Intrapericardial Delivery of Anti-arrhythmic Agents

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Dedication

This dissertation is dedicated to Tatiana Richardson, for her love and support. I am constantly amazed by her talent, goodness, dedication, hard work, cheerfulness and constant concern for others. She is an incredible wife, mother, and friend.

Thesis Abstract

Anti-arrhythmic agents are known for their narrow therapeutic window and common side effects. Delivery of anti-arrhythmic agents into the pericardium has shown to increase their efficacy and minimize their side effects. My work has focused on evaluating the clinical potential of intrapericardial (IP) anti-arrhythmic delivery. This has included working with physicians to identify appropriate clinical applications, developing devices to aid in pericardial drug delivery, and carrying out several large animal studies to test efficacy. In swine models of sinus tachycardia, atrial fibrillation, and ischemia-induced ventricular tachycardias, we have shown the pharmacodynamic benefits of IP-delivered metoprolol, amiodarone, and docosahexaenoic acid. Pharmacokinetic data show that minimal amounts of drug reach the systemic circulation. We propose that IP delivery of anti-arrhythmic agents has potential to maximize therapeutic benefits while minimizing side effects, particularly in the settings of post-operative atrial fibrillation and inappropriate sinus tachycardia.

Table of Contents

Acknowledgements i
Dedication iii
Thesis Abstract iv
Table of Contents v
List of Tables vii
List of Figures viii
Introduction 1
- Cardiac Arrhythmias 1
- Targeted Drug Delivery 1
- Intrapericardial Delivery of Anti-arrhythmic Drugs 3
- Thesis Objective 6

Section 1: Preface 9
Chapter 1: The Pericardium 12
- Introduction 13
- Anatomy 15
- Physiology of the Normal Pericardium 20
- Pericardial Disorders: Congenital, Pathological, and Iatrogenic 26
- Comparative Anatomy of the Pericardium 33
- Surgical Uses of the Pericardium 35
- Intrapericardial Therapeutics 37
- Summary 48

Chapter 2: Methods and Protocols for Cellular Electrophysiology of Single Cardiomyocytes 49

Chapter 2: Methods and Protocols for Cellular Electrophysiology of Single Cardiomyocytes 49

- Introduction 50
- Description of Apparatus/Models 55
- Detailed Methodologies 65
- Pitfalls and Troubleshooting 74
- Emerging Technologies 75
- Further Reading 75

Chapter 3: Electrophysiological Mechanisms of the Anti-arrhythmic Effects of Omega-3 Fatty Acids 77
- Introduction 79
- Background 80
- Clinical- and Animal- based evidence for Cardiac Electrophysiological Effects 83
- In Vitro Cellular Studies: In Search of Potential Mechanisms 90
- Current Hypotheses 98
- Other Possible Mechanisms 99
- Conclusions and Recommendations 105

Section 2: Preface 106

Evaluating and identifying appropriate clinical settings ... 106
Animal Models of Arrhythmias ... 108
Analytical chemistry techniques ... 110
Development of access methods to the pericardium ... 111
Chapter 4: The Use of Large Animal Models for Cardiac Electrophysiology Studies ... 114
Introduction ... 116
Choosing the Right Animal Model ... 118
Anesthetics and Monitoring ... 129
Common Cardiac Electrophysiology Interventions ... 138
Animal Models of Disease States ... 142
Summary ... 154
Chapter 5: A Novel Combination Therapy for Post-Operative Arrhythmias ... 156
Design of Medical Devices Abstract ... 157
Design of Medical Devices Poster Presentation ... 159
Section 3: Preface ... 159
Chapter 6: Pericardial Delivery of Omega-3 Fatty Acid: A Novel Approach to Reducing Myocardial Infarct Sizes and Arrhythmias ... 164
Introduction ... 165
Methods and Materials ... 167
Results ... 172
Discussion ... 182
Acknowledgements ... 186
Chapter 7: Intrapericardial delivery of metoprolol: A novel treatment for post-operative arrhythmias? ... 187
Introduction ... 188
Methods ... 191
Results ... 195
Discussion ... 201
Conclusions and Future Work ... 203
References ... 205
Introduction References ... 205
Chapter 1 References ... 208
Chapter 2 References ... 215
Chapter 3 References ... 216
Chapter 4 References ... 227
Chapter 6 References ... 233
Chapter 7 References ... 239

List of Tables

i. Table 1.1 - Normal plasma composition compared to the pericardial fluid composition of 30 patients undergoing cardiac surgery, p.20

ii. Table 1.2 – Sources of pericardial disorders, p.30

iii. Table 3.1 - A brief summary of several major clinical trials that present the electrophysiological effects of omega-3 fatty acids on the heart, p.85

iv. Table 3.2 - The Effects of eicosapentaenoic acid or docosahexaenoic acid on cardiac ion channels, p.91

v. Table 6.1 - Effects of pericardial infusion of DHA on fatty acid components of plasma, pericardial fluid, and atrial tissue, p.178

vi. Table 6.2 - Cardiac functions in control and DHA-treated groups, p.179

List of Figures

i. Figure 0.1 - Schematic of conventional and targeted drug delivery methods, p.4

ii. Figure 1.1 - Pericardial anatomy, p.14

iii. Figure 1.2 - The fibrous pericardium of a fresh human cadaver is opened to expose the epicardium of the heart, p.15

iv. Figure 1.3 – Schematic of the layers of the pericardium, p.17

v. Figure 1.4 - Percardial pressure vs. pericardial volume, p.22

vi. Figure 1.5 - Cardiac tamponade, p.31

vii. Figure 1.6 – The spectrum of pericardial disease., p.32

viii. Figure 1.7 - The PerDUCER® instrument, p.39

ix. Figure 2.1 - A simplified electric circuit model of an ion channel and cell membrane, p.52

x. Figure 2.2 - Representative current waveform (below) of a cell membrane if a voltage step across the membrane is introduced (above), p.53

xi. Figure 2.3 - A schematic diagram of the microelectrode assembly, p.55

xii. Figure 2.4 - A DMZ Universal Puller, used to fabricate micropipettes, p.56

xiii. Figure 2.5 - A tissue bath used for intracellular recording, p.62

xiv. Figure 2.6 - A typical electrophysiology setup, p.64

xv. Figure 2.7 - Various patch configurations and how each is formed from an “on-cell” configuration, p.68

xvi. Figure 2.8 - A human cardiac action potential obtained from intracellular recording with a sharp microelectrode, p.73

xvii. Figure 3.1 - The chemical structures of omega-6 and omega-3 classes of polyunsaturated fatty acids, p.81

xviii. Figure 3.2 - The microelectrode array and recorded field potentials, p.94

xix. Figure 3.3 - Spontaneous field potential rate at various concentrations of DHA, p.95

xx. Figure 3.4 - Representative waveforms of the field potential are shown at various [DHA], p.96

xxi. Figure 3.5 - Three hypotheses of how ω3PUFAs affect ion channel function, p.99

xxii. Figure 3.6 - A flow diagram of the proposed protective effect of ω3PUFAs during ischemia, p.102

xxiii. Figure 4.1 - Position of the heart within the thorax, p.122

xxiv. Figure 4.2 - Thebesian valve, p.124

xxv. Figure 4.3 - Valve in the coronary venous system, p.125

xxvi. Figure 4.4 Surgical preparation of a swine, p.131

xxvii. Figure 4.5 - Catheterization and arterial lines, p.133

xxviii. Figure 4.6 - Medial sternotomy, p.137

xxix. Figure 4.7 - Fluoroscopy, p.140

xxx. Figure 4.8: Cardiac Magnetic Resonance (CMR), p.144

xxxi. Figure 4.9 - Perfusion staining of the heart, p.152

xxxii. Figure 4.10 - TTC staining of the heart, p.154

xxxiii. Figure 6.1 - Effects of pericardial infusion of DHA on blood pressure, heart rate and arrhythmia score in myocardial ischemia/reperfusion pigs, p.173

xxxiv. Figure 6.2 - Effects of pericardial infusion of DHA on ventricular fibrillation events, morbidity of ventricular fibrillation, and mortality in myocardial ischemia/reperfusion pigs, p.175

xxxv. Figure 6.3 - Histological comparison of porcine myocardium between control (Control) and DHA-treated animal (DHA), p.176

xxxvi. Figure 7.1 - Pericardial sac tented with suture after a medial sternotomy, p.191

xxxvii. Figure 7.2. The effect of metoprolol on heart rate, delivered IV and IP, in a model of sinus tachycardia, p.194

xxxviii. Figure 7.3 - The effect of metoprolol, delivered IP and IV, on ventricular contractility, p.194

xxxix. Figure 7.4 - The effect of metoprolol, delivered IP and IV, on mean arterial pressure (MAP), p.195

xl. Figure 7.5 - Changes in inter-atrial conduction times after IP metoprolol, IV metoprolol or IP saline delivery, p.196

xli. Figure 7.6 - Changes in atrial effective refractory period (AERP) after IP metoprolol, IV metoprolol, or IP saline delivery, p.197

xlii. Figure 7.7 – Metoprolol concentrations in pericardial fluid after IP delivery, p.198

xliii. Figure 7.8 – Metoprolol concentrations in plasma after IV delivery, p.198

Introduction

Cardiac Arrhythmias

An estimated 79 million Americans live with some form of cardiovascular disease (CVD), which has become the number one cause of death in the United States(1). Interestingly, of the 900,000 lives claimed by CVD annually, about 325,000 die in an emergency department or without being hospitalized. Most of these fatalities are considered as sudden cardiac deaths (SCDs), usually caused by ventricular fibrillation.

In most cases, cardiac arrhythmias, including ventricular fibrillation (VF), can be prevented or treated using both pharmacological and/or device-based approaches. More specifically, the main classifications of anti-arrhythmic drugs include sodium, potassium, or calcium channel blockers, and β-blockers. While these agents have been used for many years, their relative effectiveness and side-effects are still under careful scrutiny(2,3). Of interest it should be noted that in a recent review article it was stated, “there is no evidence from trials that drug treatment, including the use of beta-blockers and amiodarone, or surgical interventions, such as coronary-artery bypass graft surgery or ablation procedures, prevent the recurrence of life-threatening arrhythmic events”(4).

Targeted Drug Delivery

Throughout the long history of drug design and administration, a major therapeutic obstacle has been the long and unpredictable route of the drug delivery from the injestion to the ultimate affect on the target tissues. For

example, harmful side-effects may occur when the given drug or its metabolites accumulate in tissues not intended to be treated. Futhermore, the relative concentrations of the drug in the intended tissues can vary greatly depending on numerous associated factors relative to the health and function of the gastrointestinal and circulatory systems. Also, the relative chemical forms of the given drug may be altered en route by a large number of physiologic processes. Thus, if such obstacles could be better controlled or overcome, a large number of currently-used drugs and therapies (and many more that are not in use) could be applied more effectively and safely.

Targeted drug delivery has become a major focus in the field of biomedical engineering as an effort to remove these obstacles. The basic principle of targeted drug delivery is to dispense the drug closer to its site of action. These targeted methods include employing both devices (such as implantable drug pumps) or molecular vehicles (such as drug-filled liposomes). In the future, it is considered that as pharmaceutics and biotechnology companies provide an increasing amount of viable therapies, the need for targeted drug delivery will only become greater.

More specifically, intrapericardial (IP) drug delivery is an actively growing form of targeted drug delivery. Originally, the primary clinical needs for pericardial access were: 1) to relieve the accumulation of fluid (cardiac tamponade), or 2) to treat selective diseases. Recently, the pericardial space has become a minimally-invasive route for cardiac therapeutics and/or diagnostics. For example,

angiogenic agents and nitric oxide donors have been infused into the pericardium to treat coronary vasculature with significant success(5). Other exciting applications of IP delivery being explored are: 1) the prevention of coronary restenosis, 2) the delivery of gene and cell therapies, and/or 3) the treatment of angina.

Intrapericardial Delivery of Anti-arrhythmic Drugs

Therapeutically, the IP delivery of anti-arrhythmic drugs is considered appealing because high heart tissue concentrations can be reached while maintaining minimal plasma concentrations: the latter would prevent systemic side-effects, a common problem with the administration of anti-arrhythmic drugs. As shown in figure 0.1, the conventional method of oral delivery requires the drug to negotiate the gastrointestinal (GI) tract, be transported by the circulatory system and then pass into the extracellular fluid (ECF) before arriving at the target cell. Notably, the use of IV infusion brings the drug one step closer to the target cell by bypassing the GI tract. IP infusion, a "targeted method", delivers the drug directly to the ECF, adjacent to the desired cells.

In IP delivery, the drugs are not only localized, but their concentrations at the target site can be more precisely controlled. Importantly, several anti-arrhythmic drugs have narrow windows of therapeutic concentrations, bordered by concentrations that cause adverse effects, or no effects at all. In oral and IV delivery of such agents, the ultimate concentrations at the target cells must be extrapolated based on several assumptions; typically producing poor control of

concentrations in cardiac tissue. In other words, IP approach might be much more desirable when precise control is needed.

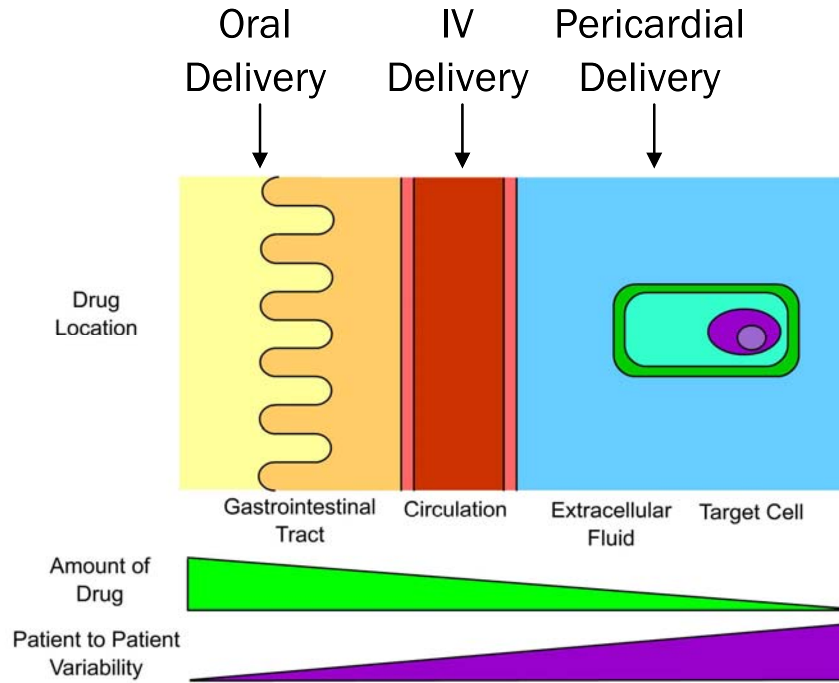

Figure 0.1 - Schematic of conventional and targeted drug delivery methods.

It is important to note that IP delivery of anti-arrhythmic drugs has been attempted recently with some success. The following agents have been administered via IP approaches to prevent or treat arrhythmias in animal models: Esmolol(6), sotalol(7), atenolol(7), ibutilide(8), procainamide(9,10), digoxin(9), amiodarone(11), arachadonic acid(12), nitroglycerin(13), and L-arginine(14) (a nitric oxide precursor). All of these studies, with one exception(7), have been acute. Many studies verify that high pericardial concentrations can be obtained with minimal plasma concentrations(8,10,11). Furthermore, the prevention of

dangerous ventricular arrhythmias has been provided by IPC delivery of nitroglycerin(13), arachadonic acid(12), and L-arginine(14). Studies employing less lipid soluble compounds(10,11) have observed higher concentrations in superficial structures such as the atria and the ventricular epicardium, with lower concentrations in the ventricular endocardium. Several groups have exploited this concentration differential so to treat the superficial SA node with beta-blockers(6)(7), assuming that there would be little penetration into the ventricular cells. Such a concentration gradient can also be used to target atrial fibrillation(8,10,11) with little ventricular effect.

To date, there have been no published clinical trials of IP-delivered anti-arrhythmic drugs. Despite the several benefits of IP delivery, most investigators appear to be somewhat cautious relative to this approach, perhaps due to unknown potential complications. For example, it has been yet to be investigated whether or not any underlying aspects of cardiac physiology may be effected by IP delivery, particularly within the cardiac autonomic nervous system(12). Perhaps the major concern of translational researchers is the potential unknown effects of the concentration gradients of less-soluble drugs across the ventricular myocardium. If the electrophysiologic responses of the outer tissue are dramatically affected by the drug but inner tissues are not, this may produce arrhythmias instead of inhibiting them(10). It should be noted that this is not only a concern with anti-arrhythmic therapies, but potentially true for any agent delivered IP.

Thesis Objective

The IP delivery of anti-arrhythmic drugs has shown great promise, but there remain several important questions that need to be addressed before beginning human clinical trials. For example, there is a clear need for critical long-term assessments of the efficacies and safeties of IP-delivered drugs. More specifically, the magnitudes of the concentration gradients across the myocardium, the relative degree of spillover into circulation, and the steady-state concentration levels (for chronic studies) should all be taken into consideration. Each of these parameters will depend on the properties of a given drug. Some pharmacological agents, therefore, may be excellent candidates for IP delivery, whereas others may not.

In addition, the clinical feasibility of accessing and utilizing the pericardial space for both acute and chronic drug administrations must be assessed. For examples, the risks and required time involved in accessing the pericardial space during an acute MI (myocardial infarction) may make IV infusion a better solution until rapid methodologies for IP delivery become available. Chronic IP delivery may be appealing for additional reasons, e.g., in order to bypass the issues of patient compliance and/or to more closely control the amount of drug delivered to the heart. Nevertheless, one must also recognize that the chronic placement of a catheter in the pericardial space may lead to other clinical complications: e.g., infection, arrhythmias (e.g., due to mechanical irritation), and/or significant fibrosis on the epicardial surfaces(9). Therefore, it is considered that other

methods of accessing the pericardial space may need to be explored.

Finally, because pilot studies on humans are obviously not feasible in early development of these techniques, it is important to validate their uses employing appropriate animal models. Not only must the electrophysiological effects of the drug be similar to that in human, but the anatomy and physiology of the pericardial space should be relatively similar.

The primary objectives of my thesis work, therefore, is:

To evaluate the efficacy, safety, and clinical potential of delivering drugs into the pericardial space so as to treat and/or prevent arrhythmias.

To accomplish these objectives, I have taken a translational approach. More specifically, I began by researching the anatomy of the pericardial space in various mammals including humans, the properties of various anti-arrhythmic agents, and even performed focused cellular experiments. This initial work composes *section one* of my dissertation. In *section two*, I report on the development of experimental methodologies and prototype devices so to study drug delivery within the pericardial space. More specifically, this included assessing access methods of the pericardium, refining analytical chemistry techniques for measuring drug concentrations, developing animal models of atrial arrhythmias, and selecting appropriate clinical applications. Finally, in *section*

three of my thesis, I report the results of our large animal experimental studies, which were performed so to assess the safety and efficacy of pericardial drug delivery.

Section 1: Preface

Section one represents the foundational research of my dissertation. The first two years of my PhD were spent learning about: 1) the pericardium, the pericardial space and the fluid within; 2) clinically used and potentially new anti-arrhythmic agents; and 3) basic and advanced electrophysiology techniques. This learning phase culminated in, and was accelerated by, the unique opportunity to write two book chapters and one review article. The *first chapter* of section one is entitled "The Pericardium" and is a chapter in the 2nd Edition of the Handbook of Cardiac Anatomy, Physiology, and Devices, edited by Paul A. Iaizzo. It was my privilege to lead the efforts of four other scientists in expanding and revising this chapter; each of which made valuable contributions. My efforts for this chapter were focused on the text pertaining to pericardial physiology, diseases and access methods.

My *second chapter* will also be published as a book chapter in Cardiac Electrophysiology Methods and Models, edited by Daniel Sigg, Paul Iaizzo, Bin He and Yong-Fu Xiao. The title of this chapter is "Methods and Protocols for Cellular Electrophysiology of Single Cardiomyocytes". I wrote this chapter under the direction of Yong-Fu Xiao, who is an expert in patch-clamp recording. The chapter describes in detail the techniques used to classify and study anti-arrhythmic agents at the cellular level. It is important to note that this chapter does not only represent my review of the literature, but also reflects my experience learning these techniques. Specifically, under the supervision of Dr.

Xiao, I learned to perform patch-clamp recording and assisted in collecting data for a manuscript entitled “Voltage Hysteresis of Human HCN4 Channels and cAMP Modification”, which will be submitted to a peer-reviewed journal in the near future. With the help of other colleagues, I also assembled an intracellular recording apparatus to record action potentials from isolated human cardiac tissues; this chapter contains data collected using this apparatus.

The *third chapter* in this section of my thesis is a review article which describes omega-3 fatty acids as novel anti-arrhythmic agents. Omega-3 fatty acids would later be used in several of my large animal protocols. This review is entitled “The Electrophysiological Basis of the Anti-arrhythmic Effects of Omega-3 Fatty Acids”, and it is currently under consideration for publication by the Journal of the American College of Cardiology. Specifically, this review carefully examines the electrophysiologic effects of omega-3 fatty acids at the cellular, whole animal and clinical levels. Furthermore, it contains original data which I collected using another electrophysiology technique: microelectrode array recording. This is a recently-developed technique which involves culturing cardiomyocytes on a Petri dish that has microscopic electrodes etched on the bottom. There are few very labs in the country that have this capability; I was fortunate enough to collaborate with a research group within Medtronic Inc. (Fridley, MN), to have access to this apparatus.

In summary, the preparation of these three chapters prepared me for my later work in large animals. They required me to critically scrutinize the past and

current literature pertinent to my overall thesis work, and provided me with understanding of both single-cell and multicellular electrophysiological techniques. The end result was greater understanding of cardiac electrophysiology.

Chapter 1: The Pericardium

Authors: Eric Richardson BS, Alexander Hill, PhD, Nicolas Skadsberg, PhD, Michael Ujhelyi, PharmD, Yong-Fu Xiao, MD PhD, Paul Iaizzo, PhD

To be published as a book chapter in The Handbook of Cardiac Anatomy, Physiology, and Devices, edited by Paul Iaizzo.

Introduction

The pericardium is a fibro-serous conical sac structure encompassing the heart and roots of the great cardiac vessels. In humans, it is located within the mediastinal cavity posterior to the sternum and cartilages of the third, fourth, fifth, sixth and seventh ribs of the left thorax, and is separated from the anterior wall of the thorax. It is encompassed from the posterior resting against the bronchi, the esophagus, the descending thoracic aorta, and the posterior regions of the mediastinal surface of each lung. Laterally, the pericardium is covered by the pleurae, and lies along the mediastinal surfaces of the lung. It can come in direct contact with the chest wall near the ventricular apical region, but varies with the dimensions of the long axes of the heart or with various disease states. Under normal circumstances, the pericardium separates and isolates the heart from contact of the surrounding tissues allowing freedom of cardiac movement within the confines of the pericardial space (figure 1.1).

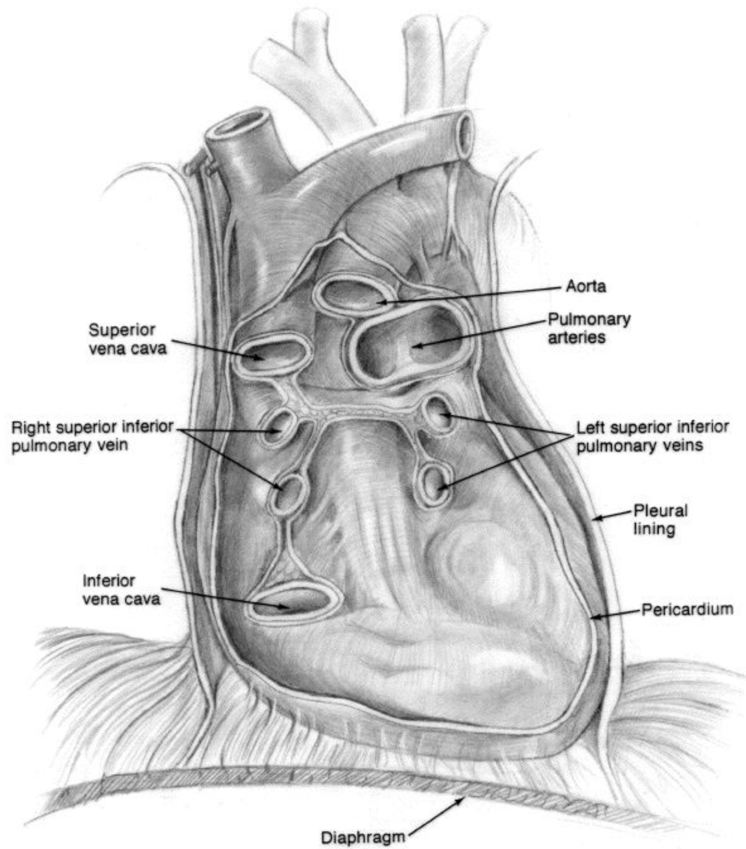

Figure 1.1 - Pericardial Anatomy. Shown is a posterior view of the pericardial sac, with the anterior surface and heart cut away. One can see that the great vessels of the heart penetrate through the pericardium, which extends up these vessels for several centimeters.

Anatomy

In humans, the 1-3 mm thick fibrous pericardium forms a flask-shaped bag. The neck of the pericardium (superior aspect) is closed by its extensions surrounding the great cardiac vessels, while the base is attached to the central tendon and to the muscular fibers of the left side of the diaphragm (figure 1.2). Much of the pericardium's diaphragmatic attachment consists of loose fibrous tissue that can be readily separated and/or isolated, but there is a small area over the central tendon where the diaphragm and the pericardium are completely fused.

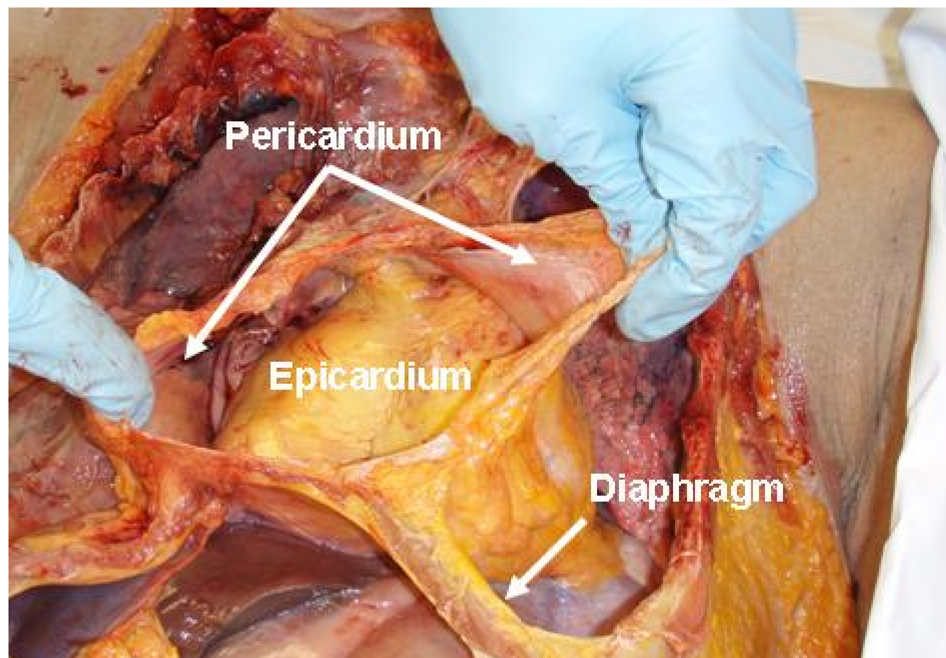

Figure 1.2 - The fibrous pericardium of a fresh human cadaver is opened to expose the epicardium of the heart. Note the attachment of the pericardium to the diaphragm.

Examination of the pericardium reveals that it is comprised of two interconnected structures, the serous pericardium and the fibrous pericardium. The serous pericardium is one continuous sac with a large in-fold that contains the heart (see figure 1.3). An appropriate analogy would be a fist (representing the heart) pushed into the side of a deflated balloon (representing the serous pericardium) and therefore enveloped by two individual layers of material. The interior surface of the pericardium is intimately connected to the surface of the heart and is known as the visceral pericardium, or the epicardium. The exterior surface of the serous pericardium is known as the parietal pericardium, and is fused with the thick lining of the fibrous pericardium. The pericardial space, where the pericardial fluid resides, is bounded on either side by the parietal and visceral pericardium. To the naked eye, however, the visceral pericardium cannot be distinguished from the surface of the heart, and the parietal pericardium cannot be distinguished from the fibrous pericardium. For this reason, the general use of the term “pericardium” refers to the composite of the parietal and fibrous pericardium, which appears to be a single sac which surrounds the heart.

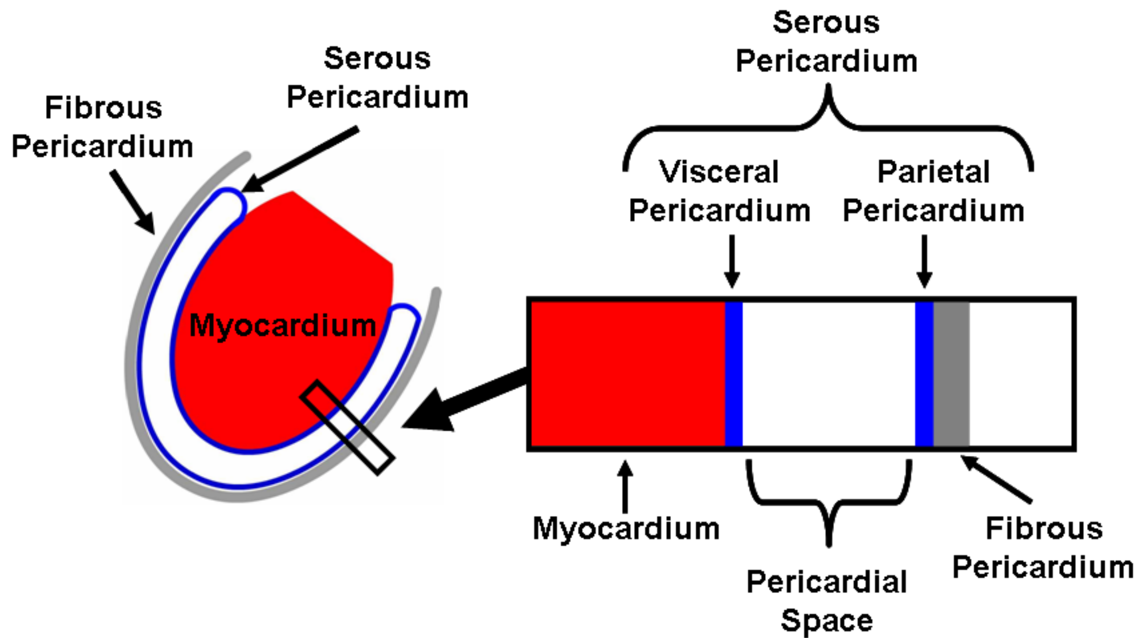

Figure 1.3 – Schematic of the layers of the pericardium. On the left, a schematic diagram of the serous and fibrous pericardium is shown with respect to the heart. On the right, an expanded cross-section view shows the attachment of two layers of the serous pericardium (visceral and parietal) to the myocardium and fibrous pericardium, respectively.

The inferior vena cava enters the pericardium through the central tendon of the diaphragm where there exists a small area of fusion between the pericardium and the central tendon, but receives no covering from this fibrous layer. Between the left pulmonary artery and subjacent pulmonary vein is a triangular fold of the serous pericardium known as the “ligament of the left vena cava” (vestigial fold of Marshall). It is formed by a serous layer over the remnant of the lower part of the

left superior vena cava (duct of Cuvier), which regresses during fetal life, but remains as a fibrous band stretching from the highest left intercostals vein to the left atrium, where it aligns with a small vein known as the "vein of the left atrium" (oblique vein of Marshall), eventually opening into the coronary sinus. The pericardium is also attached to the posterior-sternal surface by superior and inferior sterno-pericardial ligaments that securely anchor the pericardium and also act to maintain the orientation of the heart inside the thorax.

As previously mentioned, the serous pericardium is a closed sac that lines the fibrous pericardium consisting of a visceral and a parietal portion. This visceral portion that covers the heart and the great vessels is commonly referred to as the "epicardium" and is continuous with the parietal layer that lines the fibrous pericardium. The parietal portion covering the remaining vessels is arranged in the form of two tubes. The aorta and pulmonary artery are enclosed in one tube (the "arterial mesocardium"), while the superior and inferior vena cava and the four pulmonary veins are enclosed in the second tube (the "venous mesocardium"). There is an attachment to the parietal layer between the two branches, behind the left atrium, commonly referred to as the "oblique sinus." There is also a passage between the venous and arterial mesocardia, i.e., between the aorta and pulmonary artery in front and the atria behind, that is termed the "transverse sinus." The "superior sinus or superior aortic recess" extends upwards along the right side of the ascending aorta to the origination point of the innominate artery. The superior sinus also joins the transverse sinus

behind the aorta and they are both continually fused until they reach the aortic root.

The arteries of the pericardium are derived from the internal mammary and its musculophrenic branch, and also from the descending thoracic aorta. The nerves innervating the pericardium are derived from the vagus and phrenic nerves, as well as the sympathetic trunks.

Physiology of the Normal Pericardium

Pericardial Fluid

In normal hearts the pericardium is only a potential space. It contains 20-60ml of pericardial fluid, most of which resides in the major pericardial sinuses and the atrioventricular grooves (1). The fluid is an ultrafiltrate of plasma, and therefore has many similarities to plasma in its electrolyte composition. Pericardial fluid, however, contains about half the total protein concentration, one third the triglyceride and cholesterol content, and one fifth the amount of white blood cells (2). A more complete comparison of plasma and pericardial fluid composition is found in Table 1.1.

	Normal Plasma Range	Pericardial fluid mean value	Mean fluid: serum ratio
Total Protein (g/dL)	6.5-8.2	3.3	0.6
Albumin (g/dL)	3.6-5.5	2.4	0.7
Glucose (mg/dL)	70-110	133	1.0
Urea (mg/dL)	15-45	33	1.0
Calcium (mg/dL)	8.1-10.4	7.3	0.9
LDH (IU/L)	100-260	398	2.4

Creatinine (mg/dL)	0.8-1.2	0.9	0.9
Cholesterol (mg/dL)	130-240	43	0.3
Triglycerides (mg/dL)	50-170	34	0.3
White Blood Cells (K/µl)	4.0-10.8	1.4	0.2

Table 1.1. Normal plasma composition compared to the pericardial fluid composition of 30 patients undergoing cardiac surgery. Data from Ben-Horin, 2005 (2).

The details of the formation, clearance, and turnover of pericardial fluid have not yet been fully explained. It is generally agreed that pericardial fluid is plasma leakage from myocardial capillaries (3). The filtrate is eventually drained by lymphatic system. During situations of high pericardial fluid pressure such as in cardiac tamponade, investigators have found that fluid may pass through the pericardium and enter the pleural space (4). The turnover time of pericardial fluid in humans has not been established, but in sheep it is observed to be every 5.4 hours (5).

As mentioned previously, pericardial fluid distribution is not uniform. The majority of the fluid, found in the major sinuses and grooves of the heart, make up the pericardial reserve volume. The fluid is well-mixed due to the motion of the heart, and agents injected into the pericardial space quickly and evenly disperse throughout (5). Too much pericardial fluid, either due disease or intervention, may cause increased pericardial pressure and compromise cardiac performance. As shown in figure 1.4, the pericardial fluid volume acts as a buffer against increasing pressure. Once these groves and sinuses have filled, however, the pressure quickly increases with additional fluid volume.

