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Ex-Vivo Slaughterhouse Porcine Crystalloid-Perfused Beating Heart via Langendorff Method

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Rahiemin Talukder

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Title: Ex-Vivo Slaughterhouse Porcine Crystalloid-Perfused Beating Heart via Langendorff Method
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And always, thank you to my parents and brother Ryan for their sacrifices they've made along with me, keeping me going in all ways when I wanted to give up, driving me to work hard, commit my utmost best, and do good.

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List of Abbreviations

ADP	Adenosine diphosphate
AFib	Atrial fibrillation
AMI	Acute myocardial infarction
ATP	Adenosine triphosphate
AV	Atrioventricular
bpm	Beats per minute
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CF	Constant Flow
Cl-	Chloride
CN	Cranial nerve
CO	Cardiac output
CO_2	Carbon dioxide
CP	Constant pressure
E_f	Ejection fraction
Epi	Epinephrine
HR	Heart rate
I.C.	Intracoronary
IU (U)	International Units

I.V.	Intravenous
K^+	Potassium
KHB	Krebs Henseleit buffer
LA	Left atrium
LCA	Left coronary artery
LV	Left ventricle
LVEDP	Left ventricular end-diastolic pressure
Na^+	Sodium
$NaHCO_3$	Sodium bicarbonate
O_2	Oxygen
OCS	Organ Care System
pO_2	Partial Oxygen Pressure
PTT	Partial thrombin time
Q.S.	Quantity Specification
R	Row resistance
r	Tube radius
RA	Right atrium
RCA	Right coronary artery
RV	Right ventricle
SRTR	Scientific Registry of Transplant Recipients
STHS	St. Thomas Hospital Solution #2 (Plegisol)
SV	Stroke volume

TAH	Total Artificial Heart
TCAV	Transcatheter aortic valve
TU/e	Eindhoven University of Technology
UW	University of Wisconsin buffer solution
VFib	Ventricular fibrillation
VO_2	Oxygen consumption
WIT	Warm ischemic time
wt	weight
ε	Strain
η	Fluid viscosity

Chapter 1: Background

1.1 Introduction

Chapter 1 of the thesis consists of background knowledge to prepare the reader for the main study. The background section provides the anatomy and physiology foundation of the cardiovascular field, including coronary circulation and ionic physiology. The electromechanical ion gradient creates a voltage, resulting into the action potential. The background continues to widen the scope from the microcellular build-up, by summarizing how systemic action potential forms the electrical system of the heart. To monitor the cardioelectrophysiology of the natural *in vivo* heart, the reader will recognize the essentials of both regular and irregular ECG. From *in vivo* conditions, the focus redirects to *ex vivo* conditions of an isolated heart, such as heart transplantation techniques. These techniques provide an understanding on how the isolated heart will be preserved and reanimated. The resuscitative Langendorff technique is introduced to the reader, including its two methods of perfusion—constant flow or constant pressure. As human hearts hold significant more importance for operable transplantation and thus were inaccessible for the study, a comparative summary between human and pig hearts is discussed; Pig hearts may substitute human hearts as donor hearts based on anatomy and function similarities. To validate reliability of hearts for the study, advantages and disadvantages of the three types of donor hearts are discussed, proceeding to the choice made of slaughterhouse hearts for the study.

The remainder of the thesis delves into key literature review, methods and materials for the experiment, results, and discussion. Chapter 2 constructs the requirements of the elementary procedure based on preliminary Langendorff studies. The various studies provide a wide spectrum of available buffers, donor hearts, pharmaceutical agents, temperatures, cost-efficiency and the corresponding results for the formation of the procedure. Two specific studies were analyzed in terms of the recovery of slaughterhouse porcine hearts and usage of crystalloid-perfusate instead of blood. Chapter 3 discusses material and methods for the procedure, including modifications to and stabilize global cardiac contractile function and increase the duration to <1 hour. Materials reviewed are cardioplegia, antithrombotic drugs, and the perfusion buffer, concluding with the finalized procedure. However, not all materials were kept for the finalized procedure. The developed procedure is categorized by slaughterhouse harvest, preservation, and reperfusion. Chapter 4 starts with a brief overview of all 13 experimental tests (Hearts 1-26) in chronological order to visibly dictate the progress and modifications made, achieving the finalized procedure. The chapter is broken down to results for each test. Results follow a consistent structure starting with the simplified procedure, what variables were specifically studied that day, results of the experiment, and follow-up of what should be tested specifically for the following test. Confirming the finalized procedure, Chapter 5 centralizes on the discussion of hypothermic effect, and metabolic changes of the isolated heart. Limitations that ceased the heart's contractile function at 1 hour such as calcium overload and edema are addressed. Chapter 6 concludes the study with a limelight on future directions the Langendorff apparatus may take. Future works

include the next step of a convertible Langendorff/four-chamber working heart system, and a mobile Langendorff apparatus, allowing potential collaboration. As the Langendorff apparatus can serve as a research platform, future directions will investigate research and development of medical devices, injuries, and therapies. There is definitive prospective for the Langendorff apparatus based on resuscitative devices aiding cardiac transplantation in the market today. The entirety of the report includes references in support of the text that may be closely viewed at the reader's digression. References are denoted numerically in superscript brackets.

1.2 Cardiovascular Anatomy

In a natural working heart system found in mammalian species, venous deoxygenated blood enters from the superior and anterior vena cava which collects into the right atrium (RA) – the process known as atrial diastole or the relaxation-filling period. When the atria contracts, the atrial pressure exceeds the right ventricular (RV) pressure, forcing blood flow into the ventricle. This action is known as atrial systole/ventricular diastole. In ventricular systole, to avoid blood backflow towards the RA, the tricuspid valve shuts and pulmonary valve opens, allowing blood to expel towards the lungs for oxygenation via ventricular contraction. The pulmonary valve shuts to avoid backflow once pressure in pulmonary arteries exceeds right ventricle.

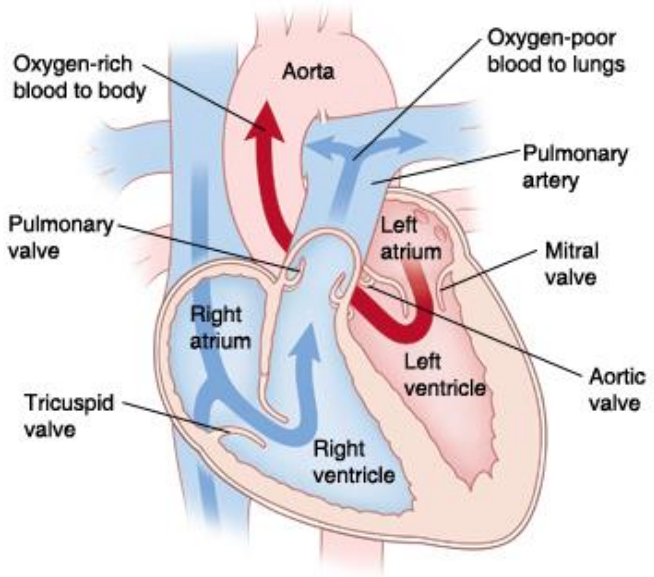


Figure 1.2.1 – Standard anatomy of the heart with deoxygenated blood from the systematic circulation on the right chambers and oxygenated blood from the pulmonary circulation [1]

Gas exchange occurs for blood at the lungs' alveoli before entering to the left atria (LA) via pulmonary veins. Once again, atrial systole occurs when LA pressure exceeds left ventricle (LV), the act known as ventricle diastole. The mitral valve also known as the bicuspid or left atrioventricular valve between the LA and LV shuts; during LV systole, blood volume is ejected via aortic valve and backflow into LA is ceased. Subsequently, pressure in aorta exceeds LV and thus shuts the aortic valve. The force of the heart contraction expels blood to the systematic circulation, exiting out the aortic valve and to the rest of the body's circulation. Figure 1.2.1 summarizes blood flow activity as the heart functions as two pumps for two separate circulations—one pulmonary, the other systemic.

1.3 Coronary Circulation

The blood supplies nutrients, gas exchange, proteins, vitamins, hormone, other metabolic substrates, and waste removal to and from every surface area of the body for continued mechanical and cellular activity. As it does so, the heart itself, and more specifically the myocardium, must also be supplied with blood as it would for any other organ. As mentioned in Section 1.2 pertaining to heart anatomy, the aortic valve shuts following ventricular diastole in the left ventricle. Roughly 5% of the cardiac output reroutes to the coronary ostia which maintain an average resting coronary blood flow at 250 ml/min via systolic pressure of <120 mmHg[2]. The coronary ostia are openings located at the base of the aortic root immediately superior to the valve, and form the entrance of the right and left coronary artery. The right coronary artery (RCA) branches into the right marginal and posterior interventricular artery whereas the left coronary artery (LCA) diverges into the circumflex artery which in a way crowns and revolves near the posterior side of the atrium, as well as the anterior interventricular artery (otherwise known as left anterior descending artery). Pacemaker cells that initiate the heart rate are found in the sinoatrial (SA) node (further discussed in Section 1.5) and are supplied by either the RCA or LCA at a 60-40% rate in the general population. Like all other capillaries, oxygen is exchanged towards the tissue as carbon dioxide enters the deoxygenated bloodstream.

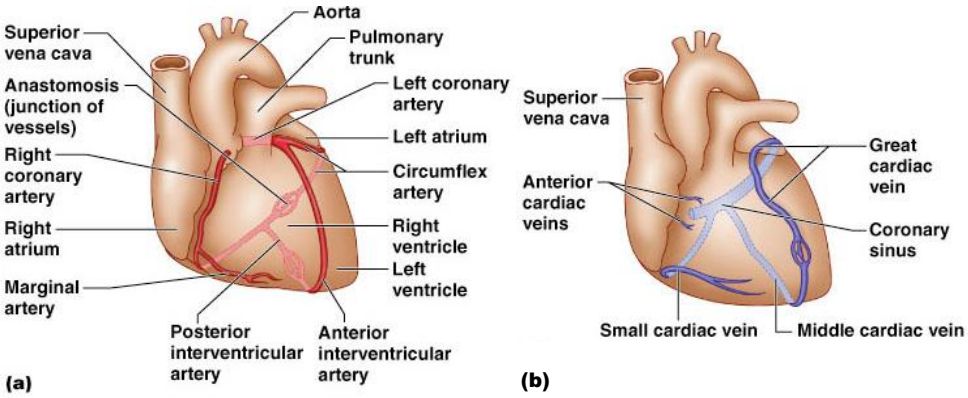


Figure 1.3.1a & b – Anatomy of coronary circulation of arterial (a) and venous (b) vessel. [3]

Deoxygenated venous blood collects at the coronary sinus found near the right atrioventricular junction and similar to other ostia, is mediated by the Thebesian valve to prevent backflow during Right Atrium systole. Blood from the sinus then empties into the right atrium along with systemic deoxygenated blood. The vasculature for coronary circulation can be viewed in Figure 1.3.1. Though pacemaker cells may initiate the electrical impulse of the heart rate, regulations are overridden by the SA node.

1.4 Systemic Cardiovascular Physiology

Cardiovascular mechanisms are regulated by the extrinsic and intrinsic responses, neuroendocrinic systems and autoregulation, respectively [4-8]. The SA node is innervated by a branch of the Vagus nerve (Cranial Nerve X) called the Cardiac Plexus. A mixture of sympathetic (excitatory or stimulatory) and parasympathic (inhibitory) nerves from the involuntary Autonomic Nervous System make up the Cardiac Plexus. The innervation moderates the heart rate. Sympathetic nerve increases heart rate, blood pressure, myocardial contractility, cardiac output and vasodilates; Parasympathetic nerves enable vasoconstriction and decreases heart rate, blood pressure, myocardial contractility, and

cardiac output. Neurotransmitters that aid in the autonomic nervous regulation are norepinephrine, epinephrine, and acetylcholine. Postsynaptic myocardial receptors such as α2 adrenergic and M2 muscarinic receptors decrease cardiac output and contractility while β-adrenergic increases it [7]. Serotonin has complex effects on both stimulating and depressing cardiac functions [9]. Drugs that are agonists or antagonist to the receptors will also affect cardiac function such caffeine and other stimulants to accelerate contractility or beta-blockers to decelerate contractility. Hormones that act as vasoconstrictors are vasopressin, angiotensin, and other catecholamines. Vasodilating hormones include corticotropin-releasing hormone and histamine. Reflex mechanisms include baroreceptors and chemoreceptors in the vessels, detecting levels of O_2, CO_2, and H^+ [4, 5]. Endothelial-derived nitric oxide also acts as a vasodilator in myocardial cells [10].

The intrinsic cardiac regulations that meet local needs are independent of the rest of the body. If the organ were to be isolated, these mechanisms would continue to serve metabolic purpose. Certain influential ion channels such as potassium, magnesium, and sodium allow vasodilation while open calcium channels enable vasoconstriction [11].

The Frank-Starling law allows the heart to change volumes of blood inflow. This law dictates that as the myocardial chambers stretch due to an increase in blood flow, a greater ventricular contractile force is required, increasing cardiac output [5, 6]. For such a system to mechanically work, the cellular and ionic level must be understood.

1.5 Ionic physiology

Electrical activity that enables spontaneous cardiac contractions can be attributed to a difference in the electrochemical gradient of the cells and thus are called cardiac action

potential. Classifications of these action potentials are dependent on the type of cells by which these potentials are produced from. Cardiac myocytes found in the atria and ventricles are present as *the fast response action potential*. As there is a fast response, there is a *slow response*, mitigated by the natural pacemaker cells found in the SA node. The ability for the action potential to occur resides in the cell membrane, specifically in its permeability of the dominant ions sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}). These ions that allow resting and action potential to occur is what develops the electrical system of the heart.

1.5.1 Ionic Influence on Resting Potential

The resting potential, or the resting state the cell naturally resides in, is based on the voltage difference across the cell membrane as seen in Table 1.1. At its resting state, cardiomyocyte, or excitable cells, is at -90 mV which holds similar for skeletal muscles. Extracellular voltage is at 0 mV while the difference in voltage in the cell itself changes, based on the difference in ionic concentrations.

Table 1.1 – Average extracellular vs intracellular composition [12-14] * not ionized

	Extracellular concentration (mM)	**Intracellular concentration (mM)**
Na^+	145	15
K^+	4	150
Ca^{2+}	1	1.4*
Cl^-	110	10
Mg^{2+}	1.5	17
ATP	0	4
Glucose	5.6	1

In Table 1.1, extracellular solutions mimic blood while intracellular mimics the interior of the cell. Blood and similar solutions are high in sodium whereas the cell interior is high in potassium as seen in Figure 1.5.1; this gradient enables Na^{+}-K^{+} active pumps to be driven via ATP usage to stabilize against the ionic diffusion.

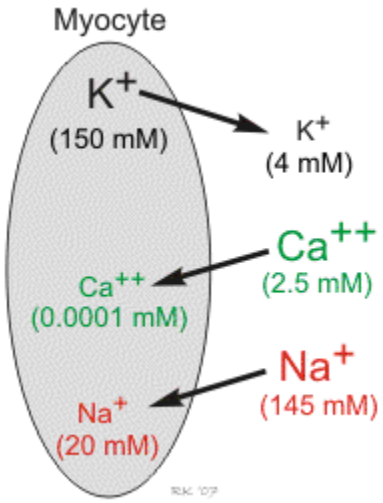


Figure 1.5.1 Cardiac myocyte resting membrane potential [15] . Ca^{2+} at 2.5 mMol reflects calcium bound to protein along with ionized Ca^{2+}.

Table 1.2 – Equilibrium potential of core ions in cell. [5, 13, 14]

Ion	Equilibrium Potential
Potassium	-94 mV
Sodium	+67 mV
Calcium	+123 mV
Chloride	-86 mV

The values found in Table 1.2 correspond with the specific single ion equilibrium charge. Thus, resting membrane potential is an aggregation of the concentration of ions within excitable cells. To sufficiently discuss how each ion plays a role in the cardiac action potential, certain transmembrane ionic channels and their properties must be taken

into effect as well as how the overall membrane potential is influenced by these channels as they selectively open and close.

The primary transmembrane ionic channels are mostly voltage-gated in which the passage is regulated by a certain intracellular voltage. While $3Na^{+}$-$2K^{+}$ ATPase pumps its respective ion against its gradient, the efflux of K^{+} diffuses outside the cell via leaky channels and inward-rectifier channels. Fast Na^{+} channels are voltage-gated, and thus are regulated at certain voltages. As fast Na^{+} approaches its equilibrium potential of +67 mV, the channel closes just as quick due to fast inactivation-gated periods.

Long-lasting Ca^{2+} channels, or L-type calcium channels, stay open at a prolonged rate. Longer Ca^{2+} waves enable higher motor muscle recruitment. Calcium homeostasis is balanced by $3Na^{+}$-$1Ca2^{+}$ exchanger. It is the flux of ions and its Ca^{2+} and Na^{+}'s potential to increase voltage of the membrane (depolarization) past a threshold with K^{+}'s potential to decrease the voltage (repolarization) that creates the action potential.

1.5.2 Action Potential

The action potential is an electrochemical mechanism in which the resting membrane potential surpasses a threshold of -90 mV to depolarize, offsetting voltage-gated ion channels to open as ions pass through. The intracellular membrane potential is -94 mV due to K^{+} being the dominant ion enabling towards equilibrium, as referenced in Table 1.2. Resting membrane potential leads the start of the cardiac action potential cycle. When the cell is at rest, K^{+} efflux occurs via K^{+} leaky channels, and membrane potential is slightly more positive than -90 mV, initiating Phase 0 and the onset of the action potential. With K^{+} diffusing out the cell, the membrane is slightly depolarized. At this

voltage, voltage-gated Fast Na^+ channels open, with Na^+ diffusing intracellularly. Due to Na^+ influx, membrane potential rapidly depolarizes, approaching Na^+'s equilibrium potential of +67 mV but the Fast Na^+ channel shuts at roughly 20 mV. On the verge of overshoot at 20 mV, voltage-gated K^+ channels open, with efflux K^+ movement. This in turn lowers the membrane voltage as K^+ exits the cell and repolarization is inclined towards K^+ membrane equilibrium at Phase 1. As it repolarizes and membrane potential is approximately +5 mV, Ca^{2+} now influences the cardiac action potential as Ca^{2+} influxes via L-type channels. Ca^{2+}'s resting potential is at roughly +120 mV, thus membrane potential is aimed towards depolarization. Meanwhile, additional slow-acting K^+ channels open, increasing extracellular passage, aiming for repolarization. With the balance of K^+ channels and Ca^{2+} channels concurrently open, their voltage effect balances and plateaus, as can be seen in Phase 2 of Figure 1.4.2. L-type calcium channels delay as they shut. By this point, inward-rectifier K^+ voltage-gated channels continue to efflux, repolarizing towards -94 mV, meeting K^+ equilibrium and resting membrane potential; thus, these channels too delay and close aside for the leaky channels. As the onset of action potential is based on the Fast Na+ channels, this specific action potential is a *fast response action potential* in cardiac muscle cells as opposed to a *slow response action potential* found in pacemaker cells of the heart.

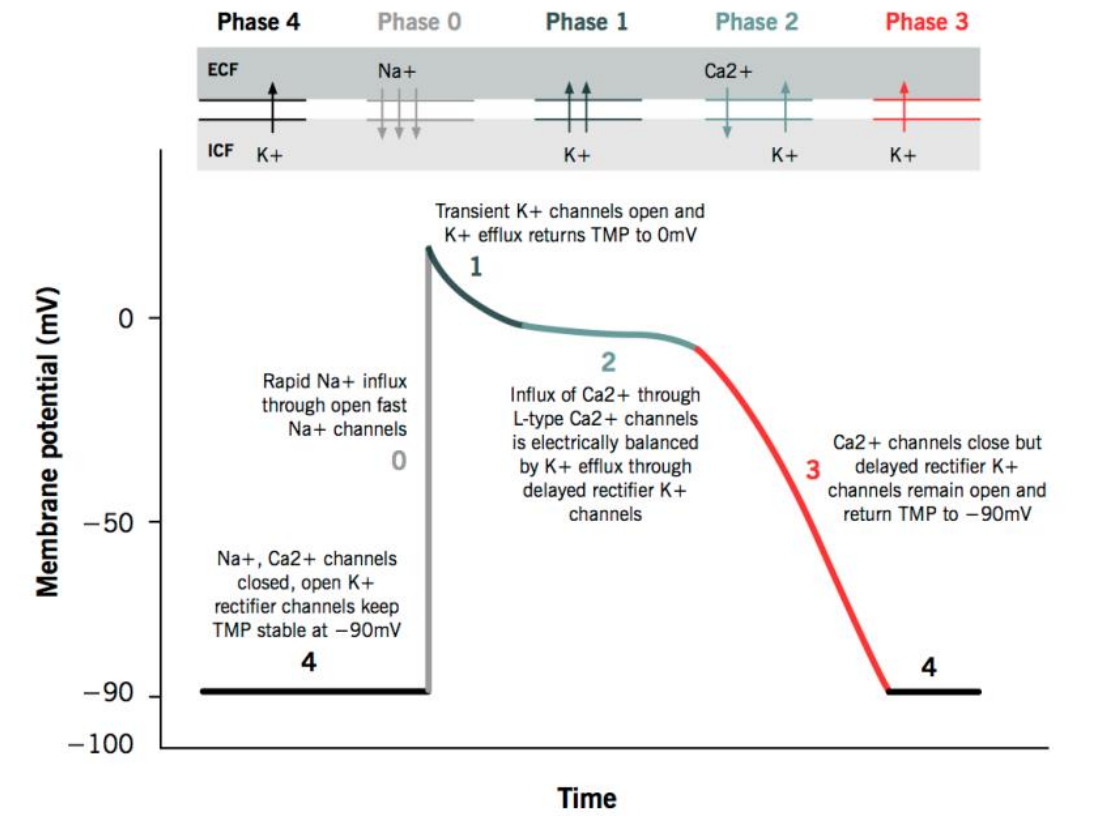


Figure 1.5.2 – Fast response action potential of cardiac myocytes. [16]

As the cell resumes resting membrane potential at Phase 4, the Na^+/Ca^{2+} exchanger aids in pumping Ca^{2+} in to the cell for reuptake by the sarcoplasmic reticulum, and in turn gets Na^+ back out of the cell to prepare for the next action potential. As the cell repolarizes, Na^+K^+-ATPase metabolic pump activate and restores equilibrium at resting potential, sending $3Na^+$ ions against its gradient extracellularly, and $2K^+$ ions intracellularly. From Phase 0-3, effective refractory period (ERP) also takes place, in which the cell cannot be restimulated for arrhythmias to occur [5] . The benefit of ERP is to coordinate simultaneous contractions.

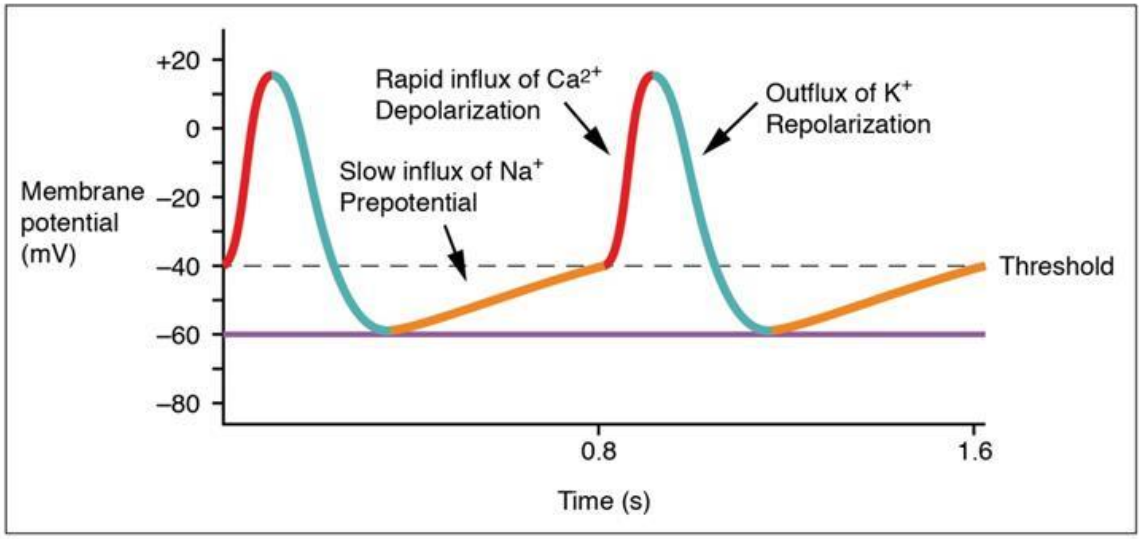


Figure 1.5.3 – Slow response action potential in autorhythmic cells. [17]

For slow response action potential which occurs strictly in pacemaker cells, resting membrane potential is -60 mV, allowing Na^+ channels to open and Na^+ to slowly influx, summarized in Figure 1.5.3. The cell is depolarized, meeting the threshold at -40 mV, which initiate Phase 0 similarly to fast response action potential shown in Figure 1.5.2. At this voltage, voltage-gated Ca^{2+} channels open, allowing rapid influx of Ca^{2+}. Ca^{2+} becomes dominant, with the cell membrane rising and continues to depolarize towards Ca^{2+}'s resting potential of +123 mV and then become gated at roughly +10 mV. While Na^+ channels are still open, some positive-dependent voltage-gated K^+ channels open as it permeates out. Membrane potential now inclines towards K^+ and as K^+ membrane equilibrium is -93mV, membrane potential repolarizes towards -90 mV. The K^+ channels shut at -60 mV and the slow action potential is complete. The derivation of which action potential is slow and fast is dependent on phase 0, or the onset of passing the threshold to the overshoot.

1.6 Electrocardiophysiology

With the initiation of the slow action potential to produce an electrical impulse, this impulse spreads through the entirety of the heart through a sophisticated conduction system with the specialized excitable cells. The monitoring of the conduction system of electrocardiograms or ECG in turn aids clinically to visualize offsets in certain chambers of the heart that undergo arrhythmias.

1.6.1 Electrical conduction system

1% of cardiac cells are not myocytes but rather its gap junctions induce a conducting system [18]. This conducting system initiating the sinus rhythm, or the electrical pathway that is then followed by contractions, starts from the SA node located in the right atrium, enabling the highest rate of depolarization across the magnitude of muscle cells. The SA nodal cells are the first order pacemaker cells. From there, the impulse travels via Bachmann's bundle in which the impulse is carried towards the left atrium, as seen in Figure 1.5.1. Along with Bachmann's bundle, the intermodal tracts conduction is carried towards the atrioventricular (AV) junction, rightfully called the AV node. Nodal conductions are inherently slower compared to conduction cells, leading for a 100 ms delay between not only the conduction between SA to AV but also atrial and ventricular contractility. The delay allows blood to move in coordinated flow based on two separate contractions either directing towards the lungs or the rest of the body[18].

