isolate the microspheres after decanting the supernatant. Microspheres were transferred to a beaker with 2 mL 0.5M glycine and 23 mL distilled water and stirred at 37°C for 1 hour. The microspheres were centrifuged a second time and distilled water was added to wash the microspheres before air-drying.

Microsphere Loading

NaHS solution was prepared by dissolving sodium hydrosulfide hydrate (NaHS salt) in deionized water. Microspheres were then soaked in the solution overnight.

Microsphere Release Profile

Microspheres loaded with NaHS solution overnight were centrifuged at 5000 rpm for 1 min at room temperature. The loading solution was removed and 2 mL PBS was added to microspheres to rinse away residual amounts of loading solution. Microspheres were centrifuged again and supernatant was decanted. Microspheres were then rinsed for a second time before 2 mL PBS were added to the tube of microspheres and the timer was started.

Zinc Acetate Assay

The concentration of H_2S released from the microspheres into the surrounding solution was evaluated by the zinc acetate assay. At each time point of 0, 15, 30, 60, 120, 180, 240, 300, 360 min, 50 µl of the solution was removed from the microspheres tube and added to a tube of 250 µl zinc acetate (1% w/v) and 450 µl of water. 50 µl PBS were added back into the tube of microspheres to replace the previously removed volume. After 7 min, 133 µl of N, N-dimethyl-p-phenylenediamine sulphate (DMPPDA; 20µM) and 133 µl of $FeCl_3$(30 µM) were added to the tube with zinc acetate and 50 µl of sample. After 45 min, 250 µl of sample were added to a 96-well plate and the absorbance was measured by a spectrophotometer at 670nm.

Cell culture

Murine cell line (H9c2) cardiomyocytes were cultured in 88% high glucose D-MEM supplemented with 10% (volume fraction) fetal bovine serum, 1% sodium pyruvate, and 1% penicillin-streptomycin. Cells were incubated in 5% by volume CO_2 at 37°C.

Media was changed every 2-3 days and cells were passaged at 80% confluence. At 80% confluence, old media was aspirated. 1 mL trypsin-EDTA was added and the flask was rocked to coat the surface and the trypsin was aspirated. 3 mL of trypsin-EDTA was added and cells were incubated at 37°C for 5 min. Cells were then checked under microscope to ensure they had been lifted from the surface. Cells were mixed to break cell clumps, and then placed in Falcon tube containing 12 mL of media with 10% fetal bovine serum, and centrifuged. The supernatant, which contained the excess media, was then removed and an additional 12 mL of media was added and cells were resuspended. Cells were seeded onto 6-well plates at density of 1.0×10^4 cells per cm^2. They were tested after they had grown to 90-100% confluence after two days. 3mL growth medium with $1x10^4$ cells/mL were added to each well of the plate. The final seeding density was $1x10^4$ cells/cm^2.

NaHS in Cell Culture

NaHS solutions of 0, 100, 1,000, 10,000 μM were prepared by dissolving sodium hydrosulfide hydrate (NaHS salt) in deionized water. 250 μL of each NaHS solution was added to seeded wells of a 6-well plate containing 3 mL growth media and cells. The concentration of H2S in solution was evaluated by the zinc acetate assay at each time point of 0, 15, 30, and 60 min. Controls of NaHS loaded microspheres in media without cells was also evaluated by the zinc acetate assay at each time point. Absorbance was read by a spectrophotometer at 670nm.

Microsphere Release profile in Cell Culture

6-well cell culture plates were pre-seeded with murine cell line (H9c2) cardiomyocytes. Gelatin microspheres were fabricated and loaded overnight with 100mM

NaHS solution. 50mg, 100mg, or 250mg of H_2S-loaded microspheres were placed in the pre-seeded 6-well cell culture plates for 0, 15, 30, 60 min. At each time point, 50 µl of the media solution was removed from the well and added to a tube of 250 µl zinc acetate and 450 µl of water. After 7 min, 133 µl of DMPPDA and 133 µl of FeCl3 were added to the tube with zinc acetate and 50 µl of sample. After 45 min, 250 µl of sample were added to a 96-well plate and the absorbance was measured by a spectrophotometer at 670nm.

Quantitative Viability Assay

A mixture of 12 mL PBS, 12 µl Calcein, and 12 µl ethidium homodimer was prepared. The wells were rinsed with PBS and aspirated. 1 mL of the imaging solution was added to each seeded well of a 6-well plate. The well plates were incubated in the dark for 30 min. The center of each well was imaged and the dead cells were counted.

Metabolic Activity Assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (MTT) assay solution was prepared using MTT reagent and PBS. Media from each well of the seeded 6-well plate was aspirated off and the wells were rinsed with 1 mL PBS before PBS was removed. 900 µl of PBS and 100 µl of MTT solution were added to each well. The cell culture was incubated at room temperature for 3 hours. 900 µl of MTT stabilizing solution was added to each well. Wells were gently and uniformly shaken in the gyratory shaker and the solution in each well was pipetted up and down to ensure dissolution of crystals. 200 µl of the solution in each well was transferred to a 96-well plate and a spectrophotometer was used to measure the absorbance at 570 nm and 690 nm. The absorbance at 690 nm was subtracted from the absorbance at 570 nm.

Ex Vivo Model

Selection of an Animal Model

In order for our findings to have significance for organ transplantations in clinical settings, the study must be carried out in an animal model with comparable anatomy. The four-chambered heart of a rat is analogous to the four-chambered hearts of humans and other mammals, whereas fish have two-chambered hearts that are not comparable in structure. Although there are species of invertebrates with hearts, they are not analogous to our circulatory system, and experimentation on the hearts of such species would not produce significant results. The possibility of using mice as a model was also investigated, but determined to be unfeasible. The small size of mice equates to a miniscule heart, which would be very difficult for the surgical team to manipulate. Additionally, this small size makes it impossible to retrieve enough tissue for all of the planned assays at all the time points from a single heart.

Various databases were searched in order to find alternatives to animal testing, using the key words "alternatives to animal donor hearts." All eleven results from both searches were critically analyzed for their relevance to our study. Of the eight hits from the Pubmed search, three of the articles discussed xenotransplantation as an alternative to heart transplantation. Since our study does not encompass organ transplantation, but only organ extraction and the *ex vivo* time period, these articles are not relevant. Three more articles discussed surgical procedures to treat heart problems in the patient, which is unrelated to the storage of harvested organs. Another article proposed a method of keeping an excised heart beating, which already assumes animal testing and therefore does not offer an alternative. The last article, although it suggests that animal testing can

be eliminated through tissue engineering of accurate models, admits that such technology is not yet fully developed. Of the results obtained through Altweb, one merely discusses the potential for alternatives to animal experimentation but does not offer specific suggestions. The second and third are chapters from a handbook on humane experimental techniques and is not specific to alternatives to excising animal hearts for experimentation. In addition, a second search was performed using different search terms. When searching with "Alternatives to harvesting animal hearts," Pubmed returned only two hits. One hit concerned alternatives to electroejaculation of bulls, and the other discussed aortic valve replacement with homografts, neither of which was relevant to our study.

Our project seeks to investigate a novel storage method to preserve isolated hearts. The heart is a complex organ whose functionality following storage can be influenced by various factors, such as metabolic rate, myocardial infarctions, and the buildup of reactive oxidative species. Computer models are not yet powerful enough to make accurate predictions for heart viability following our novel storage method because they cannot adequately account for all of the factors affecting heart viability. In addition, our proposed method focuses on the effects of H_2S, a compound that has only recently been investigated for its potential to preserve isolated tissues. Thus, its mechanism of action is still under debate and therefore cannot be represented in computer or mathematical models. Similarly, the novelty of H_2S, as well as the hydrogel method of delivery, requires experimentation in actual tissue in order to gather applicable data. Furthermore, due to the complexity of the heart, experimentation in cell cultures cannot depict how the heart would function as a whole. Some important measurements of heart

viability require the whole organ. For example, measuring left ventricular developed pressure (LVDP) requires a heart and cannot be determined from a cell culture.

To successfully evaluate the efficacy of using H_2S to prolong heart storage, an animal model must be used because of the complex interactions in the heart that cannot be modeled by computer models due to lack of information on the H_2S mechanism. As previously stated, the appropriate animal model must have similar anatomy to the human heart and feasible to experiment on. The model that fits this criteria and will contribute to a better understanding of how H_2S will interact with cardiac tissue is a rat heart. Thus, a rat heart model will be used for the *in vivo* experiments.

Surgery Methods

The appropriate sample size for the comparison of two means from independent groups of rat hearts was calculated using the formula $n \geq 2K\sigma^2/\Delta^2$, where K represents a constant that can be chosen for a given power, σ represents the standard deviation or variance in a given group, and Δ represents the difference between groups. (Daly, Bourke et al. 2000). For a 5% confidence level to detect a treatment effect of 95%, we used a K constant of 13.0. Based upon ATP values (Hu, Li et al. 2007), there was an expected difference of 150 μg/g and standard deviation of 50 μg/g. This calculation results in a sample size greater than or equal to 2.89, which was rounded to n = 3 for the *in vivo* biopsy study. A rat heart submerged in UW solution will serve as a control. The experimental groups included a rat heart submerged in UW and NaHS solution, a heart submerged in UW solution with NaHS and H_2S-loaded microspheres, and a heart submerged in UW solution with NaHS and PBS-loaded microspheres.

The rat heart submerged in UW solution with NaHS will function as a standard to which the effects of the microsphere therapy will be compared. This is the group that will test the therapeutic effects of sustained levels of H_2S in comparison to clinical standards and previous findings. A single heart submerged in UW solution with NaHS will be compared to a single heart submerged in UW solution with NaHS and PBS-loaded microspheres.

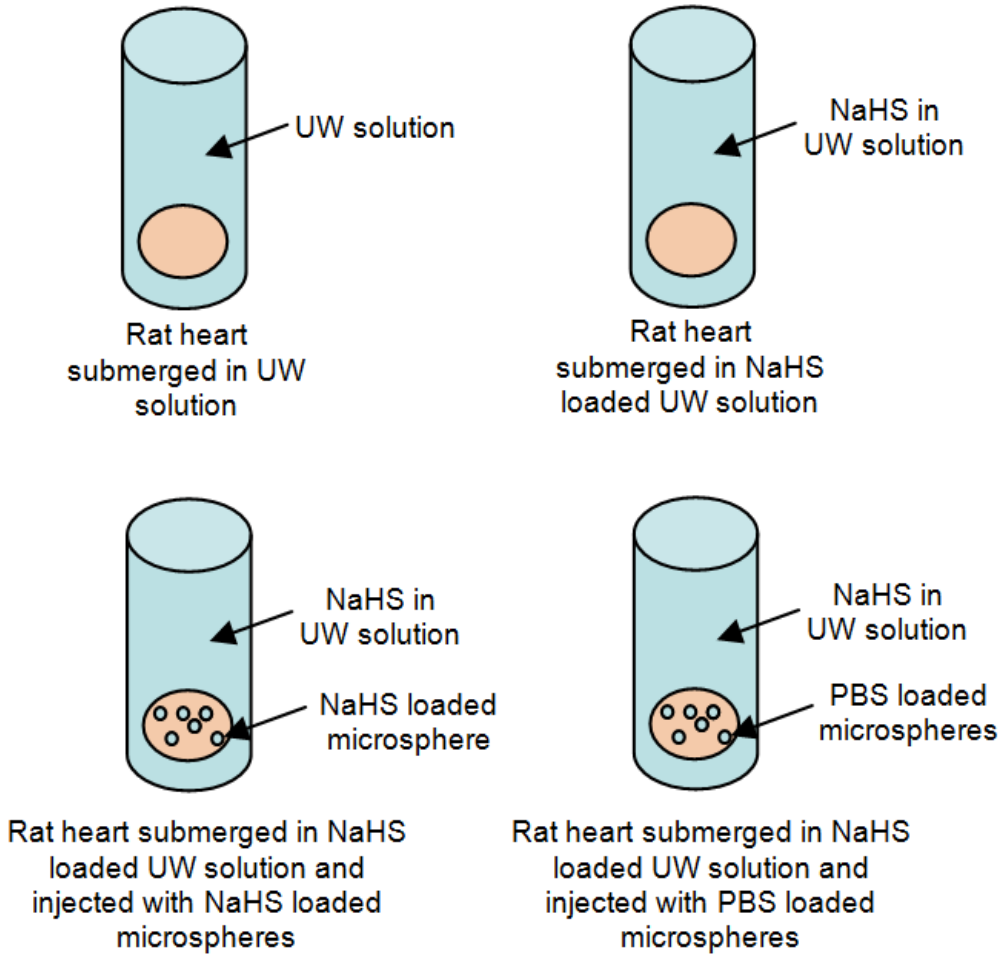

Figure III-2: The four groups of the *in vivo* studies. The groups are a rat heart submerged in UW solution (clinical standard); rat heart submerged in NaHS+UW solution (control); rat heart submerged in UW solution with NaHS loaded microspheres (experimental); and finally, rat heart submerged in UW solution with PBS loaded microspheres (microsphere control).

Heart Explantation

The anesthetic mix consisted of 1ml ketamine (100mg/mL), 0.1ml xylazine (100ng/mL), and 8.9ml sterile saline. Sprague-Dawley rats were anesthetized with the anesthetic mix (0.1mL/10g body weight). Hair was removed from the surgical site using clippers and the site was cleaned with 2 alternating scrubs of betadine soap and 70% alcohol using a sterile Q tip to remove the scrub without causing excessive heat loss due to alcohol evaporation. After an abdominal midline incision, the inferior vena cava (VCI) is isolated from surrounding tissue. 100 units of heparin were then injected into the VCI using a 30G needle. After waiting for 2 minutes for heparin to circulate, the rat was exsanguinated by cutting the infrarenal vessels. The thorax was quickly opened with a midline sternotomy and the pericardium was opened. Ice cooled saline (0.9%) was dripped onto the heart using a 10ml syringe. This was continued until the end of the harvest. The ascending aorta and the pulmonary trunk was dissected and separated. The distal ascending aorta was ligated with 6-0 silk. Cardioplegia (UW solution) was then injected into the proximal ascending aorta with a 30G needle (5-10ml over ~1 minute). The ascending aorta was then cut at the ligation, and the VCI and superior vena cava (VCS) were independently ligated with 6-0 silk. The pulmonary veins were then mass ligated using 6-0 silk and cut distal to the ligation.

Histology

Tissue Fixation and Slicing

Following the storage protocol, hearts were biopsied and frozen in OCT (optimal cutting temperature) molds and stored at -80°C. Each sample was then sectioned to 6 μm

thickness on a Cryostat (Microm HM550, Thermo Scientific), mounted on pre-cleaned Superfrost Plus slides (Fisher Scientific), and stored at -80°C.

ATP Assay

The ATP content of rat heart tissue samples was measured using the ATP Colorimetric/Fluorometric Assay Kit (Abcam, Cambridge, MA). The manufacturer's protocol was followed with the addition of deproteinizing and neutralizing steps, as outlined below.

Standard Preparation

10 µl of ATP Standard, provided with the kit, was diluted with 90 µl of dH_2O to yield 1 mM of ATP Standard. This standard was further diluted in a 1:50 ratio with dH_2O for the fluorometric assay. 0, 2, 4, 6, 8, and 10 µl of the more dilute solution was added into respective wells and the volume of each well was adjusted to 50 µl/well with ATP Assay Buffer, provided with the kit, to yield 0, 0.4, 0.8, 0.12, 0.16, 0.2 nmol/well of ATP standard.

Sample Preparation

Biopsies of the left ventricle from each rat heart were transported in liquid nitrogen and stored at -80°C immediately following the storage protocol. Approximately 10 mg of each sample was then weighed into a microcentrifuge tube. Throughout the following steps, samples were kept on ice as much as possible. Each sample was homogenized in 600 µl of ATP Assay Buffer using a pestle (Fisher Scientific) for at least 30 strokes. Samples were then centrifuged at 13,000 rcf at 4°C for 30 seconds to pellet the insoluble materials. 550 µl of the supernatant from each sample was transferred to

fresh microcentrifuge tubes, and 100 µl of ice-cold 2N perchloric acid was added. Samples were vortexed briefly to mix well, and placed on ice for 5 minutes to allow deproteinization. Samples were then centrifuged at 13,000 rcf at 4°C for 2 minutes to pellet precipitated proteins. 480 µl of the supernatant from each sample was then transferred to fresh microcentrifuge tubes and frozen at -80°C for up to a month.

ATP Detection

When ready for analysis, the frozen samples were thawed slowly on ice. Once liquid again, the samples were centrifuged at 13,000 rcf at 4°C for 2 minutes to further pellet any protein precipitation. 120 µl of each sample was then transferred to fresh tubes, and 5 µl of ice-cold Neutralization Solution containing 5M KOH and 3M $KHCO_3$ was added to neutralize the samples and precipitate excess perchloric acid. Samples were vortexed briefly to mix well and placed on ice for 5 minutes, after which samples were centrifuged at 13,000 rcf at 4°C for 1 minute. 40 µl of the supernatant from each sample was added to a well in a 96-well plate and the final volume was brought up to 50 µl/well with ATP assay buffer.

50 µl of Reaction Mix, containing 45.8 µl of ATP Assay Buffer, 0.2 µl of ATP Probe, 2 µl of ATP Converter and 2 µl of ATP Developer, all solutions provided with the kit, were added to each well of ATP Standard and sample before mixing and incubating at room temperature for 30 minutes, wrapped in foil. The signal was stable for 2 hours. Fluorescence was measured with excitation and emission wavelengths of 535 nm and 587 nm, respectively, by a spectrophotometer (Molecular Devices SpectraMax M5).