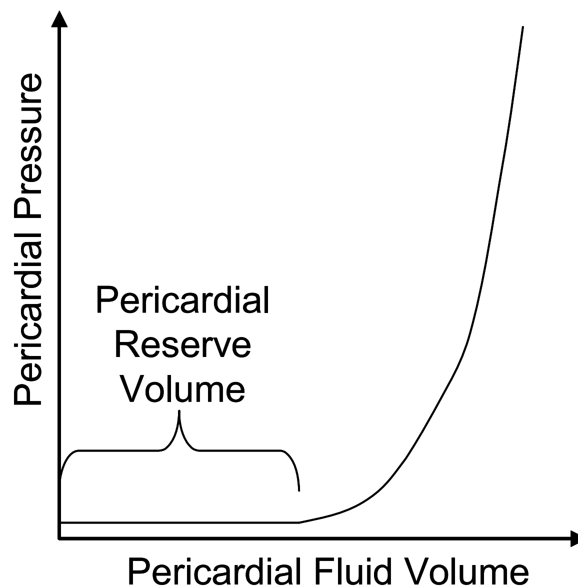

Figure 1.4 - Percardial pressure vs. pericardial volume. As pericardial fluid volume increases, the pericardial reserve volume is filled. Once the reserve volume is full, pressure within the pericardium rapidly rises and cardiac

performance may be compromised. Adapted from Spodick, 1997 (1).

Mechanical Effects of the Pericardium

The degree to which the pericardium alters wall movement(s) varies depending on the ratio of cardiac to pericardial size, loading conditions, and the degree of active and passive filling. Closure of the pericardial sac following open heart surgery has been proposed to: 1) avoid possible postoperative complications; 2) reduce the frequency of ventricular hypertrophy; and 3) facilitate future potential reoperations by reducing fibrosis (6). Differences in ventricular performance dependent on the presence of the pericardium have been reported following cardiac surgery (7,8).

The presence of the pericardium physically constrains the heart, often resulting in a depressive hemodynamic influence limiting cardiac output by restraining diastolic ventricular filling (9,10). The physical constraint by the pericardium is translated into direct external mechanical forces which alter patterns in myocardial and systemic blood flow (10,11). Direct primary and indirect secondary effects are observed as additional forces through the free wall. Because both the left and right side atria and the left and right side ventricles are bound by a common septum, geometrical changes from chamber interaction(s) are dynamic, depending on the different filling rates and ejection rates of each of

the four chambers (12,13). Thus, it is important to note that chamber-to-chamber interactions through the interventricular septum and by the pericardium further promote direct mechanical chamber interactions (14-16).

The effects of the pericardium on mechanical measures of cardiac performance are generally not evident until ventricular and atrial filling limitations are reached, changing geometrical and mechanical properties through factors such as maximum chamber volumes and elasticity. These effects become more evident as these pericardial limitations become extended (17,18). With the known force-length dependence of cardiac muscle, variation of chamber volumes through removal of the pericardium alters isometric tension, and therefore directly impacts systolic ejection. On the other hand, in specific cases where the restrictive role of the pericardium greatly increases, such as during cardiac tamponade, an increased intrapericardial fluid volume may result in critical restriction by the pericardium which then reduces cardiac performance (19).

It should also be noted that intrathoracic pressure creates an additional interaction between the ventricles, as well as between the heart and lungs in a closed chest. Thus, studying cardiac function in situ (with an opened chest) or in vitro allows elimination of the influences of intrathoracic pressures for identifying and quantifying pericardial influences on cardiac performance and ejection (20).

Such isolation of pericardial effects from diastolic filling is necessary since normal ventricular output is dependent on diastolic pressure, independently of the presence of the pericardium (21).

Pericardial Disorders: Congenital, Pathological, and Iatrogenic

Sir William Osler, considered as the father of modern medicine, referred to pericardial disease when he stated “probably no serious disease is so frequently overlooked by the practitioner” (22). Many pericardial disorders are asymptomatic and often go unnoticed throughout the patient’s life, but some may be fatal (see figure 6). Pericardial disorders may be classified as congenital, pathological or iatrogenic.

Congenital abnormalities of the pericardium are extremely rare. Partial absence of the pericardium may occur, usually exposing the left side of the heart. Complete absence of the pericardium is even less frequent (22). Cysts may also form during development in, on, or around the pericardium. These usually are not clinically significant, and need to be treated only if they are symptomatic (1). A list of major congenital abnormalities is found in Table 2.

During disease or injury, the pericardium responds with the production of either fluid, fibrin, cells, or a combinations of the three (22). The amount of each depends upon the type of disease or injury. Numerous forms of pericarditis can initiate an inflammatory response, including fibrinous pericarditis, fibrous pericarditis, infective pericarditis, and cholesterol pericarditis. Diseases unrelated to the pericardium may also trigger a pericardial response, such as a nearby

neoplasm or a myocardial infarction. After a transmural myocardial infarction, for example, patients almost always form adhesions between the necrotic area of the myocardium and the fibrous pericardium (22). Pericardial effusions, or excess fluid in the pericardium, may occur with disease. Large volumes of lymph, chyle, or blood may accumulate in the pericardial sac. If the accumulation of fluid is significant, this may result in impaired cardiac function, a condition known as cardiac tamponade (figure 5). If not treated, tamponade can be fatal. A list of several major pathologically-induced pericardial disorders is found in Table 2.

Finally, iatrogenic disorders often occur during the treatment of unrelated diseases. During cardiac surgery, the pericardium is often removed (partially or entirely), and is seldom repaired. There has been much debate as to whether closing the pericardium after surgery would be beneficial to the patient. Most surgeons believe that closing the pericardium may compromise post-operative haemodynamics and increase the risk of tamponade. Recent research has shown that there are no benefits or even adverse effects resulting from closure of the pericardium after cardiac surgery (23). If the pericardium is left open, the exposed epicardium tends to become very fibrous, complicating future interventions. Non-cardiac surgical procedures perform near the heart may also induce trauma to the pericardium and cause an inflammatory response.

Even some non-surgical interventions may damage the pericardium. Resuscitation from cardiac arrest may cause a fibrous response. Irradiation may create an effusion and subsequent tamponade (22). The pericardium may also adversely react to a number of commonly prescribed drugs such as procainamide, penicillin, doxorubicin, anticoagulants, and antithrombotics (1). The reader is again referred to Table 2 for a list of major iatrogenic pericardial disorders.

Pericardial disorders can be diagnosed using ECG, echocardiography, radiography, and/or auscultation. Pericardial fluid samples and pericardial tissue biopsies may also aid in diagnosis. Once diagnosed, pericardial disorders are treated with a number of drugs and interventions (24). In situations of large pericardial effusions, pericardiocentesis is performed by draining the effusion with a hypodermic needle inserted near the xyphoid process (figure 5). This is usually done under guidance of echocardiography or fluoroscopy to prevent myocardial puncture, but in emergency situations (such as during cardiac tamponade) it may be done without guidance ("blindly"). Chronic effusions or other diseases may necessitate a partial or complete pericardiotomy. This is done by creating an opening into the mediastinum, called a pericardial window, to view and remove portions of the pericardium. Visualization of the procedure can be enhanced by a laparoscope or thoracoscope. Balloon pericardiotomy is a more recent minimally invasive technique that uses a balloon to enlarge a small

hole in the pericardium (3).

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Congenital	Pathological	Iatrogenic
Primary:	Idiopathic pericarditis	Surgical
Pericardial absence	Due to living agents – infectious, parasitic	Instrument trauma
Cysts	Vasculitis – connective tissue disease	Cardiac resuscitation
Teratoma	Immunopathies/hypersensitivity states	Iatrogenic pneumopericardium
Lymphangioma	Disease of contiguous structures	Drug reactions and complications
Diverticulom	Disorders of metabolism	Radiation
Pericardial bands	Trauma – indirect, direct	
Secondary:	Neoplasms – primary, metastatic, multicentric	
Pericarditis due to maternal lupus	Uncertain pathogenesis	
Intrapericardial hernia of abdominal organs		

Table 1.2 – Sources of pericardial disorders. The major sources of pericardial disorders are congenital, pathological, or iatrogenic (caused by medical interventions). Listed are the major subcategories of each source. Adapted from Spodick, 1997 (1).

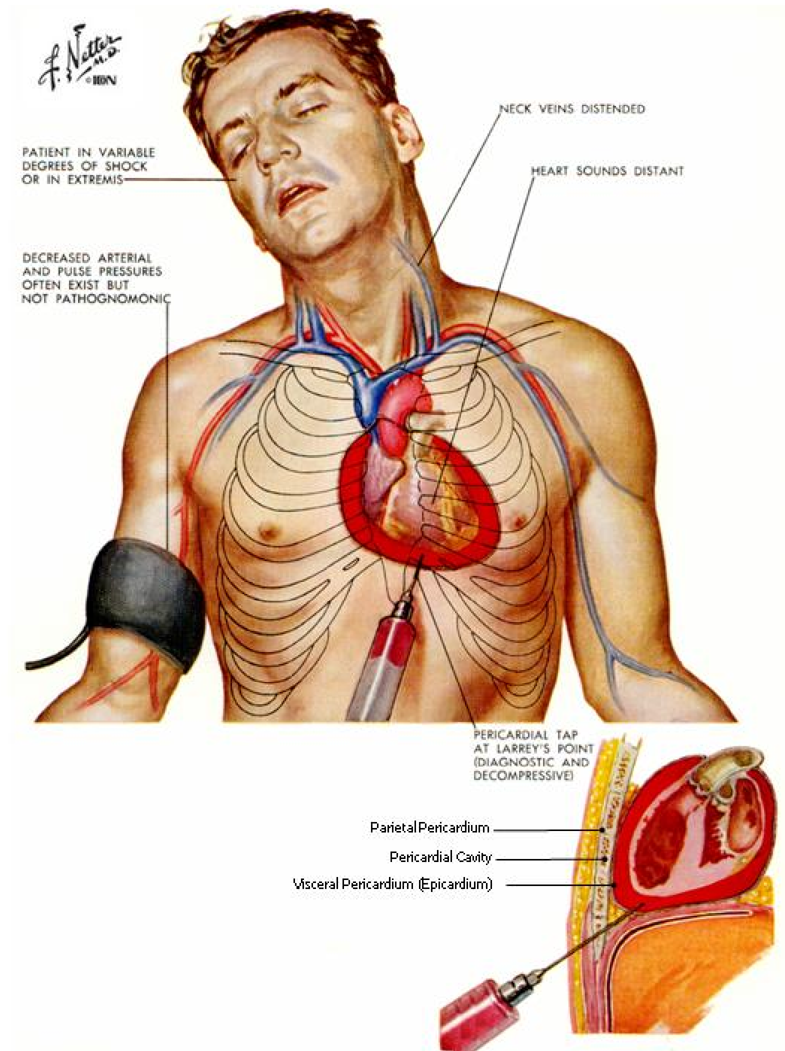

Figure 1.5 - Cardiac tamponade. Cardiac tamponade occurs when there is a large accumulation of fluid in the pericardium (Above). During tamponade, haemodynamics may be seriously compromised. Distended neck veins, decreased blood pressure, various degrees of shock, and distant heart sounds may all be symptoms of tamponade. In most cases, tamponade is treated by pericardiocentesis, or drainage of the sac with a long hypodermic needle (Below).

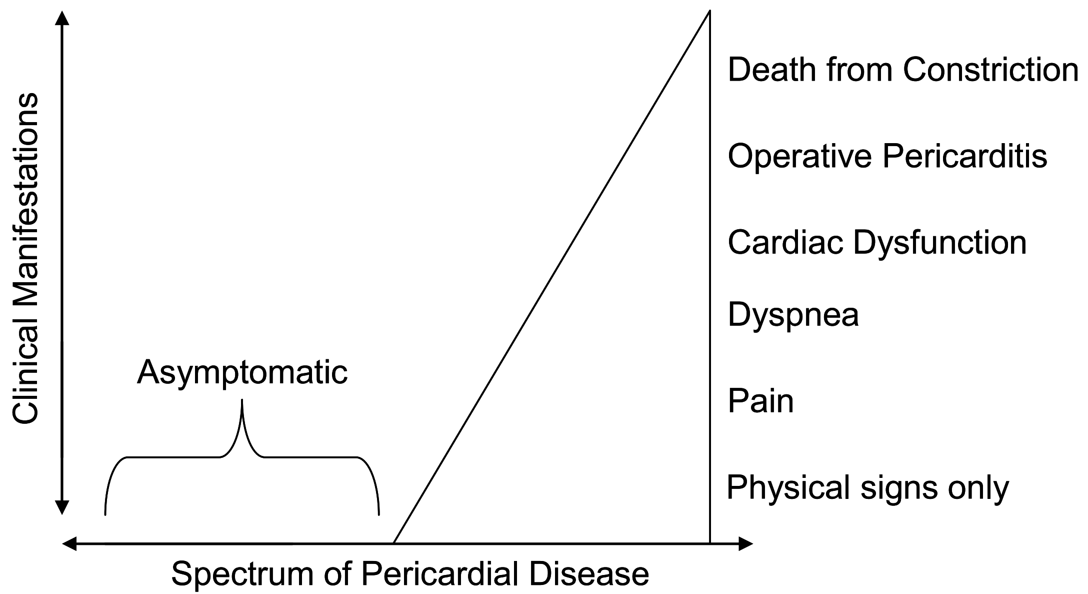

Figure 1.6 – The spectrum of pericardial disease. Most pericardial diseases are discovered post-mortem, implying that they were asymptomatic throughout the patient's life. Serious pericardial disease, however, may have many clinical manifestations, as shown in the figure. Adapted from Reddy, 1982.

Comparative Anatomy of the Pericardium

The pericardium is fixed to the great arteries at the base of the heart and is attached to the sternum and diaphragm in all mammals, although the degree of these attachments to the diaphragm varies between and within species (25,26). Specifically, the attachment to the central tendinous aponeurosis of the diaphragm is firm and broad in humans and pigs, the phrenopericardial ligament is the only attachment in dogs, and the caudal portion of the pericardium is attached via the strong sternopericadial ligament in sheep (25,26) (See Supplemental Material, MPEG2).

Although the basic structure of the pericardium is the same, differences exist between various species with respect to both geometry and structure (27-29). Generally, pericardial wall thickness usually increases with increasing heart and cavity size between the various species (27). However, humans are a notable exception to this rule, having a much thicker pericardium than animals with similar heart sizes (27). Specifically, the pericardium of human hearts varies in thickness between 1 and 3.5 mm (1), while the average pericardial thickness of various animal species were found to be considerably thinner (ovine hearts: 0.32 $\pm$ 0.01 mm, porcine hearts: 0.20 $\pm$ 0.01 mm, and canine hearts: 0.19 $\pm$ 0.01 mm (28). Differences in the volume of pericardial fluid also exist. Holt (27) reported that most dogs have between 0.5 and 2.5 ml of pericardial fluid, with some dogs

having up to 15 ml compared to 20–60 ml in adult human cadaver hearts. In our own lab, we have found that 70-80 kg swine have about 7-8 ml pericardial fluid. In selecting an appropriate animal model for pericardial access procedures or intrapericardial therapeutics, the significant differences of pericardial thickness, pericardial fluid volume, and pericardial attachments between humans and animals must be considered.

Surgical Uses of the Pericardium

Due to the inherent mechanical properties of the fibrous pericardium, it has been used in various applications during surgery and has also been used in bioprosthetic heart valves. During cardiac surgery, fresh autografts, cryopreserved homografts, or glutaraldehyde fixed xenografts can be used to as patches during reconstructive repairs in both congenital and acquired heart disease. For example, in congenitally malformed hearts, pericardial patches are used during surgical repairs of the right ventricular outflow tracts and during repair of torn aortic leaflets, among other things. Pericardial patches have also been used in acquired diseases to repair the mitral and aortic valves as well as ventricular walls (30).

The use of pericardium in heart valves is a relatively recent development. The search for an alternative to mechanical valves has led people to investigate many types of bioprosthetic valves. These bioprostheses have consisted of homografts (preserved cadaveric valves), autografts (transplant of a patients pulmonic valve to the aortic position – the Ross procedure), and xenografts. Xenograft valves have included preserved porcine aortic tissue as well as preserved bovine pericardium. More specifically, glutaraldehyde fixed bovine pericardium has been used in the design of aortic and mitral bioprosthetic valves, which have enjoyed wide clinical success. The bovine pericardium is prepared and fixed using specific processes (which typically vary slightly between manufacturers) and then cut to resemble a tri-leaflet semilunar valve and

assembled into a stent with a sewing cuff.

Intrapericardial Therapeutics

Clinical Pericardial Access

Traditionally, pericardial access has been limited to patients with pericardial effusions. The effusion gives the physician a buffer between the fibrous pericardium and the epicardium during pericardiocentesis or creating a pericardial window, preventing damage to the myocardium and coronary vessels. Recently, there has been an interest in accessing the healthy pericardium for the delivery of various therapeutics. The challenge of accessing a healthy pericardium is to puncture and catheterize the pericardium with minimal risk to the heart. This is a difficult task considering the almost negligible layer of pericardial fluid in a healthy pericardial sac.

Several unique biomedical devices or tools have been developed to aid in accessing pericardial space via use of novel catheter designs, allowing controlled myocardial penetration during fluoroscopic visualization. Specifically, a recently introduced technology has been developed that uses a sheathed needle with a suction tip designed for grasping the pericardium and accessing the pericardial space using a transthoracic approach while, at the same time, minimizing the risk of myocardial puncture; the PerDUCER® instrument (Comedicus, Inc., Columbia Heights, Minnesota) is placed following subxiphoid access into the mediastinum under fluoroscopic guidance from the apparatus positioned onto the anterior outer surface of the pericardial sac (figure 1.7). Under manual suction, the sac is retracted and a needle is inserted allowing for the placement of a guidewire into

the space via the needle lumen. The needle is then removed and a standard delivery catheter is placed into position. The Philipps University of Marburg has also been developing a similar product called the Marburg Attacher (http://cardiorepar.com) which is undergoing clinical trials. Other percutaneous subxyphoid techniques have been proposed (31), but are not in current clinical use.

A novel transatrial technique has been recently developed by Verrier and colleagues (32) which has been very successful in animal studies. In this procedure, a guide catheter is introduced into the right atrium from the femoral vein, and a needle catheter is advanced through the guide catheter to pierce the right atrial appendage. A guidewire is then passed through the needle catheter into the pericardial space. A soft delivery catheter can be passed over the guidewire and can reside in pericardial space for long-term drug delivery or for fluid sampling. Recent studies in swine have shown (33,34) that catheters left in the pericardial space over long periods of time can remain patent and cause minimal fibrosis and inflammatory response at the epicardium.

The ability to access the pericardial space has created new opportunities to further understand the role of the pericardium under normal cardiac function and following cardiac disease(s). Despite the growing literature establishing the feasibility of intrapericardial therapeutics and diagnostics, the results of clinical trials employing pericardially delivered agents directed towards angiogenesis, restenosis, and/or other coronary and myocardial indications are currently

lacking.

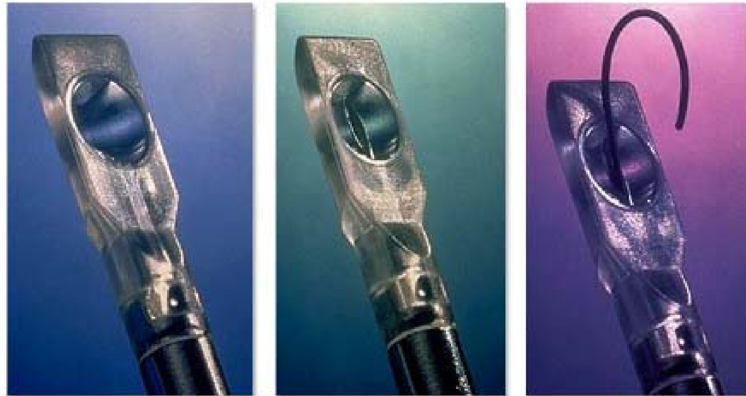

Figure 1.7 - The PerDUCER® instrument (Comedicus, Inc., Columbia Heights, Minnesota). The device uses a sheathed needle with a suction tip designed for grasping the pericardium to access the pericardial space using a transthoracic approach, thus minimizing the risk of myocardial puncture.

Potential Intrapericardial Therapies

With recent advances in minimally invasive cardiac surgical procedures, it is likely that instances and abilities in preserving the integrity of the pericardium during cardiac surgery will increase. The diminished ability of cardiac cells to regenerate under adverse loading conditions impairs the ability to regenerate lost myocardial function, making procedures reducing myocardial trauma of particular

interest. In addition, access into the pericardial space provides a new route for numerous novel treatments and therapies that can be applied directly to the epicardial surface and/or the coronary arteries. For quite some time, nonsurgical intrapericardial therapy has been employed in patients with sufficient fluid in the pericardial space allowing a needle to be safely placed within the space (1). This methodology has been used for patients with such clinical indications as, but not limited to, malignancies, recurrent effusions, uremic pericarditis, and connective tissue disease. Recently, instrumenting the pericardium has been made possible by numerous techniques that allow for the study of intrapericardial therapeutics and diagnostics by clinicians and investigators alike. More specifically, the endoluminal delivery of various agents has been found to be clinically limited due to short residence time, highly variable deposited agent concentration, inconsistency in delivery concentrations, and relatively rapid washout of agent from the target vessel (35). A desired example of targeted application includes infusion of concentrated nitric oxide donors, which could present undesirable effects if systemically delivered. Further, one is allowed increased site specificity and the delivery of label-specific therapeutic agents to target cells, receptors, and channels. A great deal of interest has been focused on delivery of angiogenic agents and various growth factors into the intrapericardial space (36-38). In particular, research has concentrated on administration in patients with ischemic heart disease (39,40). Early results indicate several benefits associated with the delivery of angiogenic agents that include increased collateral vessel

development, regional myocardial blood flow, myocardial function in the ischemic region, and myocardial vascularity.

In our lab, we have shown the intrapericardial delivery of omega-3 fatty acids can drastically reduce infarct size and lower the occurrence of ventricular arrhythmias. In a recent study (see chapter 6), 23 swine were treated with either an infusion of omega-3 fatty acids or saline in the pericardial sac prior to occluding the left anterior descending artery. Prior to, during, and after the occlusion, hemodynamic and electrophysiological data were recorded. Upon sacrificing the animal, the heart was sectioned and stained to determine infarct size. We found that in animals treated with omega-3 fatty acids, both the infarct size and arrhythmia scores were reduced by half with minimal effect on haemodynamics. Current research is underway to determine if this therapy would be feasible in a clinical setting.

Pericardial Pharmacokinetics

As described previously, the pericardium is generally believed to contain 20-60 ml of physiologic fluid (0.25±15 mL/kg) situated within the cavity space (41). Yet, dye studies suggest that pericardial fluid is not uniformly distributed over the myocardium, with the majority of pericardial fluid residing within the atriaventricular and interventricular grooves as well as the superior-transverse sinuses. Although the pericardial fluid is not uniformly distributed, pharmacokinetic studies suggest that there is complete mixing of the fluid so

that pericardial fluid content is spatially uniform (42-44). Hence, sampling pericardial fluid content should not vary by sampling location (43).

Tissue distribution and drug clearance clearly affect all drug response. Because specific pericardial pharmacokinetic data remains unknown for the majority of compounds, pericardial drug disposition must be gleaned from physical chemical properties based upon a few select studies. Pericardial fluid is cleared via lymphatics and epicardial vasculature, with the former being a very slow process (45). In addition to these passive clearance mechanisms, the epicardial tissues contain metabolic enzymes that may clear compounds via a biotransformation process. This is likely to occur with certain labile peptides and small molecules such as nitric oxide. Unfortunately there is very little known today about pericardial drug metabolism. In general, it is considered that whether or not a compound residing in the pericardial space is cleared via lymphatic drainage, passive diffusion or biotransformation will depend on its molecular size, tissue affinity, water solubility and enzymatic stability. Thus, compounds such as large proteins do not rapidly diffuse into the vascular space, and thus are slowly cleared from the pericardial space perhaps via lymphatics unless, of course, they are biotransformed (43,46). This yields a pericardial fluid clearance and residence time longer than the corresponding plasma half-life. For example, administering atrial naturetic peptide into the pericardial fluid space had a 5-fold longer clearance and residence time within the pericardial fluid space, as compared to plasma clearance of an intravenous dose (46). Similarly, small

water insoluble compounds may also have very prolonged pericardial fluid residual times.

One case report documented that the pericardial fluid half-life of 5-fluorouracil (sparingly soluble in water) was approximately 10-fold longer than plasma half-life (168 versus 16 minutes); it should be noted that the patient in this investigation had metastatic breast carcinoma with pericardial involvement (42). The patient had received a relatively large pericardial 5-fluorouracil dose (200 mg) to manage recurrent pericardial effusion. This large dose, however, was associated with nearly undetectable plasma levels indicating minimal spillover from pericardial fluid into the systemic circulation. While it was expected that 5-fluorouracil would have a longer pericardial residual time because it is water insoluble, it is unknown if these findings would occur in a healthy pericardial fluid space.

On the other hand, small water-soluble compounds have up to 5- to 8-fold shorter pericardial fluid clearance and residence times as compared to plasma (44). For example, procainamide is a water-soluble compound that has a pericardial fluid half-life ranging from 30-41±2.1 minutes as compared to the 180-minute plasma half-life; it has been reported that the procainamide rapidly diffused out of the pericardial space with a terminal elimination half-life

approximately 5-7 times shorter than plasma (47). However, procainamide spillover from pericardial fluid into plasma was considered not to produce measurable plasma concentrations because of the relatively low pericardial doses (0.5 to 2 mg/kg). Similarly, it is not surprising that the converse was also true, that intravenously administered procainamide rapidly diffused into the pericardial space, across a plasma to pericardial fluid concentration gradient, such that pericardial fluid procainamide concentrations were similar to plasma approximately 20-30 minutes following an intravenous injection. The likely explanation for these findings is that the vast ventricular epicardial blood supply served as a clearing system (pericardial administration) or a delivery system (intravenous administration) according to drug concentration diffusion gradient. Importantly, the diffusion of pericardial-administered procainamide into the vascular space will likely prevent drug accumulation in ventricular tissue, and a global pharmacologic response.

In addition to pericardial drug residence and clearance times, the determination of distribution volume may be of considerable importance, particularly to achieve desired peak drug concentrations. There is a direct and inverse relationship between peak drug concentrations and drug distribution volumes, such that a low drug distribution volume achieves higher peak concentrations. Perhaps of clinical importance, with the very small pericardial fluid volume, it is obvious that pericardial drug doses can be substantially reduced to achieve therapeutic concentrations. This was evident whereby sequential pericardial procainamide

doses of 0.5, 1 and 2 mg/kg produced peak pericardial fluid concentrations that ranged from 250-900 μg/ml; these concentrations were nearly 1000-fold greater than peak plasma concentrations of procainamide following the administration of a 2 mg/kg intravenous dose. In a follow-up study, in which a single procainamide dose was employed, similar findings were documented; it was also reported that a pericardial fluid volume of distribution of 1.6±0.16 mL/kg was observed, which is approximately 1000-fold smaller than plasma procainamide volume of distribution of 2000 mL/kg. While pericardial procainamide dosing produced very large pericardial fluid concentrations, procainamide could not be detected in the plasma given the very small doses. With such a powerful diffusion gradient, it is likely that pericardial procainamide delivery can achieve very high atrial tissue concentrations. Indirect evidence of tissue distribution is a procainamide distribution volume that is larger (40-50 mL) than the estimated pericardial fluid volume of 20-30 mL. Since the procainamide pericardial volume of distribution exceeded the expected pericardial volume, there was some tissue distribution. These pharmacodynamic data suggest that tissue distribution mainly occurs in the atrium, likely because the atrium is a very thin structure with a low blood supply. Thus, this tissue architecture is ideal for specialized therapeutic drug diffusion, and therefore differs from that of the ventricle(s).

Unfortunately, most pericardial procainamide pharmacokinetic studies performed to date have not directly measured tissue concentrations following infusion.

However, in one study which evaluated the pharmacodynamic effects of pericardial amiodarone delivery, the amiodarone tissue distribution was quantified at several myocardial locations (48). Not surprisingly, it was reported that atrial and epicardial ventricular tissue had the highest amiodarone tissue concentration, while ventricular endocardial amiodarone tissue concentrations were approximately 10-fold lower. However, importantly, the amiodarone levels were likely still within a therapeutic range. This was supported by the fact that pericardial amiodarone delivery prolonged endocardial ventricular refractory periods by up to 13%, which was equivalent to epicardial ventricular refractory period measurements and the magnitude of atrial refractory period prolongation. The similar refractory response between epicardial and endocardial measurements, with very large differences in amiodarone tissue concentrations, indicates that amiodarone effects are maximal at low tissue concentrations. Unlike pericardial amiodarone administration, pericardial procainamide had no effect on endocardial ventricular refractory periods (44). It is likely that such a beneficial ventricular tissue distribution does not occur with more water-soluble compounds such as procainamide. On the other hand, it is not surprising that amiodarone, when administered into the pericardial space, could penetrate ventricular tissue and affect global ventricular electrophysiology because it is highly lipophilic and has a huge tissue distribution including the intracellular space (48).

Lastly, perhaps it is possible to modify molecules to achieve an optimal pericardial fluid residence time and thus their therapeutic outcomes (benefits). More specifically, for some agents, it may be desirable to have a short residence time. For example, pericardial drug delivery to cardiovert atrial fibrillation may require very high drug concentrations for only a brief duration, given the acute nature of the therapy. On the other hand, the ability to manage chronic conditions such as ischemic heart disease or heart failure may necessitate longer pericardial residual times. In this regard, Baek et al. recently showed that a derivitized nitric oxide donor molecule, diazeniumdiolate, with bovine serum albumin resulted in a 5-fold increase in pericardial fluid clearance and residence time versus a small molecule nitric oxide donor (diethylenetriamine/NO)(49). This group went on to show that it may be possible that a single pericardial dose of the nitric oxide donor could inhibit in-stent restenosis. Unlike patients with any type of effusion, the normal pericardium is a very thin layer, bringing it closer to the heart, and subsequently increasing the risk of harm to the patient.

Summary

The pericardium is a unique structure that surrounds the heart and serves several important physiological roles. Removal of the pericardium, pericardial disorders, or the build-up of fluids within this space will alter hemodynamic performance. Recent therapeutic approaches have been directed to exploit the space that exists between the pericardium and the epicardial surface of the heart. New devices and techniques are being developed to access this space non-invasively. An important consideration when utilizing animal models to study such devices is that the pericardium in humans is much thicker and there is more pericardial fluid than in commonly employed animal models. The pharmacokinetics of many drugs may be greatly enhanced if the drug is delivered into the pericardium. As more is learned about the pericardium, it may play a significant role in cardiac therapies.

Chapter 2: Methods and Protocols for Cellular Electrophysiology of Single Cardiomyocytes

Authors: Eric Richardson, BS, Yong-Fu Xiao, MD, PhD

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Introduction

The Importance of Ion Channel Function in the Heart

The conduction system precisely controls the activation time of each cardiomyocyte so as to optimize the mechanical pumping of the heart. The smooth operation of this system is imperative to the proper function of the heart; in settings of damage or disease, electrical chaos can lead to death within minutes. What separates *excitable* cells in the heart, brain, and elsewhere in the body from *non-excitable* cells is the presence of certain ion channels that reside in the cell membrane. The type, population, activation, and/or inhibition of ion channels govern the whole-organ electrophysiology of the heart observed in the clinic. Heart rate, pacing and fibrillation thresholds, EKG morphologies, and the presence of arrhythmias are all products of the function (or malfunction) of these ion channels. Drugs that are prescribed to treat electrophysiological disorders are classified by which ion channels they affect. For example, Procainamide, commonly used to treat ventricular arrhythmias, is classified as a class I anti-arrhythmic, because it inhibits sodium channels.

Almost all of what we know about these ion channels and the drugs that affect them is a result of decades of research using the techniques described in this chapter. Patch clamp and other single cell electrophysiology techniques continue to be used not only to understand cardiac electrophysiology, but also to explore and screen treatments for heart disease.

Understanding Ion Channel Behavior through Electrical Analogues

Prior to discussing the methods for studying ion channel behavior, it is helpful understand the function of ion channels through electrical circuit models. This approach is convenient because we can also better understand the electrical equipment used in single-cell electrophysiology.

It is assumed that the reader has a basic knowledge of circuit elements and the physical laws that govern them. In particular, the following relationships are key to understand:

Voltage = Current x Resistance

(Ohm's Law)

Current = Capacitance x Time Rate of Change of Voltage

(Definition of Capacitance)

Voltage Drop Across Resistor (R1) = Total Voltage x R1 / (R1 + R2 + …)

(Voltage Divider Law)

If needed, a review of these relationships can be found in a basic physics text.

The simplest model of an ion channel is a resistor, as it offers resistance to the flow of ions across the cell membrane. The resistance of the lipid bilayer membrane itself is extremely large and in parallel with relation to the ion channel resistance. It may, therefore, be ignored. The membrane does play an important role, however, in providing capacitance to the system. This capacitance can be

modeled as a capacitor in parallel with the resistance of the ion channels. The transmembrane potential, typically around -90 mV in a resting cardiomyocyte, serves as the voltage source of our system. Combining these elements, the following model can be created:

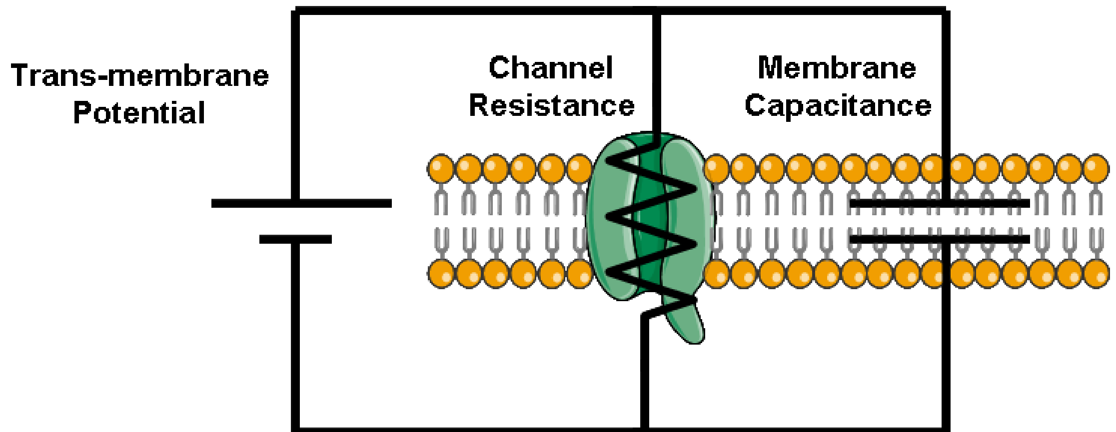


Figure 2.1 – A simplified electric circuit model of an ion channel and cell membrane.