The conducting tracts continue towards the Bundle of His, located in the interventricular septum which in turn splits into fascicles towards the left and right bundles of their respective ventricles. The bundles differentiate into Purkinje fibers

originating near the apex. The fibers allow global conduction through all ventricular cardiac fibers. The impulse is able to spread across the ventricles in 75 ms [5, 6, 18].

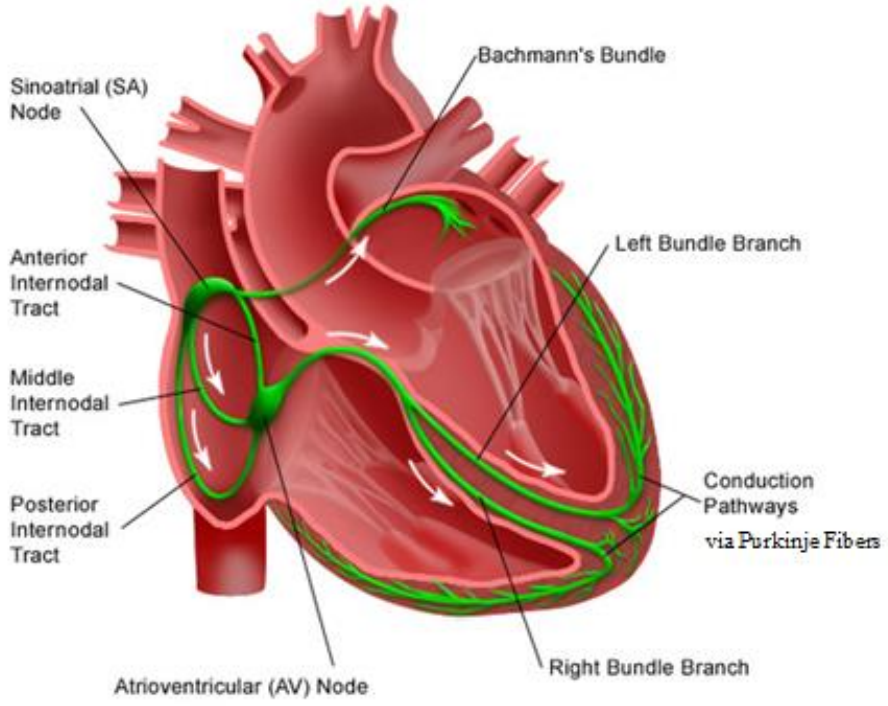


Figure 1.6.1 – Electrical system of the heart. [18]

It is the variant action potentials of all different cardiac cells and activity—atrial diastole, atrial systole, ventricular diastole, ventricular systole—that can be graphed as a full unit per 1 heart contraction cycle seen in electrocardiographs (ECG). The ECG shows the propagation of depolarization as the action potential ultimately results in contractility. The SA node's initial propagated atrial depolarization is seen as the P wave in ECG lead II and marks atrial contraction. The QRS wave as seen in Figure 1.6.2 dictates ventricular depolarization as well as atrial repolarization and overall ventricular contraction. The T wave signifies ventricular repolarization. Ventricular contractions are

delayed and occur 0.1 second after atrial conduction [5]. The ECG shown is an example of a normal sinus rhythm.

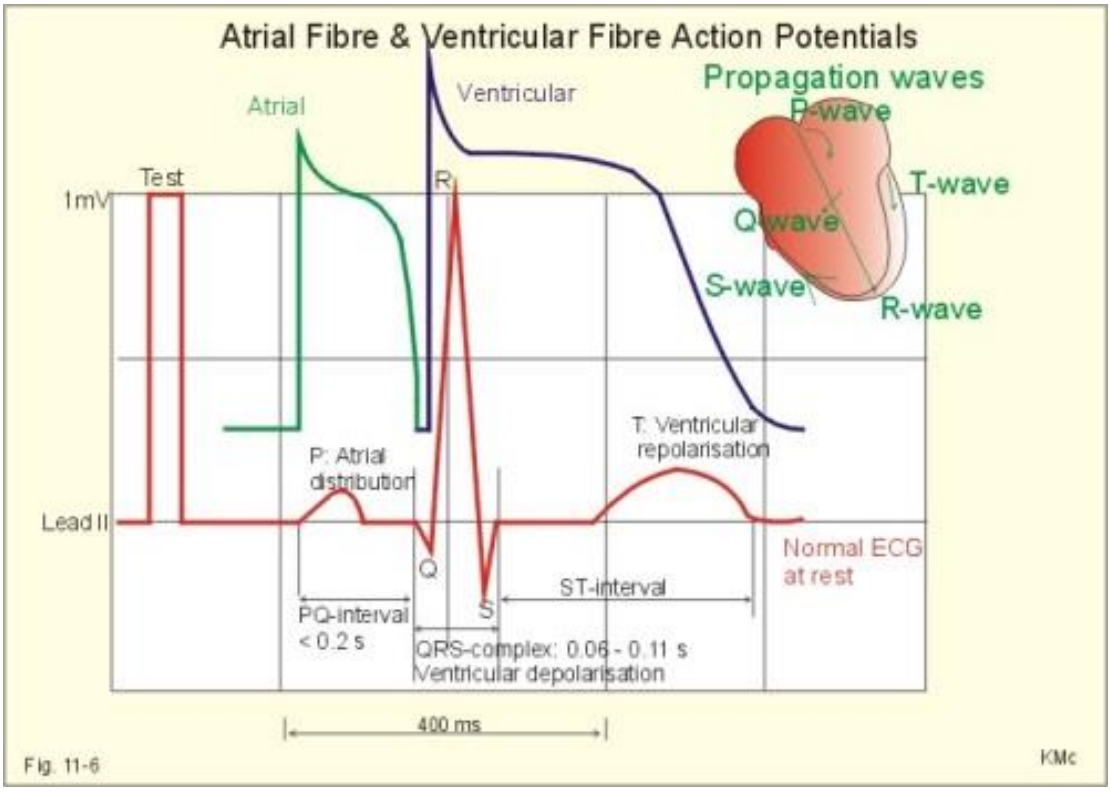


Figure 1.6.2 – Depolarization propagated waves of both atrial and ventricular function are diagramed in ECG readings. SA node initiates the impulse for the atrial contraction to occur where the impulse passes through the intranodal tract to the AV node that then initiates ventricular contraction via depolarized waves of the myocytes contractions. [19]

1.6.2 Abnormal ECG readings

ECGs can establish and chart the occurrence of a sinus rhythm base as well as types of arrhythmias dependent on certain waves that falter against the normal healthy heart. Figure 1.6.2 is an example of a normal sinus rhythm for one heartbeat. Arrhythmias that most impact an isolated heart are tachycardia and fibrillation, which may occur in both atrium and ventricles. Arrhythmias are detected in heart transplantation as the donor heart

is resuscitated by blood prior to great vessel anastomosis and must be defibrillated to restart the sinus rhythm [20] .

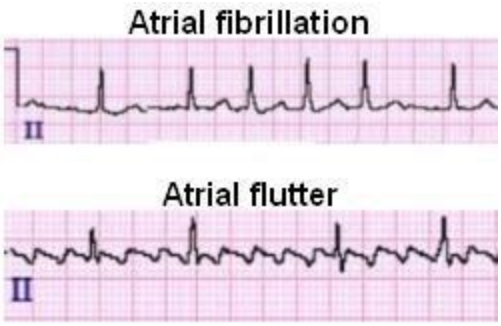
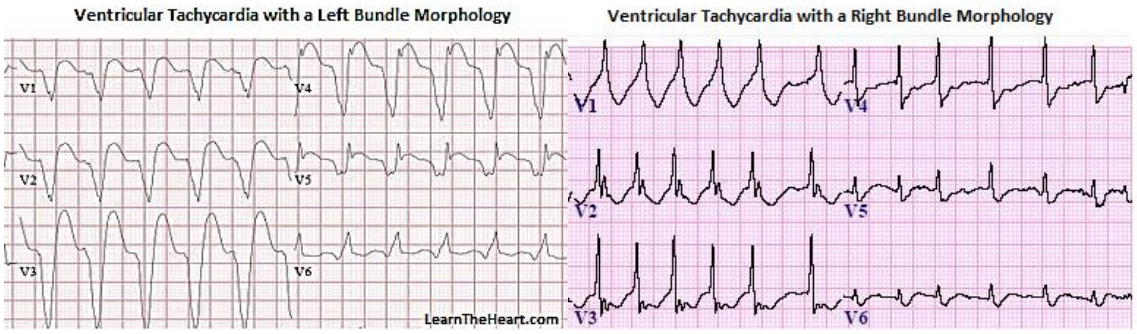


Figure 1.6.3 – Atrial fibrillation and flutter. [21]

The atria may become irregular, resulting into atrial fibrillation (AFib) [5, 6, 22]. AFib can result in heartrates of 400-600 bpm with poor blood flow. P waves won't be seen in an ECG because the action potential are repetitively shooting, with very little crossing threshold. As AFib is irregular, an atrial flutter is regularly irregular. An atrial flutter is similar to AFib but has a consistent yet increased heartbeat. The flutter can be visualized by a sawtooth pattern on the P wave of short repetitive bumps not reaching peak, seen in Figure 1.6.3. Atrial flutters are approximately 250-350 bpm. Both atrial arrhythmias are also considered as supraventricular tachycardia.

In general, ventricular tachycardia (VT) displays a widened QRS peak with ≥120 seconds duration. If VT continues, ventricular fibrillation or sudden cardiac death can result. VT can result from an injury or block to the left bundle branch or right bundle branch found along the interventricular septum. Left bundle branch block VT has an elongated QRS peak with a downward bend while right bundle branch block displays an elongated QRS peak with an upward bend in the V1 ECG reading, as seen in Figure 1.6.4. VT can range 100-250bpm[23].



(a) **(b)**

Figure 1.6.4a & b – Ventricular Tachycardia of the left (**a -** left) and right (**b -** right) bundle. [24]

While ventricular tachycardia is stabilized rapid rhythm and originates at the ventricles with operable contractions, fibrillation has no order, displays irregular rhythmia and is a superficial twitch, with ineffective contractible activity. Similarly, the atria can undergo tachycardia, flutter, and fibrillation. The arrhythmia can lead to a series of complications including clots and risk for stroke; These disorders along with many cardiac diseases and cancers can lead to end-stage heart failure, where heart transplantations remains to be the only treatment.

1.7 Heart Transplantation

Since the discovery of human heart transplantation by Dr. Christiaan Barnard's groundbreaking operation in 1967 utilizing Dr. Norman Shumway's technique [25] to the proclamation of organ donation from brain-dead patients in 1981[26], donor hearts have been widely used for heart transplants for those whose hearts are failing and incompetent to succeed in physiological body performance and circulation. By 1976 under the aid of the immunosuppressive therapy, heart rejection by the patient has significantly decreased, increasing the success of transplants [6, 26, 27].

Heart transplantation can be seen as two stages: the retrieval of the donor heart and surgical operation for the transplant recipient [12, 28]. Standard protocols for heart transplantation haven't significantly changed overtime aside from anti-rejection medications for the heart. After matching criterions for legal brain death, the donor heart undergoes several screenings to match compatibility and prevent infectious transmission. Cold preservation solutions with high potassium concentration are used for diastolic arrest, during donor cardiectomy, and for transportation. Preservation solutions and similar heart transplant protocols will be investigated in Section 3.1 [12, 27, 29-31].

The recipient is placed under anesthesia, with an incision made down the center of the chest; the sternum is divided to expose the heart. Blood flow is rerouted using a cardiopulmonary bypass machine so that the rest of the body has uninterrupted flow while vessels to the heart are clamped. The diseased heart is excised followed by the donor heart and vessels anastomosed, or reconnected. Clamps are removed to allow blood back into circulation via heart instead of the bypass machine. Defibrillation is used as needed as the cardiac functions of the new heart are carefully monitored. Excess fluids are drained; sternum is connected, followed by skin being stitched prior to sterile dressing [28].

Although the population with diseases has increased across the decades, the number of transplantations performed is roughly stagnant. Figure 1.7.1 charts total transplantations performed in the United States since 1991, including patients on active waitlist and number of donors. Government statistics show that by the ending year of 2013, less than a quarter of waitlisted patients had received a transplant. Numbers of

transplants and organ recovery has stayed stagnant for the last decade with patients waiting for a life-saving transplant at a steady incline approximately at 4.1% [32]. 18,048 organ transplants have been performed between January to July 2015 [32]. As of November 2015, 122,620 Americans are on the waitlist, 79,228 of whom are active waiting candidates [32]. 8,757 donors, both deceased and living, were recovered for the first 7 months of 2015. Compared to 121,272 waitlisted patients in 2013, there is clear indication of immobility. Heart transplants are <10% of overall procedures; there are currently 4,197 patients awaiting heart transplants. In 2014, 2,174 heart transplants were performed in the US from the total 29,532 general transplantations, with an additional >1,000 heart transplants done overseas [31-33]. To reinstate, roughly 51% of those needing a life-saving heart will get one.

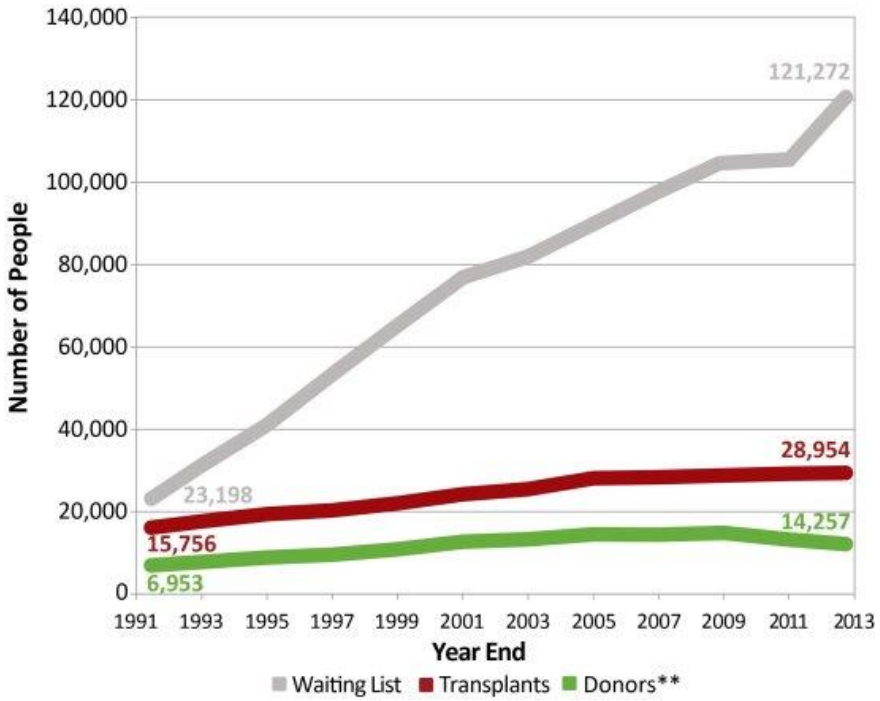


Figure 1.7.1 – Organ donors, recipients, and active patients waiting in the United States. [33]

Patients eligible for cardiac transplantation must have irreversible heart failure with age ranging between infantile to over age 65. There are slimmer chances of finding a matching heart for infants and growing children. For patients who have waited >4 months for a transplant in constant hospitalization with deteriorating conditions, the standard approach is that the patient uses a left-ventricular assist device. The device enables assistance for pumping blood in the patient's body.

With the lack of donor organs to provide viable treatments for waiting patients per year, there are slim chances for viable human hearts to be used for research instead of clinical implantations. As seen in the figure above, a global challenge is that the number of donor hearts available is less than patients seeking transplantation. Research in the cardiovascular field is progressing but is limited towards clinical tests and live animal models. The majority of human heart donors are prioritized for heart transplant rather than research, limiting the pool of hearts for research application. To expand the amount of available hearts, deceased isolated hearts can be of use if the heart is freshly preserved and then reanimated to beat. The Langendorff method shares many similarities to that of heart transplantation and moreover, the technique of preservation follows that of organ donor preservation for transplantation and clinical usage.

1.8 Langendorff Perfusion

The Langendorff perfusion to reanimate the isolated mammalian heart was developed by Oscar Langendorff between 1895-1897 in Leipzig, Germany [34]. The technique is largely used in society today on testing myocardial and coronary vasculature function, contractility, understanding cardiac diseases, injury brought upon by ischemia

or reperfusion, and leading up to the biotech industry of gene manipulation and protein expression. It has contributed advancements towards the electrophysiological, biochemical, and pharmacological fields. The advantage of using an isolated heart is that the central nervous autonomic system is eliminated by severing any neuronal coordination. Isolation of a heart offers both the advantage of separating intrinsic cardiac properties from extra cardiac regulating processes, and excellent accessibility to a wide variety of measuring techniques [13]. Many studies imposed on an isolated heart cannot be reproduced utilizing an *in vivo* experiment without serious repercussions such as global ischemia, infarction, or cardiac arrest [35]. The issues can be eliminated by optimal preservation. For ideal protection, hypothermic cardioplegia is induced as the heart's metabolism decreases along with oxygen usage.

Langendorff method has the heart resuscitated and stabilized by reintroducing glucose, ions, and oxygenation into the coronary arteries to enable action potential in both the electrical system and cardiac myocytes. With all resuscitations, an efficiently-working heart is one that has been well recovered, unstrained to injury or ischemia. The same mechanics from Section 1.3 on Coronary Circulation apply: As the pressure superior to the aortic valve increases and is more than pressure inside the left ventricle, the aortic valve shuts. Taking advantage of this cardiac physiomechanical property, the perfusate is redirected towards the left and right coronary ostia, leading to the respective coronary arteries as seen in Figure 1.8.1.

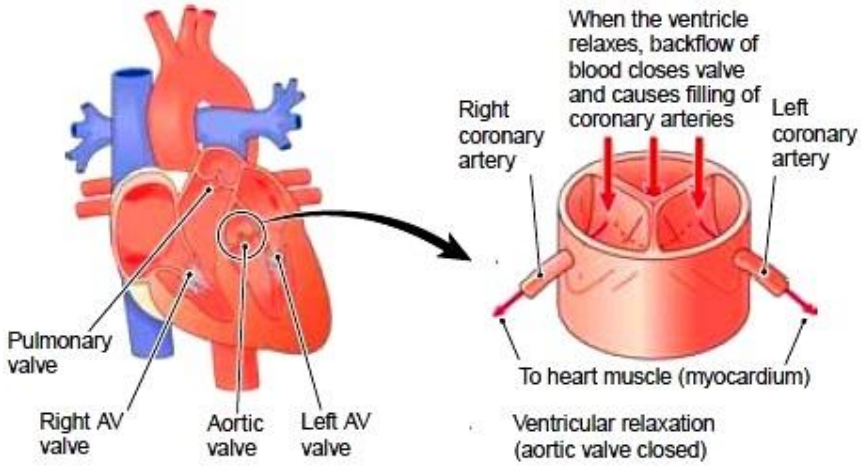


Figure 1.8.1. – Anterior view of coronary ostia located in the aortic root. [36]

After perfusion via arteries and capillaries, deoxygenated perfusate collects into the coronary sinus. Draining into the right atrium, the valve of Thespian (valve of coronary sinus) regulates the draining into the right atrium such as during atrial systole, that ejection of blood doesn't regurgitated back towards the sinus. The Langendorff perfusion echoes what physiologically occurs in the in vivo heart, minus nervous attachment and endocrinal influences. As no internal activity occurs within the chambers, an intraventricular balloon is often inserted to enable an *in-vivo* matching pressurized contractility force [13]. The balloon also enables parametric readings to distinguish global assessment of contractile function and pressure-volume relations.

1.8.1 Constant Flow vs. Constant Pressure in Langendorff System

Constant pressure and constant flow are two methods of delivering the perfusion to the heart using the Langendorff technique. Constant flow is utilized by a flow rate of 8-12 mL/min/g wet tissue weight that is continuously perfused via peristaltic/roller pump [13, 14, 35, 37-44]. The constant flow regulated by the pump speed develops a dependent perfusion

pressure in which can be monitored by a pressure transducer that should range 80-120 mmHg [5]. However, compared to an increase of blood usage as work on the heart increases as it does *in vivo*, oxygen-dependent autoregulation is hampered. Even if the heart contractility performance increases or regional ischemia occurs, the flow rate is undetermined and is stagnant unless physically adjusted. Flow should be introduced directly into the coronary ostia via catheterization.

Whereas constant flow prefers the ostia, coronary pressure advises that the flow is introduced from a cannula into the aortic root. The constant pressure is brought by a constant preload hydrostatic pressure which is influenced by placement and elevation of a compliance chamber. The importance of the chamber allows the physiologic pressure of 80-120 mmHg [5]. Constant flow systems may determine perfusion pressure and vascular resistance. With constant pressure, coronary vascular tone is maintained and autoregulated with respect to change in resistance; constant flow may shear coronary vasculature under increased inotropic conditions [13, 42, 45]. The constant pressure method has its disadvantages; with too high pressure, greater risks of forcing the perfusion undermines edema onto the cell which in turn affects global cardiac tissue [46]. The choice perfusion technique is dependent upon the resistance of coronary circulation. A system that harnesses both methods are optimal though enforces considerable effort. The Langendorff technique provides cardiac resuscitation; however, non-human hearts must be reviewed to serve as an efficient and reliable human model.

1.9 Pig and human comparisons

As described in Section 1.7, human donor hearts are prioritized for transplantation rather than research application and non-preserved human hearts offer very little as it cannot be resuscitated past necrosis. Porcine hearts can serve as an invaluable substitute. The porcine heart is of similar size, anatomy, and function and enlarges the heart candidates for testing such equipment and research, which is detailed in Table 1.3. Porcine hearts follow the classic Valentine shape, which can be explained due to broader apex with course trabeculations. Human hearts are observed to be more trapezoidal. While the porcine LA has two pulmonary veins, human hearts have four. There are differences in appendages as the one superior to the porcine right atrium is more tubular on pig whereas the tubular appendage on a human is above the left atrium [47].

Table 1.3 – Human and Pig physiological comparison [13, 14, 37, 47, 48]

	Adult human	Adult pig
Average weight (kg)	62-71	86
Average heart weight (g)	250-300	358
Resting heart rate (bpm)	60-100	100 - 150
Average temperature (^{0}C)	37	39

Pig coronary vasculature results in decreased total peripheral resistance and higher cardiac output, oncotic pressure than human [49]. Factors such as gravity that influences how the heart is situated in the body as well as pigs' tissue aging at a quicker rate certainly play a role. Studies have shown that porcine blood is hypercoagulable due to its shortened partial thrombin time (PTT) compared to that of humans [50]. In terms of action potential and heart rates, there are certain differences. P waves vary heavily, with the pig

atrial appendage contracting at 6.1-25.3 ms versus a man's appendage having a P wave of 60-100 ms [47].

With respect to the musculature, porcine LV is much thicker compared to that of homo sapiens' while the moderator band, myocardium specifically connecting a part of the AV right bundle towards the papillary muscles, is more superior [47]. It is known as a moderator band as it aids in decreasing risk of ventricular overdistension. Due to the superior location found on the pig compared to human, right ventricular activation is further intensified, influencing contractility [50].

Pig-to-human xenotransplantations have been attempted in the past, resulting into immediate death due to immunologic rejection of the transplant. Rejection can occur on a cellular level as hyperacute rejection. Hyperacute rejection results in inflammation, coagulation, fibrin deposits and platelet degranulation, hemorrhaging, and necrosis of the organ. However, if xenotransplantation succeed, the amount of heart donors would increase, eliminating shortage for patients with end-stage cardiac diseases. Current regenerative medicines are looking into pig embryos and stem cell application for patients with end-stage organ failure.

Table 1.4 – Biochemical reference range values for swine and human serum. [51-54] From The Merck Veterinary Manual, 10th edition. online version. Susan E. Aiello, ed. Copyright © 2015 by Merck & Co., Inc., Whitehouse Station, NJ. All rights reserved. Used with permission. Available at: www.MerckManuals.com. Accessed 3 Nov 2015

Parameter	Swine	Human
Albumin (g/dl)	1.8-3.4	3.5-5.2
Ca^{2+},total (mg/dl)	7.1-11.9	8.6-10.0
Ca^{2+}, ionized (mg/dl)	3.5-5.8	4.4-5.3
Cholesterol (mg/dl)	36-54	<200
Creatinine (mg/dl)	1-2.7	0.65-1.2
Glucose (mg/dl)	85-150	74-106
Phosphate (mg/dl)	7.0-10.9	2.7-4.5
TP (g/dl)	7.4-8.9	6.4-8.3
Na^{+}(mEq/l)	135-150	136-145
K^{+}(mEq/l)	4.4-6.7	3.5-5.1
Cl^{-} (mEq/l)	94-106	98-107
Mg^{2+} (mEq/l)	2.7-3.7	1.3-2.1

Biochemical parameters of the serum for the two mammals fall within the same range, shown in Table 1.4. With the main differences pertaining to Albumin, cholesterol, creatinine, and Magnesium, a simple solution similar to blood plasma can be used to resuscitate both human and pig heart. As the porcine heart is verified to serve as a human model, advantages and disadvantages of the types of accessible hearts must be reviewed.

1.10 Donor Heart Characterization for Isolated Porcine Heart Studies

Extracorporal hearts used for testings can be categorized in three ways: heart-beating donors, non-heart-beating donors, and slaughterhouse. For each category, the length of oxygen deficiency, or ischemia, must be taken into consideration. Warm ischemic time (WIT) is defined as the period from animal exsanguination and ends at cold preservation. Lesser WIT enables higher cardiac recovery and is recommended to be kept well below 5

minutes for minimal risk of ischemic injury [13, 14]. There are dispute about the start of warm ischemia starting from organ excision rather than exsanguination [55]. As there is WIT, there is cold ischemic time, though as the heart has cooled and metabolism is preserved, it is not as detrimental. Advantages and disadvantages will be discussed for each category of donor hearts.