ATP Content Calculation

To calculate the ATP concentration, the 0 ATP Standard value was first subtracted from all standard and sample readings in order to correct for the background. The standard curve was then plotted. The total amount of ATP in a sample, Ts, was then calculated in nmols by applying the ATP sample reading, minus the 0 ATP Standard reading, to the standard curve. ATP concentration was found in mM by dividing Ts by 40 µl, the amount of sample supernatant added to the well. These values were divided by 0.8 to correct for the fact that deproteinization dilutes the samples to 80% of their original concentrations. Finally, to account for variations in the mass of the tissue samples, the values were divided by the mass of the tissue sample from which it came to yield final values in mM/mg. Two independent samples from each group were measured in two independent trials, and the four values were averaged to produce a single data point per group.

Caspase

Following the storage protocol, hearts were frozen in OCT molds, transported in liquid nitrogen, and stored at -80°C. Each sample was then sectioned to 6 µm thickness on a Cryostat (Microm HM550, Thermo Scientific), mounted on pre-cleaned Superfrost Plus slides (Fisher Scientific), and stored at -80°C. When ready for analysis, slides were warmed to -20°C in the freezer and labeled with pencil. The slides were fixed by immersion in -20°C acetone for 2 minutes at room temperature, and then rinsed in 0.2% Tween-20 in 1x PBS three times, 5 minutes each. This same rinsing procedure was used between each of the following blocking and incubation steps. After fixation, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS by applying 200 µl per slide and

incubating in the humidifier box for 10 minutes at room temperature. The samples were then blocked with 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) in 1x PBS for one hour at room temperature. After rinsing, endogenous biotin was blocked by first incubating in unconjugated streptavidin (Jackson ImmunoResearch, West Grove, PA), and then in free biotin (Sigma-Aldrich, St. Louis, MO) to saturate the binding sites on streptavidin, both at 10 μg/mL in 1x PBS for 1 hour at room temperature with a rinsing step in between the two incubations. Slides were then incubated in purified rabbit anti-active caspase-3 primary antibody (BD Pharmingen, San Diego, CA) diluted 1:75 in Antibody Diluent, which was provided with an Anti-Ig HRP Detection Kit (BD Pharmingen, San Diego, CA), in the humidifier box for 1 hour at room temperature, then rinsed. The sample was then incubated in biotin-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA), 1:500 in Antibody Diluent, in the humidifier box for 30 minutes at room temperature. After rinsing, streptavidin-HRP from the Anti-Ig HRP Detection Kit was applied to the slides, which were then incubated in the humidifier box for 30 minutes at room temperature. Slides were rinsed, then incubated in DAB Substrate Solution (1 drop of DAB Chromogen per 1 mL of DAB Buffer) made from the DAB Substrate Kit (BD Pharmingen, San Diego, CA) for 5 minutes at room temperature. The sample was then rinsed in water three times for 5 minutes each. The slides were then counterstained by applying hematoxylin for 30 seconds, rinsing thoroughly in water, dipping slides twice in Bluing Reagent, and rinsing again in water. The samples were dehydrated in four changes of ethanol of 95%, 95%, 100%, and 100% for 5 minutes each at room temperature. They were then cleared in three changes in Citrosolv (D-Limonene) for 1 minute at room temperature each. A

coverslip was applied to the slide with Cytoseal mounting solution (Fisher Scientific). Samples were visualized with an Axiovert 40CFL inverted light microscope (Zeiss; Thornwood, NY).

Tissue Fixation and Slicing

Frozen hearts were sectioned to 6 μm thick sections, mounted on glass slides, and stored at -80°C as described for the caspase staining. When ready for analysis, slides were warmed to -20°C in the freezer and labeled with pencil. The slides were fixed by immersion in -20°C acetone two times for 10 minutes at room temperature, with at least 10 minutes of air-drying after each acetone incubation. Slides were then incubated in 95% ethanol for two minutes, followed by a wash in distilled water for two minutes. Then a 6.5 minute hematoxylin immersion was followed by a 2 minute rinse in tap water. Next, the slides were immersed in Clarifier 2 (Richard-Allan Scientific) for 45 seconds, followed by another 2 minute rinse in distilled water. Slides were then immersed in eosin for 15 seconds. The samples were then dehydrated in five changes of ethanol of 70%, 95%, 95%, 100%, and 100%, in order for 3 minutes each. They were then cleared in a 3 minutes incubation in Citrosolv (D-Limonene). A coverslip was applied with Cytoseal mounting solution (Fisher Scientific). Samples were visualized with an Axiovert 40CFL inverted light microscope (Zeiss; Thornwood, NY).

All samples were stained at the same time in a single experiment in order to prevent procedural variability between stainings. Slides were stained and labeled with a code by one pair of experimenters, and a third experimenter performed a blind analysis of the samples using light microscopy. The code was not revealed until all data had been collected from all samples.

IV. Results

Controlled Release of H_2S

H_2S decays over time

The objective of the experiment performed in Figure IV-1A was to determine the change in H_2S concentrations in a solution over time, as a first step in assessing the timeframe under which experiments utilizing H_2S should be performed.

It was hypothesized that H_2S, being a volatile gas, would evaporate rapidly from solution, thus presenting difficulties in containing the gas for long enough to perform assays with it. In this experiment, a 25mM NaHS solution was prepared in distilled water, as described in the methods, and subsequently stored in a glass screw-cap container at room temperature. The absorbance from a sample of the nominal 25mM NaHS solution was monitored spectrophotometrically over a time frame of 3 to 26 days after making the solution (the solution was made on day 0).

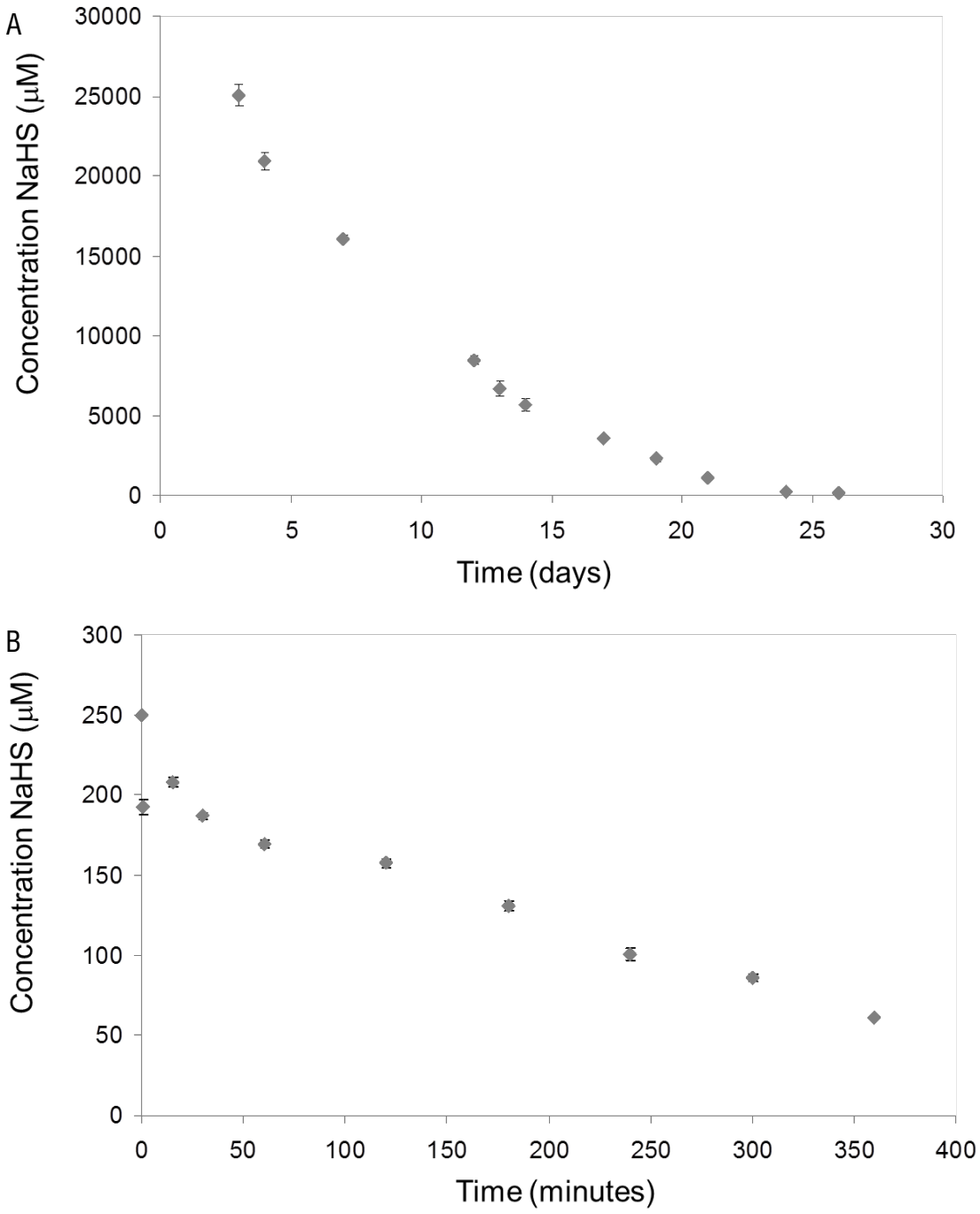

Figure IV-1: Temporal change of H_2S. (A) Decay of H_2S concentration of a stock solution initially containing 25 mM NaHS in distilled water, over time. The 25mM NaHS stock solution was stored at room temperature in a glass, screw-top container, and the absorbance was monitored over 26 days. (B) Decay of H_2S concentration of a 250 μM solution over 6 hours. Data represents the average of triplicate measurements. Error bars shown represent percent error of the triplicate measurements

As seen in Figure IV-1A, spectrophotometric absorbances were converted to H_2S concentration (mM). The figure roughly exhibits a pattern of rapid, exponential decay of

H_2S gas over the 24-day period, suggesting some dependence of the rate of decay on the concentration (higher concentration results in faster escape of H_2S from solution). By the 26th day, the concentration remaining is 0.5% of the initial concentration. Samples from each time point were collected and read in triplicates, and standard deviations were calculated for the readings. Due to unreasonably large standard deviation for the day-5 time point, this data point was removed from the set. The standard deviations for the other data points are less than 10% of the measurement, except for the two lowest concentrations, where the standard deviation amounts to 33% and 50% of the measurements, respectively. These large errors may be due to the limit in sensitivity of the spectrophotometer at distinguishing very low concentrations. The resultant pattern of H_2S decay allowed the team to determine an appropriate timeframe for which H_2S remains in solution and can be effectively worked with in vitro. Effectively, the results of this experiment determined the timeframe under which future experiments using H_2S could be performed. All subsequent experiments using H_2S were performed within three days of making NaHS solution.

The same experiment was performed to investigate the pattern of H_2S decay at an H_2S concentration and timeframe more relevant to our application. A 250 μM NaHS solution was prepared and its concentration was tracked over a period of six hours, our target period for cold-storage of the heart. The literature reports therapeutic H_2S concentrations from 1-33 μM, which is yielded by 3-100 μM NaHS solution. Due to previously observed limitations of the spectrophotometer in distinguishing very low H_2S concentrations, the study investigated 250 μM NaHS, a slightly more concentrated solution. At each time point, the absorbance of sample from the solution was measured in

duplicate. Between time points, the solution was stored in a closed 1.5 mL microcentrifuge tube. The results (Figure IV-1B) show a similar pattern of decay to the previous study. Aside from an unusual rise in concentration between 0.5 and 5 minutes, the plot shows the concentration drops continuously. After six hours, the concentration of the original 250 μM NaHS solution dropped to 60 μM NaHS, roughly a quarter of the original concentration. The standard deviations were low, all less than 5% of the measurement. This experiment confirms that H_2S depletion from solution occurs at concentrations and storage times that are protective in cold-stored hearts.

Gelatin microspheres can be fabricated using an atomizer approach

The objective of the experiment performed in Figure IV-2 and Figure IV-3 was to fabricate gelatin microspheres small enough to effectively be able to pass through blood vessels and capillaries. The team hypothesized that using a hybrid approach (where an atomizer is used to form the microspheres and a stirring oil solution is used to cure them) would yield smaller microsphere particles than previously described microsphere fabrication methods without the use of specialized equipment. Microspheres were fabricated from Type A gelatin derived from porcine skin, as described in the methods (See Section III). Results indicate that utilizing this hybrid method, yielded small microsphere particles, as depicted in Figure IV-2. A 50 μm scale bar is shown in the figure for comparison of fabricated microsphere sizes. Microsphere sizes appear to be much smaller than the 50 μm scale bar, and microsphere size was quantified using a Zeiss microscope and SPOT image analysis software. Figure IV-3 is a histogram that depicts the size distribution of microspheres fabricated by the atomizer method. The frequency of occurrence of microsphere diameters of a particular size is plotted against a

range of microsphere diameters (μm). This representative data were obtained from a single batch of microspheres, yielding an average diameter of 4.62 ± 1.54 μm, for n= 144 microspheres counted in the batch.

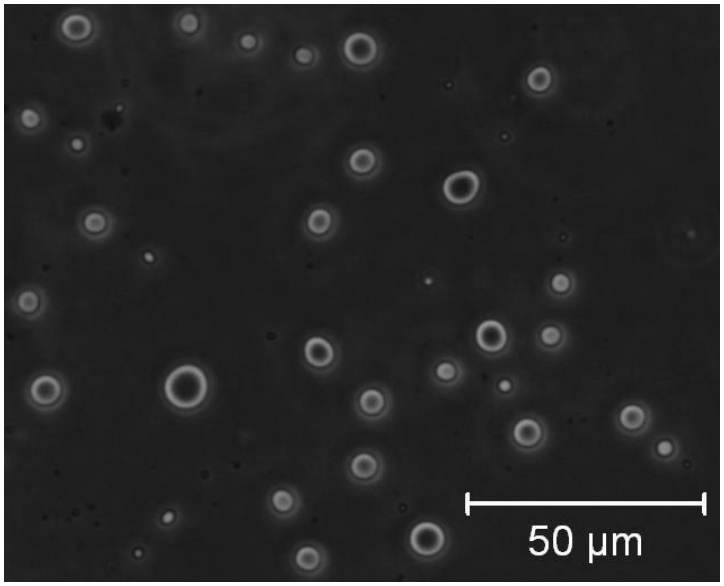

Figure IV-2: Gelatin microspheres fabricated by a hybrid oil/emulsion technique.

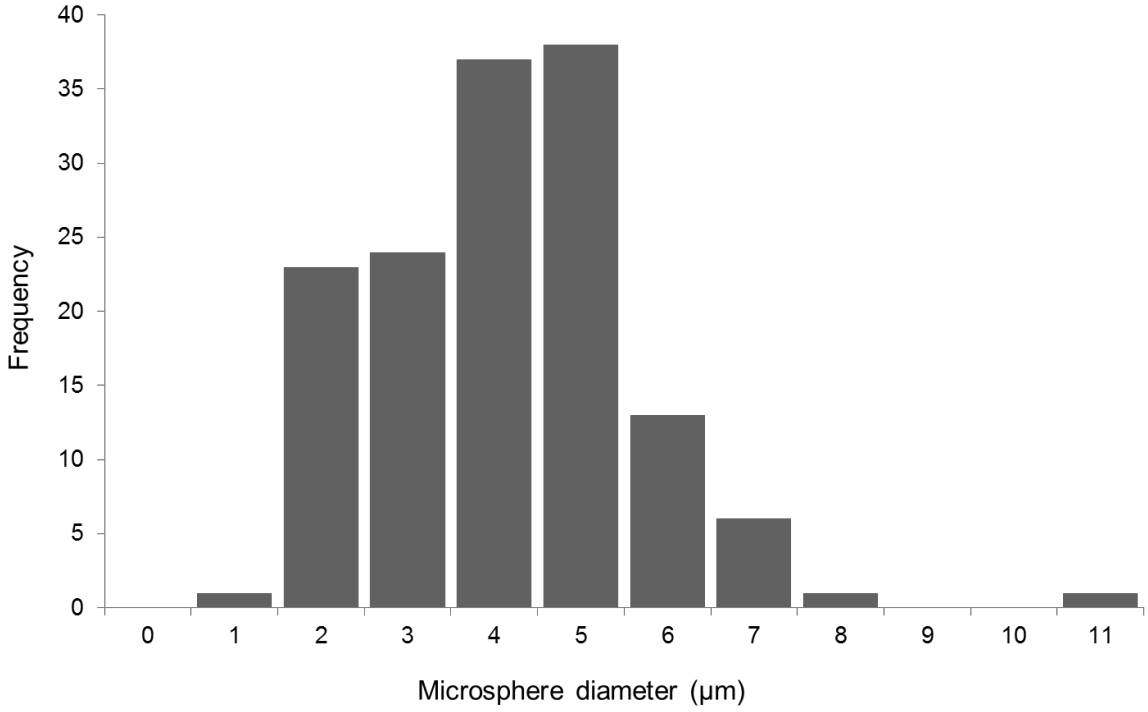

Figure IV-3: Histogram representation of microsphere size distribution. Average diameter = 4.62± 1.54 µm, n=144 microspheres counted from a single fabricated batch. Our achievement of microsphere size less than 10 µm in diameter is of importance in preventing blockage of capillaries of the rat model heart.