Should the trans-membrane potential be changed in a step-wise manner (as in voltage clamping), we can predict the resulting current that enters the cell:

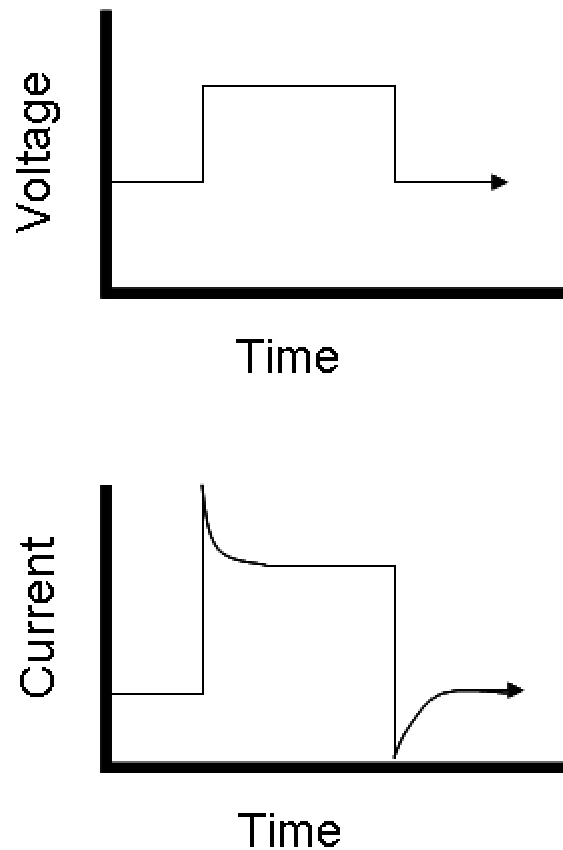


Figure 2.2 – Representative current waveform (below) of a cell membrane if a voltage step across the membrane is introduced (above).

The transient “spikes” that appear on the corners of the current waveform are due to the capacitive charge/discharge of the membrane.

In practice, most ion channels of interest are not simply resistive. They have complex voltage-gating mechanisms, selectivities, and many kinetic properties that have been model by extremely complex electrical circuits. The development of these models helps us understand the behavior of the channels to a greater

degree, and the experimental results from techniques described in this chapter help us develop and refine these models.

As the previous figure indicates, much can be learned about an ion channel if the trans-membrane voltage or current can be measured; even more can be learned when either the voltage or current is controlled. This is the opportunity that single-cell electrophysiology techniques provide. In traditional *extracellular* recording, an electrode measures the changes in the electric field surrounding an active cell. *Intracellular* recording allows us to passively measure the transmembrane voltage. With *patch-clamp* methods, we can either control the transmembrane voltage and measure the current entering the cell (voltage-clamping), or we can control the current injected into the cell and measure the transmembrane voltage (current-clamping).

Description of Apparatus/Models

Microelectrodes

The ability to probe the electrical activity of a cell requires us to place an electrode near, on, or inside of a cell that may only be a few microns wide. The function of a microelectrode is to transduce ion movement in an aqueous medium to electron movement in a wire. This is typically done by using a wire surrounded by an electrolyte solution and enclosed in glass micropippete (figure 2.3). The micropipette only allows conduction to the interior solution through a small opening at the tip. The conductive solution then transfers the electrical signal to the wire via an electro-chemical reaction.

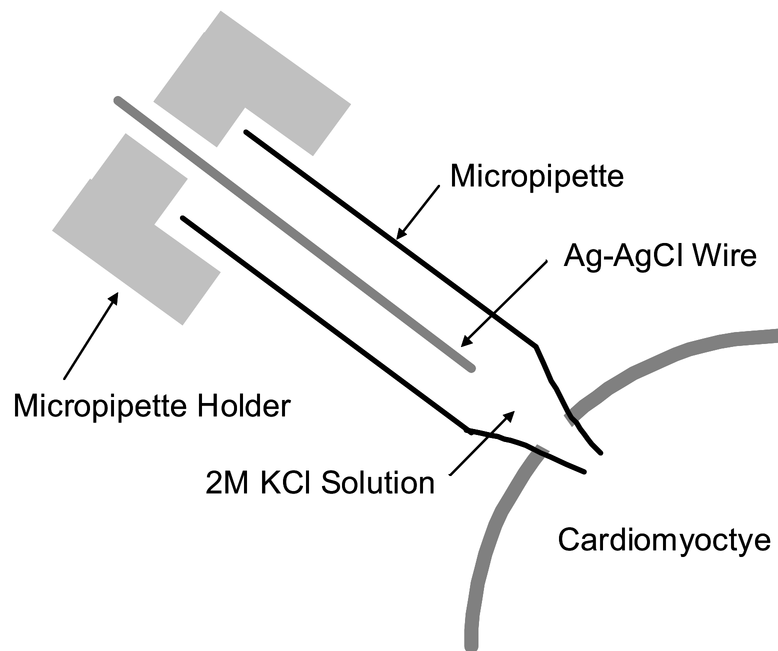


Figure 2.3 – A schematic diagram of the microelectrode assembly.

Micropipette fabrication is often a tedious, iterative process. Micropipette pullers have made this process reproducible and much easier than when it was done by hand (see figure 2.4). Micropipette pullers heat and pull small glass tubes until they neck in the middle and eventually pull apart. This produces two identical micropipettes. The force of the pull, the speed of the pull, and the intensity of the heat produce pipette tips of all varieties. Most pullers have the ability to store these parameters into a program that can be recalled when needed. Complex programs can include heat ramps, multi-stage pulls, and even small burst of air on the newly formed tip to cool the glass. The composition and thickness of the glass wall, bevel angle, length of tip, and diameter of opening are all important characteristics of the finished micropipette, and puller parameters must be adjusted to satisfy the needs of each application.

Figure 2.4 – A DMZ Universal Puller, used to fabricate micropipettes.

The solution that couples the tip of the micropipette to the wire is a salt solution that varies in composition depending on the application. For intracellular recording, high concentrations of KCl (typically 2-4M) are used because there is little leakage of ions out of the tip and into the cytoplasm of the cell. For patch clamp experiments, solutions that are more similar to the cytoplasm of the cell are used because there is more transfer of fluid through the tip. After a micropipette is pulled, it is filled with the aqueous solution using a long, flexible needle. It is important to ensure that no air bubbles are trapped in the micropipette, as this can interfere with the function of the microelectrode.

The wire inside the micropipette is typically silver, with a silver chloride coating. This wire can be prepared by using fine sand paper to abrade the surface of clean silver wire, and then immersing the abraded wire in bleach for 20-30 minutes. The wire should then be rinsed with water before placing it into the micropipette. The coating procedure may be repeated if the black silver chloride coating is scratched off after repeated insertions into micropipettes. The wire is typically mounted on a micropippete holder. The filled micropipette is placed over the wire and fastened to the holder. The assembly is then ready to be secured to the headstage.

Headstages, Amplifiers and Data Acquisition Systems

The purpose of the headstage is to provide a high impedance input immediately next to the microelectrode. This protects the very small signal from noise as it travels through a shielded cable to the amplifier, which is typically a few feet away. Headstages and amplifiers typically are sold together and are available commercially through many companies (Axon Instruments, Dagan Inc., etc). The type of headstage and amplifier is determined by the application. A simple bridge amplifier may be used for intracellular recording, but a more complex amplifier with current and voltage feedback mechanisms is needed for patch-clamp experiments. Further details about the amplifiers will be given in following sections.

The role of an amplifier is to both amplify and condition the signal received from the headstage. Voltages measured from cells are typically in the millivolt to microvolt range, and in order to maximize the resolution of standard data acquisition systems, commercial amplifiers usually have gains of both 10x and 100x. High-pass, low-pass, and notch filters may be employed to condition the signal. These filters may be adjustable, or they may be hardwired into the amplifier. Frequency responses of the filters must be taken into consideration when adjusting filter parameters. AC-coupling is almost always used in signal conditioning to prevent saturation of the downstream data acquisition system.

Additional features, such as blanking capabilities or audio monitors can also be incorporated into the amplifier. Blanking refers to the ability of the amplifier to ignore its input when given a logic-level pulse. During the pulse, the amplifier

retains the output value it gave prior to the pulse. At the end of the pulse the amplifier resumes function immediately. This feature is helpful in instances when a high voltage stimulus is used to evoke an electrophysiologic response. During the stimulus, the amplifier may easily saturate and take several milliseconds to recover, loosing valuable data from the evoked response during the recovery period. If a blanking system is appropriately configured, a logic-level pulse is sent to the amplifier for duration of the stimulus, avoiding the saturation and resuming recording as soon as stimulus is complete. Audio monitoring converts the voltage or current measured at the electrode to an audible pitch. This provides real-time feedback to an investigator who is guiding a microelectrode under microscope guidance, preventing constant glances back at the display on the amplifier.

Data acquisitions systems, commercially available for use with standard personal computers, convert the amplified, conditioned signal from the amplifier into a digital signal which can be stored, manipulated, and analyzed by the investigator. The incoming analogue signal should be amplified to maximize the full range of the acquisition system (e.g. -15V to 15V, -10V to 10V). Care should be taken, however, that the signal does not saturate the acquisition system. Many amplifiers and acquisition systems have overload-detection circuitry that informs the user that the input is beyond an acceptable range.

Choosing an appropriate sampling rate is important to prevent aliasing. While the Nyquist theorem dictates that we choose a sampling rate at least twice the fastest component of our signal, a more practical guideline is to choose a rate 2.5

times the cutoff frequency of the highest low-pass filter (typically called an anti-aliasing filter). For electrophysiology systems, this rate can be as high as 25 – 250 kHz. After the signal has been acquired, a variety of digital filters are available to further condition the signal. With the growing data storage capacities and higher maximum sampling rates that modern technology presents, it is easy to oversample to ensure that the signal is properly recorded. However, needless recording at extremely high sampling rates can lead to large data files that are hard to transfer and make post-process analysis difficult.

Most data can be analyzed with commercially available software designed for electrophysiology applications (e.g. Pclamp (Molecular Devices, Inc)). For those who would like more flexibility and the ability to apply a variety of digital filters, technical computing programs such as MATLAB (The Mathworks, Inc) have powerful signal processing capabilities.

Cell and Tissue Baths, Microscopes

For excitable cells and tissues to be studied, they must be maintained in a physiologic environment. This requires the use of a bath mounted on the stage of a microscope. The microscope is used for visualization and placement of the microelectrode in or near cells. Many patch clamp protocols call for single disassociated cells, usually attached to a coated glass coverslip. The cover slips can be taken directly from the incubator in which they were maintained and placed in a small bath mounted on the stage (see fig X). Baths typically have an inflow port on one end, a glass-bottom well in the center, and an outflow on the

other end. The inflow is attached to a reservoir of media that has a mechanically- or electrically-controlled flow meter. In applications such as pharmacology studies, there may be several reservoirs with different media compositions and a system of valves so that the investigator can quickly switch the media in the bath. The outflow of the bath can be a passive wick that transfers excess fluid to a lower drainage container, or an active suction tube that removes liquid as it reaches a certain level. As many electrophysiology processes change dramatically with temperature, high-end baths often have integrated heating elements that warm the bath to a desired temperature.

The complexity of a bath increases slightly when using tissue. Whereas single cells have little problem extracting the oxygen they need from the surrounding medium, cells within tissue rely on diffusion of oxygen from the surface of the tissue. To prevent hypoxia, therefore, the medium must be saturated with oxygen prior to entering the bath. This may be done in an adjacent chamber where fluid is bubbled with oxygen using a sparger, and then continues on to the bath. Oxygen saturation is particularly important for cardiac tissue due to its high metabolic rate and extreme sensitivity to hypoxia. Care must be taken that bubbling does not alter the pH of the medium. It is not uncommon to use a mixed gas of 95% oxygen and 5% carbon dioxide to maintain a neutral pH.

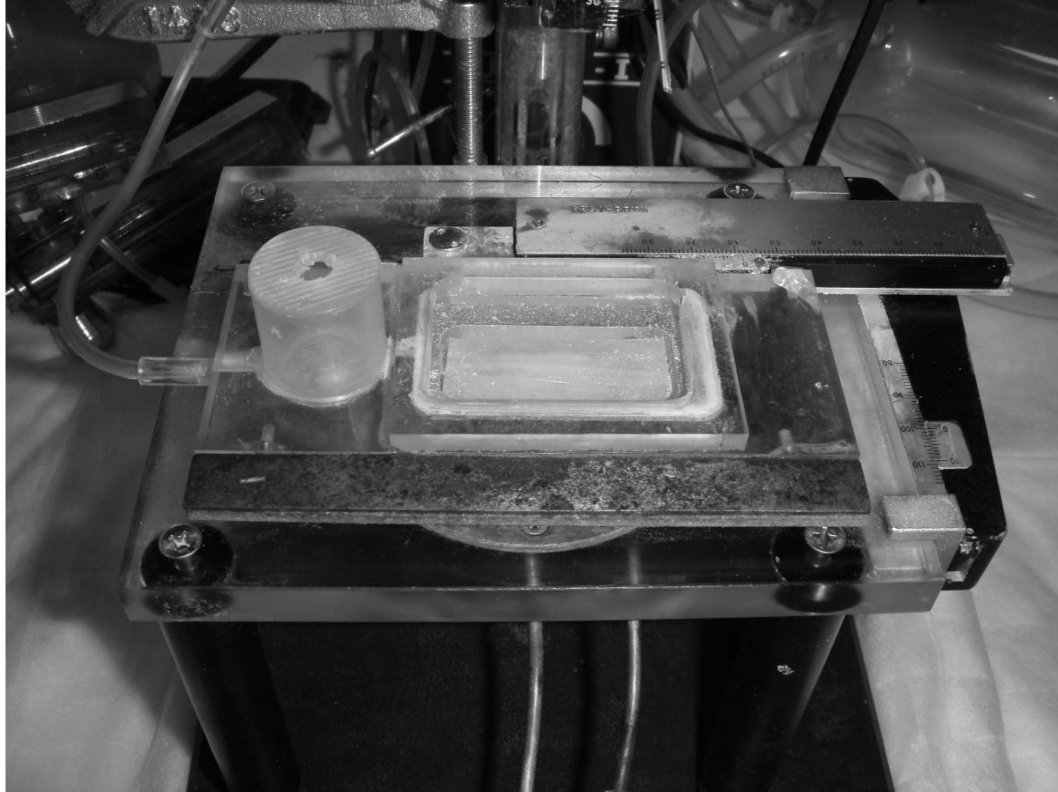


Figure 2.5 – A tissue bath used for intracellular recording.

Inverted microscopes are commonly used for patch-clamp protocols, as space is needed above the bath to introduce the microelectrode. The microscope should be capable of 100-fold magnification, and may have contrast enhancement. Electrophysiology microscopes will usually have fluorescence capabilities because cells of interest are often fluorescently-labeled. In some extracellular and intracellular applications where tissue is used, upright dissection microscopes can be used, as high resolution is not necessary.

Micromanipulators and Vibration Isolation Tables

Placement of the microelectrode near, on, or inside a micron-sized cell is

perhaps the most challenging aspect of single-cell electrophysiology. A micromanipulator allows the investigator to move a microelectrode with sub-micron precision. Micromanipulators can be mechanical, pneumatic, hydraulic, or servo-controlled. Most have three-degrees of translation with both coarse and fine controls, and a mounting surface for the headstage.

The smallest vibrations, even those of a person walking nearby, can break the contact of a microelectrode with a cell. Vibration isolation is necessary to prevent such mishaps. Vibration isolation is usually achieved by setting up the microscope, bath and micromanipulator on a very heavy slab of metal or stone. The slab is then supported by a mechanism that dampens vibrations. This can be as simple as the inner-tube of a bicycle. Commercially-available vibration isolation tables use pneumatic cylinders to achieve the same result. Additional vibrations can be avoided by mounting the micromanipulator as close to the bath as possible. Large moment arms created by equipment mounted far apart encourage vibration.

The location of the apparatus is important minimize the ambient mechanical and electrical noise. Electrophysiologists often find that the basement or lower levels of tall buildings have less mechanical vibration than higher levels. Large equipment, such as heating and cooling systems, refrigerators, and elevators can not only produce mechanical vibrations, but usually emit a large amount of electrical noise as well. Faraday cages can be used to limit the amount of electrical noise picked up by the microelectrode. More on electrical noise will be

explained later.

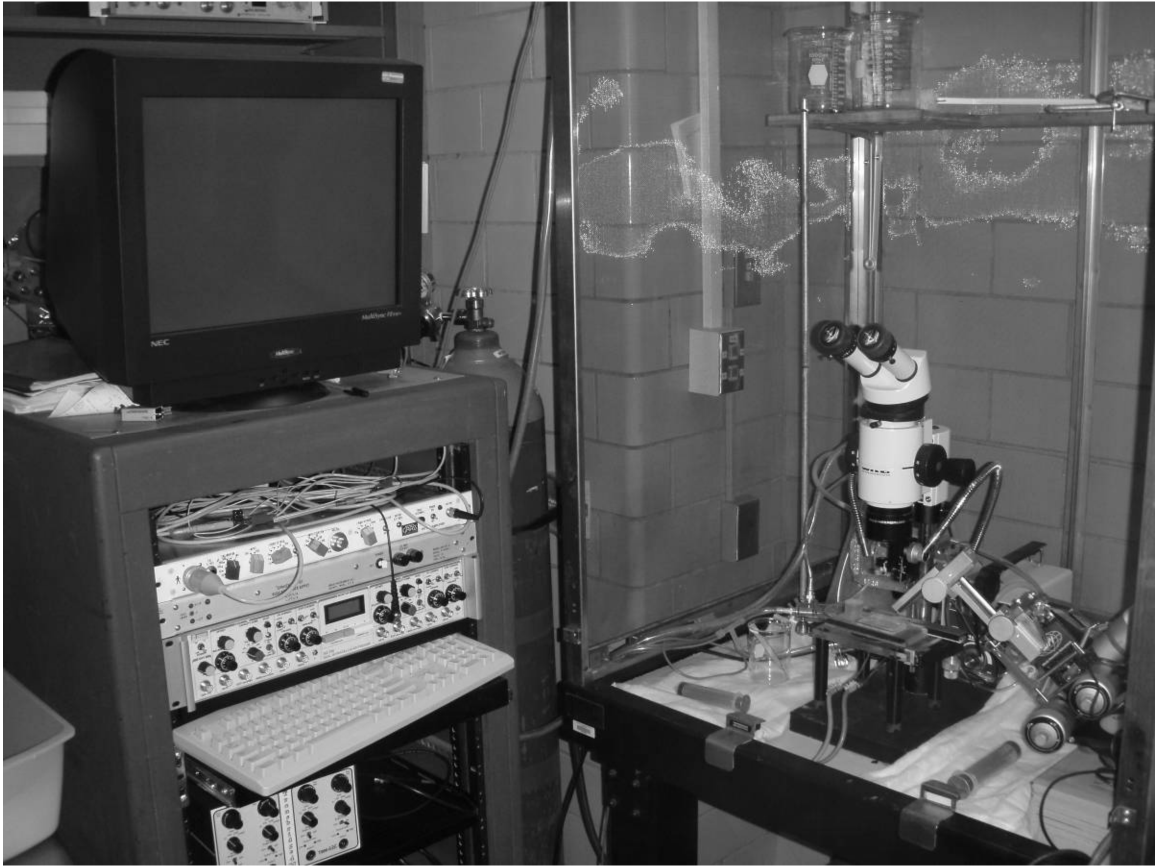


Figure 2.6 – A typical electrophysiology setup. The amplifier and acquisition system are on the left, and the vibration isolation table, faraday cage, tissue bath and microscope are on the right.

Detailed Methodologies

Methodologies will very greatly depending on the application. The following are general guidelines for the major techniques in cardiac electrophysiology. The investigator is encouraged to search the scientific literature for further details of their specific application.

Extracellular Recording

During extracellular recording, the microelectrode is placed in close proxity to one or more cells and measures the changes in the electrical field (or field potential) near the cell. This technique can provide valuable information without disturbing the cell membrane. It can be used in both tissue and disassociated cell preparations.

In this technique, a sharp microelectrode is advanced into the preparation until electrophysiological activity is detected. The microelectrode can be a liquid-filled micropipette like those described previously, or it can be made of metal (with insulation up to the tip). Size of the electrode will vary with the application, specifically if the intent is to measure the field potential from a single cell compared to several cells.

Electrical signals measured by extracellular recording are very small compared to those measured in other techniques; they are typically less than 1 mV. In addition, the amplifier is typically AC-coupled so that only dynamic changes in the electrical field at the microelectrode are recorded. Such conditions make blanking features important when using an external stimulus. External stimuli are

provided either by needle electrodes inserted into the tissue, or by paddle electrodes placed on either side of the preparations. Stimulus parameters should be tuned so that only the minimum duration, current, and voltage is used to evoke a response. This will prevent a large stimulus artifact. Typical stimulus parameters are 1-2 msec duration at 1-10V. The parameters will vary with electrode surface area, composition, and placement. Hydrolysis, or bubbling on the surface of the stimulus electrodes due to molecular separation of water, is a sure sign that stimulus parameters are too high.

Arrays of extracellular electrodes can be patterned on the bottom of a culture dish using photolithography. This can provide the investigator with temporal and spatial recording of electrical activity. This technique is discussed further in the Microelectrode Array chapter of this text.

Intracellular Recording: Sharp Microelectrodes vs. Patch Electrodes

To measure the electrical activity within the cell, two types of microelectrodes may be used: sharp microelectrodes and patch electrodes. Sharp microelectrodes were the first to be developed and consist of a fluid-filled micropipette with the tip pulled to an opening less than one micron in diameter. They are typically filled with a high-molarity salt solution, such as 2M KCl. The resistance of these electrodes is usually 1-10 megaohms. These "sharp" electrodes are thus called because they simply penetrate the cell membrane, with the membrane sealing itself around the micropipette.

In the 1980's, patch microelectrodes came into use. Patch microelectrodes are pulled to have an opening greater than one micron. The pulls are usually performed in multiple stages, with a final heat polishing of the tip to remove ragged edges. The micropipettes are filled with a solution that is similar to the extracellular or intracellular fluid of the cell because there is considerable exchange of contents with the "patched" cell. These microelectrodes are not designed to penetrate into the cell, but to be positioned such that they are just touching the membrane. These electrodes form a very tight seal with the membrane, often called a "giga-seal" because the seal resistance is typically several gigaohms.

Once the patch microelectrode is sealed to the membrane, the investigator can choose one of several patch configurations. The microelectrode can remain sealed to the intact membrane in the "on-cell" patch configuration. If suction is applied to the sealed microelectrode, the patch of membrane beneath the microelectrode will rupture, making the interior of the cell continuous with the interior of the microelectrode. This configuration is call the "whole-cell" patch. If one simply pulls the microelectrode away from a cell in the "whole-cell" configuration, a patch of membrane will separate from the cell and reform on the tip with the extracellular surface of the membrane facing away from the microelectrode. This is called the "outside-out" configuration. Finally, if a sealed microelectrode is pulled away from an intact membrane (the "on-cell" configuration), a patch of membrane will form on the tip of the microelectrode

with the extracellular surface towards the interior of the microelectrode. This is called the “inside-out” configuration (see figure 2.7).

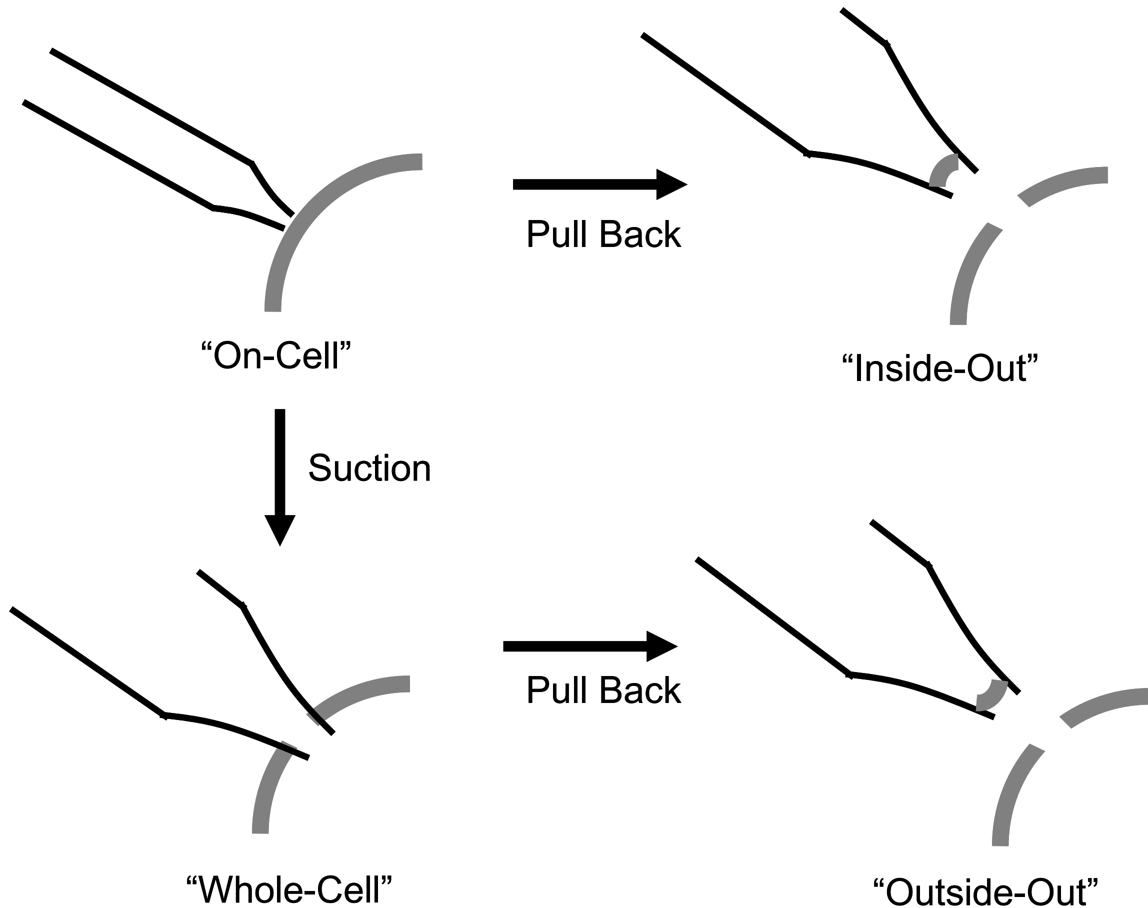


Figure 2.7 – Various patch configurations and how each is formed from an “on-cell” configuration.

Current Clamp

When using the current clamp, voltage is measured while the current passing through the microelectrode is controlled by the investigator. The simplest form of intracellular recording is a zero current clamp. In this technique, a sharp microelectrode is used to penetrate the cell and voltage is measured at the tip

when no current is passed into the cell. This measurement reflects the transmembrane voltage. A controllable current source can be connected to the electrode to simultaneously inject current and record voltage. Due to the injected current, compensation in the form of a “bridge” circuit (also know as a “voltage-follower”) must be used to prevent distortion of the signal. Such circuitry requires a bridge balance to be performed with each new microelectrode. Instructions to perform the bridge balance are usually found in the amplifier’s manual.

Voltage Clamp

The voltage clamp technique, which is one of the most commonly-used techniques in electrophysiology, serves to measure the current passing through the microelectrode while holding the voltage constant. This technique is popular because of its use in studying voltage-gated ion channels. There are several approaches to voltage clamping, the oldest of which uses two sharp electrodes place into the same cell. In this approach, call two-electrode voltage clamping (TEVC), one electrode serves as the recording electrode while the other injects current into the cell. A TEVC amplifier has feedback circuitry that allows it to read the voltage from the recording electrode and then inject current into the cell to bring it to the user-defined voltage (or command voltage). The output of the amplifier is the real-time amount of current (represented as a voltage signal) needed to maintain a cell at a certain command voltage.

Due to the difficulty of penetrating small cells with two microelectrodes, single electrode voltage clamp (SEVC) was developed. In this approach, a patch

electrode in one of the patch configurations described previously is used to both pass current through the membrane and record voltage. This can either be done in a continuous fashion (continuous single electrode voltage clamp, or SEVC-c) where the microelectrode measures voltage and injects current simultaneously or in a discontinuous fashion (discontinuous single electrode voltage clamp, or SEVC-d) where the electrode rapidly switches between only measuring voltage and only measuring current.

Applications of Models/Methodologies

Single cell cardiac electrophysiology is a very broad field with many tools and applications. The literature is best resource for protocols to achieve a certain research goal. This section presents two representative protocols: The first is a standard intracellular recording protocol for measuring action potentials in human cardiac tissue, and the second is a more complex single electrode voltage clamp protocol for measuring HCN4 channel currents (a channel commonly found in the sinoatrial node of the heart).

Example Protocol #1: Human Cardiac Action Potentials

Anti-arrhythmic medications are commonly prescribed for heart rhythm disorders. Most of these medications are classified by how they affect the morphology of the cardiac action potential. This protocol describes how human tissue samples can be used to gather this information using standard intracellular recording techniques.

Waste human cardiac tissue (usually right atrial appendage from a cardio-pulmonary bypass operation) is obtained from the operating room and immediately placed in a Tyrode's solution saturated with oxygen.

The tissue is pinned out on the bottom of a tissue bath to expose the endocardial surface. The tissue is constantly superperfused at 2ml/min with Tryodes solution supplemented with glucose and saturated with a mix of 5% carbon dioxide and 95% oxygen. The tissue bath is maintained at 37 degrees Celsius.

A sharp electrode (1-10 megaohms) filled with 2M KCl and connected to a headstage and intracellular amplifier is advanced into the bath under the guidance of a dissection microscope. When the tip of the microelectrode is submersed in the bath, the DC offset is set to 0 mV.

The microelectrode is advanced very slowly into the tissue until the voltage sensed at the tip drops below -60mV.

Plunge electrodes previously placed in the tissue then stimulate the preparation with a 2-ms square wave at 20V and 1Hz using a stimulator. The motion of contraction may cause the electrode tip to come out of the cell. This can be prevented by overstretching the cardiac tissue when it is originally pinned out in the bath.

Action potentials measured by the microelectrode and amplifier are acquired by a data acquisition card and LABVIEW program that samples at a rate of 25kHz.

After baseline data has been recorded, pharmacologic agents may be added to the superperfusate to observe changes in action potential morphology. The preparation may be viable for several hours.

Figure 2.8 shows a human cardiac action potential obtained using this protocol:

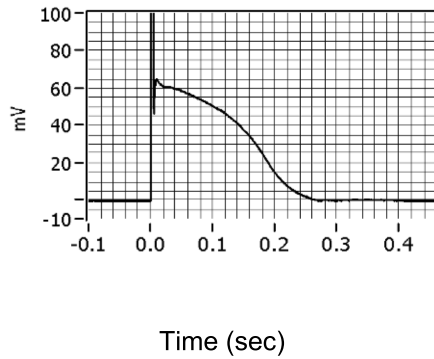


Figure 2.8 – A human cardiac action potential obtained from intracellular recording with a sharp microelectrode. The Y-axis displays mV from baseline (baseline was -40mV at time of stimulation), and the x-axis displays time in seconds. The initial spike at t = 0 is an artifact of the stimulation.

Case Study #2: Patch-clamp recording from HCN4-transfected cells

This section is currently being prepared by Dr. Xiao and will appear in the final chapter.

Pitfalls and Troubleshooting

Any investigator assembling an electrophysiology apparatus will inevitably have their patience tested. It is not uncommon to spend several hours chasing sources of noise, or trying to pull the “perfect” microelectrode. Below are just some of the many common pitfalls that are bound to occur:

Tissue/cell viability

Viability of the specimen has often prevented good results from a well-working electrophysiology apparatus. Cardiac tissue and cells, in particular, are extremely sensitive to ischemia and mechanical disruptions. Therefore, it is imperative that they are cared for properly prior to and during the experiment.

Microelectrodes

A broken microelectrode will prevent the investigator from properly penetrating or patching a cell. Constantly check the electrode impedance to be sure that it is within and acceptable range. Most amplifiers have a “Z-test” or an equivalent feature that allows one to check the resistance of the microelectrode while it is submersed in the bath.

Electrical Noise

Noise is and extremely common problem in electrophysiology. AS mentioned before, a properly grounded faraday cage, and a properly positioned apparatus (set up away from large machinery) can lower your risk of noise. If this does not solve the noise problem, ground the microscope, micromanipulator, and any other equipment within the faraday cage using clips. Sheilding the bath with

aluminum foil may also prevent noisy measurements. For good advice in troubleshooting noise, the investigator is referred to chapter 12 in the Axon Guide.

Emerging Technologies

The techniques described in this chapter can provide valuable information not only about cell physiology, but also about drug effects. Screening drugs is an important part of the pharmaceutical industry, and due to the long and tedious nature of conventional patch-clamp techniques, automated patch-clamp systems have been developed. Companies which sell automated systems include Molecular Devices, Nanion Technologies, Sophion Bioscience, and Flyion. These systems use complex microfluidic devices with suction capabilities to capture cells, seal onto the membrane, and form different patch configurations. Some systems are capable of patching 16 cells or more at the same time. These machines are costly, but they save greatly on the time that it takes to screen large libraries of potential pharmaceutical compounds. So while the basic techniques of patch clamp have not changed much over the last two decades, technology is enhancing its reach by streamlining the process.

Further Reading

This chapter is meant to be a brief introduction to single cell cardiac electrophysiology. For further information, a number of excellent resources are available. For more on the physiology of ion channels and the theory behind

electrophysiology, the investigator is referred to “Ionic Channels of excitable Membranes”, by Bertil Hill. For a thorough explanation of the laboratory setup and electronics, “The Axon Guide” is an excellent reference and was the basis for much of this chapter. Finally, for details on patch clamp protocols, “Path-Clamp Methods and Protocols” by Molnar and Hickman has many good examples. Full references are available at the end of this work.

Chapter 3: Electrophysiological Mechanisms of the Anti-arrhythmic Effects of Omega-3 Fatty Acids

Authors: Eric Richardson, BS, Paul Iaizzo, PhD, Yong-Fu Xiao, MD PhD

This manuscript is currently in preparation for submission.

Abstract

Heart rhythm disorders, or arrhythmias, are a leading cause of morbidity and mortality worldwide. Omega-3 polyunsaturated fatty acids (ω3PUFAs), commonly found in fish oils and plant seeds, have recently emerged as potential anti-arrhythmic agents. The purpose of this review is to summarize the electrophysiological basis of the anti-arrhythmic properties of ω3PUFAs from clinical, animal, and cellular research.

Evidence of the anti-arrhythmic effects of ω3PUFAs originated from epidemiological studies that correlated a low incidence of sudden cardiac death (SCD) with high dietary ω3PUFA intake. Subsequently, multiple clinical trials have confirmed the therapeutic effects of ω3PUFAs in preventing SCD and multiple other arrhythmia-related disorders. This has lead basic scientists to discover the potent effects of ω3PUFAs on several ion channels including sodium, potassium and calcium channels, as well as Na/Ca exchangers. Therefore, ω3PUFAs may hold promise as safe and effective anti-arrhythmic agents. Nevertheless, further research is needed in areas such as 1) identifying which form(s) (i.e. phospholipid, triglyceride, or free) of ω3PUFAs is/are responsible for anti-arrhythmic actions, and 2) developing reproducible methods for delivery so that the appropriate form and concentration may be present at the target site to prevent and treat arrhythmias.

Introduction

Almost 40 years ago, Bang and colleagues published a series of epidemiological papers on the diet and cardiovascular health of Inuit Eskimos. They observed a remarkably low incidence of ischemic heart disease among the Eskimos as compared to their Danish neighbors(1). They postulated that the vast differences in cardiovascular health were due in part to differences in their dietary intake of omega-3 polyunsaturated fatty acids (ω3PUFAs).

In the last four decades, an immense amount of work has been done on the physiologic effects of omega-3 fatty acids, sparked by Bang's findings. This body of research has extended beyond the cardiovascular system to the immune, gastrointestinal, and nervous systems. The following review attempts to summarize a small, yet significant part of this work: the electrophysiological effects of ω3PUFAs on the heart. This area of research continues to be highly active, in part due to the promising evidence that ω3PUFAs may prevent sudden cardiac death (SCD), which today remains as the nation's largest cause of mortality(2). Furthermore, current anti-arrhythmic drugs are known for their narrow therapeutic range, adverse side effects, and lack of efficacy(3). As ω3PUFAs are natural compounds and their effects on excitable tissues have been progressively understood, they may provide a safer, cheaper, and more effective alternative to current anti-arrhythmic drugs.

