

Aus der Klinik für Kardiologie und Angiologie
(Direktor: Prof. Dr. med. R. Braun-Dullaeus)
der Medizinischen Fakultät

der Otto-von-Guericke-Universität Magdeburg

**Investigations of pharmacological pre- and posttreatments with Omegaven and ATP in
a four-chamber isolated working swine heart model: implications for cardiac
interventions, cardiac transplantation and *ex vivo* perfusion systems**

D i s s e r t a t i o n

zur Erlangung des Doktorgrades

Dr. med.

(doctor medicinae)

an der Medizinischen Fakultät

der Otto-von-Guericke-Universität Magdeburg

vorgelegt von	Maria Sabine Seewald
geboren in	Ulm
Magdeburg	2020

Bibliographical description

Seewald, Maria Sabine:

Investigations of pharmacological pre- and posttreatments with Omegaven and ATP in a four-chamber isolated working swine heart model: implications for cardiac interventions, cardiac transplantation and *ex vivo* perfusion systems. 2020. – 88 pages, 27 figures, and appendix: 9 tables.

Abstract

Myocardial ischemia and reperfusion injury (RI) both negatively affect the outcome of cardiac interventions and transplantation. Therefore, investigations have been attempted to discover drugs for administration prior to organ procurement or for *ex vivo* cardiac perfusion, aiming to maintain and recover functional performance of the organ clinically. Ischemic preconditioning has been utilized to protect the heart from an ischemic event, whereas postconditioning is employed to minimize the consequences of ischemia at the onset of reperfusion. The underlying mechanisms and pathways of ischemic pre- and postconditioning continue to be investigated as therapeutic targets. We evaluated the effects after administration of Omegaven and adenosine triphosphate (ATP) on various parameters associated with RI upon and after reperfusion of isolated porcine hearts. An *ex vivo* four-chamber working swine heart model was utilized to monitor hemodynamic and metabolic measurements. In total, 45 trials were included in the final data analyses. Three major strategies were studied. In the first protocol, pharmacological preconditioning was performed using Omegaven, which was administered *in situ* into the pericardial cradle before the onset of ischemia. In the second and third protocol, ATP was administered either as a postconditioning agent to the perfusion buffer solution in various concentrations immediately before and/or during reperfusion, or was provided as a supplement administered incrementally to the buffer solution shortly following reperfusion *ex vivo*. For the latter, ATP was proposed to function as an inotropic and cardiosupportive drug, due to its various receptor interactions. In general, it was observed that the administration of these agents relative to a transplant scenario could improve organ recovery and functional outcomes. Preconditioning with Omegaven enhanced hemodynamic functions of the reanimated swine hearts. Furthermore, the addition of ATP as a supplement in our experimental studies also elicited beneficial effects.

Keywords

reperfusion injury, pharmacological cardioprotection, pre- and postconditioning, Omegaven, ATP, *ex vivo* perfusion, transplantation

Table of Contents

Index of Abbreviations	5
1. Introduction	7
1.1 Pioneering History in Cardiac Surgery at the University of Minnesota and the Visible Heart® Laboratories.....	7
1.2 Myocardial Ischemia	8
1.3 Reperfusion Injury.....	9
1.4 Ischemic Pre- and Postconditioning	11
1.5 Mechanisms of Ischemic Pre- and Postconditioning.....	13
1.6 Short Review on a Selection of Pharmaceutical Agents	17
1.7 Clinical Applications	19
1.8 Cardiac Transplantation and <i>Ex Vivo</i> Heart Perfusion (OCS™).....	21
1.9 The Agents ATP and Omegaven	23
1.10 Aim of the Study and Hypotheses	26
2. Materials and Methods	28
2.1 Experimental Setup	28
2.1.1 <i>In Situ</i> Setup	28
2.1.1.1 Animal Instrumentation and Parameters	28
2.1.1.2 Heart Explantation and Reanimation	31
2.1.2 <i>In Vitro</i> – Instrumentation and Parameters.....	32
2.1.2.1 Visible Heart® Methodologies	32
2.1.2.2 Instrumentation and Parameters.....	34
2.1.3 <i>In Vitro</i> Isolated Muscle Bath Studies.....	35
2.2 Study Paradigms.....	36
2.2.1 Overview Grouping of Data.....	36
2.2.2 <i>In Situ</i> – PrC Timeline and Study Protocol	38
2.2.3 <i>In Vitro</i> – PoC and Sup Treatment Timeline and Study Protocol	39
2.2.4 <i>In Vitro</i> – PrC Isolated Muscle Bath Study Timeline	40
2.3 Analysis.....	41
2.3.1 General Statistical Analysis	41
2.3.2 Python Programming Language and Application of Machine Learning Algorithms	41
3. Results	44
3.1 Study Population and General Results	44
3.2 Post-ischemic Hemodynamic and Metabolic Analyses.....	46
3.2.1 PrC with Omegaven <i>In Situ</i>	46

3.2.1.1 Hemodynamics and arrhythmia assessment.....	46
3.2.1.2 Metabolism	47
3.2.2 PoC with ATP added to reperfusion buffer.....	48
3.2.2.1 Hemodynamics and arrhythmia assessment.....	48
3.2.2.2 Metabolism	49
3.2.3 ATP Sup	50
3.2.3.1 Hemodynamics.....	50
3.2.3.2 Metabolism	51
3.2.4 PrC - Sup	52
3.2.4.1 Hemodynamics.....	52
3.2.4.2 Metabolism	54
3.2.5 PrC - PoC	54
3.2.6 Sup* - ATP (after at least 3 hr <i>in vitro</i> function)	55
3.2.6.1 Hemodynamics.....	55
3.2.6.2 Metabolism	55
3.2.7 PrC Isolated Muscle Bath.....	55
3.2.8 Support Vector Regression Model	56
4. Discussion.....	57
5. Limitations and Future Directions	63
6. Summary.....	66
7. Zusammenfassung.....	67
List of Figures	68
References.....	69
Acknowledgements.....	79
Ehrenerklärung.....	80
Erklärung zur strafrechtlichen Verurteilung	81
Publications.....	82
Curriculum Vitae	83
Appendix.....	86

Index of Abbreviations

Ach	acetylcholine
AF	atrial fibrillation
ATP	adenosine triphosphate
CK	creatin kinase
Cx43	connexin 43
DHA	docosahexaenoic acid
dPmax/dt	rate of rise in left ventricular pressures, contractility
eATP	extracellular adenosine triphosphate
EF	ejection fraction
EGF	epidermal growth factor
EHA	ecoisopentanoic acid
eNOS	endothelial NOS
ERK	extracellular signal-regulated kinases
ET	Eurotransplant
H ₂ O ₂	hydrogen peroxide
K _{ATP}	ATP-sensitive potassium channels
LAA	left atrial appendage
LV	left ventricular
MDP	median of the differential left ventricular pressures
mPTP	mitochondrial permeability transition pore
mtK _{ATP}	mitochondrial K _{ATP}
NO	nitric oxide
NOS	nitric oxide synthase
OCS™	TransMedics Organ Care™ System
OH ⁻	hydroxyl
PI3-K	phosphatidylinositol trisphosphate kinase
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PLD	phospholipase D
PoC	postconditioning group with ATP
PrC	preconditioning group with Omegaven
PrC-PoC	preconditioning with Omegaven plus postconditioning group with ATP
PrC-Sup	preconditioning with Omegaven plus ATP-supplemented group

RA	right atrium
RBF	radial basis function
RI	reperfusion injury
RISK	reperfusion injury survival kinases pathway
ROS	reactive oxygen species
SAFE	survivor activating factor enhancement pathway
STAT3	signal transducer and activator of transcription 3
Sup	ATP-supplemented group
SVM	support vector machine
Tau	diastolic relaxation
TNF	cytokine tumor necrosis factor
TNF- α	cytokine tumor necrosis factor α
TUDCA	tauroursodeoxycholic acid
UMN	University of Minnesota

1. Introduction

1.1 Pioneering History in Cardiac Surgery at the University of Minnesota and the Visible Heart® Laboratories

The University of Minnesota (UMN) has been a pioneering venue for cardiac surgery and medical device innovation. In 1951, the first open-heart procedure utilizing a heart-lung machine was performed at the UMN. Only one year later, in 1952, surgeons Drs. C. Walton Lillehei and F. John Lewis performed the first successful atrial septal defect closure using deep hypothermia. Moreover, Dr. Lillehei was a great mentor for many surgeons and coworkers who were involved in breakthrough medical advancements. One of his mentees was Dr. Christian Barnard, who completed a two-year postgraduate training in cardiothoracic surgery at the UMN in Minneapolis starting in 1959, and carried out the world's first human heart transplantation in Cape Town in 1967. During his time in Minneapolis, Barnard worked closely with Dr. Norman Shumway who was a resident at the UMN in the same group. Today, Shumway is widely regarded as the father of heart transplantation after he successfully performed the first heart transplantation operation in the United States [1]. One of his daughters, Sara Shumway, followed in her father's footsteps and is currently directing heart and lung transplantation at the UMN.

With the discovery of new surgical methods and approaches at the UMN in the 1950s, the medical device industry experienced substantial progress. On October 31, 1959 there was a power outage in the Twin Cities, causing one of Dr. Lillehei's patients, dependent on a wall circuit pacemaker, to pass away. After this incident, Earl Bakken, one of two founders of Medtronic that provided equipment maintenance services for the hospital operating rooms, was approached by Dr. Lillehei to find a workable solution for pacing the heart without electricity. Within a few weeks, Bakken developed the first battery-powered pacemaker. The device was successfully tested on dogs in the laboratory, in the same facilities that house the Visible Heart® Laboratories today. To Bakken's surprise, when he returned to the lab the next day, Dr. Lillehei had already implanted the prototype into a child overnight to save its life [2]. Medtronic, founded in 1949 as a medical equipment repair shop, rose to the largest stand-alone medical device company in the world, with more than 85,000 employees, locations in more than 140 countries, and holding more than 53,000 patents [3].

The Visible Heart® Laboratories, run by Dr. Paul A Iaizzo, are located in these historic facilities in the basement of the Mayo building. The lab is well known for the Visible Heart®, an *ex vivo* four-chamber isolated heart model that can simulate *in situ* physiological cardiac function. This project evolved from a joint collaboration established between the UMN and Medtronic in 1997. The

laboratory focuses on translational research in physiology and medical device development as well as pharmacological testing [3,4].

Human hearts deemed as nonviable for transplant are donated via LifeSource (St. Paul, MN, USA) for research purposes, making the research translational. Reanimation and formalin fixation to preserve these specimens provide unique insights of human cardiac anatomy. To honor these generous gifts from the patients and their families, the laboratory hosts an open-access heart library within the facilities of the lab and the 'Atlas of Human Cardiac Anatomy' educational website (www.vhlab.umn.edu/atlas).

1.2 Myocardial Ischemia

Myocardial ischemia is defined as a greater myocardial tissue oxygen demand than oxygen supply. During ischemic events, the heart's defense mechanism tends to depress contractility in order to conserve energy. Ischemic events can be the result of cardiac arrest during surgery, a transplant procedure, or occluded coronary arteries, and may consequently cause a variety of ischemic syndromes [5].

The *stunned myocardium* is characterized by postischemic impairment of myocardial function, however this is considered acute and completely reversible. The assumed underlying pathomechanisms are either the formation of free radicals or alterations in intracellular calcium. Additionally, intracellular acidosis can generate calcium oscillation and calcium overload upon reperfusion through the activation of the sarcolemmal sodium/hydrogen exchanger [6]. Physiologically, myocardial contractility is suppressed and can present with systolic dysfunction even though calcium levels are increased. Stimulation of the myocardium with inotropic agents is still possible. Troponin I degradation products may be used as biomarkers for occurrence of myocardial stunning [5,7].

The *hibernating myocardium* also shows reversible depressed myocardial function with different time ranges caused by mechanical occlusion of the vessel. The recovery of function occurs upon reperfusion to the ischemic region and revascularization therapy [8]. The reduction in oxygen delivery correlates to reduced local perfusion that in turn influences contractility. Usually no necrosis is observed in this state, but morphological changes in architecture such as loss of myofibrils and increased interstitial fibrosis may occur [5].

Conversely, the *maimed myocardium* is characterized by irreversible myocardial damage that follows ischemia and reperfusion. The so called myocardial infarct presents with chest pain and is characterized by ischemia-induced necrosis and loss of contractile function. It is considered the most severe ischemic syndrome. Partial recovery is possible following drug administration or mechanical reperfusion of an occluded coronary artery [9].

Silent ischemia is described by single or multiple asymptomatic ischemic episodes that can go mostly unnoticed. Detection of silent ischemia is possible with electrocardiogram monitoring for 24 hours or with exercise/stress-induced assessment, and is typically associated with ST-segment depression. Complications typically associated with these syndromes include cardiac arrhythmias, impaired contractility, reduced pump function, and/or cell death. Because of these complications, there is great clinical interest in protecting the heart from such consequences, summarized by the term *cardioprotection* [5].

1.3 Reperfusion Injury

Reperfusion injury (RI) is a phenomenon by which reperfusion to an ischemic area causes additional injury to the myocardium. Usually reperfusion is ultimately the most beneficial treatment after myocardial ischemia [10]. RI was introduced in 1973 by Hearse et al., and is known as the *oxygen paradox*. The resupply of oxygen to the hypoxic cell activates intracellular processes due to the membrane bound calcium pumps and the contractile apparatus itself. These impacts along with oscillating calcium and increasing intracellular calcium levels cause hypercontracture followed by intracellular edema [11]. Also, xanthine oxidase is considered to cause RI. ATP is catabolized during ischemia and reduced to ADP, AMP, adenosine, inosine, and finally xanthine. In combination with oxygen, the resulting xanthine oxidase releases free radicals. This can be prohibited by administering prophylactic xanthine oxidase inhibitors prior to a planned surgical procedure such as coronary artery bypass graft. Furthermore, additional delayed injury may occur as molecular signals released from injured cells can induce apoptosis in bordering healthy cells [5,10].

The assessment of RI is not trivial, especially in postoperative patients. The relative extent of injury can be indirectly determined by hemodynamic monitoring and examination of blood levels of cardiac enzymes. Myocardial viability can be tested by inotropic stimulation, because only the stunned myocardium will respond with increased heart rate and contractility. Clinically, patients after a coronary artery bypass graft require inotropic support 24-28 hours post-surgery. Importantly, RI can potentially account for an estimated 25-60% of the total infarct volume following transient ischemia and reperfusion [12,13]. Therefore, reduction of reperfusion injury is of great clinical interest not only

for cardiac bypass surgery, but also for acute myocardial infarction. Characteristics of RI after ischemia involve, but may not be limited to, myocardial stunning, arrhythmias, no-reflow phenomenon (microvascular damage), and accelerated cell death [5].

The hypocontractile state of myocardial stunning was discussed previously, and is associated with intracellular free radicals and increased intracellular calcium. Accelerated cell death refers to cells that have been irreversibly damaged. Even though cell death is not always the resulting consequence for those cells, sarcolemmal permeability increases, allowing for uncontrolled calcium influx and resulting in hypercontracture, decreased energy production, and cell death.

Arrhythmias can be caused, similar to the stunned myocardium, by free radicals and oscillating calcium at reflow. Excessive calcium cycling can cause delayed afterdepolarization and ventricular automaticity. Timing, duration, and speed of reperfusion potentially have an influence on the occurrence and severity of induced arrhythmia. Findings in the literature are controversial, but subsequent reperfusion (thrombolysis) in contrast with rapid reperfusion (transluminal coronary angioplasty) might not be as severe as arrhythmia induced during sudden reperfusion. Results obtained in humans are vague and lack evidence [5,14,15].

Microvascular damage and no-reflow occur if attempts fail to reperfuse an area of risk even though the occlusion is removed regionally or the coronary blood flow is reestablished globally. Possible pathophysiologies have multifaceted approaches. A possible reason is endothelial damage that, due to free radicals, causes edema and inhibits the release of vasodilatory agents. Also, ischemic contractures can mechanically constrict flow through the coronaries. Further, accumulated leucocytes can result in vascular plugging and eventual cellular damage caused by mechanical compression (edema). In severe cases, activated neutrophils can adhere to damaged endothelium and cause platelet activation, thus restenosis [16,17].

The post-pump syndrome is triggered by contact with nonendogeneous surfaces such as during cardiopulmonary bypass, causing a circulatory inflammatory response. Cells involved in the immune response tend to accumulate and adhere to the damaged endothelium, migrate into the interstitial space, and liberate free radicals and leukotrienes. This leads to further postsurgical myocardial damage and spread systemically, and can even cause multi organ dysfunction [5].

1.4 Ischemic Pre- and Postconditioning

After discussing the pathophysiology of myocardial ischemia, we raise the question of how to protect the heart from severe ischemia in different scenarios. Decreasing the oxygen demand by deep hypothermia, pharmacological treatments, and controlled cardiac arrest are clinically established. Other cardioprotective approaches are ischemic and pharmacological conditioning of the heart. *Ischemic preconditioning* is a biological, endogenous phenomenon characterized by brief ischemic episodes followed by adequate reperfusion to protect the heart from an extensive ischemic period. It is associated with anti-arrhythmic and anti-ischemic mechanisms of action through various receptor interactions. Also, it reduces infarct size in various animal models and human clinical trials, attenuating RI [5]. First described by Murry et al. in 1986, preconditioning was achieved by four 5-min circumflex occlusions and intermittent reperfusion before a sustained 40-min ischemic insult in a dog model. Murray and colleagues assumed that the mechanism was based on slowing the rate of ATP depletion during subsequent ischemic episodes, and also washing out accumulated catabolites. It could cause myocardial stunning, and thus reduce ATP utilization during the early phase of a sustained period of ischemia among other possible mechanisms [18]. His results were immediately replicable by other scientists in subsequent years. The common understanding at that time was that clinical implementation was not possible until the molecular mechanism could be fully understood [11].

We know today that the underlying mechanisms of ischemic preconditioning are various, including cell-surface receptor activation, survival signaling pathways, and mitochondria as the end effectors. However these pathways can be utilized as a drug delivery target to mimic survival mechanisms pharmacologically, which refers to the term *pharmacological preconditioning* [19].

While ischemic and pharmacological preconditioning have been convincingly shown to delay cell death in various experimental models, their clinical applicability may be limited to situations where the ischemic event can be anticipated (e.g., on- or off-bypass cardiac surgery, percutaneous transluminal coronary angioplasty, or stenting procedures). Therefore, another related mechanism of high clinical interest is *ischemic postconditioning* which utilizes similar molecular pathways. In 2004, Hausenloy et al. published findings about an extended ischemic preconditioning timeframe which is prolonged in the reperfusion time and even after [20,21]. Experiments by Zhao et al. in an open chest dog model found that three 30-sec cycles of reperfusion/occlusion during initial reperfusion produced the same results as ischemic preconditioning. Experiments were replicable, yet there is proven evidence that brief bouts of ischemia, subsequent to a prolonged ischemic event, can confer cardioprotection against RI [21]. The implementation of this cardioprotective therapy at the time of ischemia and shortly after reperfusion allows for precise scheduling and is more easily controlled by clinicians [19,21,22]. There are two windows of protection, the first appearing immediately after the

ischemic event for approximately 3 hours and mediated by effectors. The second window occurs 24 hours after the ischemic insult lasting up to 3 days, and is generated by an upregulation of cardioprotective proteins [5]. See Figure 1 for paradigms of ischemic pre-and postconditioning.

The concept of remote ischemic conditioning has been progressively extended. At present, it is defined as the phenomenon in which brief episodes of ischemia and reperfusion in one vascular bed, tissue, or organ render distant sites resistant to the ischemia- RI, applicable for both pre-and postconditioning. Mechanisms are not fully understood yet which makes it difficult to establish these findings clinically [23].

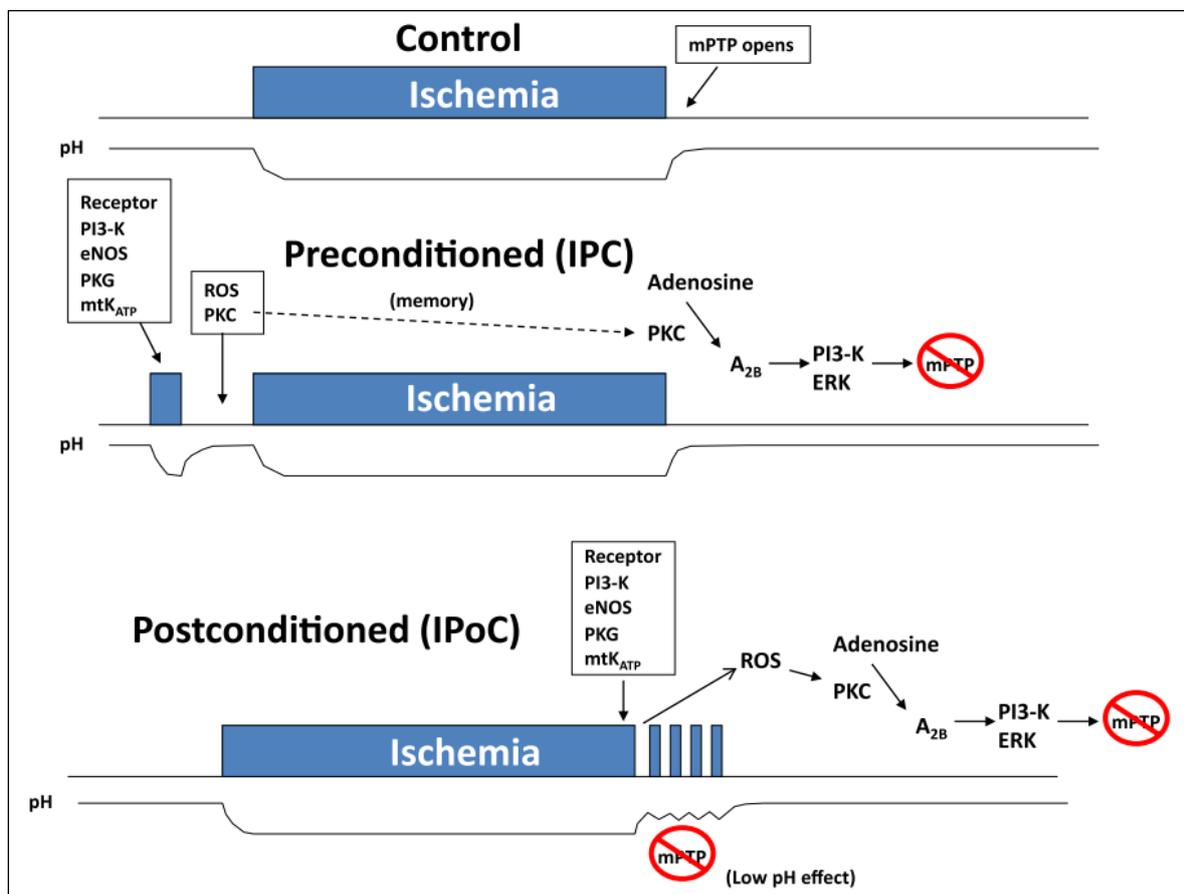


Figure 1: Paradigms of ischemic pre-and postconditioning

Modified from Cohen & Downey 2015 [11]: Signaling during ischemic pre- and postconditioning and effect of pH and transient reoxygenation on that signaling and mPTP formation; eNOS=endothelial NOS; ERK=extracellular signal-regulated kinase; mPTP=mitochondrial transition pore; mtK_{ATP}=ATP-sensitive potassium channels of the inner mitochondrial membrane; PI3-K=phosphatidylinositol trisphosphate kinase; PKC=protein kinase C; PKG=protein kinase G; ROS=reactive oxygen species

1.5 Mechanisms of Ischemic Pre- and Postconditioning

In 1991 Liu and colleagues published the first insights on the mechanism of ischemic preconditioning. They postulated that triggering receptor occupancy, specifically cardiac G_i -coupled adenosine A_1 receptor, in a rabbit model would protect against infarction [24]. In the following years, other endogenous triggers such as bradykinin and opioids were detected as preconditioning agents. All three agents are released from the myocardium during ischemia [11]. In 1995, Goto et al. discussed the necessity of a certain protective threshold to be reached, with all three receptors together or separately, to ensure preconditioning. Consequently, increased numbers of preconditioning cycles could compensate for individual blockage of those receptors [25]. Since all three triggers bind to G_i -coupled receptors, it was likely assumed that the signaling pathways converge to the same destination. Protein kinase C (PKC) was outlined as a major essential component even though the three agents use different second messenger pathways to eventually converge on PKC, see Figure 2 [11].

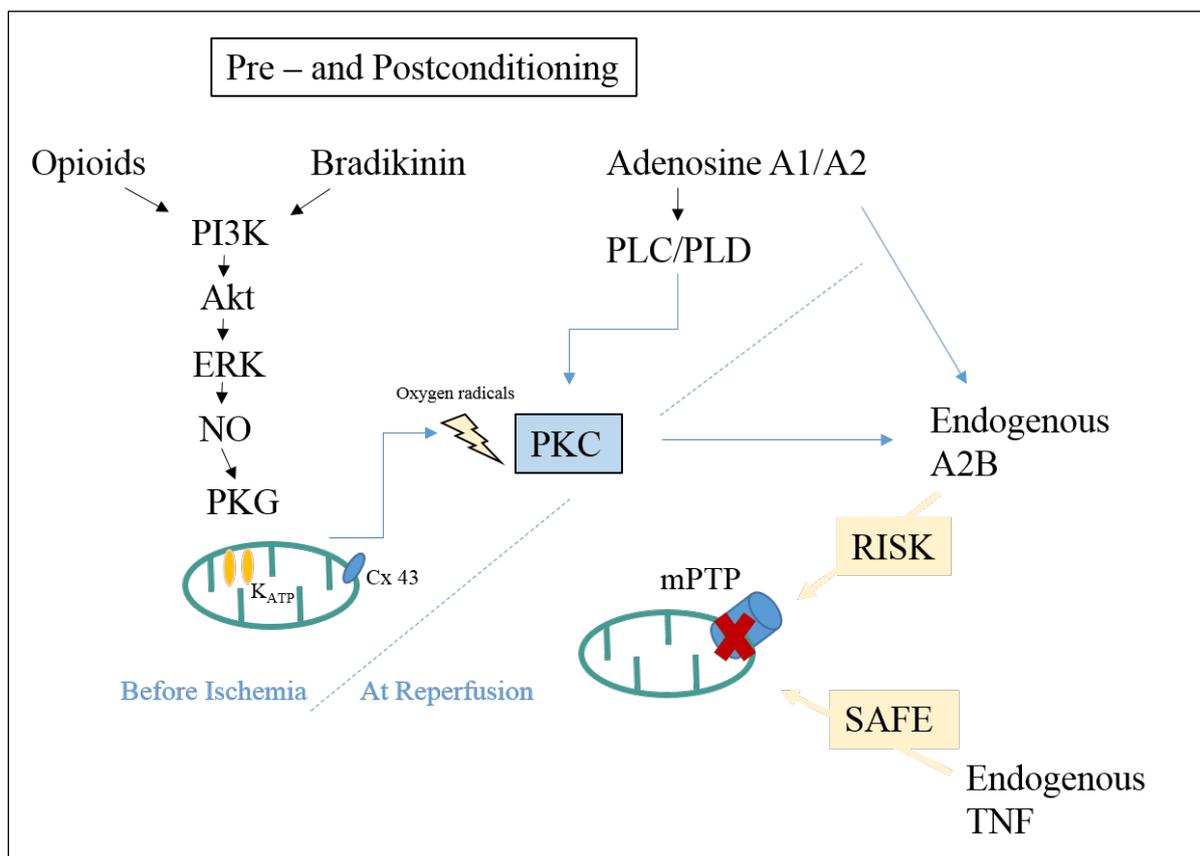


Figure 2: Proposed molecular second messenger pathways of pre- and postconditioning

Modified from Cohen & Downey 2015 [11]; Akt= protein kinase B; Cx43=connexin 43; K_{ATP} =ATP-dependent potassium channel; ERK=extracellular signal–regulated kinases (MAP kinase); mPTP=mitochondrial permeability transition pore; NO= nitric oxide; PI3-K=phosphatidylinositol trisphosphate kinase; PKC=protein kinase C; PKG=Protein kinase G; PLC=Phospholipase C; PLD=Phospholipase D; RISK= reperfusion injury survival kinases pathway; SAFE =survivor activating factor enhancement pathway, TNF=cytokine tumor necrosis factor

Opioids obtain cardioprotection through downstream metalloproteinase and epidermal growth factor (EGF) receptor activation. The same pathway was first mapped by studying acetylcholine (ACh)-receptor activation, but ACh does not endogenously occur during preconditioning. Opioids activate metalloproteinase-dependent cleavage of heparin-binding EGF-like growth factor, which can then further trigger membrane-bound EGFR dimerization. This leads to autophosphorylation of tyrosine residues on both EGFR and binding of sarcoma tyrosine kinase, which in turn attracts and activates phosphatidylinositol trisphosphate kinase (PI3-K) as an important downstream signaling module.