NaHS can be loaded into gelatin cylinders

It is of importance to emphasize the difference between NaHS and H_2S, as they are referred to in this experiment and in other experiments. NaHS is the colorless, solid reagent added to solution in our experiments. Upon dissolving, NaHS solid hydrolyzes into H_2S gas, the product that is of interest to measure in experimentation. Hence, loading values and measurements refer to NaHS, as NaHS is the compound dissolved in solution and added to the assays conducted. However, H_2S is the product of interest that is measured spectrophotometrically or by other means. Thus, values of release refer to the release of H_2S, not NaHS. The purpose of the experiment performed in Figure IV-4 is to demonstrate that NaHS can be loaded into gelatin cylinders, and to show that gelatin

cylinders can retain H_2S gas, in preparation for following experiments that entail the loading of NaHS and release of H_2S by carefully fabricated gelatin microspheres. Controls for this experiment are the same loading solutions, but without gelatin discs. These controls provide a measure of how much H_2S is lost to the atmosphere in the experimental group. Results of gelatin cylinder construct loading are shown in Figure IV-4.

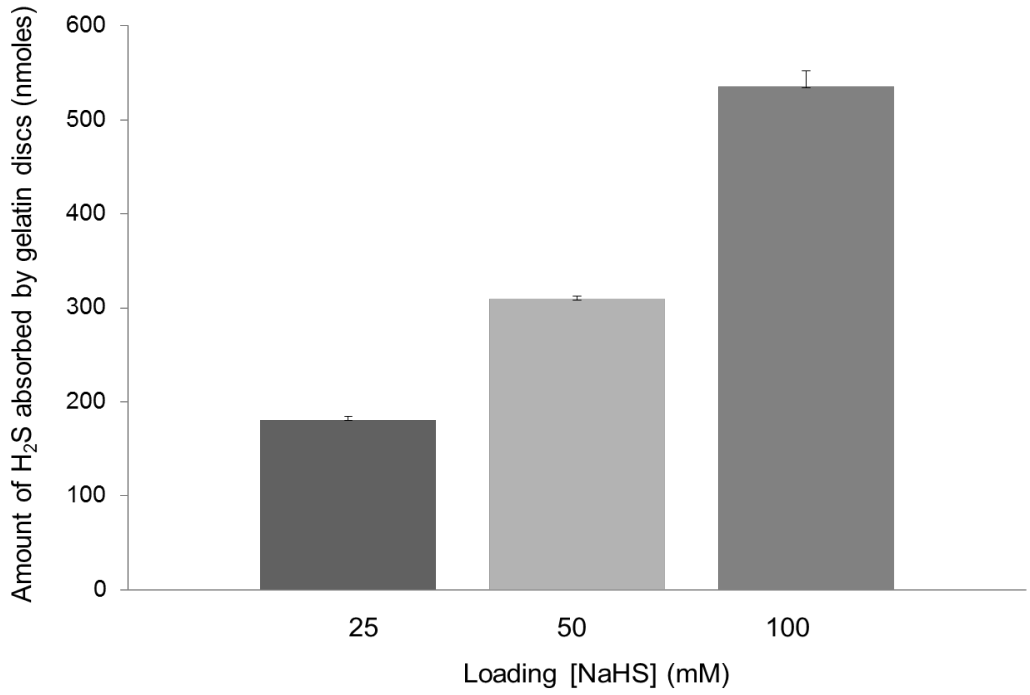

Figure IV-4: Gelatin cylinders loaded with varying H_2S concentrations. Bar graph displays concentration of NaHS loaded to gelatin cylinder constructs versus H_2S concentration (mM) absorbed by microspheres, for loading concentrations of 25, 50, and 100 mM NaHS. Uptake of H_2S was calculated as the difference in the amount of H_2S $((C_2 - C_1)$*volume) between two time points, subtracting the amount lost to the atmosphere. Standard error is shown for duplicate measurements.

The concentration of H_2S absorbed or taken up by gelatin cylinders is plotted against the loading of 3 different initial concentrations of NaHS solutions (25, 50, and 100mM NaHS solution) to gelatin cylinder-constructs. Note that H_2S uptake was

calculated from measurements of the concentration in the bulk solution. Concentrations of H_2S were measured spectrophotometrically. H_2S uptake was calculated by the following formula:

H_2S uptake = (Amt H_2S in bulk solution at t = 5 min, after sampling) - (Amt H_2S in bulk solution at t = 60 min) - (Amt H_2S lost to atmosphere)

$$= ((C_{5,e} - C_{60,e})*(200\ \mu L) - (C_{5,e})*(10\ \mu L)) - (C_{5,c} - C_{60,c})*(200\ \mu L)$$

where $C_{t,e}$ or $C_{t,e}$ represents the concentration at time t (min), for experimental or control, respectively.

The experiment was performed in duplicate (two discs loaded per loading concentration), and the standard error is shown for the two trials. T-tests indicate the percent decrease in bulk concentration is significantly higher for each experimental group compared to its corresponding control, indicating that the gelatin discs lowered the concentration of the bulk solution by absorbing H_2S. The plot also indicates that with increasing initial loading concentration of NaHS solution, the concentration of H_2S taken up by gelatin cylinders also increased. In other words, in a moderately linear fashion, increasing loading NaHS concentration corresponded to increased uptake of H_2S by gelatin cylinders. The rough linearity in H_2S absorption by gelatin cylinders indicates the capacity of gelatin material to retain H_2S, thus making gelatin a useful compound for microsphere production. Importantly, an almost 3-fold increase was seen in H_2S uptake between 25mM and 100mM initial loading concentrations of NaHS solution. Moreover, t-tests also indicate the amount sorbed in gelatin discs is significantly higher between 25

and 50 mM loading concentrations, and between 50 and 100 mM loading concentrations (p-value = 0.03% and 1.5%, respectively).

H_2S release from microspheres

The objective of this experiment was to determine whether factors in microsphere fabrication such as glutaraldehyde concentration used for crosslinkage of gelatin, and time allowed for release, affected release profiles of H_2S from microspheres. Microspheres were fabricated and crosslinked as described in methods, with crosslinking concentrations of 1M glutaraldehyde and 4.75M glutaraldehyde. In the experiments described in Figure IV-5 and B, the net release of H_2S (mmoles) per mg of microsphere was monitored over a 360 minute, or 6-hour period. The experiment was done for microspheres loaded with initial loading concentrations of 25, 50, and 100mM NaHS in micro-centrifuge tubes. The loading solution was removed and fresh 1X PBS was added to the tube at t=0. At each time point collected in the experiment, 50 µL of the bulk solution from the tubes containing microspheres were removed and replaced with the same volume in 1X PBS. The absorbance of the 50 µL sample was measured spectrophotometrically for time points of 0.5, 60, 120, 180, 240, 300, and 360 minutes. The net release of H_2S was calculated for each time point collected, and represented the cumulative release of H_2S at that particular time point. The net release was then divided by the corresponding mass of the microspheres, so that the end measurement is amount of H_2S per milligram of microspheres.

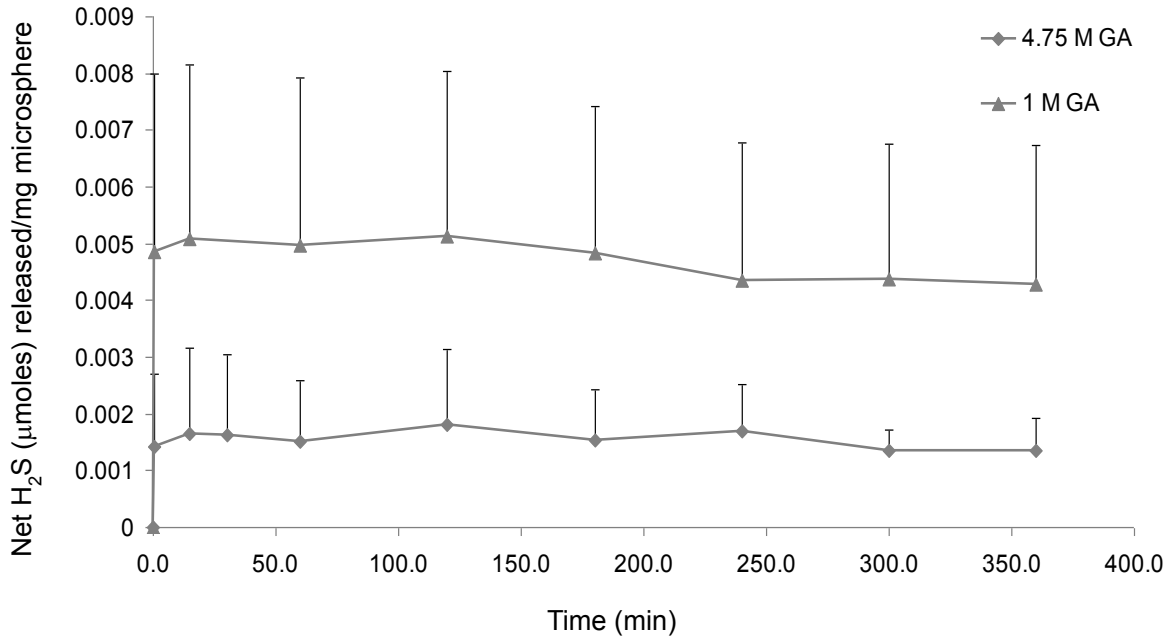

Figure IV-5: Release of H_2S from crosslinked gelatin microspheres. Net H2S (mmoles) released per mg of microspheres over time (minutes). Data shown represents microspheres crosslinked with 4.75 M and 1 M glutaraldehyde loaded with 25 mM NaHS solution. The amount of H2S released has been normalized by mass. Data demonstrates that the lower crosslinking microspheres releases more H2S, possibly because the molecule is able to diffuse from lower crosslink networks more easily.

The results can be divided into 3 categories, based on the variable of interest: effect of crosslinking density of microspheres, effect of loading concentration, and effect of microspheres.

Effect of crosslinking density of microspheres

The net release of H_2S over time was compared for 1 M and 4.75 M GA concentrations (Figure IV-5), fixing the loading concentration at 25 mM NaHS. The plot shows that 1M GA crosslinked microspheres released more H_2S than 4.75 M GA crosslinked microspheres. The range of cumulative release from 1 M GA crosslinked microspheres was 4.3 to 5.1 nmoles, while 4.75 M GA crosslinked microspheres had a

cumulative release range from 1.4 to 1.8 nmoles. The consistency of these differences between the 1 M GA and 4.75 M GA release profiles suggests the trend towards larger amount of release from 1 M GA cross-linked microspheres is significant. However, there is wide variability amongst the three identical groups at each time point for both 1 M GA and 4.75 M GA, as seen by the large standard deviations at each time point (ranging from 80% to 94% of the measurements for the 1 M GA set, and 27% to 92% of the measurements for the 4.75 M GA set). An ANOVA comparison test of the net amount H_2S released/mg microspheres between the 1 M and 4.75 M GA-cross-linking concentrations was performed at each time-point. Differences in H_2S release between 1 M and 4.75 M GA-crosslinked microspheres approach significance from the beginning time-points, and are below 10% for all time-points tested . However, only the 300 minute time-point had a p-value below 5%, to strongly support the significance of this trend (see Table IV-1 in appendix).

For microspheres crosslinked with 1M glutaraldehyde, results indicate a continual decrease of net H_2S release per mg of microspheres after 120 minutes, as shown in Figure IV-5. The cumulative amount of H_2S released by these microspheres drops by 17% of the amount released at 120 minutes, indicating the rate of H_2S lost from the system overwhelms the rate of H_2S release from the microspheres in the latter portion of the release profile. In general, the changes in H_2S concentration, shown in Figure IV-7, mirror the cumulative release profile. By the 360 minute data point, the concentration has dropped to 70% of its original concentration. In contrast, for microspheres crosslinked with 4.75 M glutaraldehyde, results do not show the same negative trend shown by 1 M glutaraldehyde crosslinked microspheres. Instead, there is an increase in net H_2S release

between time points of 0 and 60 minutes, with relatively marginal, variable increases and decreases afterwards. By the 360 minute data point, the concentration actually is higher (114%) than the original concentration.

Effect of loading concentration

The net release of H_2S over time was compared for loading concentrations of 25 mM, 50 mM, and 100 mM (Figure IV-6), fixing the crosslinking density at 4.75 M GA. The plot shows clearly that with higher loading concentration, more H_2S is released. The cumulative release has a range of 1.4-1.8 nmoles for 25 mM loading, 2.7-4.0 nmoles for 50 mM loading, and 4.4-7.7 nmoles for 100 mM loading. These large differences contribute to the significance of the trend, despite wide variability in the data The weakest significance comparison was for 25 mM and 50 mM loading concentration, where p values were significant only at the last two time points (t = 300 and t= 360 minutes), for which the standard deviation is relatively small. The differences in release between the 50 mM and 100 mM load NaHS concentrations are all significant after t = 30 minutes. Comparison of microspheres loaded with 25mM and 100mM NaHS shows the most striking significance in the difference in net release of H_2S. The significance in differences between release profiles of H_2S for the 25mM and 100mM NaHS loaded microspheres is noticed at the first time-point (p= 0.029). The significance in the difference becomes more striking at later time (p = 0.0018 at 120 minutes, p = 0.0007 at 180 minutes, p = 0.00034 at 300 minutes, and p = 0.0011 at 360 minutes).

For both 50 mM and 100 mM loading concentration, the release profile has a characteristic increase up to 120 minutes (67% and 50% of the t=0.5 min value, respectively), followed by a less consistent decrease up to the last time-point. However,

the concentration at the last time point measured still represents an overall increase (104% and 132%, respectively) over the initial concentration (though this increase does not reach significance for either group).

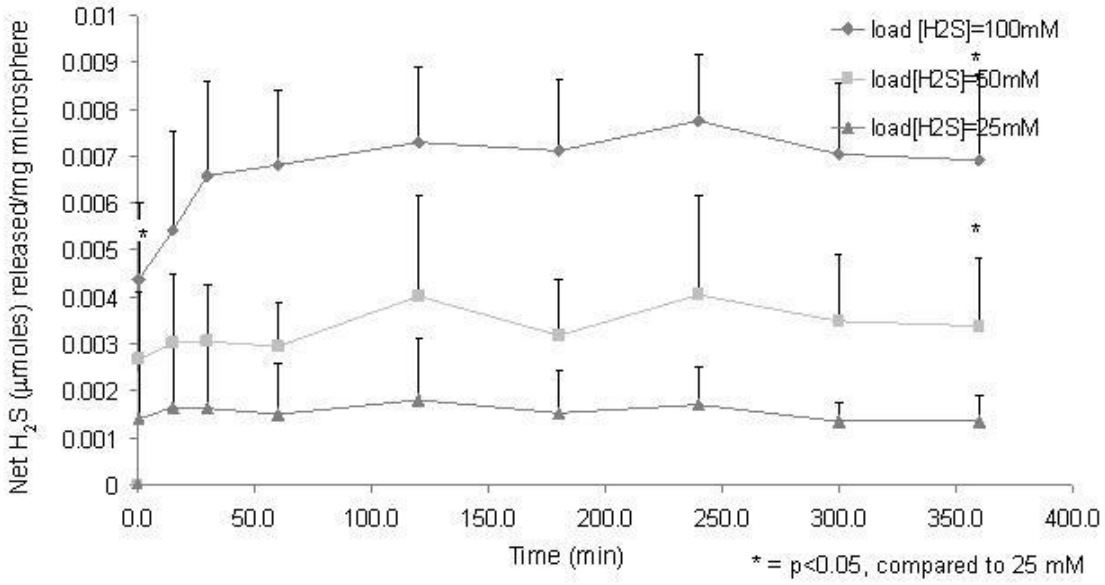

Figure IV-6: Net H_2S (mmoles) released per mg of microspheres over time (minutes). Data shown represents net H2S released from microspheres crosslinked with 4.75M glutaraldehyde, and loaded with 25 mM, 50 mM, and 100 mM NaHS solution. The amount of H2S released has been normalized by mass. Data demonstrates that we can control H2S release by changing loading concentration of NaHS.

Effect of microspheres vs. control (no microspheres)

The overall trends presented above are best understood in the context of Figure IV-7. The concentrations at each time point were normalized by the initial concentration, so that each value plotted represents the fraction of the starting concentration that remains at that time point. The plot shows the concentrations of the bulk solutions for different experimental groups compared with a control set, which was only a solution of NaHS at a particular concentration without microspheres. As expected, the concentrations in the control fall continuously, and end at 66% of the starting concentration. Notably, for the

25 mM NaHS loaded, 1 M GA crosslinked microspheres, the concentration fraction is significantly higher compared to the control between t=60 and t=180 minutes ($p = 0.0414$ at $t = 60$ min, $p=0.0321$ at $t=180$ min), but not thereafter. In contrast, for the 25 mM NaHS loaded, 4.75 M GA crosslinked microspheres, the concentration fraction appears higher than control, but only at an early time-point is this difference significant ($p = 0.0337$ at $t = 15$ min). Overall, the difference between experimental and control scenarios is most significant for 100 mM loaded, 4.75 M GA crosslinked microspheres. In this set, the concentration fraction is significantly higher than control ($p<0.05$) at all time points, with the exception of $t = 15$ min and $t = 300$ min ($p = 0.2297$ and $p = 0.0608$, respectively).