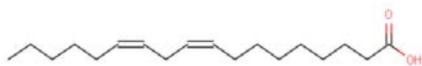
Background

Omega-3 polyunsaturated fatty acids are vital for the proper development of several critical human organs, such as the brain, and the maintenance of their normal functions. Along with the omega-6 class, these polyunsaturated fatty acids are considered as “essential,” because they cannot be synthesized in humans(4).

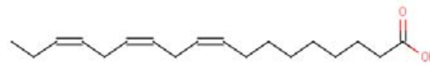
Each omega-3 fatty acid has an analogous omega-6 counterpart with the same carbon chain length, as seen in figure 3.1. The omega-3 eicosapentaenoic acid (EPA), for example, is similar to the omega-6 arachadonic acid (AA). It is important to note that humans lack the enzymes to convert between omega-3 and omega-6 classes. Within the human body the omega-3 and omega-6 fatty acids of similar length may compete for the same enzyme to further desaturate their carbon chain. For example, both omega-6 Linoleic Acid (LA) and omega-3 α-Linolenic Acid (ALA) must compete for Δ6-desaturase to form AA or EPA. This competition for enzymes underlies a clinically significant interplay between omega-3 and omega-6 fatty acids(5). This interplay may affect prostaglandin production, and may be the underlying reason for several of the non-electrophysiological effects of ω3PUFAs(6).

Omega-6 Polyunsaturated

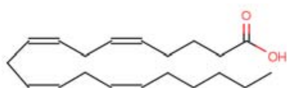
Omega-3 Polyunsaturated



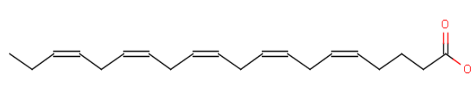
Linoleic Acid



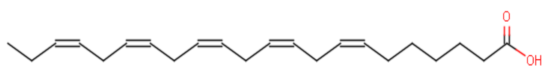
α-Linolenic Acid



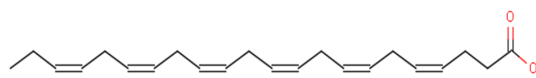
Arachidonic Acid



Eicosapentaenoic acid



Docosapentaenoic Acid



Docosahexaenoic acid

Figure 3.1 - The chemical structures of omega-6 and omega-3 classes of polyunsaturated fatty acids. Each omega-6 fatty acids on the left has an analogous omega-3 fatty acid on the right with similar chain length.

The primary source of ω3PUFAs, both directly and indirectly, is the chloroplasts of plants, plankton and algae. While plant oils such as soybean, flax seed, and canola contain some ω3PUFA, most ω3PUFA is consumed by humans through eating fish. Fish eat krill, which in turn feed on plankton, a primary source of ω3PUFAs(4). ALA is the ω3PUFA primarily found in plant oils, whereas EPA and Docosahexaenoic Acid (DHA) are found in fish. It should be

noted that the concentration of ω3PUFA depends greatly on the type of fish. Relative values may range from 1.83g per 3oz serving of Atlantic Salmon to 0.13g per 3oz serving of Pacific Cod(7). Many brands of fish oil supplements are available to the public, and recently pharmaceutical-grade purified ω3PUFAs have been approved for use in Europe and the United States.

Clinical- and Animal- based evidence for Cardiac Electrophysiological Effects

The epidemiological studies of Bang and other groups subsequently sent both scientists and physicians searching for the underlying mechanism responsible for the positive cardiovascular effects of ω3PUFAs. For the following two decades, a large volume of ω3PUFA research was focused on their anti-thrombotic, anti-inflammatory, and triglyceride-lowering effects. Some investigators, noticing therapeutic effects on SCD and psychiatric disorders, hypothesized that underlying electrophysiological mechanisms may also be at work, but only a few experiments were done in the 1980's and 1990's to test this hypothesis. In the last decade, however, there has been a large effort to uncover the electrophysiologic effects of ω3PUFAs.

A summary of several clinical trials focused on the effects of omega-3 fatty acids in cardiac electrophysiology are presented in table 3.1. Thirty of the 35 trials presented were published after 1998, indicating a growing interest over the last decade. In these trials, ω3PUFAs were shown to: 1) prevent SCD, 2) prevent ventricular arrhythmias in patients with Implantable Cardioverter/Defibrillators (ICDs), 3) lower the occurrences of premature ventricular contractions, 4) lower heart rate, 5) increase heart rate variability, 6) significantly alter EKG morphologies, and 7) prevent atrial fibrillation. Nevertheless, the outcomes are not all positive; in several instances ω3PUFAs showed no beneficial effects(9, 14, 17, 22, 24, 29, 32, 42, 43), and in a few cases they were shown to induce adverse effects(12, 20, 37). So while the results are generally positive, the NIH

in 2004 stated that “a definitive trial is still needed”(44). In response to this need, a number of large clinical trials are currently underway(45).

Table 3.1 (On the next pages) - A brief summary of several major clinical trials that present the electrophysiological effects of omega-3 fatty acids on the heart. The clinical trials are grouped by electrophysiological effect. Several parameters of the trials and important outcomes are shown to aid in comparison.

Table 1. Summary of Clinical Trials

Prevention of Cardiac Arrest and Sudden Cardiac Death (SCD) via Fish Consumption

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinant Outcomes
Siscovick et al	1995	827	Male and female case patients with primary cardiac arrest, matched by age and sex with controls	_	Fish Consumption	_	_	RBC membrane n3PUFA compostition measured	Intake of n3PUFAs is associated with a reduced risk of primary cardiac arrest.
Daviglus et al	1997	1,822	Males 40 to 55 yrs old, free of cardiovascular disease at baseline	Longitudinal Study	Fish Consumption	_	30 years	Food Consumption Survey	No significant correlation between fish consumption and SCD.
Albert et al	1998	20,551	US male physicians from 40 to 84 yrs old, free of previous MIs	Longitudinal Study	Fish Consumption	_	11 years	Food Consumption Survey	Consumption of fish at least once per week reduced the risk of SCD.
Albert et al	2002	278	SCD victims free of previous CVD from Albert 1998 study and controls matched for age and smoking	Longitudinal Study	Fish Consumption	_	17 years	n3PUFAs in whole blood measured	n3PUFA levels were strongly correlated with reduced risk of SCD.
Burr et al	2003	3,114	Males <70 yrs old with angina	3g EPA/week or two portions of oily fish	Fish oil, fish consumption	Dietary advice to eat more fruits and vegatables, or non-specific advice	3-9 years	Plasma EPA measured	Men advised to eat oily fish, and specifically fish oil capsules, had a higher risk of SCD.
Mozaffarian et al	2003	3,910	Males and females >65 years old with no known CVD	Longitudinal Study	Fish Consumption	_	9.3 years (mean follow-up)	Food Consumption Survey	Consumption of tuna or other boiled or baked fish was associated with a lower risk of arrhythmic ischemic heart disease. Consumption of fried fish and fish sandwiches showed no correlations.
Albert et al	2005	76,763	Female nurses, 30 to 55 yrs old	Longitudinal Study	Fish Consumption	_	18 years	Food Consumption Survey	ALA intake was inversely related to the risk of SCD.
Iso et al	2006	41,578	Japanese male and females 40 to 59 yrs old, free of previous CVD	Longitudinal Study	Fish Consumption	_	~10 years	Food Consumption Survey	No correlation was found between fish intake and SCD in a population whose lowest quintile of fish consumption (20g fish/day) is higher than average western consumption.

Prevention of Sudden Cardiac Death via n3PUFA Supplementation

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinent Outcomes
Singh et al	1997	360	Males and females with an acute MI within the last 24 hours	1.08g EPA, 0.72g DHA per day, or 2.9g ALA per day	Fish oil, Mustard oil	Aluminum Hydroxide	1 year	None	A reduction in total arrhythmias and ventricular ectopic beats was observed in both mustard oil and fish oil groups compared with placebo. A nonsignificant reduction in SCD was observed in both groups as well.
GISSI Investigators	1999	11,324	Males and females with recent (<3 months) MI	0.85g EPA+DHA (in a 1:2 ratio) per day	Purified ethyl ester	Vitamin E, or none (2X2 factorial design)	3.5 years	None	A reduction of 44% in SCD due to fish oil was observed.
Burr et al	2003	3,114	Males <70 yrs old with angina	3g EPA/week or two portions of oily fish	Fish oil, fish consumption	Dietary advice to eat more fruits and vegatables, or non-specific advice	3-9 years	Plasma EPA measured	Men advised to eat oily fish, and specifically fish oil capsules, had a higher risk of SCD.
JELIS Investigators	2007	18,645	Males and females with high cholesterol (>6.5mmol/L)	1.8g EPA per day	Purified ethyl ester	Pravistatin or Simvastatin (also given in EPA group)	5 years	None	No reduction in SCD was found in the EPA-treated group.

Implantable Cardioverter/Defibrillator (ICD) Cases

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinant Outcomes
Leaf et al	2005	402	Males and females with ICDs	2.6g EPA+DHA per day	Purified ethyl ester	Olive oil	1 year	RBC phospholipids measured	A significant risk reduction was observed in the fish oil group after adjustments.
Christensen et al	2005	98	Males and females with ischemic heart disease and ICDs	Longitudinal Study	_	_	1 year	Serum phospholipids measured	Patients with low n3PUFA content had more ventricular arrhythmias.
Raitt et al	2005	200	Males and Females with ICDs and a recent episode of sustained VT or VF	0.76g EPA, 0.54g DHA per day	Fish oil	Olive oil	718 days (median follow-up)	Plasma and RBC membrane n3PUFAs measured	Time to first VT/VF event was less in the fish oil group, and recurrent VT/VF events were more frequent in the fish oil group.
Jenkins et al	2006	1 (Case Report)	66 yr old male with sustained VT and an ICD	0.46g EPA, 0.38 DHA per day	Purified ethyl ester (OMACOR)	None	4 weeks	None	Prior to treatment with n3PUFAs, the patient experienced 25-40 episodes of sustained VT per day. After the start of treatement, the patient experienced only one episode in the following 4 weeks.
Brouwer et al	2006	546	Males and females with ICDs and documented malignant VT or VF	0.46g EPA, 0.34g DHA, 0.16g other n3PUFAs per day	Fish Oil supplemented with tocopherol	Sunflower seed oil	356 days (median period)	Serum cholesterol esters measured	No significant effect of n3PUFAs was observed on ventricular arrhythmias.

Prevention of Premature Ventricular Contractions (PVCs)

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinant Outcomes
Sellmayer et al	1995	79	Males and females with >2,000 PVCs per day, but without dangerous ventricular arrhythmias or pump failure	0.9g EPA, 1.5g DHA per day	Fish (Cod liver) oil	Sunflower seed oil	16 weeks	Phospholipid n3PUFAs measured	PVCs decreased by 48% percent in the fish oil group and 25% in the placebo group.
Geelen et al	2005	84	Patients with >1440 PVCs per day	0.7g EPA, 0.56g DHA, 0.26g other n3PUFAs per day	Fish Oil supplemented with tocopherol	Sunflower seed oil	14 weeks	Serum cholesterol esters measured	Fish oil supplementation did not decrease the number of PVCs.

Lowering of Heart Rate (HR)

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinant Outcomes
Dallongeville et al	2003	9,758	Males 50 to 59 yrs old, without CHD	Cross-sectional Study	_	_	_	Erythrocyte phospholipids measured	Fish consumption was correlated with lower HR.
Mozaffarian et al	2005	_	Meta-Analysis of 30 randomized, controlled trials	_	_	_	_	_	Overall, fish oil decreased HR by 1.6 bpm. Larger reductions occurred in trials with higher baseline HR, and with longer study duration. No correlation was seen between dose and reduction of HR.

Increasing Heart Rate Variability (HRV)

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinant Outcomes
Christensen et al	1996	55	Males and females < 75 yrs old with previous MI and EF <40%	4.3g EPA+DHA per day	Re-esterified triglyceride	Olive oil	12 weeks	EPA and DHA in platelets measured	n3PUFAs increased HRV.
Christensen et al	1997	52	Males and females with EF <40% and <70 yrs old	Cross-sectional Study	_	_	_	Platelet n3PUFAs measured	HRV was positively and significantly correlated with measured n3PUFA levels.
Christensen et al	2000	145	Males and females at high risk of SCD (with previous MIs or chronic renal failure), controls were healthy volunteers	Cross-sectional Study	_	_	_	Platelet or granulocyte membrane n3PUFAs measured	No significant correlation was observed between ALA levels and HRV.
Christensen et al	2001	291	Males and females with suspected ischemic heart disease	Cross-sectional Study	Fish Consumption	_	_	Food Consumption Survey and n3PUFAs in glanulocytes and adipose tissue measured	n3PUFAs were positively correlated with HRV.
Brouwer et al	2002	53	Healthy males and females	Cross-sectional Study	_	_	_	Cholesterol esters measured	DHA levels, and not EPA levels were correlated with HRV.
Hamaad et al	2006	38	Males and females with recent acute MIs	0.46g EPA, 0.38 DHA per day	Purified ethyl ester (OMACOR)	None (Open label)	3 months	None	n3PUFAs had no significant effect on HRV.

Effects on EKG Morphologies

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinant Outcomes
Geelen et al	2002	84	Healthy males and females, from 50 to 70 yrs old	0.7g EPA, 0.56g DHA, 0.26g other n3PUFAs per day	Fish oil	Sunflower seed oil	12 weeks	Serum cholesterol measured	No significant effect of fish oil was found on QTc, QRS duration, apex-to-end-T duration, T-loop morphology and spatial QRS-T angle.
Brouwer et al	2002	53	Healthy males and females	Cross-sectional Study	_	_	_	Cholesterol esters measured	Neither DHA nor EPA levels were associated with QTc.
Djousse et al	2005	3,642	Males and females free of MI, LV hypertrophy, pacemaker, and with QRS <120ms	Cross-sectional Study	_	_	_	None	QTrr and JTrr were significantly and inversely related to ALA intake.
Geelen et al	2005	74	Males and females at least 18 yrs olde with >1440 PVCs/day	0.7g EPA, 0.56g DHA, 0.26g other n3PUFAs per day	Fish oil	Sunflower seed oil	14 weeks	Serum cholesterol esters measured	QTc, T-loop width, spatial QRS-T angle and spatial U-wave amplitude were unchanged in both groups.
Chrysohoou et al.	2007	3,042	Healthy males and females from 18-89 yrs old	Cross-sectional Study	Fish Consumption			Food Consumption Survey	High consumption of fish (>300g/wk) was associated with a 13.6% reduction in QTc.
Mozzafarian et al	2006	5,096	Males and females at least 65 yrs old	Cross-sectional Study	Fish Consumption	_	_	Food Consumption Survey	Fish and n3PUFA intake and is associated with lower HR, longer PR interval, and lower likelihood of prolonged QT.

Prevention of Atrial Fibrillation

Author	Year	"n"	Patient Characteristics	Dosages	Form	Control	Length of Study/ Administration	Method of measuring/verifying n3PUFA intake	Pertinant Outcomes
Mozzafarian et al	2004	4,815	Males and females >65 yrs old	Longitudinal Study	_	_	12 years	Plasma phospholipid measured	Consumption of tuna or other boiled or baked fish, but not fried fish or fish sandwiches, was associated with a lower risk of atrial fibrillation.
Calo et al	2005	160	Males and females undergoing elective CABG	0.85g to 0.88g EPA+DHA in 1:2 ratio per day	Purified ethyl esters	None (Open label)	At least 5 days prior to CABG	None	n3PUFA administration reduced the incidence of post-operative AF, and shortened hospital stay.
Frost and Vestergaard	2005	47,949	Danish males and females from 50 to 64 yrs old	Longitudinal Study	_	_	5.7 years (mean follow-up)	Food Consumption Survey	Consumption of fish was not associated with the risk of atrial fibrillation nor atrial flutter.
Brouwer et al	2006	5,184	Males and females >55 yrs old	Longitudinal Study	_	_	6.4 years (mean follow up)	Food Consumption Survey	EPA and DHA intakes were not associated with risk of AF.

Animal studies may provide some clues as to underlying anti-arrhythmic mechanisms, and perhaps explain apparent discrepancies. A thorough meta-analysis performed by Matthan et al(46) in 2005 systematically reviewed the impact of ω3PUFAs administration on selected arrhythmia outcomes in animal models. Of the 27 relevant studies analyzed, ω3PUFAs were introduced orally in 23, and were infused intravenously in four. Interestingly, DHA and EPA were found to prevent ventricular tachycardia (VT) and ventricular fibrillation (VF) in ischemia-induced arrhythmias, but they had no effect on reperfusion-induced arrhythmias. The meta-analysis also reported no effect on the incidence of death and infarct size, and inconsistent results on 1) arrhythmia scores, 2) VF thresholds, 3) ventricular premature beats and 4) length of time in normal sinus rhythm. Finally, they also found that no significant conclusions could be drawn on the effect of ALA.

Specifically, animal studies performed by Billman and colleagues(47-49) indicate the impressive anti-arrhythmic effects of ω3PUFAs administered during ischemia. In these studies, dogs were surgically prepared with an anterior MI and implanted with an inflatable cuff around the left circumflex artery. After allowing the dogs to heal, VF could be induced reproducibly by inflating the cuff during exercise. Intravenous administration of fish oil concentrate immediately prior to inflating the cuff, however, protected 10 out of 13 dogs from developing VF. Similar results were found when the study was repeated with purified EPA (5 of 7 protected), purified DHA (6 of 8 protected) and purified ALA (6 of 8 protected).

In Vitro Cellular Studies: In Search of Potential Mechanisms

Cardiac Ion Channel Effects

Activities of cardiac ion channels are the basis of action potential initiation and propagation. Therefore, most anti-arrhythmic drugs are considered as either ion channel agonist or antagonists. In recent years, many studies using patch clamp techniques have sought to elucidate how ω3PUFAs affect the function of cardiac ion channels. A recent review by Xiao et al(50) summarizes the significant effects of ω3PUFAs on Na^{+}, Ca^{2+}, K^{+}, and other ion channels. Furthermore, ion pumps, such as the Na^{+}/Ca^{2+} exchanger, have also shown to be suppressed by ω3PUFAs(51, 52). Table 3.2 summarizes the concentrations of ω3PUFAs to induce 50% inhibition (IC_{50}) of several cardiac ion currents. The data indicate that among those affected ion channels, Ca^{2+} and Na^{+} currents are most sensitive to the ω3PUFAs. It is important to note that such a wide-spread effect on ion channels and ion pumps is not common for most anti-arrhythmic drugs.

	Rat cardiac Ca^{2+} current	Human cardiac Na^+ current	Rat cardiac I_{to} current	Rat cardiac I_K current	Canine cardiac Na/Ca exchanger
IC_{50} (μM)	2.1	3.9	7.5	20	0.82
$V_{1/2}$ (mV)	-3*	-22**	3		

Table 3.2 - The Effects of eicosapentaenoic acid or docosahexaenoic acid on cardiac ion channels. The data in each column represent the means of multiple cells (n > 7). Rat cardiac ion currents were recorded from isolated adult cardiomyocytes. Human cardiac Na^+ *currents were recorded from cultured human embryonic kidney cells (HEK293) transfected with human cardiac Na channel gene.* IC_{50}*, concentration to produce 50% inhibition of cardiac ion currents.* $\Delta V_{1/2}$*, difference of the values of the steady-state inactivation of ion channels at the point of 50% inactivation in the absence and presence of EPA or DHA.* *, $p < 0.05$; **, $p < 0.01$.

The voltage-gated Na^+ channel, which is primarily responsible for membrane excitation, plays an important role in arrhythmogenesis. Both EPA and DHA have been reported to shift the steady state inactivation curve of the voltage-gated Na^+ channel to the hyperpolarizing direction(53). In contrast, monounsaturated and saturated fatty acids at similar concentrations do not have any electrophysiological effects on these channels. The shift in the steady state

activation implies that the membrane potential must be more negative in the presence of ω3PUFAs in order to “reset” the voltage-gated Na^+ channels to an excitable state. This may explain why ω3PUFAs are effective in preventing ischemia-induced arrhythmias. During ischemia, the resting membrane potential of cells on the border of the ischemic zone slowly rises; this is considered to be due to lack of ATP to drive Na^+/K^+ pumps. Should one of these cells reach their AP threshold during a vulnerable point of the cardiac cycle, an arrhythmia could be induced. However, in the presence of ω3PUFAs, cells whose resting membrane potential has risen may not be able reset their voltage-gated sodium channels, rendering them inactive. Thus, during ischemia, the cells that often trigger arrhythmias cannot develop action potentials(54).

Action Potential Morphology

As ω3PUFAs significantly alter ion channel activity, the evidence from single cell electrophysiology experiments has likewise shown that ω3PUFA administration influences the morphologies of the cardiac action potentials (APs). For example, intracellular recordings in rat neonatal cardiomyocytes treated with EPA elicit: 1) more positive AP thresholds, 2) lower resting membrane potentials, 3) larger amounts of current needed to initiate APs, 4) slower phase 4 depolarizations, and 5) lower intrinsic beating frequencies(55, 56). Dietary fish oil has been shown to shorten AP durations and lower the plateau phase of APs in swine(52, 57). Recently, the first study in human cardiomyocytes showed that ω3PUFAs

likewise shortened the AP duration(58). The study showed similar findings in rabbit cardiomyocytes.

Multi-cellular Preparations and Micro-electrode Arrays

Basic research studies carried out at the ion channel and single cell levels have provided some insight into potential anti-arrhythmic mechanisms. As cardiomyocytes are mechanically, electrically, and chemically coupled to each other in heart, multicellular preparations are valuable to determine whether single-cell findings can be translated to animal or clinical studies. To further understand the electrophysiological basis of the anti-arrhythmic effects of ω3PUFAs in our own lab, we have recently explored the electrophysiological effect of free DHA on multi-cellular preparations of isolated murine cardiomyocytes using micro-electrode arrays (MEAs). The MEAs used in our lab (Multichannel Systems, Germany), are an 8 by 8 array of electrodes, each electrode having a 20 μm diameter and spaced 200 μm apart from the surrounding electrodes. Cardiomyocytes may be cultured directly on top of the array, allowing for the measurement of field potentials (FPs) (see figure 3.2). One advantage of using MEAs is that activity is recorded non-invasively in electrically coupled cells, as opposed to using relatively invasive measurements in a single disassociated cell, as is the case in standard patch-clamp techniques.

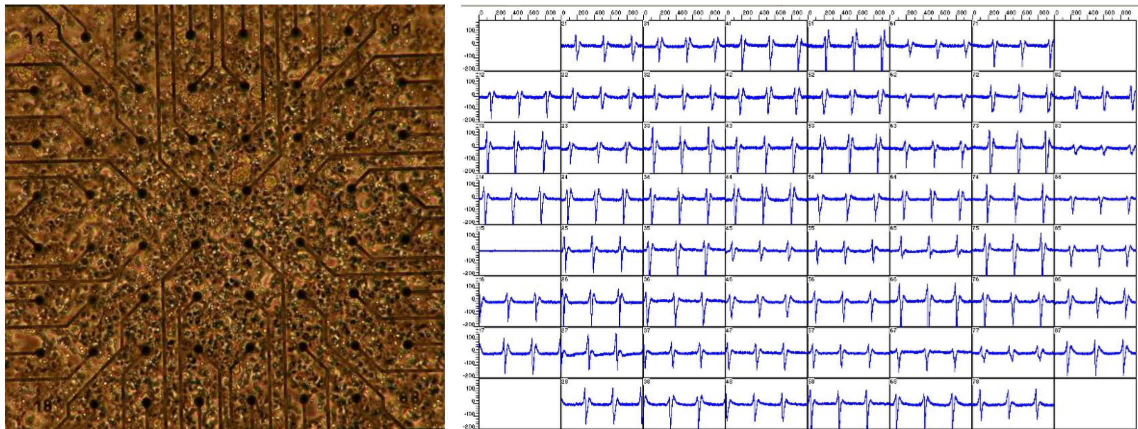


Figure 3.2 - The microelectrode array and recorded field potentials. On the left, light microscopy shows the monolayer of cardiomyocytes formed on top of the 8x8 microelectrode array. On the right, field potentials recorded from each electrode are shown (each window represents one second of electrical activity at a single electrode).

In one such study, HL-5 murine atrial cardiomyocytes were cultured for 5-7 days, after which they formed a monolayer and generated field potentials at a consistent rate. As shown in figure 3.3, the frequency of this rate lowers with exposure to increasing concentration of DHA; importantly, 100 μM DHA terminated all electrical activity in most cultures. Perfusion with a solution of 0.2% bovine serum albumin (BSA) was able to partially reverse the effects of the DHA, implying that DHA may be partitioning into the membrane under the influence of weak hydrophobic interactions.

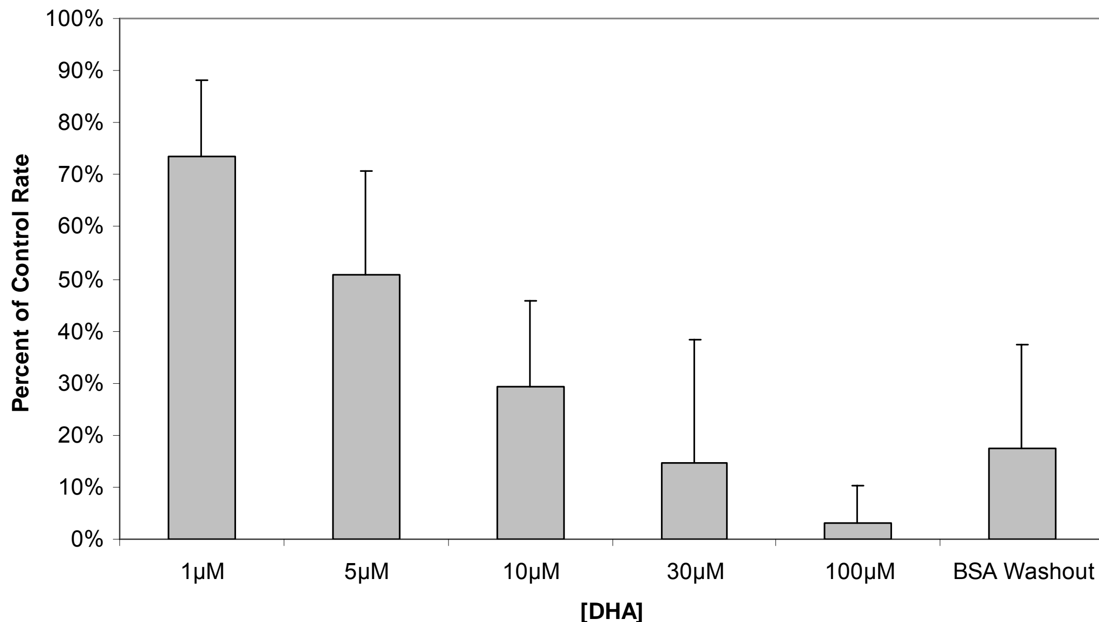


Figure 3.3 - Spontaneous field potential rate at various concentrations of DHA. Note the decrease in frequency with an increase in DHA concentration. 100µM DHA usually inhibited all activity. Bovine Serum Albumin (BSA) could be used for partial recovery. Error bars reflect the standard error.

Alterations of field potential waveforms were also observed with exposure to increasing DHA concentration. As seen in figure 3.4, waveforms appeared to lessen in amplitude, become wider, and have delayed repolarization phases. Upon quantitative analysis, the amplitudes of the waveforms were shown to decrease in a dose-dependant manner (data not shown). Study of the waveforms also showed that there was a slight widening, but due to high variability in the

morphologies no dose-dependence was determined. In addition, conduction velocities were measured and showed a slight decrease.

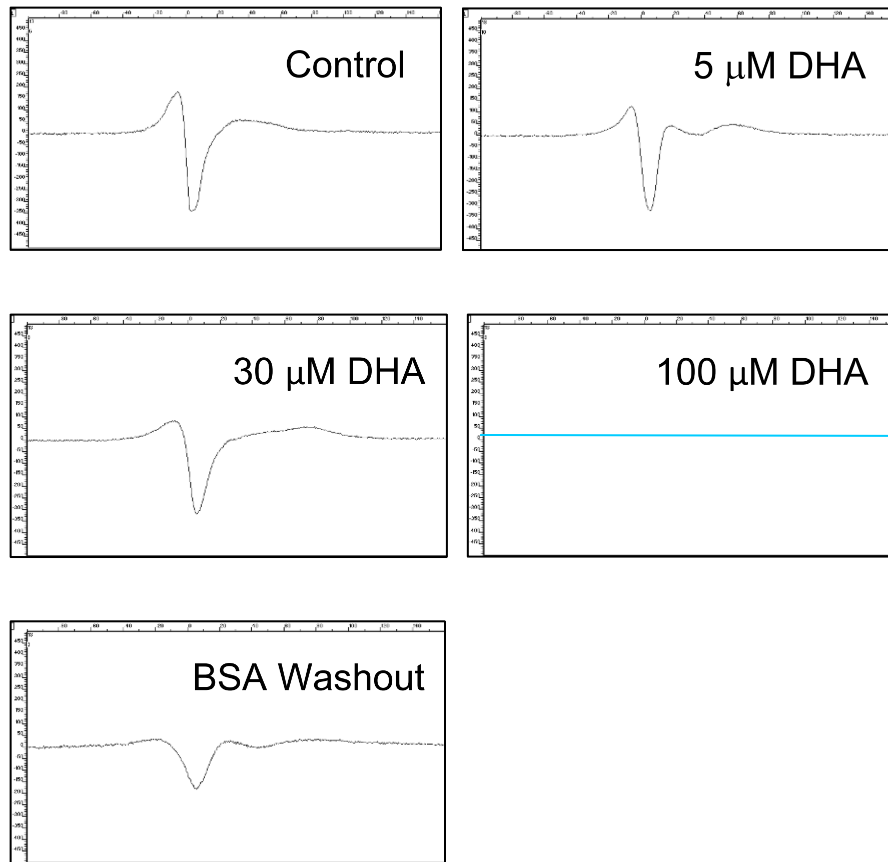


Figure 3.4 - Representative waveforms of the field potential are shown at various [DHA]. Note the decrease in amplitude, widening of the waveform, and delayed repolarization with increased [DHA].

The effects shown in these data may suggest that DHA could potentially

decrease heart rate *in vivo*. Lowering the heart rate may prevent tachycardia, increase coronary blood perfusion, and have other benefits in heart disease (e.g. reducing oxygen consumption and reserving energy). A wider field potential may also imply longer action potential durations, and this could be anti-arrhythmic. Complete inhibition of spontaneous activity at high concentrations could, however, be anti- or pro-arrhythmic. Therefore, defining an optimal dose of ω3PUFAs for therapeutic purposes is critical.

Current Hypotheses

To date, three main hypotheses attempt to explain why ω3PUFAs affect cardiac ion channels, and thus the electrophysiology of the heart (figure 3.5). First, those ion channels modified by ω3PUFAs may contain a specific binding site for ω3PUFAs that if occupied, can alter their function. This is supported by evidence from Xiao et al(59) who showed that a single point mutation in the alpha subunit of the Na^+ channel prevented EPA from inhibiting channel currents. This group also showed that co-expression of the alpha subunits with the beta subunits significantly altered the ability of ω3PUFAs to affect the channels(60).

The second hypothesis is that ω3PUFAs may be affecting membrane fluidity, and subsequently ion channel function. For example, Jahangiri et al(61) correlated the effect of ω3PUFAs on ion channels to changes in membrane fluidity by monitoring steady state fluorescence anisotropy. This hypothesis may also explain why ω3PUFAs have such wide-spread affects on membrane-dwelling channels and pumps. However, some argue that the observed effects on ion channels occur at much lower concentrations than those necessary to change membrane fluidity. Also, the lack of significant effects of ω3PUFAs on inward rectifier K^+ channels (I_{K1}) and human hyperpolarization-activated, cyclic nucleotide-gated (hHCN4) channels(50) is not along the line of this hypothesis.

The third main hypothesis considers that changes in the membrane immediately surrounding the channels might be responsible for the ion channel effects. The hydrophobic lengths of ion channels are often less than the

hydrophobic thicknesses of membrane phospholipids, so the membrane must "pinch" together around the ion channels. This compression causes tension on the channels, and affects their function(62). It is considered that ω3PUFAs may insert in the membrane around the channels, which in turn relieves some of the tension by reducing the curvature of the membrane. It has been shown that detergents which may relieve tension on ion channels induce similar effects to ω3PUFAs(63).

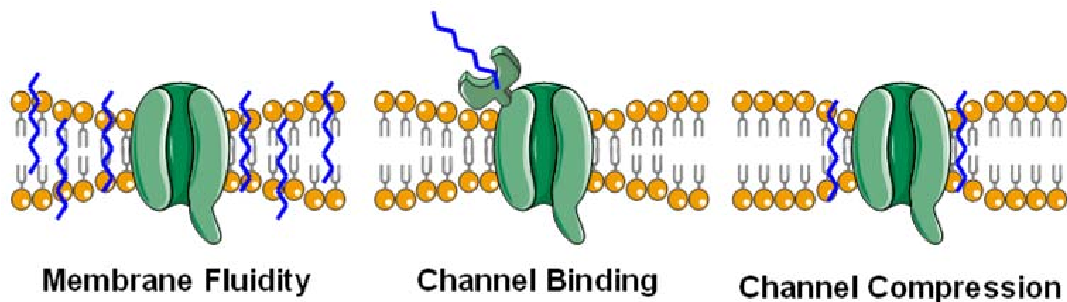


Figure 3.5 - Three hypotheses of how ω3PUFAs affect ion channel function. ω3PUFAs may directly effect the general membrane fluidity by influencing its composition (left), they may bind directly to the channel (center), or they may effect the compressive forces exerted on the channel by the membrane by allowing greater curvature near the channel.

Other Possible Mechanisms

Mechanisms beyond those mentioned here may also play a role in the cardio-protective effect of ω3PUFAs. For example, studies have shown that

ω3PUFAs can affect calcium regulation/handling. More specifically, the frequency of calcium sparks, spontaneous calcium waves, and delayed afterdepolarizations are reduced by ω3PUFAs (58, 64-66). In addition, it has been reported that DHA may lower the myocardial metabolic demand, thus protecting against ischemia(67). As mentioned before, ω3PUFAs may affect prostaglandin production due to competition of enzymes, which in turn may have several downstream effects. EPA may also inhibit apoptosis signaling during ischemia(68).

In studying all of these mechanisms, it is important to consider the kinetics of the measured effects. Acute studies may not capture long-term effects brought about by intracellular signaling processes, or gradual changes in membrane composition. These effects may prove to be just as clinically relevant as the effects seen during acute cellular or animal studies.

The Importance of Form

It is important to note that almost all of the cited *in vitro* studies used the free form of the ω3PUFA to study their anti-arrhythmic effects. However, in clinical and animal studies most ω3PUFAs are consumed and stored in the body in phospholipid and/or triglyceride form. Two important *in vitro* studies induced arrhythmias with isoproterenol in multi-cellular preparations and observed that only *free* ω3PUFAs, as opposed to other forms, could terminate the arrhythmias(55, 61). From animal studies, we learn that *ischemia* also seemed

to be necessary for one to observe anti-arrhythmic effects. The question then remains, what could be the correlation between ischemia, the free form of ω3PUFAs, and anti-arrhythmic effects? It is hypothesized that ω3PUFAs ingested by humans are transported through a series of processes to cardiac cell membranes and are stored in either triglyceride or phospholipid forms. During ischemia, phospholipase A_2 may release these fatty acids from the membrane in their free form(61). It has been shown experimentally that free fatty acid concentrations rise during ischemia(69). ω3PUFAs in their free form could then exert their beneficial effects. Those who ingest large amounts of ω3PUFAs may not only have higher concentrations of available free fatty acids, but may also have larger stores of fatty acids in cell membranes that can be released during an ischemic event(70-72) (figure 3.6).