Bradykinin's signaling uses a different metalloproteinase, but the following steps are similar to those of ACh and opioids [11,26,27].

PI3-K-produced metabolites induce protein kinase B (Akt) which is then translocated to the plasma membrane and phosphorylated. This initiates a signaling cascade by activating extracellular signal-regulated kinases (ERK) and endothelial nitric oxide synthase (eNOS); the latter produces nitric oxide (NO).

Endogenous NO is known to be an important biological regulator and signaling molecule that stimulates guanylyl cyclase, and consequently produces cyclic guanosine monophosphate and activates protein kinase G (PKG). Exogenous NO also triggers preconditioning effects even when endogenous NO signaling is inhibited, consequently the role of endogenous NO might be questioned. It is still controversial how endogenous and/or exogenous NO contribute to the ischemic preconditioning process. A PKG-independent NO-mediated signaling pathway was discovered as well, in which NO directly modifies sulfhydryl residues by S-nitrosylation. The latter is important for post-translational protein modification in signaling and increased by ischemic preconditioning. Comprising all different NO-related pathways may contribute to cardioprotection in various situations stimulated by different ischemic events or undefined factors that may determine which pathway to utilize for different scenarios [11,28,29].

The previously described triggering agents open ATP-sensitive potassium channels (K_{ATP}) and allow for redox signaling as the next step in the signaling cascade. Adenosine is the exception, nevertheless both adenosine and K_{ATP} are linked into the same chain activating PKC.

Mitochondrial K_{ATP} (mtK_{ATP}) channels located in the inner membrane were found to be PKG-dependent. The signal from cytosolic PKG is transformed through intermediate steps to reach the mtK_{ATP} . When the channel opens, potassium can enter the matrix along its electrochemical gradient, which is balanced by proton efflux and driven by the respiratory chain. The next important action is redox coupling of mtK_{ATP} channel opening and PKC activation. One theory is that the mtK_{ATP} opening causes matrix alkalization which affects complex I and/or II. This however can cause free radicals, superoxide, and its products hydrogen peroxide (H_2O_2) and hydroxyl (OH^\cdot) radicals. All of the afore-

mentioned steps happen in an ischemic cell except for the burst of reactive oxygen species (ROS) which occurs during reintroduction of oxygen during reperfusion. The relationship among mtK_{ATP} , ROS, and PKC is still poorly understood. While ROS can directly activate PKC, another suspected regulator in this chain is connexin 43 (Cx43). Cx43 makes up most of the gap junctions between cardiomyocytes, and also forms hemichannels on the inner membrane of mitochondria. While considered protective, depletion of those channels attenuates both protection and ROS production. It is suggested that mtK_{ATP} channel opening causes phosphorylation of Cx43 which is required for protection. This indicates some sort of circular signaling circuit as phosphoCx43 is needed for ROS production and ROS causes PKC activation, but PKC phosphorylates Cx43. The definite protective mechanism involving Cx43 is currently unknown [11].

The redox coupling gives an explanation why short periods of ischemia followed by reperfusion are necessary for protection, because only the resupply of oxygen enables the whole mechanism to work. If only a prolonged ischemia time occurs, redox coupling cannot take place. Recent findings indicate that the ROS species generated during ischemia are not capable of redox signaling itself. The ROS involved has not been positively identified, but seems to be a downstream product of hydroxyl radical and is likely a product of phospholipid oxidation. Reperfusion with hypoxic solution did not achieve preconditioning either [30].

Adenosine signaling is different from the aforementioned signaling pathways, bypassing the mtK_{ATP} channel and ROS production, and directly activating PKC in the sarcolemma through G_i -coupled processes and phospholipase C and D (PLC/PLD) activation [31].

Up to the point of PKC activation, all steps occur during the preconditioning cycles of short ischemic episodes followed by reperfusion. Afterwards the so called *mediator phase* is initiated during the prolonged ischemic time. In this phase, increased tissue adenosine utilizing A_{2B} receptors is required. The receptor usually presents with a low affinity to adenosine, but PKC activation most likely raises its affinity, allowing sufficient concentration during ischemia to be occupied. Even though the whole mechanism remains mostly unknown, PKC presumably sensitizes the A_{2B} receptor and responds to endogenous adenosine after sensitization. Consequently the affinity state provides information about whether or not the heart is preconditioned [11]. The so called *reperfusion injury survival kinases* (RISK) pathway is one of many mechanisms possibly involved with varied influence in different animal models. The kinase cascade triggered by endogenous adenosine involves PI3-K, Akt, and ERK [19,32].

The universal end effector of all different pathways is the mitochondrial permeability transition pore (mPTP); its inhibition is considered to be the final step in protective signal transduction. The pore is

described as a high conductance pore in the inner mitochondrial membrane that can eliminate the transmembrane electrochemical gradient, which in turn is responsible for generating ATP. The opened pore causes ATP depletion, enhanced ROS production, and failure of membrane ion pumps, among others right up to mitochondrial rupture leading to necrosis of cardiomyocytes [33]. While acidosis during ischemia can inhibit mPTP formation, increased calcium, ROS, and pH restoration after reperfusion can promote its formation. However, cardioprotective signaling aims to keep the pore closed. The final cytoplasmic kinase might be the glycogen synthase kinase-3 β , also involved in the RISK pathway. Phosphorylation of phospho-GSK-3 β inhibits the kinase, thus blocking mPTP formation. Considering this, GSK-3 β inhibitors given at reperfusion mimic preconditioning [34].

In swine hearts, RISK pathway activation was discovered to be underexpressed during ischemic postconditioning. Consequently investigations were made to find other alternatives [11]. The survivor activating factor enhancement (SAFE) pathway was discovered, but is not yet fully outlined. The identified endogenous cardioprotective agent is the cytokine tumor necrosis factor (TNF)- α , involved in ischemic pre- and postconditioning probably as part of the myocardial inflammatory response during reperfusion. Its effect is thought to be concentration-dependent, in this case only low doses are cardioprotective. The TNFR1 receptor is responsible for exogenous ligands, whereas the TNFR2 is responsible for endogenous TNF- α pre- and postconditioning. Akt or ERK are not phosphorylated, consequently the traditional RISK pathway seems not to be involved. In contrast the transcription factor signal transducer and activator of transcription 3 (STAT3) is phosphorylated, belonging to the JAK tyrosine kinase family and the associated cascade. STAT3 is associated with phosphorylation, indicating that the downstream target might be the RISK. However, RISK and SAFE pathways have the same end target and might even cross talk. Sphingosine, a membrane sphingolipid, is considered a trigger for both pathways, and catabolizes to sphingosine 1-phosphate which is associated with cell survival. It is released during ischemic pre- and postconditioning and couples to the S1P₁ receptor. Downstream activation of PI3-K and Akt indicate that cardioprotection is achieved via the RISK pathway as one potential explanation. Further, TNF- α and STAT3 play a role in this pathway [11].

Ischemic postconditioning utilizes similar signaling pathways as described for ischemic preconditioning with a slight difference in timing. The goal again is to prevent mPTP formation. During ischemia, the mtK_{ATP} channel opens and mPTPs are inhibited by acidosis, but redox signaling is not possible yet due to the lack of oxygen. Upon reperfusion, the pH is usually restored by washing out the acids and protons. For that reason, intermittent reperfusion preserves some acidosis and provides oxygen, enough to activate the PCK through redox signaling while mPTP formation is still inhibited. Consequently inhibition of the mPTP is achieved through similar signaling pathways as previously described even after the pH is normalized, thus reducing necrosis [11,35]. Cohen et al. postulated that postconditioning takes place within the first minutes of reperfusion. Once mPTP

formation occurs, no intervention dependent for its success on keeping mPTP closed would be expected to salvage myocardium in the risk zone [36,37]. Also, Roubille et al. demonstrated that the time window of protection may be larger than initially reported. Utilizing an in vivo mouse model and different protocols, Roubille and colleagues showed that delayed postconditioning up to 30 min after reperfusion did not abrogate cardioprotective effects. With that, he renewed the previous hypothesis that postconditioning is only possible within the first minute during reperfusion [24,38]. However this suggests there must be an additional mechanism of reperfusion injury other than mPTP opening, or that not all cardiomyocytes in the area at risk display the same dynamics of mPTP opening. Also it is possible that cardiomyocytes remain viable and can be rescued after partial mPTP opening [23].

The mechanisms discussed earlier would also apply to remote ischemic pre- and postconditioning. Humoral and neural mechanisms of cardioprotection are discussed in this scenario, and both are considered to be equally potent. Remote postconditioning is time dependent similar to direct postconditioning [39].

Finally, many different pathways and their interactions are involved in the mechanism of ischemic pre- and postconditioning. It is highly possible that pathways remain undiscovered. These are potential targets for pharmaceutical drug interventions to mimic endogenous pathways and better apply the mechanism of ischemic conditioning clinically.

1.6 Short Review on a Selection of Pharmaceutical Agents

Many investigations on pharmacological pre- and postconditioning agents for better clinical applicability have been conducted over the past two decades. Different animal models from mice to large mammals (*in situ* and *in vitro*) and different protocols in timing and dosage were implemented.

The first (and traditional) agents that showed promising results in attenuating myocardial reperfusion injury were adenosine, NO, bradykinin, and volatile anesthetics such as isoflurane. For example, the selective A₁/A₂ (adenosine receptors) agonist AMP 579 has been proven to attenuate reperfusion injury by reducing infarct size in swine when administered either prior to ischemia (preconditioning) or during the early reperfusion period. Similarly, in the globally ischemic and reperfused isolated rabbit heart, administration of AMP 579 at reperfusion decreased infarct size and reduced postischemic contracture [40]. In addition to those studies, J. Yu et al. found that postconditioning with adenosine inhibits inflammation by dropping myocardial NF- κ B and TNF- α expression

significantly in the rat myocardium [41]. Both bradykinin and isoflurane were found to specifically limit infarct size when administered during reperfusion as well [42,43].

Another large group of cardioprotective agents is opioids. It is known, that endogenous opioid ligands are involved in the process of ischemic pre- and postconditioning, making the opioid receptor system a logical target for drug delivery to achieve similar effects. The opioid receptor is composed of seven transmembrane domains and belongs to the class A family of G protein-coupled receptors. These are involved in cardiac stress signaling and cardioprotection by activation of PI3-K/Akt and RISK pathways components [27]. Opioid receptors (δ , μ , κ) can form homo- and heterodimeric complexes, which lead to distinction in different subgroups. Whereas the δ 1-opioid receptor is a μ - and δ -heterodimer, the δ 2-opioid receptor specifically is most likely a δ -opioid receptor homodimer. Different δ/κ -opioids (e.g., remifentanyl, fentanyl, butorphanol, etc.) showed attenuation in reperfusion injury as a pre- and postconditioning agent [16,28,44–46]. It was reported that the addition of DADLE, a δ - and η -opioid agonist, to the reperfusion buffer of a postischemic isolated heart prevented the development of ventricular arrhythmias. However, it should be noted that specific blockade of the η -opioid receptor abolished this effect [46].

Previous work from the Visible Heart® Laboratories demonstrated that preconditioning with the δ 2-specific agonist, Deltorphin D, significantly decreased infarct size in a swine coronary occlusion model, while κ -opioid receptor co-activation during preconditioning exacerbated the ischemic insult [47,48]. In addition to its infarct limiting ability, Deltorphin D preconditioning was also associated with decreased left ventricular (LV) systolic pressure and contractility (dPmax/dt) during ischemia and early reperfusion compared with controls. Similar to our preconditioning studies, a postconditioning study with Deltorphin D showed decreased systolic performance (LV systolic pressure and dPmax/dt) compared to controls during the early reperfusion period [49].

As was found with adenosine [50], Deltorphin D administration attenuated the onset of the no-reflow phenomenon exhibiting a significant increase in coronary flow in the mid-myocardial layer at the 2-hr reperfusion timepoint. Also notable was the decreased incidence of arrhythmias upon reperfusion with Deltorphin D postischemic administration, which supports a previous finding in the ischemic postconditioned isolated rabbit heart [51]. An earlier study from the Visible Heart® Laboratories, using the same isolated swine model of global ischemia, found no reduction in necrosis but improved hemodynamic performance with the opioid morphine or DADLE preconditioning [52]. It should be noted that reperfusion injury is a multi-faceted phenomenon and lack of protection against reperfusion-induced necrosis does not necessarily preclude that reperfusion injury was not attenuated.

Calcium is known to play an important role in the pathophysiology of reperfusion injury, as reperfusion-induced calcium overload is one of the primary instigators of myocardial stunning, reperfusion arrhythmias, and necrosis [5,19,53–57]. Additionally, calcium channel blockers have shown promise in attenuating microvascular damage [58]. Previous studies on isolated cardiomyocytes have demonstrated that δ -opioid receptor activation reduces L-type Ca^{2+} channel currents [59] and antagonizes β -adrenergic-induced calcium fluxes [60].

The no-flow phenomenon was lessened in patients when the K_{ATP} channel opener, nicorandil, a coronary vasodilator and pharmacological preconditioning agent, was administered as an adjunct to reperfusion therapy [61]. Similarly, results from the IONA [62] and CESAR [63] clinical trials demonstrated that nicorandil, when given to patients with unstable angina, decreased cardiovascular events associated with ischemia including a decreased incidence of arrhythmias. Murata et al. reported that opening of the mPTP during reperfusion, secondary to cytosolic and mitochondrial calcium overload, may be primarily responsible for reperfusion-induced cellular death [66]. They also showed that MPT opening is a reperfusion-specific phenomenon that does not occur during ischemia and that diazoxide, a mitochondrial K_{ATP} channel opener, attenuates mitochondrial Ca^{2+} loading, and consequently MPT opening during reperfusion [64].

The aforementioned agents represent a very small selection of agents first identified as effectively proven pharmaceuticals.

1.7 Clinical Applications

Pre- and postconditioning are considered as well-established phenomena, but their use is not yet established in clinical practice. The identification of possible targets, in terms of signaling pathways and their corresponding pharmaceutical agents to follow similar mechanisms, remains a challenging task. Clinical studies have shown controversial results when attempting to translate animal research for human use [65]. Problematic for preconditioning is the timepoint of administration, however there are several applications for both pre- and postconditioning.

An application for ischemic and pharmacological conditioning would be cardiac surgery including coronary artery bypass grafts and valve replacements among many others. Operations using cardiopulmonary bypass offer exact timepoints of aortic cross-clamp and induced ischemia. Clinical studies have been performed, including one by Lu et al. in 1997, where 30 patients undergoing aortic and mitral valve replacements for rheumatic valve disease experienced beneficial effects from ischemic preconditioning. Researchers applied two cycles of 2-min occlusion of the vena cava and

aorta followed by 3 min of reperfusion. Higher ATP levels during cardiac arrest, lower troponin levels, and better myocardial recovery after reperfusion were expressed with better contractility after 30 min [66]. Other studies focused on free radical generation during surgery that could correlate higher concentrations of free radicals in preconditioned hearts with better left ventricular function after recovery from surgery [67]. Adenosine is one of the pharmacological agents mentioned above that underwent many clinical trials for pharmacological preconditioning. Evidence that adenosine has reduced perioperative ischemic myocardial cell death is mixed, even though many clinical studies suggest this correlation. Therefore adenosine is not routinely used by most cardiac surgeons [22].

Patients presenting with myocardial infarction may benefit from pre-infarct angina as ischemic preconditioning. In a clinical setting, post-treatment prior to or during thrombolysis or revascularization is possible to mitigate reperfusion injury [68]. Percutaneous coronary intervention and minimally invasive coronary artery bypass surgery without cardiopulmonary bypass or cardioplegia showed promising results, reducing peri-procedural myocardial infarction and decreasing biomarker levels when ischemic preconditioning was applied before procedures [22]. Heart transplantation is another scenario where pre- and postconditioning could be used effectively, and will be discussed in more detail.

On the other hand, some studies failed to show positive results (or results were ambiguous) when implementing various preconditioning protocols. Surgeons' concerns about causing ischemia stems from the fear of distal embolization of atherosclerotic plaque debris. Furthermore, the current cardioplege technique is thought to adequately protect the heart. More research is required and the demand for pharmacological agents continue to grow [19]. It is controversial whether certain underlying pathologies in addition to cardiac diseases (e.g., diabetes, hypercholesterolemia/ atherosclerosis, etc.) abolish or alter preconditioning effects [22,25].

1.8 Cardiac Transplantation and *Ex Vivo* Heart Perfusion (OCS™)

The Annual Eurotransplant (ET) report from 2018 states that only 619 hearts out of 896 (69 %) reported hearts in the ET zone were actually transplanted, and that the total number of patients waiting for heart transplant is increasing every year [69]. The challenges of heart transplant are not only related to the surgical procedure and follow-up patient care. Waiting lists constantly exceed the number of available hearts. There is a trend worldwide that the number of transplants is declining annually, thus increasing mortality. This is the case for Europe, not as much in the U.S. where the number is fairly stable. In 2018, 318 heart transplants were performed in Germany whereas 719 German patients were actually on a waiting list.

Transplant is widely considered the only long-term therapy and treatment of choice for advanced heart failure, but only a small fraction of patients can be treated with this modality [69,70].

For donor heart selection, there are different criteria that allow potential consideration for transplant, and the criteria vary by country. Safe criteria traditionally include: donor age <40 years, normal cardiac anatomy and left ventricular function, no infection, no coronary artery disease, and appropriate matching in size. Absolute contraindicative criteria are donor sepsis and certain severe infections such as HIV, Hepatitis C, and West Nile Virus. More difficult to weigh are relative contraindications, and these can place the surgeon in an uncomfortable situation. Within the past few years the need for donor hearts has justified extensions of the criteria donation after brain death such as: older donors, from further distances, with lower cardiac function, and higher risk features, such as hepatitis C [71]. Extracranial malignancy, myocardial toxins, advanced age, undersized heart, and prolonged ischemic time are a few examples that make the decision whether to accept the organ for transplant difficult. The challenge is to predict actual organ functionality, which is already variable within the hearts that meet the safe criteria for transplant [72,73]. In these cases, emerging technology can help to assess *ex vivo* function prior to and as a bridge to transplantation.

The TransMedics Organ Care™ System (OCS™) is one system used for clinical trials in this field [74,75]. The OCS™ is a portable perfusion and monitoring system that maintains donor organs in a near physiologic, metabolically active, and functioning state *ex vivo*. The heart is perfused with warm, oxygenated, nutrient-enriched blood and is maintained in a living state until the organ is ready to be transplanted (see Figure 3). Novel research suggests achievement of metabolic homeostasis rather than attempting to limit metabolic activity, which reduces IR injury and improves graft preservation [76]. The number of transplants can be increased due to the ability to preserve more organs and monitor the status of harvested organs. The system further enables *ex vivo* resuscitation of donor organs from the insult of brain death as well as metabolic and functional assessment. Moreover,

ischemic time is significantly reduced. Organs that would have been initially rejected can be verified and rehabilitated before making a final decision on whether the organ meets transplantation criteria. The timeframe between cross-clamp and transplantation significantly increases, allowing for longer transportation time, better recipient recruitment, and preparation for surgical procedures [77,78].

The OCS™ Heart consists of different functional modules. The console is portable and fits within all modes of transportation, originally measured by the size of airplane trolleys. The heart perfusion module provides a sterile blood circuit and protected environment for the donor heart. To optimize heart perfusion, a heart solution set is provided for infusion into the blood that could be considered as a target for pharmacological intervention. A wireless monitor enables the operator to control and display hemodynamic parameters such as coronary blood flow, aortic pressure, heart rate, and aortic blood flow, as well as metabolic parameters such as serial lactate levels, serial arterial-venous lactate differential, coronary sinus saturation, and temperature.

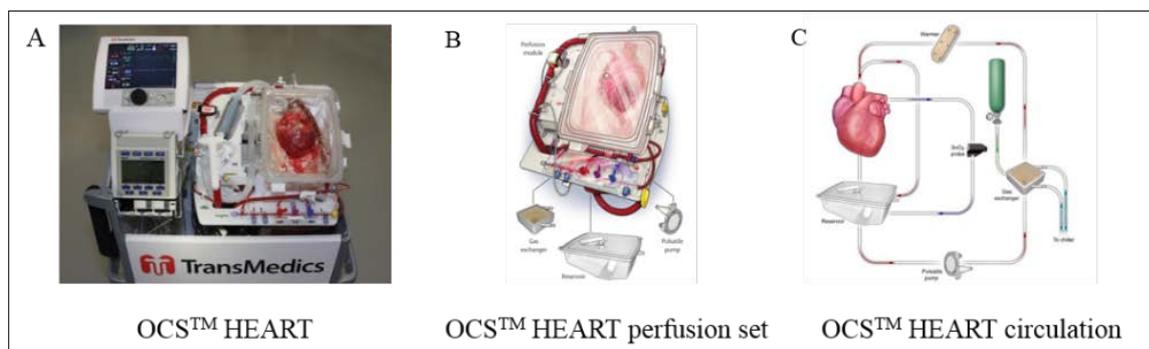


Figure 3: OCS™ Heart

In practice, heparin (10,000 IU) is initially administered and 1200-1500ml blood are collected in 60-90 sec immediately prior to donor cross-clamp from the superior vena cava, utilizing a 34F single stage venous cannula. After cross-clamp, the heart is arrested with 500-750ml cardioplegic solution, immediately explanted, cannulated (aorta and pulmonary artery), and connected to the OCS™ device. Once the heart is reanimated to normal sinus rhythm, the pump flow and solution flow rates can be adjusted to maintain mean aortic pressure of 60-90mmHg and coronary blood flow of 650-850mL/min. Adequate perfusion is verified if the concentration of venous lactate is lower than the arterial lactate concentration [74,77].

As of October 2011, there have been 125 OCS™ -assisted heart transplants on both commercial and trial basis in Europe, and on a trial basis in the United States. Results from the randomized clinical trial PROCEED II, published in 2015, confirm that this technology is highly promising for the future. Short-term outcomes of 30-day patient and graft survival treated with OCS™ and standard cold ischemia were equally successful [74].

1.9 The Agents ATP and Omegaven

Adenosine-5'-triphosphate (ATP) is a highly polar and negatively charged purine nucleotide (molecular weight: 507.18g/mol) and its structure is shown in Figure 4. ATP has long been known as the main intracellular energy source for both mechanical and chemical processes [79]. ATP is formed by glycolysis in the cytosol and under aerobic conditions formed by the mitochondria by oxidative phosphorylation and the electron transport chain. Through the work of Burnstock and colleagues, it has also been found to act as an extracellular signaling agent through purine receptors [80]. There are two main types of purine receptors $P1_n$ (also called $A_{n=1-3}$) whose ligand is adenosine, and $P2$ receptors that use primarily ADP as a ligand. $P1$ receptors are metabotropic (G-protein coupled) and there are both metabotropic and ionotropic $P2$ receptors. Ionotropic $P2$ receptors are designated as $P2X_{n=1-8}$ and metabotropic $P2$ receptors are designated as $P2Y_{n=1,2}$ [81]. Through these receptors ATP has been shown to mediate inflammatory processes [82] and pharmacologic preconditioning [11]. These extracellular responses are the basis for newfound interest in the potential clinical applications and use of ATP as a cardioprotective agent.

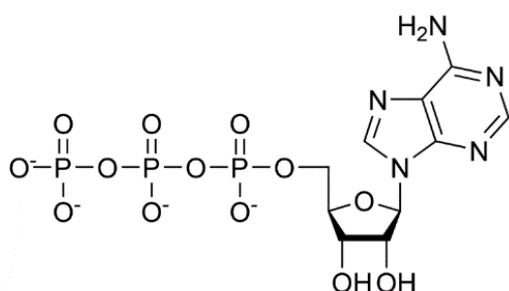


Figure 4: Chemical structure of adenosine-5'-triphosphate

ATP has been shown to activate $P2Y_1$ receptors leading to a G-protein mediated increase in PLC which activates PKC to produce ROS. This action could potentially mediate conditioning via subsequently sensitizing the adenosine receptor A_{2b} [83]. Adenosine then initiates the RISK signaling cascade that ultimately closes the mPTP pore, the beneficial step of postconditioning in skeletal muscle. It was hypothesized that ATP may sensitize this receptor in swine skeletal and cardiac muscle and potentially mediate conditioning in a reperfusion injury model. However, little work has been done in the area of defining ATP as a pre- and postconditioning agent in the porcine model.

The pharmacology of extracellular ATP (eATP) is complex, in that there are many receptors that utilize eATP as a ligand and also break down products of eATP that activate other G-protein coupled receptors. In order to understand how eATP works, it is beneficial to know which receptors are present in the human myocardium and their relative abundance. A study by Musa and colleagues

quantified mRNA transcripts of subtypes of ionotropic (P2X) and metabotropic (P2Y) receptors in the human sinoatrial node and right atrium. They found that P2Y₁, 2 and 14 were the most common metabotropic receptors in the human myocardium, and that the P2X₄ was the most common ionotropic purine receptor in the human myocardium [84].

ATP has been shown to activate the ionotropic P2X₄ receptor, and this activation mediates cardioprotection in mice that were induced to overexpress this receptor [85,86]. In this study, cardiac function and survival improved in the group of mice that overexpressed P2X₄. Also, in a murine model of heart failure treatment with MRS2339, a P2X₄ non-hydrolyzable agonist was found to confer protection from heart failure [87]. All purine receptors are found to be expressed at the mRNA level in the human left ventricle. It should also be noted that in human right atria and sinoatrial node tissue, the most expressed ionotropic purinergic receptor is P2X₄, followed by P2X₇ with notably lesser amounts of P2X₁, 2, 3, and 5. This was found utilizing quantitative r-polymerase chain reaction techniques [84]. Together, these results suggest that P2X₄ activation is one of many pieces of the puzzle as to how endogenous extracellular ATP exerts its protective effects in attenuating IR injury. It is thought that activation of P2X₄ increases the activity of the Na/Ca exchanger, thus increasing the level of intracellular NO. It subsequently leads to the signaling cascade that mediates the closure of the mPTP [88].

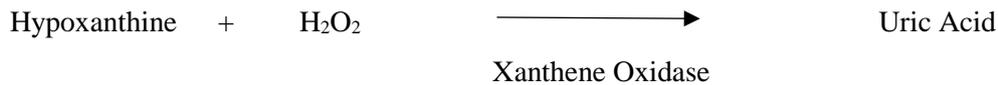
The second most abundant receptor in the human sinoatrial node and right atrium, P2X₇, was found to mediate both preconditioning and postconditioning in rat hearts on the Langendorff apparatus and was observed to interact with pannexin-I hemichannels to release various cardioprotective cytokines [89]. Taking into account the relative abundance of this receptor in the human heart, this may be yet another cardioprotective mechanism that eATP could be activating in the human myocardium. In addition to mediating the closure of the mPTP pore, ATP mediates beneficial immunomodulatory actions that may help to limit vascular injury. ATP is rapidly degraded in the vasculature by the ecto-ATPases CD39 and CD73 to adenosine which inhibits the production of cytokines implicated in inflammation [90].