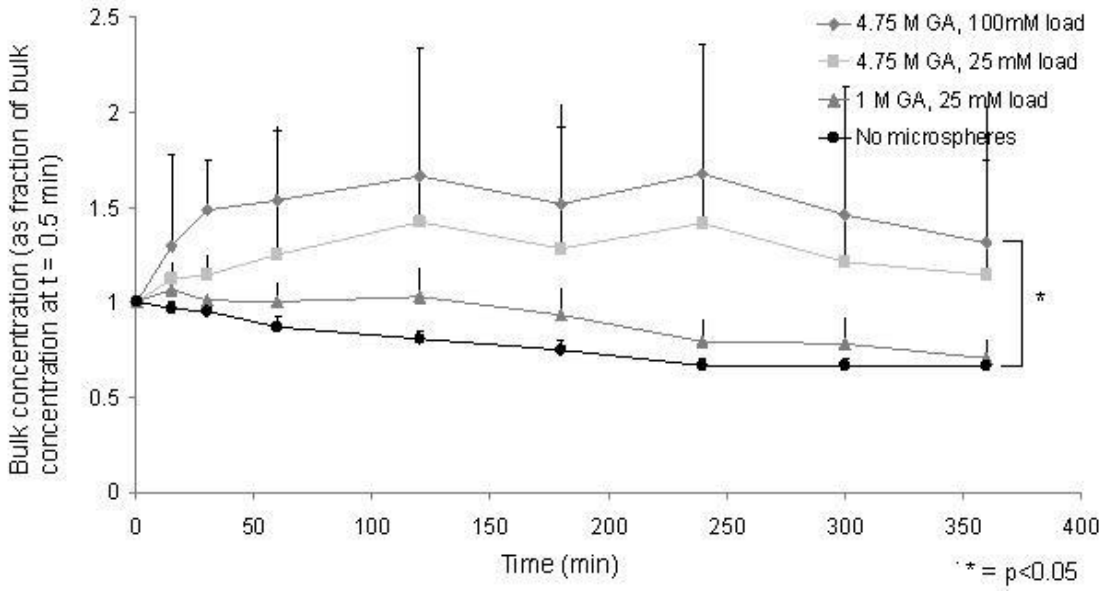

Figure IV-7: Change in bulk solution concentration over time for experimental and control (no microspheres) groups. Bulk concentration (as fraction of bulk concentration at t= 0.5 min) over time (minutes). The concentration in the control group continuously falls. 4.75 M GA, 100 mM loaded microspheres keep the bulk solution from decreasing in concentration, presumably by releasing H2S. The other two experimental groups are not as effective throughout the timeframe. Data demonstrates the efficacy of high crosslink density and high loading concentration at keeping the bulk solution concentration from dropping.

The results presented in Figure IV-5 to Figure IV-7 indicate that the crosslinking concentration of GA and the loading concentration of microspheres have an impact on the release-profile of H_2S over time. Namely, as our results indicate above: a higher crosslinkage (4.75M GA) yields less release of H_2S, higher loading concentrations (100mM NaHS) yield more release of H_2S, and microspheres with high crosslinkage and high loading concentration yield significantly more stable release profiles than groups without microspheres.

Controlled Release of H_2S *in vitro*

H_2S-loaded microspheres do not induce significant apoptosis

The objective of this experiment was to investigate the effect of introducing blank microspheres and microspheres loaded with NaHS on cell survival, in order to determine the concentration of H_2S that is safe for use on the heart organ in the rat model system used. Microspheres were fabricated and crosslinked with 1M glutaraldehyde. Sets of 50 mg, 100 mg, and 250 mg microsphere masses were weighed and loaded with 100 mM H_2S overnight. H9c2 murine cardiomyocytes were seeded at 1.0 X 10^4 cells/cm^2 on 6 well plates and grown to 90% confluence. 0 mg, 50 mg, 100 mg, and 250 mg of loaded and blank/non-loaded microspheres were added to the cells and incubated for 1 hour. After 1 hour incubation, cells were stained with ethidium homodimer and calcein AM and live-dead cell counts were performed through microscopy and imaging. Live-dead images are shown in Figure IV-8 where Row A depicts non-loaded control microspheres and Row B depicts loaded experimental microspheres. Masses are 0 mg, 50 mg, 100 mg, and 250 mg, respectively left to right.

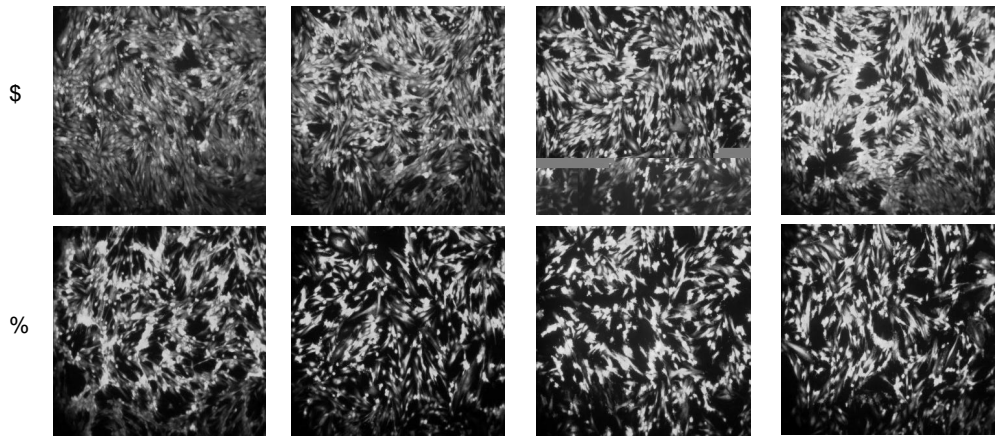

Figure IV-8: Viability assay results for cardiomyocytes incubated with varying [H_2S]. All cells were approximately 90% confluent at the time of the assay. Cells were seeded 2 days prior to conducting the assay.

Row (A): Representative control samples, microspheres incubated in absence H_2S. Images depict cardiomyocytes incubated with 0, 50, 100, and 250 mg of microspheres (from left to right).

Row (B): Representative experimental samples, microspheres incubated in presence of 100mM H_2S. Images depict cardiomyocytes incubated with 0, 50, 100, and 250 mg microspheres (from left to right). Assay was conducted upon 1 hour incubation of cells with H_2S.

Live-dead cell counts were performed in triplicate and cell survival is shown as a percentage of live cells per total number of cells in the assayed well. For 0 mg of microspheres, the experimental group, which consists of microspheres loaded with NaHS, displayed significantly higher levels of cell survival than the 100 mg microspheres of the experimental group ($p<0.001$). The control and experimental groups show comparable levels of cell death to the 50 mg microsphere sample, but the experimental group has lower death than the control group. The 100 mg experimental group has significantly more cell death than each of the other experimental groups and only 85% of the cells retain viability. The 50 and 250mg experimental groups show comparable levels of apoptosis to the 0mg experimental group ($p>0.05$). However, the experimental group has a large standard deviation, thus it is difficult to tell how reliable the result is. The 250

mg experimental group has lower cell death compared to the 250 mg control group, but the 250 mg control group has a large standard deviation. The control group displayed increased cell death as the amount of microspheres added increased. The 0 and 50mg control groups did not display significantly different amounts of cell death; however the remainder of the groups differed from the 0mg control ($p<.002$). Differences were assessed using ANOVA and Tukey's two-tailed multiple comparison test.

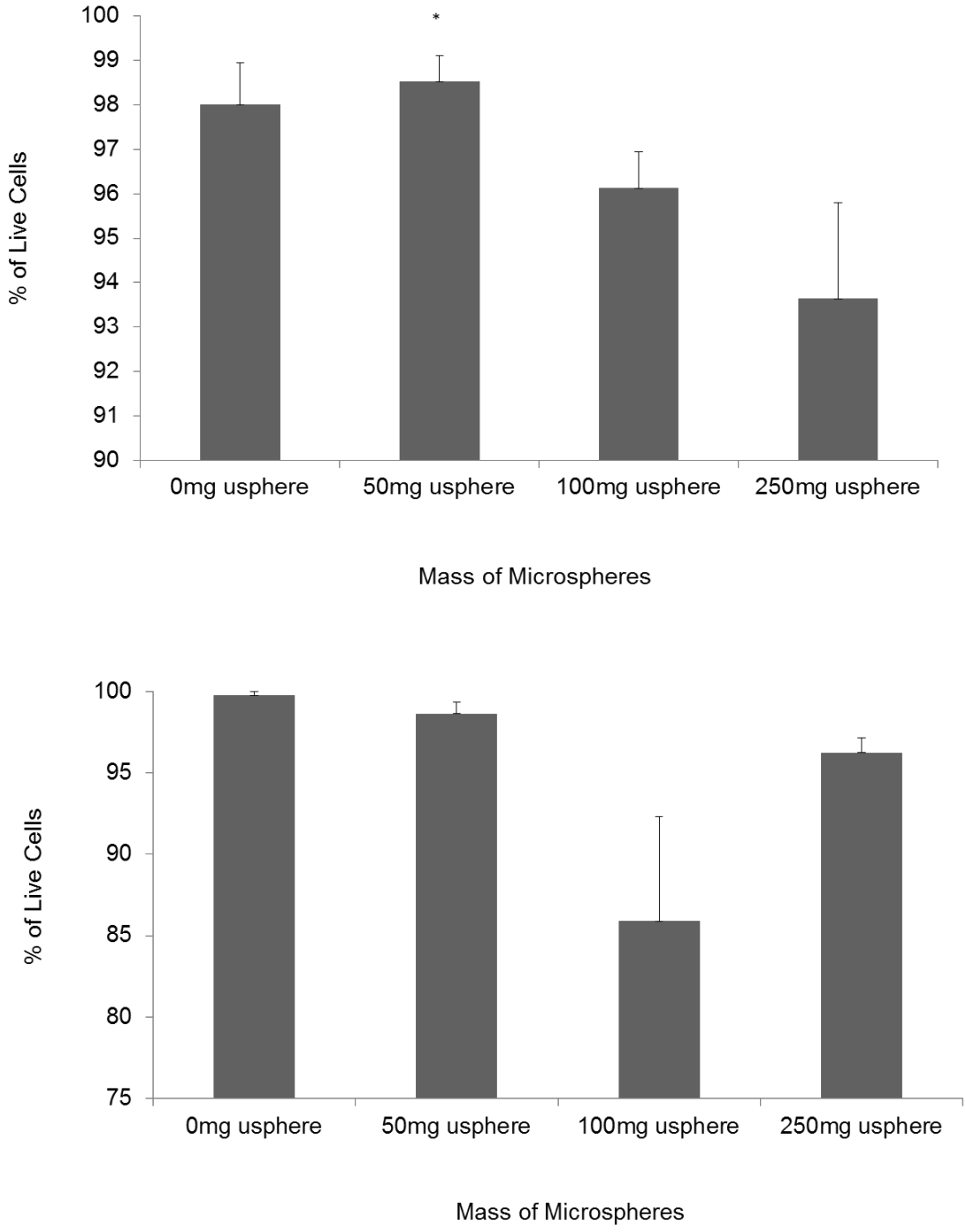

Figure IV-9: Quantitative representation of cell death for control cell samples. Each bar depicts the average of biological triplicate measurements. Error bars are presented as standard deviations.

Rat cardiomyocytes display similar metabolic activity

The objective of this experiment was to investigate the effect of NaHS on cytotoxicity and ensure that there are no detrimental effects of NaHS. H9c2 murine cardiomyocytes were seeded at 1.0 X 10^4 cells/cm^2 on 6 well plates and grown to 90% confluence. Varying volumes (0 μL, 10 μL, 1000 μL, and 10000 μL) of 100 mM NaHS were added to the cells and incubated for 3 hours. After incubation the MTT assay was performed as specified in the methods and the results are depicted as relative change in spectrophotometrically-measured absorbance.

Three biological replicates and three technical replicates were performed and standard deviations are shown (Figure II-1). It can be seen that there is no significant change in relative metabolic activity between the control group and the 10 and 1,000 μL groups. Decreased metabolic activity was observed in the 10,000 μL group ($p<.005$). The trend was a slight decrease in metabolic activity as the concentration of NaHS increased past 1,000 μL.

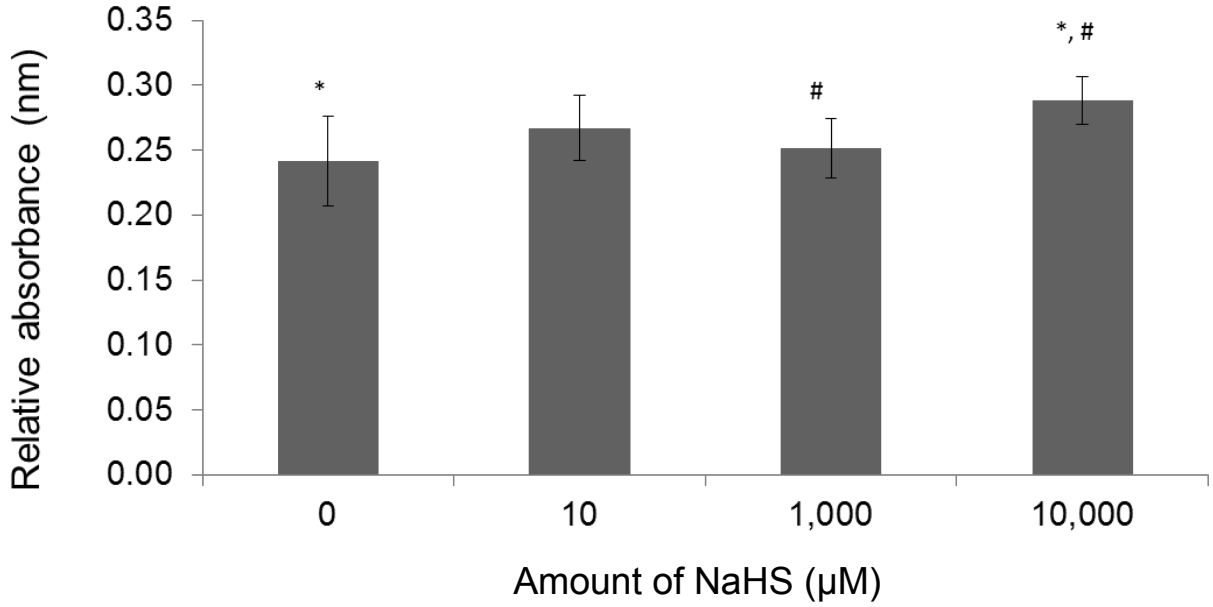

Figure IV-10:.Effect of H_2S on cardiomyocyte viability. Relative metabolic activity, measured by means of MTT assay, for cardiomyocytes incubated with 0, 10, 1,000, and 10,000 µL volumes of 100mM NaHS for 3 hours. Data for each bar depicts the average of three biological replicates, with standard deviations shown. ANOVA revealed a significant difference between groups (with a p value of 1.37E-03). Tukey's two-tailed multiple comparison test revealed that there was no significant difference in the percent of live cells between the 0mg control and the 50mg group, but the remainder of the groups varied from each other. Groups marked with a * do not vary statistically.

H2S temporal release from microspheres with murine cardiomyocytes varies with mass

The temporal release profile of H_2S into cell media using H_2S-loaded microspheres in the presence of murine cardiomyocytes was investigated. Microspheres were fabricated and crosslinked with 4.75M glutaraldehyde as previously mentioned. Varying microsphere masses of 50 mg, 100 mg, and 250 mg were weighed and loaded with 100 mM H_2S overnight. The microspheres were added to cell media and H_2S concentration in media was measured at 0, 15, 30, and 60 minutes. Results are shown as a

percentage decrease from the initial H_2S concentration in the media, in order to account for differences in initial H_2S concentration between the control and experimental groups. The control experiment in Figure IV-11 demonstrates the release profile of H_2S-loaded microspheres in media without the presence of murine cardiomyocytes. The experiment in Figure IV-12 investigates the effect of cells on the release profile of H_2S-loaded microspheres. H9c2 murine cardiomyocytes were seeded at 1.0 X 10^4 cells/cm^2 on 6-well plates and grown to 90% confluence prior to microsphere addition. Six biological replicates were performed and averages with standard deviations are shown.

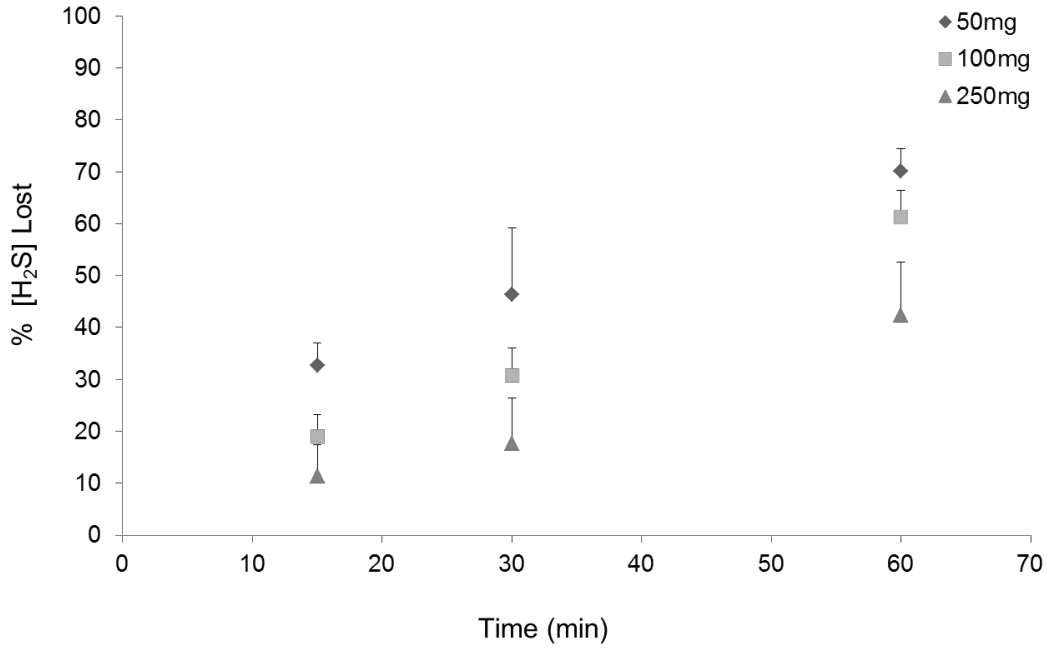

Figure IV-11: Cellular release profiles of H_2S. Net [H_2S] released in cell media only over time with microspheres loaded with NaHS. Microspheres were crosslinked with 4.75M GA.