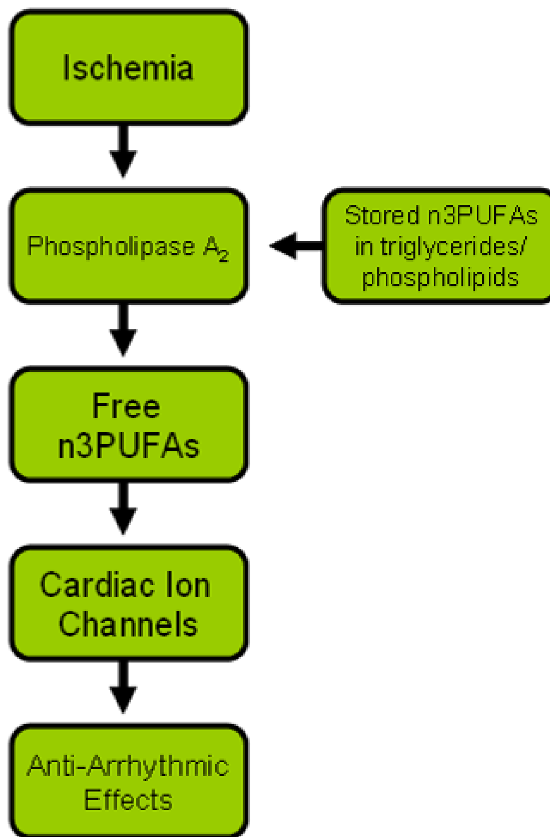


Figure 3.6 - A flow diagram of the proposed protective effect of ω3PUFAs during ischemia. During ischemia, phospholipase A_2 is activated and releases ω3PUFAs from their stored triglyceride or phospholipid form. This raises the concentration of free ω3PUFAs, which in turn exert their effects on cardiac ion channels. The effected ion channels produce anti-arrhythmic effects.

The distribution, interconversion, and resulting forms of ω3PUFAs are very complex, with interplay between environment, age, certain disorders, and/or the presence of competing fatty acids(71). If the presence of free DHA in the heart

tissue is the best indicator of arrhythmia protection as *in vitro* studies would suggest, then it may be hard to correlate the presence of other forms of ω3PUFAs in the plasma with anti-arrhythmic effects. As seen in table 3.1, clinical trials thus far have each tried to correlate anti-arrhythmic effects with levels of ω3PUFAs in red blood cell phospholipids, plasma phospholipids, platelet phospholipids, and serum cholesterol esters. Yet, these indirect measurements may not represent well the free fatty acid concentrations in the heart. A recent study has explored the correlation between cardiac tissue, red blood cell (RBC), and plasma levels of ω3PUFAs and proposes that an “omega-3 index” derived from RBC levels should be used to infer cardiac tissue levels(73). It is obviously challenging to measure free fatty acid concentration in heart tissue, so the development of a standard biomarker may explain some of the disparity in reported animal and clinical studies.

In order to have more of the free form available prior to or during an arrhythmia for therapeutic purposes, consumption of ω3PUFAs can be increased, as many of the clinical studies have shown. Other approaches may include manipulating the body’s own mechanisms to release stored phospholipid ω3PUFAs, or even delivering free ω3PUFAs directly to the heart tissue. In a recent study performed in our lab, we delivered free DHA into the pericardial sac surrounding the heart of swine, so as to bathe the heart tissue in free DHA. Arrhythmias were then induced by occluding the left anterior descending artery for 45 minutes, and after allowing reperfusion of the artery for 3 hours. We found

that the DHA treatment, as compared to saline-treated controls, reduced the incidence of VF and lowered both arrhythmia scores and infarct sizes by almost 50% (74). Further work remains to compare these results to treatments with other forms of DHA and other ω3PUFAs.

Conclusions and Recommendations

The administration or increased consumption of omega-3 polyunsaturated fatty acids may fill a large, unmet clinical need for a safe, effective anti-arrhythmic therapy. Clinical trials have shown, for example, reductions of up to 44% in the incidence of sudden cardiac death among those that take fish oil supplements(16). Such a reduction by a pharmacologic intervention could be considered unprecedented. Future studies will continue to advance our understanding the mechanism(s) of the anti-arrhythmic effects of ω3PUFAs. To date, the following two questions, in our opinion, lack sufficient evidence to be answered confidently: Which form(s) (phospholipid, triglyceride, or free) of ω3PUFA is(are) responsible for these positive anti-arrhythmic effects? And, how can the appropriate form(s) be readily available to myocardial tissue to prevent or treat arrhythmias? Answering these questions would further enable the use of ω3PUFAs clinically, and move toward preventing and treating one of the largest causes of morbidity and mortality worldwide.

Section 2: Preface

Evaluating and identifying appropriate clinical settings

After gaining a foundational knowledge of cardiac electrophysiology, I next determined to select certain clinical settings where intrapericardial delivery of anti-arrhythmic held promise. To do so, I initially consulted with several individuals who provided me with valuable insights. In the engineering terminology of product development, I performed several "VOC" or "Voice of the Customer" interviews. The following is a list of several clinicians, scientist and engineers with whom I have consulted with throughout my PhD project:

Lisa Shafer, PhD (Nueromodulation, Medtronic)

Greg Stewart, PhD (Nueromodulation, Medtronic)

Daniel Sigg, MD, PhD (CRDM, Medtronic)

Mike Ujlyhe, PharmD (CRDM, Medtronic)

James Coles, PhD (CRDM, Medtronic)

David Benditt, MD (Dept. of Medicine, U of M)

Kenneth Liao, MD (Dept. of Surgery, U of M)

William Elmquist, PharmD, PhD (Dept. of Pharmaceutics, U of M)

Bin He, PhD (Dept. of Biomedical Engineering, U of M)

Ernesto Molina, MD, PhD (Dept. of Surgery, U of M)

Bryan Whitson, MD, PhD (Dept. of Surgery, U of M)

Angela Craig, DVM (Research Animal Resources, U of M)

Bob Patterson, PhD (Dept. of Biomedical Engineering, U of M)

Tom Bischoff (Neuromodulation, Medtronic)

Paul Iaizzo, PhD (Dept. of Surgery, U of M)

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I am deeply grateful to these individuals for their input and guidance. With their help, I was able to target three clinical settings. The three settings are the following:

Docosahexaenoic Acid (DHA, an omega-3 fatty acid,) to treat ischemia/reperfusion–induced ventricular arrhythmias. As reviewed in section 1, DHA may have a potent anti-arrhythmic effect in its free form. When taken orally or intravenously, however, DHA collects in the cardiac tissue as a phospholipid

or triglyceride. Thus, by delivering free DHA into the pericardium, one may maximize its effects. Additionally, it is a good candidate to treat ventricular arrhythmias due to its high lipid solubility and ability to penetrate the ventricular wall.

Metoprolol to treat sinus tachycardia. Metoprolol administration in humans has the potential unwanted side-effects of both hypotension and bronchoconstriction; nevertheless, it is often used in post-operative settings to treat various tachycardias. By delivering metoprolol directly into the pericardial space, higher concentrations of this drug could collect in superficial structures, such as the SA node, providing potent rate-lowering effects. In contrast, only low concentrations would be present in the ventricular myocardium or in the lungs, thus minimizing hypotension and/or bronchoconstriction.

Amiodarone to treat atrial fibrillation. Amiodarone administration in humans also has many potential side-effects including pulmonary toxicities and hypotension. Furthermore, the pharmacokinetics of amiodarone make it hard to obtain high cardiac tissue concentrations rapidly. Therefore, intrapericardial delivery of amiodarone during atrial fibrillation, particularly in the post-operative setting, could potentially both maximize this drug's effectiveness and limit some side-effects.

Animal Models of Arrhythmias

Developing appropriate and reproducible animal models of cardiac arrhythmias was the next step so to allow the subsequent study of the relative effectiveness

of our proposed therapies. We had previously developed a model of ischemia/reperfusion-induced ventricular arrhythmias which involved clamping the left anterior descending artery. This induces serious ventricular tachycardias, and in untreated animals, the incidence of fatal ventricular fibrillation was as high as 38%.

As an appropriate model of sinus tachycardia, we considered a hemorrhagic model but the variability and risks involved were too high. Next, we attempted a slow infusion of epinephrine in a swine model, but the systemic pressures were excessive. Subsequently, we identified that a continuous infusion of isoproterenol, a beta-agonist, was the easiest way to induce a sustained tachycardia in these animals: we were able to induce and hold their relative heart rates above 180bpm for extended periods of time (i.e., hours).

When we began our work, it was well-known that a reliable model of atrial fibrillation was still a challenge. However, after trying many stimulus protocols, we found that a 2-second burst pace at 50 Hz of the left atrial appendage, induced reproducible bouts of AF for several seconds. If the episode lasted longer than one minute, we called it a “sustained episode” of AF. If three of these 2-second bursts were performed, about half of the animals would have at least one sustained episode. In an effort to obtain a more reliable model of AF, we studied retrospectively the number of sustained episodes induced by burst pacing in 19 swine. Within this cohort of animals, two main parameters were varied: animal weight and the administered general anesthesia. Interestingly, we

did not find any strong correlation of AF inducibility with either parameter. Therefore, we settled on using our simple burst-pacing protocol to measure relative AF inducibility. To supplement this study protocol, we performed complete electrophysiologic assessments on these animals so to measure atrial, ventricular, and AV nodal refractory periods. These experimental methods are explained in greater detail in the third section of this work.

Chapter 4 of section two of my thesis contains a review of large animal models in cardiac electrophysiology. This was written in collaboration with Jason Quill, Michael Eggen, and Paul Iaizzo as another book chapter in the Cardiac Electrophysiology Methods and Models. The compendium of information described in this chapter includes our experiences in developing and using large animal models for applied cardiac electrophysiological investigations. In other words, these experimental methods, learned over the course of several years, were also the foundation for the animal studies of section three of my thesis.

Analytical chemistry techniques

In general, the primary benefit of drug administration via pericardial delivery is in the fundamental pharmacokinetics (PK) of delivery. Thus within our experimental designs, in addition to showing the electrophysiological benefits, we also wanted to show the PK advantages. However, his would require us to measure drug contents in plasma, pericardial fluid, and/or target tissues of interest. In collaboration with several other students and our in house laboratory scientist/biochemist, Bill Gallagher, we developed HPLC protocols to measure

metoprolol concentrations. This was a very tedious and long process, especially in calibrating and validating our measurements. Michael Woo, PhD, a current medical student who has been performing research in our laboratory played an integral role in carrying out these experiments. In addition, HPLC protocols were also developed for the measurement of Amiodarone and Esmolol. Finally, GC protocols were developed for the measurement of omega-3 fatty acids. By developing these procedures in-house it also allowed us to cut quite a bit of cost and time.

Development of access methods to the pericardium

A review of currently used pericardial access methods was presented in chapter one of section one. We decided to test each of these described methods in large swine so to assess their relative safety and ease of use. Our results for each method are briefly described as follows:

The transatrial approach seemed to be straightforward, but we often found it difficult to know when we had entered the pericardial space. Additionally, in several trials we pierced through the pericardium and our catheter was directed into the lung. Our attempts in these swine were through the external jugular vein and onward into the superior vena cava, so anatomically perhaps a femoral approach might have made it easier.

A subxyphoid puncture, similar to what is done during pericardiocentesis proved to be very difficult in a healthy swine; it should be note that the volume of pericardial fluid in a swine is much less than in a human. The long needle used

in this procedure often punctured into the myocardium, and this in itself caused arrhythmias. We concluded that without any pericardial infusion, this procedure is close to impossible in swine with current technologies.

A subxyphoid surgical approach worked well, but a part of the xyphoid often had to be removed in order to get good visualization of the pericardium. Nevertheless, the use of a large incision exposed the pericardium and we were able to introduce a catheter into the pericardial space with little effect on the heart.

After trying existing access methods, we became convinced that better tools and methods could be developed to access the pericardium. Subsequently, we applied for a grant from the Medical Device Center at the University of Minnesota and we received money to pursue development of such pericardial access devices. Importantly, the support from this grant led to the design and implementation of a post-operative delivery catheter. This catheter is placed within the pericardial space just before closing the chest. *Chapter 5* of the second section of my thesis contains an abstract and a poster presented at the 2009 Design of Medical Devices Conference. It should be noted that this abstract was selected for oral presentation, and subsequently won the "3-in-5" competition as one of the three top presentations. We have disclosed our design to the University of Minnesota Office of Technology Commercialization, and development of this device and other pericardial access devices are currently

ongoing in our laboratory.

In summary, the work of this second section of my thesis includes: 1) “expert” advice in choosing clinical settings for pericardial delivery, 2) the results of developing various animal models for appropriate clinical settings; 3) descriptions as to analytical chemistry methods that were developed to perform pharmacokinetic analyses, and 4) examples of developed prototype devices for the access of the pericardial space in various clinical settings.

Chapter 4: The Use of Large Animal Models for Cardiac Electrophysiology Studies

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Abstract

Large animal models can be utilized to recreate many cardiac electrophysiological diseases states and procedures, in addition to being used to test new devices and imaging techniques. In this chapter, a brief summary of regulatory principles regarding animal models is presented. Factors for choosing an animal model, such as growth rates, ease of handling, and comparative cardiac anatomy are presented, with the cardiac anatomy presented in terms of common electrophysiologic procedures: lead placement, His pacing, and ablation studies. General anesthesia information is then provided, along with common methods of accessing the heart and invasive monitoring techniques. Finally, common electrophysiologic interventions are discussed. This includes different techniques for creating common pathologies, such as: congestive heart failure models, acute and chronic atrial fibrillation models, ventricular fibrillation, and myocardial infarction (ischemia).

Introduction

Animal experimentation has resulted in numerous discoveries including: the need for insulin, the creation of vaccines for polio and rabies, skin grafts for burn victims, and CT scanning technology. Furthermore, both every drug and any invasive medical device in use today, has undergone some form of animal testing prior to human clinical studies and/or medical usage. Importantly, these advances in medical technologies have not only benefited countless patients, but veterinary science as well. More specifically, it has been reported that dogs can now receive heart valves and hip replacements thanks to surgical research, vaccines to benefit pets have been developed, and heart disease treatments for pets have all resulted from medical research through animal experimentation.[1]

Currently, research on large animals primarily exists at Universities in the regulatory environment described by the Animal Welfare Act of 1966 and the Health Research Extension Act of 1985. The Public Health Service Policy on Humane Care and Use of Laboratory Animals published through the Office of Laboratory Animal Welfare in 2002 supplements the Health Research Extension Act. In brief, the goals of these policies are to require:

Procedures to be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society

The selection of appropriate species and the minimum number of animals to be

used to achieve valid results (including using alternate forms of testing, such as in vitro testing and computer simulations).

The avoidance or minimization of animal discomfort, distress, and pain when consistent with sound scientific practices.

The appropriate use of sedation, analgesia, or anesthesia during procedures that involve more than momentary or slight pain to the animal. Acute studies should terminate with a painless ending of the animal's life.

Appropriate living conditions and care for the animals and for investigators to be properly trained.

Prior to animal experimentation, the study protocol must be approved by an independent body that ensures investigators comply with the above goals. If the study is found to comply, all animals are kept in facilities overseen by a veterinarian and provided adequate food, housing, and medical care.

With these regulations in place, every effort is made to maximize health advancements while minimizing the number of animal experiments performed and the distress experienced during the protocol. Since there are not computer simulations and/or adequate in vitro tests that are yet capable of mimicking the system responses of the human body, animal testing is not only necessary, but the best way to screen potential therapies and/or devices prior to human use.

Choosing the Right Animal Model

There are many factors that go into the careful decision of selecting the proper/ correct animal model for pharmacological or device investigations, including: (1) the species (and breed), (2) size of the animal (or target organ of interest), and (3) whether or not the study is to be acute or chronic. The three most common large animal models for cardiac investigations are the dog, pig, and sheep. Thus, this section will focus and attempt to compare these three models to human anatomy, so to aid in the choice of which species is appropriate for the type of proposed study. Nevertheless, both the relative expertise and experiences of the given investigator with the chosen animal model are important factors in the ultimate success of the planned experiments. Additionally, an experienced investigator may be able to recall a previous study in which data from control animals could be used (i.e., historic data, thereby reducing the number of newly required experiments.

Rate of growth

Pigs, dogs, and sheep all can be appropriate models for use in acute studies, but the study design for chronic studies must accommodate the natural growth of the animal when interpreting results. Dogs reach mature size by approximately one year of age. Sheep are also relatively slow growing and reach a standard mature weight with little growth beyond that size. Their final size is highly heritable and thus breed specific; but can also be nutrition dependent. The large rate of growth of swine makes the results of chronic

studies with these animals more difficult to interpret; e.g.,the heart continues to enlarge with the size of the animal (within the age range of most swine during cardiac studies). There are mini-pigs available that have a much lower growth rates2, and are available for chronic studies.

In studies where a specific heart size is required, estimates have been done for each species comparing the excised heart weight to body weight. In general, the heart weight to body weight ratio for adult dogs, pigs, and sheep are 7 g/kg, 2.5 g/kg, and 3 g/kg, respectively3. For comparison, the heart weight to body weight ratio of humans is 5 g/kg. It should be noted that for the pig model, a more realistic heart weight to body weight ratio may be obtained by using younger animals (25-30 kgs, several months old)4. However, these numbers are variable based upon breed of the animal or lifestyle of the population, so they should be used in the context of an animal experiment only as a rule of thumb for ordering animals.

Arrythmogenicity

There have not been any detailed studies comparing the relative onsets of arrhythmias in canine, swine, and sheep. Nevertheless, many investigators have noticed a qualitative order to the sensitivity with which the animal develops arrhythmias. In general, dogs are thought to be relatively insensitive to arrhythmias, followed by sheep, and ending with pigs. Of interest, one highly experienced animal researcher (Dick Bianco, Experimental Surgical Services, University of Minnesota, at the Bakken Surgical Symposium, 2008) was

discussing the benefits of each animal model, and stated that pigs would "fibrillate if you looked at them funny."

Comparative Anatomy

Detailed descriptions regarding comparative cardiac anatomies between canine, sheep, swine, and humans has become recently available[5]. In this section of this chapter, we will describe the major differences in cardiac anatomies between animals and humans, but will describe the anatomy in the context of how it most likely affects studies in cardiac electrophysiology, such as lead placement and ablation studies.

It should be emphasized that all of the animal models discussed in this chapter have cardiac anatomy that are generally similar to that of humans. All hearts contain four chambers, with two atrio-ventricular valves and two semilunar valves and the same pathways of blood blow. The systemic blood enters the right atrium through the superior and inferior vena cava, while the coronary venous system returns blood into the right atrium through the coronary sinus. The blood then flows through the tricuspid valve during diastole to the right ventricle, where it is pumped through the pulmonary valve during systole and into the pulmonary artery and the lungs. Upon oxygenation, the blood returns via the pulmonary veins to the left atrium. During diastole, the blood passes through the mitral valve and into the left ventricle. Finally, the left ventricle pumps the blood into the systemic arteries through the aortic valve. The coronary arteries are fed through backflow in the ascending aorta during diastole that enters the coronary

system via the left and right coronary ostia, located within the sinus of Valsalva. The right and left heart pumps work in series in a coordinated fashion: in a healthy heart, the right and left atria contract in concert and subsequently so do the ventricles.

One of the most important things to recognize when choosing an animal model is that there are differences between such, and the choice of an incorrect model can produce misleading results. An often stated example of incorrect model choice comes from pharmaceutical studies using an infarction model in dogs during the 1970's. These protocols intended to create infarcts in sub-selected regions of the coronary arteries. However, canines have a high degree of collateralization within their coronary arteries compared with humans. Therefore, in these trials, the dog myocardium was still being fed in areas thought to be ischemic because of these collateralizations, and it was reported that various drugs were effective at reducing damage caused by myocardial ischemia. Unfortunately, these drugs then failed in trials of human patients because the animal study data was not transferable[6]. It is considered that this could have been avoided if an animal model was chosen, such as pigs or sheep that had a similar degree of collateralization as humans and produced consistent infarcts (not just myocardial stunning) as the protocol required.

The remainder of this section will describe some common devices and therapies that are used in animal models, and describe how the structural anatomy of the differing species can and will influence ones ultimate

experimental design.

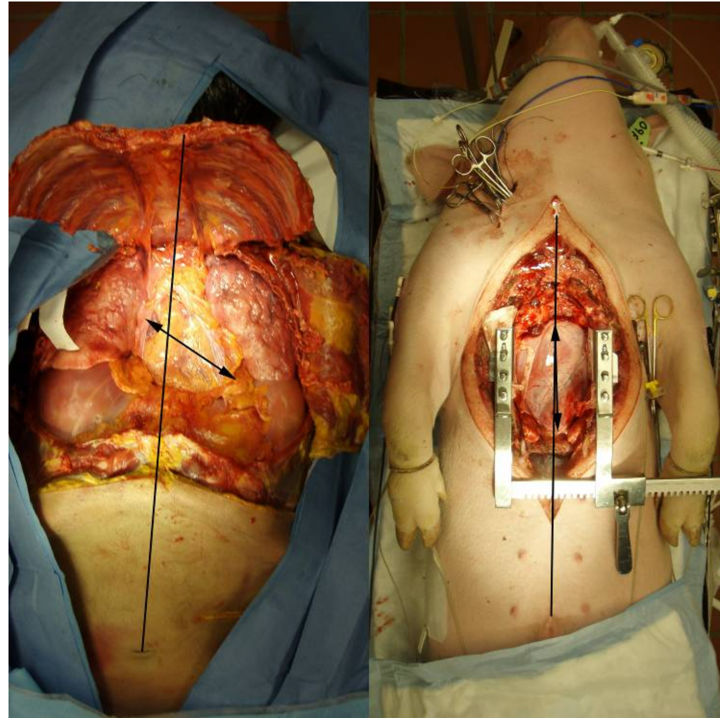

Figure 4.1 - Position of the heart within the thorax.

The long axis of the heart in humans (left) is at approximately a 60 degree angle with the midline of the thorax, whereas quadrupeds, such as the pig (right), have a long axis of the heart more in line with the midline of the thorax.

Lead Placement

The relative orientation of the heart within the thorax in quadrupeds differs from that of humans[7]. In humans, the right ventricle rests upon the diaphragm, with the long axis of the heart at approximately a 60 degree angle from the sternum, in such a manner that one third of the heart is in the right half of the

thorax and two thirds of the heart is in the left half of the thorax. In contract, the long axis of the heart in quadrupeds is much more in line with the sternum (Fig. 4.1). Thus, the relative orientation of a given heart within the thorax will ultimately affect the entrance angles of the superior vena cava into the right atrium. In humans, the superior and inferior vena cava are essentially in line with each other, while in pigs, sheep and dogs, they enter the atrium at nearly right angles. These differences become even more obvious and important when viewing a lead placement in a given heart location via fluoroscopy or any other imaging modalities. For example, a physician/scientist may at first, have difficulties adjusting to these anatomical differences when placing leads in various animal models.

When a research protocol calls for left-sided lead implantation, anatomical variabilities of the the Thebesian valve, coronary venous valves, and left azygous veins can all complicate such deliveries. More specifically, the Thebesian valve covers the coronary sinus ostium and helps prevent backflow from the right atrium into the coronary sinus. In humans, this valve was found to be present in a variety of morphologies: remnant, non-obstructing valves, and prominent valves covering more than 50% of the ostium[8]. In hearts with prominent Thebesian valves (Fig 4.2), it has been shown using an isolated heart apparatus that coronary sinus access can be obstructed. While comparative anatomy studies of the Thebesian valve in animal models were not found, observation of swine hearts in isolated heart preparations in our laboratory, have shown that

prominent Thebesian valves are extremely rare and in most cases, non-existant. When testing devices requiring coronary sinus access in animal models, it should be noted that access may be more difficult in humans due to the Thebesian valve. The ostium of the dog is much smaller than those of the other animal models hence it would be most appropriate to use if one wants to increase the difficulty of assessing the coronary sinus by various means (e.g., studies in which on is trying to optimize catheter designs).

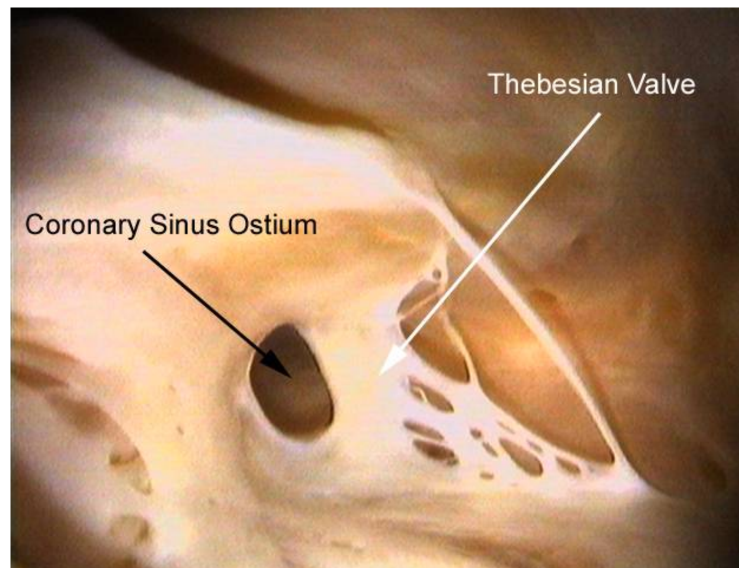

Figure 4.2 - Thebesian Valve

A thebesian valve covers the coronary sinus ostium within the right atrium of a human

Although the relative access to the coronary sinuses in pigs and sheep is usually not impaired by the presences of Thebesian valves, the left azygous veins in each species drains into their coronary sinuses near the ostium[7], and thus this

will often complicate left-sided lead implantations.

Left-sided pacing is usually accomplished by positioning a lead within a venous branch which feeds into one of the larger main coronary veins: the posterior interventricular vein, the posterior vein of the left ventricle, the left marginal vein, or the anterior interventricular vein. It should be noted that the sub-selection process of implanting a lead can be helped or hindered by the presence of cardiac veins within the venous system. More specifically, intra-luminal ridges and valves (Fig 4.3) have been observed using fiberscopes (1.5 or 2.5 mm diameters) within the major cardiac veins of perfusion-fixed human hearts[9]. Therefore, during a left-sided lead implantation, the location of these ridges and valves can angle a guidewire into a branch or hinder the sub-selection process by blocking access[10]. No published comparative anatomy studies have been found on cardiac venous valve anatomy, but qualitative observations by our group of swine hearts, have indicated that these valves to be less prevalent than in humans.

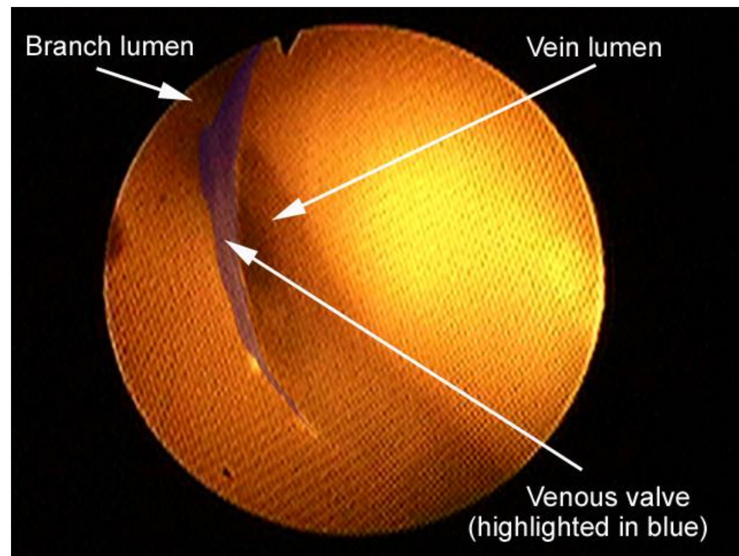

Figure 4.3 - Valve in the coronary venous system

A valve (blue) within the human coronary venous system is shown in between the vein lumen (right) and the branch lumen (left)

His bundle pacing

All of the major large mammalian animal models have similar conductive pathways as humans, with the presence of an SA node, AV node, bundle of His, left and right bundle branches, and purkinje cells. Yet, there are subtle differences between species in all portions of the conduction system; we will discuss a few of the more notable ones here. For example, sheep have an os cordis, which is a bone at the base of the heart near the central fibrous body. This bone blocks the usual route of the His bundle to the crest of the ventricular septum, instead passing underneath the os cordis into the right ventricular septum before branching[11]. Pigs and dogs do not have an os cordis, and neither

do humans, with the His bundle located just beneath the membranous septum at the crest of the interventricular septum[11].

Additionally, the cells present in the His bundle and bundle branches of sheep differ from that of humans. In sheep, the Purkinje cells are most prevalent, whereas in human His bundles and bundle branches, they are composed of mainly transitional cells. Additionally, human hearts elicit Purkinje cells throughout the pathways to the subendocardium. In contrast, sheep have comparatively more prominent cells that are present throughout the mural myocardium of the right and left ventricles[12]. As such, these differences in conduction pathways and cellular makeup should be noted for researchers attempting to pace from this site.

Ablation Studies

There are numerous published reports describing ablation studies in large mammalian models, hence this section is by no means comprehensive. Yet, listed here are some important anatomical differences between the various animal models and that of humans which should be kept in mind for any type of ablation study.

If a trans-septal procedure is to be performed as part of the procedure, then the fossa ovalis is usually punctured via a catheter delivered from the inferior vena cava. As noted above, in quadripeds, the inferior and superior vena cava enter the right atrium at right angles to each other: which differs from that of humans where the entrances of the vena cava are in-line, but with flows in opposite

directions[7]. Yet, while the entrance angles of the vena cava may differ slightly, the relative locations of the fossa to the inferior vena cava in quadripeds is similar to human anatomy, and thus should have little effect on trans-septal punctures.

It should also be noted that in humans, there exists an interatrial groove for communication of electrical signals between the right and left atrium, named Bachman's bundle. Bachman's bundle has been claimed to not exist in sheep[11], but more recent studies have claimed to pace at Bachman's bundle as part of their methodology[13]. This apparent discrepancy between anatomical literature and electrophysiology studies is quite interesting, but is noted without further comment. Nevertheless, Bachman's bundles have been reportedly found in both pig and canine hearts[11].

Pulmonary vein isolation via ablation has been the focus of multiple papers as sites to eliminate atrial fibrillation. Humans typically have four pulmonary vein ostia, whereas pigs typically have two[7], and dogs have five to six[14]. In addition, it has been noted that there is a more prominent myocyte border between the pulmonary veins and atria in the swine heart: in other words there is no migration of myocytes into the lumen of the pulmonary veins in the swine heart.

Finally, an alternative ablation strategy is to use fractionation analysis and to ablate at the site of dominant frequency. This technique is mentioned because it provides an excellent example of the value of animal models. These sites of dominant frequencies were located and electrograms were simultaneously

recorded with optical high-speed imaging within isolated sheep hearts: the employed clear perfusate allowed imaging within these hearts, and the electrical signals were correlated to regions with fast vortex-like re-entry around miniscule cores[15]. This novel approach allowed the electrical signals to be compared with mechanical function of the atrial tissues through the use of a well-thought out experiment in an animal model, helping to validate this technique being used on human patients.

Anesthetics and Monitoring

The choice of anesthetic(s) to be employed in a given study protocol will depend on several factors, including: (1) the nature of the planned intervention, including the amount of pain or distress involved, (2) if the study is acute or chronic, (3) the known effect of the agents on suppressing hemodynamic function, and/or (4) if electrophysiology measurements will be taken during the intervention. It should be noted that general guidelines for the choice of anesthesia can be found on AWIC website, under the section entitled “Anesthesia.” Nevertheless, the use of profound anesthesia will typically require intubation and ventilation of the animal, and therefore a proper ventilator will be needed. If the agent to be used is a volatile anesthetic, such as Isofluorane, a vaporizer will also be required.

At the beginning of a typical acute animal study, the conscience animal is sedated with an induction agent (e.g. Telazol, Thiopental Sodium), which then allows the investigator to gain intravenous access to provide supplemental agents if needed and then to subsequently intubate the animal. Typically, at this

point the animal is moved to the operating table, where mechanical ventilation and anesthesia is initiated and levels monitored. Nevertheless, all animals should be properly secured to the operating table to prevent movement or falls from the table, if anesthesia levels become too low. This is usually done by using straps or ropes. Yet, care should be taken so that the restraints do not prevent proper limb circulation or alter respiration, especially if it is planned that the animal will be recovering from the intervention.

During anesthesia, all animals should be closely monitored. At a minimum, a 3-lead ECG should be continuously monitored and blood pressure should be assessed often (e.g., either a non-invasive cuff or a direct arterial line can be used to do so). An excessively high heart rate, high blood pressure, and movement of the animal can be signs that the anesthesia is too low (the animal is “light”). Conversely, a low pressure and a low heart rate may be indicators that the animal is receiving too much anesthesia. Typically, high-end anesthesia machines provide on-line inhaled and exhaled anesthetic levels, which can be assessed to ensure the animal is within an appropriate range of anesthesia. Given that many anesthetics can cause arrhythmias, the ECG should be monitored to detect dangerous arrhythmias. It should be noted that swine a particularly susceptible to rhythm abnormalities. Yet, this characteristic can be utilized to develop models to study various treatment regimes. Note that our laboratory always has a defibrillator available in case arrhythmias or fibrillation develops.

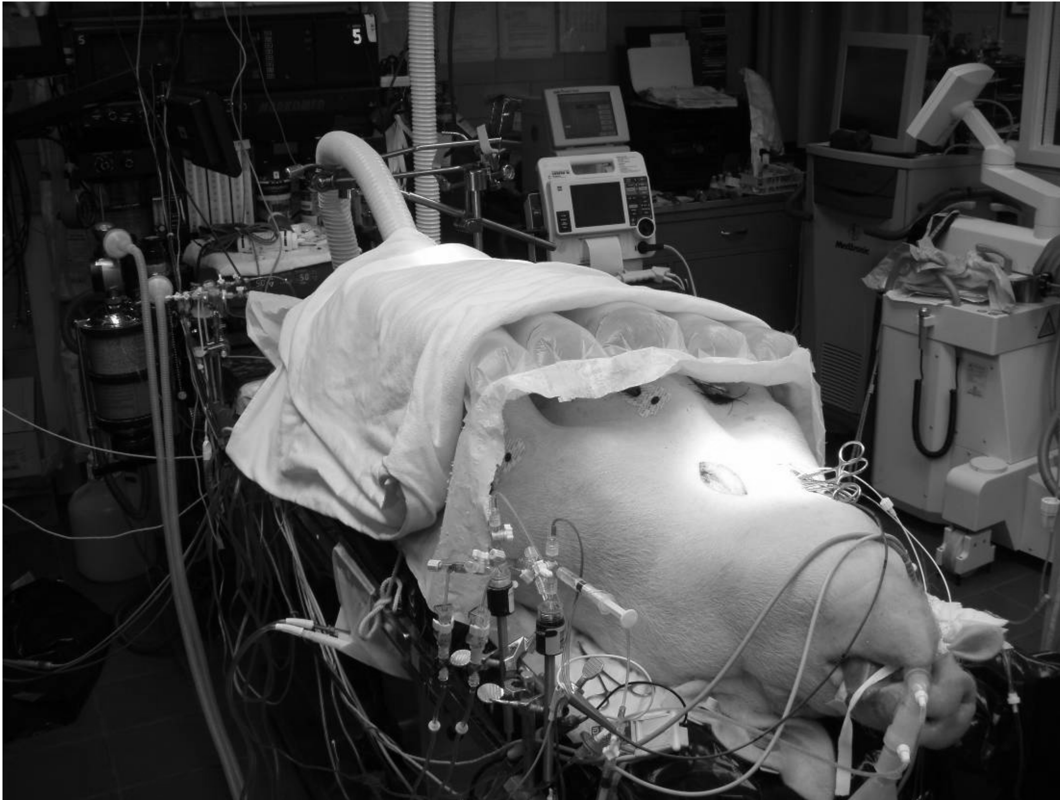

Figure 4.4 Surgical preparation of a swine

A 90kg swine has been properly sedated and intubated. The animal is under general anesthesia (Isofluorane) and secured to the operating table using chords. A commercial warming system (The Bair Hugger, Arizant Medical) is placed over the abdomen covered with a cotton blanket.