To our knowledge, ATP use as a cardioprotective agent has not been investigated in a porcine model of IR injury. The numerous potential benefits of ATP and its analogs in previous studies guided the decision to condition swine hearts pharmacologically with ATP and investigate its potential in the current study.

Omegaven was chosen as a preconditioning agent to limit oxidative stress on the heart given prior to explantation, to limit reperfusion injury as oxidative stress has been implicated as one of the many mechanisms behind this pathology. Omegaven is an emulsion of unsaturated fatty acids that is

available for parenteral use and contains mostly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [91].

Under hypoxic conditions, ATP in the myocyte is depleted and converted to the nucleotide precursor hypoxanthine. When reperfused, the accumulated xanthine is rapidly converted to uric acid and hydrogen peroxide (H₂O₂) by the enzyme xanthine oxidase which requires oxygen. It is thought that Omegaven sequesters itself in the cell membrane and acts as a free radical scavenger.



Previous studies in the Visible Heart® Laboratories reported antiarrhythmic and infarct limiting effects of omega-3-fatty acids [14,92]. It was also observed that omega-3-fatty acids prevented edema on an *ex vivo* apparatus and maintained cardiac function for a longer time.

Pericardial delivery avoids possible systemic side effects such as reduced blood pressure or hemolysis when given intravenously. Omegaven has been investigated as a potential beneficial agent in ovarian ischemic reperfusion injury in rats. Grungor and colleagues found that high doses of Omegaven reduced the severity of injury measured by tissue scores and reduced the levels of reactive oxygen intermediates [93]. A study by Byne and colleagues evaluated neutrophil adhesion and diapedesis in rats undergoing cardiac procedures. They observed a fourfold decrease in neutrophil adhesion with Omegaven treatment compared to control. It was also found that damaging enzymes released through degranulation were minimized by treatment with Omegaven, most notably myeloperoxidase. Omegaven can also work by reducing inflammation induced by ischemia. This is thought to be initiated by an upregulation of neutrophil adhesion molecules and suppression of inflammatory processes. By inhibiting neutrophil adhesion and degranulation, myocytes are not subject to high concentrations of myeloperoxidase, elastase, and cytokines [94]. Furthermore, another study by Sukhotnik et al. demonstrated a beneficial role of omega-3-fatty acids in an ischemic rat model of intestinal IR injury. It was found that there was an increase in proliferation of intestinal cells, indicating that the tissue was more protected compared to control animals [95].

In summary, drawing upon previous studies it is likely that in cardiac tissue Omegaven may have a dual mechanism. It may primarily act as an oxygen radical scavenger of breakdown products (H₂O₂) from intracellular ATP metabolism. It may also decrease the inflammatory responses that are triggered by an ischemic event.

1.10 Aim of the Study and Hypotheses

Since the discovery of ischemic pre- and postconditioning, there has been extensive interest in elucidating the biochemical pathways involved and finding pharmacologic agents that can be used clinically to mimic the benefits without occluding a vessel. In general, preconditioning agents have the potential to be used as a pretreatment for organ procurement and in open-heart surgeries. Postconditioning agents could be administered after an acute myocardial infarction and during reperfusion in several clinical scenarios to limit tissue damage and infarct size. It is important to review the present state of knowledge and involved pathways to understand which pharmacophores would be most beneficial. All of these pathways have been implicated in increasing the activity of PKC and subsequently ROS. This leads to sensitization of the A2b receptor which activates the postconditioning pathway and mPTP closure. Other posttreatment protocols are beneficial when administered after surgical procedures and on *ex vivo* perfusion devices.

This thesis contains several aspects of the above described mechanisms and models in different study approaches. We utilized *in situ* and *ex vivo* swine models to investigate the role of:

- Omegaven as a potential preconditioning agent;
- ATP as a postconditioning agent;
- ATP as supplement administered immediately after reanimation incrementally over 2 hours to preconditioned hearts;
- ATP as a supplement administered immediately after reanimation incrementally over 2 hours to non-preconditioned hearts;
- ATP as a posttreatment after time on an *ex vivo* apparatus (preliminary data); and
- ATP as preconditioning agent in isolated tissue bath studies (preliminary data).

This study mirrors a cardiac transplantation scenario. While the *in situ* setup studies applications for the donor, the *ex vivo* heart perfusion setup investigates potential for the recipient or the organ on an *ex vivo* perfusion device. Therefore, *in vitro* hemodynamic and metabolic data were analyzed for both approaches to evaluate potential cardioprotective effects. Additionally, swine diaphragm obtained *in situ* was utilized to study ATP as a preconditioning agent in isolated tissue bath studies.

Due to the lack of red blood cells and hemoglobin in the buffer, oxygen supply over time on the apparatus is insufficient. Thus the apparatus can be utilized as an acute progressive, global ischemic heart failure model. Another rewarding aspect of this study was to improve the Visible Heart®

apparatus, to sustain organ function longer in order to support a variety of other research projects that utilize this model (e.g., device testing and imaging).

The obtained dataset was applied to various analysis tools beyond standard methods. Machine learning algorithms, support vector machines, and cross-validation techniques were used to create predictive models, investigate correlation between certain aspects of the data, and gain further insight into the predictive capacity of measured parameters.

The main hypotheses for this study were to test the potential benefits of: (1) Omegaven as a pharmacological preconditioning agent, and (2) ATP as a pharmacological posttreatment targeting different mechanisms of action, including traditional pharmacological postconditioning and cardiosupportive postischemic administration therapies *ex vivo*.

2. Materials and Methods

2.1 Experimental Setup

2.1.1 *In Situ* Setup

2.1.1.1 Animal Instrumentation and Parameters

This research was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota, and followed principles in the Guide for the Care and Use of Laboratory Animals.

For the first part of the *in situ* study, Yorkshire pigs (70-90 kg) were sedated and anesthetized. An intramuscular injection of 5-7mg/kg Telazol™ was followed by 5-7mg/kg methohexital intravenously utilizing an ear vein catheter. After intubation with an endotracheal tube, anesthesia was maintained with isoflurane (> 1.2 MAC) delivered in a mixture of house air and oxygen (S/5 Avance; GE Healthcare, USA). Then instrumentation of both sides of the heart was executed (Figure 5A-C).

The right external jugular and carotid arteries were accessed with cautery and blunt dissection. An 8.5F Swan-Ganz catheter (Optiq; ICU Medical, San Clemente, CA, USA) was placed in the external jugular, then transversed through the right atrium, the right ventricle, through the pulmonary valve down the pulmonary artery until a wedge pressure was noted. This enabled monitoring of the right atrial pressure, pulmonary artery pressure, and wedge pressure (or left atrial pressure). An additional 5F balloon pressure catheter was placed in the external jugular and into the right ventricle to continuously record pressure (Attain 6215; Medtronic, Minneapolis, MN, USA). The left heart was instrumented with a 5F pressure catheter (Venous; Oscor, Palm Harbor, FL, USA) into the carotid artery, and prolapsed through the aortic valve to record left ventricular pressure. Pressure catheters were flushed with heparinized saline, connected to a pressure transducer, and all data were recorded (iox2, EMKA Technologies, Falls Church, VA, USA). For peripheral monitoring, a branch of the femoral artery was accessed for real-time blood pressure or blood gas monitoring (18F Gelco-catheter). Heart rate and temperature were recorded during the procedure (Ultraview SL; Spacelabs Healthcare, Snoqualmie, WA, USA).

Most data were recorded, stored, and processed with the EMKA technologies system to enable powerful data acquisition and real-time analysis. The program includes predetermined calculations of various measurements and parameters. For example, left ventricular pressure data included maximal/minimal systolic/diastolic pressures, maximal/minimal slope, and differential pressure, among many other calculated features. For this study, the program was set to average obtained data

over a 5-sec time interval. Both real-time data and processed data were included in our final analysis.

The next steps involved a median sternotomy and creation of a pericardial cradle (Figure 5C). After the medial surgical incision, a sternal saw was used to remove the anterior protrusion from the sternum (keel bone) and for partial medial dissection of the sternum from the xiphoid process to near the insertion of the sternocleidomastoid muscles. Dissection of the sternal-pericardial ligament and retraction of the sternum followed. Blunt dissection was used to separate the pericardium from the pleural lining. Then a 3-5cm incision was made into the pericardium, and a pericardial cradle was created with four square knot sutures.

A unipolar plunge temporary pacing lead (Atrial 6492; Medtronic) was placed into the left atrial appendage (LAA) to induce atrial fibrillation (AF) according to the study paradigm. This was connected to the breakout box (Ensite®, St. Jude Medical, St. Paul, MN, USA) and to a Grass stimulator (Grass Technologies, West Warwick, RI, USA), which in turn was attached to an oscilloscope (MSO; Tektronix, Beaverton, OR, USA) for electrical verification. The parameters for the Grass stimulator were optimized to deliver 4 H with a 2-sec pulse duration to the LAA via the plunge pacing lead. A single pulse was delivered to the LAA to induce AF. To determine relative AF burden, the animal's heart was considered to be in sustained AF after 1 min, and was allowed to remain in AF for up to 10 min. If it exceeded the predetermined timeframe of 10 min, a 5 Joule shock was conducted to terminate AF (LIFEPAK®; Medtronic). AF was induced up to 5 times at scheduled timepoints in cases where the heart did not stay in AF for up to 1 min.

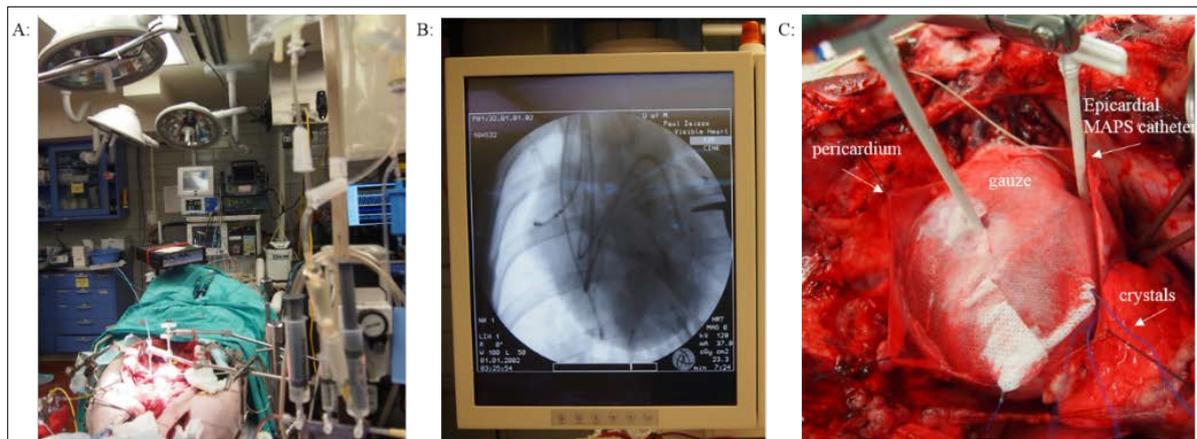


Figure 5: In situ setup

A. Instrumentation in situ; B. Instrumentation under fluoroscopy; C. Pericardial cradle

Contractility data were collected by placing 4 piezoelectric sonomicrometry crystals (Figure 5C). Piezoelectric crystals transmit ultrasound signals through tissue to other crystals that receive the signal. Between two crystals, a distance measurement could be determined based upon the travel time

of the ultrasound signal and fiber orientation of the tissue. Crystals were placed on the epicardial surface of the left ventricle to obtain pressure-volume loops and ejection fraction (EF), ejection volume, and minimum/maximum contractility; relaxation was determined utilizing an oval-shaped three-dimensional model for calculations.

Furthermore, echocardiography utilizing a Philips Ultrasound machine (Amsterdam, The Netherlands) calculated EF% (Figure 6). Therefore, a transthoracic echo probe was placed directly on the heart, capturing a long- and short-axis view. The EF was calculated in long-axis view at predetermined timepoints over the course of the study. Therefore, left ventricular volume was traced and the following equation was applied: $LV \text{ end-diastolic volume} - LV \text{ end-systolic volume} \times 100 / LV \text{ end-diastolic volume}$.

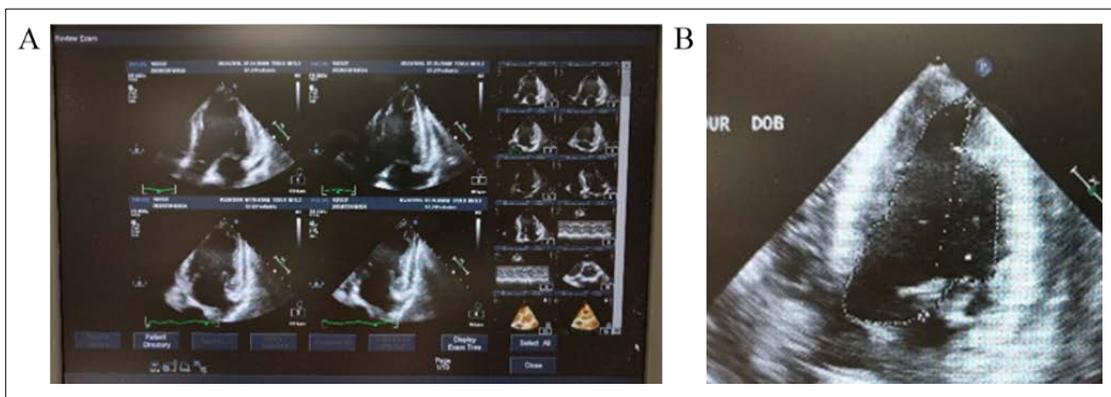


Figure 6: Calculation of ejection fraction using echocardiography images

For an electrophysiological study on arrhythmia and monophasic action potentials, MAPS4-catheters (Medtronic prototypes) were placed endocardially into the right atrium and ventricle utilizing fluoroscopy, and epicardially on the surface of the left atrium and ventricle (Figure 5B). Myocardial metabolism was monitored by arterial and venous blood sampling every 10 min, as well as after AF burden. Therefore, a steerable catheter (C304; Medtronic) was placed in the coronary sinus. To sample arterial blood, an outline on the right femoral artery was created. A Radiometer ABL 90 blood gas analyzer (Brea, CA, USA) was used for final assessment and analysis of lactate, glucose, bicarbonate, and various electrolytes [96].

2.1.1.2 Heart Explantation and Reanimation

After the predefined time of the *in situ* protocol, the heart was explanted utilizing clinical transplant standards. Pericardial tissue around the ascending aorta was dissected and the pericardium was removed. Two suture points (2-3cm apart) of 2.0 Ethibond suture were placed in the ascending aorta, and 30,000U of heparin were administered intravenously. An aortic root cannula (DLP®; Medtronic) was placed between the suture points and secured to the aorta. The stylet-bevel was removed and a clamp placed on the cannula to cut off flow, with the delivery system pressurized. To arrest the heart, a high potassium cold St. Thomas cardioplegia solution was administered into the ascending aorta under a pressure of 150mm/Hg with a pressurized bag [97]. The inferior vena cava and ascending aorta (superior to the aortic root clamp) were each clamped with a DeBakey 1-2cm clamp. Next, we removed the clamp from the aortic root cannula to allow cardioplegia to flush the heart at 150mm/hg towards the aortic valve. The pressure forced the valve to stay closed, hence perfusing the coronary arteries. The superior vena cava was also clamped, and a small incision was made in the pulmonary artery to prevent overpressurization of the heart. The heart typically stopped within 1 min, at which time it was removed from the chest. While the heart was placed in a bath of cold modified Krebs-Henseleit buffer (3-8°C) [98], the inferior vena cava, pulmonary artery, right and left superior pulmonary veins, and aorta were cannulated with clear tygon tubing. In addition, the superior vena cava and innominate artery of the aorta were cannulated to serve as camera ports for filming right and left side anatomy, respectively.

The cannulas were then attached to corresponding tubes on the Visible Heart® apparatus, and the heart was perfused with a clear buffer solution and warmed up. The heart was slowly rewarmed over 15-30 min until it reached a temperature of 35.5°C, then a full buffer change was conducted to replace the buffer in the reservoir and circulating tube system. Once the perfused heart reached 37±5°C, native sinus rhythm was restored by delivering 34 J shocks to the ventricles via an epicardial patch electrode [96].

2.1.2 *In Vitro* – Instrumentation and Parameters

2.1.2.1 Visible Heart® Methodologies

The Visible Heart® apparatus is an *ex vivo* heart perfusion apparatus that allows the heart to function in different perfusion and working modes under physiologic conditions (Figures 7 [99] and 8). The apparatus experimentally simulates a heart transplant recipient. Oxygen and metabolic substrates are delivered in the clear perfusate. The concentrations of the ingredients of the modified Krebs-Henseleit buffer for this setup remain confidential. The perfusate enters a standard reservoir (Minimax; Medtronic) and oxygenator (3381 Hollow Fiber; Medtronic) from where it circulates throughout the apparatus. The circulating volume is about 5.5 liters. A water jacket maintaining circulated water at 39°C (BioCal 370 BioMedicus; Medtronic) envelopes the perfusate chambers and the oxygenator, regulating perfusate temperature. This jacket maintains measured myocardial temperatures at 37.0°C±0.5°C [96–100].

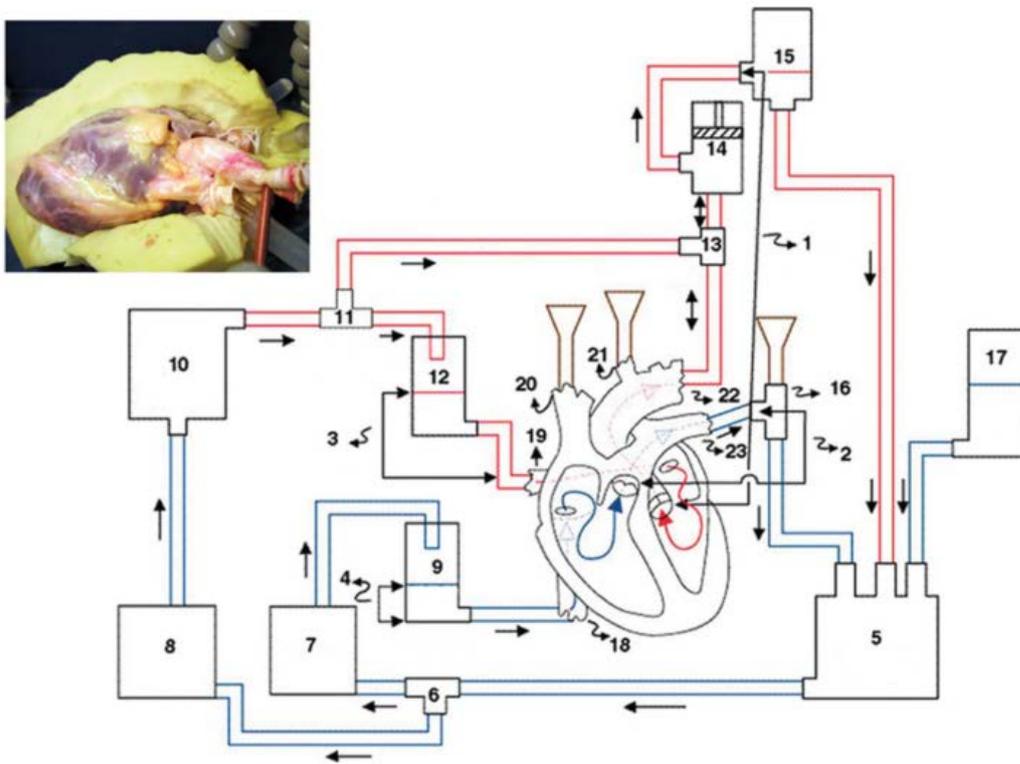


Figure 7: Scheme of Visible Heart® Methodologies



Figure 8: Ex vivo preparation Visible Heart® Methodologies

Initially, the heart is perfused in Langendorff mode (Figure 9A) as this provides the myocardium with oxygen and metabolites that were depleted during the ischemic period [101]. This is achieved through retrograde perfusion into the ascending aorta which keeps the aortic valve closed, and allows for fluid flow into the coronary arteries during the diastolic period as it would during a normal cardiac cycle. After moving through the coronary system, the fluids eventually exit through the coronary sinus into the right atrium. During Langendorff perfusion, the left chambers of the heart remain filled and there is no fluid exchange [102]. As soon as sinus rhythm was sustained, the heart was transitioned into a four-chamber working mode.

The four-chamber working mode (Figure 9B) allows blood flow through all four chambers of the heart as it would naturally occur. The atria empty into the ventricles, then eject during systole into the pulmonary artery and aorta, respectively. Two-way flow through the aorta distinguishes this physiological perfusion from the Langendorff mode. In this case, the aortic valve opens and closes as the heart contracts, and flow into the coronaries is determined by contraction of the heart itself and not by an external pump. Preload and afterload can be adjusted to represent a normal or impaired heart. In our study, we considered the hearts to present under normal conditions with corresponding optimized preload and afterload for each individual heart.

The right side working mode is an intermediate mode whereby the right side of the heart is perfused only in combination with Langendorff perfusion. This mode still represents physiological function of the heart, but also allows it to rest or recover after being in four-chamber working mode [99,101,103].

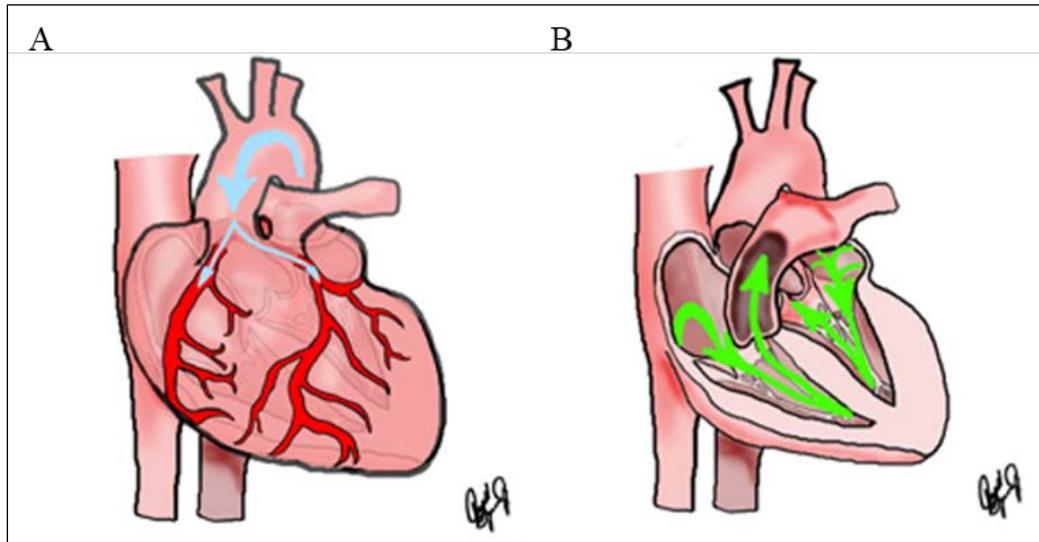


Figure 9: Working modalities on the Visible Heart® apparatus

A. Langendorff perfusion; B. Four-chamber working mode

2.1.2.2 Instrumentation and Parameters

In general, parameters were monitored as previously described for the *in situ* setup. Full hemodynamic monitoring was achieved by placing 5F balloon pressure catheters (Venous; Oscor) into the ventricles and connecting pressure lines to the in- and outflow vessels of the heart. Myocardial metabolism was monitored by arterial and venous buffer sampling every 10 min, as well as after AF burden. Again, a steerable catheter (C304; Medtronic) was placed in the coronary sinus. The aortic root cannula was used to sample arterial perfusate, and the Radiometer ABL 90 blood gas analyzer was utilized for final assessment. Additionally, troponin and creatin kinase (CK) were determined in the central laboratory of the Fairview Hospital, University of Minnesota.

Echocardiography and piezoelectric crystals were utilized to obtain contractility data. Pressures and crystal data was continuously recorded (iox2; EMKA Technologies), and echocardiography was applied at predefined timepoints. Moreover, heart weights before reanimation and after completion of the experiment were collected to determine relative percentage weight change (edema development) [96]. Data on electrophysiological properties, especially on monophasic action potentials, were analyzed by another graduate student from the laboratory.

2.1.3 *In Vitro* Isolated Muscle Bath Studies

The mechanography muscle bath apparatus contains two adjustable hooks that hold each muscle bundle at both ends, so that it is in a vertical position (Figure 10). Bundles are stimulated to contract by a pair of platinum electrodes that send out an electrical pulse of 1 Hz every 10 sec. The top hook is connected to a spring and force transducer which amplifies a signal that is recorded using LabVIEW 3.0 software (National Instruments, Austin, TX, USA). The bundle is immersed inside a glass bath containing Krebs buffer, and the temperature is kept constant at 37°C through a constant flow pump which circulates water through the glass bath.

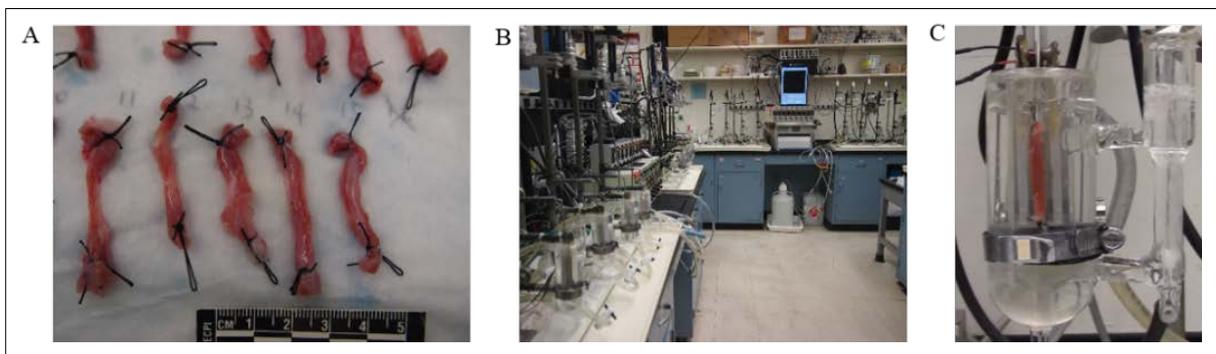


Figure 10: Muscle bath studies

A. Isolated muscle; B. Muscle bath laboratory; C. Single muscle bath

Swine diaphragm tissue was obtained and immediately immersed in carbogenated (95% O₂/5% CO₂) Krebs-Henseleit buffer. The diaphragm was dissected into bundles approximately 4cm long and 2-3mm wide and tied at each end with sutures. Bundles were subsequently hung on the apparatus and allowed to relax for 10 min. After relaxation, muscles were optimized according to their length-tension relationship using a standard lab protocol. In this protocol, the baseline force is adjusted to produce the maximum peak force, thus ensuring optimal overlap of sarcomeres. Muscle bundles that did not achieve a minimum peak force of 1g were excluded from final analysis. After incubation with ATP, the buffer was changed and a 10-min drug recovery period was observed. After the recovery period, carbogen (95% O₂/5% CO₂) was changed to 95% N₂/5% CO₂ and muscle bundles were subjected to a state of ischemia for 1 hr. Muscles were then reperfused with carbogen and buffer was changed. Bundles were observed and allowed to recover for 4 hr. During this period, fresh Krebs buffer was added to the baths every 30 min.