Heart-beating donors are unconscious mammalian laboratory animals under anesthetic such as pentobarbital in which thoracotomy is performed and the heart is excised. Between animal research regulation and constant monitoring, usage of laboratory donors has high cost and heavy equipment usage. Long-term cases of pentabarbitol include impaired myocardial contractility. The advantages allow greater benefits than its shortcomings as it allows the facility with controlled experimentation. As the heart may take 3-5 minutes from excision to experiment reperfusion, its WIT is dramatically reduced. However, the institution did not have access to laboratory heart-beating donors.

Contrasting to beating animal donors, there are nonbeating heart donors. Nonbeating donors are defined as brain-dead cadavers with 25 minutes minimum WIT, having already undergone cardiac arrest. Advantages are that cadavers are easily accessible for other organ harvests and are a cheap alternative. However, necrosis and myocardial damage have already taken place on the tissue, allowing for poor resuscitation for the heart itself.

Slaughterhouse-harvested hearts are the third possible candidates, though the method is uncontrolled for the experiment. Under the United States Department of Agriculture (USDA) inspection for all lawfully-abiding livestock slaughter at abattoirs must comply

with food safety and humane handling laws. Contrary to anesthesia administration which is unlawful, brain death is commenced by either stunning or concussion. Though many slaughterhouses administer stunning and shocks, mechanical stunning determines brain-death but allows systemic circulation to occur, and cardiac function is not compromised. An example of mechanical stunning is using a captive bolt pistol in which the skull is penetrated, cerebrum destroyed but brain stem intact for regulatory functions uninterrupted. The carotid artery is severed and the swine is ensanguined. Pericardium is inspected immediately after excision by a USDA officer for diseases. Advantages for slaughterhouse hearts are that the process is cost-effective. Disadvantages include uncontrolled settings as there is no access to the swine prior to USDA treatment. Also, as anesthetization is not allowed, pig stress levels are increased and thus stress hormones and neurotransmitters of certain catecholamine are increased. Compared to laboratory swine, slaughterhouse swine will have a faster HR. WIT will be greater than laboratory porcine hearts. Ischemia overall will lengthen by slaughterhouse location to the lab. However, WIT can be reduced by procedure, in which acting similarly to laboratory settings. Based on the choices, for DU's in vivo resuscitation, slaughterhouse pigs were used.

Chapter 2: Preliminary Studies

The resuscitative technique produced by Oskar Langendorff in 1895 allows for *in vitro* hearts to continue beating after taken out of the animal's body by reperfusion of an oxygenated warm nutrient-rich solution directed into the coronary arteries. Numerous studies have widely used this technique in observance for cardiac functions, diseases, pharmaceutical applications, myocardium necrosis, and other broad spectrum of the biology, chemistry, and medicine disciplines [12-14, 34, 39]. Biomedical equipment design and development has also progressed due to the cost-efficiency of the Langendorff method and its easy access [13, 14, 29, 30, 35, 37, 38, 40-44, 46, 48, 56-76]. In this chapter, preliminary studies were reviewed, which have constructed the foundations for the research in which assimilated the Langendorff procedure for this thesis. The following studies will discuss the laboratory animal used for heart reanimation, methods, and results. Most investigated studies had *in vitro* hearts resuscitated for >1 hour prior to working-heart conversion for approximately 4 hours [13, 14, 29, 35, 37-44, 48, 57, 58, 68, 70, 72, 73, 75-84].

2.1 The Isolated Blood and Perfusion Fluid Perfused Heart

Sutherland and Hearse 2000 [35] developed a guide of the different methods to prepare isolated perfused hearts. The guide details on what types of studies can be tested using the Langendorff heart and discusses optimal usages for the following: mammalian hearts, temperatures, measurements and parameters, apparatus set-up, perfusion duration, buffer

composition, oxygen delivery. Options for the excision stage were explored: common advantages for small mammalian hearts such as rats, guinea pigs, mice, hamsters, and rabbits are that handling is easier and perfusion volume is smaller. Murine hearts are most frequently used but display the disadvantage of using a small heart by having small surface area and volume. Recording pressure measurements can be difficult with the small organ. Action potentials are indefinitely smaller than that compared to a large mammal, leading to high heart rates (HR). For example, an *in vitro* rat HR is 250-320 bpm [35]. The advantage for large mammalian hearts is that results follow similarly to human physiologic rates as anatomy is similar. However, there is an increase in variables to monitor, resulting in high cost, more equipment, and a larger volume of perfusion buffer used. With larger mammalian hearts, pressure recordings are easier to trace and differentiate from noise though some are heavily affected and functionally depressed due to anesthesia compared to others. The guide reviews types of inhalation agents, dosage, and application for certain animals. Different approaches for heart excision is discussed and brought to light and careful consideration of correct heparin dosage should be applied for intravenous-applied anesthetized animals.

Reanimation stage discusses asanguinous and blood-perfused buffers and respective advantages, apparatus set-up, procedure. This study suggests that pure crystalloid perfusate be directed at 8-12 mL/min/g [35] of wet tissue weight whereas blood flow should be 3-4 mL/min/g tissue weight. Saline solutions may replace blood with regards to similar ionic levels. Crystalloid buffers have low oxygen carrying capacity unlike hemoglobin in red blood cells. Oxygen rate in saline has partial pressure of O_2 (pO_2) of

≥500mmHg [35] compared to the blood alternative of 80-120mmHg [35] and is compensated in coronary flow rate. Though most studies choose one perfusion method over the other of constant pressure vs. constant flow, a convertible set-up between the two modes is most beneficial; however, a convertible set-up is not always feasible. Types of different aortic root cannula are covered, transitioning over to the set-up of the Langendorff equipment including water-jacketed reservoir with sintered glass oxygenator in which gas is distributed evenly to lower risk of air emboli within the coronary vasculature. Sutherland and Hearse recommend that a recirculated perfusate uses a 5 μm porosity filter. Pacing of hearts is left to the experimenter, with full regards to the knowledge that autorhythmicity is aided and augmented. If pacemakers are to be used, a bipolar stimulator should be chosen to lower the risk of electrolysis from the perfusion fluid and that position of the lead should be on the ventricle and while the reference electrode is on the cannula within the aorta. However, because the SA node is perfused by vessels outside of coronary perfusion, it is a necessary element to be taken.

Optimal cardiac function requires an inflexible 37-37.5°C myocardial temperature. Nonetheless, hypothermia is preferred to hyperthermia as temperatures above 39°C will cause tissue injury. With the heart in isolated conditions, contractility deterioration occurs at 5-10%/hour though is influenced by preservation via static storage or continuous perfusion. When describing the best perfusion fluid, Sutherland and Hearse explain that the $CaCl^2$ amount of 2.5 mMol is theoretically wrong despite matching physiological levels. In the blood, Ca^{2+} is bound to protein; plasma carries the ionized Ca^{2+} which is half the amount of 2.5 mMol and thus in the past, twice the amount of carbon chloride

has always been used when using non-protein related buffers. With these findings brought to light, ionized calcium concentrations are now brought to 1.2 – 2 mMol [35] in most isolated heart studies. Calcium should be last to be added to the buffer as it may precipitate, forming bonds with phosphate, heightening risk for coronary occlusions. Ways these occlusions can be disrupted is by adding carbogen before calcium addition. High addition of glucose at a near diabetic rate of 11 mMol [35] is utilized as to compensate for the lack of fatty acids. Fatty acids are first priority when harnessed for energy consumption *in vivo* though insulin may also be added. Though physiologically sought as the predominant energy consumption, fatty acids should not be used in crystalloid buffers as dissolving is limited and adds to frothing and foam creation when certain proteins undergo direct oxygenation. Whether to add non-dissolvable oncotic agents such as albumin or mannitol lowering the risk of edema and increase in global contractile function is at the experimenter's discretion.

The notion of bacterial or particles contamination should not be eliminated as it could develop and culture in the equipment itself if not properly sterilized in an autoclave. The risk can be eliminated by making fresh buffer the day of the experiment and not storing buffers with glucose or calcium titrated in an aqueous solution past 48 hours without vacuum filtration. It is not necessary to use antibiotics in the solution. Such equipment care will enable apparatus usage for multiple years. The last section of the report goes into detail of optimal animal anesthetization. Anticoagulants must always be used with blood [35].

2.2 The Visible Heart Lab

The focus on the following studies narrows specifically on large mammalian hearts. The collaborative effort between University of Minnesota and Medtronic, the Visible Heart Lab, has designed one of the most highly developed and elaborate isolated heart platforms on a massive research and educational scale. In Chinchoy et al. 2000 [37] , beating swine donors were used. Once anesthetized and heparinized (10,000-30,000 U), a sternotomy was performed. The cardioprotective extracellular cold buffer St. Thomas Hospital solution #2 (Plegisol) with 25 mg adenosine mechanically arrested the heart via retrograde flow which was applied into the aorta with topical saline and ice induced. After excision, the hearts were placed in an ice and saline slurry mixture and transported for 5 minutes and attached to the Langendorff set-up for 40 minutes with continuous perfusion of a crystalloid buffer. Length of time between cardioplegic applications to reperfusion was averaged 74 minutes, with all hearts resuscitated and converted to working-heart mode. A clear oxygenated crystalloid 37°C solution of modified Krebs-Hensleit buffer (KHB) was utilized instead of blood for camera probes and visual activity of the flow and physical intracardiac function. Additives to the KHB was chelating agent EDTA (0.32 mMol/L), insulin (10 U/L), sodium pyruvate to serve as fatty-acids for energy utilization (2.27 mMol/L), and mannitol (16 mM/L) to decrease swelling as perfusion pressure was set to 65 mmHg. The perfusion was requisite for spontaneous conduction and contraction. This specific preparation led to4 hours of global contraction as well as hemodynamic measurements near *in vivo* values. Pacing and defibrillator was as needed as the hearts beat spontaneously until autorhythmicity was stabilized. In the

Visible Heart Lab studies, dobutamine was used to manipulate hemodynamic characteristics to reach *in vivo* conditions, though lidocaine and epinephrine can be used as a substitute. Though edema progressed throughout the *in vitro* testing, cardiac performance was near stabilized even at 4 hours [37].

Continuing investigations in the Visible Heart Lab, Alexander Hill and colleagues in 2005[48] compared *in vitro* human hearts to the previous *in vitro* porcine hearts from 2000[37]. Hearts obtained from human cadavers were not all diseased-free. Again, Langendorff perfusion was situated first to enable cardiac resuscitation and autorhymicity stabilization by constant flow before converting to working heart mode. Plegisol was used as cardioplegia, directed into the aortic root cannula above 100 mmHg with the heart topically cooled by ice. Storage solutions were cold saline or UW solution during transport, with the container placed in ice. At the lab, hearts were placed in ice slurry of modified KHB as the vessels were cannulated. 7L of 37°C modified KHB was used as reperfusate, with 4L replaced throughout the reperfusion for clear quality of transparency for visual activity, disposing catabolites. Buffer was shown to be warmed at location 17 in Figure 2.1.1. Water jackets were maintained at 40°C, keeping perfusion at 37°C. Despite the non-usage of blood, a filter was used to separate large particles from the circulating KHB (5 and 10 in Figure 2.2.1). Defibrillation was used as needed for stabilization at 34J with the electrode placed on the exterior left ventricle epicardium. It was recommended that inotropic support be used before each defibrillating shock administered, such as epinephrine or dobutamine at the respected amount of 0.5 mg/mL

and 0.1 mg/mL. Systolic pressures were applied at 70-90 mmHg throughout the 4 hours of testing.

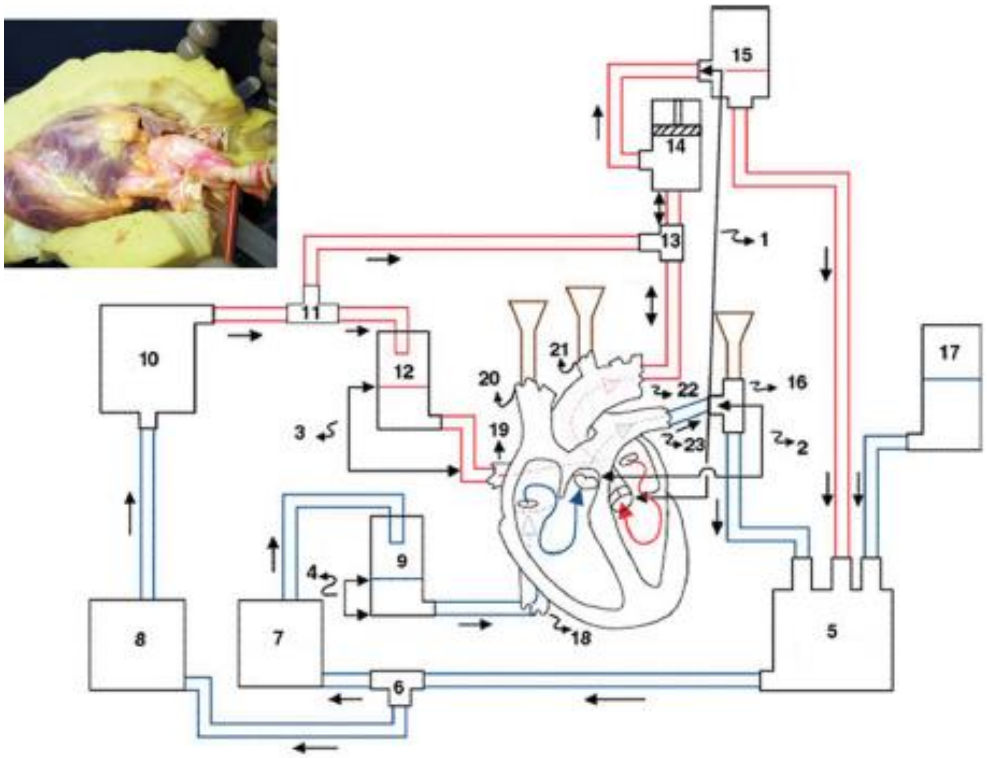


Figure 2.2.1 – Langendorff schematic of the Visible Heart Lab apparatus pertaining human hearts [48]. Reprinted with permission by Elsevier – (license number 3677490412112).

Locations 1-4 in Figure 2.1.1 of the Visible Heart apparatus are preload and afterload columns to enforce systemic and pulmonary circulation. Centrifugal pumps are located in 7 and 8. Cannulation was at 18, 19, 22, and 23 of the pulmonary vessels, vena cava, and aorta while camera ports are located at 20 and 21. The divider at location 11 was what converts the upper Langendorff mode to the working-heart mode [48].

2.3 Eindhoven University of Technology

Eindhoven University of Technology utilized slaughterhouse pig hearts instead of an animal-donor lab in 2005 [13, 14]. Hearts obtained from a slaughterhouse led to less control over the specific factors contributing towards the pigs' death such as WIT. Pigs obtained were of weights 90-100 kg and stunned by electroshock while blood was collected as perfusate mixed with 2500U heparin. Porcine hearts were excised and flushed by 1L modified Plegisol applied in retrograde fashion via aortic root. Warm and cold ischemic time were 5 minutes and 70-90 minutes, respectively; for optimal preservation, WIT was kept within or under five minutes. Hearts were transported in 1L STHS at 4°C immersed within an ice slurry transport bag. KHB was used as warm wash before reanimation at the lab with the addition of mannitol in the 37°C blood-KHB mixed reperfusion buffer which increased colloid-osmotic pressure. 3L of the recirculating blood reperfusion (25% hematocrit) was stored in a reservoir and transported by a centrifugal pump, passing through a membrane oxygenator that enabled radial blood flow [13]. Along with carbogen induced into the perfusate (95% oxygen, 5% carbon dioxide), the Langendorff circulation system utilized a filter to remove white blood cells before oxygenation as to not damage the cardiac muscle, as seen in Figure 2.3.1. Perfusion rate was set at a constant flow of 1 mL/min/g tissue [35]. Defibrillator was as needed, with shocks administered at 50, 70, or 100 J; once stabilized with pacemaker application of 100-120 bpm, Langendorff hearts were assessed at 1, 2, 3, and 4 hours before transitioning to working ejecting mode. Pressure measurements were induced by pressure transducers connected to a fluid-filled latex balloon in the left atrium, as seen in the schematic. By hour 4, developed pressures

and heart rates deteriorated. One of the pilot studies for the thesis had KHB reperfusion and the heart was continuously paced for an hour before undergoing cardiac arrest [13].

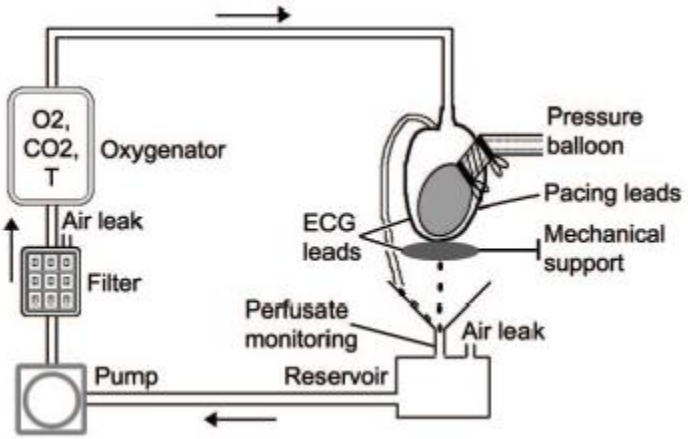


Figure 2.3.1 – HemoLab 2005 Langendorff schematic. [13]

Schampaert et al. also of HemoLab, Eindhoven University of Technology developed a study in 2013 using slaughterhouse pigs [43]. Heart weights averaged 472± 51 g. After electrocution, a sternotomy was performed and the heart was excised where it was then rapidly cooled by 1L Plegisol with the addition of 5000U heparin. Heparin application in cardioplegia was the first study found in literature review that did not have heparin added to the animal's circulation itself. As before, WIT was within 5 minutes while 1L Plegisol was administrated hourly. Oxygenated 37ºC blood was perfused [43]. Beating pig hearts mimicked *in vivo* flow and pressure. HR ranged 70-120 bpm, averaged 70 mmHg for coronary perfusion pressure and 0.7L/min coronary flow. As the aim of the study was autoregulation with regards to brief occlusion, length of reanimation time was not focused on.

2.4 Additional studies

Grosse-Siestrup et al. 2002 study [38] highlighted the usage and non-sacrificial advantages of using slaughtered pigs. Out of the total 492 organs harvested, 191 were hearts. In addition to the organs, 1500L of autologous donor blood was collected and used for perfusion, with pigs slaughtered consecutively. Hearts were excised and cannulated within 6-7 minutes WIT prior to remaining organ harvest. Placed on ice, 500 mL of cardioplegia was flushed prior to 500 mL of cold modified KHB flush. Modified KHB contained insulin and buadione monoxime additive were perfused via aortic retrograde infusion. Hearts were stored at 4^0C during transportation. Altogether, standard procedures for hearts took 12 minutes, with heart excision prioritized compared to other organs harvested for the same lab. Autologous blood contained sodium citrate and 10,000U/L heparin. Unfortunately, results were not annotated in the study as the emphasis was on abattoir porcine organs to benefit and improve the field of transplantation techniques and research [38].

Petrucci Jr et al. 2003[85] developed a study in which explanted a porcine heart was perfused by a parabiotic circulation using another anesthetized swine's blood and body as seen in Figure 2.4.1. Mean donor weight was 22 kg. The animal donor that supplied the isolated heart was heparinized (500 IU/kg) as the heart was excised. WIT was approximately 14 minutes. The constant perfusion set-up was similar to the University of Minnesota apparatus in which blood was collected in a reservoir that was rewarmed at 37°C, oxygenated, and pumped into the coronary arteries. The support animal was anesthetized by IV injection of fentanyl (12.5 µg/kg), pentobarbital (15 mg/kg),

pancuronium bromide (8 mg) as well as heparin. Jugular and carotid vasculature were cannulated. Onset of Vfib led to defibrillating shocks at 5 J. At 30 minutes, hearts were induced with regional ischemia and by global ischemia at 90 minutes along with reperfusion. Atrial pacemakers were used at 160 bpm for stimulation. Hematocrit of the perfused blood averaged 31.30 ± 3.40% while oxygen pressure was consistent at ≤ 300 mmHg. Systolic pressure was 131.70 ± 6.4 mmHg for the first 60 minutes and decreased to 100.70 ± 9.62 mmHg.

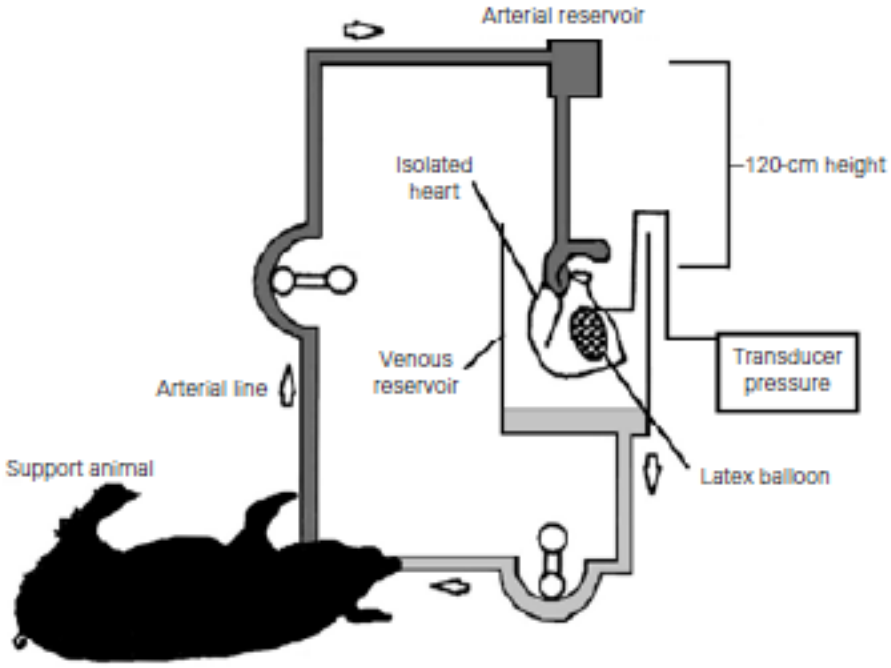


Figure 2.4.1 – Perfusion system of Petrucci Jr's 2003 study in a parabiotic model. Heart explanted and blood circulating through the support animal. [85]

This study sheds light on the Gregg phenomenon or the Anrep effect—in which the rate of change in coronary perfusion results in a change in myocardial contractility and oxygen reuptake consumption [86]. With the parabolic circulation, fatty acids increase in the blood, following results close to clinical settings, increasing metabolism [85].

M.A. Schechter et al. 2014[80] created a similar apparatus to previous studies that included a centrifugal pump, bubble trap, and heating apparatus. Red blood cells were reconstituted in saline resulting in 20-25% hematocrit. An advantage to this study were calcium level details: Ca^{2+} was initially kept low at 0.3-0.5 mM/L prior to heart mounting via aortic cannulation with slow trickle flow to rid the system of air. The system steadily had reperfusion warmed to 37°C while the heart was attached and calcium added to the reperfusion. By stabilization and global contractile function, ionic Ca^{2+} was ≥0.8 mM/L [80]. Aortic pressure ranged 40-42 mmHg, with fluctuations resulting from coronary resistance. The preload chamber seen in Figure 2.4.2 was used in working-heart system and not Langendorff. The centrifugal pump allowed for control over pressure though transition between constant flow and pressure were employed. The heart was resuscitated for >20 minutes [80].

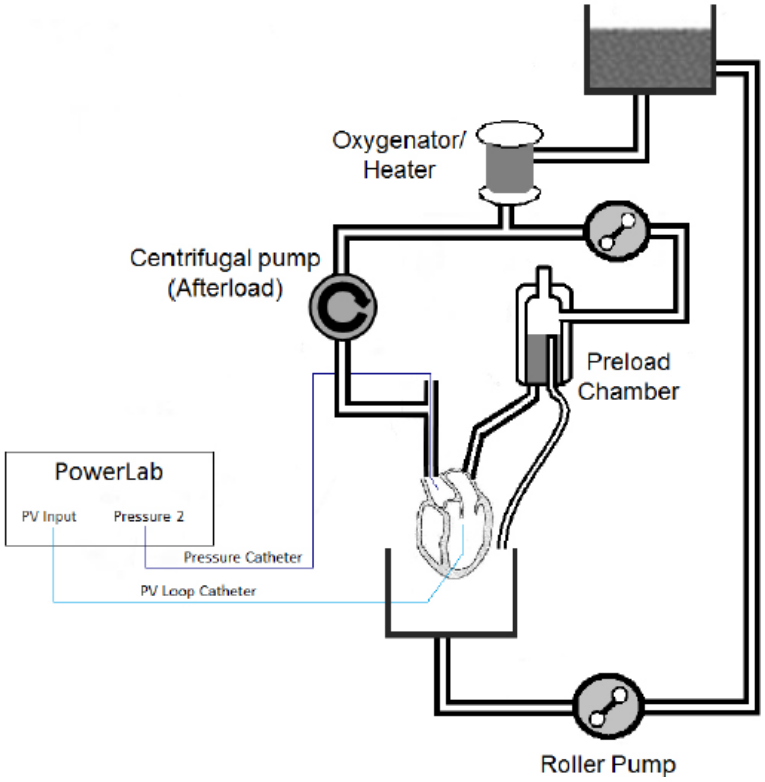


Figure 2.4.2 – Apparatus schematic of M.A. Schechter 2014 study with red blood cell reconstituted by saline [80]. Reprint under Creative Commons Attribution-NonCommercial License.

Though University of Denver does not have immediate access to lab donor animals with monitored aesthesia and the equipment for excisions, the crystalloid salt reperfusion was a beneficial finding from the University of Minnesota studies due to its transparency. Key features taken from HemoLab [13, 14] was that slaughterhouse pigs can be utilized as well as anticoagulant could be administered into the cardioplegia instead of the donor animal's circulation. Both Visible Heart Lab and HemoLab studies laid foundation to the research of this thesis, with specific investigation of additional literature [3, 5, 6, 8, 11-14, 27-30, 35, 37-44, 46-48, 50, 55-68, 70-78, 81-85, 87-109]. However, not all details could be found in literature due to proprietary license and thus had to be investigated to design and develop an

efficient Langendorff apparatus that allowed stabilized sinus rhythm for ≥1 hour and global cardiac contractile function.

Chapter 3: Materials and Methods

Over the course of the two year research period, modifications provided a near-consistent protocol to improve and increase global cardiac activity and preservation of porcine heart obtained from a slaughterhouse. The protocol entails the details of myocardial preservation, transportation, and reperfusion. Materials are explored, signified by cardioplegia (Section 3.1), Pharmaceutical antithrombotic drugs (Section 3.2), and Krebs-Henseleit buffer (Section 3.3). Based on types of solutions and agents available, Section 3.4 discusses the procedure detailed in part by 3 stages: Slaughterhouse harvest, preservation, and resuscitation at the laboratory.