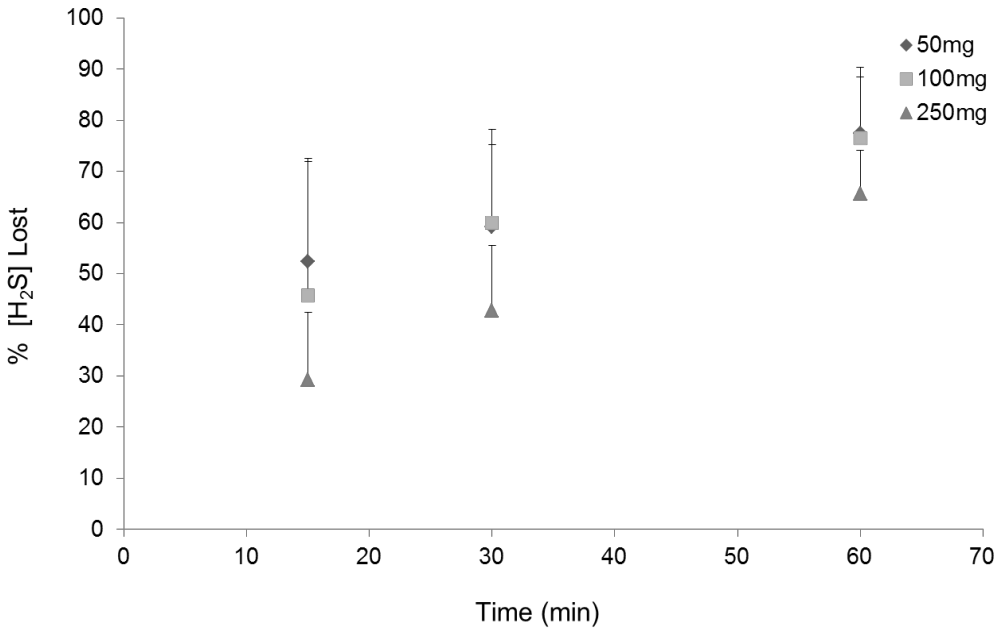

Figure IV-12: Net [H_2S] released over time microspheres loaded with NaHS in cell media with cardiomyocytes. Microspheres were crosslinked with 4.75M GA.

Without cardiomyocytes in culture, as the mass of microspheres increased in solution, the percent decrease from the initial H_2S concentration also increased in a time-dependent manner. The addition of 50 mg microspheres into media resulted in the greatest percent decrease from initial H_2S concentration for all time points with percent decreases of 32.7%, 46.3%, and 70.1% at 15, 30, and 60 min, respectively (Figure IV-11).The 250 mg microsphere group had the smallest percent change for all three time points, with percent changes of 11.3%, 17.6%, and 42.3% for 15, 30, and 60 min, respectively (Figure IV-11).In between, 100 mg microspheres had percent decreases of 19.0%, 30.7%, and 61.3% for 15, 30, and 60 min, respectively (Figure IV-11).

To determine the effect of the mass of the microspheres on release of H_2S into solution without cells, a statistical analysis was performed. Each of the three different masses of microspheres was compared against another group. The differences in percent

decreases from initial H_2S concentration over time between 50 mg and 100 mg were statistically significant ($p<0.05$), with the percent change in 50 mg microspheres statistically greater than that in 100 mg microspheres. Similarly, when comparing 100 mg and 250 mg microspheres, the percent decreases from initial H_2S concentration were statistically different across all time points ($p<0.05$). The two groups with the greatest difference in mass, 50 mg and 250 mg microspheres, yielded statistically significant differences ($p<0.001$). As the difference in mass of microspheres increased, so did the difference in percent changes in H_2S concentration, as shown by the decrease in p-values at the respective time point.

H_2S-loaded microspheres when added to cell media with cardiomyocytes exhibited the same trend of increasing percent decrease from the initial H_2S concentration over time, or decreasing H_2S concentration in media over time (Figure IV-12). As in the control, 50 mg of microspheres had the greatest percent change for all three time points with percent decreases of 52.4%, 59.1%, and 77.4% for 15, 30, and 60 min, respectively (Figure IV-12).250 mg again had the smallest percent decrease for all three time points with percent decreases of 29.2%, 42.8%, and 65.6% for 15, 30, and 60 min, respectively (Figure IV-12).In between, 100 mg had percent changes of 45.8%, 59.9%, and 76.6% for 15, 30, and 60 min, respectively (Figure IV-12).

A statistical analysis was performed comparing the percent decreases from the initial H_2S concentration between different masses of microspheres. Although there was not a statistically significant difference between 50 mg and 100 mg of microspheres, there was a statistically significant difference between 100 mg and 250 mg of

microspheres at 30 min (p<0.05). The greatest disparity in mass, 50 mg and 250 mg of microspheres, yielded statistically significant differences in percent decreases from initial

The presence of cardiomyocytes in cell media resulted in greater percent decreases from initial H_2S concentration at all time points and at all three masses of microspheres tested, compared to the control without cardiomyocytes. With the delivery of H_2S to cardiomyocytes, the decrease in H_2S concentration in media was noticeably greater than in the control. 50 mg of H_2S-loaded microspheres delivered to cardiomyocytes resulted in higher percent decreases from initial H_2S concentrations at all time points (p<0.05). 100 mg of microspheres into cardiomyocyte culture compared to just media also had statistically significant differences in percent decreases from initial H_2S concentration at 30 min and 60 min (p<0.01). Statistically significant differences for 250 mg of microspheres were found at 30 min (p<0.05) and 60 min (p<0.001).

Controlled release of H_2S *in vivo*

ATP content of stored hearts over time

The objective of this experiment was to measure ATP content in tissue samples taken from the left ventricle of hearts that were stored in control and experimental conditions. The three control groups were C-frozen, C-ischemia+UW, and C-UW. The three experimental groups were E-UW+NaHS, E-UW+μS, and E-UW+μS+H_2S. All hearts were stored in solutions for 8 hours and sampled every 2 hours, with the exception of the C-frozen group. Tissue samples were frozen and stored at -80°C after surgery, and were subsequently thawed and assayed as described in the methods section. The C-frozen group consists of hearts that were frozen immediately after explantation, while C-

ischemia+UW hearts were subjected to warm ischemia to stimulate apoptosis, and C-UW hearts were preserved in UW solution, as is the current clinical standard. Hearts in the E-UW+NaHS group were stored in UW solution containing 25μM H_2S. The E-UW+μS group, where "μS" is an abbreviation for microspheres, was stored in UW solution with 25 μM H_2S in solution and injected with PBS-loaded microspheres. The E-UW+μS+H_2S group was stored in UW solution with 25μM H_2S and injected with H_2S-loaded microspheres.

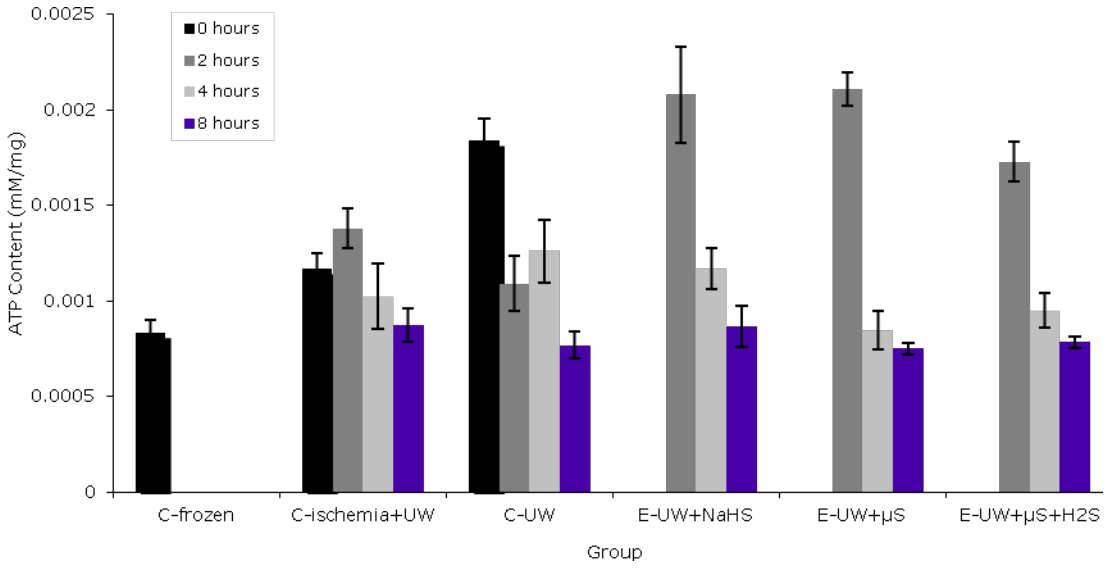

Figure IV-13: ATP retained in whole rat hearts over time plotted by group. Error bars represent +1 standard error of the mean. *C-frozen* represents sample taken immediately after stopping the heart. *C-ischemia+UW* represents hearts that underwent warm ischemia, stimulating apoptosis. *C-UW* represents the clinical standard, static storage with UW solution. *E-UW+NaHS* represents hearts stored with a modified UW solution which contained 25μM H_2S. *E-UW+μS* represents hearts perfused with gelatin microspheres loaded with PBS, then stored in UW solutions with 25μM H_2S. *E-UW+μS+H_2S* represents hearts stored in modified UW solution with 25μM H_2S and perfused with gelatin microspheres that were loaded with H_2S overnight.

Two independent biological samples were assayed in technical duplicate for every group. Thus each data point in Figure IV-13 and Figure IV-14 is the average of four distinct readings and is shown with its respective standard error of the mean. Constraints in time and funding limited the sample size to 2; larger sample size may find trends not observable using the smaller sample size, or may reinforce the significance in these differences. The ATP content is reported as mM/mg in order to control for varying tissue mass between samples. Tissue samples of hearts in the three control groups were taken at 0, 2, 4, 6, and 8 hours, while tissue samples of hearts in the three experimental groups were taken at 2, 4, 6, and 8 hours. Although tissue samples from the 6-hour time point were not assayed for ATP content due to constraints in resources, the data indicates that there is no significant difference between ATP content at 4 hours and 8 hours in any of the groups.

Data in each group was analyzed using ANOVA across time points to find significant decreases in ATP content over time (Figure IV-13). All groups demonstrated at least one significant difference in ATP content between time points ($p < 0.05$). Tukey's multiple comparison test was then used to find that in the C-ischemia+UW group, only the ATP content at 2 hours was found to be significantly greater than the ATP content at 8 hours ($p < 0.05$). In the C-UW group, the ATP content at 0 hours was found to be significantly greater than that at 2 hours, 4 hours, and 8 hours ($p < 0.05$). No other significant differences were found in the C-UW group. In all three experimental groups, E-UW+NaHS, E-UW+μS, E-UW+μS+H_2S, Tukey's test showed that the ATP content at 2 hours was significantly greater than that at both 4 and 8 hours, but there were no significant differences in the ATP content at 4 hours and 8 hours for any of the groups (p

< 0.05). The C-frozen group was not analyzed across time points because hearts in the C-frozen group were frozen immediately following explantation and therefore were only sampled at the 0 hour time point, before hearts were frozen.

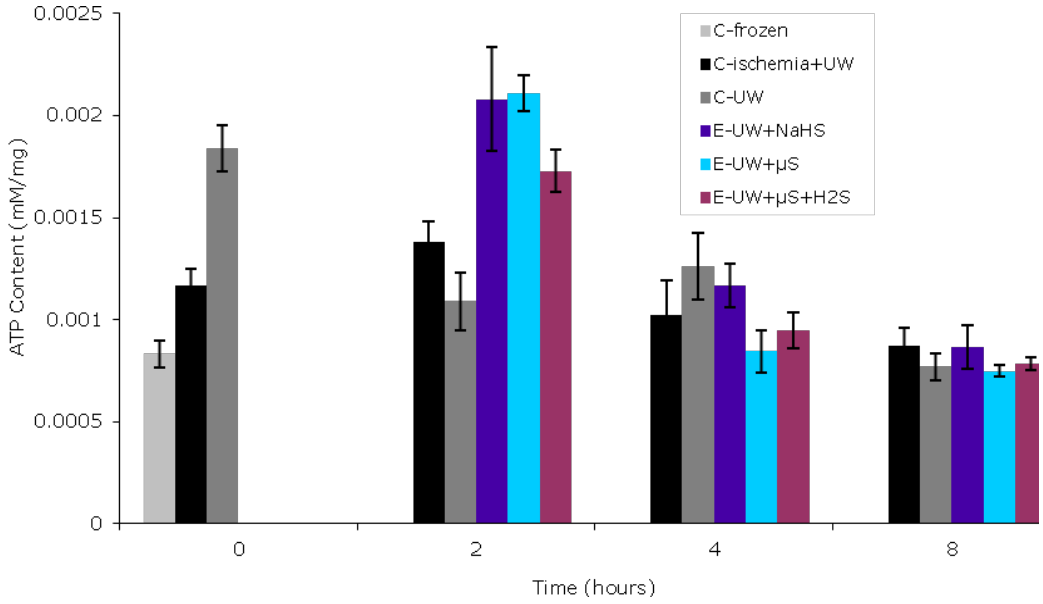

Figure IV-14: ATP retained in whole rat hearts across groups plotted by time. Error bars represent ±1 standard error or the mean. *C-frozen* represents sample taken immediately after stopping the heart. *C-ischemia+UW* represents hearts that underwent warm ischemia, stimulating apoptosis. *C-UW* represents the clinical standard, static storage with UW solution. *E-UW+NaHS* represents hearts stored with a modified UW solution which contained 25μM H_2S. *E-UW+μS* represents hearts perfused with gelatin microspheres loaded with PBS, then stored in UW solutions with 25μM H_2S. *E-UW+μS+H_2S* represents hearts stored in modified UW solution with 25μM H_2S and perfused with gelatin microspheres that were loaded with H_2S overnight.

Data across time points was also analyzed in each group using ANOVA, which showed that significant differences existed between groups at 0 hours ($p < .0001$) and 2 hours ($p < .001$) (Figure IV-14). To determine which specific pairs of means were significantly different, Tukey's multiple comparison test was used to analyze the data at 0 hours and 2 hours. Results showed that at 0 hours, the average ATP content of C-UW hearts was significantly greater than that of both C-frozen and C-ischemia+UW hearts ($df = 9$, $\alpha = 0.05$, $q = 3.95$), but there was no significant difference between C-frozen and C-

ischemia+UW groups. At two hours, Tukey's test showed that both E-UW+μS and E-UW+NaHS hearts had a significantly greater ATP content than C-ischemia+UW and C-UW hearts ($df = 15$, $\alpha = 0.05$, $q = 4.37$), but there were no significant differences in ATP content between C-ischemia+UW and C-UW groups, or between E-UW+NaHS, E-UW+μS, and E-UW+μS+H_2S groups. One-way ANOVA tests showed that there were no significant differences in ATP content at 4 hours ($p = 0.22$) or at 8 hours ($p = 0.66$) of storage.

Apoptosis levels from caspase-3 staining

The objective of this experiment was to qualitatively determine the amount of apoptosis in the tissue samples taken from the same rat heart experimental groups as those in the ATP protocols. Representative pictures from the caspase-3 assay are shown in Figure IV-15. As can be seen, the C-UW and E-UW+μS+H_2S groups show deeper staining than that of the other groups. Also, the tissue of the C-frozen, C-ischemia+UW, E-UW+μS, and E-UW+μS+H_2S groups show more tears and discontinuities while those of the C-UW and E-UW+NaHS groups are relatively intact, suggesting that the C-UW and E-UW+NaHS groups are better at protecting the morphology of the heart tissue. Although there is little discernable difference in the amount of tissue stained by the caspase-3 assay, a Western blot could verify any differences in caspase-3 levels.

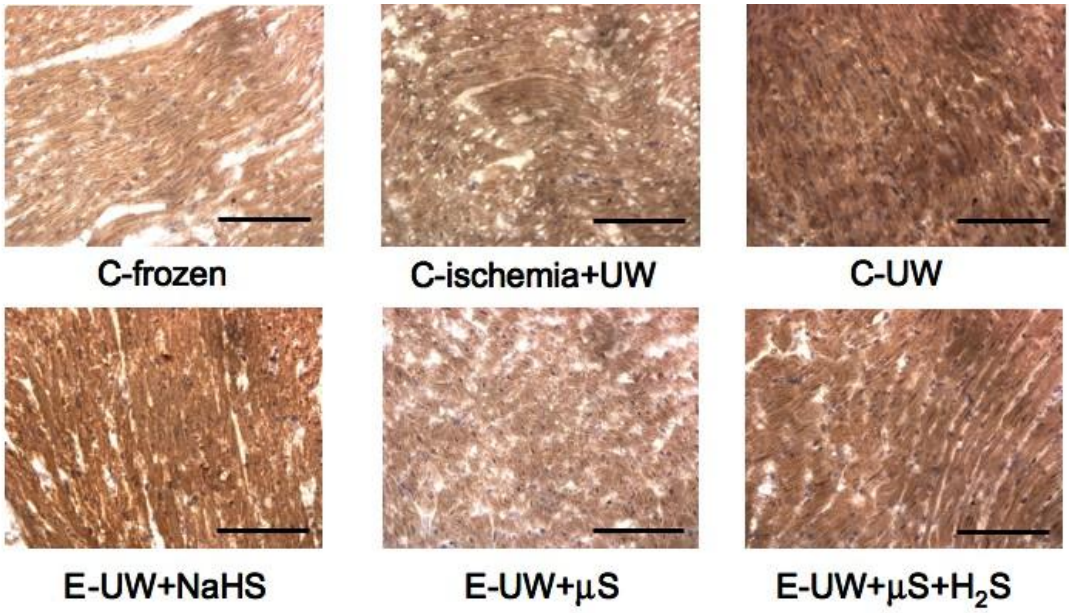

Figure IV-15: Representative sections of immunochemistry staining for tissue caspase 3. Frozen sections from heart tissue incubated with various experimental treatments for 4 hours were stained for activated caspase 3. *C-frozen* represents sample taken immediately after stopping the heart. *C-ischemia+UW* represents hearts that underwent warm ischemia, stimulating apoptosis. *C-UW* represents the clinical standard, static storage with UW solution. *E-UW+NaHS* represents hearts stored with a modified UW solution which contained 25μM H_2S. *E-UW+μS* represents hearts perfused with gelatin microspheres loaded with PBS, then stored in UW solutions with 25μM H_2S. *E-UW+μS+H_2S* represents hearts stored in modified UW solution with 25μM H_2S and perfused with gelatin microspheres that were loaded with H_2S overnight. Images were taken with a 40x objective lens. Scale bars represent 100μm.