In the preconditioning phase, muscles were randomized and either pretreated with 10μM ATP (n=11), 5μM ATP (n=6), or control (Krebs only, n=13) for 1 hr. After reperfusion, bundle length (mm), weight (mg), and two diameter measurements (mm) were recorded for each muscle bundle. All force

data were normalized to the peak baseline force achieved by each muscle bundle in the baseline phase (Ratio = Peak force / Baseline peak force). Data are reported as mean \pm standard deviation. Further, data from each concentration were compared to control using student's t-test function in Xcel.

2.2 Study Paradigms

2.2.1 Overview Grouping of Data

In addition to controls, six groups of animals were used to investigate the potential benefits of Omegaven as a preconditioning agent and ATP as a post-treatment (see Figure 11):

- 1) **PrC**: Preconditioning with Omegaven *in situ*; 10 ml into the pericardial sack (n= 6)
- 2) **PrC-PoC**: Preconditioning with Omegaven, 10 ml into the pericardial sack plus postconditioning with ATP 3.5 μmol = 10 mg (n= 2) or 10.75 μmol = 30 mg (n= 1)
- 3) **PoC**: Postconditioning with ATP supplemented to reperfusion buffer; concentrations administered were 3.5 μmol = 10 mg (n= 3) or 10.75 μmol = 30 mg (n= 3)
- 4) **PrC-Sup**: Preconditioned heart supplemented with ATP incrementally every 10 min over the course of 2-hr study period; concentrations studied were 2 mg (n= 3), 5 mg (n= 3), and 10 mg (n= 3)
- 5) **Sup**: ATP was incrementally administered every 10 min over the course of 2-hr study period to hearts with no pretreatment; concentrations studied were 2 mg (n= 3), 5 mg (n= 4), and 10 mg (n= 3)
- 6) **Sup***: ATP was incrementally administered every 10 min over the course of 1-hr study period to hearts that were already pretreated with Omegaven after at least 3 hr *in vitro* function; concentrations studied were 2 mg (n= 2), 5 mg (n= 3), and 10 mg (n= 3)
- 7) Muscle baths: Control (Krebs only, n= 13), 10 μM ATP (n= 11) and 5 μM ATP (n= 6)

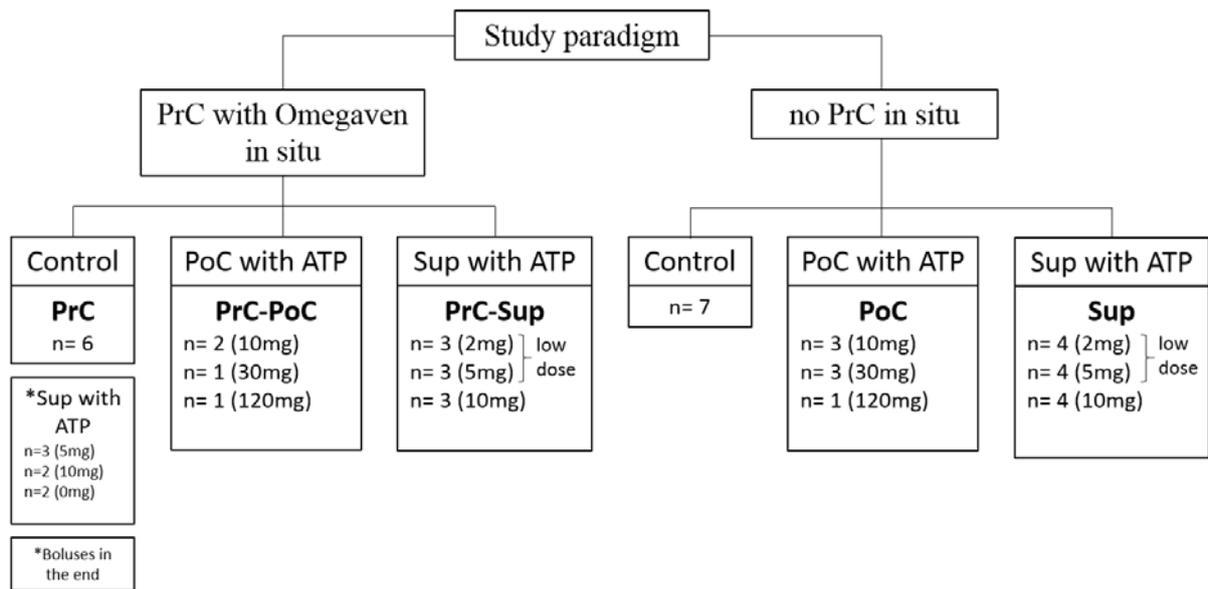


Figure 11: Overview of study groups

2.2.2 *In Situ* – PrC Timeline and Study Protocol

The timelines (*in situ* and *in vitro*) include the protocol's major procedures. Since the Visible Heart® Laboratories conduct animal research associated with high costs, several students analyzed different parts of the study. For a full overview, but also justification of my data analysis, all parts of the study paradigm are included. During *in situ* and *in vitro*, constant hemodynamic monitoring was applied. Moreover metabolic sampling was performed (every 10 min starting at baseline (BL), before and after (AF) during working mode. Figure 12 displays the *in situ* PrC study paradigm:

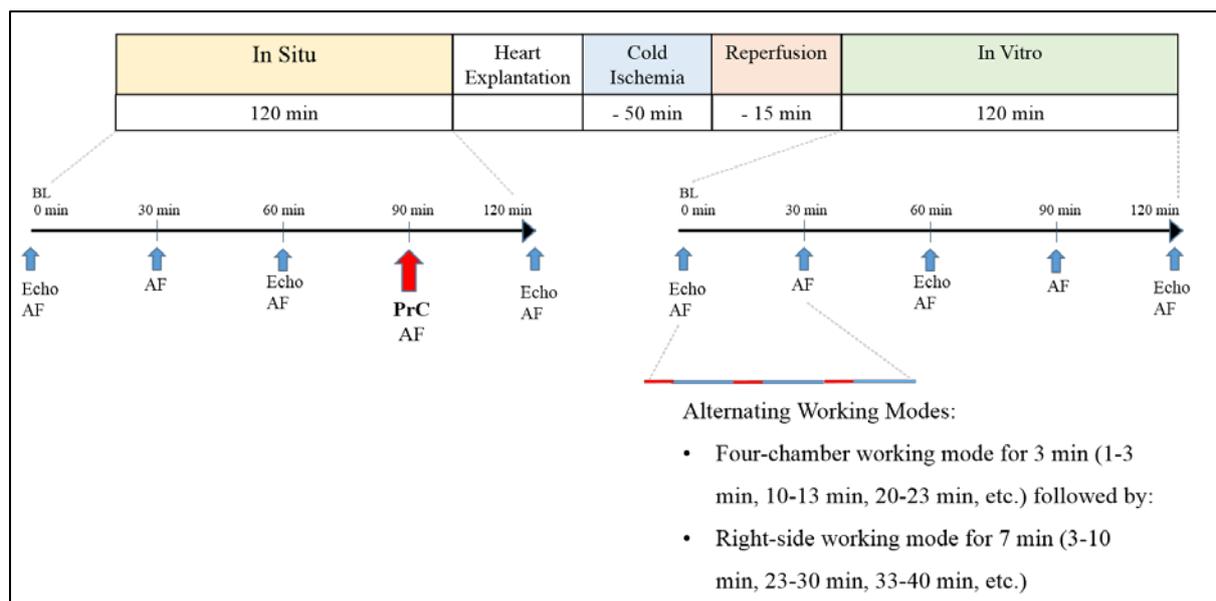


Figure 12: *In Situ* - PrC timeline and study protocol

AF=atrial fibrillation; BL=baseline

2.2.3 *In Vitro* – PoC and Sup Treatment Timeline and Study Protocol

Constant hemodynamic monitoring was applied as well as metabolic sampling was performed (every 10 min starting at BL, before and after AF) during working mode. Figure 13 displays the study paradigm for the *in vitro* PoC and Sup protocols:

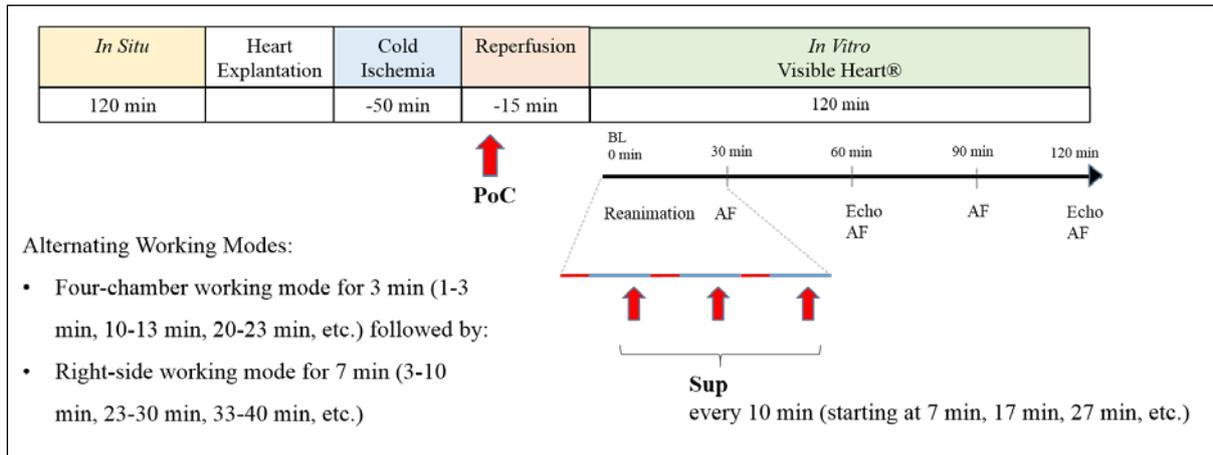


Figure 13: *In Vitro* – PoC and Sup treatment timeline and study protocol

AF=atrial fibrillation; BL=baseline

A second **Sup*** protocol *in vitro* (Figure 14) was run after the previously discussed *in vitro* protocol or after the heart was on the apparatus for at least 150 min. These pilot studies were used to gain early insights and dosing information about ATP as a posttreatment.

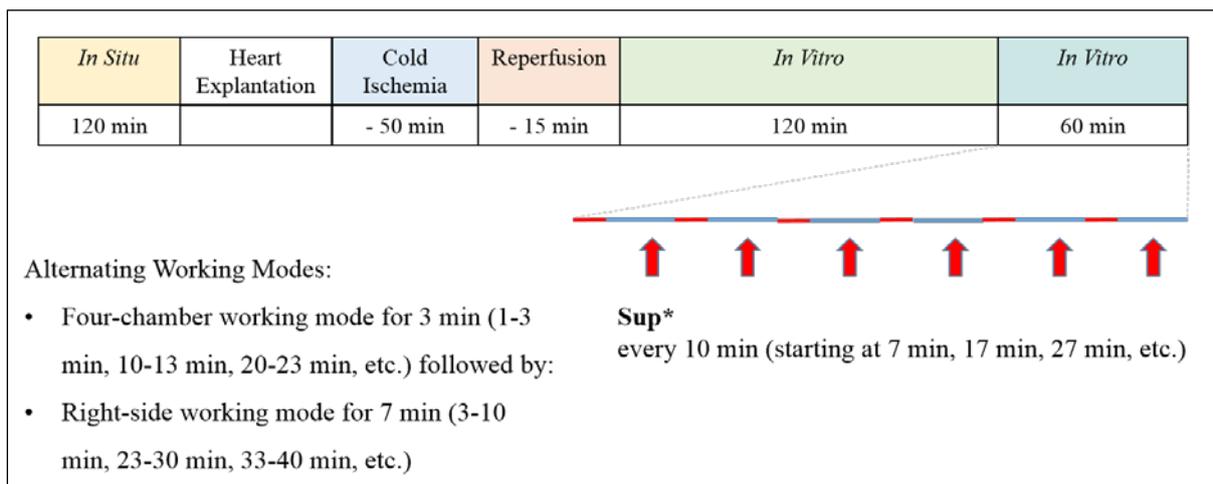


Figure 14. *In Vitro* – Sup* treatment timeline and study protocol

2.2.4 *In Vitro* – PrC Isolated Muscle Bath Study Timeline

Figure 15 displays the timeline for the *in vitro* PrC muscle bath studies:

<i>In situ</i>	Preparation	Baseline	Preconditioning	Pre-Ischemia	Ischemia	Reperfusion
Previously described preconditioning protocol	Bundle dissection	10-20 min	60 min	10 min	60 min	4 hr
	95% O ₂ /5% CO ₂	95% O ₂ /5% CO ₂	ATP (5μm or 10μM) or control	95% O ₂ /5% CO ₂	95% N ₂ /5% CO ₂	95% O ₂ /5% CO ₂

Figure 15: *In Vitro* - PrC isolated muscle bath study timeline

2.3 Analysis

2.3.1 General Statistical Analysis

Two-sided t-tests without a Bonferroni correction was performed and multiple variance analyses were used to determine the significance between different test groups. Additionally, a time point variance analysis, including post-hoc comparison/Bonferroni correction, was performed. Working with that preliminary and explorative data set, this statistical analysis aimed to see trends within the groups and paradigms for further experimental approaches.

Among many others, parameters included median of differential left ventricular pressure (MDP), Tau, dP_{max}/dt , venous pO_2 , and venous lactate; see appendix. A P value < 0.05 was considered to indicate significance, unless otherwise noted. The displayed graphs show median values \pm STD. The predefined specific timeframes of working mode include averaged values over 5 sec, processed by EMKA.

The evaluation of all *in situ* and *in vitro* hemodynamic parameters was made in “Python” programming language utilizing the Python library for applications and statistical tests [104]. Data for tissue bath studies are reported as mean \pm STD, and data from each concentration were compared to control using student’s t-test function in Excel.

3.3.2 Python Programming Language and Application of Machine Learning Algorithms

Python is a high-level, general purpose programming language that supports multiple programming paradigms as an open source. It was implemented in 1989 by Guido van Rossum as an interface to embed existing applications in a highly readable language. It is used for data management and preparation, but also web development, simulation, algorithm design and database interfaces among others [105].

There are large standard libraries that provide suitable tools to facilitate data analysis and modeling capacities. One example is Pandas as an open source analysis toolkit used in this work. It provides excellent handling of labeled data [106]. Another open source that we used was Scikit Learn, a free software machine learning library that is largely written in Python and features various classification, regression, and clustering algorithms [104]. This also includes support vector machines such as those used in this data analysis (Figure 16). We also used different fundamental packages for scientific computing including NumPy and SciPy [107,108].

```

##Importing Libraries: external Libraries and internal Libraries (code created to import functions seperately)
import importlib
##Code for our ATP data
import atp_prot
import pandas as pd
#Utility code
import atp_util
import warnings
warnings.filterwarnings('ignore')
importlib.reload(atp_prot)
importlib.reload(atp_util)
%matplotlib inline

props_of_interest = sum([[ 'Arterial ' + i, 'Venous ' + i] for i in ['K+ (mmol/L)',
'Na+ (mmol/L)',
'Ca++ (mg/dL)',
'Cl- (mmol/L)',
'Glu (mg/dL)',
'Lac (mmol/L)',
'pH',
'pCO2 (mmHg)',
'pO2 (mmHg)']], [])

props_of_interest = ['Venous Lac (mmol/L)']
wms = pd.read_csv('working_modes_bg.csv')
## Lambda is a function applied to all the columns of the data frame
wms = wms.apply(lambda x: (x - x.mean()) / x.std() if 'Venous' in x.name or 'Arterial' in x.name or 'Minus' in x.name else x)
#print(wms)
from sklearn import svm
from sklearn.linear_model import LinearRegression
from sklearn.linear_model import Ridge
##custom features defined for creating the model
#cLf = svm.SVR(kernel='rbf',gamma=0.5, C=100)
cLf= LinearRegression()
#cLf= Ridge(alpha=100)
#props_of_interest = ['Timepoints (min)', 'Baseline Median Differential Pressure (mmHg)']
atp_prot.analyze_model_predictions(wms, 'Median Differential Pressure (mmHg)',
props_of_interest, [], 'Combined',
['Start', 'End', 'Reperfusion'],
['Saline', 'Omegaven', 'Saline+ATP', 'Omegaven + ATP'],
cLf, shuffle=True)

```

Figure 16: Extract of the script written in Python showing comparison linear regression and support vector regression

Machine learning is a subfield of artificial intelligence closely related to computational statistics. It focuses on prediction making based on mathematical algorithms that are embedded in a loss function. A loss function is a mathematical function to optimize a problem that involves various variables, but eventually tries to objectively answer the question and estimate a parameter, for instance, the difference between estimated and true values. In this context, the dataset is usually split to include a training set and test set that allows one to create a model. The training set is used to train the model and, based on those observations, data-driven predictions can be made on the test set [104]. How well a model performs is expressed by the coefficient of determination R^2 ; the value represents how well observed outcomes are replicated by the model. This is based on the proportion of total variation of outcomes explained by the model.

One type of machine learning is the concept of support vector machine (SVM), a tool that can be used to fit classification and regression models (Figure 17). When data analysis exceeds three dimensions or is non-linear, kernels help to create higher dimensional operations to allow for the data to be linearly separated. The kernel is an operating system and central module that expresses a measure of similarity between vectors; it loads first and remains in main memory. Linear support vector machines separate the dataset into two classes by fitting two hyperplanes in between to create a maximum margin. For the dataset presented in this thesis, the Gaussian radial basis function (RBF) kernel (Figure 17), commonly used in SVM, was used as the best performing model. This particular kernel projects in infinite dimensions and focuses on the Euclidian distance between two data points to the

square. The γ parameter can be tuned and it sets the width of the bell-shaped curve. The larger the value of γ , the narrower will be the bell [104,109,110].

Gaussian kernel: $k(x, x_i) = \exp(-\|x - x_i\|^2/2\sigma^2)$; $1/2\sigma^2 = \gamma$ for $\gamma > 0$

Radial Basis Function SVM: $f(x) = \sum_i^N \alpha_i y_i \exp(-\|x - x_i\|^2/2\sigma^2 + b)$

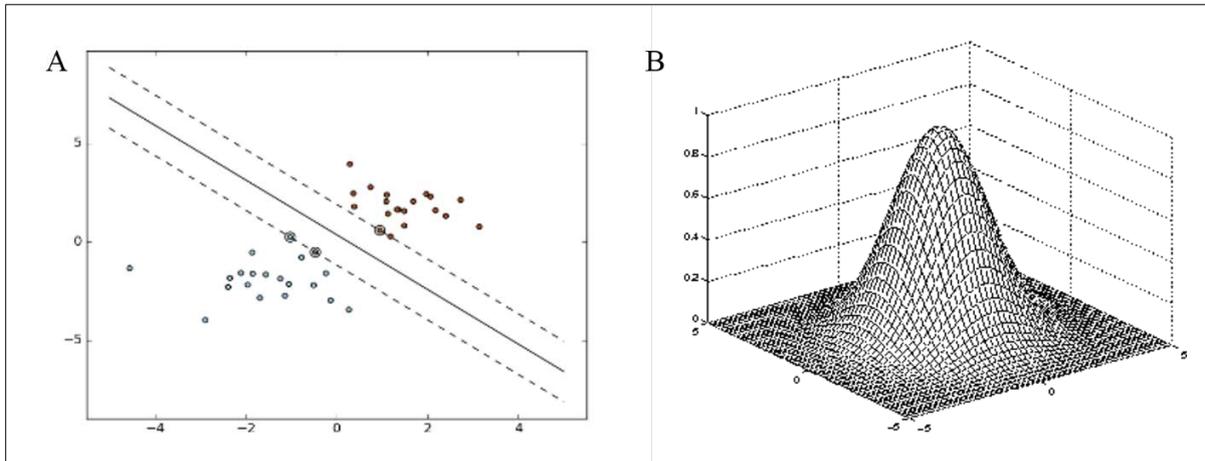


Figure 17: A. Linear support vector machine; B. Radial basis function kernel

Due to the complexity of models used in statistics and machine learning, random error or noise occur, supporting the phenomenon termed *overfitting*. The potential for overfitting depends not only on the number of parameters, but also the conformability of the model structure with the data shape and the magnitude of model error. Consequently, overfitting is expressed by low performance of the model on the validation dataset.

Therefore, the cross-validation technique (Figure 18) was used by dividing the dataset into five subsets. Within these subsets, each portion of the dataset was tested by performing an analysis on one subset while using the other four subsets as a training set. Afterwards, the average overall trials were computed and variance of the resulting estimate was reduced [111].

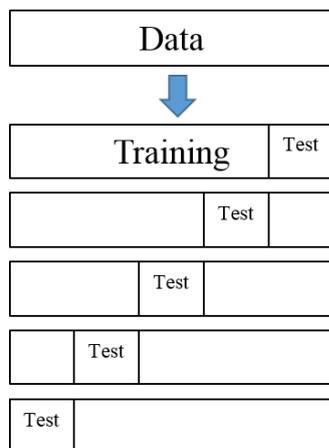


Figure 18: Cross-validation scheme

3. Results

3.1 Study Population and General Results

Forty-nine animals were initially included in the study protocol. Data recordings and analysis started at the beginning of *ex vivo* time. Seven additional animals were included for the Sup* protocol. Animals presenting with physiological abnormalities *in situ* (severe arrhythmias requiring drug interventions or hemodynamic instability) were excluded from the functional analysis (n= 2). Additionally, hearts suffering from insult during the surgical explantation or from systemic errors during the experiment in either *in situ* or *in vitro* protocols were not considered for further analysis (n= 2), which adds up an exclusion of four data sets in total. Furthermore, due to study complexity and variable functions, not all parameters were obtained in some studies. Therefore, occasionally we report differing sample numbers relative to the total number of valid experiments per group.

On average, heart weights were 483.3 g just prior to *in vitro* reanimation and 657.6g after at least 3 hr *ex vivo*. We considered these increases in weight were due to edema. Heart weights increased up to 50% of initial measurements, but did not differ significantly between groups. Further, developed edema correlated with the percentage increase in wall thickness observed in all groups. Wall thickness in saline (control) hearts increased 16.6% within the first hour. Insufficient data points after 2 hr were collected to perform statistical analysis. The average ATP-Sup heart wall thickness increased by 31.5% after 1 hr, and further increased to 37.3% within the subsequent hour. The wall thickness of hearts receiving a bolus dose of ATP as a postconditioning agent increased 14% after 1 hr, and 36% after 2 hr. We found no significant differences within the various concentrations of both groups. Further, data suggest no substantial difference in the relative formation of edema between controls and any of the treatment groups.

For functional analyses *in vitro*, the primary hemodynamic parameter was maintenance of LV pressures in the four-chamber working mode. It should be noted that within a given 3-min interval during working mode, the LV pressure gradually decreased. Therefore, in these experiments, the median value of the MDP, calculated by subtracting the diastolic pressure from the systolic pressure, was reported. The median value (unlike the mean) is robust to outliers, and MDP normally produces consistent results regardless of baseline drift. Also, diastolic relaxation (Tau), rate of rise in LV pressures (dPmax/dt), and raw systolic and diastolic pressures, when appropriate, were determined.

For contractility data, four sonometric ultrasound crystals (Sonometrics, London, ON, Canada) were placed within the LV (base, apical, mid-anterior, and mid-posterior locations); this allowed for

estimation of an idealized contracting ellipsoid and also acted as a proxy for ejection volume measurements [96].

Metabolic measurements followed expected physiological trends, showing increasing levels of lactate as well as gradual decreases in glucose concentration. Note, a full buffer change was performed after 70 min that reset the concentrations of all ingredients and metabolites within the buffer solution. Lactate as a parameter showed differences in behavior within the groups. Therefore, both venous lactate and venous pO_2 will be discussed further among other parameters that were obtained. The chosen hemodynamic and metabolic parameters with corresponding STD for each group and each timepoint are shown in the appendix.

Arrhythmic analysis could only be performed for the Omegaven-preconditioned (PrC), ATP-postconditioned (PoC), and ATP-supplemented (Sup) hearts; see appendix. In general, measurements were variable and multifactorially influenced. Results are reported but cannot be generalized, and must be considered carefully.

The data sets comparing PoC and Sup groups with controls were published in the Journal of Experimental Biology and Medicine by Seewald et al in 2019 [112].

3.2 Post-ischemic Hemodynamic and Metabolic Analyses

3.2.1 PrC with Omegaven *In Situ*

3.2.1.1 Hemodynamics and arrhythmia assessment

For the PrC hearts, hemodynamic LV function presented with decreased initial MDPs after reanimation in comparison to saline controls, as shown in Figure 19. The pressures remained below the control group throughout the first hour of the study period, but were not within the range of significance. After the buffer change at 70 min, PrC hearts seemed to maintain function better and even showed an upward trend at 110 min. At the 120-min timepoint, the Omegaven group presented with increased MDPs ($p= 0.177$). The buffer change at the 70-min timepoint did not result in long-term recovery, but rather a continued decrease in function was seen in both groups.

Similarly, diastolic relaxation, as reflected in the time constant Tau, was not affected by this pretreatment. Contractility measurements, expressed by dP_{max}/dt but also EF and differential volume changes, elicited the same response behaviors but were not significantly different from the control group.

Cumulative time in induced AF at the 30-min timepoint was 66.9 sec which was higher than in the control group at that measurement timepoint (31.4 sec). At all subsequent timepoints, pretreated hearts had lower times in AF and more stable hemodynamics, in comparison to the saline controls that tended to be more vulnerable. At 60 min, time duration in AF was 16.8 sec, at 90 min 14.7 sec, and at 120 min the average cumulative time was 13.0 sec.

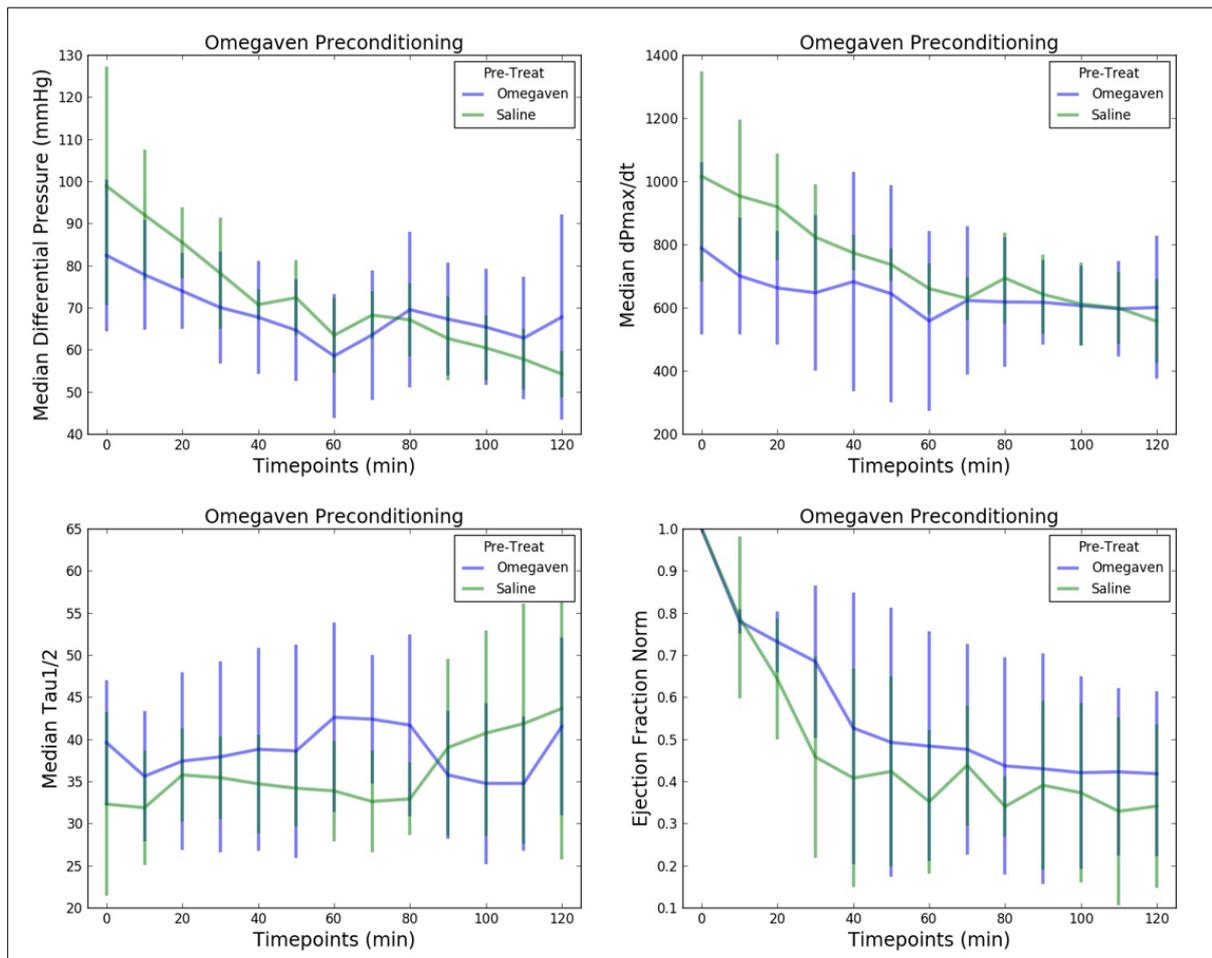


Figure 19: PrC with Omegaven hemodynamics

3.2.1.2 Metabolism

In general, metabolic measurements followed expected physiological trends, showing increased levels of lactate as well as gradual decreases in glucose concentration in venous and arterial samples over time. More specifically, the Omegaven PrC group showed lower lactate levels than the saline control group at the 60-min timepoint ($p=0.07$, Figure 20). After the full buffer change at 70 min, lactate levels started off with similar low values at 90 min. The following lactate slope flattened in comparison to the beginning of the experiment and did not reach similar values, as seen after the same amount of time immediately after reanimation.