Under general anesthesia, both humans and animals have impaired abilities to properly regulate their core temperatures; therefore while under general anesthesia, it is important to both constantly measure core temperatures and provide proper thermal management. In most large animal studies, core

temperatures are monitored via a temperature probe placed in the rectum. However, in those investigational studies in which a Swan-Ganz catheter is employed, this system will provide continuous pulmonary artery temperatures; of which our laboratory considers the “gold standard” measurement of core temperature. Nonetheless, active warming or cooling may be necessary to maintain the animal within an appropriate range for a given research protocol. For example, swine have a normal core temperature of 38 º C and our group always attempts to control core temperature within 0.5 º C. For a simplistic approach to provide therapy, blankets around the abdomen and the chest can help raise temperature, whereas wet cloths (water or rubbing alcohol) allowing for evaporative cooling placed in the same locations can actively lower the temperature (the use of fans can also expedite convective losses). Additionally, various heating (Fig 4.4) and cooling systems are available commercially. It has been our experience that electrophysiological parameters of the heart are extremely sensitive to temperature, particularly refractory periods and the induction of atrial fibrillation. Furthermore, mild hypothermia, even several degrees C, is considered to confer some degree of cardio-protection against ischemic damage.

Invasive Monitoring

If more sophisticated hemodynamic monitoring is desired to accomplish the purposes of a given study protocol, appropriate venous and arterial accesses will be necessary to introduce appropriate catheters into the heart for sensing.

Access to large vessels, such as the femoral arteries, the carotid arteries, the femoral veins or the jugular veins, can be done 1) with a blind stick, 2) under ultrasound guidance, or 3) after a direct surgical cut-down. In most, acute investigational studies on large animal models, cut-downs, or incisions of the skin and exposures of the vessel walls, are commonly performed. Next, the vessels are then accessed using the Seldinger technique with introducers; such sheaths typically have hemostasis valves which allows instruments to be passed in and out of the vessels without the loss of blood. In acute surgeries, where a given catheter will be placed in the vessel but will not be moved during the intervention, an introducer is not necessary. In such cases, a given vessel is isolated and then nicked and the catheter placed directly within; suture (ligatures) may then be used to cinch the vessel closed around the catheter (Fig. 4.5).

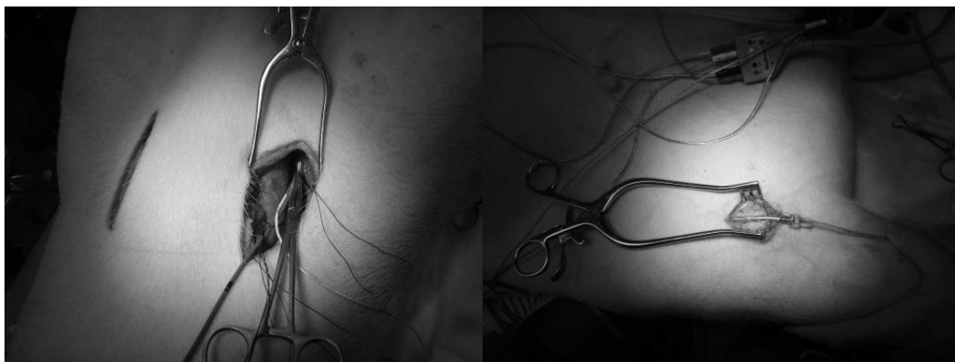

Figure 4.5 - Catheterization and arterial lines

On the left, catheters are placed into the right jugular vein of a swine without an introducer. Suture is used to cinch the vein around the catheter after it is placed. On the right, an arterial pressure line is shown after placement into a branch of

the femoral artery. A cut-down was performed prior to placement, exposing the vessel for easier access.

With venous and arterial access, a variety of quantitative monitoring can be performed to obtain pressures, flows, and electrical information from each chamber of the heart. With a Swan-Ganz catheter, for example, the balloon tipped catheter is advanced into the superior vena cava, and then continues through the right atrium, through the tricuspid valve into the right ventricle, and passes through the pulmonic valve into a small branch of the pulmonary artery (i.e., within the lungs). This catheter system can provide valuable real-time information as to right atrial pressures, wedge pressures (an estimate of left atrial pressures), cardiac outputs, core temperatures, and relative oxygen saturations. Also commonly employed in large animal cardiovascular research are Millar pressure catheters, which are small-diameter catheters (5 to 7 F) that sense pressures at their tips via a diaphragm that covers a piezoelectric crystal sensor. For example, in our laboratory, we typically place one each within the right and left ventricles to obtain continuous pressure waveforms; e.g., from these one can obtain parameters of cardiac contractilities and relaxations. Pacing leads, defibrillation leads, and electrophysiology catheters can also be placed within these hearts using similar approaches; these will be discussed later.

Accessing the Heart

If epicardial access to the heart is needed, there are a variety of approaches one may choose. Perhaps the least invasive of these is a "sub-xyphoid puncture"; this typically allows for access to the pericardial space at or near the apex of the heart. It should be noted that a number of commercially available tools and methods have been developed to facilitate a sub-xyphoid puncture. An added benefit of this procedure is that it can be done without perforating the pleural lining, preventing the need for intubation, general anesthesia, or post-operative chest tubes. However, it can be technically challenging because there are the added risks of puncturing the myocardium or an epicardial vessels, which can result result in cardiac tamponade. A "pericardial window" is a more-invasive version of the sub-xyphoid approach. In this case, instead of a small puncture, the investigator makes a fairly large incision at the base of the rib cage and exposes the pericardium. Then a portion or the whole xyphoid process is removed allowing for direct visualization of the pericardium and heart within.

A thoracotomy is generally described as a surgical window between two ribs, in which a spreader (i.e., a rib retractor holds the ribs apart) is placed to gain access to the heart. Such performed experimental procedures typically require intubation and also may necessitate collapsing one of the lungs to gain better visualization and/or access to the heart. The planned placement of the incision is important, because only a section of the heart is exposed during such a thoracotomy procedure.

A medial sternotomy is the most invasive method of accessing the heart, but gives the investigator complete access to almost all surfaces. In general this experimental approach would include placing the animal under a deep surgical plane of general anesthesia, with a scalpel or electro-cautery utilized to expose the sternum (cutting through skin, muscle and other connective tissues). Next, the sternum is transected down its center using a bone saw. Note that extreme, care must be taken when cutting the sternum because animals such as swine have ligaments that hold the heart close to the sternum (a sternal-pericardial ligament). Then, a retractor is positioned to spread the opening of the rib cage, exposing the beating heart and lungs. The pericardium may be kept intact, it may be excised, or it may be sutured open to provide mechanical support (cradle). Hemodynamic parameters may change dramatically when the thoracic cavity is open, and also when the pericardium is removed.[16] Nevertheless, it is important to monitor all animals carefully during any of these procedures. Examples of parameters to be monitored include: end tidal CO_2 levels, heart rates, blood pressures, cardiac outputs, anesthetic concentrations, core temperatures, arterial O_2 and pH values, renal outputs, hemoglobin levels, and/or other parameters of specific interest to a give particular investigation (Fig 4.6).

If the animal is to be recovered from a thoracic surgery, extreme care must be taken to use aseptic technique, as any subsequent chest infections (mediastinitis) have a very high associated mortality rate for most large animal cardiovascular models. In general, the closure procedure can be complicated,

including the placement of chest tubes, required post-operative monitoring, and the investigator is referred to the literature for these details. Also, recovery from thoracic surgery may take anywhere from days to several weeks and be associated with considerable pain. Therefore, appropriate pain medication and antibiotics must be given to help ensure both the comfort and survival of these animals. An excellent resource for more detailed information on these aforementioned procedures can be found in “Animal Models in Cardiovascular Research” by David Gross.[17]

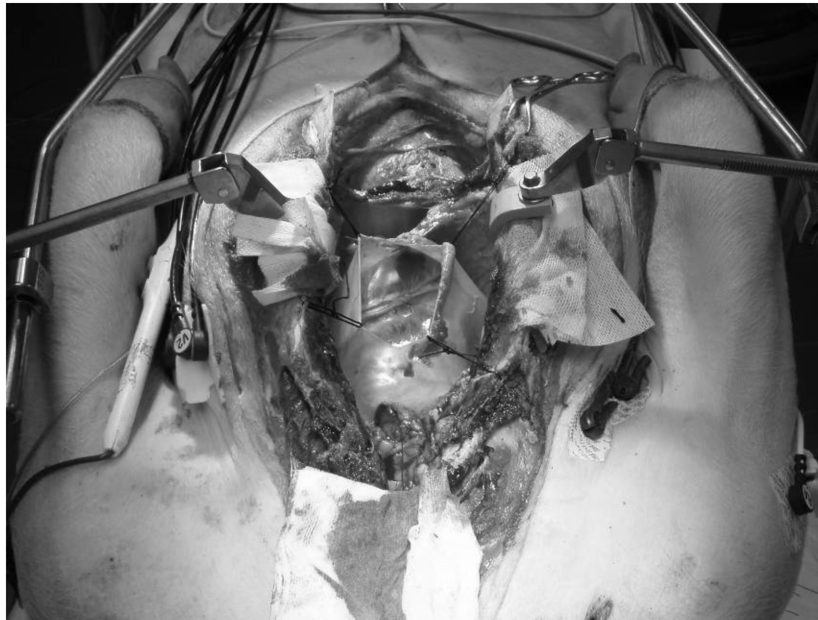

Figure 4.6 - Medial sternotomy

A full medial sternotomy in an anesthetized 90kg swine is shown. A retractor is used to hold the ribs apart, and a pericardial sling was created with suture.

Common Cardiac Electrophysiology Interventions

Many common electrophysiology procedures and/or interventions performed by physicians in the hospital or clinical can be readily reproduced (or developed) in large animal models. For example, it is quite typical for our laboratory to perform the basic electrophysiology (EP) studies one would perform in a standard hospital catheter lab on our large animal models employing the same equipment. In general, for such an EP study to be performed on an animal, they are first are administered an anesthetic that has minimal hemodynamic affects, such a Pentobarbital. Venous access is commonly achieved via an introducer, and then electrophysiology (EP) catheters are placed in the right side of the heart under the guidance of fluoroscopy. Pacing leads may also be implanted at various cites to provide stimulation and sensing. Left-sided leads and EP catheters can be placed through arterial access, or a trans-septal puncture may be performed to allow catheters to pass from the right atrium to the left atrium.[18]

After catheters are placed, various stimulation protocols are commonly employed to measure various desired electrophysiological parameters. For example, a common protocol is to stimulate the right atrium eight times with a stimulus interval of 400 to 600ms. After these eight stimuli (called S1 stimuli) have captured the rhythm of the heart, a ninth stimulus (the S2 stimulus) is given at a much smaller interval called the S1-S2 interval. This protocol is then repeated, each time shortening the S1-S2 interval. When the S1-S2 interval

becomes so short that the S2 stimulus fails to propagate through the AV node, the S1-S2 interval is called the AV node effective refractory period. If the interval is shortened further, the S2 stimulus will eventually fail to evoke any response from the atria, and this S1-S2 interval is the atrial effective refractory period. The same stimulus protocol can be performed in the ventricle to measure the antegrade AV node refractory period, and the ventricular effective refractory period.

It should also be noted that burst pacing, where a high-frequency train of stimuli are delivered to the heart, can be useful in testing fibrillation thresholds in both the atrium and the ventricle. Thus, importantly a defibrillator should always be nearby if sustained ventricular tachycardia or ventricular fibrillation might be induced. While atrial fibrillation is stable in most humans, some animals (particularly swine) may develop dangerous ventricular arrhythmias or become hemodynamically unstable if allowed to elicit atrial fibrillation too long.

Implantation of pacemaker and ICD leads in various large animal models is also a common procedure. As such, leads can either have passive fixation, where the lead is lodged into trabeculae, or active fixation, where the lead tip has a helix and is screwed into the tissue. Yet, the relative degree of veltricular trabeculae greatly differs amongst the commonly employed animal models: e.g., swine can be considered to have minimal trabeculation relative to the dog. Nevertheless, after the lead is placed under fluoroscopy in the desired cardiac location, the introducer is removed and the lead is subsequently connected to the pacemaker

or ICD. These devices are then implanted into a pocket formed under a skin or muscle flap. It is recommended that these devices be implanted where they will not be subjected to excessive external impact or motion (Fig 4.7).

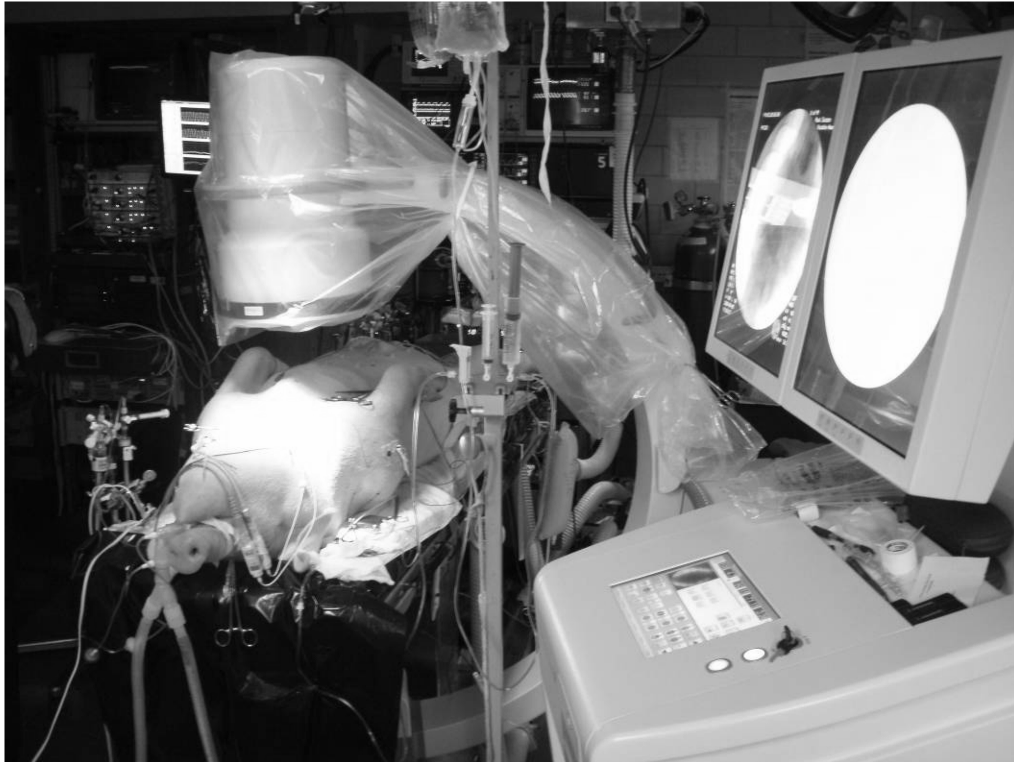

Figure 4.7 - Fluoroscopy

Fluoroscopy is a commonly used imaging modality for placing leads and EP catheters.

Ablation is another common electrophysiology procedure to have been incorporated into numerous animal study protocols. More specifically, an EP catheter can be used to find the appropriate site of ablation, and typically the

same catheter can be used to deliver therapy from an ablation system to the target tissue. Today, the therapy can be delivered either in the form of RF energy (which burns the tissue) or via cryo-therapy (where the tissue fails due to freezing). The parameters or duration, energy and temperature of the therapy will vary depending on the application, the thickness of the tissue, the relative tissue viability, etc.

Large mammalian models have been extensively used to develop the technolgies of cardiac mapping, which in general can measure the relative activation patterns within the heart, to study and/or subsequently treat arrhythmias. Typically, in such studies, a multi-electrode EP catheter placed on the right side of the heart; they can be considered the most basic tool to map activation patterns. For examples, properties of atrial, His, and ventricular activation and repolarization potentials can be measured using this technique. To better understand the global activation patterns within a given heart chamber, mapping systems have been developed to measure three dimensional patterns. To date, St. Jude's Ensite System, Medtronic's Localisa system, and Biosense Webster's Carto system are all commercially available cardiac mapping systems that have obtained clinical use. It should be noted that recent research from our lab has shown that body surface potential mapping may also be used to get similar 3-D cardiac activation patterns, but also begin to provide transmural information; these emerging technologies and procedures may in the future provide a cheaper alternative diagnostic tool, only requiring an array of ECG

electrodes on the chest.[19]

Furthermore, it should be also noted that a number of more advanced biological techniques have begun to be employed in a growing number of large animal electrophysiology studies. More specifically, cell and gene therapies are emerging as a potential "cure" for heart rhythm disorders: e.g., cells, genes, and extracellular matrices can be injected with special needle catheters into various anatomical areas of the heart as specific means to treat arrhythmias.[20] In such cases, imaging beyond fluoroscopy, including cardiac MRI and CT, can provide valuable anatomical information which is helpful in analyzing electrophysiology data.[19] As noted in Chapter (Skadsberg) isolated heart models, such as those developed in our Visible Heart Lab (www.visibleheart.com) allows for direct endoscopic visualization of catheters, leads and other devices while still maintaining the electrical and mechanical function of the heart.[21] Finally, post-mortem histological analyses may also provide valuable insights into the structure of the cardiac conduction system.[22]

In summary, a number of techniques and methods are available for investigators to study the efficacy and feasibility of electrophysiology therapies and large animal models enable the translation of cell- or tissue- level discoveries to become a viable therapy that can be used in the clinic to benefit people's lives.

Animal Models of Disease States

In any type of translational research it is desirable to have an appropriate animal model which exhibits similar physiology and pathophysiology to the targeted

disease state in humans. In the realm of heart failure, animal models have been developed by numerous laboratories to mimic a variety of myocardial disease states or conditions such as: congestive heart failure, hypertrophic heart failure, myocardial infarction, atrial fibrillation, ventricular fibrillation, tachycardia, etc. Such models have been developed for use in either acute or chronic investigations. The following section briefly covers some of the more common disease states which can be created (modeled) in the common large mammals used for translational studies on associated cardiac electrophysiology.

Congestive Heart Failure Models:

Dilated Cardiomyopathy (DCM), a form of systolic heart failure, is characterized by ventricular dilation and functional impairment. Commonly, DCM is associated with conduction disturbances in the myocardium; for example, a left bundle branch block (LBB) causes significant interventricular and intraventricular dyssynchrony in such patients. Futhermore, it is current clinical practice in these cases, to provide cardiac resynchronization therapy (CRT), the implantation of a biventricular pacing system that is used to resynchronize the ventricles. Because there remains no consensus as to the optimal anatomical site for lead placement in CRT (or lead placement in general for pacing in a failing heart), numerous large animal models of DCM have been developed and employed to study the validation and optimization of such therapies.

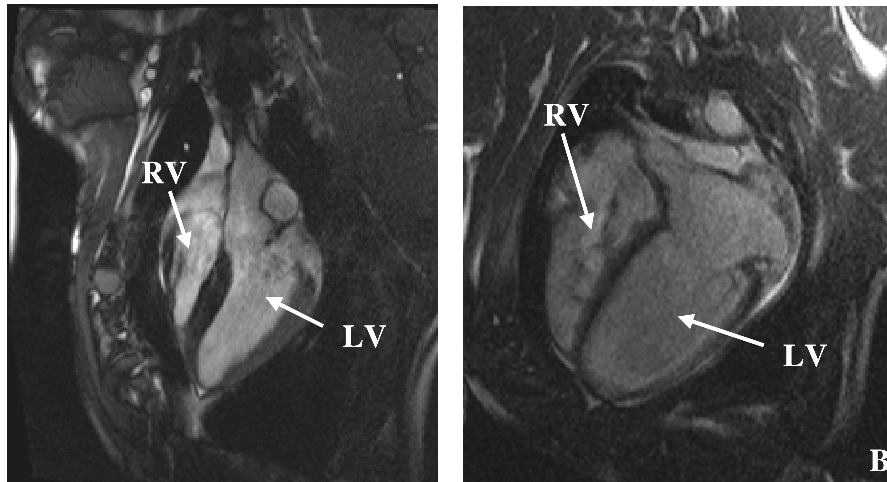

Figure 4.8 - Cardiac Magnetic Resonance

A four-chamber MR image of a normal swine heart is shown in end-diastole (A), and after three weeks of high rate pacing at 200 beats/minute (B). The increase in chamber size and wall thinning after high rate pacing can easily be discerned.

Chronic high rate pacing, or tachypacing, is considered the gold standard in creating animal models of DCM, is highly reproducible, and technically simple to produce[1]. By pacing the heart at 2-4 times the intrinsic rate from either the atria or ventricles, this will produce marked changes in both cardiac function and anatomy within a matter of weeks; the extent of remodeling can be controlled by both the pacing rate and duration (Fig. 4.8). Typically, such a high rate pacing model of DCM will cause the following functional and anatomical changes in the heart: [23-25]

- Increased filling pressures
- Left and right ventricular systolic dysfunction (low cardiac output, contractility, and ejection fraction)
- LV, RV, RA, and LA chamber dilatation, spherical LV chamber geometry
- Increase in cardiac mass (not uniform across species)
- Mitral valve regurgitation
- In addition, high rate pacing is associated with the following systemic effects:
- Neurohumoural activation
- Increase in systemic vascular resistance
- Ascites (accumulation of fluid in the abdomen)
- Peripheral edema

As an example, Wilson and colleagues studied the effects of high rate pacing in canines at endpoints of three and eight weeks.[26] They demonstrated that high rate pacing at a rate of 240-260 beats/minute in the canine model reduced cardiac outputs (control 130 ± 20 ml/min/kg, 8 week pacing 116 ± 14 ml/min/kg), elevated pulmonary wedge pressures (control 10 ± 3 mm Hg, eight week pacing 26 ± 8 mm Hg), and increased right atrial pressures (control 4 ±1 mmHg, eight week pacing 9 ± 3 mm Hg) (all $p < .01$ vs. control). They also noted that after

the study termination, the dogs in both pacing groups also had left and right ventricular volumes which were approximately twice those of the controls. Additionally, after three weeks of pacing, a functional assessment with echocardiography revealed a decreased percent left ventricular shortening (34 ± 6% to 17 ± 7%, $p < .01$).

To date, the canine model has been the primary large-mammalian model of pacing-induced DCM, with porcine and ovine models also utilized. It should be noted that the pacing time period necessary to reach the same hemodynamic endpoints varies by species, with pacing periods approximately twice as long in canines as in swine, partly due to the increased collateral coronary circulation in canines. Peripheral edema and ascites is common with this type of heart failure model, and the use of diuretics should be considered during the progression of the disease state for animal comfort and also to reduce mortality. In addition, for any procedure or measurement that can not be made while the animal is awake, consideration should be given to the choice of anesthesia; a high dose of any administered anesthetic which may be a cardiovascular depressant (such as thiopental or isoflurane) could be morbid in such a heart failure model. Furthermore, there is general agreement that the cardiac and systemic effects of high rate pacing are largely reversible after cessation of pacing, where the time period for the recovery can be in a matter of weeks.

It should also be noted that tachypacing-induced heart failure, can also be fine tuned in order to more closely represent specific heart failure mechanisms in

man. In one variation, for the study of ventricular timing and pacing site in CRT therapy, Helm et al first created a left bundle branch block, followed by three to four weeks of tachypacing (210 beats/minute) in canines to produce the combination of DCM and ventricular dyssynchrony.[27] In another example, Shen and colleagues sequentially clipped two regions of the left circumflex artery, followed by intermittent periods of chronic rapid pacing (220 beats/minute).[28] Subsequently in this canine model, severe LV dysfunction resulted, and it was noted that LV function and peripheral vasoconstriction did not recover after the cessation of pacing. In other words, the irreversibility of this latter model indicates a different underlying mechanism for the induced heart failure; than the tachy pacing model alone, which is largely reversible.

Acute and Chronic Atrial Fibrillation Models:

Atrial fibrillation (AF) is an uncoordinated tachyarrhythmia associated with a high level of morbidity and mortality as previously described (See Benditt's chapter). One necessary feature in a large animal model of AF is that the arrhythmia must be sustained for a period of time long enough to permit the electrical characterization of the rhythm and application of the therapy designed to correct the condition. To date, both acute and chronic large animal models have been developed for the study AF.

There are a wide range of methods to generate AF in an animal model, which can be grouped into the following categories:

Surgical

Pharmacological or Chemical

Electrical

Surgical methods used in the induction of AF involve creating an injury to the atrial tissue, such that there is a subsequent barrier for the propagating action potentials.[29] For example, this barrier can be created by clamping (traumatic injury), making an incision, or ablating the tissue. Once the anatomical block is formed, AF can typically be initiated by brief, rapid electrical stimulation and the presence of the created block allows the formation of a reentry loop. Yet, it should be noted that results can vary in this type of model of AF; as they are sensitive to the locations and sizes of the injuries, and also the relative placements of the stimulating electrodes.

In general, pharmacological and chemical methods of inducing AF involve intravenous or local/targeted delivery of an agent which will increase the susceptibility of the heart to AF. Typically, pharmacological agents are chosen to alter specific ionic channels and their ionic currents; in order to prolong the myocyte refractory periods, making it easier to electrically initiate AF. For example, in one study by Anadon et al, AF and atrial flutter were induced in swine with an intravenous delivery of ethanol followed by rapid stimulation of the right atrium to initiate an arrhythmia.[30] Furthermore, it was noted that as the intravenous concentration of alcohol was increased, the ability to generate

sustained AF was increased. However, this model was an acute model as AF was only sustained for a matter of minutes. In contrast, Kijtawornrat and colleagues have been successful in creating an acute animal model in canines, where AF was sustained for approximately 40 minutes.[31] In this study, AF was achieved through a combination of rapid atrial pacing and an infusion of Phenylephrine. In general, rapid pacing decreases the atrial effective refractory period, slows atrial conduction, and increases electrophysiologic heterogeneity, where the phenylephrine infusion increased the difference between left and right atrial and intra-atrial refractory periods. More specifically, AF in these canines was sustained for approximately 40 minutes following the infusion of Phenylephrine and 20 minutes of tachypacing at 40 hz.

Similar to animal models for DCM, high rate pacing has been successfully utilized in developing chronic models of AF. For example, Bauer and colleagues used burst pacing at a frequency of 42 hz in the right atrial appendage to cause AF induced heart failure in swine.[32] In this case, the burst pacing resulted in very high ventricular rates, where an average ventricular rate of 270 ± 5 bpm was reported during sustained AF. Furthermore, after three weeks of burst pacing, chronic heart failure was evident in these animals. In another experimental approach, Wijffels et al created a chronic model of AF in goats through the use of an external fibrillator/pacemaker attached to epicardial electrodes on the atria.[33] Upon detection of a sinus rhythm, external stimulation of the atria was induced for 1 s at a rate of 50 hz, which automatically maintained AF 24 hours/day. It was

reported that the repetitive induction of AF for a period of 1 week by this pacing method led to a progressive increase in the duration of AF, and subsequently AF became sustained for periods >24 hours without any maintenance pacing.

Animal Models of Ventricular Fibrillation:

Common methods to induce ventricular fibrillation in large animals include electrical stimulation at high frequencies and/or the induction acute ischemia. Since there are different mechanisms that cause VF, an appropriate animal for VF should be chosen based on the patient population for the therapy being studied (simulated). For instance, if targeting VF in children, which commonly occurs because of asphyxiation or circulatory shock, one would use a model where the trachea is clamped in a anesthetized animal.[34] However, if the therapy is targeting VF due to an ischemic event, an acute or ischemic model would be obviously more appropriate.[35] Additionally, VF can be caused by an overdose of certain medications, and the appropriate animal model to mimic this type of mechanism can in many cases be achieved by treating an animal with the same medications. The methods and considerations involved in creating ischemic conditions in animal models is described in the next section below, however, for a detailed description of other aforementioned methods we refer the reader to the vast body of literature.

Animal Models of Myocardial Infarction, Ischemia

Myocardial infarction (also commonly known as a heart attack) refers to the focal or global damaging of myocardial tissue due to ischemia, a lack or stoppage of

blood flow. Importantly, there are many electrical changes and resultant arrhythmias in the heart that arise from this condition that are of interest to the electrophysiologist (i.e. ventricular fibrillation, ventricular tachycardia, etc.) as previously described in this book.

Historically, there have been a wide variety of species employed and methods used to create large animal models of cardiac ischemia. Most common are acute experiments in which a targeted coronary artery is occluded in anesthetized animals. Typical methods of creating such an occlusion include opening the chest to gain access to the heart and the subsequent surgical ligation of the targeted branch of a coronary artery, or by placing a removable clip around the artery which gives the ability to study cyclic periods of ischemia (i.e., ischemia-reperfusion injury)(Fig. 4.9). As such, the size and location of the infracted region will ultimately be dependent on the relative location of the artery occluded and also the duration of the ischemic time.

If it by investigational design that a closed chest model be preferred, then in such cases a coronary occlusion can be induced via a balloon catheter placed into that given vessel (e.g., via access from the femoral artery). Although the potential for temperature variations are reduced in a closed chest model, there is limitation as to the type of physiological measurements that can be readily obtained. For example, in the open chest model, flow probes can be placed around the pulmonary artery and the aorta for the on-line continuous measuremenst of cardiac output and total coronary artery flows, and additionally sonomicrometry

crystals can be sutured to the epicardium for the assessments of focal wall motions and/or regional functions.

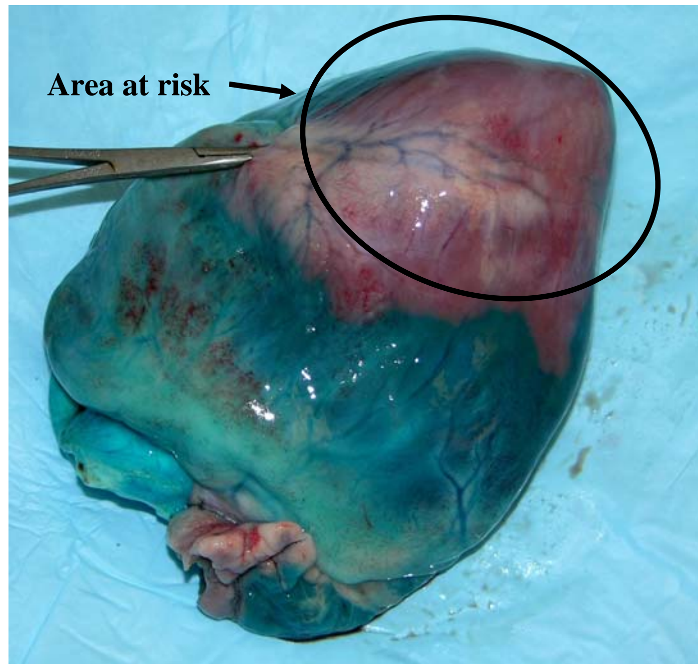

Figure 4.9 - Perfusion staining of the heart

An excised swine heart from an acute occlusion study after the injection of patent blue dye is shown, with regions stained blue indicating where perfusion occurred during the occlusion, while non-dyed regions indicate the area at risk.

There are multiple methods to quantify the size and location of an induced infarcted region. Common methods used to identify the area at risk, or an area that is not perfused, is through the use of the injection of microspheres or dyes

while the vessels are still occluded. More specifically, the injected microspheres will then congregate in the capillaries of any tissues that are perfused. Therefore, following tissue digestion, any areas which contain microspheres can be identified as regions which were perfused, and visa versa. In contrast, if a dye injection is employed, the areas which were perfused will turn the color of the dye and areas which were not perfused will remain unaffected (Fig. 4.9). Triphenyletrazolium chloride (TTC) staining is a common histo-chemical method which is often used to delineate normal and infarcted myocardial tissue (post mortem), as the stain only adheres to infarcted (damaged) tissue[36] (Fig. 4.10). If an in vivo assessment of perfusion and myocardial infarction is desired, such as during a chronic study with progressive occlusion, if available, a contrast enhanced, cardiac MRI can be used to do so.[37,38]

In an acute large animal infact study, it is only possible to simulate where a myocardial infarction would likely be created. However, for scar formation (the replacement of dead myocardium), a chronic study would be necessary. An investigators decision as to whether an acute or chronic study should be performed is dependent on the relative pathophysiology and underlying mechanisms of the arrhythmia that is of primary interest. For example, in the case of a chronic study, where the animal is woken up after the occlusion, careful consideration must be made as to the location of the region occluded and also to the choice of the species used. Historically, dogs have been used in chronic ischemia models, as the increased collateral coronary circulation improves

survivability (they elicit minimal arrhythmias). However, as previously mentioned, the swine model has coronary anatomy more similar to that of a human heart; yet, an induced large ischemic area would be more likely fatal in this species as compared to a dog.

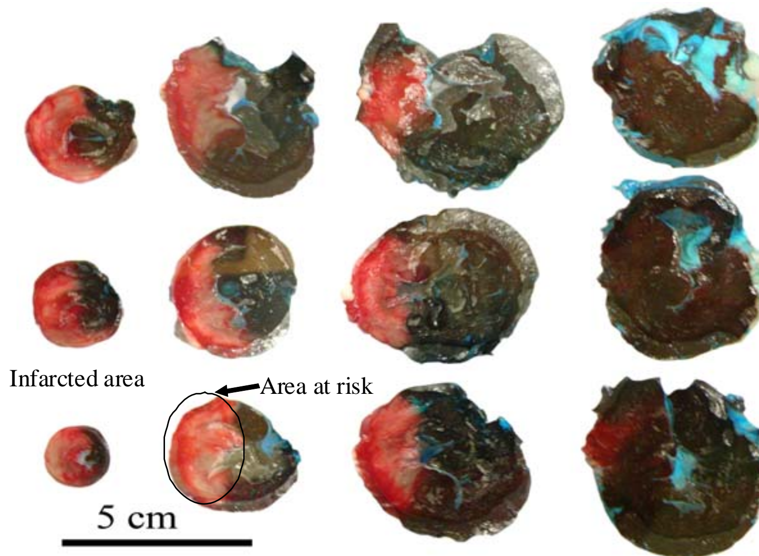

Figure 4.10 - TTC staining of the heart

TTC staining after an acute infarct and the injection of patent blue dye in an acute model of myocardial infarction is shown. In the non-dyed regions, or area at risk, the infarcted regions are pale in color.