Tissue morphology from H&E staining

The objective of this experiment was to analyze tissue samples for markers of gross tissue damage, including general morphology and infiltration with inflammatory cells. Representative images of hematoxylin and eosin (H&E) staining of tissue samples are shown in Figure #. One sample from each control (C-frozen, C-ischemia+UW, and C-UW) and each experimental group (E-UW+NaHS, E-UW+μS, E-UW+μS+NaHS) was imaged.

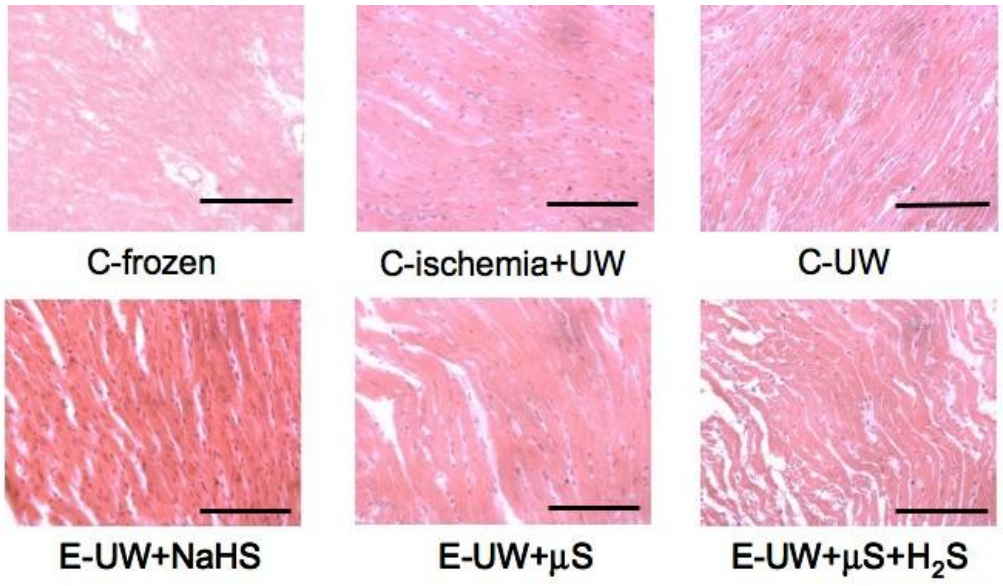

Figure IV-16: Representative sections of tissue morphology. Frozen sections from heart tissue incubated with various experimental treatments for 4 hours were stained with hematoxylin and eosin. *C-frozen* represents sample taken immediately after stopping the heart. *C-ischemia+UW* represents hearts that underwent warm ischemia, stimulating apoptosis. *C-UW* represents the clinical standard, static storage with UW solution. *E-UW+NaHS* represents hearts stored with a modified UW solution which contained 25μM H_2S. *E-UW+μS* represents hearts perfused with gelatin microspheres loaded with PBS, then stored in UW solutions with 25μM H_2S. *E-UW+μS+H_2S* represents hearts stored in modified UW solution with 25μM H_2S and perfused with gelatin microspheres that were loaded with H_2S overnight. Images were taken with a 40x objective lens. Scale bars represent 100μm.

V. Discussion

Controlled release of H_2S

Fabricating microspheres

The ability to fabricate hydrogel microspheres under the size of 10 μm is crucial in the future application to human models as any larger would cause infarctions. We have been able to consistently create hydrogel microspheres well below the limit, averaging 4.62±1.54 microns. Although outliers greater than ten microns occasionally appear, the application of a vacuum filter removes any microspheres >10μm. Equally important is the ability for the microspheres to release H_2S at a constant rate and in controlled amounts.

It is important to note that while this study provided a successful manner to produce these microspheres within the 10 μm limit, the procedures executed in previous studies produced microspheres of more than 50 μm, approximately five times the size of our target microspheres. Therefore, in utilizing the previous study's procedures, several alterations to optimize the procedure to the needs of the current experiments were added. In particular, the porcine gel was distributed evenly using an atomizer, which was continually washed in between experiments to prevent aggregation of the porcine gel microspheres. Aggregation could lead to an increased microsphere radius and clumping of the microspheres. As a result, it is advised that between experiments, the atomizer be heated and rinsed to remove any excess porcine gel. In an effort to ensure the accuracy of these results, it is important to measure the size of the microspheres prior to the crosslinking procedure.

H_2S depletes from solution

Figure IV-1 shows the dramatic drop in H_2S concentration from a closed solution over time, for both a large (days) and small (hours) time window, as a result of the H_2S escaping the solution as a volatile gas. The experiment was performed by taking a sample from the solution at the stated time-point and measuring H_2S concentration. Two concentrations (25 mM and 250 μM) were tested to show that H_2S loss can also be observed at therapeutically relevant concentrations.

Between time-points, the solution was closed in a 1 L glass screw-top container, or in a 1.5 mL plastic micro-centrifuge tube (Figure IV-1). The likely contributing process to the drop in H_2S levels over time is H_2S volatility. A certain concentration (or pressure) of H_2S exists at the air-solution interface, which is proportional to the solution's H_2S concentration, according to Henry's Law. At 25^o C, the Henry's Law constant for H_2S is 9.8 atm/M, or, for every mole of H_2S per liter of solution, there exists around 0.4 moles of H_2S per liter air. With higher concentrations, more H_2S would exist in the head-space above the solution; the steeper rates of loss at higher concentrations in Figure IV-1 support this concentration dependence. Moreover, the amount of H_2S in the head-space above the solution is related to the volume of the head-space. Thus, the head-space volume was controlled by ensuring the solution (1.5 mL) filled the 1.5 mL micro-centrifuge tubes to the brim.

However, due to imperfect seals in the containers, H_2S in the head-space can continuously leak out of the system, despite the containers being closed. In addition, the solution is repeatedly opened and closed at each sample point, which also contributes to the loss of H_2S. To confirm that the observed H_2S loss is not simply due to the container

being opened at the sampling times, we compared two situations. In one case, the concentration of a 250 μM NaHS solution in a 1.5 mL micro-centrifuge tube was sampled at t = 5 and t= 360 minutes only, and closed in the in-between interval. In the other case, 250 μM NaHS solution was sampled at t= 5, 15, 30, 60, 120, 180, 240, 300, and 360 minutes, and otherwise remained closed. Despite the solution being closed after the initial sampling in the first case, the concentration decreased to 63% of its initial value by the 6 hour time-point (data not shown). This decrease over 6 hours is significantly larger than the concentration decrease between two time-points spaced just 15 minutes apart (10%). Importantly, this result suggests that H_2S is being lost even when the container is closed. As expected, the concentration decrease at the 6-hour time point (24% of initial value) was significantly larger for the tube that is opened repeatedly to obtain samples. Thus, the continuous loss of H_2S during the period that the tube is closed is roughly half of the total, observed loss when taking samples throughout the six-hour period. At lower temperatures, it is expected that the depletion of H_2S due to volatility will also be lower, due to the inverse dependence of the Henry's Law constant on temperature. However, H_2S will continue to be depleted through other processes, including catalyzed oxidation of sulfide and biologically mediated metabolism (see Section 0 of this chapter, a discussion on cardiomyocyte H_2S consumption). Thus, if a stable H_2S concentration is desired for therapeutic applications, simply using a closed solution of NaHS is not an effective option.

Our observations of H_2S depletion from closed containers is consistent with observations made by other researchers. Typically, experimenters using H_2S from NaHS solution prepare the solution fresh on the day of the experiment (Chen and Morris, 1972),

or create a stock solution that is used within 3 days (Dombkowski et al., 2003). Following this standard, our experiments involving NaHS solution were done within three days of preparing the stock solution.

H_2S can be sorbed to gelatin discs

Figure IV-4 shows the capacity of 1 M glutaraldehyde crosslinked gelatin discs to uptake hydrogen sulfide. Dried gelatin discs were loaded with varying concentrations of NaHS. There is a clear trend towards higher uptake of sulfide with higher loading concentration. The mechanism by which H_2S is associated with the gelatin may be physical or chemical. Tabata et al. describes an application of gelatin microspheres that incorporates basic fibroblast growth factor (bFGF). They tested release profiles of bFGF from microspheres composed of basic or acidic gelatin, which have isoelectric points of 9 and 5, respectively. During loading, solution with bFGF was added to dried gelatin microspheres. The swelling of the microspheres in solution allowed bFGF to enter. The negative charge of bFGF formed polyionic interactions with the acidic type of gelatin, and so the release of bFGF was observed to be prolonged and dependent on the degradation of the gelatin carrier. In contrast, bFGF in basic gelatin microspheres had no other forms of association and simply diffused out at a much faster rate of desorption.

NaHS dissociates into SH-, in equilibrium with H_2S (and small amounts of S^{2-}). The gelatin used in our studies was porcine gelatin (G2500, Sigma Aldrich) which, due to the acidic pretreatment, likely has an isoelectric point that would allow it to interact with the SH- anion. It is possible that both ionic interactions and physical constraints, provided by the glutaraldehyde crosslinking, contribute to keeping sulfide in the microspheres.

Future work may target what effect ionic interactions have on the release of H_2S from microspheres.

Increased crosslinking density slows initial release of H_2S

Figure IV-5 shows the release profiles of H_2S from microspheres fabricated at two different crosslinking densities, 1 M and 4.75 M glutaraldehyde (GA). The 1 M GA microspheres, on average, released more than three times the amount of H_2S per milligram than 4.75 M GA microspheres, starting from the first measured time-point. As the ANOVA comparisons show, this difference did not reach the significance level, but approached it. The wide variability, particularly in the 4.75 M GA data, contributed to the high p-values. It is likely that repeated trials will strengthen this trend and move the p-value below 5%. In both groups, the first bout of release from 0 to 0.5 minutes is by far the largest amount released between two time-points. This burst release is set up by the large concentration gradient between the inside and outside of the microspheres in the beginning of the experiment, when there is no H_2S in the bulk solution. There is relatively little change measured in net amount released per milligram thereafter. However, in the case of 1 M GA microspheres, the rate of decrease is more pronounced; by the end of the experiment, they had lost 12% of the amount released at the first time point. In contrast, the 4.75 M GA microspheres had a release profile characterized by periods of loss and periods of release throughout the time window. The net result by the end of the experiment was only a 4% loss from the amount released at the first time point. As the ANOVA test did not yield significant results, this comparison, too, needs more repeated trials for validation.

These results suggest that 1 M GA microspheres had a larger initial "burst" release of H_2S compared to 4.75 M GA microspheres, but stopped releasing H_2S before the end of the time-window. 4.75 M GA microspheres had a smaller burst release, and preserved enough H_2S in the microspheres to continue releasing H_2S to the bulk solution throughout the time-window. This result may not be readily apparent from the plots, which do not reflect an actual increase in net amount of H_2S released for 4.75 M GA microspheres. However, it is important to note that net amount of H_2S released *underestimates* the actual amount of H_2S released from the microspheres. The net amount of H_2S released between two time points is calculated by the difference between the concentration after sampling at the time point and the concentration at the next time point (see methods). If these two concentrations are the same, the net amount of H_2S released is calculated to be zero. However, this only indicates the rate of H_2S release by microspheres is exactly balanced by the rate of H_2S loss to the atmosphere. Thus, if the amount of H_2S lost to the atmosphere were reliably incorporated into the calculations, the plot of total H_2S released would show a steadily increasing trend-line for the 4.75 M GA microspheres.

Because of the normalization by mass, the observed difference between the burst release from the two types of microspheres must be attributed to characteristics of the microspheres themselves. The crosslinking density of the microspheres is expected to be proportional to the GA concentration used in their fabrication. A higher crosslinking density would reasonably lower the amount of H_2S that can diffuse out of the microspheres, through a caging-type effect that traps the H_2S inside the microspheres. Iwanaga et al observed a similar inverse relationship between the release rate of insulin

from gelatin microspheres and the crosslinking density of the microspheres in similar *in vitro* release experiments. Moreover, it is likely the initial burst release from 1 M GA microspheres depleted the H_2S in the microspheres, so the net amount released decreases thereafter.

Higher concentration of loading solution results in higher net H_2S released

Figure IV-6 shows the direct relationship between the concentration of the loading solution and the net amount of H_2S released per milligram of microspheres, fixing the crosslinking density at 4.75 M GA. Microspheres loaded in 100 mM and 50 mM NaHS solution released more than 4 and 2 times the amount of H2S per milligram than microspheres loaded in 25 mM NaHS solution. This dependence on the loading solution concentration is reasonable because diffusion of H_2S into microspheres is related to the concentration gradient set up between the loading solution and inside of the microspheres. Thus, more H_2S is loaded into microspheres with higher loading concentration. When the loading solution is replaced by fresh PBS at the start of the experiment, the concentration gradient between the inside and outside of the microspheres this time motivates diffusion of H_2S out of the microspheres.

Moreover, with more H_2S loaded in microspheres, the microspheres release more H_2S *throughout* the time window, not only during the first few minutes. By the end of the experiment, the 4.75 M GA crosslinked, 100 mM NaHS loaded microspheres had released 70% more than the initial amount released in the first 0.5 minutes and the 50 mM NaHS loaded microspheres had released 35% more than the initial amount.

4.75 M GA, 100 mM NaHS loaded gelatin microspheres preserve H_2S concentration

The above trends are corroborated by data comparing the actual concentrations of the solution in the tube, with or without microspheres. The end goal of creating H_2S-loaded microspheres is to continuously release H_2S to the bulk solution, such that the concentration of the bulk solution does not drop. Our control group thus was a NaHS solution without microspheres, with a starting concentration comparable to the concentration measured in the first time-point of the experimental groups with microspheres (150 – 640 μM H_2S). As expected, the concentration of H_2S drops in the control throughout the experiment, due to two contributions: sampling (replacing 50 μL of sample at each time point with an equal volume of PBS) and loss to the atmosphere. By the end of 6 hours, the concentration remaining is 66% of the starting concentration.

Figure IV-7 shows that the experimental groups with microspheres in the solution variably improved the stability of the concentration. The 1 M GA, 25 mM loaded group had a higher concentration relative to its initial concentration compared to the control; this difference reached significance in the time period from 60 to 180 minutes. Thereafter, the fraction of the concentration remaining approaches that of the control. This trend matches our explanation that 1 M GA crosslinked microspheres release H_2S near the beginning of the time-window, but not towards the end.

In contrast, both 50 mM and 100 mM NaHS loaded, 4.75 M GA microspheres kept the H_2S concentration higher than their respective initial concentrations. However, due to large variability in the data, the difference was significant only for 100 mM NaHS loaded microspheres. For this group, the concentration of the bulk solution relative to the

initial concentration stayed higher than the same ratio for the control group for all time-points after 30 minutes, with the exception of the data point at t= 300 minutes. This observation also supports the trend towards continuous release of H_2S apparent in the data for net amount released of H_2S per milligram.

The variation in the experiments is quite large. An underlying factor that confounds the trends is the difficulty in controlling the residual loading solution in the experimental tubes. As explained in the Methods, at the start of the experiment, the loading solution is removed from the microcentrifuge tube by a micropipetter. Fresh PBS is then added to the tube twice to wash the microspheres and remove residual loading solution. However, the procedure cannot guarantee the complete consistency in removing the residual loading solution. This factor affects the first measurement and all calculations of the net release thereafter. A more reliable means to measure H_2S originating exclusively from microspheres (instead of residual loading solution), would improve the precision and accuracy of our results.

The release experiments thus show that crosslinking density and loading concentration are parameters that can be changed to produce different types of release profiles. Future work can test intermediate or higher crosslinking concentrations to see what type of release profile results. The data suggests that microspheres with higher loading concentration and higher crosslinking density improve the stability of the bulk solution concentration. Thus, 4.75 M GA, 100 mM microspheres were selected for the subsequent studies to produce a cold-storage solution with relatively constant H_2S levels, towards the preservation of rat hearts.

H_2S released from crosslinked microspheres is taken up by cardiomyocytes

Cardiomyocytes were demonstrated to uptake H_2S released from crosslinked, H_2S-loaded microspheres into solution. The rate of decrease of H_2S in solution is higher in the presence of cardiomyocytes (Figure IV-11 and Figure IV-12), indicating that the cells must be taking up H_2S. For the release of H_2S in purely cell media, the loss of H_2S from solution over time can be attributed to the evolution of H_2S as a gas, which escapes from solution. When cardiomyocytes are added to the media, the percentage decrease from initial H_2S concentration increases, meaning there is an additional pathway of escape of H_2S from the media into the cells.