The partial pressure of oxygen measured in venous blood showed physiologic and similar trends in the Omegaven-preconditioned group and saline control group, which was a steady increase of venous pO_2 over the course of 2 hr. The outliers in the Omegaven graph are due to measurement errors and high variability within the dataset (e.g., STD of 167.66 at 70 min).

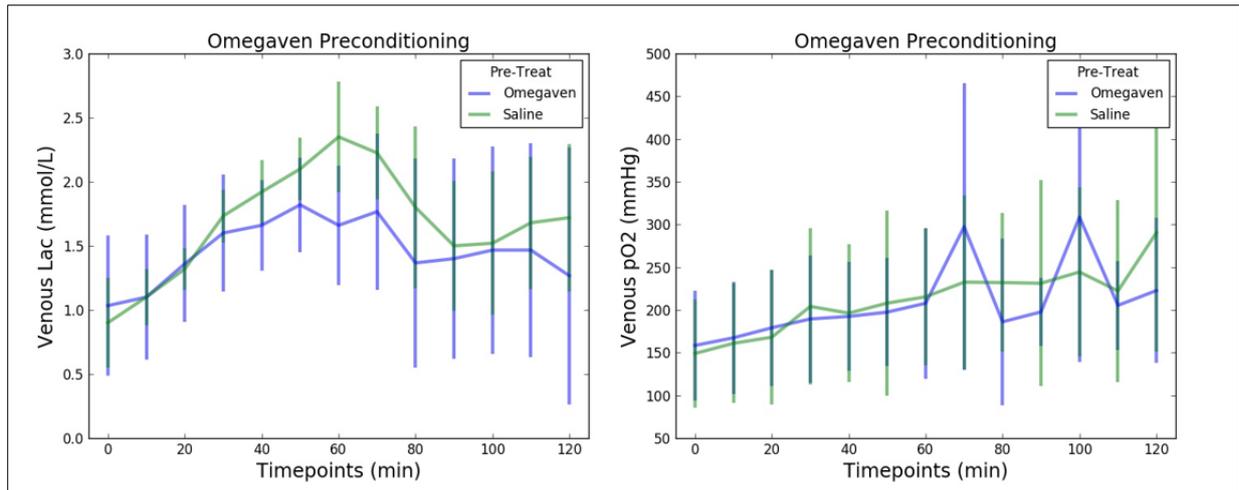


Figure 20: PrC with Omegaven metabolism

3.2.2 PoC with ATP added to reperfusion buffer

3.2.2.1 Hemodynamics and arrhythmia assessment

In the PoC groups, hearts elicited a significantly decreased MDP compared with the control group (Figure 21). This observation remained significant throughout the duration of the protocol for both the 10mg and 30 mg PoC dosing subgroups. Yet, the extent of LV functional depression appeared to be dose-dependent; MDP was lower with 30 mg than with 10 mg (for both groups $P < 0.05$ after 90 min). These effects were not statistically significant within the two PoC subgroups after 2 hr *ex vivo*. We found no evidence of functional stabilization of hemodynamic parameters after the 70-min full buffer change, perhaps implicating an acute reaction to ATP within this paradigm. Note that the PoC groups did not differ from the control group in terms of Tau, dPmax/dt, or normalized contractility measurements.

Our measurements of arrhythmogenicity were highly variable. At the 30-min point, the cumulative duration of AF was 307.2 sec in the 10mg PoC subgroup and 264.1 sec in the 30mg PoC subgroup, as compared with 31.4 sec on average in the control group. At the 90-min point, the cumulative duration of AF was 89.3 sec in the 30mg PoC subgroup.

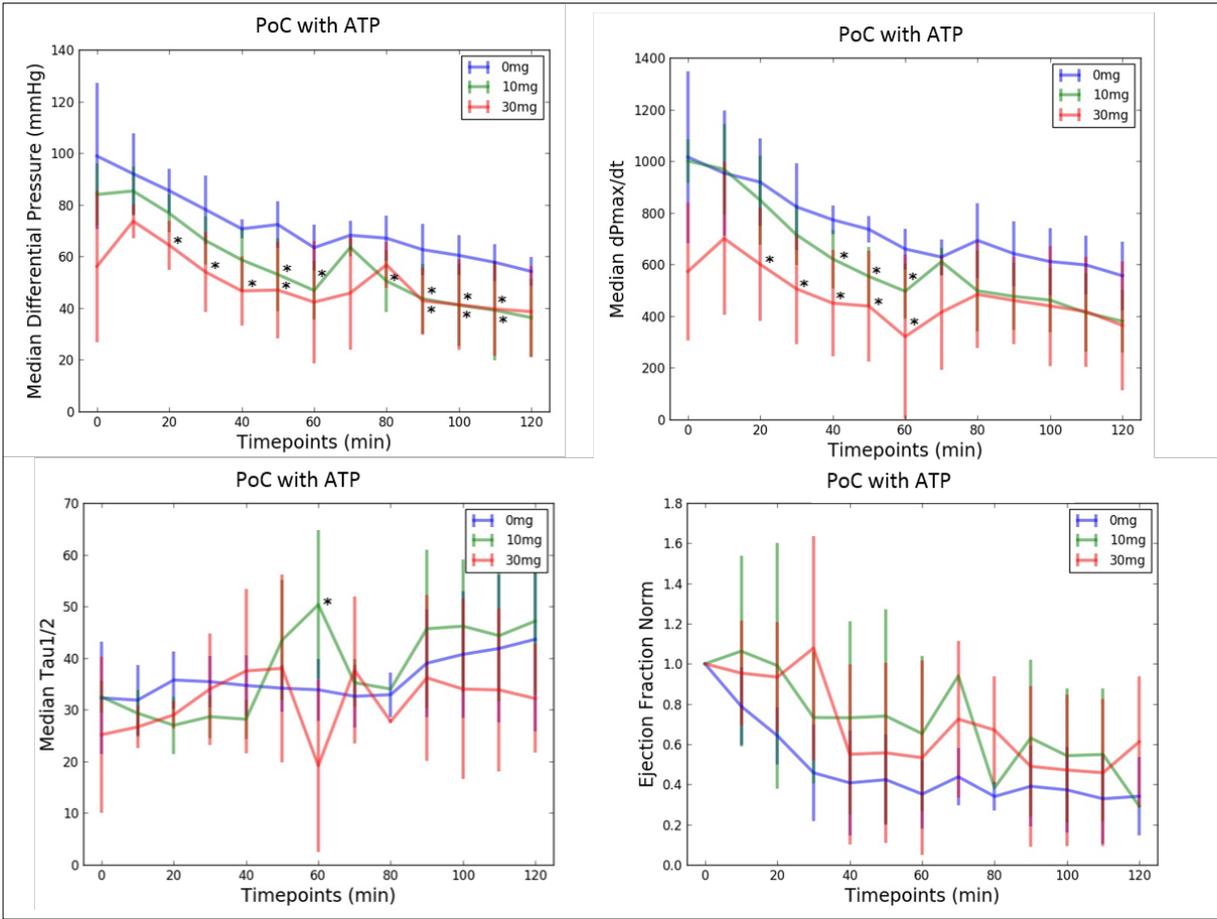


Figure 21: PoC with ATP hemodynamics

3.2.2.2 Metabolism

As mentioned before, metabolic measurements followed expected physiologic trends, showing increases in lactate level as well as gradual decreases in glucose concentration over time. Overall, venous lactate and pO_2 tended to be lower in the PoC group than in the control group (Figure 22).

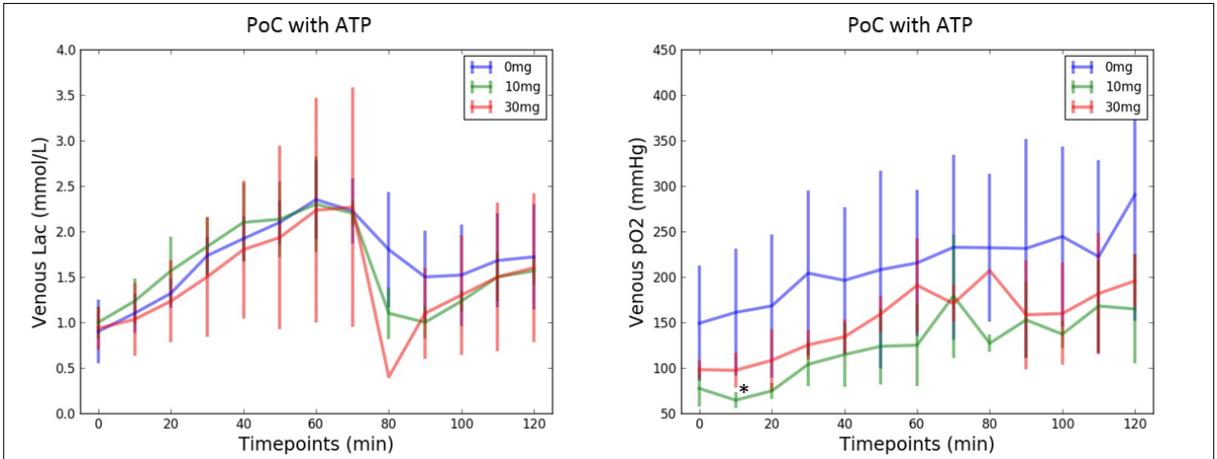


Figure 22: PoC with ATP metabolism

3.2.3 ATP Sup

3.2.3.1 Hemodynamics

ATP was incrementally administered every 10 min over the course of the 2-hr study period to hearts with no pretreatment; single amounts per ATP administration studied were 2 mg (n= 4), 5mg (n= 4), and 10 mg (n= 4). Hemodynamic differences between 2 mg, 5 mg, and 10 mg ATP post-treated hearts compared to saline controls were most pronounced 2 hr post reanimation/*in vitro* functioning on the Visible Heart® apparatus (Figure 23).

We found that dosages could be separated into low (2 mg and 5 mg) and high (10 mg) concentrations. The low concentrations were superior to the high; the most pronounced difference between the Sup group and control group was in MDP at the 90-min point (p= 0.02). The difference remained significant at the 120-min point (p= 0.05).

We noted no significant differences in dPmax/dt between the low-concentration Sup subgroup and control group at any time point. However, the low-concentration Sup subgroup generally had lower Tau, indicating improved diastolic relaxation (p< 0.05). The same was also true for contractility measurements obtained by sonometric crystals.

The 10 mg Sup concentration presented with high variability, so its utility in determining optimal dosage should be considered carefully. Better sustained differential volume changes and normalized EFs further supported the improved contractile function of the reanimated heart administered with low-dose bolus therapies.

Arrhythmia induction in the Sup group showed only minor deviations from the control group. At the 90-min point, arrhythmia was prolonged in the low-concentration Sup subgroup: 218.98 sec on average. It was also prolonged in the high-concentration Sup subgroup: 77 sec on average.

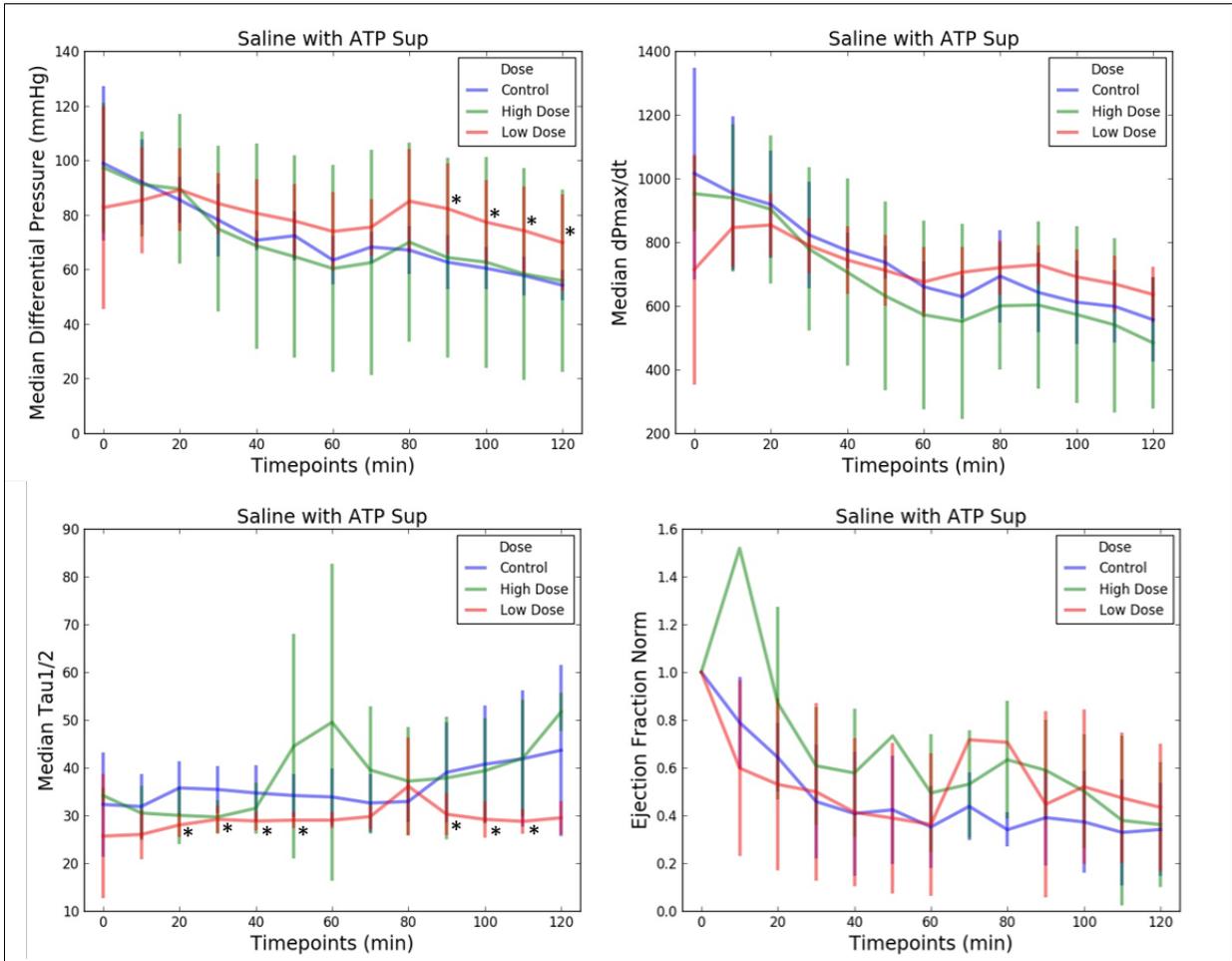


Figure 23: Sup with ATP hemodynamics

3.2.3.2 Metabolism

Changes in hemodynamic function in the low-concentration Sup subgroup were consistent with metabolic changes, with a significantly smaller increase in lactate levels over time compared with the control group (Figure 24). This difference continued at the 120-min point ($p= 0.01$). For both Sup subgroups, pO_2 stayed lower than in the control group, with the most pronounced difference at the 120-min point ($p= 0.05$).

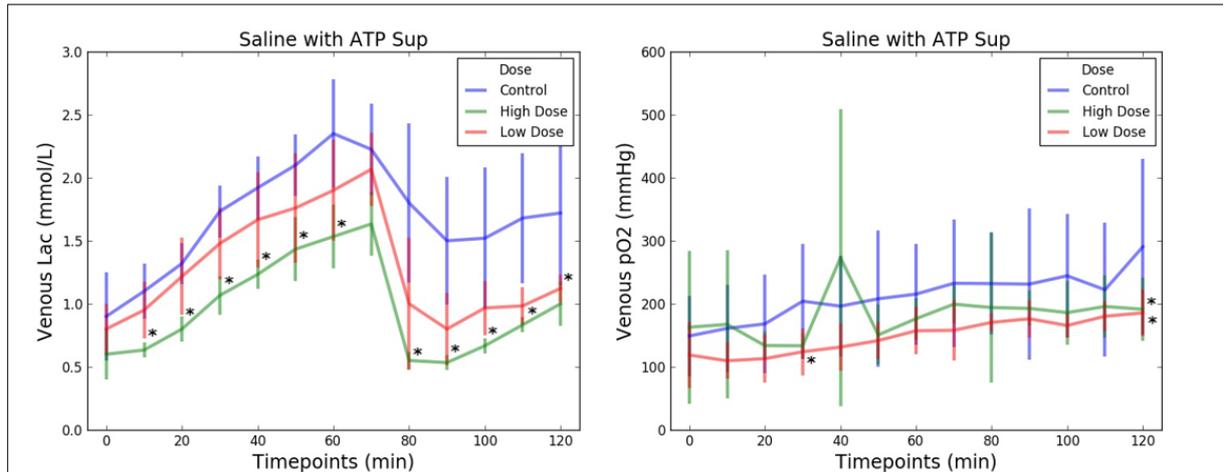


Figure 24: Sup with ATP metabolism

3.2.4 PrC - Sup

3.2.4.1 Hemodynamics

ATP was incrementally administered every 10 min over the course of the 2-hr study period to hearts that were already pretreated with Omegaven; concentrations studied were 2 mg (n= 2), 5 mg (n= 3), and 10 mg (n= 2). Again 2 mg and 5 mg were grouped as low-dose (n= 5) and 10 mg remains considered as high dose (n= 2).

Hemodynamic measurements from PrC-Sup hearts behaved similar to their control group (Omegaven-PrC), with trends of improvement in function (Figure 25). PrC-Sup low-dose MDPs started off at the highest levels in comparison to both PrC-Sup high-dose and just preconditioned hearts. All groups showed a decrease of function over time in a similar pattern. At the 60-min timepoint just before the buffer change, MDPs in the low-dose group were significantly better ($p= 0.01$). At the 120-min timepoint, all concentrations leveled out at the same pressure zone. The same functional gain was indicated by the constant Tau, even though at the 120-min timepoint, the variance was very high and did not allow for assured inquiry with that dataset. Contractility (dp_{max}/dt) showed higher values for PrC-Sup hearts. Best performing was the low-dose group PrC-Sup in comparison to the control without being significant though in this dataset. EF measurements obtained through sonometric ultrasound crystals do not support that finding.

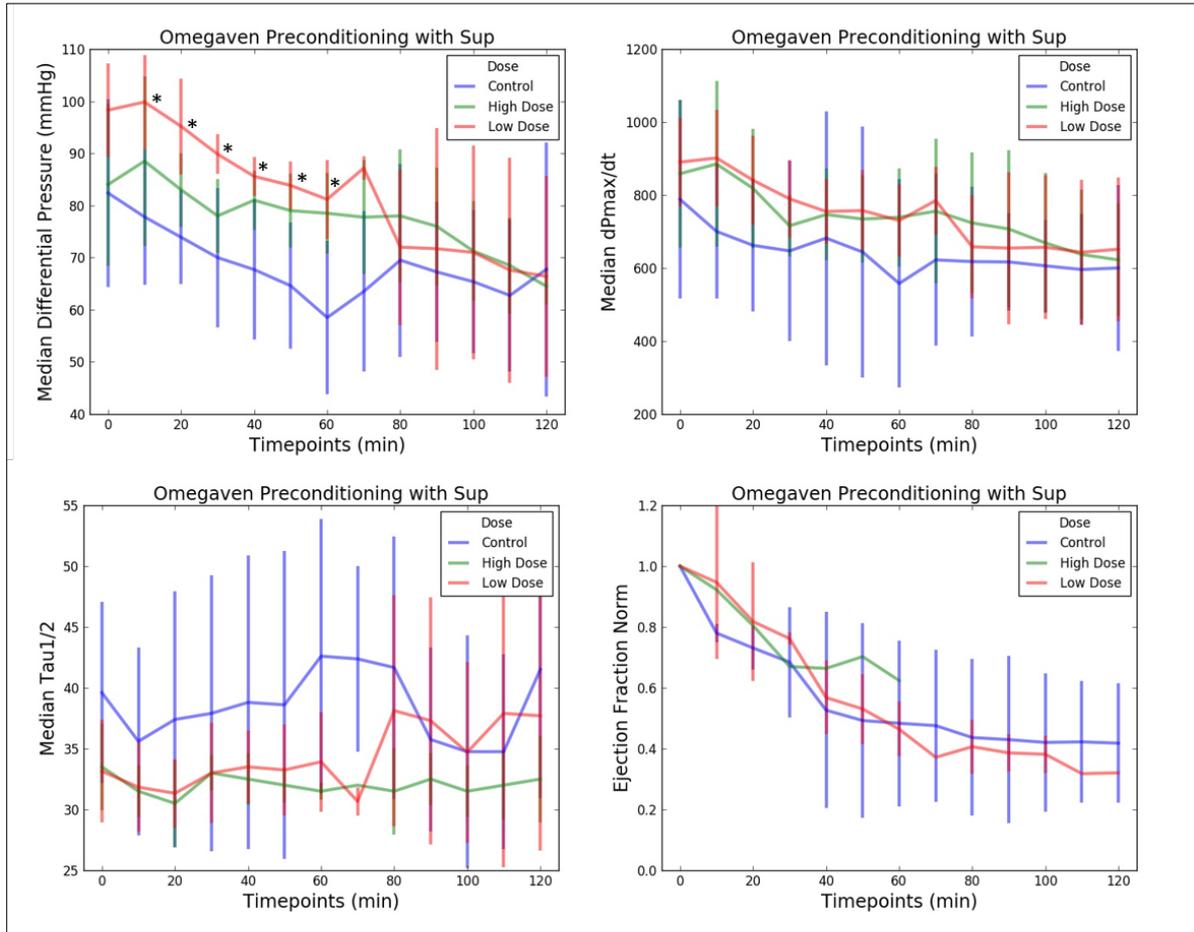


Figure 25: PrC-Sup with Omegaven and ATP hemodynamics

Arrhythmia analysis showed the best group results overall for the preconditioned hearts and those supplemented with 5mg every 10 min over the course of the study. Hearts presented as stable and less susceptible to arrhythmic activity over the course of 2 hr. At the 30-min timepoint, the hearts had a cumulative time in AF of 10.7 sec, 8.4 sec at 60 min, 18.4 sec at 90 min, and 9.0 sec at 120 min.

3.2.4.2 Metabolism

Observations for metabolic parameters in the PrC-Sup group were identical to the Sup group (Figure 26). Changes in function in the low-concentration PrC-Sup subgroup were consistent with metabolic changes, very similar to the control group. For the low-dose PrC-Sup subgroup, venous lactate values stayed lower than in the control group until the 50-min timepoint. After that, the low-dose treated hearts and controls maintained similar levels of venous lactate until the end of recordings. For the high-dose PrC-Sup group, a substantial increase of venous lactate was found after the 90-min timepoint.

The venous pO₂ recordings obtained from the low-dose PrC-Sup group remain below the control for the 2-hr timeframe. The high-dose data are variable (also consider low sample size) and presented with similar venous pO₂ values as the controls, including outliers.

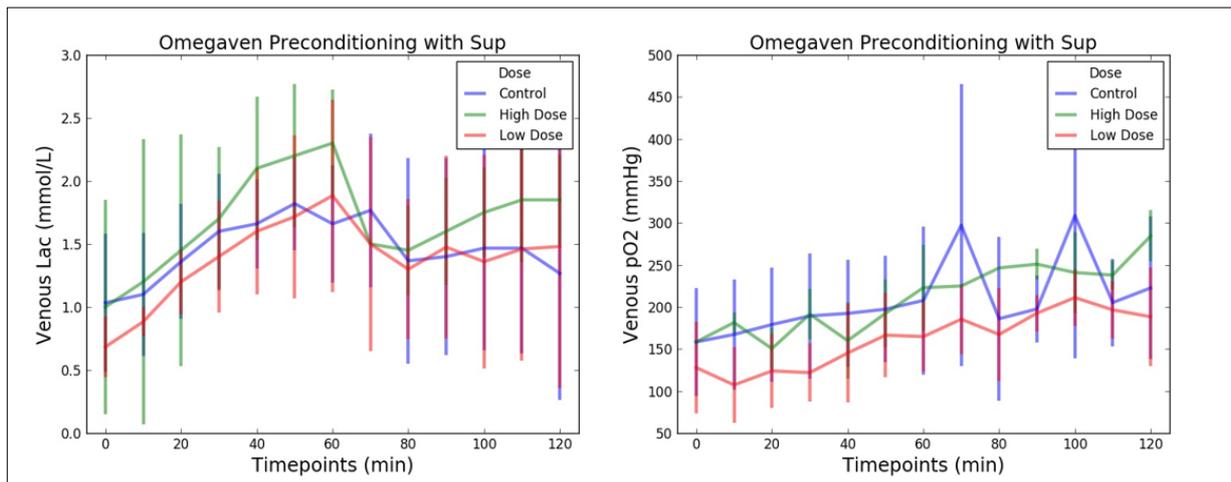


Figure 26: PrC-Sup with Omegaven and ATP metabolism

3.2.5 PrC - PoC

Both hemodynamic and metabolic parameters indicated that combining these two approaches to cardioprotection is harmful to the organ. Within our timeframe of observation at the beginning of the experiment, the full number of animals in each subgroup was not tested. The minimal number of animals though was not representative for statistical analysis.

3.2.6 Sup* - ATP (after at least 3 hr *in vitro* function)

3.2.6.1 Hemodynamics

This study approach tested the ability of ATP administered at least 150 min after reanimation on the apparatus, incrementally over the course of 1 hr or as a bolus, to maintain and/or recover functional performance after progression of global ischemia. Early studies provided preliminary data and helped to define ATP toxicity dosage at that stage of the experiment.

Also, different bolus dosages were applied as to determine toxic effects of very high ATP concentrations. No significant changes after applications were seen.