3.1 Cardioplegia

The application of cardioplegia is to induce rapid cessation of electrical activity, mechanical arrest, and paralysis of the heart [12]. Cardioplegia is a generally hyperkalemic crystalloid solution above the physiologic level of 4 mMol K^+, which enables depolarization. As referred to in Section 1.5, resting membrane potential for the myocytes is -90 mV via fast response action potential. Due to depolarization and Na^+ influx, one last contraction occurs. However, K^+ efflux ceases as its concentration gradient is higher extracellularly and no longer diffuses out. Sarcoplasmic reticulum Ca^{2+} reuptake occurs in the cell. Instead of the physiologic extracellular 4 mMol/L, cardioplegia involves 16 - 125 mMol/L of potassium [89]. As membrane voltage threshold has been crossed at -60

mV, cardiac fibers are prevented to stimulate. As the resting membrane potential nears -50 mV, Na^+ channels inactivate due to cellular diastole. Global diastole prevents ATP utilization. It is pertinent that the solution is customized specifically to the organ being preserved. Despite ischemia, blood-based or crystalloid-based cardioplegic buffer application provides cellular protection. Optimal preservation utilizes cardioplegia along with hypothermia to restore as much as 80% ±17% cardiac function [30]. The cardioplegic solution washes out metabolic waste (catabolites) such as free hydrogen ions, lactate, and any byproducts of anaerobic activity.

Cardioplegia is categorized between two classes: extracellular and intracellular buffers, as charted in Table 3.1. Extracellular hyperkalemic solutions mimic the extracellular physiological fluid such as blood and plasma and thus are high in sodium whereas intracellular solutions are similar to the ionic concentration of a cell and high in potassium [12]. The *extracellular* concept however is relative as K^+ concentration remains high with respect to average physiologic K^+ concentrations.

Table 3.1 – Commonly used intracellular and extracellular crystalloid solutions for organ preservation [12, 14]

Extracellular (high Na^+, high Ca^{2+}, low K^+)	Intracellular (low Na^+, low Ca^{2+}, high K^+)
Plegisol (St. Thomas Hospital #2)	University of Wisconsin solution
Krebs-Henseleit buffer (KHB)	Bretschneider's solution
Celsior	Euro-Collins
Custodiol (HTK) (low Na^+-low K^+)	Roe
Tyrode	Stanford
Ringer's solution	Collins
UW (modified)	Collins-Sachs
NIH	EuroCollins

Disadvantages in extracellular solutions include inducing edema [6]. Intracellular fluids enable rapid cardiac arrest and include lower risks of edema, preventing ionic toxicity during hypothermia. Based on these benefits, many studies favor intracellular preservation solution compared to extracellular[29, 61, 77, 79, 84, 90, 108]. However, intracellular fluids can initiate endothelial damage [110].

The solution may be applied in antegrade or retrograde perfusion. Antegrade flow follows *in vivo* circulation while retrograde is done against the natural order of flow. Flow from the coronary ostia to the coronary arteries or blood flow from the aorta to the systemic circulation is considered antegrade. An example of retrograde is to introduce flow from the coronary sinus to the cardiac veins. It should be taken into consideration that the flow direction is with respect to the vessel; retrograde flow can be induced towards the aortic root, but antegrade in the coronary arteries. Some studies perform a retrograde perfusion, using catheters past the right atrium and into the coronary sinus. Though long debated, antegrade flow is preferred though increased washout is supplied by administering both antegrade and retrograde flow. The additional flow secures washout of metabolites and contact with cardioplegia. To not entail tissue damage due to high pressure, perfusion should stay set at 120 – 140 mmHg for all solutions, whether for preservation, transplantation, or reperfusion, as it physiologically occurs in a living *in vivo* body [12, 66, 70].

Static storage is a one-time application of the solution while continuous cardioplegia consistently circulates through coronary vasculature. The classic cold ischemic storage preserves hearts for 4-6 hours [41]. Compared to continuous flow, static storage is easily

manageable with no operational equipment needed. Dependent on the storage for heart preservation, continuous cold perfusion enables greater recovery for the heart rather than static ischemic storage [111]. Jahania et al. [12] promotes continuous cold perfusion in which 93% Left Ventricular End-Diastolic Pressure (LVEDP) was recovered in rabbit hearts after 24 hours of continuous perfusion using modified UW as compared to 35% LVEDP with static storage. Several literatures have compared not just the effects of static versus continuous application but also warm and cold-blood cardioplegia against crystalloid cardioplegia [11, 29, 61, 84, 111, 112]. Continuous warm-blood cardioplegia provides higher recovery of left ventricular global contractile function and higher sarcoplasmic reticulum reuptake of intracellular Ca^{2+}compared to its static cold counterpart [111]. The reuptake of intracellular Ca^{2+} ceases edema. Other reports state that non-depolarizing cardioplegia provide optimal recovery of ventricular function such as cardioplegic HTK compared to Celsior, both extracellular solutions [78]. The method for non-depolarizing cardioplegia is attributed by ceasing Na^{+} influx as well as removing extracellular Ca^{2+} to decrease contractile function [113]. The main cardioplegia reviewed was the extracellular St. Thomas Hospital #2 solution and intracellular University of Wisconsin solution.

3.1.1 St. Thomas Hospital Solution #2 (Plegisol™)

In this thesis, St. Thomas Hospital solution #2 (STHS) is referred by the specific brand-name, Plegisol [89]. Manufactured by Hospira Inc., the hyperkalemic extracellular solution mechanically arrests the heart, with Magnesium addition so that calcium overload risk are decreased, as seen in Table 3.2[89] . Similar to saline ionic concentration, Plegisol is cost-effective and highly used in clinical and laboratory settings [29, 44, 60, 61, 77,

[94]. However, sterilized pH is 3.8 and must be adjusted to pH 7.4 modified by 8.4% sodium bicarbonate in 10 mL aqueous solution.

Table 3.2 – Composition of St. Thomas Hospital Solution #2 (Plegisol) [89]

Active Ingredient/Active Moiety (g/L)	
Ingredient Name	**Strength (g/L)**
Potassium Chloride (Potassium Cation and Chloride ion)	1.193
Sodium Chloride (Sodium cation and chloride ion)	6.43
Calcium Chloride (Calcium cation and Chloride ion)	0.176
Magnesium Chloride (Magnesium cation and Chloride ion)	3.253
Inactive Ingredients	
Ingredient Name	**Strength**
Hydrochloric Acid	
Sodium Hydroxide	
Water	q.s.

3.1.2 University of Wisconsin (Viaspan) solution

Manufactured by Organ Recovery Systems and also known as SPS-1 (Static Preservation Solution) or Belzer UW solution, the solution was developed by Drs. F.O. Belzer and J. Southard at the eponymous university [114, 115]. UW solution is not suited for continuous coronary perfusion due to its viscosity enabling vasoconstrictuion and endothelial damage [110]. However, it is most effective for static storage, leading to 80-85% recovery based on several studies [99]. High pressure flow of 120mmHg is recommended to compensate for vasoconstriction [110]. Mohara et al 2002 had dog hearts transplanted with 103% cardiac output for 120 mmHg coronary pressure and 58% at 60 mmHg [110].

Table 3.3 – Composition of Static Preservation Solution (SPS-1) UW Solution [99]

SPS-1 Static Preservation Solution (UW Solution) Composition (g/L)	
Pentafraction	50
Lactobionic Acid (as Lactone)	35.83
Potassium Phosphate monobasic	3.4
Magnesium Sulfate heptahydrate	1.23
Raffinose pentahydrate	17.83
Adenosine	1.34
Allopurinol	0.136
Total Glutathione	0.922
Potassium Hydroxide	5.61
Sodium Hydroxide/Hydrochloric Acid	Adjust to pH 7.4
Water for Injection	q.s

Based on Table 3.3, varied additives enable cardiac arrest, protection, and slow metabolism. Lactobionic acid and pentafraction offer osmotic support, and decreases edema. Potassium phosphate monobasic serves as the potassium source. While Magnesium sulfate heptahydrate is a desiccant, raffinose pentahydrate enables the hypertonicity for cell desiccation to occur before cooling. Adenosine reduces ATP catabolic rate, inhibits platelet aggregation and inflammatory cells, decreasing superoxides, and increases contractility [99], recovering developed pressure [13, 42]. Allopurinol lowers uric acid in blood plasma. Glutathione reduces oxidative agents by oxidizing free radicals and is highly recommended in most cardioplegia. Hydroxethyl starch prevents shock based on blood loss. UW supplies have high costs especially from Organ-Recovery with a case of ten 1L bags. Other alternatives to decrease costs were 1-2L samples from Bridge to Life Solutions Ltd (Columbia, SC) and CoStorSol™ sold under Preservation Solutions, Inc (Elkhorn, WI). Table 3.4 highlights and compares the two cardioplegia discussed by ionic values as well as an extracellular crystalloid solution that will be discussed in Section 3.3.

Table 3.4 – Ionic concentration comparisons of STHS, UW, and KHB [89, 99]

	In Vivo Parameters (mMol)		Plegisol (Extracellular) (mMol)	UW (Intracellular) (mMol)	KHB (Extracellular) (mMol)
	Intracellular	Extracellular			
Na^+	5-15	150	120	30	142.5
K^+	150	5	16	120	4.4
Ca^{2+}	10^{-4}	2.5	1.2	-	1.76
Mg^{2+}	-	-	16	5	1.2
H_2PO_4	-	-	-	25	1.2
SO_4	-	-	-	5	1.2
HCO_3	-	-	10	-	25
pH	-	-	7.8	7.4	7.4

Cardioplegic solutions are supplied in 1L bags, with up to the full liter administrated to arrest the heart. Glutathione, insulin, and dexamethasone—an inflammatory inhibitor—are highly recommended as additives in whichever cardioplegia is used. Similarly, the extracellular HTK solution is similar to UW for short-term usage only [78]. Intracellular solutions used for long term is Celsior, which is essentially modified UW solution. While UW sells on average at $230-$438 per liter, HTK (Custodiol) sells at $124-$211 per liter. Due to HTK's viscosity, twice of the amount is flushed to compensate flow rate. Plegisol is the most cost-efficient and averages $90/L.

Todo et al. [108] compared UW to varied extracellular solutions to preserve dog livers. 80% of both subjects lived for more than 7 days. Comparisons between UW and other cardioplegia such as EuroCollins, Standford, and HTK solution shows that highest content of ATP storage and best preservation of myocyte was due to UW solutions, in Schmidt et al. [116]. A study by Wicomb et al. [84] used rabbit hearts and mechanically arrested the heart via coronary perfusion with 15-25 mL either Plegisol or UW. Hearts were topically cooled in iced saline and immediately tested. Cardiac output (mL/g heart

wt/min) for Plegisol was 20.5 and UW at 34.7. When cardioplegia was applied for 4 hours of cardioplegia, cardiac output was the following: Plegisol at 17.4, UW at 25.2 [84] .

A study by Lareau et al. investigated % recovery by developed force between 4°C and 12°C at 24 hours. Plegisol had higher recovery at both temperatures, with 110± 17% recovery at 12°C, UW at 32± 10% [117]. UW and STHS in prolonged ischemic comparison of 12 and 24 hours showed that UW was ultimately the better preservation solution as right ventricular systolic pressure was 125.6% versus STHS being 65.5%. Post-ischemic recovery of left ventricular rate of pressure increased as well as myocardial preservation had also improved due to UW [79]. STHS had better myocardial recovery at tepid temperature than UW solution [112].

UW solution offers more than the simple electrolytes that Plegisol does such as glutathione and allopurinol to limit oxygen radical injuries and decrease reperfusion injury; hydroxyethyl starch, lactobionate, raffinose limit edema. Adenosine has cardioprotective effects in aiding phosphorylation potential converting stored ADP to ATP [12]. Even though they're shown for long-term effects, the short-term effect from the link above showed higher cardiac output with UW. UW solution however impairs EDHF (endothelium derived-hyperpolarizing factor) function in porcine or human coronary arteries in which EDHF hyperpolarizes endothelium cells to vasodilate; As hyperkalemic cardioplegia initiates depolarization, hyperpolarizing cardioplegia with K+ channel openers are suggested. Mg^{2+} preserves EDHF relaxation and restores hyperkalemia impairment [87]. Though most literature were based on long-term storage of ≥4 hours, the procedure developed in the thesis had 1-2 hour storage. When choosing cardioplegia,

long-term studies didn't apply and ultimately was chosen on cost-effectiveness and efficiency. Static Plegisol was employed as cardioplegia, with UW solution used for transport towards the first half of tests and Plegisol used for the latter half of tests.

3.2 Heparin and Streptokinase

To effectively wash out blood, heparin is used and recommended in studies with any blood usage, either in the animal's circulation or in topical wash to prevent coagulation [13, 37, 39, 42-44, 46, 57, 75, 118-120]. Many studies use only 5,000 U/L heparin in either cardioplegia or blood-based reperfusion [13, 43, 64, 75]. Other studies use 10,000-25,000 U/L or 300U/kg of tissue weight for isolated hearts of several species [30, 37, 41, 42, 72, 73, 84, 94]. However, as heparin causes for preventative measure, the drug does not affect formed thrombi. Instead of increasing heparin dosage, thrombolytic agents were looked at, specifically Streptokinase.

Streptokinase (SK) has a high affinity to human plasmin and is immunogenic [109]. Cost-effective and efficient, the medicine is the only thrombolytic drug listed in the World Health Organization List of Essential Medicines [104]. The drug is a white, lyophilized sterile powder. As powder, the drug can be stored below 25^0C and at -20^0C for prolonged storage time, ranging from weeks to months. Once induced into a serum and reconstituted by saline or dextrose, it can be stored at 4-10^{o}C for a maximum of 8 hours only [103]. At 37^0C at a pH of 7.5, 1U of streptokinase can deteriorate a standard clot.

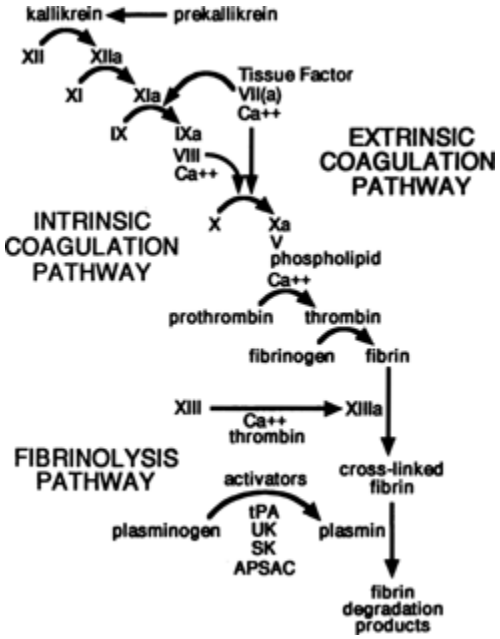


Figure 3.2.1 – Coagulation pathways of plasma factors. [121]

These thrombolytic agents are fixed protocols for treatment of acute myocardial infarctions (AMI) and embolisms and are activated by tissue plasminogen activator (tPA). Plasmin is an active enzyme that when active in the blood, degrades fibrin, a protein used in blood clotting. The proenzyme preceding the active plasmin is the inactive plasminogen, which activates in the presence of tPA, or naturally in the body by kallikrein and factor XII as diagramed in Figure 3.2.1. Plasminogen cleaving occurs at the Arg-Val amine bond, forming plasmin that breaks fibrin from thrombin.

Enzymes optimally perform at regular warm-body temperature of 37.5°C and exponentially decrease during hypothermia. Similar effects occur for pH, with optimal function at pH 7-8, dependent on the particular enzymes and proteins, as seen in Figure 3.2.2.

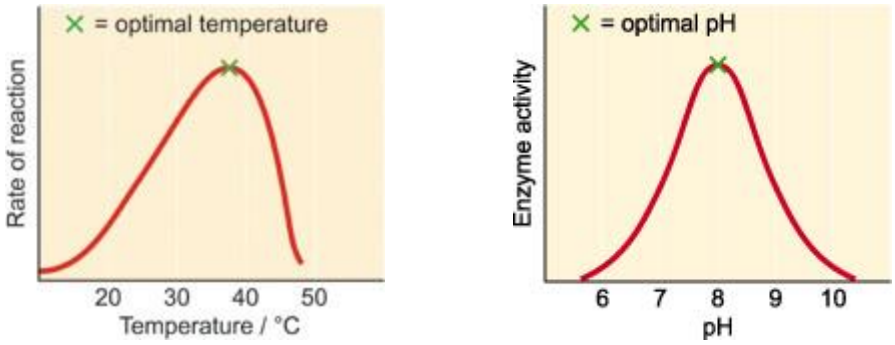


Figure 3.2.2 – Temperature (left) & pH (right) influences enzymatic activity. [122]

Pharmaceutical agents in 4^0C solution have a minimal reaction as that is regarded as storage temperature. Although used in several studies and is recommended for experiments with blood usage, the anticoagulant Heparin was not used in our studies. Increasing cardioplegic temperature from 4^0C to 20^0C enables higher reaction rates of SK with clots dissolved 2 minutes after administration. Though optimal temperature of cardioplegia has 97% recovery at 4-10°C, cardioplegia at 20^0C was at 88% recovery [112].

Table 3.5 – Streptokinase dosage for Intracoronary(I.C.) and Intravenous (I.V.) in clinical settings [6]

Route	Dosage/Duration
Intravenous infusion	1,500,000 IU/60 min
Intracoronary infusion	20,000 IU (bolus) 2,000-4,000 IU/min for 30-90 min (60 min average)

For AMI, Intracoronary infusion is administered into the target coronary artery via coronary catheterization[6] , seen in Table 3.5. The listed doses are for clinical and patient application only and not for the isolated heart. Streptokinase is also widely used for pulmonary embolism, deep vein thrombosis, arterial thrombosis—each with its own set of dose and duration for treatment. Heparin should be administrated prior to SK during arrhythmias such as atrial fibrillation. Heparin is administered to a target to decrease PTT [6].

Streptokinase literature was reviewed to determine a concentration of streptokinase in an organ of similar mass and flow rate of the heart. Austrian's G. Stark's 1988 abstract discussed 2500U SK concentration/100 mL influencing the conduction circuit of the *in vitro* heart. SK resulted in a faster sinus node recovery time and ventricular border frequency and atrial border frequency had increased [106] . Mickelson's 1988 study had of rabbit myocardial function protected by 150 U/mL Streptokinase in KHB, preserving left ventricular function and prevented increasing LVEDP [67]. Mumme 1993 investigated temperature-dependent fibrinolytic activity for optimal temperatures of SK-induced cardioplegia. Fibrinolytic activity was 100% at 40°C and 7.9% at 25^0C [96]. Hachenberg 2001 investigated non-heart-beating livers that were preflushed with streptokinase at 7,500U in 20 mL Ringer's solution (concentration of 375,000 U/L) [62]. Structural integrity was improved as metabolism recovered for efficient cardiac function. Szyrach 2011 had warm ischemic porcine kidneys preflushed with streptokinase, with Group C being administered 12,500 U/L and Group D at 50,000 U/L. Group D resulted in toxic effects [76]. A study most similar to the research of this thesis was Mownah 2014 on reanimated porcine heart with 375,000U SK introduced into 250 mL AQIX (concentration of 1.5MU/L) [68]. Limited peer-reviewed articles of streptokinase application on isolated organs were found. Based on the results of SK dosages from both isolated organ studies and patient *in-vivo* studies, the dosage of SK was determined for University of Denver's isolated Langendorff study. The studies listed above show that concentration may vary and is not a factor on an isolated heart compared to a patient in which SK therapy can lead to tissue injury. In University of Denver's testing, 20,000 U

on average is applied in 1-L cardioplegia, determined by the heart's coronary artery flow rate of 225 mL/min to not induce high-pressure tissue injury.

3.3 Krebs-Henseleit Buffer

Krebs Henseleit Buffer (KHB) is a physiological solution that increases and stabilizes metabolism from the cardioplegic effects [13, 37, 39, 42, 44, 46, 48, 57, 123]. Though heparinized blood or red blood cells reconstituted in saline are preferable due to hemoglobin utilization for oxygen consumption and are performed in majority of Langendorff-based studies, saline-based solutions are investigated due to cost-efficiency and transparency to observe mechanical cardiac functions [14, 35]. Mimicking blood, the buffer is generally composed of oxygen, salts, glucose, principle ions involved in initiating action potential (potassium, sodium, calcium, chloride) and other additives at one's own preference that must follow a pH of 7.4 when oxygenated at physiological temperature [123]. Buffer preparation provided by the manufacturer recommends 3.12 mMol $CaCl_2$ which exceeds physiological level of 2.5 mM. The perfusate's ionized calcium utilized in the procedure on average was half the value of physiological protein-bound calcium at 1.2 mM. Glucose is increased to 11 mMol/L as opposed to the normal *in vivo* 3-6 mMol/L range to compensate for lack of fatty acids in KHB as explained in Section 2.1. Insulin is recommended for heightened myocardial metabolism increasing developed pressure[13]. Due to the transparency, crystalloid buffers are best for video and camera footage of interior cardiac activity such as valve action.

Table 3.6 – Comparison of studies with KHB usage, with or without blood mixture. A supplemental comparison of modified KHB found from additional literature review can be found in Appendix A.

Author	Rosenstrauch et al 2003 [47]	Van den Akker 2005 [13]	Skrzypiec-Spring et al 2007[44]	Bell, Mocanu and Yellen 2011[57]	DU Current Buffer
Reperfusion type	1:1 heparinized blood & KHB	25% hematocrit of blood and KHB	Modified KHB, Blood	KHB, Whole Blood, Red Blood Cell augmented Crystalloid Buffer	KHB
NaCl	118.1 mM	118 mM	118.5 mM	118.5 mM	118.1 mM
$NaHCO_3$	25.0 mM	25.0 mM	25.0 mM	25.0 mM	25.0 mM
KCl	4.69 mM	4.5 mM	3-4 mM	4.7 mM	4.7 mM
$MgSO_4$	1.17 mM	0	1.2 mM	1.2 mM	1.2 mM
KH_2PO_4	1.17 mM	0	1.2 mM	1.2 mM	1.2 mM
Glucose	11.1 mM	11 mM	5.5 mM	11 mM	11 mM
$CaCl_2$	2.54 mM	1.25 mM	1.2 mM	1.2-1.8 mM	1.5-2.5 mM
Mannitol	-	16 mM	-	-	16 mM
pH	7.4	7.4	7.4	7.4	7.4

1L of KHB was prepared by adding 9.6g of Krebs Henseleit powder (Sigma-Aldrich, St. Louis, MO; Product Number K3753) to 900mL Millipore water. $CaCl_2$ was added, ranging from 1-3.12 mMol as listed in Table 3.7 (Sigma-Aldrich, Product Number: C7902-500G) prior to the addition of 2.1g Sodium Bicarbonate (Sigma-Aldrich, Product Number S5761-500G). All buffers were refrigerated prior to use. Any necessary buffer additives were mixed during magnetic stirring before Millipore water was added to complete 1L. All perfusates were delivered at 37-35.7°C and gassed with 95% Oxygen

and 5% Carbon dioxide. Table 3.6 notes the comparisons regarding core ions within the solutions of each study, with the majority of values being exact across studies. Manageable and cost-effective, KHB was the selected buffer for the DU Langendorff study and procedure, with variable calcium amount. The following section elaborates each section of the procedure.

3.4 Procedure

3.4.1 Slaughterhouse Harvest

Hearts harvested and used for this study (26 total hearts) were obtained from Yorkshire, Berkshire, and Hampshire pigs (136 kg ± 18.14 kg) that were slaughtered for human consumption. Hearts were isolated immediately after brain death via captive bolt pistol with brain stem intact to allow uninterrupted blood circulation prior to exsanguination. Sternotomy was performed under USDA protocol. Before access to the heart, the pericardium was inspected immediate to excision by a USDA officer for conditions such as tapeworms and tumors. WIT ranged from 90 seconds – 8 minutes between exsanguination to complete cardiac arrest via cardioplegia, decreasing in length of time over the course of the research due to slaughterhouse accommodations. Brief yet strict observance was undertaken prior to cold saline submergence so that the heart was actively contracting to accept the heart as a reliable test subject in which necrosis hasn't taken effect [20] and that the electrical conduction system was systematically intact.

Figure 3.3.1 – Cannulated tubing structure to connect syringe for retrograde coronary flow towards the aorta. 1/3" diameter tubing for syringe insert was connected to ¾" to ½" transition cannula which was further connected to ¾" cannula that was secured into the aorta.

3.4.2 Preservation

Given the heart-lung block, the heart was excised from the lung with aorta trimmed to 2 inches and immediately cooled in a 4^0C saline bath (Phosphate Buffered Saline tablets in aqueous solution, Research Products International Corp., Prospect, IL; Product Number P32080-100T) as blood was washed out and cardioplegia cannula was prepared to the aorta secured by string and zipties. As application of any solution via aortic root starts, aortic valve must be checked to visualize that it is shut, intact, and in no way compromised. 1L STHS (Plegisol; Hospira, Inc, Lake Forest, IL) was poured into a 1L beaker as cardioplegia was applied using a 60-mL syringe via aortic root by a makeshift tubing system. The cannulated device was composed of a ¾" cannula held and secured to the aortic tissue by string, zipties and clamps to ensure backflow didn't occur as seen in Figure 3.3.1. A total of 13 tests were accomplished with two subject hearts per experiment. For the first 6 tests, the antithrombotic method used 5KU-15KU/L Heparin Sodium Injection, USP (Sagent ™ Pharmaceuticals, Schaumburg, IL. Product Number: NAPPLA6K23D4, Lot Number: WJ3138N) during cardioplegic administration. Streptokinase was applied as antithrombotic agent for Tests 6-13 (Sigma-Aldrich, St.

Louis, MO, Product Number: S3134-50KU). As dosage on an isolated heart was independent of concentration, 10KU–25 KU Streptokinase was used per heart reconstituted in 500ml–1L 25^0C Plegisol per heart. The tubing device was used for Tests 1–10. Tests 11–13 replaced the cannula-insert device with Medtronic perfusion cannulae coronary catheterization (Medtronic Inc., Minneapolis, MN) lodged into the left and right coronary ostia (Figure 3.3.2), sealed by balloon inflates to direct 1-way flow, bypassing the aortic valve. Catheterization harnessed Medtronic DLP ® Multiple Perfusion Set (Model Number 14007) and DLP®/Gundry ® Retrograde Coronary Sinus Perfusion cannula with manual-Inflate Cuff (Model Number 94615).