A mass effect of microspheres is also demonstrated with the percent decrease of H_2S in solution. Increasing the mass of H_2S-loaded microspheres added to solution results in a smaller percent decrease of H_2S (Figure IV-11 and Figure IV-12) because there is a higher initial amount of H_2S in solution. For the same seeding density of cells, the amount of H_2S metabolized should be similar with varying masses of microspheres. It is reasonable to expect the percent decrease of H_2S to be smaller with higher masses of microspheres. This experimental trend indicates a H_2S uptake threshold limit by the cells.

A review of current literature regarding the consumption of exogenous H_2S correlates with the acquired results. In trout gills, it was demonstrated that H_2S is metabolized in an oxygen-dependent manner as H_2S diffuses through chemoreceptive neuroepithelial cells (Olson, Healy et al. 2008). Thus, H_2S uptake is known to require oxygen and diffusion through the cellular membrane. Another study showed the existence of dynamic steady-state cellular H_2S levels balanced between H_2S production and

consumption (Doeller, Isbell et al. 2005). In rat aorta smooth muscle cells, consumption rates are dependent on H_2S concentration with saturation kinetics of a K_m of less than 5μM H_2S and V_{ma} of approximately 40 pmol s^{-1}mg $protein^{-1}$(Doeller, Isbell et al. 2005). These kinetics demonstrate a concentration range in which H_2S is consumed by cells. For H_2S concentrations less than 30μM, cellular consumption rates were about 10-fold greater than the spontaneous H_2S oxidation rates found without the presence of enzymatically-active cells (Doeller, Isbell et al. 2005). Greater than 100μM H_2S, the H_2S consumption rate was comparable to the oxidation rate, showing that H_2S consumption is inhibited at higher H_2S levels (Doeller, Isbell et al. 2005). Although the primary enzymes for H_2S consumption in animals still remain unknown, the study showed that these enzymes have an operating range of 0.5 to 50μM H_2S, which is in line with the concentration of H_2S in mammalian blood and tissues (Doeller, Isbell et al. 2005).

In the context of these existing studies, the results showing uptake of H_2S by the cardiomyocytes can be explained by the concentration-dependent consumption of H_2S by cells. With the uptake mechanism of H_2S via diffusion through the cellular membrane, there was a noticeable difference in rate of decrease of H_2S from solution with varying masses of H_2S-loaded microspheres. The lower rate of H_2S depletion from solution with greater masses of microspheres (and thus higher concentration of released H_2S) demonstrates that there is saturation kinetics involved in the consumption of H_2S, as defined in the previously mentioned study. Thus, it is apparent that the cell has mechanisms to maintain a certain H_2S level through control of H_2S production and consumption.

Cardiomyocyte Viability

The MTT cytotoxicity test was chosen to determine the relative metabolic activity of a flat culture of cardiomyocytes due to its objectivity and ease of spectrophotometric measurement. It worked well for the NaHS-only incubation experiment. However, it could not be similarly utilized for experiments involving microspheres because the microspheres could not be entirely removed from the liquid; microspheres would have artificially increased spectrophotometric measures and would have yielded inflated measurements. The live/dead assay (also known as a viability assay) was chosen instead for relative viability experiments involving microspheres. As an intercalating agent, ethidium homodimer yielded a colorimetric response after penetrating the cells with permeabilized membranes in the culture. Calcein AM was taken up by functioning cells and metabolized to yield a fluorescent green color in viable cells. This assay was quantified by taking pictures under a fluorescence microscope and counting the number of dead cells. The total number of cells was determined using an event counter on the program Image J (developed by NIH). The percent of live cells was then determined and was utilized to compare the effects of the drug delivery system on cardiomyocyte viability.

A small baseline of cell death was expected from the live/dead stain because the cells were incubated in their respective H_2S treatments for one hour in a chemical fume hood at room temperature (25°C), not a biological hood. Addition of fresh media was used to minimize cell death. After one hour incubation, the cells were washed and again incubated for a half-hour with the live/dead stain reagents, which could have caused further cell death.

Microspheres Alone have a Negative Effect on Viability

Microspheres were fabricated using non-toxic, biodegradable materials, so it is unlikely the baseline of cell death in this group could be attributed to the products of microsphere degradation or from the intact gelatin spheres themselves. The downward trend in cell survival in the "Microsphere Only" group could be due to decreased oxygen delivery to cells residing under microspheres (Figure IV-8and Figure IV-9). The surface area of the non-circulating fluid was partially blocked by the microsphere mass, which most likely settled to the bottom of the wells. Therefore, a slight decrease in viability in a flat culture is expected. This problem is unlikely to occur in dynamic whole-heart conditions but may be expected in this system.

The Cardioprotective Effects of H_2S

The relative viabilities of the microsphere group were subtracted from the H_2S-loaded microsphere group to observe the sole effect of H_2S on cell viability. After accounting for the effect of the microspheres, the trends in viability are different. The NaHS-cultured groups demonstrated a slight increased ability to survive the culture period; percent of live cells increased by 0.1% for the 50mg group and by 2.6% for the 250mg group Figure IV-9. However, the percent of viable cells decreased by 10.2% in the 100mg group. The 100mg group of microspheres alone had significantly more cell death than the 0 and 50mg groups, but not significantly more cell death than the 250mg group of microspheres alone. The NaHS-alone studies showed a general protective trend (decreased cell death) as concentration increased (Figure IV-8 and Figure IV-10). It is possible that the 100mg group represents the worst combination

tested of amount of microspheres and releasing and cytoprotective ability. Possibly the 250mg group represents the point at which the slight detrimental effect of microspheres was overpowered by the stronger protective effect of NaHS. When the groups are normalized to the 0mg group, the 50mg and the 250mg group demonstrate enhanced viability.

Cardiomyocyte Viability is enhanced by H_2S

Mitochondria are an important focus because they both sustain and cause injury to cardiac tissue during ischemia and reperfusion. Controlling mitochondrial oxidative output may act to decrease heart tissue death. H_2S acts by decreasing mitochondrial electron transport and is associated with cytochrome c retention and lower amounts of ROS (Chen, Camara et al. 2007). The metabolic activity assay was chosen for use in this study specifically to quantify metabolic recovery. Furthermore, metabolic function is correlated with cell survival. The metabolic activity assay therefore gives insight into both the cell viability and metabolic activity.

The metabolic activity test results show a significant increase in cell metabolic activity as the amount of NaHS added to the media increases. Surprisingly, although the 10,000uL group demonstrates higher viability than both the 0 and 1,000uL groups, respectively, it is not significantly higher than the 10uL group. To further investigate the effect of NaHS on cell metabolic activity, increasing amounts/concentrations of NaHS should be tested to find the threshold where adding more NaHS becomes toxic to the cells and decreases the cell metabolic activity. Learning this threshold will allow better optimization of NaHS and maximize the cell metabolic activity.

This result should also be viewed in the context of H_2S's effects on metabolic function. Inhalation of H_2S caused a decrease of metabolic rate by 90% in murine subjects within 10 minutes. Mice were exposed to H_2S for a total of 6 hours. Afterward, metabolic rate returned to baseline levels within an hour (Blackstone, Morrison et al. 2005). In this study, cardiomyocytes were incubated for 1 hour with NaHS, and afterward were immediately incubated with the MTT reagent for 4 hours. Cardiomyocytes in the experimental groups therefore began the incubation period with suppressed metabolic function. The ability of the experimental groups to recover metabolic function and surpass the control group is more impressive when viewed from this context.

Drug Delivery Implications

Our results replicate the findings of prior studies, which conclude that H_2S has significant cardioprotective effects. We further quantify this effect at levels of NaHS that are safe for clinical use and present a novel drug delivery vehicle for use *in vivo*.

There is a threshold between the 50mg and 100mg groups where the effect of microspheres overrides the protective effects of H_2S and causes increased cell apoptosis. The microspheres should be limited to less than 100mg per well in a 6-well plate because the cell viability trends downward beyond this point. Cell survival with NaHS trends upward with more NaHS enhancing the viability potential of cells. The limiting factor appears to be the total amount of NaHS that can be loaded into microspheres. To produce the highest protective effects, microspheres should be loaded with a maximum concentration of NaHS, but a minimum of microspheres should actually be injected.

It appears that not only is our drug delivery system safe for use *in vivo*, but NaHS may confer additional protective effects at normothermic temperatures. Because each incubation period with NaHS, microspheres, and a combination of both was only one hour long, H_2S did not have time to work over a long period of time. Incubation with the MTT reagents was four hours long, which is four times longer than the NaHS incubation period, and incubation with the live/dead reagents was a half-hour long. As previously suggested these prolonged incubation times outside an incubator and inside a chemical fume hood could lead to increased cell death. Thus this would artificially decrease the percent of viable cells. Further research with incubation periods greater than one hour with NaHS and microspheres could illuminate long-term protective abilities of the drug delivery system. Furthermore, neither interactions between NaHS incubation and temperature were not studied, nor was NaHS used in conjunction with UW solution. With the basic safety and efficacy established, these studies may now be completed.

Controlled release of H_2S *ex vivo*

The heart is a unique organ in that its ability to contract is essential to its function. Thus, unlike cells in other organs, the maintenance of ATP levels during preservation is especially crucial to cardiomyocytes, which use ATP not only to maintain intracellular homeostasis, but also to maintain a functional contractile apparatus (Hegge, Southard et al. 2001). ATP content in stored hearts has been widely used as an indicator of heart tissue preservation and myocyte recovery (Peltz, He et al. 2005; Hu, Li et al. 2007; Zheng, Min et al. 2008). Here, we looked at ATP content as a representative measure of how well the heart might function following storage with NaHS both in solution and delivered through microspheres. H_2S is predicted to preserve ATP content by inducing a

state of suspended animation, during which hypometabolic conditions can save energy through suppression of both ATP-consuming and ATP-generating processes (Storey 2004; Hu, Li et al. 2007).

ATP Content Changes Suggest Prolonged Preservation in the Presence of H_2S

The comparison between the UW control (C-UW) and experimental groups across time points suggest that NaHS can prolong heart viability in storage. In the C-UW group, the ATP content was significantly greater at 0 hours compared to 2, 4, and 8 hours into storage (Figure IV-13). In comparison, all three experimental groups, in which hearts were stored in UW solution with NaHS (E-UW+NaHS), stored in UW solution with NaHS and injected with PBS-loaded microspheres (E-UW+μS), or stored in UW solution with NaHS and injected with H_2S-loaded microspheres (E-UW+μS+H_2S), showed a significantly greater ATP content in tissue samples at 2 hours into storage than at 4 and 8 hours (Figure IV-14). These results suggest that while a significant portion of ATP was lost between 0 and 2 hours in hearts stored in UW solution alone, the significant drop in ATP content occurred following 2 hours of storage in all hearts that were stored in UW solution with NaHS. This comparison overall would indicate that the presence of NaHS with microspheres in the experimental groups' storage solutions prolonged ATP preservation, which is subsequently suggestive of prolonged heart preservation. Although the ATP content of the C-UW group seemed to be depleted before the 2 hour time point, this result is in line with previous research that has shown that organs lose 95% of their ATP content within 2-4 hours during cold storage (Stringham, Southard et al. 1992; Southard and Belzer 1995). Furthermore, these analyses suggest that ATP content of

tissue samples biopsied at 6 hours of storage would not be significantly different in any group. Although tissue samples of 0 hr time points from the experimental groups were not available for comparison with the C-UW 0 hour time point, it is still noteworthy that the significant drop in ATP content occurs at different times between the UW control hearts and hearts treated with NaHS. Additionally, statistical analyses between groups at each of the time points, discussed below, provide further support for the conclusion that NaHS in UW solution preserves ATP content of hearts in storage.

Although overall, the decrease across time in ATP content was expected, an unexpected finding from our analysis was that, in the C-ischemia+UW group, only the 2 hour time point was found to be significantly greater than the 8 hour time point (Figure IV-13). The reliability of the 2 hour time point may be questionable when considering that the one-way ANOVA only yielded $p = 0.0496$, which is extremely close to the threshold of $p = 0.05$ for significance. It is unlikely that ATP content would increase in the C-ischemia+UW group hearts, which were subjected to a storage protocol meant to encourage apoptosis. However, there has been some evidence that ischemic preconditioning could confer protective benefits to explanted hearts against ischemia-reperfusion injury (Kandilci, Gumusel et al. 2006; Ghadhanfar and Juggi 2007). One study has shown that ATP steadily increases over a 6 hour reperfusion period in hearts that were subjected to 45 minutes of global ischemia (Freude, Masters et al. 2000). Thus, the increase in ATP content at 2 hours may reflect an actual effect from ischemic preconditioning. However, the increase in ATP content was likely not sustained because the C-ischemia+UW hearts were stored in static UW solution, compared to the continuous reperfusion that followed the ischemia in Freud et al (2000). Furthermore,

there have been discrepancies in reports of functional recovery following global ischemic preconditioning to hypothermic cardioplegia (Ghadhanfar and Juggi 2007). Our results are more in line with previous research that showed preconditioning with alternating episodes of ischemia and reperfusion reduced starting levels of ATP in canine hearts, but slowed ATP depletion by decreasing ATP utilization such that ATP content was significantly greater than in controls up until 40 minutes of ischemia (Murry, Richard et al. 1990). Although reperfusion was not utilized in this present study, our findings present an interesting avenue for further research.

Statistical Analyses of ATP Content

Statistical analyses were performed between groups in order to determine whether NaHS and microsphere delivery of NaHS not only prolonged, but also enhanced cardioprotection during cold storage. The results show that among the control groups at 0 hours, the ATP content of C-UW hearts was significantly greater than that of C-frozen and C-ischemia+UW groups (Figure IV-14). The difference between C-UW and C-ischemia+UW groups was expected due to the clinical precedent of using UW as a preservation solution, which would suggest better ATP preservation, and the ischemic conditions that the C-ischemia+UW group was subjected to, which has been shown to produce ATP depletion at the onset of storage (Murry, Richard et al. 1990). However, the difference between the C-UW group and the C-frozen group, which was flash frozen immediately after explantation with the expectation of preserving the heart in its most viable state, was unexpected. These results might suggest that the storage protocol itself could contribute to ATP depletion or the decreased detection of ATP content. However,

if true, this effect would be systematic and would therefore have little effect on comparisons between groups.

More interestingly, the ATP content for tissue samples from the 2 hour time point, at which all six groups can be compared, suggest that the presence of NaHS in UW solution significantly enhances energy preservation during storage. Tukey's multiple comparison test showed that both E-UW+μS and E-UW+NaHS hearts, which have in common submersion in UW solution with NaHS, but without H_2S-loaded microspheres during storage, showed significantly greater ATP content than C-ischemia and C-UW hearts (Figure IV-14). In contrast, there were no significant differences in ATP content among C-ischemia+UW, C-UW, and E-UW+μS+H_2S groups, or among E-UW+NaHS, E-UW+μS, and E-UW+μS+H_2S groups. These results complement previous research that has found similar benefits for ATP conservation in rat hearts stored with 1μmol/L NaHS in a bicarbonate buffer, Krebs-Henseleit solution (Hu, Li et al. 2007). Our results indicate that NaHS in UW solution can enhance ATP preservation to greater levels than UW solution alone. However, no significant differences were found between the E-UW+μS+H_2S group and any other group at 2 hours (Figure IV-14). While significant differences were found between E-UW+NaHS, E-UW+μS, and C-ischemia+UW and C-UW, the ATP content of E-UW+μS+H_2S hearts lies in the middle of these two pairs and did not reach a significant difference. This result may suggest that the E-UW+μS+H_2S group also experienced the preservative benefits of NaHS in UW solution, similar to E-UW+NaHS and E-UW+μS groups, but that confounding conditions insignificantly decreased ATP content. The relatively high ATP content of E-UW+μS hearts would

indicate that the microspheres themselves do not have detrimental effects on the heart after injection.

Overall, results from assaying ATP content suggest that NaHS in solution can prolong heart viability as measured by energy reserves, and also enhance the effects of UW solution in storage. These results are limited, however, in that significant differences are only seen at 0 and 2 hours of storage, and the differences are no longer significant at 4 hours (Figure IV-14) or at 8 hours (Figure IV-14). Also, a major limitation is the small sample size. As only duplicate biological samples were taken, our experimental design may be insufficient to show the actual underlying differences. The inconsistency with the currently accepted clinical standard of 4-6 hours in UW solution is not supported by our data. A potential systematic decrease in ATP content during storage and experimentation might contribute to this discrepancy by rendering differences in ATP content between groups too small to reach statistical significance. There is, albeit statistically insignificant, a trend in which the E-UW+NaHS group displays slightly higher ATP content at 4 and 8 hours. Further experimentation with more replicates might strengthen this trend. Additionally, future research may investigate different measures of energy content to provide a more complete picture of how microsphere-controlled delivery of H_2S can enhance energy preservation for recovery of contractile function following reperfusion. Alternate measures of energy reserve could include glycogen content, or the ratio of adenosine nucleotides rather than just ATP, which research has shown might be less correlated to heart function than high phosphate potential in general (Freude, Masters et al. 2000; Hegge, Southard et al. 2001).

Activated caspase-3 is a marker of apoptosis

Caspase-3 is a protease that is activated during the early stages of apoptosis, or programmed cell death. Activation of the caspase family of proteins is believed to be a major mechanism of cell death and has been studied extensively in the context of I/R injury (Murphy and Steenbergen 2008). Histological stains with specific antibodies are commonly used to detect caspase-3 activity and visualize apoptotic cells (Freude, Masters et al. 2000; Elrod, Calvert et al. 2007; Kafa, Uysal et al. 2010).