Results were inconclusive but showed trends of stabilization and minor hemodynamic improvements without any significant support. It was noted that hearts presenting with better viability at the beginning of this protocol seemed to benefit from the additional treatment. Indeed, function did not worsen in those specimens. At the same time, hearts that presented with decreased function after 3 hr *in vitro*, in comparison to others, experienced no benefit or recovery but also did not seem to be influenced negatively. We were not able to observe any toxic ATP concentration after bolus application at this point.

Those rather punctual observations without extensive statistical analysis represented preliminary experiments with ATP as a posttreatment and initiated all other consideration and treatment protocols (see above).

3.2.6.2 Metabolism

Metabolism analysis showed similar trends as the hemodynamic parameters, and supported the use of ATP as a potential cardiosupportive agent.

3.2.7 PrC Isolated Muscle Bath

Both concentrations of ATP significantly reduced normalized peak force compared to control bundles after the ischemic period. The 5 μM group reduced peak force by $15.4 \pm 14.0\%$ ($p= 0.025$) and the 10 μM reduce peak force by $11.2\% \pm 13\%$ ($p= 0.034$). However, there was not a significant difference in recovery after the reperfusion phase in the 5 μM ($p= 0.24$) or the 10 μM group ($p= 0.21$), although there appeared to be a decrease in function in both treatment groups. The treated bundles returned to a higher force quicker than control bundles; however, over the course of the 4-hour reperfusion cycle force began decrease in the 10 μM group. In contrast, the 5 μM group appeared to do better than control after the 4-hr reperfusion cycle though this difference was not significant.

3.2.8 Support Vector Regression Model

We explored the utility of Support Vector Regression in the prediction of hemodynamic data from a set of relevant metabolic parameters measured with the blood gas equipment. In these studies, ATP and Omegaven were administered as possible cardioprotective and signaling agents (see paradigms above).

A linear model was fit between venous lactate levels and median left ventricular differential pressure for all 243 timepoints. Although there was a negative relationship between lactate level and hemodynamic performance, the R^2 value only reached 0.06. Venous glucose concentration was more strongly correlated to performance, yielding a R^2 value of 0.147. Inputting a standard parameter profile of venous and arterial glucose, potassium, sodium, calcium, chloride, glucose, lactate, pH, pCO_2 , and pO_2 levels achieved a R^2 value of 0.38, with an average fivefold cross-validation score of 0.31, performing better than any individual parameter. See Figure 27 as an example of support vector regression model that displays the prediction of median differential pressures based on the given data set. The model's robustness to overfitting was verified by testing the C regularization parameter on a validation curve.

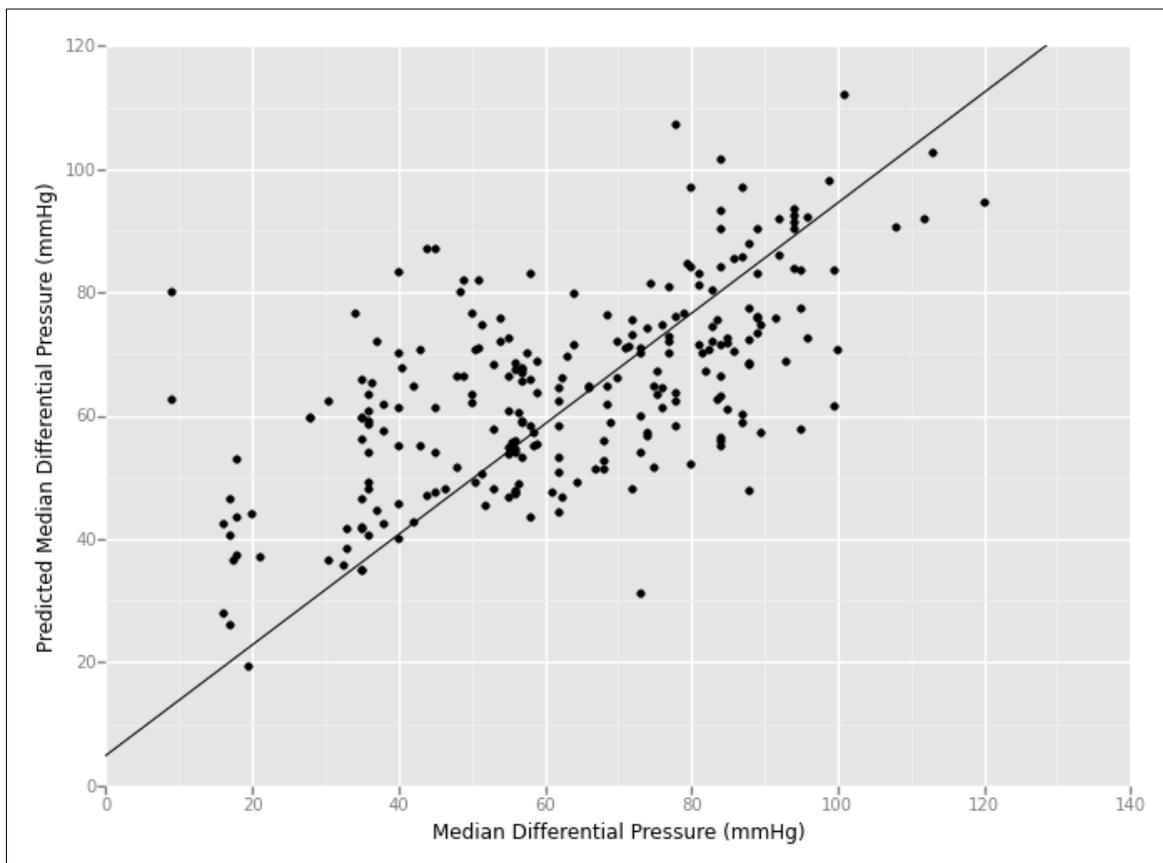


Figure 27: Example of support vector regression model to predict median differential pressure

4. Discussion

In this thesis, the utility of Omegaven as a preconditioning agent administered *in situ* and ATP administered post ischemia on an *ex vivo* perfusion apparatus was demonstrated using isolated working swine hearts. Multiple study paradigms, including differences in timing and treatment concentrations, were used to address various molecular targets and mechanisms. The overall goal was to mitigate reperfusion injury and reinforce the heart's ability to function in a simulated transplant scenario, recovering on an *ex vivo* perfusion device following a hypothermic ischemic period.

The paradigms considering the ATP treatment was developed under different points of supposition:

- (1) Under the assumption that ecto-ATPase would break up ATP into its building components immediately after ATP-tissue contact. Therefore, incremental ATP doses at the given concentration were supplied before every working mode. There was no analysis of the ATP concentration or its breakdown products in the buffer to give credence to this assumption. As a result, we had to consider further alternative modes of action.
- (2) The accumulation of ATP in the perfusate. Preliminary data from our laboratory showed that ATP given after an extended *ex vivo* time, even in high concentrations, does not have a negative effect on the heart's function. Therefore, after complete buffer changes at 70 and 80 min, the amount of ATP given throughout the protocol was replaced. We did not observe any negative impacts on the hemodynamic measurements after administration of a bolus subsequent to the buffer change.
- (3) ATP as an external energy source was debated. Chaudry discussed ATP's ability to cross cell plasma membranes, and supported the theory [79]. The intracellular ATP concentration is defined as 1-10mmol [113]. Administering the various dosages of ATP does not increase the extracellular ATP concentration in the same order of magnitude as the intracellular ATP concentration, which means the concentration remains very low in comparison to intracellular. Due to ATP's chemical features and negative charge, as well as the small amount administered, we decided to prioritize ATP's role for extracellular signaling [114].

PrC Omegaven

Omegaven is an agent that was already utilized in the Visible Heart® Laboratories to treat explanted hearts on the *ex vivo* apparatus. It showed beneficial effects in reducing arrhythmia and limiting infarct size [14,92]. This thesis work focused on hemodynamic performance *ex vivo* in combination with a metabolic assessment, building on previous findings. Omegaven was proposed as a preconditioning agent for the purpose of limiting oxidative damage to the heart.

Hemodynamic measurements showed decreased initial MDPs, as expected, within the preconditioning paradigm to preserve energy and avoid calcium recycling. The relative functional recovery of the pretreated group in comparison to control after the buffer change at 70 min indicates that Omegaven has beneficial long-term effects on that model. Also previous studies utilizing the same experimental setup support that hypothesis by showing decreased arrhythmia susceptibility when pretreated with omega-3-fatty acids.

Omegaven was further studied in the combination with tauroursodeoxycholic acid (TUDCA) and DHA, which have previously shown positive effects in this isolated model. Several combinations were delivered to the pericardial space as a convenient and effective method of improving protection of the heart against AF as well as improving post-transplantation hemodynamic function, with very positive results (data obtained within The Visible Heart® Laboratories, currently being reviewed).

Omegaven is thought to decrease oxidative stress through its double bonds reacting with free radicals generated during ischemia [91]. This was the rationale behind choosing Omegaven as a prophylactic treatment prior to ischemia. Omegaven is also thought to sequester in the cell membrane. Thus, by loading cells with Omegaven prior to ischemia, damage from free radicals formed after the ischemic event would be attenuated. The work by Yellon added to the growing body of evidence that neutrophils and an increase of inflammatory enzymes were a contributing factor to IR injury [10]. This is a potential therapeutic application for Omegaven in addition to its activity as a free radical scavenger, as it was recently found to decrease neutrophil adhesion, transmigration, and myeloperoxidase levels [94]. In this study of IR injury in the rat intestine by Bryne and colleagues, neutrophil adherence was increased fourfold in IR injury, and this effect was reduced with treatment with Omegaven 5ml/kg i.v. given one day prior and four hours before IR injury (30 min occlusion followed by 90 min reperfusion). Also, transmigration of neutrophils was found to be significantly reduced compared to IR injury with pretreatment with Omegaven. This study shows that Omegaven may confer additional benefits in attenuating inflammatory responses. However, further studies are needed to determine if pretreatment or posttreatment with Omegaven has anti-inflammatory properties in humans.

Omegaven's beneficial effect can only be assumed by combining different studies performed on the Visible Heart® apparatus, even though hearts seem more viable during longer device testing experiments on the device. For this reason, Omegaven is used as a preconditioning agent in the Visible Heart® Laboratories.

PoC ATP

ATP has been shown to activate P2Y₁ receptors leading to a G-protein mediated increase in PLC, which activates PKC to produce ROS in various studies. This action could potentially mediate conditioning via subsequent sensitization of the adenosine receptor A_{2b} [83]. Adenosine then initiates the RISK signaling cascade that ultimately closes the mPTP pore, the beneficial step of postconditioning in skeletal muscle.

It was hypothesized that ATP may sensitize this or, in fact, any receptors in swine skeletal and cardiac muscle and potentially mediate conditioning in a RI model. However, little work has been done in the area of defining ATP as a pre- and postconditioning agent in the porcine model.

Hemodynamic measurements during postconditioning on the Visible Heart[®] apparatus showed decreased initial MDPs compared to control hearts. Since PoC aims to preserve functional resources to avoid reperfusion injury and vascular damage, decreased pressures are expected. However, we would have predicted the hearts to significantly overcome initially suppressed function after reperfusion, but there was no recovery or increase in function seen over time. Therefore, the beneficial effects as a postconditioning agent remain uncertain. ATP had a depressed effect on cardiac function, which could mean either that these concentrations of ATP are toxic during initial reperfusion, or that hearts enter a beneficial state of lower metabolic output that does not recover within our experimental timeframe. However, the observations were not statistically significant in this experiment.

One limitation to our protocol is that we only allowed the heart to recover on the apparatus for 2 hr in a relatively ischemic and hypotonic solution. It is possible that prolonging our experimental time would reveal an improvement in the pretreated and postconditioned hearts over the controls. It is also worth considering that our experiments are designed to detect functional differences in relatively harsh transplantation environments. Given the improved functional performance for Sup hearts in this study, we believe that better outlining the role of PoC pathways and their corresponding molecular and physiological consequences is an important goal for further research.

Sup ATP

ATP administered incrementally to the reperfusion buffer every 10 min showed improved cardiac functionality and cardiosupportive benefits. This was seen in all related hemodynamic parameters, most pronounced at the 120-min timepoint. The data suggest that Sup helped to maintain cardiac function and preserved contractile ability better in comparison to saline controls. This might be related to ATP's role as an inotropic, extracellular signaling agent targeting the P2X₄ receptor. This activation has been shown to mediate cardioprotection in mice which were induced to overexpress this receptor [85,86]. In the same study, cardiac function and survival were improved in the group of mice that

overexpressed P2X₄. Also in a murine model of heart failure treatment with MRS2339, a P2X₄ non-hydrolyzable agonist was found to confer protection from heart failure [87]. All purine receptors are found to be expressed at the mRNA level in the human left ventricle. It should also be noted that in human right atria and sinoatrial node tissue, the most expressed ionotropic purinergic receptor is P2X₄ followed by P2X₇ with notably lesser amounts of P2X₁, 2, 3, and 5. It has also been found that endothelial nitric oxide synthase (eNOS), as a result of P2X₄ activation, is a possible interacting protein activated by calcium and calmodulin. This suggests a potential crosslink with the conditioning mechanisms. Together, these results infer that P2X₄ activation is one of many pieces of the puzzle as to how endogenous extracellular ATP exerts its protective effects in attenuating IR injury.

Decreased lactate levels in combination with the lower pO₂ levels compared to controls indicate some metabolic differences between the treated and untreated groups. It can be assumed, that treated hearts utilized more aerobic metabolism and/or were able to metabolize the accumulating lactate better than the untreated group. At the same time, those observations could be linked to some signaling pathways, intracellular and/or extracellular, which cannot be defined in this work.

Induction of arrhythmia appears to be most pronounced at the 90-min timepoint, when there is a comparatively high concentration of ATP in solution after the buffer change and the supplemented bolus. That is supported by the literature and indicates high ATP concentrations to be a pro-arrhythmogen.

The varied responses to ATP suggest complex modes of interaction with the underlying biology. This is unsurprising given the prominence of ATP as a ubiquitous energy-storing molecule. The advantages of ATP having diverse targets must be weighed with the abstruseness of its mode of action. We found that regular cardiosupportive administration of ATP increased LV pressure, lowered lactate levels, and decreased venous oxygen levels; in marked contrast, bolus administration of ATP in the PoC decreased pressure throughout the experiments.

In experimental protocols, it is advantageous to maintain function for device testing, but this is not necessarily the case for clinical practice if this added function leads to long-term cell death. Future work needs to determine which experimental outcome is most beneficial for clinical practice. If both strategies of ATP administration are beneficial, then we need to explore this question: Can both be used in the same paradigm for added benefit?

Directly comparing the PoC and Sup groups, the low-concentration Sup subgroup had significantly better functional values (MDP and Tau) after 120 min than the PoC group, but no hearts treated with ATP had significantly worse function than in the control group. Both treatment strategies, if appropriate, could be important in clinical transplant scenarios, but their dosage and application times

are different. Further research should determine how these two strategies interact in the same protocol. Our data suggest a potential to apply the Sup strategy to an *ex vivo* heart perfusion scenario, which technologic advancements could soon make a reality.

Our data may also suggest that the interaction of different ATP receptors and signaling pathways is probably dose- and time-dependent. In the Sup group, because we administered ATP shortly after reperfusion, PoC pathways could have also been activated. The very low doses of ATP administered during reperfusion may not have had a negative effect on hemodynamic function, but rather might have been cardioprotective to the mPTP utilizing different mechanisms of action simultaneously.

PrC-Sup

ATP administered incrementally to the reperfusion buffer every 10 min to hearts that were already pretreated with Omegaven showed a combined effect of both treatments. Cardiac function improvement was not as pronounced as seen in the Sup group due to the influence of Omegaven, but showed significantly better functionality after 60 min compared to controls. This indicates ATP's additional beneficial effects primarily during the first hour of function post reperfusion and initiation of working, when there is an accumulation of metabolites and Omegaven's lowering effect on MDP. At the same time, after 2 hr there was no trend indicative of long-term relative functional recover or sustainability.

These results indicate that the combination of both agents might not be a beneficial cocktail after all. Again, it is necessary to determine clinical needs on *ex vivo* perfusion and other settings to adapt pharmacological protocols to achieve best clinical long-term outcomes.

PrC-PoC

Hearts pretreated with Omegaven that were additionally postconditioned with ATP experienced hemodynamic dysfunction. These results were expected, as the underlying molecular mechanisms for both PrC and PoC depress function to avoid calcium cycling. Due to the predictable failure of this experimental paradigm within our timeframe of observation, we terminated this group.

Both hemodynamic and metabolic parameters indicated that combining these two approaches to cardioprotection are harmful to the organ within this experimental setup. Knowing that each treatment option by itself depressed MDPs *ex vivo*, the accumulated effect was not reconcilable on our *ex vivo* ischemic perfusion model that focuses on hemodynamic viability as a predictor of overall function.

Sup*

This dataset was the very first obtained that involved ATP as any sort of posttreatment. Preliminary data suggested positive results of administering ATP for better hemodynamic maintenance. It also became clear that ATP does not have a worsening effect, even though it approaches different receptors and therefore intrinsic mechanisms. Preliminary data collection also included boluses that were administered after this protocol to test ATP's toxicity, showing negative results. Based on those observations in combination with a literature review, our dosages for the different treatment paradigms were chosen.

PrC ATP Isolated Muscle Bath

Force recovery from ischemia was not effected from treatment of 10 μM ATP. Treatment with 5 μM ATP appeared to slightly improve function. More work is needed to complete this dose response study. A larger sample size for the 5 μM group and an adequately powered 2.5 μM and 1.0 μM group are needed to complete the assessment of the utility of ATP as a preconditioning agent.

This experimental approach remains part of the explorative and preliminary and data set together with Sup*.

Support Vector Regression

Using machine learning models to infer hemodynamic function from metabolic readings is a promising method for improving monitoring of heart viability. Machine learning paradigms could be implemented into medical devices such as the OSCTM. It is important to note that despite the limited number of input variables, our model was able to predict hemodynamic function reasonably well, even without knowledge of timepoint.

Clinically, very often only a few parameters are chosen to make assumptions about organ viability. In OCSTM hearts, one of the main predictors during *ex vivo* perfusion is lactate. Together with lactate assessment, the full blood gas profile which includes differentiating numerous parameters, will result in more accurate predictions on organ recovery and function.

Why RBF/What model to choose? The decision making process of which model to choose was based on domain knowledge and assumptions of how the data might behave. With a dataset considering all different blood gas parameters within a biological study setup, there is no linear behavior to be expected. Moreover, numerous recorded parameters interact with each other, assuming high complexity of the created model. Therefore the RBF was chosen and was validated by the R^2 values of the points resulting from the cross-validation prediction.

5. Limitations and Future Directions

In biomedical engineering, including device testing and pharmacological studies, the swine model has emerged as an alternative to dogs and monkeys as a choice of non-rodent species. The swine presents with major similarities in anatomy to human hearts. In general, the size of the heart can be matched with human species and age group, and the coronary arteries and blood vessels are similar. Exceptions are the large left azygos, which empties into the coronary sinus in the pig, and the presence of only two pulmonary arteries. Histologically, there are more prominent Purkinje cells and vasa vasorum in swine than in a human [115,116].

Molecularly, in the context of pre- and postconditioning of the heart, swine hearts have been considered to deviate from human signaling pathways. According to recent findings, the RISK pathway, deemed as the key to cardioprotection, is underexpressed in pigs. Still, Musiolik et al. demonstrated that PoC effects were achieved in a pig model even though the RISK pathway was pharmacologically blocked, indicating that there must be other mechanisms involved in ultimately inhibiting the mitochondrial transition pore. That could include reduction in ROS and calcium overload, improving maintenance of acidosis during early reperfusion [117]. Another study by Skyschally and colleagues indicated that RISK activation during both gentle and complete reperfusion protocols presented with similar expression, even though PoC reduced infarct size. Those same results were achieved by blocking the RISK pathway pharmacologically [118].

These findings suggest a minor role of RISK for PoC and cardioprotection in pigs, and similarly point to the SAFE pathway involving the signal transduction of TNF- α through sphingosine kinase and STAT3 as the primary PoC pathway. However, both pathways may interact and also converge to the same target—the mitochondria. Further molecular investigations are needed [119].

This diversity in pharmacological action is consistent with our contrasting results with ATP administration in different study paradigms. Future studies should therefore aim to elucidate how these mechanisms of action participate within model organisms of different cellular biology, for instance, testing a similar protocol in a species with known RISK utilization with additional studies using a RISK antagonist.

Although our experimental design implicates the benefit of intermittent ATP administration following a period of ischemia, our protocol leaves many unanswered questions as to how an appropriate dosage would be found for translational use. Downregulation of porcine RISK pathways, as well as different pharmacological half-life values of ATP in oxygenated buffer solution and blood traversing complex

vasculature, remain as important barriers to successful clinical introduction. In any case, better understanding of ATP dynamics in our experimental setup would allow for a protocol with more consistent dosage administration. For example, our administration of a bolus at buffer change to “replace” lost ATP does not take into account natural degradation. Meanwhile our initial hour of experimentation of incremental addition of ATP was meant to take into account this degradation. Given that the bolus did not drastically change expected function of the heart after buffer change, we believe that ATP stayed in buffer solution, but without more thorough ATP monitoring, we cannot be certain.

Both posttreatment approaches to the isolated heart are of great clinical interest for emerging technology, particularly the assessment of *ex vivo* function prior to and as a bridge to transplantation (e.g., OCS™). Reduction of ischemic insult ultimately strives to increase the number of viable organs for transplantation. With thorough metabolic and functional monitoring of organ function over time, more informed assessments can be made from a research and clinical perspective, giving opportunities for the administration of targeted drugs for cardioprotection. ATP as a cardioprotective agent could be one such candidate, supplemented during and after reperfusion, or as part of the OCS™ maintenance solution which constantly circulates through the system.

Organ preservation in an active metabolic state represents a precipice among the current active goals in transplantation research. A primary marker of such homeostasis is the consistency to which native intracellular ATP is maintained from explant to donation. Within the context of mitochondrial transport processes, calcium influx, ROS, and pertinent PoT and PoC mechanisms reflect the critical step towards preventing irreversible IR injury [76].

We employed a clear, acellular buffer, which is routinely used to take endoscopic internal images (Visible Heart® methodologies); this buffer could be considered as less physiologic than utilizing whole blood. Due to the lack of red blood cells and hemoglobin in the buffer, oxygen supply over time on the apparatus is insufficient. Thus the apparatus can be utilized as an acute progressive, global ischemic heart failure model. This is also shown by the development of edema over time and loss of hemodynamic and metabolic functionality.

Moreover, our protocol involved a relatively short study period. OCS™ *ex vivo* times may be more than 10 hr, thus longer application of ATP administration where global ischemia may be ongoing needs to be studied.

ATP is an agent presenting with many biochemical features, indicating the variability of side effects. It acts as a fundamental intracellular energy source and endogenous signaling agent, but its usage as a drug on an *ex vivo* cardiac perfusion device requires competent dosage for beneficial effects. High

extracellular concentrations may induce arrhythmic effects and can cause atrioventricular blockage [114]. Furthermore, ecto-ATPases cause rapid break-up, so further downstream metabolites such as AMP and adenosine require future attention. ATP is also a known vasodilator and contributes to a state of hypotonicity. Consequently, finding an optimal dosage, potentially combined with another agent to mitigate side effects or to enhance ATP's effect, would be highly beneficial. A study paradigm that would elucidate ATP's features in an extracellular environment, in whole blood or a buffer solution, would provide useful information relative to its role in cardioprotection. This could include using ATP analogues such as MRS2339 that present with stronger characteristics towards tissue interactions and enzymes.

6. Summary

Ex vivo perfusion is an emerging technology aimed to improve both graft preservation and future device development. Pharmacological treatment paradigms are highly applicable therapies to *ex vivo* devices as they can mitigate reperfusion injury, consequently foster graft longevity and increase the numbers of viable organs for transplant worldwide. The Visible Heart® methodologies, used as the experimental *ex vivo* four-chamber working swine heart model in these studies, enabled the investigations of Omegaven and ATP as pre- and posttreatments utilizing different administration paradigms. Real-time monitoring of both hemodynamic and metabolic parameters allowed for critical insights into the physiological status of the given isolated organs.

The primary hypotheses for these studies were to investigate the potential benefits of Omegaven as a pharmacological preconditioning agent. Moreover ATP was administered either as a postconditioning agent to the perfusion buffer solution in various concentrations immediately before reperfusion, or was provided as a supplement administered incrementally to the buffer solution shortly following reperfusion *ex vivo*. The administration of either or both agents in a transplant and cardiac intervention scenario could improve organ recovery and patient outcomes.

Our findings suggest that preconditioning with Omegaven enhances hemodynamic function of reanimated swine hearts. Pretreated hearts elicited better left ventricular functions even two hours after reanimation, compared to controls. Hence, during an organ procurement procedure, one could readily deliver such a pericardial therapy as a precise method to directly target the heart and not systemically. The applications of ATP as a postconditioning treatment showed rather ambiguous effects, whereas ATP as supplement after reperfusion as a continuous infusion elicited beneficial effects, and even in addition to those related to Omegaven. The clinical incremental administrations of ATP, directly to isolated human hearts, e.g., those recovered for transplantation could be applicable such as utilizing the Organ Care System™ as a bridge to transplant.

In the future, additional investigations are ongoing so to determine the molecular and underlying mechanisms associated with conditioning pathways. Nevertheless, our obtained study data presented in this thesis work is highly promising with numerous potential clinical applications which could improve patient outcomes.

Parts of this data set included in this thesis have been published:

Maria Seewald, Erik N. Gaasedelen, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iazzo: Effects of ATP administration on isolated swine hearts: Implications for *ex vivo* perfusion and cardiac transplantation. *Experimental Biology and Medicine* 2019, 244: 915–922. DOI: 10.1177/1535370219850786.

7. Zusammenfassung

Die Wiederherstellung des Blutflusses nach myokardialer Ischämie ist eine wichtige therapeutische Maßnahme und beeinflusst maßgeblich das Gesamtüberleben und das klinische Ergebnis nach einer Intervention an den Herzkranzgefäßen oder einer Herztransplantation. Paradoxe Weise kann die Reperfusion selbst zu Schädigungen führen.

Die in dieser Arbeit untersuchten Behandlungsstrategien werden als pharmakologische Prä- und Postkonditionierung bezeichnet. Dieser Therapieansatz zielt darauf ab, präventiv Pharmaka zu applizieren, um die oben genannten Reperfusionsschäden einzuschränken. Beide Ansätze unterliegen identischen intrazellulären Signalkaskaden und Rezeptorinteraktionen mit dem Ziel, die mitochondriale Transitions-pore, welche sich als Folge des ischämischen Ereignisses öffnen würde, geschlossen zu halten.

Omegaven, eine Omega-3-Fettsäureemulsion, und Adenosintriphosphat (ATP) wurden aufgrund ihrer vielversprechenden kardioprotektiven und kardiostützenden Eigenschaften für diese Arbeit ausgewählt, und unter verschiedenen Gesichtspunkten getestet. Zusätzlich zu den bereits erläuterten Mechanismen wurde ATP als supportives, inotropes und antiinflammatorisches Agens näher betrachtet und in verschiedenen Protokollen *ex vivo* untersucht. Ziel aller Protokolle war die pharmakologische Stabilisierung des Organs nach der Reperfusion sowie die physiologische Evaluation der Organe über mindestens zwei Stunden *ex vivo*. Dazu wurden die Daten von 45 Versuchen an isolierten Schweineherzen ausgewertet und verglichen.