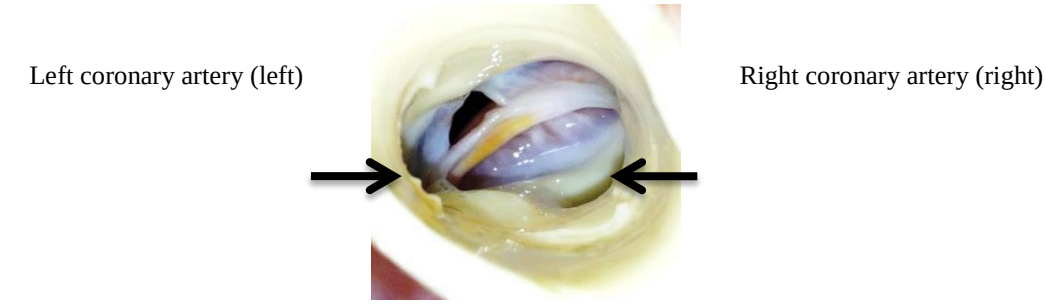


Figure 3.3.2 – Superior view of the aortic valve closed under 72 mmHg. Perfusion was rerouted through coronary ostia and into the arteries.

Amount of air in the manual-Inflate cuff was determined by insert or outtake of air via 3-ml syringe from the inflation assembly with female slip luer and one-way valve (blue air chamber) as seen in Figure 3.3.3. Guide wires seen in Figure 3.3.4 directed the catheter past the ostia. Procedure was repeated for the second heart.

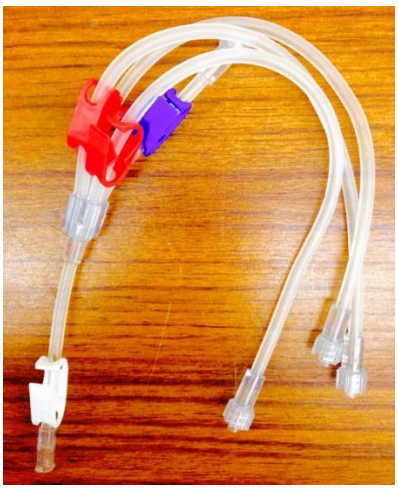


Figure 3.3.3 – Medtronic DLP ® Multiple Perfusion Set.

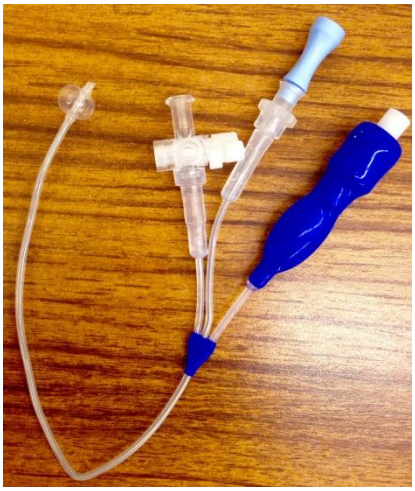


Figure 3.3.4 – DLP®/Gundry ® Retrograde Coronary Sinus Perfusion cannula with manual-Inflate Cuff.

3.4.2.1 Transportation

The hearts were stored in an airtight plastic bag, each immersed in either 1L Plegisol or UW solution and placed on ice for hypothermic protection. Transportation was 60-130 minutes in cold static ischemia, 70 minutes on average. UW solution was originally used as transport solution for the first half of experiments to implement the cardioprotective effects as found in Section 3.1.2. Based on cost-efficiency, Plegisol enacted as the dominant transport solution. In Test 4, to enable results similar to continuous perfusion than static storage, hearts were reperfused with 500 mL cardioplegia 30 minutes into transport.

3.4.3 Resuscitation

3.4.3.1 Water purification and pH

The Millipore system (Direct-Q® 3 UV with Pump Water Purification System, EMD Millipore, Billerica, MA; Serial Number F5MN93607E) was operational with 18 MΩ-ch

being the highest filtration via 0.2μm absolute final filter and used by Test 3 to ultra-purify tap water. Water used prior was DI water. Intermittent measurements were taken by Oakton pH/Conductivity/Temperature meter (Eutech Instruments, Singapore; Product Number 54X002608). Water quality not only stayed consistent but pH of KHB used for experimental purposes no longer needed HCl for adjustment and was consistent at a pH of 7.46 ±0.03 once oxygenated at room temperature by Test 2.

3.4.3.2 Reperfusion

The Langendorff apparatus is an *ex vivo* circulation to introduce flow to the cardiac aortic root with the schematic found in Figure 3.3.5. The aerated glass reservoir (HSE Glass Perfusate Reservoir for use with Peristaltic Pumps, Harvard Apparatus, Holliston, MA, Item Number 730322) contains 5L modified KHB (Sigma-Aldrich, St. Louis, MO), with 37°C buffer sustained by the heat exchanger pumped through the water thermocirculator. Oxygenation was 95% Oxygen 5% Carbon Dioxide, Carbogen, supplied at 9psi (General Air Service & Supply, Denver, CO, Serial Number: 2201299RS). ¼ inch and 10 mm Tygon 2375 tubing allowed oxygenated crystalloid buffer flow pumped by a second roller pump (Masterflex® L/S ™ Easy-Load® II; Model Number 77200-62). The heart lays within a chamber at a height of 45cm for added pressure column. A transfer cannula was inserted into the cardiac aorta and clamped in a homemade acrylic chamber. Exhausted buffer exited the heart into the chamber, collecting back into the reservoir. An additional 2L were used for rewarming purposes at 15°C and 25°C, sequentially, to prevent temperature-shock and reperfusion tissue-injury.

Infrared Thermometer (EtekCity ETC8380) measured temperature of the organ intermittently throughout testing.

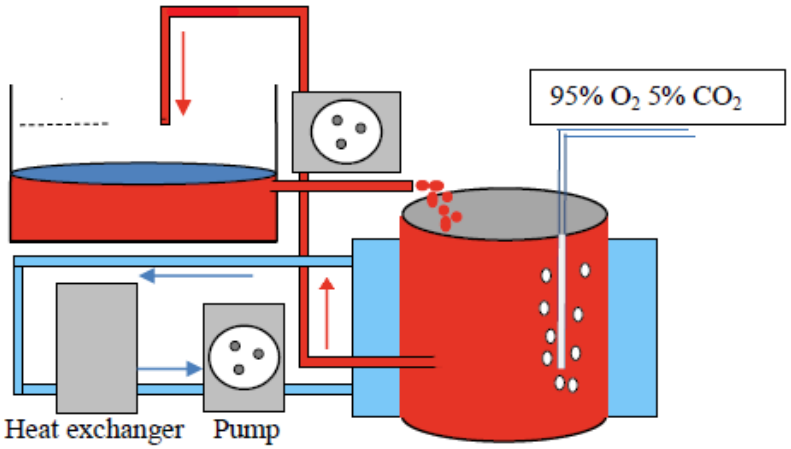


Figure 3.3.5 – DU Cardiac Langendorff constant flow schematic in Test 1-2.

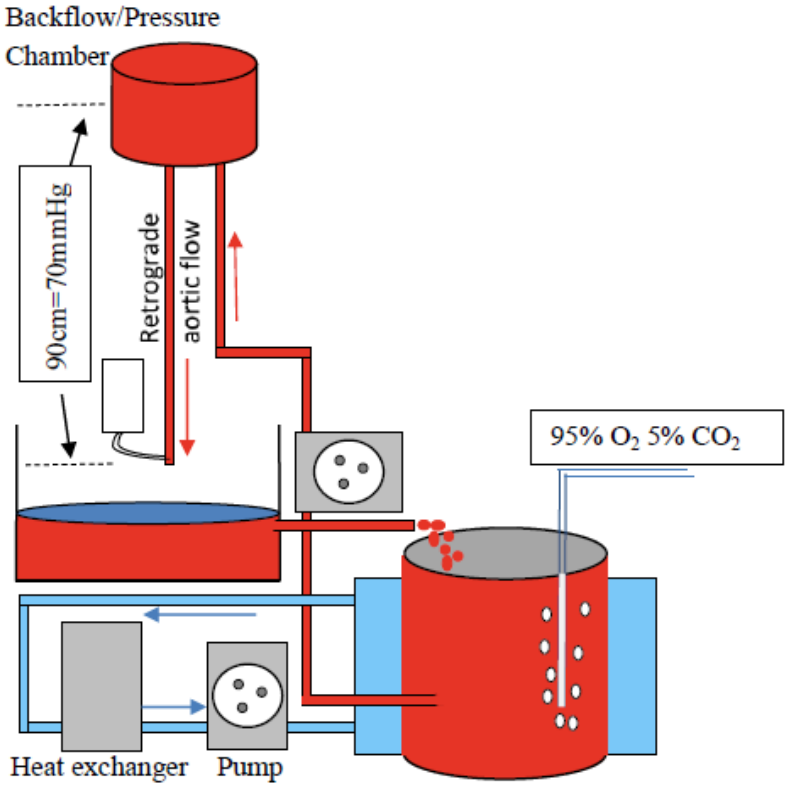


Figure 3.3.6 – DU Cardiac Langendorff constant pressure schematic used in Test 3-13.

Modifications in KHB over the course of the experiments required the components found in Table 3.7.

Table 3.7 – University of Denver's Cardiac Biomechanics Lab Modified KHB (Sigma-Aldrich ® Products K3753, C7902-500G, S5761-500G, and M4125-100G)

Component	Amount
NaCl	118.1 mM
$NaHCO_3$	25.0 mM
KCl	4.7 mM
$MgSO_4$	1.2 mM
KH_2PO_4	1.2 mM
D-Glucose	11 mM
$CaCl_2$	1-3.12 mM
pH	7.4
Reperfusion type	KHB
Mannitol	16 mM
Insulin	0.25 ml/L
Epinephrine (1 mg:1 ml)	0.25 ml/L
Oxygen	9 psi

Reconstituted KHB should not be used after 2 days as glucose deteriorates, increasing risk of bacterial contamination. Ultimately, KHB lowers in acidity and becomes a pH of 6.8 after 7 days. After 1-hour transport, the water heater (10L Fisher Water Bath, Serial Number 928579, FisherScientific, Pittsburgh, PA) encompassing the perfusate reservoir was heated to roughly 45°C ± 5°C and kept the circulating KHB at 37°C so that it was available for immediate use once hearts were mounted. Additional set up took 5-20 minutes, prolonging cold ischemic time. Though usage of cardioplegia allows hearts to stay preserved 4-6 hours, the study was aimed to keep minimal length of cold ischemia to roughly one hour in which included minimum transport time and preservation

application. The most recently-excised and preserved heart with the shortest cold ischemic time was cannulated to the Langendorff extracorporeal system with carbogen pressure at 9psi. The Langendorff apparatus over the course of the experiments was originally set up to constant flow at roughly 120 mmHg for Tests 1 and 2, with the schematic shown in Figure 3.3.5. From Test 3 - 13, constant pressure mode was determined with aortic pressure averaging 82 ± 10 mmHg with the schematic shown in Figure 3.3.6. Retrograde aortic pressure was measured by a pressure transducer (Statys™, BDC Laboratories).

Insulin (ProZinc ® protamine zinc recombinant human insulin, Boehringer Ingelheim, Ingelheim am Rhein, Germany) was administered for Tests 4-6 when UW solution was utilized as the transportation medium. Recommended rate is 10 U/L. 1 mL is equivalent to 40 U and with 7L reperfusion, 1.75 mL/heart was administered. Both hearts per test used the same circulating perfusion, risking oversaturation.

Flow rate via speed pump was determined by 1 ml/g of heart muscle [35] and thus, flow rate was 175 - 300 mL/min under oxygenation. Pump settings determined the flow speed at a setting of 2-3. The flowrate was kept constant for all test subjects. Temperature must always be monitored and regulated; 37.5°C is optimal body temperature—too low temperature decreases contractility, and suppresses enzymatic activity, whereas ≥39°C and above can lead to tissue injury and causes burns. An air trap was highly recommended for lethal air emboli blocking flow and must be cleared out through the circulation. Monitoring was key as fluctuating levels of pressures, temperature, pH and ions was a sure sign of reperfusion-injury damage, calcium-overload, and can ensure

further damage. All components are significant to the aid and function of the heart though moderation in certain ions must be considered such as calcium which will be discussed in Section 5.2.

3.4.4 Pacing and Defibrillation

All hearts underwent ventricular tachycardia prior to fibrillation in which hearts were then defibrillated using by ZOLL ® R-Series ALS manual external defibrillator and ECG (ZOLL, Chelmsford, MA) with spoon paddles to stabilize cardiac sinus rhythm. 5-7 shocks were administrated with a minimum of 5 minutes in between at 10-20 J. Pacemaker rate was set to 100 ppm, output at 15 mA, and sensitivity at 2.5 mV. Once stabilized, second-hand Single-Chamber temporary pacemaker (Medtronic Inc., Minneapolis, MN) was used to augment and aid the impulse during pacing and monitoring. Epinephrine (1:1000 1mg/mL, Amphastar Pharmaceuticals, El Monte, CA, Lot Number DTO38J3) or any other types of β-adrenergic agonists are recommended for injection prior to defibrillation at a rate of 0.5-1 mL of 1mg:1 mL every 3-5 minutes or intermittently [39, 48]. As the agents are in sterile vials, blunt BD PrecisionGlide needles, 21 G 1 ½ were used for withdrawing the medication.

Ventricular fibrillation (VFib) is easily induced in large mammalian hearts and can be initiated by reentrant tachycardia and other sources of cardiac arrhythmia. Episodes of reentry is involved when propagation fails after excitation of the heart, resulting in atrial fibrillation, atrial flutters, and ventricular tachycardia after such myocardial ischemia [124]. With such occurrence which disables efficient contractility, a defibrillator must be used to reset the electrical impulse to regular beating rhythm. The defibrillator used in the

study is biphasic in which VF is ceased with a lower current [125]. Typically, the ECG is measured from the surface of the body by placing two electrodes directly on the skin and reading the potential difference between it. An isolated heart will have epicardial ECGs differentiate from full-body ECG as resistance differs between each medium. The detected waveform features depend on the amount of cardiac tissue involved in the contraction, as well as the orientation of the electrode placement with respect to the heart. A number of different ECG lead configurations exist based on electrode location [22] .

Pacing was vastly recommended, securing the SA node to initiate consistent heart rates. Isolated heart beats are below that of normal physiological rate [35]. Though pacing does aid regulation in contractility, in an isolated heart, the organ will ultimately undergo necrosis, certain tissues at its current state undergoing global ischemia over time. Though the nodal cells are self-initiating, the specific rate of *in-vivo* impulses are implemented by the right vagus cranial nerve (CN X) [14]. Pacing should not go over 30 minutes for a living patient nor *ex vivo* heart as burns can occur with transcutaneous pacing [125]. Pig hearts that averaged 250g in weight were recommended to pace at 100-120 bpm [75, 85, 126].

Cardiac performance increases with reperfusion time and studies show that peak performance and stabilization occurs 30-90 minutes, dependent upon the additives and composition of the perfusate [37, 39, 48]. Equipment was washed using Alconox® (Alconox, Inc, White Beach, NY) and diluted bleach prior to air-dried sanitation.

Chapter 4: Results

4.1 Overview of Experimental Work

Results were collected for all 13 experimental tests. The following results are organized by all tests in chronological order. With Test 1 serving as a baseline, each test was modified based off of the inadequate results of the preceding test. This thesis includes all experiments in chronological order with a simplified procedure included per test. Results and data are categorized with the respective test, as each test discusses results and suggestions for the following test. Key progression within each test was fine-tuning the procedure as well as calcium concentration. WIT per test ultimately decreased. In the study, WIT was defined as the lack of oxygenated normothermic blood from swine exsanguination to a complete 1L 4°C antegrade cardioplegic flush. Test 10 was the introduction of <5 minutes WIT with the heart immediately submerged in 4°C saline slurry from excision.

Table 4.1 is a summary of all tests, highlighting Langendorff mode, cardioplegic additives, and mean heart rate for all experiments. Table 4.1 illuminates the progress of all tests towards the final satisfactory results which meet stabilized and functional cardiac activity. Physiological cardiac activity was achieved at 1 hour minimum with global contractile function. After heart stabilization via defibrillation of 15-20J and epinephrine application, stabilized heart rates ranged 60 - 110 bpm for an hour; hearts were compliant

during the duration of the first hour of activity with rigor induced by edema at the 1 hour mark. Cardiac function was monitored by the ECG functions of the external defibrillator. ECG reports are available from Test 8 thereafter. ECG readings specify prominent QRS complex predominantly accompanied by Polymorphic Ventricle Tachycardia (PVT) prior to fibrillation in which is commonly caused by myocardial ischemia. PVT has multiple ventricular foci in which resultant QRS complexes vary that prominently leads R waves onto the T wave and degenerates to ventricular fibrillation, otherwise known as the "'R on T' phenomenon. *Torsades de Pointes* (TdP) is a type of PVT in which QT intervals are prolonged [125]. Mean aortic pressure was 101.3 ± 1.94 mmHg.

Table 4.1 – Stabilized heart rate per experimented hearts. All hearts originally underwent atrial or ventricular fibrillation. As testing progressed, hearts stabilized in contractile function for ≤1 hour on average in Test 11-13. *Appendage only

Test	Test Date	Experiment heart test (Heart #)	Constant Flow (CF) or Constant Pressure (CP)	Cardioplegic additives: Heparin (H) or Strepotkinase (SK)	Ca^{2+} addition to KHB (mMol)	T = ¼ hour (avg) (bpm)	T= ½ hour (avg) (bpm)
1	Aug 20 2013	1	CF	H	1.2	52*	0
		2	CF	H	1.2	78*	0
2	Sep 24 2013	3	CF	H	1.2	54*	30
		4	CF	H	1.2	-	0
3	Oct 8 2013	5	CP	H	1.2	AFib	0
		6	CP	H	1.2	N/A	0
4	Feb 16 2014	7	CP	H	1.25	8	0
		8	CP	H	1.25	10	0
5	May 6 2014	9	CP	H	1.25	86	VF
		10	CP	H	1.25	AFib	0
6	July 8 2014	11	CP	H & SK	1.2	N/A	0
		12	CP	H & SK	1.2	N/A	0
7	Nov 4 2014	13	CP	SK	1.2	120	0
		14	CP	SK	1.2	30	160
8	Jan 27 2015	15	CP	SK	2.5	145	79
		16	CP	SK	2.5	N/A	0
9	Feb 3 2015	17	CP	SK	2.52	100	0
		18	CP	SK	2.7	60	0
10	Feb 17 2015	19	CP	SK	0.5	100-180	0
		20	CP	SK	1.2	150-180	0
11	Mar 24 2015	21	CP	SK	1.66	176	24
		22	CP	SK	1.66	0	0
12	Mar 31 2015	23	CP	SK	1.66	143	126
		24	CP	SK	1.66	185	58
13	Apr 7 2015	25	CP	SK	2	172	110
		26	CP	SK	2.5	150	92

13 tests were run, each with 2 subjects (26 total hearts). Only in a specific test was a heart not viable for testing (Heart 16) due to inappropriate tissue excision. This section dictates the consistent procedure used with minor modifications for each test, discussing the significant results of each test, and concludes with modifications to be utilized for the next experiment. Results continued to improve as additional tests were conducted, increasing efficiency for certain procedures kept constant. Certain details not annotated in literature review had to be sought out via experimental testing to design and develop a working Langendorff platform that allowed ≥1 hour global resuscitation.

4.1.1 Test 1: August 20, 2013 (Hearts 1, 2)

Hearts were washed topically with 2L 2°C saline with 1L 4°C Plegisol (STHS) flushed via aortic cannulation before stored in 1L 4°C UW in plastic bags with indirect ice contact. Procedure and overview results may be viewed in Table 4.2. Cold ischemic time averaged 67 minutes.

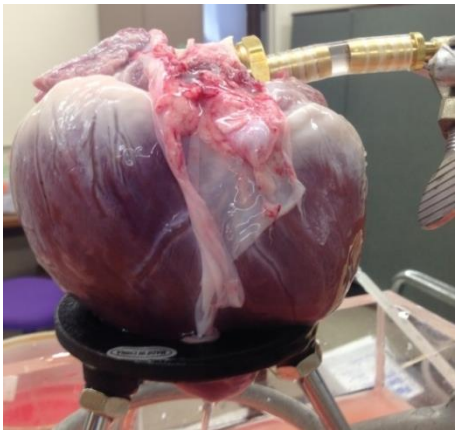
Table 4.2 – Procedure with modifications and results for Test 1

Parameter	Heart 1	Heart 2
Warm Ischemic Time (min)	15	13
Topical wash	2°C saline	2°C saline
Cardioplegia	Plegisol	Plegisol
Cardioplegia Additive	5KU Heparin	5KU Heparin
Transport	4°C UW	4°C UW
Flow technique	cannula	cannula
Reperfusion mode (CF or CP)	CF	CF
Krebs Henseleit Additives	Epinephrine	Epinephrine
Calcium (mM)	1.2	1.2
Resuscitated period (min)	17	14
Average Hear Rate (bpm)	52 (appendage)	78 (appendage)

Langendorff setup was at constant pressure with gravity directed flowrate roughly 250 ml/min. Heparin and epinephrine application was provided by literature review and

suggested by physicians. $CaCl_2$ was modified in KHB to be half of the physiologically-recommended 2.5 mMol found in literature review and thus added at 1.2 mMol (0.176g) per Liter. KHB composition may be reviewed in Section 3.3. Movement in right appendages only contracted rhythmically at 52 bpm and 78 respectively for Hearts 1 and 2. No contractions were monitored in the left ventricle, atrium, or apex. Hearts were not compliant. Coronary emboli and thrombi were apparent in the vasculature despite heparin preflush. Figure 4.1a & b illustrates the general laboratory and procedural set-up. Dissolving coronary thrombi becomes priority to allow unobstructed perfusion flow.

(a)



(b)

Figure 4.1a & b – Heart 1 Langendorff set-up (**a**) Constant Pressure mode Langendorff set up with reservoir column raised roughly 80 cm above heart. (**b**) Heart applied via constant pressure Langendorff perfusion with cannula attached towards aorta for Heart 1.

4.1.2 Test 2 – September 24, 2013 (Hearts 3, 4)

Modifications to the general procedure (Table 4.3) include application of Medtronic Model 5348 pacemaker and rewarming immersion was first introduced at 20°C before aortic cannulation to the Langendorff system. Procedure was kept similar as Test 1 with the addition of pacing the heart in Async to initiate signal towards the SA node. Pacemaker settings were set at 100 bpm. Oxygenation was at 5psi and flow rate

averaging 500 mL/min. Pacemaker lead was administered once atrial appendage contractions stabilized. *Test 2 highlights as the first test with physical activity in a cardiac region.* Contractions proceeded with superficial left ventricle contractions at 30 bpm; global contraction did not occur. Modifications to alternate the constant pressure system into a constant flow system were reviewed for future testing. Procedure and overview results may be viewed in Table 4.3. Coronary thrombi and air embolism were still located and prioritized to remove for future testing.

Table 4.3 – Procedure with modifications and results for Test 2

Parameter	Heart 3	Heart 4
Warm Ischemic Time (min)	9	8
Topical wash	4^0C saline	4^0C saline
Cardioplegia	Plegisol	Plegisol
Cardioplegia Additive	10KU Heparin	10KU Heparin
Transport	4^0C UW solution for 72 min	4^0C UW solution for 72 min
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CF	CF
Krebs Henseleit Additives	Epinephrine	Epinephrine
Calcium (mM)	1.2	1.2
Resuscitated period (min)	10	5
Average heart rate (bpm)	54 (appendage)	N/A

4.1.3 Test 3 – October 8, 2014 (Hearts 5, 6)

Test 3 was the onset of Langendorff perfusion set from constant pressure to constant flow, which was maintained for all future testing. The conversion allowed a minimal risk of air emboli in coronary vasculature, overriding autoregulated shifts in perfusate volume. Minor modifications include the initiation of immersing hearts in a 1L beaker bath in which increased hypothermic effect of Plegisol as previous tests had flushed Plegisol pass through the vasculature only once prior to disposal. However, cold ischemic storage was 2 hours. No visual activity on contractile function was significant.

Pacemaker was not used. Future testing would shorten ischemic storage from 2 hours to 60-80 minutes. Procedure and overview results may be viewed in Table 4.4.

Table 4.4 – Procedure with modifications and results for Test 3

Parameter	Heart 5	Heart 6
WIT (min)	7	5
Topical wash	4^0C saline	4^0C saline
Cardioplegia	Plegisol	Plegisol
Cardioplegia Additive	5 KU Heparin	5 KU Heparin
Transport	4°C UW for 117 minutes	4°C UW for 117 minutes
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	Epinephrine	Epinephrine
Calcium (mM)	1.2	1.2
Resuscitated period (min)	-	-
Average heart rate (bpm)	AFib	-

4.1.4 Test 4 – February 16, 2014 (Hearts 7, 8)

Modifications include new cold ischemic procedure and insulin addition to the recirculating 37°C KHB. For Test 4 only, intermittent flushing of 500mL UW solution occurred every 30 minutes between initial preservation flush and reperfusion 70 minutes after, mimicking continuous flow. However, no significant increase in contractile function occurred. Recombinant human insulin (ProZinc ®, Boehringer Ingelheim, Germany) served as an additional energy source. Procedure and overview results may be viewed in Table 4.5.