Summary

Large animal models can be utilized to recreate many cardiac electrophysiological diseases states and procedures, in addition to being used to test new devices and imaging techniques. The three most common large animal models are dogs, sheep, and swine, but the choice of animal model is dependent

upon many factors: anatomy of interest, skill and experience of the investigator, ease of handling, study design, and the ability to create specific pathological models. Used correctly, animal models are the best available method to predict systemic responses to therapies, while poorly designed experiments can lead to misleading and non-transferable results. Any animal studies conducted with government funding or at a major corporation or university, is highly regulated with the aim to maximize human benefit while minimizing animal use, pain, and suffering. Every invasive device used clinically today was once tested in a large animal model, and the continued use of animal models will make available the next generation of devices and therapies to patients in a safe and efficacious form.

Chapter 5: A Novel Combination Therapy for Post-Operative Arrhythmias

Authors: Eric Richardson, BS, Bryan Whitson, MD PhD, Paul Iaizzo, PhD

The following abstract was selected for oral presentation at the Design of Medical Devices Conference in April 2009. The oral presentation won the “3 in 5” competition as one of the top three presentations. The poster, also contained in this chapter, was presented at the same conference.

Design of Medical Devices Abstract

A Novel Combination Therapy for Post-Operative Atrial Fibrillation

Eric Richardson BS, Bryan Whitson MD PhD, Paul Iaizzo PhD

Atrial fibrillation (AF) is a common heart rhythm disorder, effecting about 20% of cardiac surgical patients. While often benign, it leads to a prolonged hospital stay, and can potentially develop into more dangerous ventricular arrhythmias. Many anti-arrhythmic drugs have been used to both prevent and treat post-operative AF, but they often cause side effects (such as hypotension) that are harmful to recovering patients. Cardioversion is often the therapy of last resort to restore the patient to sinus rhythm. We propose a novel yet simple device that allows for local pharmacological and electrical therapy of the heart. We have shown in animal models that such delivery increases the efficacy of the therapy and reduces side effects.

Several variations of the device have been conceived and prototypes are currently being developed. The simplest version is a multi-port infusion catheter incorporated into a temporary pacing lead. Placement of the device would be trivial for surgeons, who routinely place temporary pacing leads prior to closing the chest. Removal of the device post-operatively would be equally simple. More complex iterations include an expandable wick to maintain the infused drug in a desired location. Designs may also include epicardial defibrillation capabilities, which would lower the energy required for defibrillation.

To demonstrate that drug would accumulate in the desired location when delivered through such a device, a sternotomy and pericardiotomy were performed on a fresh human cadaver. A catheter was placed in the pericardium, and an infusion of radio-opaque contrast was administered under fluoroscopy. The majority of the contrast collected near the pulmonary veins, which are often the origin of atrial arrhythmias.

To compare the effectiveness of drugs administered into the pericardium to drugs administered through the conventional route (intravenously), animal studies in swine (n=8) were performed. Results showed that when metoprolol, a common beta-blocker, was administered in the pericardium, it had a greater and more lasting effect on heart rate than when given intravenously. In addition, during pericardial delivery myocardial contractility was better preserved, and only trace amounts of metoprolol were found in the circulation. Thus pericardial delivery may enhance certain therapeutic effects of drugs while limiting side effects.

Currently, studies are underway to compare the effectiveness of another drug, amiodarone, when delivered into the pericardium. Should our animal studies continue to show promising results, we anticipate moving into clinical trials. Development and refinement of prototype delivery devices will also continue as we pursue this promising new therapy for post-operative AF.

A Novel Combination Therapy for Post-Operative Supraventricular Arrhythmias

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Abstract

Atrial fibrillation (AF) is a common heart rhythm disorder, effecting about 20 % of cardiac surgical patients. While often benign, symptoms can lead to a prolonged hospital stays, and can potentially develop into more dangerous ventricular arrhythmias. To date, many anti-arrhythmic drugs have been used to both prevent and treat post-operative AF, but they often cause side-effects (such as hypotension) that are harmful to recovering patients. Cardioversion is often the therapy of last resort to restore the patient to sinus rhythm.

We propose here a class of novel yet simple devices, that allow for the delivery of focal pharmacological and electrical cardiac therapies. In our animal trials, such delivery increases therapeutic efficacies while reducing side-effects.

Several variations of these devices have been conceived and prototypes are currently being developed: e.g., the simplest version is a multi-port infusion catheter incorporated into a temporary pacing lead. Placement of such a device would likely be relatively trivial for surgeons, who routinely place temporary pacing leads prior to closing the chest. By design, the removal of the device post-operatively would be equally simple. Several of our more complex iterations include an expandable wick to maintain the infused drug in a desired location. Additional designs have also included epicardial defibrillation capabilities, which would lower the energy required for defibrillation.

To demonstrate that administered drugs would accumulate in the desired location when delivered through such a device, a sternotomy and pericardiotomy were performed on a fresh human cadaver. A catheter was placed in the pericardium, and an infusion of radio-opaque contrast was administered under fluoroscopy. The majority of the contrast collected near the pulmonary veins, which are often the origin of atrial arrhythmias. To compare the effectiveness of drugs administered into the pericardium to drugs administered through the conventional route (intravenously, IV), animal studies in swine (n=8) were performed. Results showed that when Metoprolol, a common beta-blocker, was administered in the pericardial space, it had a greater and more lasting effect on heart rate than when given IV. In addition, during pericardial delivery myocardial contractility was better preserved, and only trace amounts of metoprolol were found in the circulation. Thus pericardial delivery may enhance certain therapeutic effects of drugs while limiting side-effects.

Currently, studies are underway to compare the effectiveness of another drug, Amiodarone, when delivered into the pericardial space. Should our animal studies continue to show promising results, we anticipate planning clinical trials. Development and refinement of prototype delivery devices will also continue as we pursue this promising new therapy for post-operative AF.

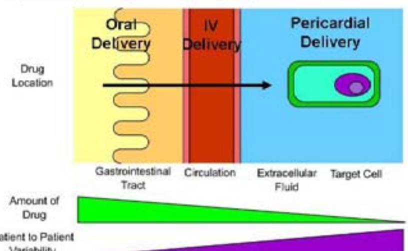


Figure 1. The principle of targeted drug delivery. With oral delivery, the drug must pass through many steps to finally arrive at the target cell, with each step introducing more variability and reducing the amount of drug. Targeted delivery, such as pericardial space delivery, moves the site of administration closer to the site of action.

Methods

Feasibility studies were carried out in anesthetized swine (70-100kg) with surgical median sternotomies and pericardial cradles. In the first study (n=23), the left anterior descending arteries were clamped to induced ventricular arrhythmias. Animals either received saline or docosahexaenoic acid (DHA) into the pericardial space. In the second study group (n=21), isoproterenol was administered to raise these animals heart rates above 180bpm, and/or AF was induced. Animals received Metroprolol IV or into the pericardial space (IP). A third study is currently ongoing (n=9 to date) in which atrial fibrillation is induced in swine and Amiodarone is given either IV or IP. In addition to these pharmacodynamic studies, prototypes of post-operative delivery catheters were built and tested in both swine, as well as a fresh human cadaver studies. In the human cadaver trials, contrast agent was injected through the delivery catheter, and fluoroscopy was used to determine where the contrast typically collected.

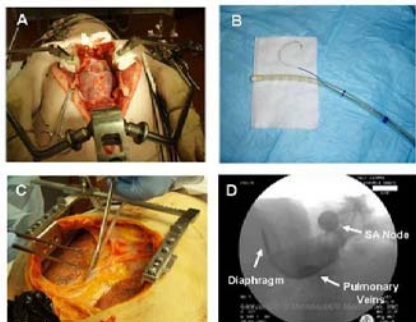


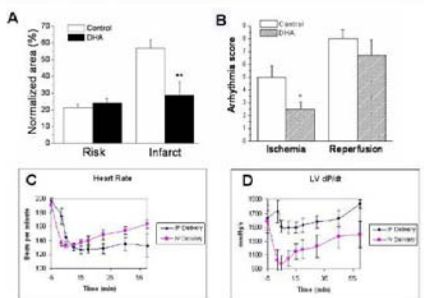
Figure 2. A. A swine open-chest model of pericardial delivery. B. A prototype post-operative delivery catheter. C. Testing of catheters in human cadavers. D. Fluoroscopy of a human cadaver receiving contrast through delivery catheter. Note the collection of fluid near the pulmonary veins, which are often the site of arrhythmias.

Results and Discussion

Animal studies with DHA, an omega-3 fatty acid, showed that pericardial delivery reduced arrhythmia scores by 50% , and lower associated infarct sized by 49% (figures 3A and 3B). In addition, the incidences of VF and mortality rate were also lowered (1). IP delivered Metoprolol lowered heart rates to the same extent as IV metoprolol, but these effects were enhanced (figure 3C). Also, IP Metoprolol did not adversely effect contractility, whereas IV Metoprolol did (figure 3D). Studies on the administration of Amiodarone are currently ongoing. In general, initial animal trials have shown that IP delivery was as effective or even more effective than IV delivery; importantly IP delivery was also associated with fewer side-effects.

Prototype devices were able to deliver drugs and contrast successfully into the pericardial space after the chest cavity was re-closed in both human and swine trials. Fluoroscopic imaging in the supine individuals showed that the contrast tended to collect on the posterior side of the heart, near the pulmonary veins (figure 2D). This is likely advantageous, because arrhythmias (e.g., atrial fibrillation) often originate from the pulmonary veins. In swine, metoprolol delivery with our devices was able to lower heart rates, following which the rates were raised using pacing. This shows the ability to both raise and lower rates without effecting contractility.

Results and Discussion



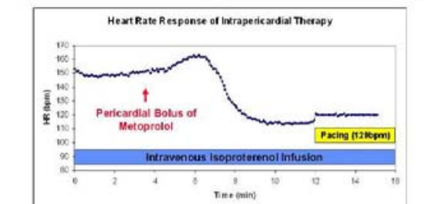


Figure 3. A and B: The reduction of both arrhythmia scores and infarct sizes after DHA was delivered into the pericardial spaces of an ischemic swine model. C and D: The effect of a bolus of Metoprolol given either IV or IP in swine with induced sinus tachycardia. E. The heart rate responses of an animal receiving therapy with a prototype pericardial delivery catheter. Isoproterenol was used to induce sinus tachycardia, following which a bolus of metoprolol through the catheter dropped the heart rate. The rate was raised again and maintained using the pacing feature of the catheter.

Conclusions and Future Work

- **Intrapericardial delivered DHA and Metoprolol can effectively prevent arrhythmias while limiting side-effects.**
- **Prototype post-operative delivery catheters allow drug to accumulate in areas of the heart that are prone to initiate arrhythmia.**
- **Prototype catheters can both lower and raise the heart rate, with a minimal effect on blood pressure.**
- **Future Work:**
  - **Refine the designs of the various delivery catheters.**
  - **Complete animal trials with amiodarone and other agents**
  - **After efficacies and safety are established, initiate human clinical trials**

(1) "Pericardial delivery of omega-3 fatty acid: a novel approach to reducing myocardial infarct sizes and arrhythmias" Yong-Fu Xiao, Daniel C. Sigg, Michael R. Ujhelyi, Joshua J. Wilhelm, Eric S. Richardson, Paul A. Iaizzo. *American Journal of Physiology – Heart and Circulatory Physiology*. 2008 May;294(5):H2212-8.

Section 3: Preface

Finally, with the background research developed in section one, and the tools and models developed in section two, we performed three large animal studies to determine the effectiveness and safety of our therapy. Section three of my thesis includes these experimental studies and the following are brief abstracts from each study. Each study constitutes one chapter of section three of my thesis:

Chapter 6: Intrapericardial omega-3 fatty acids and ventricular arrhythmias. This study investigated if pericardial delivery of n-3 PUFAs protects the myocardium from ischemic damage and arrhythmias. *Methods*: Acute myocardial infarction was induced in 23 pigs with 45 min balloon inflations or clamp occlusions of the left anterior descending coronary artery and 180 min reperfusion. Docosahexaenoic acid (C22:6n-3, DHA, 45 mg), one of the main n-3 PUFAs in fish oil, was infused to the pericardial space only during the 40 min stabilizing phase, 45 min ischemia and 1st 5 min reperfusion. *Results*: Hemodynamics and cardiac functions were very similar between two groups. However, DHA therapy significantly reduced infarct sizes from 56.8 ± 4.9% for control animals ($n = 12$) to 28.8 ± 7.9% ($p < 0.01$) for DHA-treated pigs ($n = 11$). Compared with controls, DHA-treated animals significantly slowed heart rates and reduced ventricular arrhythmia scores during ischemia. Further, three (25%) control animals experienced 8 episodes of ventricular fibrillation (VF), and 2 died subsequent to unsuccessful defibrillation. In contrast, only one (9%) of eleven DHA-treated pigs had one episode of VF and was successfully converted to normal rhythm after

electric defibrillation; thus mortality was reduced from 17% in the controls to 0% in the DHA-treated animals. *Conclusions*: These data demonstrate that pericardial infusion of n-3 PUFA DHA can significantly reduce malignant arrhythmias and infarct sizes in a porcine infarct model. Pericardial administration of n-3 PUFAs could represent a novel approach to treating myocardial infarction. This study was led by Dr. Yong-Fu Xiao of Medtronic, and was recently published in the *American Journal of Physiology – Heart and Circulatory Physiology*. My role in the study was to carry out several of the animal studies, analyze the hemodynamic data, and stain for infarct sizes.

Chapter 7: Intrapericardial metoprolol and sinus tachycardia/atrial fibrillation. The purpose of this study was to measure the pharmacokinetics (PK) and pharmacodynamics (PD) of metoprolol when given IP as compared to when given intravenously (IV). *Methods*: Male swine (70-90 kg) were anaesthetized and instrumented with a pressure catheter in the left ventricle, a Swan-Ganz Catheter, and ECG leads. A median sternotomy was performed and a pericardial sling was created with suture. In nine animals, Isoproterenol (0.5-1.0 μg/min) was continuously infused to achieve a heart rate (HR) > 180 bpm. In another eight swine, the animals were instrumented to measure atrial, ventricular, and AV node effective refractory periods, and AF inducibility. Metoprolol (20 mg in 20 ml saline) was administered IV or IP to animals in both groups. Hemodynamic and electrophysiologic parameters were continuously measured for one hour, and samples of blood and pericardial fluid were taken at predetermined intervals.

Metoprolol concentrations were measured using HPLC. *Results:* HR was lowered significantly ($p<0.05$) in both IP and IV delivery (70% and 73% of control rate, respectively) for duration of the study. Ventricular contractility (as measured by dP/dt) was significantly ($p<0.05$) reduced in IV-treated animals (73% of control measurements) but were not significantly lower after one hour. In contrast, cardiac output and contractility were never significantly affected in IP-treated animals. PR and QRS intervals were affected similarly in both groups, but QT intervals were only affected in the IP-treated group. No significant trends were seen with regards to trans-mural activation times, AVNERP, VERP, or ability to induce AF. Both IV and IP delivered metoprolol did significantly stabilize intra-atrial conduction times and AERPs compared to saline-treated controls. When given IP, the elimination half-life of metoprolol in the pericardial fluid was 18.1 minutes, with negligible amounts observed in the plasma. When given IV, the elimination half life of metoprolol in the plasma was 11.1 minutes, with negligible amounts in the pericardial fluid. Pericardial fluid drug concentrations during IP delivery were approximately 1,000-fold higher than plasma drug concentrations during IV delivery. *Conclusion*s: Intrapericardial delivery of metoprolol may enhance electrophysiologic effects without affecting hemodynamic parameters. These effects can be achieved with negligible systemic drug concentrations. The manuscript of this study is in preparation for submission to *Circulation Research*. My role was to conceive of the study, develop the animal model, and design the experiments. I also carried out the animal studies and analyzed the data with the

help of the Visible Heart Lab staff and students (especially Christopher Rolfes and Michael Woo).

Chapter 8 (to be completed): Intrapericardial amiodarone and atrial fibrillation. The purpose of this study was to compare the effects of IV- and IP- delivered amiodarone on cardiac electrophysiology, including the susceptibility to atrial fibrillation. This study is currently underway, with data from nine animals completed. *Methods.* Male swine (40-110kg) were anesthetized and instrumented as described in the previous study. A pericardial sling was created, and the animals were allowed to stabilize. After baseline electrophysiology measurements were taken, the animals received amiodarone IP or IV. Over the next 3 hours, haemodynamics were monitored, electrophysiology parameters were measured, and sample were taken from the blood and pericardial fluid. At the end of the study, the heart was excised to observe drug concentration in various structures of the heart. *Results*: Data is still being collected for this study. Two formulations for intrapericardial amiodarone will be tested in this study, because most formulations of amiodarone are very acidic and require a large amount of solubilizing agent. We currently are developing collaborations to find the appropriate formulation for pericardial delivery. Because data is still being collected for this study, there is no chapter dedicated to this last study. However, all available data will be presented at my Oral Defense (May 5th, 2009).

Chapter 6: Pericardial Delivery of Omega-3 Fatty Acid: A Novel Approach to Reducing Myocardial Infarct Sizes and Arrhythmias

Authors: Yong-Fu Xiao, MD PhD, Daniel Sigg MD PhD, Michael Ujhelyi, PharmD, Joshua Wilhelm, MS, Eric Richardson, BS, Paul Iaizzo, PhD.

This chapter has been published in the *American Journal of Physiology – Heart and Circulatory Physiology*, 2008 May;294(5):H2212-8. Permission has been granted to publish this work in my dissertation.

Introduction

Since the original epidemiological studies were published three decades ago (2), dietary n-3 polyunsaturated fatty acids (PUFAs) have been correlated with a reduced risk of coronary heart disease. The primary sources of n-3 PUFAs in the human diet are mainly plant-derived α-linolenic acid (LNA, C18:3n-3) and marine-derived eicosapentaenoic acid (EPA, C20:5n-3) plus docosahexaenoic acid (DHA, C22:6n-3). Recently, increased n-3 PUFAs intake has been shown to have reduction of ischemia-induced myocardial damage (9, 19, 28) and arrhythmias (14, 21, 24). A major benefit of n-3 PUFAs is that unlike several cardiac pharmaceuticals, they have only minor side effects (15, 18), and are on the FDA's Generally Accepted as Safe (GRAS) list. While dietary supplementation is the most common administration of n-3 PUFAs in both clinical and animal studies, the resulting myocardial concentrations of free n-3 PUFAs depend on several factors in both the gastrointestinal system and in blood. Although n-3 PUFAs have been intravenously administrated in experimental animals for cardiac arrhythmia prevention (3), and in humans for anti-inflammatory therapy (7), it is important to point out that in blood, albumins bind to n-3 PUFAs and greatly reduce their free form concentrations. Thus in the present study, we hypothesize that a more localized and better controlled approach to deliver n-3 PUFAs to the heart is through the pericardial space. Compared with blood, pericardial fluid contains lower amounts of proteins and is only about 1% of the blood volume. Therefore, pericardial infusion of n-3 PUFAs

was considered to effectively build up a high concentration around the heart. High concentrations of n-3 PUFAs should enhance their effects on myocardium, as they are highly lipid-soluble. Furthermore, previous studies with the pericardial administration of amiodarone and procainamide have shown that the active motion of the heart within the pericardium mixes and distributes the drugs throughout the epicardium and that deep penetrations can occur (31).

Basic research and clinical trials have shown that n-3 PUFAs reduce the risk of acute myocardial infarction.(9, 19, 21-24, 34, 37) Dietary supplementation of fish oil attenuated myocardial infarct size, dysfunction, and lethal ventricular arrhythmias in ischemia/reperfusion animal models (22, 23, 34, 37). However, the effects of n-3 PUFAs being directly infused to the heart on ischemic myocardium were not assessed. In the present study, we thus delivered DHA to the pericardial space in an acute ischemia/reperfusion porcine model. Our results indicate that pericardial delivery of the n-3 PUFA (DHA) reduces ischemia-induced ventricular arrhythmias, infarct sizes and mortality. Thus, this promising novel approach may have clinically relevant applications as a number of new devices allow for less-invasive access to the pericardial space through sub-xyphoid or trans-vascular catheters (16).

Methods and Materials

This study protocol was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* [Department of Health and Human Services Publication No. (NIH) 85-23, Revised 1996], and was approved by the Institutional Animal Care and Use Committee of the University of Minnesota. This ischemia/reperfusion-induced myocardial infarction model was performed using male Yorkshire swine. These animals were randomly assigned into two groups, control (n = 12) and DHA-treated (n = 11).

Surgical procedures

Otherwise healthy swine with an averaged body weight of 42.3 ± 1.1 kg (mean ± SE, n = 23, $p > 0.05$ between control and DHA-treated groups) were sedated with midazolam intramuscularly (2 mg/kg) and anesthetized with intravenous pentobarbital sodium (20 mg/kg initial bolus, followed by a continuous infusion at 5 - 20 mg/kg/hr). After endotracheal intubation, ventilation (2:1 air to oxygen mixture) was adjusted to maintain an arterial PCO_2 of 40 ± 2mmHg, and rectal temperatures were maintained at 38 ± 0.5°C using convective air warming as needed (Bair Hugger®, Arizant Medical; Eden Prairie, MN, USA). Two Mikro-Tip catheter transducers (5 Fr, model MPC-500, Millar Instruments; Houston, TX, USA) were placed for ventricular pressure monitoring: 1) via the right jugular vein into the right ventricle; and 2) the other via the right carotid artery into the left ventricle. A Swan-Ganz catheter was placed into the pulmonary artery via a jugular vein and hooked up to a "Q2 Continuous Cardiac Output Computer"

(Abbot Critical Care, IL, USA) to measure the cardiac output. Two approaches were used to occlude the left anterior descending (LAD) coronary artery. In one group (n = 6), a guide catheter was inserted into the left coronary artery via the left carotid artery. A balloon (3.5 $\times$ 6 mm) catheter was advanced via a guide catheter to the site between the second and third branches of the LAD under fluoroscopic guidance. To reduce potential variabilities caused by the balloon method, the 2nd group (n = 17) was also used in our study. A small incision was made into the anterior portion of the pericardial sac in each animal and approximately 0.5 cm of the LAD between the second and third branches was dissected for placing a vessel clamp (Sklar Vascular Size 2 Single Clamp, Sklar Instruments, West Chester, PA, USA). A femoral artery was cannulated for measurement of blood pressure and withdrawal of blood samples.

In all animals, a medial sternotomy was performed to expose the heart and pericardial sac. A soft infusion catheter was placed into the sac near the apex of the heart for perfusion of the control saline or DHA (45 mg, ~1 mg/kg) solutions. The animals were fully heparinized following surgical preparation and throughout the subsequent experimental protocol (300 IU/kg intravenous bolus of heparin followed by an infusion of 67 IU/kg/hr). After 60 min stabilization following completion of surgery, a balloon was inflated or a vascular clamp was placed to induce ischemia. To verify the effectiveness of occlusion, microspheres (E-Z TRAC 15 μm diameter blue ultraspheres, Interactive Medical Technologies, Irvine, CA, USA) were injected via the guide catheter into the left atria 30 min

after inflation of a balloon or placement of a clamp to block the artery flow. The balloon was deflated or the clamp was removed after 45 min ischemia. The auto-reperfusion (physiologic reperfusion) period lasted 180 min. DHA (Cayman Chemical Co., Ann Arbor, MI, USA) was obtained as a stock solution of 250 mg DHA/ml ethanol and diluted to 3 mg/ml in 0.9% saline solution for subsequent pericardial infusion. The reason to choose DHA in our study is that DHA is present in all organs and is the most abundant n-3 PUFA in membranes, including in heart tissue (1). The control animals received the same amount of the saline solution plus the equivalent amount of the vehicle (ethanol). Blood pressures, heart rates, left and right intraventricular pressures, dP/d*t*s, and electrocardiograms were recorded during experiments to monitor hemodynamics, cardiac function and rhythm. Data were acquired with SonoLAB software (Sonometrics, London, Ontario, Canada). Post-acquisition analysis was performed using both CardioSoft software and datanalys software (EMKA Technologies, Falls Church, VA, USA).

Measurement of infarct size and risk area

At the end of each experiment, the LAD was re-occluded (i.e., the arterial balloon was reinflated or the clamp was repositioned) and patent blue dye was injected into the left atrial appendage to verify the ischemic area (area-at-risk) as previously described (8, 26). Briefly, the animals were sacrificed and the hearts were removed and frozen at -20°C. Infarction was determined on the next experimental day. The hearts were sectioned to 4 mm transverse slices. The

slices were then incubated with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) at 37°C for 10 min, and fixed in 10% formalin for 1 min. Triphenyltetrazolium chloride forms a red formazan derivative when reacting with viable tissue, whereas necrotic tissue is pale white. A person blinded to the applied treatments photographed the slices and quantified the morphology of infarct size and area-at-risk of the left ventricles using Adobe Photoshop (CS version 8.0, Adobe Systems, Inc., San Jose, CA, USA). In addition, microspheres disposed from homogenized infarct, area at risk and non-risk left ventricular tissues were counted under microscope (8, 26). The results were 0.8 ± 0.5, 1.0 ± 0.7 and 98.3 ± 31.0 for infarct, area at risk and non-risk, respectively. These results indicate the effectiveness of LAD occlusions.

Arrhythmia assessment

A standard peripheral, five lead electrocardiogram was used to monitor arrhythmias during each experiment (Model 9030, SpaceLabs Inc., Redmond, WA). Analysis using the Ponemah Physiology Platform Version 3.1 software (Gould Instrument Systems; Valley View, OH, USA) was completed by a person who was blinded to the experimental treatment. A modified scoring system (8, 26) was used to quantify arrhythmias by an individual blinded to the treatment: 0 = ≤10 premature ventricular contractions (PVCs) in 9 min; 1 = 11 to 50 PVCs in 9 min; 2 = >50 PVCs in 9 min; 3 = 1 episode of ventricular fibrillation (VF) in 9 min; 4 = 2-5 episodes of VF in 9 min; and 5 = >5 episodes of VF in 9 min. Ventricular fibrillation was treated using 30-J shocks administered via internal

paddles with biphasic DC cardioversion (Lifepak 12, Medtronic, Inc., Minneapolis, MN, USA) and repeated if necessary.

Fatty acid measurement

Two ml blood samples were drawn after ceasing pericardial solution perfusion. Pericardial fluid (1.5 ml) and right atrial tissues (5–10 g) were collected at the end of each experiment before patent blue dye was injected into the left atrial appendage. These samples were stored at -80°C immediately after collection. Analysis was performed by a laboratory (Lipid Technologies, LLC, Austin, MN, USA) in blinded fashion. Plasma, pericardial fluid, and atrial tissue lipids were extracted and analyzed according to methods described previously (17).

Data analysis

Data are presented as mean ± SE. The unpaired Student's *t*-test and Chi-square test were applied. Statistical significance was considered if a p value was < 0.05 between two means.

Results

After an operation, each animal was stabilized in the first 20 min plus additional 40 min with pericardial solution delivery (inset, Fig. 1A, 1B). The pericardial space of each animal received a 2-min injection (bolus injection) of 6 ml saline solution contained either DHA for the treatment or the same amount vehicle (alcohol) for the control. Then, pericardial infusion started and lasted 90 min with a speed of 0.1 ml/min. Each DHA-treated animal received 45 mg DHA in 15 ml solution (3 mg/ml). Mean blood pressures in both groups averaged ~100 mmHg during stabilization and gradually decreased after initiation of ischemia and reperfusion (Fig. 1A). The recorded time courses showed that pericardial bolus infusions induced increases in heart rates in control animals, but not in DHA-treated pigs (Fig. 1B). LAD occlusions further increased the heart rates in the controls (Fig. 1B, $p < 0.01$). With the exception of the heart rates, the maximal left ventricular systolic pressures, the left ventricular end diastolic pressures, the maximal rate of positive and the negative left ventricular pressure changes (±dP/d*t*), and the cardiac outputs displayed no significant differences among DHA-treated and untreated animals.

Xiao et al.,
Figure 1

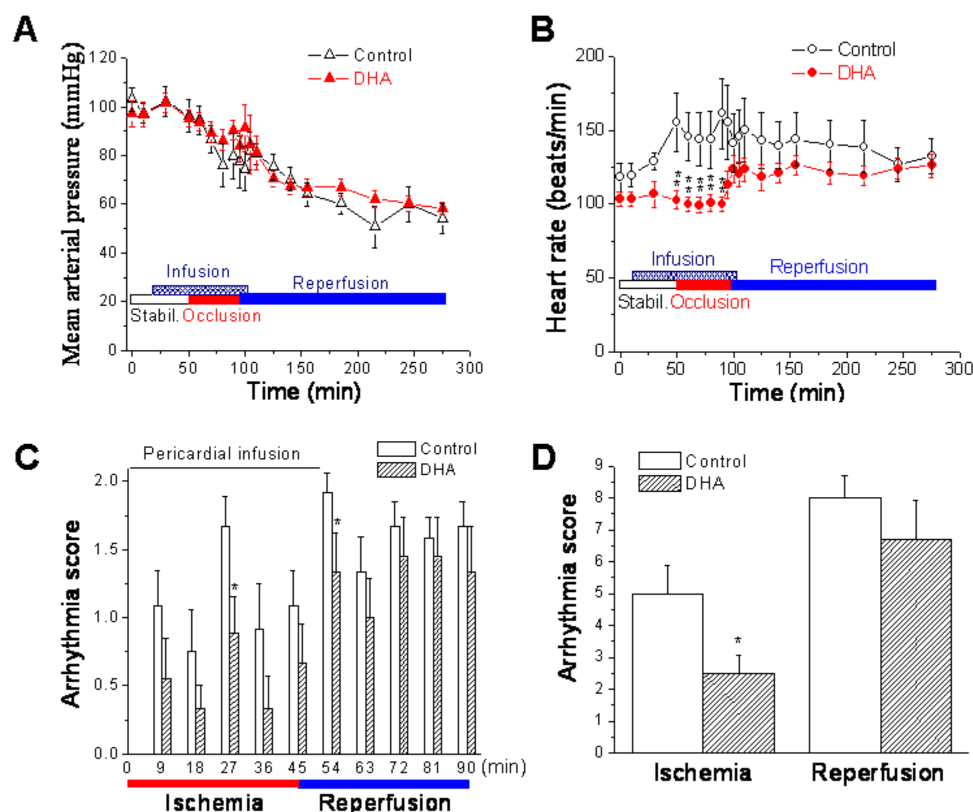


*Figure 6.1 - Effects of pericardial infusion of DHA on blood pressure, heart rate and arrhythmia score in myocardial ischemia/reperfusion pigs. Compared to controls, the DHA-treated animals did not significantly alter blood pressures (**A**), but significantly lowered heart rates (**B**) and arrhythmia scores (**C**,**D**) during pericardial infusion. Panel **C** shows the means of the arrhythmia scores every 9 min during ischemia and reperfusion of the first 45 min. Panel **D** shows the means of the total accumulated arrhythmia scores during ischemia and the 1st 45 min reperfusion. Infusion in panel **A** and **B** was the duration of pericardial perfusion with the control or DHA solution. Stabil., stabilization. *, $p < 0.05$; **, p <*

0.01; versus control.

The arrhythmia scores determined during 45 min of LAD occlusion and the first 9 min of reperfusion were markedly lower in DHA-treated pigs than in controls (Fig. 1C). The pericardial infusions of DHA significantly reduced the sum of accumulated arrhythmia scores during occlusion (Fig. 1D-Ischemia, $p < 0.05$), but not during the first 45-min reperfusion (Fig. 1D-Reperfusion, $p > 0.05$). Importantly, three out of twelve control animals elicited 8 episodes of ventricular fibrillation (VF) during reperfusion. Applied electric defibrillation initially restored their cardiac function, but in two animals secondary episodes of VF were occurred 2 hr after reperfusion. Defibrillation failed to restore an effective rhythm (Fig. 2). In contrast, only one out of eleven DHA-treated pigs showed an episode of VF during reperfusion. In this animal electric defibrillation successfully restored the heart rhythm without further reoccurrence of VF (Fig. 2). Thus, the overall morbidity from VF was decreased from 25% in the controls to 9% in the DHA-treated animals (Fig. 2C, $p < 0.01$); most of these VF episodes occurred within 15 min following reperfusion. The overall animal mortality was reduced from 17% in the controls to 0% in the DHA-treated pigs (Fig. 2D, $p < 0.01$).

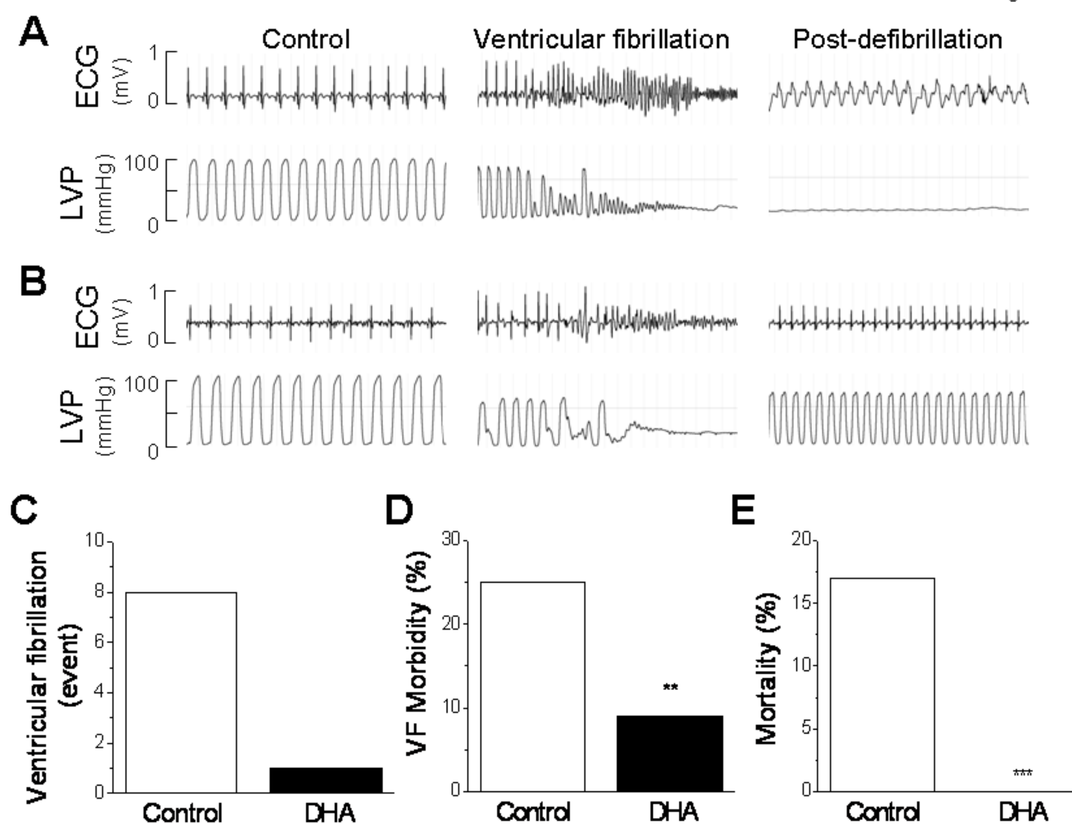


*Figure 6.2 - Effects of pericardial infusion of DHA on ventricular fibrillation events, morbidity of ventricular fibrillation, and mortality in myocardial ischemia/reperfusion pigs. The original Lead-II ECG and left ventricular pressure traces from a control (**A**) and a DHA-treated (**B**) pigs are shown. The recordings were taken during stabilization (Control) and coronary reperfusion (Ventricular fibrillation and post-defibrillation). Electric defibrillation restored heart function of the DHA-treated pig, but failed in the control animal. Panels **C**, **D** and **E** show the events of VF, morbidities of VF and mortalities in control and DHA-treated*

*animals. **, p < 0.01; ***, p < 0.001; versus control.*

The animals treated with pericardial DHA-perfusion had significantly lower infarct sizes compared to controls (Fig. 3). Importantly, the areas-at-risk for ischemia of the left ventricle for controls was 21.2 ± 2.3% (n = 12) and this was not significantly different from that for DHA-treated animals which was 24.2 ± 2.5% (n = 11, $p > 0.05$). Nevertheless, DHA treatment significantly reduced the resultant infarct sizes (Fig. 3, $p < 0.01$).