Prä- und Postkonditionierung sowie kardiostützende Pharmaka stellen ein großes Potential in vielen Bereichen der Herzmedizin dar. In dieser Arbeit konnte gezeigt werden, dass Präkonditionierung mit Omegaven eine verbesserte hämodynamische Funktion der Organe zur Folge hatte. Während Postkonditionierung mit ATP im Rahmen dieser Analyse ambivalente Ergebnisse zeigte, war die inkrementale Supplementierung mit ATP nach Reperfusion vorteilhaft bezüglich der hämodynamischen und metabolischen Funktion der isolierten Organe. Weitere Untersuchungen der Agenzien bezüglich der optimalen Konzentrations-Wirkungs-Beziehungen sowie der molekularen Interaktionen in diesem Versuchsaufbau sind notwendig, um sichere Schlussfolgerungen abzuleiten.

Ein Teil der Daten dieser medizinischen Doktorarbeit wurde bereits veröffentlicht:

Maria Seewald, Erik N. Gaasedelen, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iaizzo: Effects of ATP administration on isolated swine hearts: Implications for *ex vivo* perfusion and cardiac transplantation. *Exp Biol Med* (Maywood). 2019 May 27:1535370219850786. doi: 10.1177/1535370219850786.

List of Figures

Figure 1: Paradigms of ischemic pre-and postconditioning.....	12
Figure 2: Proposed molecular second messenger pathways of pre- and postconditioning.....	13
Figure 3: OCS™ Heart.....	22
Figure 4: Chemical structure of adenosine-5'-triphosphate.....	23
Figure 5: In situ setup	29
Figure 6: Calculation of ejection fraction using echocardiography images.....	30
Figure 7: Scheme of Visible Heart® Methodologies.....	32
Figure 8: Ex vivo preparation Visible Heart® Methodologies.....	33
Figure 9: Working modalities on the Visible Heart® apparatus.....	34
Figure 10: Muscle bath studies	35
Figure 11: Overview of study groups	37
Figure 12: In Situ - PrC timeline and study protocol.....	38
Figure 13: In Vitro – PoC and Sup treatment timeline and study protocol.....	39
Figure 14. In Vitro – Sup* treatment timeline and study protocol	39
Figure 15: In Vitro - PrC isolated muscle bath study timeline	40
Figure 16: Extract of the script written in Python showing comparison linear regression and support vector regression	42
Figure 17: A. Linear support vector machine; B. Radial basis function kernel.....	43
Figure 18: Cross-validation scheme.....	43
Figure 19: PrC with Omegaven hemodynamics	47
Figure 20: PrC with Omegaven metabolism.....	48
Figure 21: PoC with ATP hemodynamics	49
Figure 22: PoC with ATP metabolism.....	49
Figure 23: Sup with ATP hemodynamics	51
Figure 24: Sup with ATP metabolism.....	52
Figure 25: PrC-Sup with Omegaven and ATP hemodynamics	53
Figure 26: PrC-Sup with Omegaven and ATP metabolism.....	54
Figure 27: Example of support vector regression model to predict median differential pressure	56

References

- [1] D.A. Cooley, C. Walton Lillehei, the “Father of Open Heart Surgery,” *Circulation*. 100 (1999) 1364–1365. <https://doi.org/10.1161/01.CIR.100.13.1364>.
- [2] E.E. Bakken, Earl E. Bakken Life Timeline 1924, (1943) 1–10. <http://www.earlbakken.com/content/timeline/timeline.html> (accessed January 28, 2020).
- [3] Medtronic, (2016). <http://www.medtronic.com/us-en/about.html> (accessed January 1, 2016).
- [4] Visible Heart® Laboratories, VHL history, (n.d.). <http://www.vhlab.umn.edu/about.html> (accessed January 1, 2016).
- [5] B.T. Howard, T.L. Iles, J.A. Coles, D.C. Sigg, P.A. Iaizzo, Reversible and Irreversible Damage of the Myocardium: Ischemia/Reperfusion Injury and Cardioprotection, in: P.A. Iaizzo (Ed.), *Handb. Card. Anatomy, Physiol. Devices*, 3rd. Editi, 2015. <https://doi.org/10.1007/978-3-319-19464-6>.
- [6] M. Karmazyn, The Myocardial Sodium-hydrogen Exchanger (NHE) and Its Role in Mediating Ischemic and Reperfusion Injury, *Keio J. Med.* 47 (1997) 65–72.
- [7] R. Bolli, E. Marba, E. Marba, Molecular and Cellular Mechanisms of Myocardial Stunning, *Am. Physiol. Soc.* 79 (1999) 609–634.
- [8] Y.-T. Shen, S.F. Vatner, Mechanism of Impaired Myocardial Function During Progressive Coronary Stenosis in Conscious Pigs, *Cardiovasc Res.* 76 (1995) 479–488. <https://doi.org/https://doi.org/10.1161/01.res.76.3.479>.
- [9] W.E. Boden, W.W. Brooks, C.H. Conrad, O.H.L. Bing, W.B. Hood, Incomplete , delayed functional recovery late after reperfusion following acute myocardial infarction : " Maimed myocardium ", *Am. Heart J.* 130 (1995) 27–29.
- [10] D.M. Yellon, D.J. Hausenloy, Myocardial Reperfusion Injury, *N. Engl. J. Med.* 357 (2007) 1121–1135. <https://doi.org/0.1056/NEJMra071667>.
- [11] M. V. Cohen, J.M. Downey, Signalling pathways and mechanisms of protection in pre- and postconditioning : historical perspective and lessons for the future, *Br. J. Pharmacol.* 172 (2015) 1913–1932. <https://doi.org/10.1111/bph.12903>.
- [12] A. Farb, F.D. Kolodgie, M. Jenkins, R. Virmani, Myocardial Infarct Extension During Artery Occlusion : Pathologic Evidence sion After Coronary 1 to III . HRP I min to, *J Am Coll Cardiol.* 21 (1993) 1245–1253.
- [13] K. Matsumura, R.W. Jeremy, J. Schaper, L.C. Becker, Progression of Myocardial Necrosis During Reperfusion of Ischemic Myocardium, *Circulation.* 97 (1998) 795–805.
- [14] Y. Xiao, D.C. Sigg, M.R. Ujhelyi, J.J. Wilhelm, E.S. Richardson, P.A. Iaizzo,

- Pericardial delivery of omega-3 fatty acid : a novel approach to reducing myocardial infarct sizes and arrhythmias, *Am J Physiol Hear. Circ Physiol.* 294 (2008) 2212–2218. <https://doi.org/10.1152/ajpheart.91502.2007>.
- [15] X.H.T. Wehrens, P.A. Doevendans, T.J.O. Ophuis, H.J.J. Wellens, A comparison of electrocardiographic changes during reperfusion of acute myocardial infarction by thrombolysis or percutaneous transluminal coronary angioplasty, *Am J Physiol Hear. Circ Physiol.* 139 (2000) 430–6.
- [16] G. Minguet, J.F. Brichant, J.J. Oris, Opioids and protection against ischemia-reperfusion injury : From experimental data to potential clinical applications Opioids and protection against ischemia-reperfusion injury : from experimental data to potential clinical applications, *Acta Anaesth. Belg.* 63 (2012) 23–34.
- [17] P.B. Shereif H. Rezkalla, MD; Robert A. Kloner, MD, No-Reflow Phenomenon, 105 (2002) 656–662.
- [18] C.E. Murry, R.B. Jennings, K.A. Reimer, Preconditioning with ischemia : injury delay of lethal cell isChemical myocardium, *Circulation.* 74 (1986) 1124–1136. <https://doi.org/https://doi.org/10.1161/01.cir.74.5.1124>.
- [19] T. Yetgin, O.C. Manintveld, D.J. Duncker, W.J. van der Giessen, Postconditioning against ischaemia-reperfusion injury : ready for wide application in patients ?, *Neth Hear. J.* 18 (2010) 389–392. <https://doi.org/https://dx.doi.org/10.1007%2Fbf03091803>.
- [20] D.J. Hausenloy, A. Tsang, M.M. Mocanu, D.M. Yellon, Ischemic preconditioning protects by activating prosurvival kinases at reperfusion, (2005) 971–976. <https://doi.org/10.1152/ajpheart.00374.2004>.
- [21] Z. Zhao, J.S. Corvera, M.E. Halkos, F. Kerendi, N.-P. Wang, R.A. Guyton, J. Vinten-johansen, Inhibition of myocardial injury by ischemic postconditioning during reperfusion : comparison with ischemic preconditioning, *Am J Physiol Hear. Circ Physiol.* 285 (2003) 579–588.
- [22] R.A. Kloner, S.H. Rezkalla, Preconditioning , postconditioning and their application to clinical cardiology, *Cardiovasc. Res.* 70 (2006) 297–307. <https://doi.org/10.1016/j.cardiores.2006.01.012>.
- [23] M. Basalay, V. Barsukevich, S. Mastitskaya, A. Mrochek, J. Pernow, P. Sjöquist, G.L. Ackland, A. V Gourine, A. Gourine, Remote ischaemic pre- and delayed postconditioning – similar degree of cardioprotection but distinct mechanisms, *Exp Physiol.* 97 (2012) 908–917. <https://doi.org/10.1113/expphysiol.2012.064923>.
- [24] G.S. Liu, J. Thornton, D.M. van Winkle, A.W.H. Stanley, R.A. Olsson, J.M. Downey, Protection Against Infarction Afforded by Preconditioning is Mediated by A1 Adenosine Receptors in Rabbit Heart, *Circulation.* 84 (1991) 350–357.
- [25] M. Goto, Y. Liu, X.-M. Yang, J.L. Ardell, M. V. Cohen, J.M. Downey, Role of Bradykinin in Protection of Ischemic Preconditioning in Rabbit Hearts, *Cardiovasc Res.* 77 (1995) 611–621.
- [26] M. V Cohen, S. Philipp, T. Krieg, L. Cui, A. Kuno, V. Solodushko, J.M. Downey,

- Preconditioning-mimetics bradykinin and DADLE activate PI3-kinase through divergent pathways, *J. Mol. Cell. Cardiol.* 42 (2007) 842–851. <https://doi.org/10.1016/j.yjmcc.2007.01.004>.
- [27] J.P. Headrick, L.E.S. Hoe, E.F. Du Toit, J.N. Peart, Opioid receptors and cardioprotection – ‘ opioidergic conditioning ’ of the heart *Tables of Links, Br. J. Pharmacol.* 172 (2015) 2026–2050. <https://doi.org/10.1111/bph.13042>.
- [28] C. Penna, G. Alloatti, S. Cappello, D. Gattullo, G. Berta, B. Mognetti, G. Losano, P. Pagliaro, G. Alloatti, S. Cappello, G. Berta, B. Mognetti, G. Losano, P. Pagliaro, Platelet-activating factor induces cardioprotection in isolated rat heart akin to ischemic preconditioning : role of phosphoinositide 3-kinase and protein kinase C activation, *Am J Physiol Hear. Circ Physiol.* 288 (2005) 2512–2520. <https://doi.org/10.1152/ajpheart.00599.2004>.
- [29] C.S. Junhui Suna, Angel M. Aponte, Mark J. Kohra, Guang Tonga, E. Murphy, Essential role of nitric oxide in acute ischemic preconditioning: S-Nitros(yl)ation versus sGC/cGMP/PKG signaling?, *Free Radic Biol Med.* 54 (2012) 105–112. <https://doi.org/10.1016/j.freeradbiomed.2012.09.005.Essential>.
- [30] A.O. Garlid, M. Jaburek, J.P. Jacobs, K.D. Garlid, Mitochondrial reactive oxygen species : which ROS signals cardioprotection ?, *Am J Physiol Hear. Circ Physiol.* 305 (2013) 960–968. <https://doi.org/10.1152/ajpheart.00858.2012>.
- [31] M. V. Cohen, X. Yang, G.S. Liu, G. Heusch, J.M. Downey, Acetylcholine, Bradykinin, Opioids, and Phenylephrine, but not Adenosine, Trigger Preconditioning by Generating Free Radicals and Opening Mitochondrial KATP Channels, *Circ Res.* 89 (2001) 273–279. <https://doi.org/10.1161/hh1501.094266>.
- [32] D.J. Hausenloy, D.M. Yellon, New directions for protecting the heart against ischaemia – reperfusion injury : targeting the Reperfusion Injury Salvage Kinase (RISK) -pathway, *Cardiovasc Res.* 61 (2004) 448–460. <https://doi.org/10.1016/j.cardiores.2003.09.024>.
- [33] D.J. Hausenloy, H.L. Maddock, G.F. Baxter, D.M. Yellon, Inhibiting mitochondrial permeability transition pore opening : a new paradigm for myocardial preconditioning ?, *Cardiovasc. Res.* 55 (2002) 534–543.
- [34] K. Foerster, I. Paul, N. Solenkova, A. Staudt, M. V. Cohen, J.M. Downey, S.B. Felix, T. Krieger, NECA at reperfusion limits infarction and inhibits formation of the mitochondrial permeability transition pore by activating p70S6 kinase, *Basic Res Cardiol.* 101 (2006) 319–326. <https://doi.org/10.1007/s00395-006-0593-4>.
- [35] C. Penna, D. Mancardi, G. Losano, Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism , mitochondrial ATP-sensitive K + channel and protein kinase C activation, 189 (2006) 180–189. <https://doi.org/10.1007/s00395-006-0584-5>.
- [36] M. V. Cohen, X.-M. Yang, J.M. Downey, The pH Hypothesis of Postconditioning Staccato Reperfusion Reintroduces Oxygen and Perpetuates, *Circulation.* 115 (2007) 1895–1903. <https://doi.org/10.1161/CIRCULATIONAHA.106.675710>.

- [37] M. V. Cohen, X.-M. Yang, J.M. Downey, Acidosis, Oxygen, and Interference with Mitochondrial Permeability Transition Pore Formation in the Early Minutes of Reperfusion Are Critical to Postconditioning's Success, *Basic Res Cardiol.* 103 (2008) 464–471. <https://doi.org/10.1007/s00395-008-0737-9>. Acidosis.
- [38] H. Kin, Z. Zhao, H. Sun, N. Wang, J.S. Corvera, M.E. Halkos, F. Kerendi, R.A. Guyton, J. Vinten-johansen, Postconditioning attenuates myocardial ischemia – reperfusion injury by inhibiting events in the early minutes of reperfusion, *Cardiovasc Res.* 62 (2004) 74–85. <https://doi.org/10.1016/j.cardiores.2004.01.006>.
- [39] M. Basalay, V. Barsukevich, S. Mastitskaya, A. Mrochek, J. Pernow, P. Sj, G.L. Ackland, A. V Gourine, A. Gourine, Remote ischaemic pre- and delayed postconditioning – similar degree of cardioprotection but distinct mechanisms, *Exp Physiol* 97.8. 8 (2012) 908–917. <https://doi.org/10.1113/expphysiol.2012.064923>.
- [40] Z. Xu, J.M. Downey, M. V Cohen, AMP 579 Reduces Contracture and Limits Infarction in Rabbit Heart by Activating Adenosine A 2 Receptors, *J Cardiovasc Pharmacol.* 38 (2001) 474–481.
- [41] J.-J. Ke, F.-X. Yu, Y. Rao, Y. Wang, Adenosine postconditioning protects against myocardial ischemia – reperfusion injury though modulate production of TNF- α and prevents activation of transcription factor NF- κ B, *Mol Biol Rep.* 38 (2011) 531–538. <https://doi.org/10.1007/s11033-010-0137-8>.
- [42] G.F. Baxter, Z. Ebrahim, Role of bradykinin in preconditioning and protection of the ischaemic myocardium, *Br J Pharmacol.* 135 (2002) 843–854.
- [43] P.C. Chiari, M.W. Bienengraeber, D. Ph, P.S. Pagel, D. Ph, J.G. Krolikowski, Isoflurane Protects against Myocardial Infarction during Early Reperfusion by Activation of Phosphatidylinositol-3- Kinase Signal Transduction : Evidence for Anesthetic- induced Postconditioning in Rabbits, *Anesthesiology.* 102 (2005) 102–109.
- [44] Y.-C. Xu, R.-P. Li, F.-S. Xue, X.-L. Cui, S.-Y. Wang, G.-P. Liu, G.-Z. Yang, C. Sun, X. Liao, κ -Opioid receptors are involved in enhanced cardioprotection by combined fentanyl and limb remote ischemic postconditioning, *J. Anesth.* 29 (2015) 535–543. <https://doi.org/10.1007/s00540-015-1998-8>.
- [45] Y. Wu, J. Wan, W.-Z. Zhen, L.-F. Chen, J. Zhan, J.-J. Ke, Z. Zhang, Y.-L. Wang, The effect of butorphanol postconditioning on myocardial ischaemia reperfusion injury in rats, *Interact. Cardiovasc. Thorac. Surg.* 18 (2013) 308–312. <https://doi.org/10.1093/icvts/ivt516>.
- [46] N. V Naryzhnaya, L.N. Maslov, Y.B. Lishmanov, G.J. Gross, Receptor Specificity of the Antiarrhythmic Effect Produced by Opioid Peptides Dalargin and DADLE during Myocardial Reperfusion, *Bull Exp Biol Med.* 133 (2002) 391–393.
- [47] J.A. Coles, D.C. Sigg, P.A. Iaizzo, Role of kappa-opioid receptor activation in pharmacological preconditioning of swine, *Am J Physiol Hear. Circ Physiol.* 284 (2003) 2091–2099. <https://doi.org/10.1152/ajpheart.00843.2002>.
- [48] D.C. Sigg, J.A. Coles, P.R. Oeltgen, P.A. Iaizzo, C. Daniel, J.A. Coles, P.R. Oeltgen,

- P.A. Iaizzo, Role of μ -opioid receptor agonists on infarct size reduction in swine, *Am J Physiol Hear. Circ Physiol.* 282 (2002) 1953–1960.
- [49] M. Seewald, J.A. Coles, D.C. Sigg, P.A. Iaizzo, Featured Article: Pharmacological postconditioning with delta opioid attenuates myocardial reperfusion injury in isolated porcine hearts, *Exp. Biol. Med.* 242 (2017) 986–995.
<https://doi.org/10.1177/1535370216684041>.
- [50] B. Olafsson, M.B. Forman, D.W. Puett, A. Pou, C.U. Cates, G.C. Friesinger, R. Virmani, Reduction of reperfusion injury in the canine preparation by intracoronary adenosine : importance of the endothelium and the no-reflow phenomenon, *Circulation.* 76 (1987) 1135–1146.
- [51] M. Galagudza, D. Kurapeev, S. Minasian, G. Valen, J. Vaage, Ischemic postconditioning : brief ischemia during reperfusion converts persistent ventricular fibrillation into regular rhythm, *Eur J Cardiothorac Surg.* 25 (2004) 1006–1010.
<https://doi.org/10.1016/j.ejcts.2004.02.003>.
- [52] D.C. Sigg, J.A. Coles, W.J. Gallagher, P.R. Oeltgen, P.A. Iaizzo, Opioid Preconditioning : Myocardial Function and Energy Metabolism, *Ann Thorac Surg.* 72 (2001) 1576–82.
- [53] G.T.C. Wong, R. Li, L.L. Jiang, M.G. Irwin, Remifentanil post-conditioning attenuates cardiac ischemia-reperfusion injury via κ or δ opioid receptor activation, *Acta Anaesthesiol. Scand.* 54 (2010) 510–518. <https://doi.org/10.1111/j.1399-6576.2009.02145.x>.
- [54] R. Bolli, Mechanism of Myocardial " Stunning ", *Circulation.* 82 (1990) 723–738.
- [55] L.H. Opie, W.A. Coetzee, Role of calcium ions in reperfusion arrhythmias: relevance to pharmacologic intervention, *Cardiovasc. Drugs Ther.* 2 (1988) 623–636.
<https://doi.org/https://doi.org/10.1007/bf00054202>.
- [56] H.M. Piper, D. Garcia-Dorado, Prime Causes of Rapid Cardiomyocyte Death During Reperfusion, *Ann Thorac Surg.* 68 (1999) 1913–1919.
- [57] M. Ovize, G.F. Baxter, F. Di Lisa, D. Garcia-dorado, D.J. Hausenloy, G. Heusch, J. Vinten-johansen, D.M. Yellon, R. Schulz, Postconditioning and protection from reperfusion injury : where do we stand ? Position Paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology, *Cardiovasc. Res.* 87 (2010) 406–423. <https://doi.org/10.1093/cvr/cvq129>.
- [58] J.A. Watts, E.M. Hawes, S.H. Jenkins, T.C. Williams, Effects of nisoldipine on the no-reflow phenomenon in globally ischemic rat hearts, *J Cardiovasc Pharmacol.* 16 (1990) 487–494. <https://doi.org/https://doi.org/10.1097/00005344-199009000-00020>.
- [59] R.-P. Xiao, H.A. Spurgeon, M.C. Capogrossi, E.G. Lakatta, Stimulation of Opioid receptors on Cardiac Ventricular Myocytes reduces L Type Ca²⁺ Channel Current, *J. Mol. Cell. Cardiol.* 25 (1993) 661–666.
<https://doi.org/https://doi.org/10.1006/jmcc.1993.1079>.
- [60] R. Xiao, S. Pepe, H.A. Spurgeon, M.C. Capogrossi, E.G. Lakatta, Opioid peptide

- receptor stimulation reverses P-adrenergic effects in rat heart cells, *Am. J. Physiol.* 272 (1997) 797–805. <https://doi.org/https://doi.org/10.1152/ajpheart.1997.272.2.H797>.
- [61] H. Ito, Y. Taniyama, K. Iwakura, N. Nishikawa, T. Masuyama, T. Kuzuya, M. Hori, Y. Higashino, K. Fujii, T. Minamino, Intravenous Nicorandil Can Preserve Microvascular Integrity and Myocardial Viability in Patients With Reperused Anterior Wall Myocardial Infarction, *J. Am. Coll. Cardiol.* 33 (1999) 604–4.
- [62] H.J. Dargie, I. Ford, K.M. Fox, W.S. Hillis, Effect of nicorandil on coronary events in patients with stable angina : the Impact Of Nicorandil in Angina (IONA) randomised trial, *Lancet.* 359 (2002) 1269–1275. [https://doi.org/https://doi.org/10.1016/S0140-6736\(02\)08265-X](https://doi.org/https://doi.org/10.1016/S0140-6736(02)08265-X).
- [63] D.J. Patel, H.J. Purcell, K.M. Fox, Cardioprotection by opening of the K (ATP) channel in unstable angina Is this a clinical manifestation of myocardial preconditioning? Results of a randomized study with nicorandil, *Eur. Heart J.* 20 (1999) 51–57.
- [64] M. Murata, M. Akao, B. O'Rourke, E. Marbán, Mitochondrial ATP-Sensitive Mitochondrial ATP-Sensitive Potassium Channels Attenuate Matrix Ca²⁺ Overload During Simulated Ischemia and Reperfusion. Possible Mechanism of Cardioprotection, *Circ. Res.* 89 (2001) 891–899. <https://doi.org/10.1161/hh2201.100205>.
- [65] J.M. Downey, M. V. Cohen, Why Do We Still Not Have Cardioprotective Drugs?, *Circ J.* 73 (2009) 1171–1177. <https://doi.org/https://doi.org/10.1253/circj.cj-09-0338>.
- [66] E.-X. Lu, S.-X. Chen, M.-D. Yuan, T.-H. Hu, H.-C. Zhou, W.-J. Luo, G.-H. Li, L.-M. Xu, Preconditioning Improves Myocardial Preservation in Patients Undergoing Open Heart Operations, *Ann. Thorac. Surg.* 64 (1997) 1320–1324. [https://doi.org/10.1016/S0003-4975\(97\)00838-2](https://doi.org/10.1016/S0003-4975(97)00838-2).
- [67] Z. Wu, M.R. Tarkka, J. Eloranta, E. Pehkonen, L. Kaukinen, E.L. Honkonen, S. Kaukinen, Effect of Ischemic Preconditioning on Myocardial Protection in Coronary Artery Bypass Graft Patients*. Can the Free Radicals Act as a Trigger for Ischemic Preconditioning ?, *Chest J.* 119 (2001) 1061–1068.
- [68] Y. Nakagawa, H. Ito, M. Kitakaze, H. Kusuoka, M. Hori, T. Kuzuya, Y. Higashino, K. Fujii, T. Minamino, Effect of angina pectoris on myocardial protection in patients with reperused anterior wall myocardial infarction: Retrospective clinical evidence of “preconditioning,” *J. Am. Coll. Cardiol.* 25 (1995) 1076–1083. [https://doi.org/10.1016/0735-1097\(94\)00542-X](https://doi.org/10.1016/0735-1097(94)00542-X).
- [69] Eurotransplant International Foundation, Annual Report 2018, Leiden, 2018.
- [70] UNOS, Transplant trends UNOS, (2019). <https://unos.org/data/transplant-trends/> (accessed January 2, 2020).
- [71] T.K. Rajab, S.K. Singh, Donation After Cardiac Death Heart Transplantation in America Is Clinically Necessary and Ethically Justified, *Circ. Hear. Fail.* 11 (2018) 10–12. <https://doi.org/10.1161/CIRCHEARTFAILURE.118.004884>.
- [72] A. Klein, C. Lewis, J. Madsen, Organ Transplantation: A Clinical Guide. Section

Heart, Cambridge University Press, 2011.
<https://doi.org/https://doi.org/10.1017/CBO9780511994876>.

- [73] S.R. Zeissig, F. Polster, N.R. Fruehauf, G. Kirste, I. Schmidtman, Current Practice of Heart Donor Evaluation in Germany : Multivariable Risk Factor Analysis Confirms Practicability of Guidelines, *J. Transplant.* 2013 (2013) 7.
<https://doi.org/https://doi.org/10.1155/2013/701854>.
- [74] A. Ardehali, F. Esmailian, M. Deng, E. Soltesz, E. Hsich, Y. Naka, D. Mancini, M. Camacho, M. Zucker, Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective , open-label , multicentre , randomised non-inferiority trial, *Lancet.* 385 (2015) 2577–2584. [https://doi.org/10.1016/S0140-6736\(15\)60261-6](https://doi.org/10.1016/S0140-6736(15)60261-6).
- [75] M.J. Taylor, C. Baicu, Current State of Hypothermic Machine Perfusion Preservation of Organs : The Clinical Perspective, *Cryobiology.* 60 (2011) 20–35.
<https://doi.org/10.1016/j.cryobiol.2009.10.006.Current>.
- [76] D.A. Schipper, K.M. Marsh, A.S. Ferng, D.J. Duncker, J.D. Laman, Z. Khalpey, The Critical Role of Bioenergetics in Donor Cardiac Allograft Preservation, *J. Cardiovasc. Transl. Res.* 9 (2016) 176–183. <https://doi.org/10.1007/s12265-016-9692-2>.
- [77] TransMedics, The Organ Care System (OCS TM) HEART Overview The Organ Care System (OCS TM), (2015) 1–14.
- [78] TransMedics, OCSTM Heart, (2020). <https://www.transmedics.com/ocs-heart/> (accessed February 1, 2020).
- [79] I.H. Chaudry, Does ATP Cross the Cell Plasma Membrane ?, *Yale J. Biol. Med.* 55 (1982) 1–10.
- [80] G. Burnstock, Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future, *BioEssays.* 34 (2012) 218–25. <https://doi.org/10.1002/bies.201100130>.
- [81] G. Burnstock, A. Pelleg, Cardiac purinergic signalling in health and disease, *Purinergic Signal.* 11 (2015) 1–46. <https://doi.org/10.1007/s11302-014-9436-1>.
- [82] D. Shah, F. Romero, W. Stafstrom, M. Duong, R. Summer, Extracellular ATP mediates the late phase of neutrophil recruitment to the lung in murine models of acute lung injury, *Am J Physiol Lung Cell Mol Physiol.* 306 (2013) 152–61.
<https://doi.org/10.1152/ajplung.00229.2013>.
- [83] A. Díaz-Vegas, C.A. Campos, A. Contreras-Ferrat, M. Casas, S. Buvinic, E. Jaimovich, A. Espinosa, ROS Production via P2Y 1 -PKC-NOX2 Is Triggered by Extracellular ATP after Electrical Stimulation of Skeletal Muscle Cells, *PLoS One.* (2015) 1–14. <https://doi.org/10.1371/journal.pone.0129882>.
- [84] H. Musa, J.O. Tellez, N.J. Chandler, I.D. Greener, M. Macewski, U. MacKiewicz, A. Beresewicz, P. Molenaar, M.R. Boyett, H. Dobrzynski, P2 purinergic receptor mRNA in rat and human sinoatrial node and other heart regions, *Naunyn. Schmiedebergs. Arch. Pharmacol.* 379 (2009) 541–549. <https://doi.org/10.1007/s00210-009-0403-2>.