Optimization of caspase-3 staining

In this study, we started with a standard histological method that takes advantage of the enzymatic reaction between horseradish peroxidase (HRP) and 3,3'-diaminobenzidine (DAB). When oxidized, DAB produces a brown end product that can be visualized through light microscopy (Key 2006). The general staining protocol involves first binding the antigen of interest with a specific primary antibody, in this case, anti-active caspase-3 (BD Pharmingen, San Diego, CA). The primary antibody is then bound by a biotinylated secondary antibody. A streptavidin-HRP conjugate is applied next, in which the streptavidin molecule binds to the biotin of the secondary antibody. The final step in visualization is reacting the HRP with DAB, which will produce brown staining of the antigen of interest.

As with any immunohistochemistry, the protocol must be optimized to match the specific tissues and antibodies being investigated. One particularly persistent issue encountered in our optimization process was high levels of non-specific staining. Such "noise" can be caused by a variety of factors that stem from non-specific binding of each reagent. Both the primary and secondary antibodies may bind non-specifically if applied

at too high concentrations. Additionally, streptavidin can bind to endogenous biotin molecules, which are found in a wide variety of tissues. Finally, DAB, which reacts with HRP, can also react with endogenous peroxidases.

To prevent such background staining, blocking and optimization steps must be performed. In this study, we investigated various permutations of staining protocols that included hydrogen peroxide (0.3 – 3%) to block endogenous peroxidase activity, streptavidin and biotin (10 – 15 μg/mL each) to block endogenous biotin, 2% bovine serum albumin (BSA) to block non-specific staining due to hydrophobic interactions and normal goat serum (1.5 – 10% in 1xPBS) to block non-specific staining from the secondary antibody, which were goat anti-rabbit immunoglobulins (Key 2006). Each antibody was also titrated, or tested at a range of suggested dilutions, in order to optimize our protocol. Additionally, the fixation protocol to preserve tissue morphology must also be optimized since incomplete or over-fixation can interfere with antigen retrieval. Samples in this study were fixed in -20°C acetone for 10-20 minutes. However, the investigators were unable to produce negative stainings with the exclusion of the primary antibody, indicating a high level of background signal.

Caspase-3 results discussion

Due to unforeseen difficulties in staining optimization, the results from staining tissue samples from each control and experimental group are largely inconclusive. The C-UW and E-UW+NaHS slides, upon preliminary examination, seem to indicate higher numbers of apoptotic cells due to the greater intensity of brown staining when imaged at the same light intensity as the other samples. However, these results are contrary to previous research that has shown the reduction of apoptosis, specifically of caspase-3

activity, in UW solution (Hegge, Southard et al. 2001) and in the presence of H_2S (Elrod, Calvert et al. 2007). Variations in the density of the tissue may have contributed to the seeming variation in staining density. Alternately, the tissue sections may have degraded while in the -80°C freezer. Not only was there a freezer malfunction during the time of our experiments, but our slices were frozen directly after being sliced on the cryostat. Tissue quality and morphology may have been better preserved had the slices been fixed following sectioning and then stored in the freezer, rather than being fixed immediately before the staining procedure. Furthermore, a higher objective lens with greater resolution may be used to more closely examine the slices. Until additional protocol optimization and standardization can be performed, it is difficult to make conclusions about whether the injection of H_2S-loaded microspheres conferred additional protective benefits to the heart in storage.

Hematoxylin and Eosin staining reveals no significant differences

Samples from each group were analyzed under an inverted light microscope and examined for the above mentioned markers of tissue damage. Although a few isolated sites of lymphocytic infiltration were found, they were scattered across the groups such that no one sample had a significantly greater number of inflammatory occurrences. Overall, the H&E stain revealed no significant differences in tissue damage across the control or experimental groups (Figure IV-16).

Although these results might be interpreted to mean that neither the H_2S nor the presence of microspheres caused tissue damage, there are several experimental limitations that must be taken into consideration. One of the most significant limitations is the small sample size. Only one sample from each group was stained and analyzed due

to restrictions in time and resources. Subsequently, the effects of random error and variations between individual samples would be significantly exaggerated. One such individual difference is the actual tissue size. By basic principles of probability, it is more likely that a larger tissue section would have a greater absolute number of inflammation sites, even if the overall percentage of inflammation sites per unit of area. Furthermore, without both biological and technical replicates, the data is not reliable enough to make firm conclusions. Therefore, although these preliminary results appear promising, they must be confirmed by additional replications of this study.

Future directions for tissue viability tests

Previous studies indicate that caspase-3 histological stains are a viable method of quantifying apoptotic cells. Thus, with further optimization of our caspase-3 protocol, we may visualize and quantify apoptotic nuclei. Additionally, there are alternate histological assays that can be applied to measure indicators of cell damage. One histological assay commonly seen in the literature is the TUNEL assay, which labels fragmented DNA and indicates late stage apoptosis (Peltz, He et al. 2005; Yang, Wu et al. 2006; Hu, Li et al. 2007). Hematoxylin and eosin (HE) staining is also a standard histological stain used in a wide variety of research contexts and tissue types. Although HE staining is not specifically intended to reveal apoptotic markers, it can be used to visualize tissue and cell morphology. The morphology can be subsequently analyzed for indications of cell damage. This method has been previously applied to analyze myocardial inflammation, and thus could similarly be used in this study to compare myocardial damage after storage in UW solution with or without NaHS, and with or without the injection of H_2S-loaded microspheres (Elrod, Calvert et al. 2007). To determine caspase-3 activity, the

protein can also be measured by Western blot, which allows quantification of protein levels from tissue homogenates as compared to histological visualization of caspase-3-positive nuclei in tissue sections (Rinaldi, Gobbi et al. 2006; Jha, Calvert et al. 2008).

Furthermore, there are other markers of cell damage besides apoptosis. Necrosis and autophagy have also been shown to play a role in myocardial infarctions (Murphy and Steenbergen 2008). Additionally, creatine kinase is a macromolecule released upon cell lesion, when membrane integrity is lost. Therefore, determination of creatine kinase levels, specifically the cardiac isoenzyme of creatine kinase, CK-MB, can mark myocardial injury and detect myocardial infarction. CK-MB is also significant for its role in energy processes, since it catalyzes the conversion of phosphocreatine to ADP (de Souza, Olivieri et al. 2000).

Perhaps the most clinically relevant tests of viability are functional tests upon reperfusion. Functional parameters commonly measured to determine heart viability are left ventricular developed pressure (LVDP), arrhythmias, coronary flow, heart rate, inflow, and pulmonary artery perfusate oxygen concentration (Peltz, He et al. 2005; Zhang, Huang et al. 2007). These tests often require a Langendorff perfusion apparatus, which was not available during this study. Therefore, future experiments after obtaining the Langendorff apparatus could measure heart function after storage and thus provide a more comprehensive view of the potential preservative advantages of storage in UW solution with controlled delivery of NaHS through microspheres.

VI. Conclusions

We have successfully manufactured microspheres that will not block capillaries, and we have shown that these microspheres, when crosslinked using 4.75M glutaraldehyde solution and soaked overnight in 100mM NaHS, successfully sustain H_2S concentrations. We found that while the upper limit to microspheres in a 6-well culture plate is 100mg, the H_2S released by these microspheres is taken up by cardiomyocytes. In addition, when higher levels of H_2S were introduced via the microspheres, protective effects increased. This suggests that the optimal configuration of microspheres is those with the highest H_2S loading, but with the lowest mass of microspheres possible. Our results also show that at a minimum, the H_2S delivery method developed here is comparable to current clinical preservation methods.

Potential future directions include taking this work and performing functional assessments in a small mammal, such as the Langendorff perfusion apparatus, which assesses heart function *ex vivo* after preservation. Further biological assessments may also be conducted. Additionally, this work may be expanded to other organs, like the liver and kidneys. This work can also be expanded to other preservation techniques, like adding further compounds to improve preservation, or by adding microspheres and using continuous perfusion during the *ex vivo* period. Sustaining release of H_2S has the potential to significantly expand *ex vivo* storage times and improve transplant outcomes.

Future directions can also include modifications to the microspheres. The gelatin backbone could be modified to have specific H_2S affinity. Additionally, the aldehyde-amine group of GA crosslinkage is not stable, so the microspheres should be synthesized

using sodium borohydride. Also, analyzing the perfusate would demonstrate if there are microspheres left in the heart and if continuous perfusion of H_2S has any harmful effects to the heart. Microspheres can be examined after multiple reperfusions to determine if any changes occur between reperfusions. Further steps are required to implement this method in a clinical setting. Prior to patient exposure, the microspheres can be treated with UV light for sterilization.

VII. Appendix

Time (min)	1 M vs. 4.75 M GA, 25 mM NaHS	4.75 M GA, 25 vs. 50 mM NaHS	4.75 M GA, 25 vs. 100 mM NaHS	4.75 M GA, 50 vs. 100 mM NaHS
0.5	0.0893	0.2400	0.0297	0.1710
15	0.0915	0.2389	0.0270	0.1108
30	X	0.1780	0.0067	0.0227
60	0.0697	0.0884	0.0015	0.0056
120	0.0834	0.1292	0.0018	0.0487
180	0.0530	0.0669	0.0007	0.0068
240	0.0825	0.0850	0.0003	0.0262
300	0.0463	0.0269	0.0003	0.0138
360	0.0602	0.0409	0.0011	0.0234

Table VII-1: P-values from the ANOVA comparisons of the net H_2S released per milligram microsphere for various microsphere types (X denotes a data point was not collected at the 30 min time-point).

Time (min)	Control vs. 1 M GA, 25 mM NaHS	Control vs. 4.75 M GA, 25 mM NaHS	Control vs. 4.75 M GA, 100 mM NaHS	1 M vs. 4.75 M GA, 25 mM NaHS
15	0.1794	0.0337	0.2297	0.3144
30	X	0.0688	0.0086	X
60	0.0414	0.4143	0.0124	0.4001
120	0.0231	0.1656	0.0497	0.7780
180	0.0321	0.3135	0.0100	0.8377
240	0.0645	0.1503	0.0261	0.7463
300	0.1192	0.3529	0.0608	0.8554
360	0.2536	0.4137	0.0236	0.7768

Table VII-2: P-values from the ANOVA comparisons of the bulk solution concentration from various experimental groups (X denotes a data point that was not collected at the 30 min time-point).

Glossary

Term	Definition
Acidosis	A condition in which there is excessive acid in a body fluid
Aerobic Metabolism	Intracellular chemical reactions requiring oxygen in order for the organism to properly function
Allograft	A transplant that originates from a member of the same species as the transplant recipient.
Anabolism	Energy-requiring reactions that result in the synthesis of larger molecules from smaller units
Anaerobic Metabolism	A form of respiration using electron acceptors and instruments other than oxygen
ANOVA	ANalysis Of VAriance - statistical test that measures the means of several groups
Antegrade	Moving in a forward direction
Anti-port	An integral membrane protein that involved in secondary active transport of two or more different molecules or ions across a phospholipid membrane in opposite directions.
Antioxidant	A molecule capable of inhibiting the oxidation of other molecule
Apoptosis	Programmed cell death
Arrhythmias	An irregular heartbeat

Term	Definition
Assay	A procedure for testing or measuring the activity of a drug or biochemical
Atomizer	A spray bottle with a nozzle for producing a fine spray
ATP	Adenosine Triphosphate; the main source of energy to power metabolic processes in the cell
Cardiomyocytes	Involuntary, striated muscle cells found in the heart
Cardioplegia	Intentional and temporary cessation of cardiac activity, primarily for cardiac surgery
Caspase	An enzyme that plays an important role in apoptosis
Catecholamines	"Fight-or-flight" hormones released by the adrenal glands in response to stress (sympathetic nervous system response). Catecholamines include epinephrine and norepinephrine.
Cell	The functional basic unit of life
Collagen	A group of naturally occurring proteins that is the main component of connective tissue
Colloid	Substance dispersed evenly throughout another substance on a molecular level
Concentration	Strength of a solution; measure of how much of a given substance there is mixed with another substance
Coronary	Relating to the arteries that supply the heart tissues and originate in the aorta

Term	Definition
Crosslinking	The process of bonding that links one polymer chain to another by covalent bonds or ionic bonds.
Cytokine	A small cell-signaling molecule protein molecule used during intracellular communication
Cytoprotective	The quality of being protective to cells against harmful agents
Defibrillation	The action of delivering an electrical current to a heart in acute failure in order to reestablish normal sinus rhythm
Density	Mass per unit volume (e.g. g/mL)
Diastole	The period of time when the heart fills with blood after systole (contraction)
DNA	Deoxyribonucleic acid; contains the genetic instructions for the development and function of living organisms.
Echocardiogram	A sonogram of the heart to assess blood flow and cardiac valve function
Edema	Swelling caused by fluid retention in the body's tissues
Electrocardiogram	An external capture of the electrical activity of the heart measured using skin electrodes. A common diagnostic tool for abnormal rhythms of the heart or myocardial infarction.
Electron Transport Chain	Couples electron transfer between an electron donor (such as NADH) and an electron acceptor (such as O_2) to the transfer of H^+ ions (protons) across a membrane

Term	Definition
ELISA	Stands for Enzyme-linked immunosorbent assay. ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample
Endothelial	Referring to the thin layer along the insides of blood vessels, separating blood from the vessel wall
Enzyme	A protein that increases the rate of a chemical reaction in living organisms
Ex Vivo	Outside the organism
Explantation	The removal of cells, tissues, or organs of animals and plants
Fixation (tissue)	A chemical process by which biological tissues are preserved from decay, either through autolysis or putrefaction
Gasotransmitters	Gaseous molecules synthesized in the body, such as hydrogen sulfide and nitric oxide
Gelatin	A solid substance, derived from the collagen inside animals' skin and bones; commonly used in food, pharmaceuticals, photography, and cosmetic manufacturing
Glutaraldehyde	An organic compound with the formula CH2(CH2CHO)2 used for crosslinking gelatin into polymers
Histology	The study of the microscopic anatomy of cells and tissues of plants and animals
Hydrogel	A network of polymer chains that are hydrophilic, commonly used in tissue engineering

Term	Definition
Hydrogen sulfide (H2S)	A colorless, very poisonous, flammable gas with the characteristic foul odor of rotten eggs that is endogenously produced in human body in small amounts as signaling molecules
Hyperkalemic	Elevated intracellular potassium levels
Hypothermia	A condition in which the body temperature falls below the necessary temperature for proper metabolism and body functions, usually around 35.0C
In Situ	to examine the phenomenon exactly in place where it occurs
In Vitro	When a procedure is performed in a controlled, non-living environment, such as in a test tube
In Vivo	When a procedure is performed in a living organism
Infarction	The process which causes an area of dead tissue due to a loss of blood supply
Ion	A charged atom or molecule
Ischemia	A restriction in blood supply leading to tissue death
Ischemia/Reperfusion Injury	Tissue damage caused when blood supply returns to the tissue after a period of ischemia
Membrane	A flexible enclosing or separating tissue forming a plane or film that separates two environments in a living organism
Microflora	A collective bacteria and other microorganisms in an ecosystem

Term	Definition
Micron/Micrometer	10^{-6} meters
Microspheres	Small spherical particles with diameters in the micrometer range and manufactured from various natural and synthetic materials
Mitochondria	A membrane-enclosed organelle found in most eukaryotic cells that is known as the "powerhouse" of the cell, as it produces ATP
Molar	A concentration, also called molarity, that measures of the concentration of a solute in a solution in mols/L
Morphology	Relating to the form or structure of organisms and their specific structural features
Myocardial infarction	The interruption of blood supply to a part of the heart, causing heart cells to die; commonly known as heart attack
Myocardium	The middle of the three layers forming the wall of the heart
NaHS	Sodium hydrosulfide
Necrosis	The premature death of cells and living tissue
Negative Control	A control that confirms that the procedure is not observing an unrelated effect
Normothermic	To be at normal human body temperature
Organ Care System	A device by Transmedics that stores donor organs in a normothermic environment prior to transplantation.

Term	Definition
Osmolarity	The measure of solute concentration in number of osmoles (Osm) of solute per liter (L) of solution
Perfusate	A fluid (as a solution pumped through the heart) that is circulated through blood vessels or other natural channels
Plasma	Component of blood
Polymer	A large molecule composed of repeating structural units
Reperfusion	When blood supply returns to the tissue after a period of ischemia
Retrograde	Moving in a direction contrary to the normal direction
RNA	Ribonucleic acid; catalyzes biological reactions, controls gene expression, synthesizes proteins
ROS	Radical oxidative species - chemically-reactive molecules containing oxygen such as oxygen ions and peroxides
Sarcolemma	The cell membrane of a muscle cell
Spectrophotometer	Machine that quantitatively measures the reflection or transmission properties of a material as a function of wavelength
Spectroscopy	The study of the interaction between matter and radiated energy
St. Thomas Solution	A preservation medium for use in organ transplantation
Static Cold Storage	The conventional method of preserving organs for transplantation at cold temperatures, typically at 4°C.

Term	Definition
Suspended animation	A process in which biological activities are slowed down through external means without loss of life. Involuntary functions, breathing, and heart beating may still occur.
Systole	A phase of contraction during the cardiac cycle
Transcapillary	Through the capillary
Transmembranal	Through the cellular membrane
Transplantation	The moving of an organ from one body to another to replace the recipient's damaged or absent organ
Troponin	A complex of three regulatory proteins that is integral to muscle contraction in cardiac muscle
Trypan Blue Exclusion Assay	A visual dye test to determine cell viability. Dead cells stained a blue color can be distinguished from live cells under a microscope.
TUNEL	Terminal deoxynucleotidyl transferase; a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids
University of Wisconsin Solution	the first intracellular-like preservation medium designed for use in organ transplantation; the current "gold standard" solution in organ storage

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Index