Table 4.5 – Procedure with modifications and results for Test 4

Parameter	Heart 7	Heart 8
WIT (min)	9	6
Topical wash	4°C saline	4°C saline
Cardioplegia	4^0C Plegisol	4^0C Plegisol
Cardioplegia Additive	7.5KU heparin	7.5KU heparin
Transport	4^0C UW in 70 min	4^0C UW in 70 min
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	1.75 ml insulin 1.75 ml epinephrine	1.75 ml insulin 1.75 ml epinephrine
Calcium (mM)	1.25	1.25
Resuscitated period (min)	10	5
Average heart rate (bpm)	8	10

Further inquiry looked into alternating Plegisol to another cardioplegic source. Subsequently, Plegisol specifications were reviewed and sodium bicarbonate was requested to activate certain additives to the solution, increasing pH to 7.4. Though it was assumed the sterilized Plegisol was 7.4, pH of the sterile solution was 3.4, causing detrimental effects on the isolated hearts. *The pH of 3.4 explains the failure in preservation for all previous tests.*

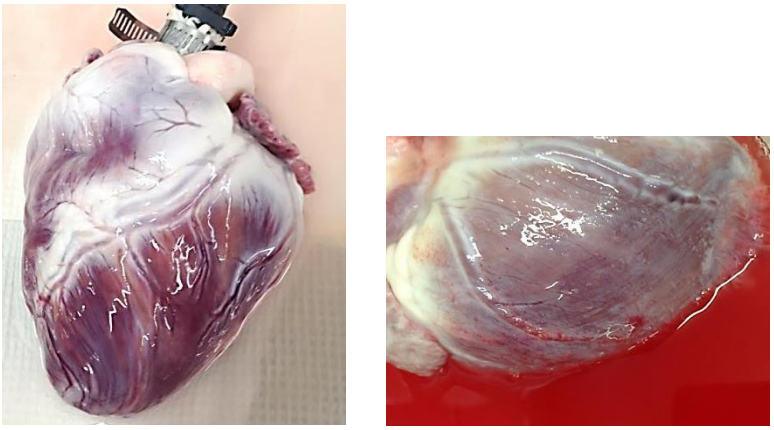
4.1.5 Test 5 – May 6, 2014 (Hearts 9, 10)

From Test 5 thereafter, Plegisol was modified with 8.4% sodium bicarbonate to stabilize pH to 7.4 and was the key modification. 8.4% sodium bicarbonate buffer was formed by adding 0.84g sodium bicarbonate to 10 mL water; the buffer was then added to the sterile Plegisol. Heparin was increased based on similar isolated porcine studies [43]. As heparin was in storage temperature of 4^0C and administrated to 4^0C STHS and not at a physiologic temperature, half-life exponentially increased, slowing enzymatic activity; combined with hypercoaguability [50, 55, 127] of swine blood, the low dose of heparin was

insignificant. Thrombi formation was evident in vasculature, as seen in Figure 4.2. Procedure and overview results may be viewed in Table 4.6.

Table 4.6 – Procedure with modifications and results for Test 5

Parameter	Heart 9	Heart 10
WIT (min)	8	7
Topical wash	4^0C saline	4^0C saline
Cardioplegia	Modified Plegisol	Modified Plegisol
Cardioplegia Additive	7.5KU heparin	7.5KU Heparin
Transport	4^0C in UW Solution	4^0C in UW Solution
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	1.75 ml insulin, 1.75 ml Epinephrine	1.75 ml insulin, 1.75 ml Epinephrine
Calcium (mM)	1.25	1.25
Resuscitated period (min)	40	20
Average heart rate (bpm)	86	AFib



(a) **(b)**

Figure 4.2a & b – Buffer flow visualization for Test 5. (**a**) Coronary arteries indicate buffer flow in Test 5. However, other minor vessels closer towards the apex determine partially occluding thrombi. (**b**) Heart 10 shows hypothermic tissue damage and scarring once oxygenated KHB flow was introduced. Heart had a shortened resuscitated period.

Mean atrial contractions averaged 77 bpm as myocardial temperature reached 37°C although no apical contractions were seen. Significant right ventricle contractions were apparent, leading to a follow up of equally dividing flow between the two coronary ostia.

Thrombi and air emboli disintegration are looked upon for future testing, specifically a fibrinolytic pharmaceutical agent.

4.1.6 Test 6 – July 8, 2014 (Hearts 11, 12)

Primary modification was application of the fibrinolytic drug, Streptokinase. While Heparin was used as a preventative measure, Streptokinase treated coronary thrombi already formed. Based on previous tests, keeping the coronary vasculature clear of thrombi was crucial as flow would not reach near the apex. Isolated hearts were gently massaged in order to aid vasculature flow to lower risk of coronary thrombi formation. In Table 4.7, WIT was increased due to training of experimental procedures and may have caused detrimental effects with lack of immediate preservation. Streptokinase was shown to be highly effective though visual cues indicated thrombi near the apex. Similarly to prior testing, epinephrine was used as needed prior to pacing. Pacing was set to rapid atrium pacing using Medtronic Single-Chamber temporary pacemaker with output at 50-70 mA, rate at 100 ppm, sensitivity at ASYNC with the pacing lead fed through the right atrium, on contact with the base of the right ventricle. Procedure and overview results may be viewed in Table 4.7.

Table 4.7 – Procedure with modifications and results for Test 6

Parameter	Heart 11	Heart 12
Warm Ischemic Time (min)	15	13
Topical wash	4°C Plegisol	4°C Plegisol
Cardioplegia	Modified Plegisol	Modified Plegisol
Cardioplegia Additive	5KU heparin 10KU SK	5KU heparin 10KU SK
Transport	4°C in UW solution	4°C in UW solution
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	2 ml insulin, Epinephrine	4 ml insulin, Epinephrine
Calcium (mM)	1.2	1.2
Resuscitated period (min)	26	19
Average heart rate (bpm)	50	65

Following the test, Streptokinase was determined to not be as efficient due to environmental temperature being equivalent to storage temperature, rendering inactivity. Assessing biochemical foundations of the pharmaceutical agents, Streptokinase and heparin had little to none effect at 4^0C compared to a more physiological temperature (Section 3.2). Dosage of Streptokinase was not a factor as dilutions in studies ranged from 2mL to 500mL (Section 3.2). For future testing, 20^0C of 250-300 mL would be applied prior to the 4^0C Plegisol flush, with storage solution still constant at 4^0C. A longer period of rewarming over the course of 30 minutes to prevent temperature shock and a colloid agent to decrease osmotic swelling was likewise proposed for future testing.

4.1.7 Test 7 – November 4, 2014 (Hearts 13, 14)

Mannitol was the primary modification of Test 7 in order to lower edema as well as preflushing the hearts with Streptokinase and heparin in 25°C saline. Dosage of heparin and streptokinase (SK) were increased from the previous examination and titrated to tepid saline. Streptokinase was diluted in 500 mL saline at room temperature per heart. Heart

13 was topically rinsed with tepid saline. The heart was massaged to prolong coaguability and increase kinetic activity. Warm SK/heparin-infused saline was applied in retrograde fashion via aortic root to slightly cool the heart and lower the metabolism in order to prevent ischemic shock[20] as well as have the biologic enzymatic activity performing at near-optimal level in a physiological temperature. WIT was noted as the timeframe between swine concussion to 4°C cardioplegic application, including the tepid pharmacologic pretreatment. By 4°C cardioplegic flush, no thrombi was visible within the vasculature. A slow flush was utilized to minimalize risk of ischemic shock, then flushed briefly with 150-300 mL UW solution prior to storage within 1L Belzer UW solution (Bridge2Life, WI). However, UW was provided by solution already contacted with the heart and not sterilized; there was high risk of catabolites and blood cells re-entering the vasculature. UW solution in retrograde flow provided internal contact within the vasculature as well as exterior myocardium contact and to resolve apical underperformance. Procedure was repeated for Heart 14. Overview results and procedure may be viewed in Table 4.8.

Table 4.8 – Procedure with modifications and results for Test 7

Parameter	Heart 13	Heart 14
WIT (min)	8	11
Topical wash	6°C saline	6°C saline
Cardioplegia	4°C Modified Plegisol	4°C Modified Plegisol
Cardioplegia Additive	25KU SK, 7.5KU heparin in 500 mL 25°C saline	25KU SK, 7.5 heparin in 500 mL 25°C saline
Transport	4°C UW solution	4°C UW solution
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	Mannitol (2.92g/l), 1.1 ml epinephrine	Mannitol (2.92g/l), 1.5 ml epinephrine
Calcium (mM)	1.2	1.2
Resuscitated period (min)	38	35
Average heart rate (bpm)	75	30

Subsequently, UW solution was perfused as a way to mimic continuous perfusion: the temperature of an object in a cooler of ice does not reach 4°C and may remain above 8°C in semi-submerged positions; the muscle mass at the apex is thick and likely required persistent flow in order to penetrate the entire tissue and facilitate diffusion of ion concentrations.

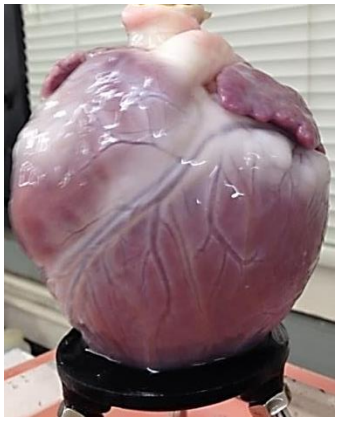


Figure 4.3 – Buffer flow visualization for Test 7 of KHB within coronary arteries (Heart 13). Vasculature had a bluish tint and more translucent appearance compared to an *in vivo* heart.

Prior to buffer reperfusion, 2.62g/L Mannitol was added to the buffer. Test 7 highlighted the decrease in flow speed to a trickle as the aortic cannula was mounted to

the heart aorta to decrease risk of air emboli before flow rate was increased back to 200 ml/min. KHB allowed the veins to appear transparent as thrombi was cleared, allowing no occluding flow, as seen in Figure 4.3. Once reperfused by KHB, right atrium displayed regular and stabilized contractions, circulated by the anterior coronary artery. However, the remaining regions of the heart bore no contractions which may signify a lack of signal transduction and/or dysfunctional ion balances precluding myocyte cell membrane potentials. Pacing was as needed with an output of 50-70 mA, rate of 100 ppm, and sensitivity at ASYNC. Pacing method was done with the (-) lead clipped near the SA node (internal pacing) (-) and the other (+) near the apex instead of using a pacing wire. Pacing was determined at the immediate onset of contractile performance prior to atrial or ventricular tachycardia. The technique was updated from applying the pacemaker once cardiac activity ceased. Decreasing flow rate into a trickle while mounting was used for all future tests. Heparin and Streptokinase were effective agents and coronary vasculature coagulation was eliminated as an obstacle while edema and ventricular function continued.

Post-experimentation, Dr. Ashok Babu, cardiac surgeon of Anschutz Medical Campus and Dr. Christopher Orton, veterinary cardiac surgeon of Colorado State University offered various insights into the isolated heart experimentation and recommendations on the procedure. Similarly to heart transplantation procedures, hypothermia should be immediate for excised hearts undergoing contractions prior to cardioplegic application. The surgeons enforced the usage of streptokinase and heparin due to swine hypercoagulability [50]. Pressure applied during cardioplegic flush can

enable leakage past the shut aortic valves. While not detrimental to the left ventricle, muscle compliancy was impacted. Overdistension should be prevented and thus for future tests, mitral valve was kept open as working heart mode would not be utilized. Overdistension of the ventricle will increase the oxygen demand and have a negative effect on contractility [39, 55].

Cardioplegia-induced ischemia, contact with ice, and reperfusion of KH Buffer can all lead to edema, and surgeons do agree that mannitol or any other source of colloids be used for osmotic pressure. A defibrillator was highly recommended, due to history of fibrillation with every test ultimately leading to shortened reanimation length. With smaller mammalian hearts than pig hearts, a pacemaker at a high enough setting can initiate defibrillation onto the heart. Though the classic and most popular AED equipped in buildings for emergency usages was suggested and is cost-effective, the device automatically traces the ventricular fibrillation and management was not certain for manipulating energy output. Usual AED exert 120J onto the general human body. 10-30J was recommended for isolated hearts, a value similarly used for open heart surgery once blood is re-introduced. The heart should not be paced when during fibrillation but once stabilized and at a standstill [55].

December 9, 2014 (Heart N/A)

Though no hearts were collected on this day, attempts were futile as time-sensitive buffers and equipment were prepared, as well as activated Plegisol with SK reconstituted into solution and thus with a life of 8 hours. However, pigs underwent scalding rather than regular exsanguination based on livestock availability. Hearts obtained from scalded

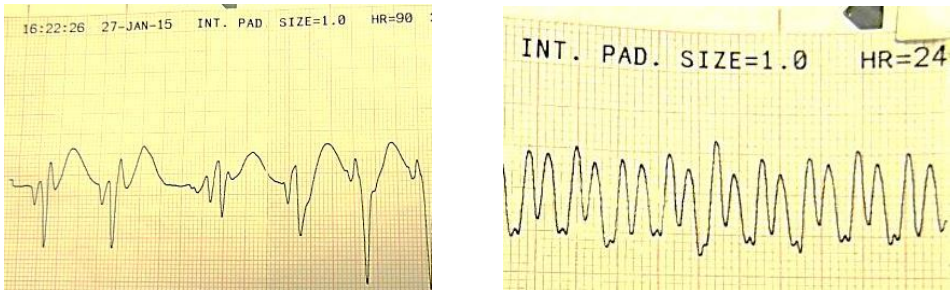
pig would have underwent temperature of 56-60°C with a WIT of ≥ 15-30 minutes [128]; the concept was disregarded. Key results from the test was to have the SK reconstituted only once hearts were accessible and prior to excision. Blood-cardioplegia was suggested as it was most efficient than pure crystalloid cardioplegia, with 1:4 heparinized blood, although never utilized during the series of test.

4.1.8 Test 8 – January 27, 2015 (Hearts 15, 16)

Prior to Test 8, defibrillation was inaccessible. Modification made was a defibrillator used prior to pacing for contractile stabilization. Factors tested was compliancy and contractile recovery based on preservation in saline vs. preservation in Plegisol. Though saline is the gold-standard for hypothermia and cardiac preservation, a direct comparison was sought for the effects. Heart 15 had saline storage while Heart 16 was stored Plegisol. Heart 16 was significantly compliant compared to Heart 15 during rewarming. 5L KHB were prepared for circulation while 2L expired-KHB was used for rewarming purpose only to minimalize expired glucose effect in the vasculature. Calcium was increased from 1.5 mMol to 2.5 mMol to follow recommended literature dictating half the physiologic range. However, Ca^{2+} measurement was inaccessible to determine utilization rate and if the amount or lack thereof was a factor in the decrease in contraction stability. The increase in Ca^{2+} indicated no significant increase in stable contractile function. Calcium overload influencing ATP utilization was an inhibiting factor that the experimentation aims to resolve. Procedure and overview results may be viewed in Table 4.9.

Table 4.9 – Procedure with modifications and results for Test 8

Parameter	Heart 15	Heart 16
WIT (min)	7	7
Topical wash	4°C saline	4°C saline
Cardioplegia	500mL 20°C saline prior to 500mL 4°C Modified Plegisol	500mL 20°C saline prior to 500mL 4°C Modified Plegisol
Cardioplegia Additive	10K in 20°C saline	10K in 20°C saline
Transport	4°C Saline	4°C Plegisol
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	Mannitol (2.92 g/L)	Mannitol (2.92 g/L)
Calcium (mM)	2.5	2.5
Resuscitated period (min)	37	25
Average heart rate (bpm)	79	150



(**a**) (**b**)

Figure 4.4a & b – Heart 15 ECGs. Key observance of definite course fibrillation with (**a**) at heart rate of 90bpm and (**b**) of 240bpm. [125]

(**a**) (**b**)

Figure 4.5a & b – Heart 16 ECGs. (**a)** Ventricular fibrillation can be determined by the second inclined wave in the QRS portfolio. (**b)** 'Normal' stabilized heart rate ECG peaks.

With respect to the ECG curves, there was no activity in Heart 15. The ECG curves agreed well with observation that there was no muscle contraction in the heart. Heart 16 was stable and displayed "Torsade de Pointes" which is a distinctive form of polymorphic ventricular tachycardia (VT) as seen in Figure 4.5b. The reperfusate solution possibly being hypomagnesemic was a possible factor in which magnesium level should be increased >2 mg/dl. Though SK did clear coronary vasculature, blood had coagulated within the inner cardiac chambers in the duration of Test 8.

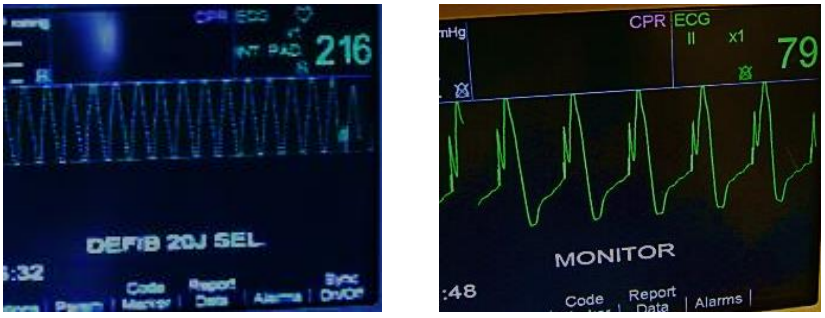
4.1.9 Test 9 – February 3, 2015 (Hearts 17, 18)

A comparison between Plegisol and saline preservation was repeated (Heart 17 Plegisol, Heart 18 saline). For both hearts, from animal concussion to SK preflush averaged 3.5 minutes of total WIT. Prior to mounting on the Langendorff circulation, Heart 17 was compliant. Both hearts fibrillated upon reperfusion (Figure 4.6a and Figure 4.7a). Defibrillation was applied at 10-50 J, shocks administrated 2-3 minutes apart until stabilization (Figure 4.6b and Figure 4.7b). Heart 18 stabilized only to return to fibrillation before inactivity with HR ranging 12-65 on Lead II. Mitral valve was kept

open to prevent left ventricular overdistension into the chambers. Procedure and overview results may be viewed in Table 4.10. The acclimating range for defibrillation was significantly varied; for future testing, 20J shocks will strictly be administrated.

Table 4.10 – Procedure with modifications and results for Test 9

Parameter	Heart 17	Heart 18
WIT (min)	8	8
Topical wash	4°C saline	4°C saline
Cardioplegia	4°C Modified Plegisol	4°C Modified Plegisol
Cardioplegia Additive	SK in 20°C Plegisol preflush	SK in 20°C saline preflush
Transport	4°C Plegisol	4°C saline
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	1.25 mL epinephrine, Mannitol (2.92 g/L)	1.25 mL epinephrine, Mannitol (2.92 g/L)
Calcium (mM)	2.52	2.70
Defibrillation	10-50J	10-50J
Resuscitated period (min)	30	30
Average heart rate (bpm)	100	60



(a) **(b)**

Figure 4.6a & b – Heart 17 ECGs (**a)** Ventricular tachycardia at 216 bpm. **(b)** Stabilized HR 79 bpm.

(**a**) (**b**)

Figure 4.7a & b – Heart 18 ECGs. (**a)** Stabilized with global atrial contraction at HR 112 bpm prior to pacing. (**b**) Prominent wide wave can be seen for Heart 18. Superficial right atrial contraction can only be seen however to produce this ECG reading.

Samples of KHB were taken to the local medical campus under observation of an Atomic Absorption Spectrometer (AAS) for samples ranging from fresh KHB to KHB at 60 minutes to calculate calcium utilization over the course of the Langendorff testing. Results from the AAS would indicate if cardiac death was based on ionized calcium depletion. AAS test ran with 0.05 mL KHB sample titrated with 5mL $LaCl_3$ in which it was diluted 101 times.

Table 4.11 – KHB Calcium Utilization under observation of AAS

Time of Sample collection (min)	AA (mg/L)
0	77.77
10	74
40	73
60	69

Based on Table 4.11, Calcium myocardial uptake had occurred, with 0.2 ± 0.1 mg/minute utilized; calcium depletion was not a factor. Schechter et. al suggested that calcium should steadily increase to avoid toxic effects [80]. Perfusion began with 0.46 mMol ionized Ca^{2+} [80]. 0.8 mMol was recommended in order to have the system operate in a working-heart state. Converting initial KH Buffer prior to reperfusion, 77 mg/L Ca^{2+}

was 0.694 mMol/L Ca^{2+} (1.94 mMol $CaCl_2$). A slow increase of Ca^{2+} in reperfusate was proposed for future testing despite Duke's study using blood and how pure crystalline solutions would differ. Another study with whole blood/KHB-diluted perfusion also showed calcium levels at time 0 at 0.45 mMol. After 15 minutes, calcium level increased to 1.16-1.3 mMol [75]. In du Toit's 1992 study on rat hearts, a low extracellular cardioplegic with calcium level at 0.75 mMol improved cardio output to 91.8%. Reperfusion medium contained 1.25 mMol $CaCl_2$ [129].

4.1.10 Test 10 – February 17, 2015 (Hearts 19, 20)

Highlight of Test 10 compared the effects of Ca^{2+} levels at 0.5mM/L gradually increasing in Heart 19 vs. 1.2 mM/L Ca^{2+} in Heart 20. The results based off the 6L KHB were compared against Duke University's study [80]. 10KU SK in 20°C cardioplegia was also assessed compared to previous tests using 20KU. Also, cardiac recovery and contractile function based on immediate hypothermia (Heart 19) was compared to recovery based on delaying hypothermia to ensure Streptokinase was effective at 20^0C (Heart 20). Heart 19 was cannulated to the Langendorff system with oxygenated KHB at 20°C instead of 37^0C, preventing optimal function. Function recovered with temperature increased after 30 minutes of resuscitation. Heart 19 had limited contractility including appendage contraction displayed. The heart continuously underwent tachycardia at 150-200 bpm despite defibrillation. Procedure and overview results may be viewed in Table 4.12.

Table 4.12 – Procedure with modifications and results for Test 10

Parameter	Heart 19	Heart 20
WIT (min)	2	3
Topical wash	4°C saline	4°C saline
Cardioplegia	4^0 Modified Plegisol	4^0Modified Plegisol
Cardioplegia Additive	10K SK in 4°C Plegisol	10K SK in 20^0C Plegisol
Transport	4°C Plegisol	4°C Plegisol
Flow technique	Aortic cannulation	Aortic cannulation
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	Mannitol (2.92 g/L)	Mannitol (2.92 g/L)
Calcium (mM)	0.5	1.2
Defibrillation	10-50J	10-50 J
Resuscitated period (min)	45	30
Average heart rate (bpm)	100	150

Figure 4.8 – Heart 20 ECG. Course VF was visualized by the two peaks located in QRS wave.

Compared to Test 9, blood clots decreased in post-resuscitation dissection. With 10KU SK directed into each heart as opposed to the 20K-25K previously used, the dosage may not had been effective. For Heart 19, 10,000U Streptokinase at 4°C had no significant effect. Heart 19 resulted with limited contractile function more so than the appendage contraction displayed though the heart continuously underwent tachycardia at 150-200 bpm despite defibrillation. Tepid SK-infused cardioplegia resulted in the highest global contractile function and was to be used for all other future testing. Emboli were

still consistent within coronary vasculature. Heart 19 was added to apparatus at 20^0C and temperature wasn't modified to 37°C until half hour later. Procedure included mounting the heart at starting flow rate of 100 mL/min to avoid reperfusion injury and incrementally increase to 300 mL/min. Investigation on how metabolites in Plegisol affected the hearts' recovery was addressed and would be looked into for the following test. A Medtronic representative suggested that warm SK-infused cardioplegia would provide efficient results and that using a small-point needle will alleviate vasculature emboli. Also, epinephrine or lidocaine was recommended for future testing; epinephrine had been used in Test 1-6 and Test 9 but not for Test 7, 8, and 10 as no positive effects were seen. For all future tests, 37°C buffer must be immediate prior to heart mounting.

4.1.11 Test 11 – March 24, 2015 (Hearts 21, 22)

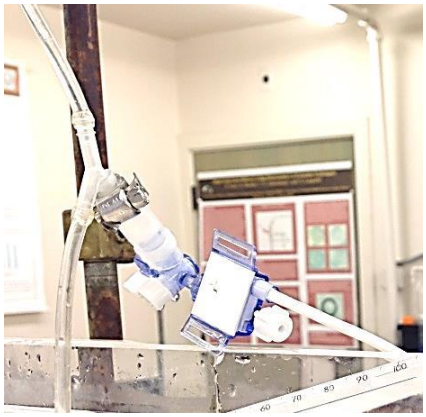
Test 11 compared 36°C saline washout (Heart 21) vs. 4°C saline (Heart 22) for higher cardiac recovery. The test was to compare the immediate cooling to stabilize and lower metabolism output on a cellular level or to keep the vessels dilated in warm saline for blood, metabolite, and air washout and pass easily from microvasculature in heart before cooling. Keeping the vessels dilated in warm saline would increase WIT. Both hearts were still beating when excised and topically bathed in saline. Heart 22 had hypercoagulation, higher resistance, enabling higher pressure during cardioplegic flush prior dilation in arteries due to flow compared to Heart 21. All visible blood in the vasculature was ultimately removed. However, Plegisol flush was recycled from the transport bag mixed with blood and tissue particulates instead of using fresh sterile

Plegisol; It was probable that biologic debris from the nonsterilized Plegisol rerouted back into the vasculature. Procedure and overview results may be viewed in Table 4.13.

Table 4.13 – Procedure with modifications and results for Test 11

Parameter	Heart 21	Heart 22
WIT (min)	3	4
Topical wash	36°C saline	4°C saline
Cardioplegia	Plegisol	Plegisol
Cardioplegia Additive	20KU SK in 20°C	20KU SK in 20°C
Transport	4°C	4°C
Flow technique	Aortic cannulation and catheterization	Aortic cannulation and catheterization
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	Mannitol (2.92 g/L)	5ml epinephrine, Mannitol (2.92 g/L)
Calcium (mM)	1.66	1.66
Defibrillation	10-30J	10-30 J
Resuscitated period (min)	51	0
Average heart rate (bpm)	75	0
Average aortic pressure (mmHg)	N/A	94.8

Heart 21 displayed global contractions during contact with warm saline wash. Appendages continued beating during the cold cardioplegic flush. Contractions indicated ATP usage, depleting its reservoir. Heart 21 was mounted first to the Langendorff system. All previous tests had the most recent heart excised to be the first heart tested on the Langendorff apparatus, decreasing cold ischemic time for the first heart, extending the cold ischemic time for the latter. With this change in chronologic order, both hearts had similar ischemic times.



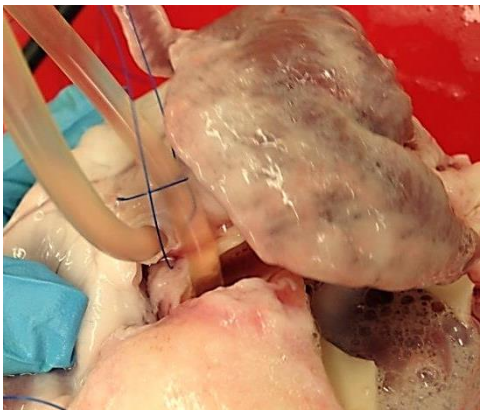


Figure 4.9 – Pressure transducer utilized in Test11. **Figure 4.10** – catheterization via 4 mm tubes inserted directly into coronary ostia.