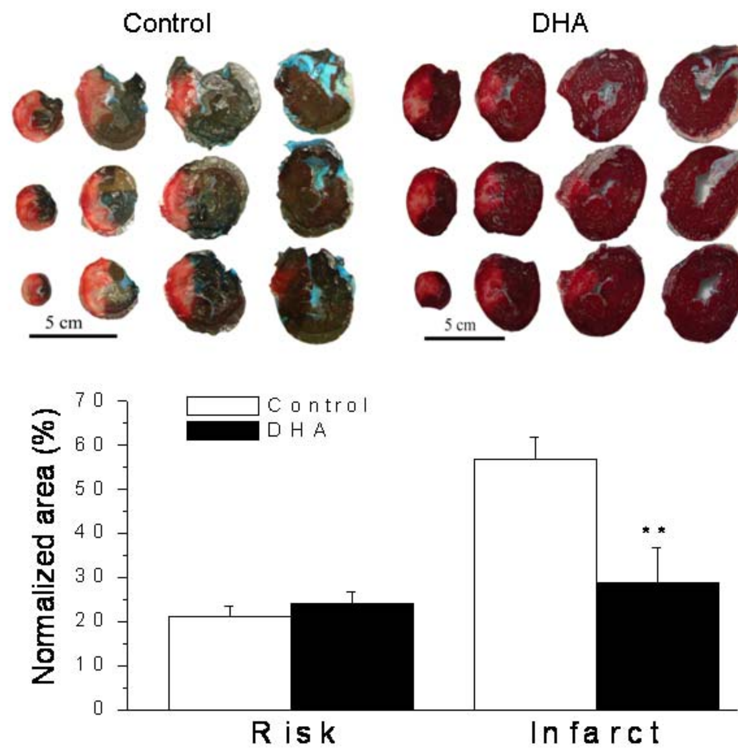


*Figure 6.3 - Histological comparison of porcine myocardium between control (Control) and DHA-treated animal (DHA). Top panels show the slices of the left ventricles from one control (left) and one DHA-treated (right) animals. Ischemic area was red (area at risk) after triphenyltetrazolium chloride (TTC) staining and the necrotic area was pale white after 10% formalin fixation. Low panel shows that the normalized values (risk = ischemic area/left ventricular area × %) of area-at-risk were similar between control and DHA-treated groups, but the normalized infarct size (infarction = infarct area/ischemic area × %) was significantly reduced, from 56.8 ± 4.9% for the controls to 28.8 ± 7.9% for DHA-treated animals. **, $p < 0.01$; versus control.*

To determine the effects of pericardial perfusion of DHA on fatty acid components during ischemia and reperfusion, we collected blood, pericardial fluid, and atrial tissue at various time points. Table 6.1 summarizes the results of fatty acid components in the collected samples. The data show that DHA concentrations varied among different tissues and also between the control and DHA-treated groups, but the differences were not significant ($p > 0.05$). The contents of n-3 and n-6 PUFAs, as well as monounsaturated and saturated fatty acids for control samples were also not significantly different from those for DHA-treated tissues (table 6.1).

Table 6.1 - Effects of pericardial infusion of DHA on fatty acid components of plasma, pericardial fluid, and atrial tissue

	DHA	EPA	n-3	n-6	Monounsaturated	Saturated
Blood plasma						
Control	3.8 ± 1.9	1.2 ± 0.5	14.9 ± 5.3	99.3 ± 31.1	252.8 ± 73.3	250.1 ± 32.5
DHA-treated	1.7 ± 0.8	1.5 ± 0.5	7.6 ± 2.6	54.7 ± 17.1	96.8 ± 32.3	119.6 ± 36.6
Pericardial fluid						
Control	6.6 ± 1.1	1.8 ± 0.2	18.1 ± 2.4	220.7 ± 5.9	190.6 ± 17.2	168.2 ± 19.9
DHA-treated	8.0 ± 2.2	2.5 ± 0.8	18.7 ± 4.2	206.0 ± 34.0	157.9 ± 23.6	149.5 ± 23.6
Atrial tissue						
Control	2.3 ± 0.4	1.1 ± 0.2	9.1 ± 0.6	117.3 ± 9.5	324.9 ± 50.3	326.1 ± 49.9

DHA-treated	2.9 ± 0.2	1.4 ± 0.2	11.0 ± 1.1	130.9 ± 8.9	360.5 ± 61.7	366.2 ± 48.7

Values are expressed as means ± SE of μg fatty acid per ml blood plasma and pericardial fluid of the control (n = 3) and DHA-treated (n = 4) animals, and mg fatty acid per 10 g atrial tissue of the control (n = 3) and DHA-treated (n = 6) animals. Blood samples were collected 30 min after termination of pericardial solution perfusion. Atrial tissue and pericardial fluid samples were collected at the end of each experiment. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; n-3, total amount of n-3 polyunsaturated fatty acids; n-6, total amount of n-6 polyunsaturated fatty acids; Monounsaturated, total amount of monounsaturated fatty acids; Saturated, total amount of saturated fatty acids.

Table 6.2 - Cardiac functions in control and DHA-treated groups

		Ischemia		Reperfusion		
	Preischemia	20 min	40 min	60 min	120 min	180 min
LVSP (mmHg)						
Control	107.3 ± 4.9	93.5 ± 6.7	91.1 ± 8.5	79.1 ± 3.4	68.0 ± 7.7	72.0 ± 3.8
DHA	109.4 ± 3.2	101.3 ± 3.7	98.4 ± 6.1	82.4 ± 4.5	80.2 ± 4.6	76.3 ± 2.5
LVEDP						

(mmHg)						
Control	6.9 ± 1.0	8.5 ± 1.9	8.6 ± 1.9	7.5 ± 2.0	8.6 ± 1.5	7.4 ± 1.8
DHA	8.1 ± 0.9	9.3 ± 1.3	9.0 ± 1.3	8.0 ± 1.2	7.8 ± 1.4	7.7 ± 1.5
LV dP/d*t*$_{max}$ (mmHg/s)						
Control	1683 ± 228	1389 ± 185	1499 ± 254	1163 ± 135	946 ± 55	920 ± 79
DHA	1327 ± 92	1229 ± 89	1465 ± 238	1104 ± 65	1042 ± 83	1070 ± 85
RVSP (mmHg)						
Control	26.3 ± 1.2	24.9 ± 1.1	23.2 ± 1.2	23.3 ± 0.9	21.5 ± 1.6	22.3 ± 1.0
DHA	20.8 ± 1.9	19.8 ± 1.6	21.8 ± 2.7	19.4 ± 2.0	20.8 ± 2.3	21.8 ± 2.3
RVEDP (mmHg)						
Control	3.9 ± 1.1	3.8 ± 1.3	3.7 ± 1.3	3.4 ± 1.4	3.9 ± 1.0	3.0 ± 0.9
DHA	3.4 ± 0.3	3.4 ± 0.3	3.7 ± 1.0	3.1 ± 0.5	3.1 ± 0.4	2.9 ± 0.4
CO						
(L/min)						
Control	4.26 ± 0.61	3.76 ± 0.22	3.76 ± 0.37	3.10 ± 0.32	2.23 ± 0.30	2.38 ± 0.20
DHA	4.02 ± 0.34	3.46 ± 0.22	3.44 ± 0.29	3.16 ± 0.30	3.13 ± 0.44	2.92 ± 0.32
MPAP						
(mmHg)						
Control	13.9 ± 1.7	13.4 ± 1.5	11.8 ± 1.9	10.4 ± 1.4	12.7 ± 1.6	12.8 ± 1.7
DHA	10.5 ± 1.2	10.6 ± 0.9	10.8 ± 2.2	10.6 ± 1.5	11.4 ± 1.4	11.8 ± 1.2

Values are means ± SE. Animals were treated with either DHA or vehicle (Control). LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, the maximum first derivative of LV pressure; RVSP, right ventricular systolic pressure; RVEDP, right ventricular end-diastolic pressure; CO, cardiac output; MPAP, mean pulmonary artery pressure.

Discussion

Millions of patients die annually from sudden cardiac death in the world. More than half of them occur within one hour following an acute myocardial infarction and this is associated with sustained ventricular arrhythmias. In this study, we report that compared to control animals, pericardial infusion of DHA at a time before and during ischemia significantly reduced infarct sizes and ventricular arrhythmias. Reduction of myocardial injuries and/or induced arrhythmias has been reported for rats (34, 35), rabbits (23) and humans (6), but this has been associated with chronic dietary intake of n-3 PUFAs. One advantage of a dietary approach is non-invasive and n-3 PUFAs can be absorbed and transported to heart cells. Membrane-stored n-3 released promptly to perform their antiarrhythmic action (20). However, the process of ingested n-3 PUFAs to having cardioprotection is more complicated than intravenous or pericardial perfusion (20). Many factors may affect dietary PUFAs absorption, transportation, membrane storage and release from heart cells. In addition, accumulation of these ingested compounds in heart cells to a cardioprotective level may take weeks (27, 36). Also, myocardial concentration of accumulated n-3 PUFAs with dietary approach can be quite variable and unpredictable in a given individual. Here, we report that a novel delivery approach with dosed DHA to the pericardial space have a significant cardioprotective effect.

Previously, the antiarrhythmic effects of intravenous infusion of DHA or EPA were assessed in an ischemic canine model (4). In that study n-3 PUFAs had to

be mixed with albumin before infusion as infused DHA or EPA causes hemolysis. Unfortunately, infused albumin would expand the intravascular volume acutely, which might in itself induce acute congestive heart failure in a subject with an already compromised cardiovascular system (4). Importantly, our described pericardial approach does not require additional albumin administration and thus would help avoid the serious consequences mentioned above. The amount (45 mg) of DHA employed in our present study is only 5.2% of the dose (860 mg) of that used in the aforementioned canine study (4) and 1.2% of the dose (3.8 g) used in a clinical study that blood infusion of n-3 PUFAs results in reductions of sustained ventricular tachycardias in those patients at high risk of sudden cardiac death (25). Thus, the pericardial approach can maximize the protective effects of n-3 PUFAs and minimize systemic side effects, such as hemolysis (4).

Interestingly, the reductions of the arrhythmia scores calculated every 9 min during occlusion did not reach statistical significances except one at the third 9-min data point (Fig. 1C), but the sum of the individual 9-min arrhythmia scores during occlusion was significantly reduced in DHA-treated animals (Fig. 1D). However, the sum of the arrhythmia scores during the initial 45-min reperfusion was not significant between two groups (Fig. 1D). The significant reduction of arrhythmia score only occurred during the 1st 9 min after cessation of DHA perfusion (Fig. 1C). These results indicate that the DHA effects subsided after termination of DHA perfusion. In addition, the heart rates were significantly lower in DHA-treated animals during occlusion. These results are consistent with the

previous observations that intravenous infusion of n-3 fatty acids evoked significant reductions in heart rates both before and during the coronary occlusion (3). As the blood samples were collected 30 min after cessation of DHA perfusion and as the atrial tissues and pericardial fluids were sampled at the end of experiments, DHA levels in these samples were not significantly different between control and DHA-treated groups (table 6.1). As the effects of DHA were quickly on and off during this acute study, it may act more likely as a pharmacological agent which may differ from dietary supplements of n-3 PUFAs. A possible explanation is that as DHA is highly lipophilic the infused DHA in the pericardium might rapidly diffuse and dilute into myocardium, blood, and other intra- and extra-cellular fluids. As the incidence of VF and mortality were significantly lower in DHA-treated animals (Fig. 2), the cardioprotective effect of DHA was most likely extended to the whole reperfusion period. This is consistent with the dramatic reduction of infarct sizes in DHA-treated animals (Fig. 3). However, the arrhythmia scores during reperfusion were not significant between two experimental groups (Fig. 1). The underlying mechanism of such differences is unknown, but the parameters measured in this study may reflect their different sensitivities to cell damages during and after ischemia.

While the precise mechanisms how n-3 PUFAs protect the heart from ischemic damage need to be further investigated, previous cellular studies suggest that n-3 PUFAs could suppress inflammation (7, 32), reduce platelet aggregations (37), decrease resultant ischemic oxidative injury (12), and/or inhibit apoptotic signals

(11). A recent study reported that in rabbits, dietary-fed EPA reduced subsequent induced myocardial infarct sizes via the activation of K_{Ca} channels and NO-mediated mechanisms (23). It has been noted that inhibition of Ca^{2+} and Na^{+}/Ca^{2+} exchanger currents by EPA or DHA prevents an increase in intracellular Ca^{2+} concentrations which when elevated are likely main cause of cell death during ischemia (30, 33). However relative to this latter finding, the contractility of cardiomyocytes is not affected in the presence of n-3 PUFAs (19). Reducing cardiomyocyte damages by n-3 PUFAs from free radicals generated during ischemia or hypoxia have also been reported in other studies (5, 29).

The direct actions of n-3 PUFAs on cardiac ion channels may explain their antiarrhythmic effects (33). The n-3 PUFAs have been shown to inhibit voltage-gated Na^{+}, K^{+} and Ca^{2+} channels, as well as Na^{+}/Ca^{2+} exchangers (33) and Ca^{2+}-activated K^{+} channels (23). These combined effects would serve to stabilize the membrane excitability of cardiomyocytes (13). It has been considered that the cardiomyocytes in an ischemic border zone have relatively depolarized resting membrane potentials and possibly trigger ventricular fibrillation due to easily being excited (19). Elevation of n-3 PUFAs stabilizes excitability of partially depolarized cells (33) and thus prevent arrhythmias. This hypothesis is supported by the recent finding that dietary fish oil reduces the occurrence of early afterdepolarizations in porcine ventricular myocytes (10). Although there is no clinical report on pericardial delivery of n-3 PUFAs, such approach is potentially beneficial to an ischemic heart and may prevent sudden cardiac death in the

event of an infarction. In summary, our current data demonstrate that pericardial infusion of the n-3 PUFA DHA significantly reduces infarct sizes and malignant arrhythmias (including ventricular fibrillation) in an ischemia/reperfusion porcine model.

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Chapter 7: Intrapericardial delivery of metoprolol: A novel treatment for post-operative arrhythmias?

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Introduction

Anti-arrhythmic drugs are known for their narrow therapeutic range and harmful side effects. Intrapericardial (IP) delivery of anti-arrhythmic drugs has been proposed to achieve high myocardial concentrations with minimal plasma concentrations. Low plasma concentrations would prevent systemic side effects, a common problem with anti-arrhythmic drugs. IP delivery also has the benefit that the concentration at the target site can be more precisely controlled. Several anti-arrhythmic drugs have narrow windows of therapeutic concentrations, bordered by concentrations that cause adverse effects or no effects at all. In oral and IV delivery, concentration at the target cell must be extrapolated based on several assumptions, typically producing poor control of concentration in the heart tissue. IP delivery is much more desirable when precise control is needed.

IP delivery of anti-arrhythmic drugs has been attempted recently with some success. Esmolol[1], sotalol[2], atenolol[2], ibutilide[3], procainamide[4, 5], digoxin[4], amiodarone[6], arachadonic acid[7], nitroglycerin[8], and L-arginine[9] (a nitric oxide precursor) have been administered IP to prevent or treat arrhythmias in animal models. All of these studies, with one exception[2], have been acute. Many studies verify that high pericardial concentrations can be obtained with minimal plasma concentrations[3, 5, 6]. Prevention of dangerous ventricular arrhythmias has been shown with nitroglycerin[8], arachadonic acid[7], and L-arginine[9]. Studies with less lipid soluble compounds[5, 6] observed high concentrations in superficial structures

such as the atria and the ventricular epicardium, but lower concentrations in the ventricular endocardium. Some have exploited this concentration gradient to treat the superficial SA node with beta-blockers[12], assuming that there is little penetration into the ventricles. The concentration gradient can also be used to target atrial fibrillation[3, 5, 6] with little ventricular effect.

Despite the several benefits of IP delivery, most investigators appear to be somewhat cautious relative to this approach, perhaps due to unknown potential complications. For example, underlying aspects of cardiac physiology may be effected by IP delivery, particularly the cardiac autonomic nervous system[7]. Perhaps the major concern of researchers, however, is unknown effect of the concentration gradient of less-soluble drugs across the ventricular myocardium. If the electrophysiology of the epicardium is affected by the drug but endocardium is not, this may produce arrhythmias instead of inhibiting them[5]. This is not only a concern with anti-arrhythmic therapies, but with any agent delivered IP that may have electrophysiological effects.

The objective of this study was to assess the efficacy of intrapericardial metoprolol and to compare this efficacy to the current standard, intravenous delivery. Metoprolol was chosen because of its moderate lipid solubility, likely causing a gradient across the ventricular myocardium. In addition, we feel that the most likely initial application of intrapericardial delivery will be in the post-operative setting. Placement of an intrapericardial catheter would be trivial in this setting, and the catheter could be removed before discharge from the hospital.

Post-operative arrhythmias are extremely common, and metoprolol is often used in the event of supraventricular tachyarrhythmias (SVTs). However, metoprolol has an adverse effect on cardiac contractility, which is particularly dangerous to the recovering cardiac surgical patient. We hypothesized that intrapericardial delivery of metoprolol would be effective in treating post-operative SVTs without effecting contractility.

Methods

Twenty-one male Yorkshire swine (70-90kg) were sedated with Telazol (500mg) and Thiopental (500mg). The animals were then intubated and maintained on isofluorane (>1.2 MAC). An arterial line was placed in a branch of the femoral artery, and a 5-lead EKG monitored heart rhythm. Cut-downs were performed to expose the jugular vein and carotid artery. A swan-ganz catheter (Hospira, IL) was introduced through the jugular to measure cardiac output, right atrial pressure and pulmonary artery pressure. A pressure catheter (Millar Instruments, TX) was introduced into the left ventricle (LV) through the carotid to measure LV performance. A medial sternotomy was performed on all animals, and a small incision was made on the anterior surface of the pericardium. The pericardium was then "tented" with suture to form a reservoir for the drug, as further described by Ayers et al.[6] and shown in figure 7.1. All surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

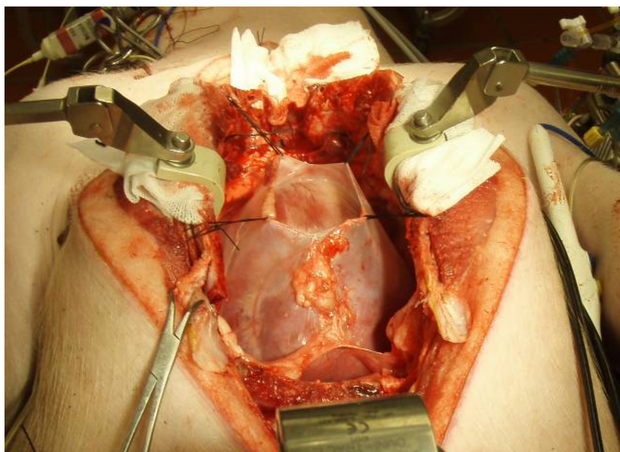

Figure 7.1 - Pericardial sac tented with suture after a medial sternotomy.

Animals underwent one of three protocols after stabilization. The first group (n=8) received a slow infusion of isoproterenol (0.5-1.0 mcg/min) through the distal port of the swan-ganz catheter which was titrated until the heart rate was over 180 bpm. This infusion was kept constant for the remainder of the study. It has been shown (ref) that this is a reliable model of sinus tachycardia which can be sustained for hours in dogs. Additional electrodes were placed on the chest and limbs to measure a full 12-lead EKG. After the heart rate stabilized, a bolus of 20mg of metoprolol (1mg/ml standard IV injection, Bedford Labs, OH) was given IP or IV. When metoprolol was given IV, 20ml of saline was given into the pericardial space. Hemodynamic and 12-lead EKG data were taken prior to and up to one hour after the bolus of metoprolol. Samples of 500 μl were drawn from the pericardial space at -5, 5, 10, 20, 30, 45, and 60 minutes. Blood samples (5ml) were drawn from the arterial line at -5, 2, 5, 10, 15, 20, 30, 45, and 60 minutes. HPLC was used to analyze the content of metoprolol in both plasma

and pericardial fluid using previously published techniques (reference needed).

The second group (n=8) received additional instrumentation to study refractoriness and susceptibility to atrial fibrillation. Fluoroscopy was used to place endocardial pacing leads (Medtronic 3830 Bipolar Pacing Lead, MN) in the high lateral right atrium and the right ventricular apex. These leads were connected to a GEM III defibrillator (Medtronic, MN). A Medtronic 2090 programmer/analyzer was used to control the defibrillator during the measurement of refractory periods. Epicardial pacing leads (Medtronic, MN) were also placed on the left atrial appendage (LAA) and the left ventricular (LV) epicardium. A decapolar electrophysiology catheter (Medtronic, MN) was plunged through the LV myocardium to record activation times at different depths of the myocardium. Any changes in the delay between endocardial and epicardial activation times could indicate that the drug is affecting the myocardium unevenly. The signals from the programmer, epicardial pacing leads, decapolar catheter, and EKG leads were recorded simultaneously by an ENSITE electrophysiology system (St. Jude Medical, MN).

After instrumentation, control electrophysiology data was taken. Atrial effective refractory periods (AERP), ventricular effective refractory periods (VERP), and AV nodal effective refractory periods (AVNERP) were measured following a standard protocol explained by Ayers et al[6]. Susceptibility to atrial fibrillation was measured by delivering 2-second, 50Hz burst pace of 2-msec, 20V pulses to the LAA using a stimulator (GRASS Technologies, RI). This was done three times,

each time measuring the duration atrial fibrillation. If the episode was sustained (meaning that it lasted longer than one minute), the animal was defibrillated with a 5J epicardial shock (Medtronic Lifepak, MN). Finally, to measure hemodynamic stability during AF, a 30-second burst pace in the LAA maintained the animal in AF while hemodynamic measurements were taken.

After the control data was taken, animals received 20 mg of metoprolol (1mg/ml standard IV formulation) in the pericardial space (n=3), intravenously (n=3), or they received 20ml of saline (n=3) in the pericardium. Saline controls were used in this group because it was uncertain how the electrophysiological parameters would change over the course of the study. Blood samples and pericardial samples were taken throughout the study as previously described. Electrophysiology parameters were taken at 5, 30, and 60 minutes after drug delivery.

In the final group, metoprolol was given IP (n=20 or IV (n=2) and blood and pericardial fluid samples were taken as previously described.

Results

Metoprolol in Sinus Tachycardia

After both IP and IV delivery of metoprolol, HR was lowered significantly to 70% and 73% of control rate (respectively). While the onset of action was slightly slower in IP delivery, the effect was considerably lengthened (figure 7.2). The effect of IV metoprolol was considerably reduced after one hour.

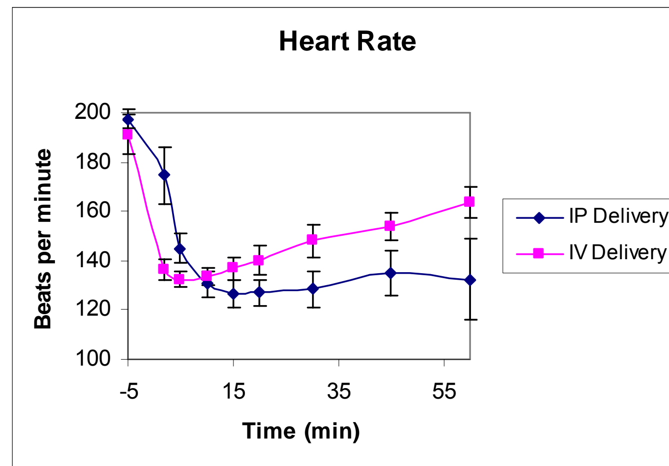

Figure 7.2. The effect of metoprolol on heart rate, delivered IV and IP, in a model of sinus tachycardia.

Ventricular contractility (as measured by dP/dt) was significantly reduced in IV-treated animals to 73% of the control measurement, but returned to baseline after one hour. In contrast, contractility was never significantly affected in IP treated animals (figure 7.3). Likewise, mean arterial pressure (MAP) was lowered considerably during IV delivery, but was nearly unaffected during IP delivery (figure 7.4).

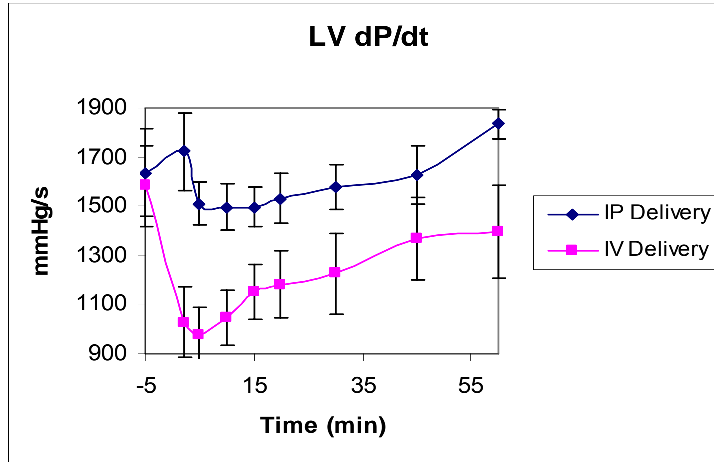

Figure 7.3 - The effect of metoprolol, delivered IP and IV, on ventricular contractility.

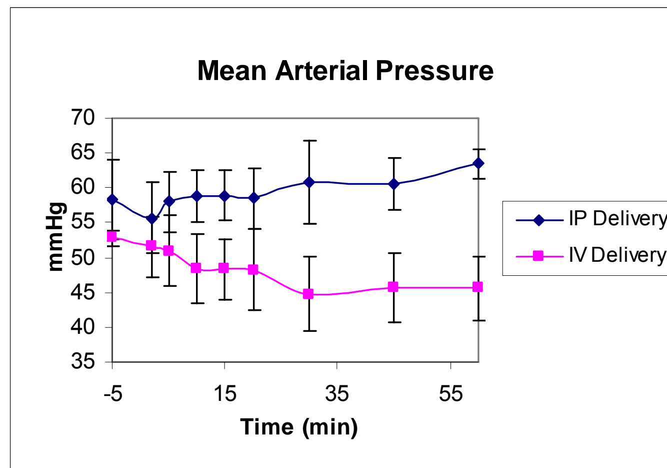

Figure 7.4 - The effect of metoprolol, delivered IP and IV, on mean arterial pressure (MAP).

No significant differences between the groups were seen in cardiac output, and

PR and QRS intervals.

Refractoriness and Susceptibility to Atrial Fibrillation

Electrophysiology measurements were highly variable, so no significant trends were seen with regards to AVNERP, VERP, or ability to induce AF. AV node physiology and perhaps the presence of AV node “gap” made it difficult to measure the expected increase in AV node refractoriness with intravenous delivery. A His catheter was placed in the animals to elucidate AV node function, but His potentials were difficult to measure. Transmural activations times, measured as the difference in activation time between the most epicardial and most endocardial electrode, did not significantly change in any of the groups.

There were slight trends in atrial electrophysiology, however. Inter-atrial conduction times increased in saline control animals, but remained closer to their control values in IV and IP treated animals. Similarly, AERPs decreased in saline-treated animals, but remained the same in IP and IV treated animals (figures 7.5 and 7.6).

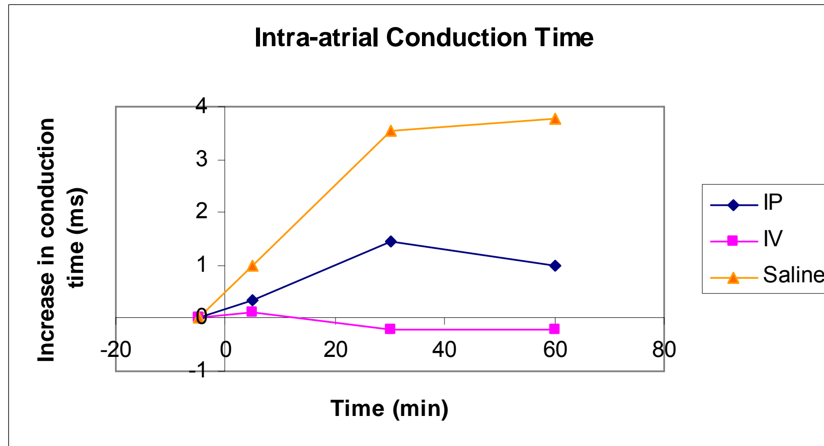

Figure 7.5 - Changes in inter-atrial conduction times after IP metoprolol, IV metoprolol or IP saline delivery.

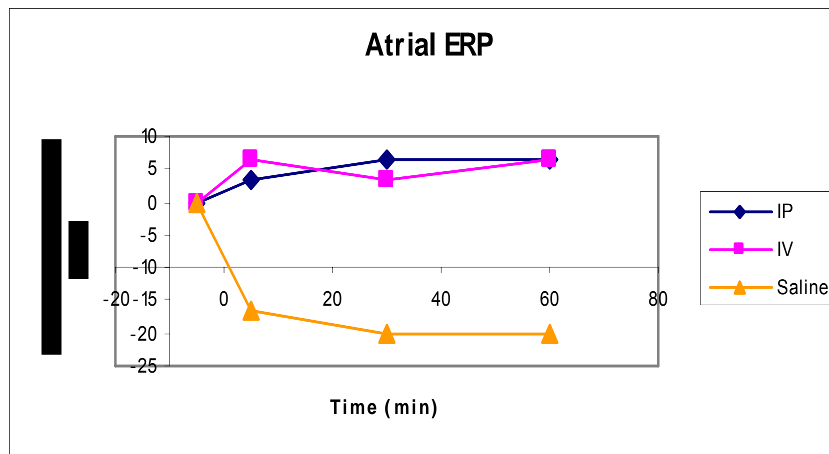

Figure 7.6 - Changes in atrial effective refractory period (AERP) after IP metoprolol, IV metoprolol, or IP saline delivery.

Pharmacokinetics

When given IP, the elimination half-life of metoprolol in the pericardial fluid was 18.1 minutes, with negligible amounts observed in the plasma (figure 7.7). When

given IV, the elimination half life of metoprolol in the plasma was 11.1 minutes, with negligible amounts in the pericardial fluid (figure 7.8). Pericardial fluid drug concentrations during IP delivery were approximately 1,000-fold higher than plasma drug concentrations during IV delivery. Samples from only 8 of the 21 studies have been analyzed thus far. Samples from the remaining 13 studies will be done in the next week. After the data is collected, a pharmacokinetic model will be fit to the data, and added to the results section.

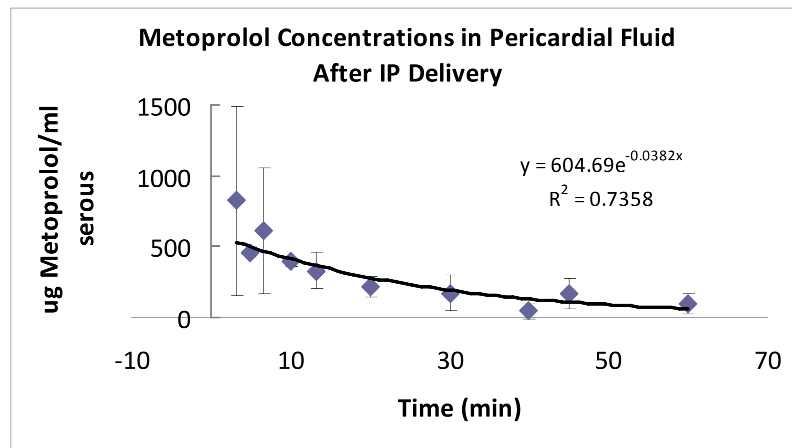

Figure 7.7 – Metoprolol concentrations in pericardial fluid after IP delivery

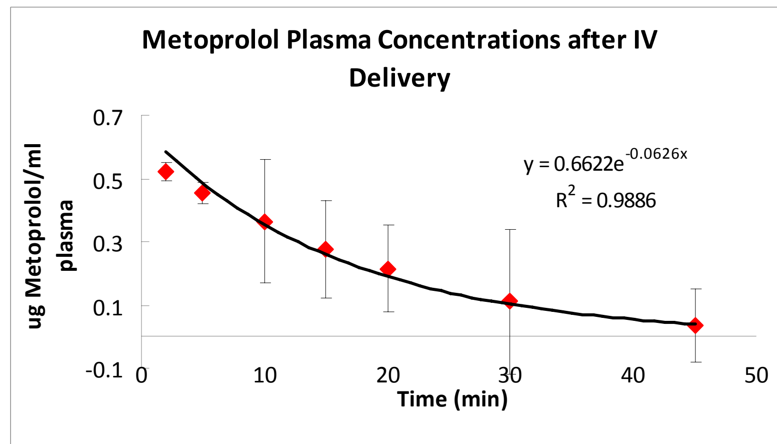

Figure 7.8 – Metoprolol concentrations in plasma after IV delivery

Discussion

Intrapericardial delivery of metoprolol effectively lowers the heart rate during sinus tachycardia for a sustained period of time without affecting ventricular contractility or mean arterial pressure. With regards to refractoriness, the small sample size and high variability make it hard to draw conclusions from our data. The prevention of change in both inter-atrial conduction time and atrial refractoriness may or may not be clinically significant. Activations times did not change across the myocardial wall, which may suggest that transmural concentrations gradient will not induce ventricular arrhythmias, though more complete mapping should be pursued. It appears, therefore, that IP metoprolol might best be suited for sinus tachycardia and not necessarily atrial fibrillation. The AV node is not as superficial as the SA node, so IV delivery may accomplish rate control better than IP delivery.

A small catheter placed within the chest after cardiac surgery to delivery drugs into the pericardium would add minimal time and cost to the procedure. Given that the pericardium is almost always left open or removed completely during cardiac surgery, a wicking material or other mechanism to maintain the drug close to the targeted area could be incorporated into the catheter. Excess drug, particularly if the drug is constantly infused, could be drained by the chest tubes. This delivery would be similar to mediastinal irrigation of antibiotics that is often performed for mediastinitis.

Several important limitations must be considered. The metoprolol delivered IP in

this study was an IV formulation. Eventually, the formulation must be optimized for IP delivery, accounting for characteristics such as pH. Also, the dose in this study was chosen to be the same as standard clinical IV dose. Further dose-response studies are needed to optimize the dosage. As previous studies have shown (ref), *concentration* of the drug may be more important than the *amount* of drug given into the pericardium. In spite of the remaining work, pericardial delivery of metoprolol in the post-operative setting may prove to enhance the efficacy of the drug while mitigating unwanted side effects.

Conclusions and Future Work

Intrapericardial delivery can increase the effectiveness of certain anti-arrhythmic agents while decreasing side effects. I have come to this conclusion after:

- Completing background research and basic electrophysiology experiments to understand the function of anti-arrhythmic agents,
- Selecting appropriate clinical settings for intrapericardial delivery after talking with several physicians and scientists,
- Assessing and developing tools to deliver agents into the pericardium,
- Developing animal models of arrhythmias,
- Developing analytical chemistry techniques to measure pharmacokinetics, and finally
- Designing and carrying out three large animal studies to determine the efficacy and safety of intrapericardial delivery.

There is, however, much work to be done before this would be ready for clinical implementation. Much of this work will be done in at the Visible Heart Lab. This work includes:

- Development of better devices to deliver drugs into the pericardium,
- Refinement of intrapericardial drug formulations,

- Continuation of large animal studies with other potential agents, and finally,
- Carefully planned and controlled clinical trials.

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