- [85] D. Sonin, S.-Y. Zhou, C. Cronin, T. Sonina, J. Wu, K.A. Jacobson, A. Pappano, B.T. Liang, L. Bt, Role of P2X purinergic receptors in the rescue of ischemic heart failure, *Am J Physiol Hear. Circ Physiol.* 295 (2008) 1191–1197. <https://doi.org/10.1152/ajpheart.00577.2008>.
- [86] J.-B. Shen, R. Shutt, M. Agosto, A. Pappano, B.T. Liang, Reversal of cardiac myocyte dysfunction as a unique mechanism of rescue by P2X 4 receptors in cardiomyopathy, *Am J Physiol Hear. Circ Physiol.* 296 (2009) 1089–H1095. <https://doi.org/https://dx.doi.org/10.1152%2Fajpheart.01316.2008>.
- [87] S.-Y. Zhou, M. Mamdani, K. Qanud, J.-B. Shen, A.J. Pappano, T.S. Kumar, K.A. Jacobson, T. Hintze, F.A. Recchia, B.T. Liang, Treatment of Heart Failure by a Methanocarpa Derivative of Adenosine Monophosphate: Implication for a Role of Cardiac Purinergic P2X Receptors, *J Pharmacol Exp Ther.* 333 (2010) 920–928. <https://doi.org/10.1124/jpet.109.164376>.
- [88] D. Sonin, S.-Y. Zhou, C. Cronin, T. Sonina, J. Wu, K.A. Jacobson, A. Pappano, B.T. Liang, Z. S-y, Role of P2X purinergic receptors in the rescue of ischemic heart failure, *Am J Physiol Hear. Circ Phys-Iol.* 295 (2008) 1191–1197. <https://doi.org/10.1152/ajpheart.00577.2008>.—Evidence.
- [89] D.A. Vessey, L. Li, M. Kelley, P2X 7 receptor agonists pre- and postcondition the heart against ischemia-reperfusion injury by opening pannexin-1 / P2X 7 channels, *Am J Physiol Hear. Circ Physiol.* 301 (2011) 881–887. <https://doi.org/10.1152/ajpheart.00305.2011>.
- [90] R. Yang, B.T. Liang, Targets in Ischemia and Heart Failure ?, *Cardiovasc Res.* 111 (2012) 397–401. <https://doi.org/10.1161/CIRCRESAHA.112.265959>.
- [91] M. Veljovic, A. Popadic, Z. Vukic, R. Ilic, Z. Trifunovic, M. Antunovic, V. Mandaric, S. Tisma, Z. Markovic, Myocardial protection during elective coronary artery bypasses grafting by pretreatment with omega-3 polyunsaturated fatty acids, *Vojnosanit. Pregl.* 70 (2013) 484–492. <https://doi.org/10.2298/VSP1305484V>.
- [92] E.S. Richardson, P.A. Iaizzo, Y. Xiao, Electrophysiological Mechanisms of the Anti-arrhythmic Effects of Omega-3 Fatty Acids, *J. Cardiovasc. Trans. Res.* 4 (2011) 42–52. <https://doi.org/10.1007/s12265-010-9243-1>.
- [93] A.N.C. Gungor, H. Turkon, A. Albayrak, M. Ovali, M. Islimye, M. Gencer, S. Hacivelioglu, S. Cevizci, I. Cesur, E. Cosar, Does Omegaven have beneficial effects on a rat model of ovarian ischemia/reperfusion?, *Eur. J. Obstet. Gynecol.* 181 (2014) 240–245. <https://doi.org/10.1016/j.ejogrb.2014.08.001>.
- [94] J. Byrne, J. Mcguinness, G. Chen, A.D.K. Hill, M.J. Redmond, Intravenous omega-3 , a technique to prevent an excessive innate immune response to cardiac surgery in a rodent gut ischemia model, *J. Thorac. Cardiovasc. Surg.* 141 (2010) 803–807. <https://doi.org/10.1016/j.jtcvs.2010.04.030>.
- [95] I. Sukhotnik, N. Slijper, Y. Pollak, E. Chemodanov, R. Shaoul, A.G. Coran, J.G. Mogilner, Parenteral omega-3 fatty acids (Omegaven) modulate intestinal recovery after intestinal ischemia-reperfusion in a rat model, *J. Pediatr. Surg.* 46 (2011) 1353–

1360. <https://doi.org/10.1016/j.jpedsurg.2010.09.051>.
- [96] T.L. Iles, B. Howard, S. Howard, S. Quallich, C. Rolfes, E. Richardson, H.R. Iaizzo, P.A. Iaizzo, Testing the Efficacy of Pharmacological Agents in a Pericardial Target Delivery Model in the Swine, *J. Vis. Exp.* 113 (2016) 1–7. <https://doi.org/10.3791/52600>.
- [97] P. Jynge, D. Hearse, F. D, W. Mahalu, S. Canković-Darracott, K. O’Brien, M. Braimbridge, The St. Thomas’ hospital cardioplegic solution: a characterization in two species., *Scand J Thorac Cardiovasc Surg Suppl.* 30 (1981) 1–28.
- [98] L.E. Bailey, S.D. Onc, Krebs-Henseleit Solution as a Physiological Buffer in Perfused and Superfused Preparations WCOs-1, *J. Pharmacol. Methods.* 1 (1978) 171–175.
- [99] A.J. Hill, T.G. Laske, J.A. Coles, D.C. Sigg, N.D. Skadsberg, S.A. Vincent, C.L. Soule, W.J. Gallagher, P.A. Iaizzo, In Vitro Studies of Human Hearts, *Ann Thorac Surg.* 79 (2005) 168–77. <https://doi.org/10.1016/j.athoracsur.2004.06.080>.
- [100] S.P. Bell, C. Bs, M.N. Sack, A. Patel, L.H. Opie, D.P. Hil, D.M. Yellon, C. Ds, Delta Opioid Receptor Stimulation Mimics Ischemic Preconditioning in Human Heart Muscle, *J Am Coll Cardiol.* 36 (2000) 2296–302.
- [101] Visible Heart® Laboratories, Methodologies/Visible-Heart/Methods, (n.d.). <http://www.vhlab.umn.edu/atlas/methodologies/visible-heart/methods.shtml> (accessed January 28, 2020).
- [102] R.M. Bell, M.M. Mocanu, D.M. Yellon, Retrograde heart perfusion : The Langendorff technique of isolated heart perfusion, *J. Mol. Cell. Cardiol.* 50 (2011) 940–950. <https://doi.org/10.1016/j.yjmcc.2011.02.018>.
- [103] E. Chinchoy, C.L. Soule, A.J. Houlton, W.J. Gallagher, M.A. Hjelle, T.G. Laske, P.A. Iaizzo, Isolated Four-Chamber Working Swine Heart Model, *Ann Thorac Surg.* 70 (2000) 1607–14.
- [104] F. Pedregosa, G. Varoquaux, A. Gramfort, V. Michel, B. Thirion, O. Grisel, M. Blondel, Scikit-learn : Machine Learning in Python, *J. Mach. Learn. Res.* 12 (2011) 2825–2830.
- [105] Python Software Foundation, Python Software, (2016). <http://www.python.org> (accessed February 1, 2020).
- [106] W. Mckinney, Data Structures for Statistical Computing in Python, in: *Proc. 9th Python Sci. Conf.*, 2010: pp. 51–56.
- [107] T.E. Oliphant, Python for Scientific Computing, *Comput. Sci. Eng.* 9 (2007) 10–20. <https://doi.org/http://dx.doi.org/10.1109/MCSE.2011.36>.
- [108] S.C.C. and G.V. Stéfan van der Walt, The NumPy Array: A Structure for Efficient Numerical Computation, *Comput. Sci. Eng.* 13 (2011) 22–30. <https://doi.org/http://dx.doi.org/10.1109/MCSE.2011.37>.
- [109] scikit-learn developers, Support Vector Machines, (n.d.). <http://scikit->

- learn.org/stable/modules/svm.html#svm (accessed August 7, 2016).
- [110] M.N. Bernstein, The Radial Basis Function Kernel, (n.d.) 1–4.
<http://pages.cs.wisc.edu/~matthewb/pages/notes/pdf/svms/RBFKernel.pdf>.
- [111] scikit-learn developers, Model evaluation: quantifying the quality of predictions, (n.d.).
http://scikit-learn.org/stable/modules/model_evaluation.html (accessed February 1, 2020).
- [112] M.S. Seewald, E.N. Gaasedelen, T.L. Iles, L.M. Mattison, A.R. Mattson, M.M. Schmidt, R.C. Braun-dullaesus, P.A. Iaizzo, Effects of ATP administration on isolated swine hearts : Implications for ex vivo perfusion and cardiac transplantation, *Exp. Biol. Med.* 244 (2019) 915–922. <https://doi.org/10.1177/1535370219850786>.
- [113] H. Huang, X. Zhang, S. Li, N. Liu, W. Lian, E. McDowell, P. Zhou, C. Zhao, H. Guo, C. Zhang, C. Yang, G. Wen, X. Dong, L. Lu, N. Ma, W. Dong, Q.P. Dou, X. Wang, J. Liu, Physiological levels of ATP negatively regulate proteasome function., *Cell Res.* 20 (2010) 1372–1385. <https://doi.org/10.1038/cr.2010.123>.
- [114] G. Vassort, Adenosine 5 J -Triphosphate : a P2-Purinergic Agonist in the Myocardium, *Physiol. Rev.* 81 (2001) 767–806.
- [115] M.M. Swindle, A. Makin, A.J. Herron, F.J.C. Jr, K.S. Frazier, Swine as Models in Biomedical Research and Toxicology Testing, *Vet. Pathol.* 49 (2012) 344–356.
<https://doi.org/10.1177/0300985811402846>.
- [116] L.G.A.R.H.A. SIMON J. CRICK , MARY N. SHEPPARD , SIEW YEN HO, Anatomy of the pig heart : comparisons with normal human cardiac structure, *J. Anat.* 193 (1998) 105–119.
- [117] J. Musiolik, P. Van Caster, A. Skyschally, K. Boengler, P. Gres, R. Schulz, G. Heusch, Reduction of infarct size by gentle reperfusion without activation of reperfusion injury salvage kinases in pigs, *Cardiovasc. Res.* 85 (2010) 110–117.
<https://doi.org/10.1093/cvr/cvp271>.
- [118] A. Skyschally, P. Van Caster, K. Boengler, P. Gres, J. Musiolik, D. Schilawa, R. Schulz, G. Heusch, Ischemic Postconditioning in Pigs, *Circ. Res.* 104 (2009) 15–18.
<https://doi.org/10.1161/CIRCRESAHA.108.186429>.
- [119] G. Heusch, No RISK , no ... cardioprotection ? A critical perspective, *Cardiovasc. Res.* 84 (2009) 173–175. <https://doi.org/10.1093/cvr/cvp298>.

Acknowledgements

I am incredibly thankful I was able to perform this research project and gather all the great and diverse experiences within the curriculum of my medical school education.

This work could not have been completed without the support of Professor Paul A. Iazzo of the Visible Heart Laboratories. In his laboratories and facilities I was given the opportunity to perform experiments, be part of a dedicated team and extend my knowledge in many varied directions. Dr. Iazzo's mentorship is invaluable and I hope to continue to share his dedication for research and medicine.

I would also like to acknowledge Professor Tinen Iles and Dr. Erik Gaasedelen. Professor Iles provided immense support during my time in the lab and continued that support as we published the results of our research.

Erik, a PhD student at the time, introduced me to modern approaches of data analysis, and was an essential part of helping me master my research-related responsibilities.

I thank all my fellow labmates for all the support, stimulating discussions, and long evenings at the lab, cleaning and enjoying our time together. My time in Minneapolis was challenging and unforgettable in terms of my medical education and interdisciplinary work; I am very grateful not only for the academic achievements but also for the long-lasting friendships.

I would also like to express my gratitude to Professor Rüdiger Braun-Dullaeus from my home faculty who supported my intentions starting in 2014. His cooperativeness opened the door to perform my research abroad, gain international academic experiences, and built a sustainable relationship with Professor Iazzo from my home faculty Magdeburg.

Both Professor Iazzo and Braun-Dullaeus supervised my subject-specific and content-related work, became role models, and influenced my future medical career in cardiology.

Last but not least, I would like to thank my family and friends for their great support and understanding along the rough road of my studies and scientific career over the past years.

Ehrenerklärung

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

Investigations of pharmacological pre- and posttreatments with Omegaven and ATP in a four-chamber isolated working swine heart model: implications for cardiac interventions, cardiac transplantation and ex vivo perfusion systems

in der Klinik für Kardiologie und Angiologie mit Unterstützung durch Prof. Dr. med. R. Braun-Dullaes

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 18.02.2020

Maria Seewald

Erklärung zur strafrechtlichen Verurteilung

Ich erkläre hiermit, nicht wegen einer Straftat verurteilt worden zu sein, die Wissenschaftsbezug hat.

Magdeburg, den 18.02.2020

Maria Seewald

Publications

Journal Articles

- Maria Seewald, Erik N. Gaasedelen, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iaizzo: Effects of ATP administration on isolated swine hearts: Implications for *ex vivo* perfusion and cardiac transplantation. *Experimental Biology and Medicine* 2019, 244: 915–922. DOI: 10.1177/1535370219850786.
- Maria Seewald, James A Coles Jr, Daniel C Sigg and Paul A Iaizzo: Pharmacological postconditioning with delta opioid attenuates myocardial reperfusion injury in isolated porcine hearts. *Experimental Biology and Medicine* 2016; 0: 1–10. DOI: 10.1177/1535370216684041.

Oral Presentations

- 10/2016 Biomedical Engineering Society Annual Meeting, Minneapolis/USA
Maria S. Seewald, Erik N. Gaasedelen, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iaizzo: Improving Cardiac Transplantation Outcome Utilizing an *Ex Vivo* Heart Perfusion Model: Pharmacological Post-Treatment and Functional Assessments
- 02/2016 Mimics® Innovation Conference, Tampa/USA
Maria S. Seewald, Erik N. Gaasedelen, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iaizzo: 3D Modeling of Adult and Congenital Human Heart within the Visible Heart® Laboratories

Posters

- 08/2019 European Society of Cardiology (ESC), Paris
Paul Werner, Marco Russo, Maria S. Seewald, Iuliana Coti, Sabine Scherzer, Thomas Haberl, Guenther Laufer, Alfred Kocher, Martin Andreas: Mid-term results of bioprosthetic aortic valve replacement with the Trifecta valve: A word of caution.
- 10/2016 Acute Cardiovascular Care Conference (ESC), Lissabon/Portugal
Maria S. Seewald, Erik N. Gaasedelen, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iaizzo: Investigation for pre-and post-treatments with an omega-3-acid ethyl esters emulsion and ATP in a four-chamber isolated working heart model (moderated poster presentation)
- 10/2016 Acute Cardiovascular Care Conference (ESC), Lissabon/Portugal
Erik N. Gaasedelen, Maria S. Seewald, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iaizzo: Predicting hemodynamics from metabolic measurements using Support Vector Regression in an isolated ex-vivo four chamber working heart model
- 02/2016 Mimics® Innovation Conference, Tampa/USA
Maria S. Seewald, Erik N. Gaasedelen, Tinen L. Iles, Lars M. Mattison, Alexander R. Mattson, Megan M. Schmidt, Paul A. Iaizzo: Anatomical Case Study: Imaging and Three Dimensional Modeling of a Perfusion Fixed 10-month-old Human Heart-Lung Bloc

Curriculum Vitae

Der Lebenslauf ist in der Version aus Datenschutzgründen nicht enthalten.

Appendix

A. Tables of recorded values

Saline Control

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	98.857	28.379	1015.857	332.316	32.286	10.92	148.983	63.504	0.9	0.352
10	92	15.521	953.5	241.098	31.857	6.768	160.917	69.658	1.1	0.219
20	85.417	8.458	918.667	167.992	35.75	5.529	168.1	78.632	1.32	0.164
30	78.143	13.259	823.071	167.625	35.429	4.962	204.1	91.153	1.733	0.207
40	70.7	3.701	773.2	55.692	34.7	5.869	196.2	80.288	1.92	0.249
50	72.333	8.959	736	51.548	34.167	4.491	207.96	108.551	2.1	0.245
60	63.429	8.9	660.071	79.17	33.857	5.956	215.333	80.107	2.35	0.432
70	68.2	5.675	628.9	68.55	32.6	6.066	232.5	101.661	2.225	0.359
80	67.083	8.709	693.25	144.53	32.917	4.318	232	81.151	1.8	0.632
90	62.643	9.986	642.071	125.119	39	10.488	231.2	120.506	1.5	0.505
100	60.429	7.716	611.5	130.75	40.714	12.213	244.4	98.713	1.52	0.559
110	57.714	7.135	598.143	114.138	41.857	14.241	222.2	106.149	1.68	0.517
120	54.214	5.544	556.357	132.41	43.643	17.9	290.4	139.102	1.72	0.576

PrC Omegaven

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	82.4	18.022	788	271.916	1	7.436	158.417	64.545	1.033	0.547
10	77.8	12.969	700.4	184.191	0.898	7.733	167.34	65.789	1.1	0.485
20	73.9	8.975	662.1	180.827	0.936	10.526	179.12	68.178	1.36	0.456
30	70	13.379	646.9	246.297	0.946	11.36	189.42	74.281	1.6	0.458
40	67.7	13.415	681.9	347.749	0.966	12.071	192.4	63.948	1.66	0.351
50	64.6	12.095	643.8	343.19	0.96	12.661	197.4	63.426	1.82	0.37
60	58.5	14.722	557.7	285.505	1.076	11.283	207.8	88.288	1.66	0.467
70	63.5	15.4	622.25	235.631	1.158	7.631	297.333	167.661	1.767	0.611
80	69.5	18.514	617.67	204.234	1.106	10.786	186.1	97.481	1.367	0.814
90	67.25	13.432	616.63	134.07	0.972	7.588	197.667	40.054	1.4	0.781
100	65.38	13.768	606	126.139	0.942	9.57	308.667	169.768	1.467	0.808
110	62.75	14.523	595.88	151.053	0.944	7.974	205.333	51.859	1.467	0.833
120	67.75	24.364	600.25	226.309	1.131	10.536	222.667	84.949	1.267	1.002

PrC-Sup low dose (2+5mg) ATP

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	98.286	9.013	890.143	120.531	1.008	4.23	127.933	54.588	0.683	0.24
10	99.833	9.02	901.5	132.772	0.978	3.656	107.25	45.082	0.883	0.117
20	95.167	9.174	840.417	122.076	0.969	2.805	123.94	43.775	1.2	0.255
30	89.833	3.804	789.417	105.608	1.025	4.099	121.9	34.638	1.4	0.443
40	85.583	3.774	754.833	89.168	1.038	3.017	145.217	58.6	1.6	0.498
50	83.833	4.546	757.417	111.588	1.03	3.738	166.5	49.955	1.717	0.646
60	81.167	7.594	730.167	99.449	1.049	4.104	164.833	41.84	1.883	0.763
70	87.167	2.255	784.833	92.809	0.918	1.155	185.5	41.719	1.5	0.849
80	72	1.414	683.25	107.834	1.189	6.364				
90	72	14.9	658	141.414	1.158	9.508	167.333	55.591	1.3	0.557
100	71.7	23.205	654.6	208.663	1.162	10.159	192.75	21.407	1.475	0.723
110	71	20.506	656.9	196.859	1.088	7.446	211.2	33.892	1.36	0.844
120	67.6	21.568	643.2	199.248	1.182	12.651	196.6	34.239	1.46	0.885

PrC-Sup high dose (10mg) ATP

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	84	15.556	858.5	202.94	1	3.536	158.5	0.707	1	0.849
10	88.5	16.263	885	227.688	0.942	2.121	181.5	12.021	1.2	1.131
20	83	7.071	818	164.049	0.91	3.536	150.5	24.749	1.45	0.919
30	78	7.071	715.75	85.206	0.988	1.414	191.5	30.406	1.7	0.566
40	81	5.657	746.5	126.572	0.972	2.121	160	45.255	2.1	0.566
50	79	7.071	734.25	119.147	0.958	1.414	192.5	40.305	2.2	0.566
60	78.5	7.778	739	134.35	0.944	0.707	223	50.912	2.3	0.424
70	77.75	10.96	755.75	198.343	0.961		225		1.5	
80	78	12.728	723.5	193.04	0.94	3.536	246.5	0.707	1.45	0.354
90	76	11.314	707	214.96	0.972	2.121	251	18.385	1.6	0.424
100	71.25	9.546	668.75	191.272	0.942	2.121	241	48.083	1.75	0.354
110	68.5	9.192	637	176.777	0.956	2.828	238	16.971	1.85	0.495
120	64.5	3.536	622.25	154.503	0.97	3.536	284.5	30.406	1.85	0.354

PoC ATP (10mg)

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	84	12	1000.667	84.271	32.5	3.041	77.367	19.679	1	0.173
10	85.333	9.504	968.333	175.021	29.333	4.509	64.467	8.808	1.233	0.252
20	76.667	7.234	848.667	172.004	27	5.568	74.633	8.693	1.567	0.379
30	66.167	9.412	714.833	116.153	28.667	4.163	103.9	24.218	1.833	0.321
40	58.667	12.22	621.5	113.454	28.167	3.753	114.567	35.607	2.1	0.436
50	53	14.177	553.833	114.963	43.5	11.63	123.7	41.7	2.133	0.416
60	46.833	11.361	496.333	106.514	50.333	14.364	124.9	44.851	2.3	0.529
70	63.5	3.536	611	53.74	35.25	4.596	179	67.882	2.2	0.141
80	50.5	12.021	498	155.563	34	0	127	9.899	1.1	0.283
90	43.667	13.614	476.333	130.5	45.667	15.308	152.667	41.429	1	0.173
100	41.167	15.893	462.167	123.333	46.167	12.829	137	14.933	1.233	0.115
110	39.167	19.276	413.5	151.196	44.333	12.662	168	50.09	1.5	0.265
120	36.333	15.119	380.333	121.352	47.167	14.18	164.667	60.044	1.567	0.153

PoC ATP (30mg)

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	56.167	29.199	572.833	265.792	25.167	15.036	98.067	9.995	0.933	0.231
10	73.667	6.658	701	295.192	26.667	4.041	97.3	19.359	1.033	0.404
20	64.333	9.504	600.333	218.555	29	2.646	108.333	34.298	1.233	0.451
30	54	15.395	505.667	212.665	34	10.817	125.333	16.042	1.5	0.656
40	46.667	13.512	450.333	204.882	37.5	15.882	134	19.157	1.8	0.755
50	47	18.735	439	214.784	38	18.193	159.333	20.033	1.933	1.007
60	42.333	23.714	320.833	318.77	19.167	16.646	190.667	51.423	2.233	1.234
70	45.833	21.773	415.167	221.218	37.667	14.224	170.667	20.108	2.267	1.32
80	56.75	8.839	484.5	207.889	27.75	0.354	207		0.4	
90	42.833	12.984	460.833	168.479	36.167	16.003	158.333	60.368	1.1	0.5
100	41.333	17.502	439.667	231.241	34	17.436	159.667	56.048	1.3	0.656
110	39.667	18.148	417.667	213.017	33.833	15.751	181.667	66.606	1.5	0.819
120	38.667	17.559	364.5	249.429	32.167	10.492	195.667	29.006	1.6	0.819

Sup ATP low dose (2mg and 5mg)

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	82.667	37.087	713.667	37.087	25.667	12.987	118.78	52.629	0.8	0.2
10	85.333	19.367	845.5	19.367	26	5.099	109.6	28.877	0.95	0.226
20	89.167	15.171	853.833	15.171	28	2.53	113.017	38.327	1.217	0.306
30	84.167	11.112	789.667	11.112	29.167	2.858	123.889	37.967	1.478	0.282
40	80.583	12.5	744	12.5	28.833	2.041	131.3	37.342	1.667	0.378
50	77.75	13.564	711.083	13.564	29	1.673	141.6	29.091	1.76	0.434
60	73.917	14.417	675	14.417	29	1.673	157.167	37.413	1.9	0.4
70	75.5	10.376	705.25	10.376	29.75	2.5	158	48.125	2.067	0.289
80	85	19.023	719.9	19.023	36.1	10.286	170.5	14.248	1	0.523
90	82.25	16.516	728.667	16.516	30.25	4.356	176.167	29.342	0.8	0.29
100	77.333	15.501	690.917	15.501	29.167	3.817	165.5	19.827	0.967	0.216
110	74.167	16.154	668.667	16.154	28.75	2.564	180.167	23.828	0.983	0.147
120	69.833	17.668	635.5	17.668	29.5	3.507	185.556	36.678	1.122	0.109

Sup ATP high dose (10mg)

WM at [min]	MDP [mmHg]	STD	dPmax/dt	STD	Tau	STD	venous pO2 [mmHg]	STD	venous lactate [mmol/L]	STD
0	97.333	23.756	952.167	117.363	34.167	1.607	162.8	121.44	0.6	0.2
10	91.167	19.251	938.833	231.452	30.5	5.635	167.333	117.053	0.633	0.058
20	89.5	27.509	903.167	232.885	30	6.083	133.667	22.546	0.8	0.1
30	74.833	30.333	778.167	256.279	29.667	3.512	133.333	20.502	1.067	0.153
40	68.667	37.634	705.667	293.115	31.5	5.268	273	235.217	1.233	0.115
50	64.667	37.072	630.667	295.595	44.5	23.574	150.667	47.501	1.433	0.252
60	60.333	37.981	571.833	296.493	49.5	33.095	176.333	32.13	1.533	0.252
70	62.5	41.27	551.333	305.268	39.5	13.332	199.333	30.567	1.633	0.252
80	82.5	36.592	600.167	199.615	37.167	11.295	194	118.794	0.55	0.071
90	64.333	36.692	602.5	262.659	37.833	12.79	192.667	28.29	0.533	0.058
100	62.667	38.631	572.833	277.193	39.333	10.97	186	50.478	0.667	0.058
110	58.333	38.682	539.833	273.591	42	12.166	195.667	48.952	0.833	0.058
120	55.833	33.415	483.5	205.364	51.667	4.041	191.333	50.302	1	0.173

B. Arrhythmia Assessment

Study Paradigm	Amount ATP	30 min	60 min	90 min	120 min
	[time in sec]	[time in sec]	[time in sec]	[time in sec]	[time in sec]
Saline Control		31.39	14.49	115.75* (20.2)	12.3
PrC		66.9	16.8	14.7	13.0
PoC	10	307.2	13.4	12.5	28.6
	30	264.1	14.1	89.3	12.9
Sup	2mg	0	7.7735	24.191	6.292
	5mg	16.46	18.94	218.98	15.85
	low dose (2+5mg)	8.23	13.35675	121.5855	11.071
	high dose (10mg)	20.642	32.241	76.684	29.03
PrC-Sup	5mg	10.7	8.4	18.4	9

* The increased time in atrial fibrillation (AF) is caused by a single experiment outlier, where the heart was in AF for 7 min. Excluding this study, the control group presented with an averaged time of 20.2 sec for that particular timepoint.