Heart 21 displayed the first observance of significant apical myocardial movement via fibrillation. However, aortic tissue for Heart 21 was trimmed, severing the ostia towards the Right Coronary Artery. Unable to use an aortic cannula, the aortic valve were sutured and the coronary arteries were directly catheterized though unstable (Figure 4.10). Heart 22 was remarkably less compliant than Heart 21. Aortic pressure could not be determined as the Y-catheter tubing fluctuated for a reliable reading. Despite the catheters sutured into the ostia, back flow prevented normal flow rate into the coronary vasculature and thus no stable contractile function was observed for Heart 21. The pressure transducer was first utilized in Test 11 (Figure 4.9).

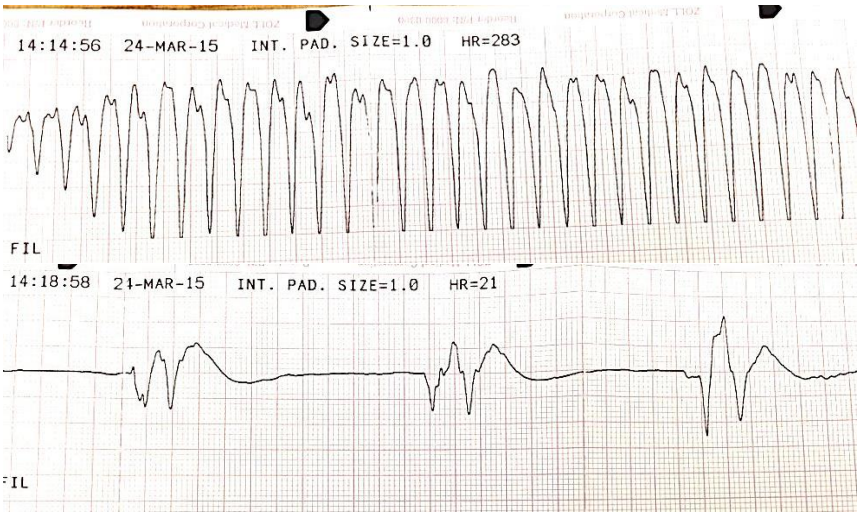
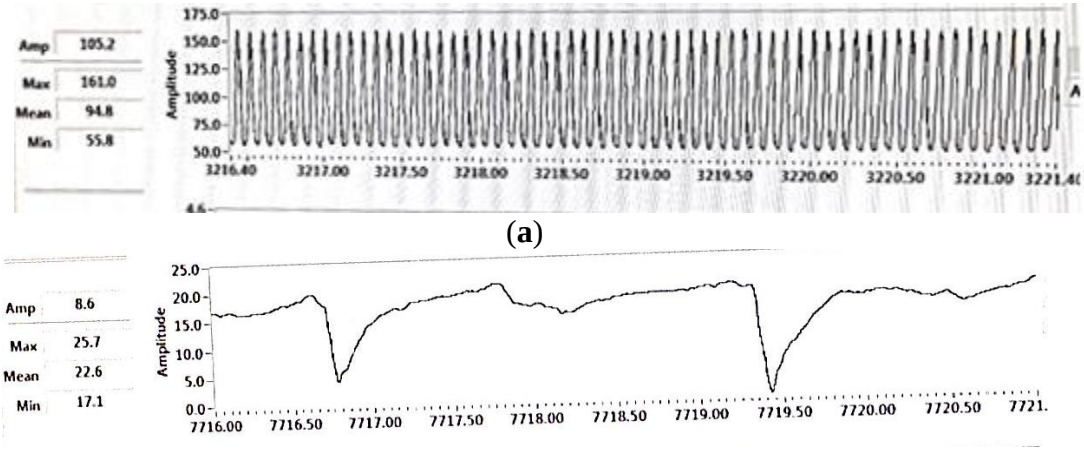


Figure 4.11a & b – Heart 22 ECG. (**a**) Ventricular fibrillation ECG prior to shocking (Heart 22). (**b**) Stabilized bradycardia HR 21 bpm (Heart 22).

Figure 4.11a & b above visualizes both VF with a HR of 283bpm and the onset of poor electrical conduction resulting in bradycardia in the heart within minutes. Cell death progressed via lack of ATP utilization with HR 21 bpm in Figure 4.11b. Calcium overload was a certain factor due to muscular rigor and onset of edema. Partial contractions were absent; osmotic swelling increased.



(**a**)

(**b**)

Figure 4.12 a & b – Heart 23 ECGs. (**a**) Aortic pressure mean data acquired. (**b**) Heavy resistance with decrease in pressure (Heart 22).

Aortic pressure for Heart 22 can be compared from start time at 3,217.0 seconds (53.6 minutes) from Figure 4.12a to end time at roughly 77 minutes, or 7716.75 – 3217 seconds from Figure 4.12b. Average aortic pressure was 94.8, well in the normal physiologic pressure range of 80-120 mmHg. Figure 4.12b has pressure decreasing at a downward rate of becoming approximately 10% of its original pressure with a mean pressure of 22.6mmHg.

4.1.12 Test 12 – March 31, 2015 (Hearts 23, 24)

As Heart 21 was unable to be operated efficiently due to incision of the coronary ostia, warm vs. cold wash saline test was repeated along with finding changes dependent on metabolite washout in recycled Plegisol vs. fresh Plegisol. Similar to the previous experiment, Heart 23 underwent warm saline wash; Heart 24 had cold saline wash. No pacemaker was used after Heart 23 was defibrillated twice. Both hearts showed contractile performance for ≤60 minutes. Procedure & results are shown in Table 4.14.

Table 4.14 – Procedure with modifications and results for Test 12

Parameter	Heart 23	Heart 24
WIT (min)	3	4
Topical wash	30°C saline	4°C saline
Cardioplegia	20°C Plegisol preceded by 4°C Plegisol	20°C Plegisol preceded by 4°C Plegisol
Cardioplegia Additive	20K SK in 20^{0}C Plegisol	20K SK in 20^{0}C Plegisol
Transport	4^{0}C Plegisol	4^{0}C Plegisol
Flow technique	Aortic cannulation and catheterization	Aortic cannulation and catheterization
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	Mannitol (2.92 g/L), 2 ml epinephrine	Mannitol (2.92 g/L), 2 ml epinephrine
Calcium (mM)	1.66	1.66
Defibrillation	10-20J	10-20 J
Resuscitated period (min)	>60	>60
Average heart rate (bpm)	126	58
Average aortic pressure (mmHg)	117.9	N/A

Modifications for Test 12 include Coronary catheterization (Medtronic) at the slaughterhouse and a KHB preflush via Langendorff circulation. Langendorff apparatus continued using an aortic cannula to mount the heart. Test 12 signified the first observance of right ventricle contractility over the left ventricle due to flow dispersion between direct left and right coronary catheterization. Average aortic pressure was 117mmHg, well in the normal physiologic pressure range of 80-120 mmHg (Figure 4.13). Catheterized perfusion of 200 mL was directed in an ostium before switching to perfusion in the other ostium. Both atrial contractions were stabilized.

Loss of contractile function can result from necrosis, decrease in temperature, and obstructed flow in vasculature. Decreased heart activity was compensated by increasing perfusion temperature towards 37^{0}C again. The following test would focus on keeping the lung block intact with the heart during cardioplegic flush as an additional source of oxygen compared to previous tests of immediately excising lungs off.

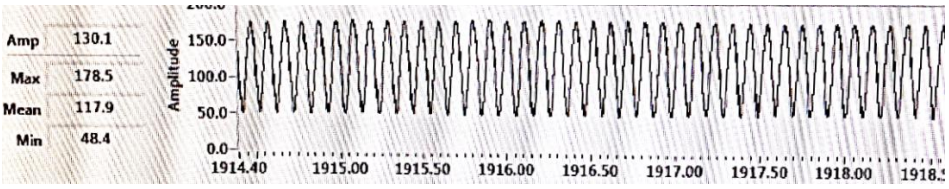


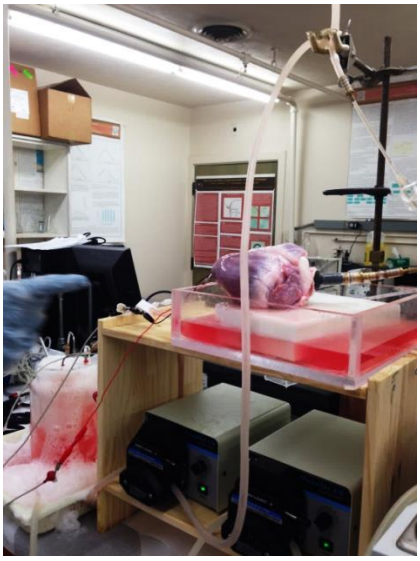
Figure 4.13 – Heart 23 mean aortic pressure.

4.1.13 Test 13 -- April 7, 2015 (Hearts 25, 26)

Test 13 was modified to keep the lung block intact with the heart during cold saline immersion. Balloon catheters with inflates induced antegrade flow towards right and left coronary artery via ostia simultaneously. 100 mL tepid SK-induced cardioplegia was then applied to the ostia one by one before excising lungs to allow direct Plegisol circulation and clear blood from the microvasculature. Preceding lung excision, both arteries were perfused concurrently. During single ostium-perfusion, topical washing and saline sterility was maintained. Workbench chamber where hearts were perfused and submerged in cold solution was intermittently changed and replenished with fresh saline to remove all contact with blood. Hearts stayed beating while flushed with tepid SK-induced cardioplegia with 4°C topical saline. The set up may be viewed in Figure 4.14. Procedure and results may be viewed in Table 4.15.

Table 4.15 – Procedure with modifications and results for Test 13

Parameter	Heart 25	Heart 26
WIT (min)	1.5	3
Topical wash	4^0C saline	4^0C saline
Cardioplegia	20^0C Plegisol preceded by 4^0C Plegisol	20^0C Plegisol preceded by 4^0C Plegisol
Cardioplegia Additive	20KU SK in 20^0C Plegisol	20KU SK in 20^0C Plegisol
Transport	4^0C Plegisol	4^0C Plegisol
Flow technique	Aortic cannulation and catheterization	Aortic cannulation and catheterization
Reperfusion mode (CF or CP)	CP	CP
Krebs Henseleit Additives	4.5 mL epinephrine, Mannitol (2.92 g/L)	4.5 mL epinephrine, Mannitol (2.92 g/L)
Calcium (mM)	2	2.5
Defibrillation	20J	20J
Resuscitated period (min)	72	63
Average heart rate (bpm)	110	92
Average aortic pressure	101	95



(a)

(b)

Figure 4.14a & b – (**a**) Heart 25 Langendorff apparatus set up. (**b**) Anterior zoomed view of Langendorff apparatus and set-up for Heart 25 with emphasis on aortic cannulae placement.

Ionic calcium levels increased from 2.0 - 2.5 mMol prior to Heart 26 mounted on the apparatus. Valves were stitched together as access to the left ventricle was unnecessary

and to eliminate ventricular overdistension. Mitral valve was kept open to eliminate additional pressure. Heart 25 had the highest rate of compliancy compared to Hearts 1-26; Heart 26 had higher resistance. Test 13 enabled both hearts to act as two separate circuits, limiting risk of vessel occlusion and efficient buffer and gas delivery to both right and left myocardial chambers. Reentry heart activity of fibrillation affected Heart 25 and 26. Heart 25 marked the first observance where intermittent defibrillation had the heart stabilized to sinus rhythm without additional aid before pacemaker was applied; global myocardial contractions were observed and constant. Air embolism was successfully removed by the bubble trap. Single-chamber pacemaker with lead inserted into the right ventricular chamber was set at a rate of 100 ppm, output at 15 mA, and sensitivity at 2.5 mV. Under these settings, the pacemaker successfully augmented the pulse. Using the pacemaker, the heart rate was modified by rate setting manipulation, decreasing or increasing stable contractions.

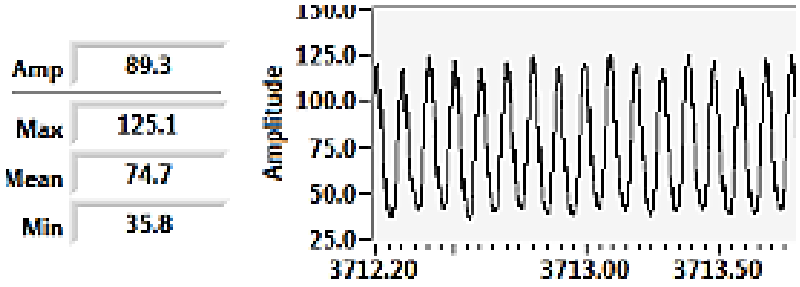
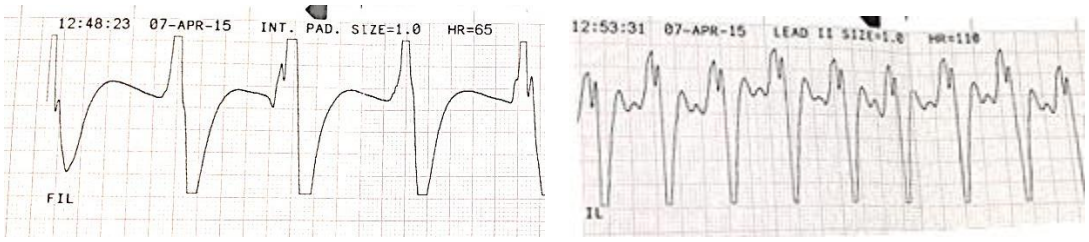
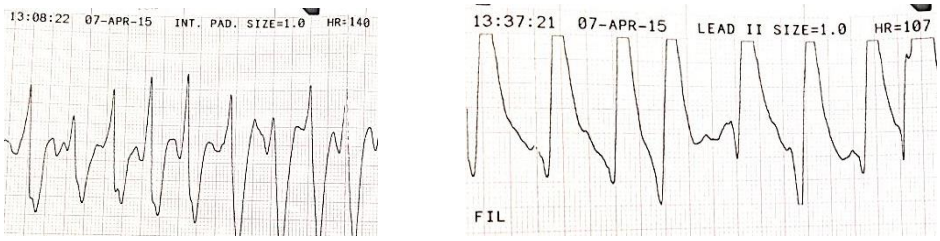


Figure 4.15 – Heart 26 aortic pressure stabilized at 74.7 mmHg at t = 62 minutes.



(a) **(b)**

Figure 4.16a & b – Heart 25 ECGs. (**a)** Stabilized ECG reading over a 15 minute period. (**b)** Ventricular tachycardia leading to fibrillation.



(a) (**b**)

Figure 4.17a & b – Heart 26 ECGs. (**a**) Ventricular tachycardia leading to fibrillation. (**b**) Stabilized heart rate proceeding defibrillation.

Chapter 5: Discussion

Over the duration for the development of the slaughterhouse porcine isolated Langendorff heart procedure, the lab had achieved a beating isolated heart using crystalloid solution with cardiac function restored, global contractions for 1 hour resuscitation, and sinus rhythm at 90-110 bpm. Buffers used for all preservation and resuscitation advancements were stabilized to pH 7.4. The final procedure was secured to use Plegisol only as both preservation and transportation solution. Test 13 achieved <95% global ventricular recovery and compliance. The procedure for the highest recovery entailed the excised slaughterhouse porcine heart with lung block intact. After verifying contractility occurrence, the heart was immediately submerged in 4°C saline to wash out blood as well as lower cardiac metabolic state. WIT was 2 - 3 minutes. Lungs and tissues were originally trimmed pre-cardioplegic treatment for Tests 1 - 12 before the last test and thereafter pulmonary tissue was trimmed after the flush with the aorta trimmed for access of cardioplegic application. 500 mL tepid Plegisol modified with $NaHCO_3$ infused with 20KU Streptokinase was first applied through coronary ostia catheterization retrograde flow via aortic root and washed out blood and metabolic waste. 1L cold Plegisol completed the preservation flush per heart. Hearts were each stored in 1L 4°C modified Plegisol in plastic bags with indirect ice contact, leading to 1 hour cold ischemia during transport back to the lab. 2L modified KHB were used for slow

normothermic rewarming at 15°C and 25°C before the organ was cannulated to the Langendorff apparatus with a 5L modified KHB circulation at 37°C. Flow rate would be lowered to a trickle while mounting heart via aorta before the flow rate was increased to 200 ml/min. The KHB composition may be viewed in Table 5.1. Mean aortic constant pressure was 101.3 ± 1.94 mmHg.

Table 5.1 – Modified KHB per liter

NaCl	118.1 mM
$NaHCO_3$	25.0 mM
KCl	4.7 mM
$MgSO_4$	1.2 mM
KH_2PO_4	1.2 mM
D-Glucose	11 mM
$CaCl_2$	2 mM
pH	7.4
Reperfusion Method	Retrograde with respect to Aorta
Mannitol	16 mM
Oxygenation	95% O_2 and 5%CO_2
Oxygen Pressure	10 ± 1 psi

All hearts underwent ventricular tachycardia prior to fibrillation; hearts were defibrillated at 20 J/shock with 5-7 shocks applied intermittently with a 2-minute delay. Once stabilized, the heart was continuously paced and monitored. Objective of developing a Langendorff apparatus using a blood substitute for slaughterhouse porcine hearts was met with global myocardial contractions and apparent QRS complex in ECG output was paced at 100 bpm. Edema was procured at 1 hour of cardiac activity. The result of the procedure mimicking average *in vivo* heart function exemplifies resuscitation through the effect of hypothermia on metabolism.

5.1 Hypothermia and metabolism

Hypothermia does not cease tissue metabolism but rather prolongs reaction rates of enzymatic activity including degradation. As enzymatic activity declines, the mitochondrial coenzyme used as an energy transfer knowns as Adenosine triphosphate (ATP) is depleted as its precursor Adenosine diphosphate (ADP) inhabits the cytoplasm instead of the mitochondria and thus leans away from homeostatic levels [12]. Hypothermia, though delays general necrosis and can enable intracellular and extracellular edema, acidosis, and glucose utilization. Active pumps are suppressed and oxygen uptake decelerates so that reactions shift towards anaerobic needs. Ischemia-reperfusion injury is derived from anaerobic processes such as lactate acid formation due to inadequate oxygen, or oxidative stress from sudden onset of oxygen [14]. Such injuries can also derive from calcium overloading and acidosis. Examples of oxidative stress are hydroxide radicals, superoxides, hydrogen peroxides, and peroxynitrites. Oxygen stress can lead to alterations of proteins, DNA, and large molecules in select areas of the membrane. To alleviate oxidative stress, glutathione and allopurinol serve as antioxidants for stabilization. Glutathione decreases the toxic free radical effects. Radicals however do open potassium channels, increasing permeability for potassium so that repolarization for action potential occurs. Permeability shifts towards potassium and away from calcium; calcium channels stay closed, decreasing chances for calcium overload. Oxygen delivery must be consistent to decrease the risk of oxygen-related injury. Delivery output of oxygen and other ions are regulated by either constant pressure or constant flow perfusion mode. Factors such as temperature also influence metabolism. A temperature

study on Plegisol indicated that tepid solutions increased contractile function over hypothermia after heart transplantation (89% ± 18% vs 63% ± 25%, *P* = .046) [112].

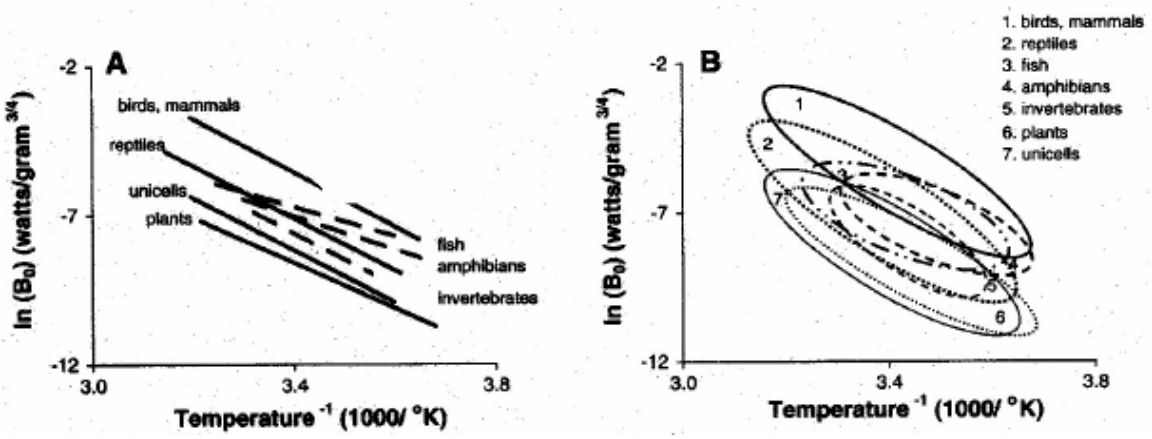


Figure 5.1a & b – Logarithmic values displayed for metabolism of endothermic species with respect to temperature (1000/K) values where ln(B_0) signifies mass-normalized metabolic rates as function for the inverse of temperature. [92]

Figure 5.1 refers to the rate of metabolic process dependent on environment, ranged from 1000/(3 to 3.8) Kelvin (60^0C, 21.1^0C, and 9.8^0C respectively) for resting metabolic rate for warm-blooded organisms. Mammalian values average 39^0C prior to overheating and heat strokes exceeding physiological temperature for humans at 37^0C. As temperature decreases, oxygen consumption and uptake (VO_2) too, decreases. Van't Hoff equation dictates that VO_2 will drop by 50% per every 10°C decrease in physiological temperature. Cardiac arrest via 4°C cardioplegia then enables 97% MVO_2. In Figure 5.2, as a result of cardiac work rather than temperature, the positive correlation on VO_2 is displayed [12].

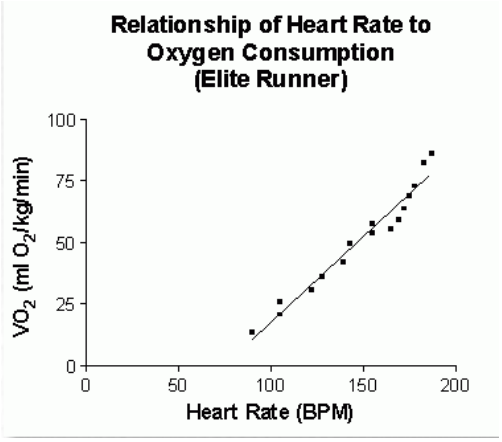


Figure 5.2 – Heart Rate vs oxygen consumption. [130]

Oxygen saturation is based on hemoglobin affinity for O_2 in blood. Saline solutions replicate the physical saturation level by an increase in O_2 pressure. Lower pH is an indicator of anaerobic glycolysis with the increase in H^+ in circulation. However, metabolic changes serve a miniscule effect compared to the over pH of the buffer [13, 14] .

Even the hypothermic process must be looked into as direct contact of ice without solution on an isolated heart can cause freeze damage and tissue injury to myocytes [131]. The following statement from HemoLab was only one example of cooling the isolated organ with both ice and solution contact:

> "The root of the aorta was cross-clamped, and 1,000 mL of cold (4 °C) Plegisol was delivered antegrade to stop the heart. The heart was cooled externally with 4 °C normal saline solution. Once cardioplegia was achieved, the heart was flushed with 1,000 mL of 4°C normal saline solution to remove potassium. Finally, the heart was excised, weighed, wrapped in a towel to avoid direct contact of heart tissue with ice, and placed in an insulated container filled with ice-cold saline solution. The cadaver of the pig was then discarded, and all further experiments were performed on the isolated heart." [42]

Another example was the procedure highlighted by the Texas Heart Institute:

> "The heart may be cooled in 2 ways:
>
> - Blood is cooled as it passes through the heart-lung machine. In turn, this cooled blood lowers body temperature when it reaches all of the body parts.
> - Cold salt-water (saline) is poured over the heart.

> After cooling, the heart slows and stops. Injecting a special potassium solution into the heart can speed up this process and stop the heart completely. The heart is then safe from tissue injury for 2 to 4 hours." [28]

The literature provided from Texas Heart Institute and HemoLab's work were just a few examples in which topical hypothermia was routine for cardiac transplantation. A factor driving resuscitation and normal-level metabolism was calcium.

5.2 Calcium and Calcium Overload

Calcium enables contractions to occur via attachment to the troponin complex that allows myosin to bind to actin in the cardiomyocytes. Physiologically, total calcium concentration in human ranges at 1.4-2.7 mMol/L. The range further breaks down to plasma ranging 2.1-2.55 mMol/L and ionized calcium averaging 1.5 mMol/L [6]. However, physiological proteins are bounded to calcium as well as ionized calcium in the plasma. Without these proteins available, calcium amount was cut in half. Calcium addition to the tested KHB was ranged 1.2-1.25 mMol/L from Test 1 to 7. A summary of the calcium levels for all tests may be reviewed in Table 4.1. Post-study, parameters for biochemical ranges indicated that pig calcium levels were higher than humans, as it ranged 1.78-2.9 mMol/L [53].

The first 7 tests used the ionized value as it was expected only half the calcium level would be needed to eliminate the amount bound to protein. Further investigation proved that the total calcium concentration of 2.1-2.55 mMol/L was dissociated from calcium-bound protein. Calcium was a particular factor that had to be thoroughly analyzed and monitored to prohibit calcium overloading effects. Overloading effect was experienced in the testing via edema production, and decrease of Calcium in the KHB, as referred to in

Table 4.11. By Testing 8 and 9, Ca^{2+} was at 2.5mMol/L but cardiac performances failed at immediate fibrillation. Further calcium was added to the buffer for the second heart for Test 9 (Heart 18) in the even that calcium from Heart 17 performance had decreased calcium concentration. However, the addition of the Defibrillator was not manifested optimally with only a few shocks administrated without training. The onset of Test 10 was to reproduce Duke University [80] test with the recommendation of ionized calcium at 0.4 mMol/L and raised to 0.8 mMol/L prior to stabilized performance. After dissuading results, comparisons to the Duke study were revised and Test 11 and 12 provided 1.66mMol/L. By the last test, Heart 25 was submerged in KHB with 2 mMol/L and Heart 26 to 2.5mMol/L, both with satisfactory cardiac performance.

Calcium overloading may occur are due to a variety of reasons, the main being edema, hyperkalemia, and oxidative stress. Edema is the water diffusion into the mitochondria. Cl^-, Ca^{2+}, and K^+ in the cell draws water in due to ionic bonds and hydrogen polarity[13]. Hyperkalemic solution, in which extracellular Na^+ is high, allows the sodium gradient intracellularly. However, as the abnormally higher intracellular levels occur, Na^+-Ca^{2+} pump forces Na^+ back extracellularly and increased Ca^{2+} are pumped inside the cell. *KHB is an extracellular solution.* Low potassium is unable to repolarize to send Ca^{2+} extracellular and thus more Ca^{2+} enters from both the Na^+-Ca^{2+} pump and the sarcoplasmic reticulum. Reperfusion-induced myocardial injury via oxidative stress leads to mitochondrial permeability transition pore opening [107]. Reperfusion injury enables inflammatory responses towards reactive oxygen species. The induced response may increase the myocardial injury [107]. Other factors include the excess of hydrogen ions [13]

and lactate formation. Oxidative stress on the isolated hearts was possible as oxygenation binding wasn't as competent as *in vivo* nature with regards to red blood cells and will be discussed further in Section 6.1.1. Risk of edema was immediate, which is further explained in the following section.

5.3 Edema

The rate of edema is valuable information that relates to cardiac function and ventricular compliance in which if edema rate is significant, coronary perfusion may increase in resistance and flow becomes obstructed, leading to depressed performance and contractility. Myocardial weight gain percentage can be deduced due to edema. Edema can be determined by pre-experiment heart weight and post-experiment heart weight (Wt_{start} and Wt_{end} respectively) to indicate water content [13]. With an inverse correlation to compliance as the distension of volume due to increased pressure as opposed to a distending force [132], elastance measurements in the form of E_{max} and E_{min} should be sought. E_{min} is the end-diastolic elastance and E_{max} is the end-systolic elastance when used with a three-element time-varying Windkessel model. The model includes arterial compliance and peripheral resistance during any given point of the cardiac cycle [13].

Calcium overloading can also causing edema [5, 6, 8, 13, 14, 55]. Colloid or other oncotic agents can lower water accumulation, such as albumin and mannitol. Albumin—otherwise known as Bovine Serum Albumin (BSA)—eliminates edema by binding to the endothelial glycocalyx, reducing capillary hydraulic conductivity. Poelzing et al. [127] discussed edema in detail in which mannitol in the excised heart could diffuse into the

extracellular matrix; when mannitol accumulation in the extracellular compartment exceeded the vascular mannitol concentration, fluid gradient osmotically leaned towards extracellular space [127]. Edema is without preventability, as it was triggered and advanced during every step of the procedure, from harvest to preservation, to resuscitation. With an osmotic increase in cellular volume, less ADP was produced, causing for lesser heart contractility. In the isolated test experiments, although mannitol was added at 2.62 mMol/L per test and had prolonged edema onset, it ultimately set in by the 1 hour mark. For future studies, mannitol will be replaced by albumin.

5.4 Limitations

Limitations with severe accountability included the inaccessibility of living animal donors under a well-controlled system. Limited by option, slaughterhouse porcine hearts were used. With fresh slaughtered porcine, there was still the advantage of removing the heart with manageable WIT kept under 5 minutes rather than have the ischemic and necrotized frozen hearts supplied to the lab. However, the average 72 minute transport time in lieu cold ischemic time can be decreased, if not eliminated altogether, by using beating-heart donor instead of slaughterhouse-harvested donor. With cardiac resuscitation platform being developed in the founding stage of University of Denver's cardiac biomechanics lab, parameter measurements were limited. Though measurements for hemodynamic flow rates, internal pressures on chambers such as LVEDP, preload, afterload were inaccessible, the parameters are indispensable for the translation from Langendorff to four-chamber working heart mode.

Procedure in preservation tactics while at the abbatoir as well as rapid cannulation became efficient with practice and thus earlier stages of the experiments with longer WIT certainly had negative effects. Practice on such tactics on either 3D heart models or non-fresh non-tested hearts should be necessarily. Equipment such as pressure transducer, defibrillation, ECGs were not established until Heart 13, and pressure transducer even later from Heart 19. Originally heart rate was determined visually until ECG access via defibrillator monitored all readings. Despite internal paddle spoons able to measure heart rate, these were held by hand and readings weren't constant. Thus any friction made by the holder easily disrupted heart rate reading. ECG leads were used for constant measurement and recording but both ECG wire connection shared the same connection as the defibrillating spoons; both could not be operated simultaneously.

Assessment with data acquisition is commonly used as cardiac parameters such as pressure-volume curves and atrial inflow. Left ventricular end-diastolic pressure (LVEDP) is a global indicator for cardiac activity. This pressure is the difference between end-systolic pressure (Psmax) and end-diastolic pressure (EDP): PLVdev= Psmax – EDP. Similar to tension and compression, the heart contraction (dP/dt), relaxation (–dP/dt), and ejection fraction also dictate the rate of cardiac recovery. Other inconvenience included foam particulate production for all tests at an ample rate of the KHB being utilized for the heart. Protein and fatty acid interference on KHB is a viable factor and currently foam formation has been unresolved. Foam may be eliminated by anti-foam agents, usage of a membrane oxygenator, and albumin [35].

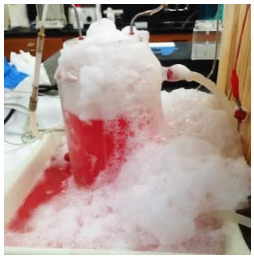


Figure 5.3 – Foam accumulation during experiments for Hearts 22 & 23.

Unlike the standard computational simulation that is easily accessible to modify, the DU Langendorff study collaborated with Innovative Food LLC's slaughterhouse which was undermined by the slaughterhouse's schedule. Though swine was harvested on Monday and Tuesdays, their hours are flexible—pigs may come in the morning only to be succeeded by cattle followed by alternating back to swine harvest, particularly hog scalding at the end of the day; numbers of swine harvest varied week by week and wasn't always accessible. Scalding would occur for the last few pigs harvested and was not an option for the experiments. Due to the distance between lab and slaughterhouse resulting in an hour of driving, the opportunity of testing proved rigid. Windows of opportunity were missed at times resulting in better communications between company and lab. These are not isolated events—expanded time in transit due to external circumstances further delayed testing and increased cold ischemic time for the isolated hearts.

Chapter 6: Future Research Developments

As the Langendorff method of cardiac resuscitation was an initial step towards a physiological function to serve as a platform for further investigative purposes, different fields of tests will be possible. WIT was strictly monitored as slaughterhouse authoritative procedures were inflexible. Hearts were inspected by USDA in which that insight would be harder to obtain if done within lab operatives. With an *ex vivo* platform, the length of experiments will be acute, with resuscitation lasting 4-6 hours if not continuously perfused with blood as it naturally occurs in the mammalian body, such as the properties of the Organ Care System, discussed further in Section 6.3.1. With such limitations annotated in the Langendorff apparatus as discussed in Chapter 5, DU's research aims to eliminate limitations and outline more efficient methods that will enable the beating heart to serve as a platform for further testing and extend the resuscitated duration.

6.1 Optional Pharmaceutical Additives to Langendorff System

Many other agents and additional solution may be added to elongate and enhance current resuscitation time without compromising global contraction function. Under *in vivo* conditions, energy supplied to the heart may derive fully from fatty-acids prior to resorting to secondary substrates such as glucose [133]. Studies show that Heparin, fatty-acids, and moderations in calcium in a working heart configuration were utilized to

recover and stabilize cardiac function up to a minimum of 4 hours [14, 45, 67, 80, 133-135]. However, with an aqueous solution, fatty-acids stress the isolated heart and are insoluble to crystalloid perfusion. Albumin can replace mannitol as a colloid agent, eliminating foam production. Potassium channel openers are also suggested to lessen risk of ischemic-injury and increase cardiac protection [14]. KHB components should be freshly prepared in the lab itself instead of using store-bought KHB mix. Correspondents from University of Alberta [136, 137] recommended keeping the inactive components of KHB in stock, as well as diluting and adding substrates ei. glucose, palmitrate, and sodium bicarbonate prior to usage.

The current procedure utilizes 5L KHB to examine two hearts in sequential order; the second heart tested was in contact with unfiltered soluble metabolic waste from the first heart. Modifications should include fresh modified KHB introduced to the apparatus in regards to the second heart tested: 3-5L per heart will replenish ionic concentrations as well as filter out catabolites from the previous heart. KHB was recommended to be vacuum filtrated. Filters sized 2μm fixed prior to the perfusate entering the oxygenated reservoir can eliminate large particles, tissue debris, toxins, and catabolites.

Oxygen delivery will be further investigated to enhance global contractility so that the heart may serve as a platform beating longer than an hour. An increase in oxygen delivery may be satisfied by the addition of red blood cells or perfluorocarbons. Red blood cells maximize oxygen transport due to the protein hemoglobin. Up to four oxygen molecules are carried on a single hemoglobin unit [5, 6, 14]. Based on its bi-concave shape and lack of nuclei, a single red blood cell can encompass greater quantities of

hemoglobin, specifically 280 million per cell [6]. The release and carrying capacity of oxygen to a hemoglobin protein is dependent on increasing partial oxygen pressure (pO_2). The addition of the natural proteins in blood would alleviate edema by increasing oncotic pressure; coronary flow rates would match physiological speeds. Even if blood is not used, red blood cells in KHB should be filtered as risk of shearing via roller pumps is high. Membrane oxygenators are required for blood use [35].

Oxygen delivery was futile and less than efficient in KHB. Perfluorochemical or perfluorocarbon (PFC) enable twice as much pO_2 carrying capacity in aqueous solutions and may be used as an additive to either the reperfusion or the cardioplegia to lower the risk of acute myocardial infarction (AMI) [35]. PFCs have been implemented in a myriad of preclinical trials as a blood replacement oxygen carrier, where the literature reflects minor side-effects. PFCs are cost efficient, deliver ample oxygen at normal pressures, and prevent edema. For PFC to be soluble in aqueous solutions, an emulsion with surfactant is required. However, PFCs are not FDA approved and are currently under preclinical trials. PFCs are intended for ischemia zones attenuated from postischemic reperfusion injury and prevent ischemic contractures [91]. Increasing oxygen delivery, sterile perfusion, and the directed pharmaceutical agents will allow and enhance the resuscitated heart to serve as a platform for an increased length of time.

6.2 Four-chamber working heart

The Langendorff method can be converted into a four-chamber working heart system in which right and left chambers of the heart are synchronous in cardiac activity, conserving reperfusate in a closed-cycle. Similar to *in vivo* function, blood-filled atria

compress to empty blood into the ventricles, where each ventricle contracts to direct flow from the RV towards the pulmonary system and the LV to the aorta towards the systemic circulation, respectively. Preload and afterloads conditions must be prepared for optimal working heart function. The configuration requires higher energy usage, thus Langendorff perfusion should render cardiac resuscitation stabilized prior to the working heart mode.

The four-chamber working heart perfusion enables complete usage of all chambers and musculature. Whereas retrograde flow of perfusate was the only route via aortic root in Langendorff, the working heart enables antegrade flow out of the left ventricle, past the open aortic valve; as the valve shuts, flow is then directed towards the coronary vasculature. Figure 6.2.2 illuminates the details of a working-heart system that will maximum myocardial performance and global contractile function. Valve activity is based on heart contractions and thus coronary flow is induced by a natural flow and not the roller pump originally used in Langendorff. Based on the Frank-Starling law, increase in preload regulates an increase in muscle contractions via higher chamber filling. Preloads and afterloads must be regulated by the apparatus. Afterload pressure is the resistance to the output ejected via diastolic aortic pressure and will increase cardiac output; however, both overdistention and hypotension can decrease flow and impair contractility. Higher preload and lesser afterload is optimal for a stable cardiac performance [35, 37]. Ejection fraction and stroke volume can decline with the loading pressures reversed with a high afterload and low preload [37, 39]. Global myocardial functions may be assessed by stroke volume and ejection fraction. The apparatus will be

designed to have Langendorff and Working Heart mode easily be converted from one to the other as shown in Figure 6.2.2.

Table 6.1 – Loading pressure constants for cardiac chambers mimicking physiological values [5, 6, 138].

Preload	RA	2-10 mmHg
	LA	5-15 mmHg
Afterload	RV	10-20 mm Hg
	LV	70-110 mm Hg

If 1 mmHg is equivalent to 1.13 cm of the reperfusion height column, aortic afterload must be almost a meter above the heart and be even greater in length if a hypertensive circulation is to be tested. An intraventricular balloon with a catheter attachment acts as a pressure transducer with an end-diastolic pressure of 5-10 mmHg as Table 6.1 indicates so that contractions are easily sustained. The balloon is inserted into the left ventricle via mitral valve; the left appendage may be removed if needed [35]. The intraventricular balloon aids in preloading as it implements the Frank-Starling law (Section 1.4) while accurately measuring LVEDP, contractile function, and heart rate (Section 1.7) [13, 35]. Passive filling of the atria will not determine elastance [37] and thus either a three-point windkessel model or ratio of end-systolic pressure to stroke volume will calculate arterial elastance [14].

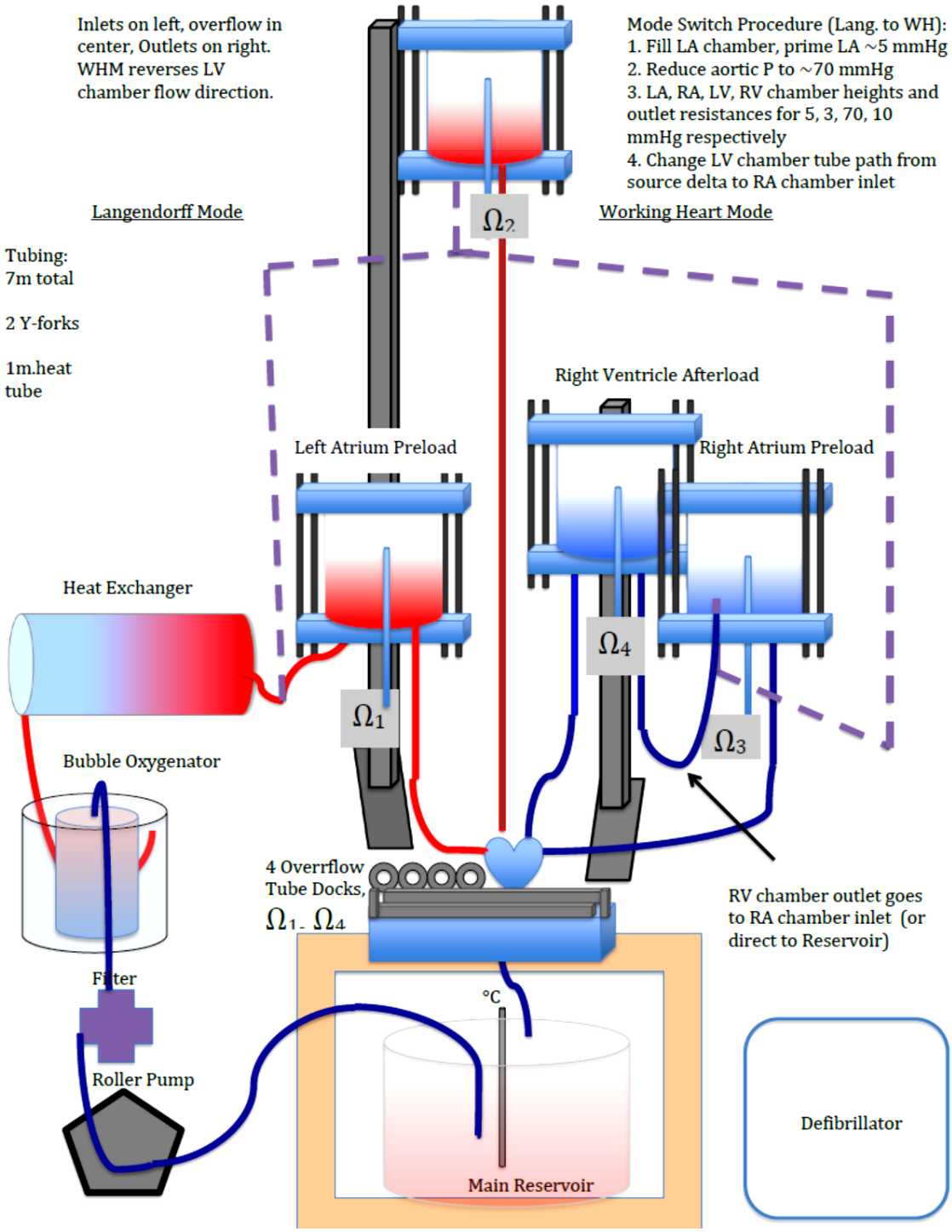


Figure 6.2.2 – Proposed schematic of four-chamber working heart model [139].Used with permission.

6.3 Outreach for collaborations and human hearts

Using the Langendorff configuration that converts to working heart configuration exemplifies further research purposes by becoming a platform to study specific physiology, histology, or mechanical interactions with certain medical devices. Currently, the DU Langendorff system is gaining attention with local clinics interested in investigating ablation therapy specifically to the right ventricle, *in vivo* flow measurements with MRI, and other medical testing and such therapies. Permission was obtained from Colorado Donor Network to use human donor hearts rendered unsuitable for transplants based on satisfactory results with respect to the procedure. Investigations on human hearts may be compared to porcine heart results.

6.3.1 Mobile Langendorff/Working model for faster accessibility and usage

As a single-heart-testing lab-stationed swine model leads to various complexities regarding ionic concentrations, metabolites, and whole cardiac function due to a 1-hour ischemic time, a mobile system would increase not only efficient resuscitation but extend the resuscitation period. To have a mobile system that could be brought into the slaughterhouse would eliminate transport time and by extension, cold ischemic time; the isolated organ would be continuously perfused with warm modified and oxygenated KHB, decreasing the amount of cardioplegic usage. Based on literature found in Section 2.5.1 on varied cardioplegic solutions, warm-blood solution led to 70% functional recovery of left ventricle contractile function as opposed to 46% via cold blood solution [111]. Increasing options for cardioplegic methods allow greater postischemic recovery. Other studies show similar reports with continuous warm cardioplegia such as the mobile

Organ Care System located in the following section (Section 6.3.1.1) with 80-93% LVEDP recovered. For such mobile systems to work, access to set up a station at either the slaughterhouse or in vehicle is required, including but not limited to oxygen tanks, refrigerated solutions that can easily be heated, a sterilized environment including electrical sources.

The proposed mobile device is similar to a product currently in clinical trials in the US and out in market in international countries. Transmedic Organ Care System: Heart ™ (OCS) utilizes continuous warm blood in pulsatile flow of an isolated donor heart. Produced in 2006, instead of hypothermia lowering the metabolism, the OCS continuously keeps the heart beating, ensuring the organ at 37°C and consistently monitors all physiologic activity as it is held in the device's chamber[100]. The heart continues pumping oxygenated blood until transplanted [14]. The OCS leads to net improvement compared to traditional transportation and reduces ischemic time. Using this new technology, hearts can now be transported for successful transplantation past a distance of 450 km (280 mi). Hearts may be transported past local junctions and towards densely populated cities; organs that are able to be preserved and not needed in isolated towns/countries can now be transplanted across water boundaries in which hasn't been done before in the past. Not only does the pool of donor hearts increase in terms of location distribution but also hearts from non-beating donors can be utilized as well as beating-heart donors, including previously-diseased hearts for transplantation. Suggested increase in the amount of donor hearts is expected to be 25% in Europe alone [100, 140]. Total cost of care will be reduced.

The ‘box’ contains OCS monitors, cables, connectors, portable defibrillator, solution transport bags, iStat (ionic analyzer), Alaris infusion pump, pure oxygen cylinder, and filling buffers. The system’s internal memory records all equipment system error and alarm notifications for the heart rate though an SD memory card can be used. Updates are saved automatically every 15 minutes. To add to its accessibility, the box is mobile with wheels. Clinical testing was done in Padua, Italy under Dr. Gino Gerosa, as well as Austria and France; Select hospitals in the US have initiated clinical trials in 2015.

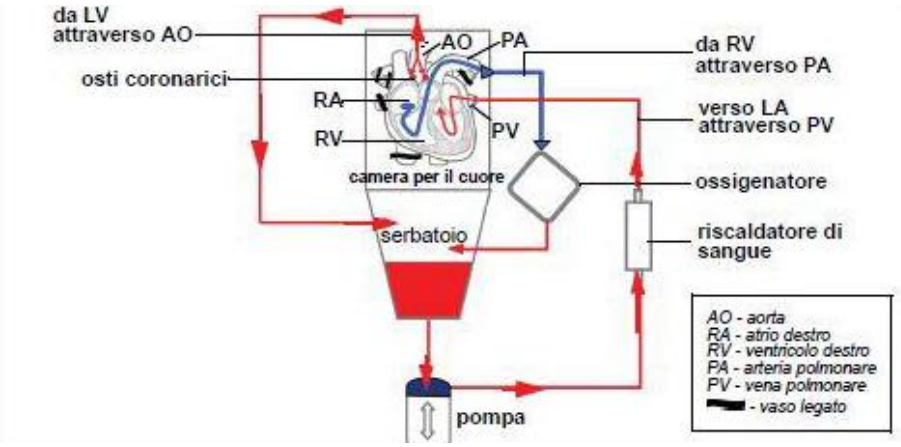


Figure 6.3 – Resting Mode of TransMedic Organ Care System ™.

In the OCS studies, 1.2L blood was taken from the donor at the outlet of the aortic arch. 500mL-1L Plegisol was used as the cardioplegia. Defibrillation was set at 5J and occurs twice and 10 joules once. Catabolite removal hasn’t been secured in OCS until end of transport. However, TransMedic have established a membrane to attach to the interior of the heart walls itself in the OCS in which catabolites diffuses into a dialysis solution to purify the blood in the system cycle [100]. The OCS enforces that mobile Langendorff resuscitation is futile and manageable, increasing the door for heart donors of cardiac transplantation.

Chapter 7: Conclusion

A procedure conditioning the slaughterhouse porcine isolated Langendorff heart for resuscitation using a crystalloid buffer, stabilized global cardiac function, and compliance for 1 hour was achieved. 250-300g hearts were retrieved at the local slaughterhouse, with 2-3 WIT. The lung-heart block was kept intact and was submerged in 4°C saline wash, which cleared blood and decreased metabolic temperature. 20KU Streptokinase in 500ml 20°C modified Plegisol of pH 7.4 was antegrade flushed through the coronary arteries via coronary catheterization. Lung block was dissected prior to storage. Test subjects were stored in 4°C Plegisol with indirect ice contact under roughly 1 hour cold ischemic transport time as it was brought back to the lab. 5L of 37.5°C modified KHB with 2-2.5 mMol calcium chloride dihyrdate served as the reperfusion buffer. Buffer was oxygenated at 9psi carbogen and pH was 7.4. Prior to cannulation to the Langendorff system, the hearts were normothermally raised in temperature at increments of 10°C per 7 minutes. Langendorff mounting system was 45 cm high, under constant pressure with mean aortic constant pressure as 101.3 ± 1.94 mmHg. Air emboli were eliminated and constant ECG was monitored. At start of tachycardia for the mounted heart, ZOLL® defibrillator was applied at 20J per 5 minutes with induced epinephrine until stabilization and normalized heart rhythm. Heart rate averaged 90-110 bpm. At onset of stabilization,

internal pacing was commenced and augmented at 100 ppm, output at 15 mA, and sensitivity at 2.5 mV.

This thesis breaks in piece, part by part of University of Denver's two-year study from background, and preliminary studies which evidently set up the experimental materials and methods. Results from the 13 tests inspired discussion. An isolated ex-vivo slaughterhouse beating model was developed using Langendorff methods and can easily be converted to a four-chamber Working Heart Model. The beating model meets qualifications to serve as a model to mimic the physiological heart in which medical devices and therapies can be tested on to assess design conditions and improvement. Future works include the Langendorff system as a platform for quantitative flow dynamic testing for an implanted transcatheter aortic valve. The thesis written about the Langendorff model does not depict alternatives that were reviewed when determining a future step of action to modify the procedure. An array of over 200 literature was reviewed not only for the procedure modifications but as separate mini studies about smaller fields of the study, whether it be cardioplegia, temperature, pharmaceuticals, procedure, cost-effective alternatives, ordering equipment, and intricate detail that sometimes could not be found in literature. The experience of research in an interdisciplinary field was rich and there can only be progress made as the Langendorff system serves as a stepping stone for greater work.

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Appendix A: Modified Crystalloid Reperfusion Buffer

The table provides additional crystalloid buffer comparisons to the DU Cardiac Lab preparation. The appendix is an extension of Table 3.6 but is by no means exhaustive. All reperfusates are oxygenated with pH 7.4 and temperature of 37°C.

Study	Animal Heart	Defibrillated/ Paced?	Reperfusion Type (Blood, Mixture, KHB)	Langendorff mode (CP, CF)	Reperfusion component (mMol/L)								
					NaCl (mM/L)	$NaHCO_3$ (mM/L)	KCl (mM/L)	$MgSO_4$ (mM/L)	KH_2PO (mM/L)	Glucose (mM/L)	$CaCl_2$ (mM/L)	Colloid agent	Additions
Hans [135] 1978	Wistar rats	300 bpm	Modified Tyrode solution	CF 9.0 ml/min	139	17	3	1 ($MgCl_2$)	-	11 mM, & glycerol-3-phosphate	3	0.08 mM Fatty-acid BSA complex	0.5 mM K^+ palmitate
Visible Heart lab [37, 48, 141] 2000, 2005, 2007	Mongrel swines and human	86 bpm as needed with lidocaine/ epi	Modified KHB	CP 65 mmHg	118	25	4.7	1.2	1.2	11	1.2	16 mM/L Mannitol	10 U/L insulin, 2.27 mM/L Na-pyruvate, 0.32 mM/L EDTA
Varga et al.[142] 2004	Sprague-Dawley rats	Defibrillated	Modified KHB	CP 75 mmHg	118	25	4.7	1.2	0.36	10	1.7	-	2 mg/kg Dexamethasone pretreatment
Sermasappasuk, P. [46] 2007	Rats	-	Modified KHB	CF 9.7 ml/min	118	24.88	4.7	1.66	1.18	5.55	1.5	0.1%w/v BSA	2 mM Na-pyruvate
Minasian et al.[143] 2013	Wistar rats	-	Modified KHB	CP 85 mmHg	118.5	25	4.7	1.2	1.2	11	0.1-2.5 avg 1.5	-	-
Poirier, M., Wagg, C.[136, 137, 144] 2014	Rat	360-400 bpm	Modified KHB	CP 60 mmHg	118	25	4.7	1.2	1.2	5	1.75 or 2.5	3% BSA	0.8 mM palmitate, insulin, 0.5 EDTA