

Evaluation of the Sodium Calcium Exchange Inhibitor
2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline as a Cardioplegic Additive

by

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for the degree of Master of Science

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ABSTRACT

Arresting the heart with cardioplegia solution is the usual strategy to protect the myocardium during cardiac surgery. However, ischemia-reperfusion injury, due in part to Ca^{2+} overload, remains a clinical problem. Ca^{2+} influx during ischemia occurs through reverse mode action of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. We therefore tested the hypothesis that delivering the $\text{Na}^+/\text{Ca}^{2+}$ exchanger blocker SEA0400 to a cardioplegia solution would result in superior myocardial protection during ischemic-cardioplegic arrest. Studies were performed on isolated hearts and individual cardiomyocytes from young adult male Fisher Rats. Hearts arrested with cardioplegia containing SEA0400 showed improved recovery of left ventricular function after reperfusion. The onset of reperfusion arrhythmia was delayed, troponin release was reduced, and mitochondrial damage was minimized. In the isolated cell model, contraction amplitudes were higher during reperfusion in the SEA0400 group without a change in Ca^{2+} transients. This suggests that cells arrested with cardioplegia containing SEA0400 developed improved myofilament sensitivity to Ca^{2+} .

LIST OF ABBREVIATIONS & SYMBOLS

PCI	percutaneous catheter interventions
Ca ²⁺	calcium ion
NCX	sodium calcium exchanger
SEA0400	2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline
ATP	adenosine triphosphate
NHE	sodium hydrogen exchanger
Na ⁺	sodium ion
O ₂	oxygen
Tn-I	troponin-I
CABG	coronary artery bypass graft
H ⁺	hydrogen ion
HOE694	cariporide
KB-R7943	2-(2-(4-(4-nitrobenzyloxy)phenyl)ethyl)-isothioureamethanesulfonate
IC ₅₀	half maximal inhibitory concentration
°C	degree celsius

ml	millilitre
min	minutes
mM	millimolar
kg	kilogram
ECG	electrocardiogram
DMSO	dimethyl sulfoxide
EM	electron microscope
μM	micromolar
μm	micrometer
Hz	hertz
LV	left ventricle
LVDP	left ventricle developed pressure
$^+\text{dp}/\text{dt}$	positive instantaneous change in pressure
$-\text{dp}/\text{dt}$	negative instantaneous change in pressure
LVW	left ventricular work
CVR	coronary vascular resistance
MPTP	mitochondrial permeability transition pore

ECGP	electrochemical gradient of protons
SR	sarcoplasmic reticulum
Max	maximum
Nm	nanomoles

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CHAPTER I: INTRODUCTION

Overview:

Heart disease is a major cause of illness, and death. Cardiovascular disease claims more than 70,000 lives in Canada annually. It is estimated that 1.6 million Canadians are affected and living with heart disease (Health Canada, 2010). In the United States cardiovascular disease claims more than 800,000 lives annually, 34.3% of all deaths. It is estimated that over 80 million people are affected with heart disease in the United States alone (American Heart, 2006). Despite the severity and scale of the disease there are many medical therapies and treatments available.

Treatment of heart disease begins with prevention. Many forms of heart disease can be prevented through minor lifestyle changes. These changes include performing daily exercise, avoiding smoking and excessive drinking, eating a healthy balanced diet, and maintaining a healthy body weight. These changes can help reduce risk factors such as high cholesterol, diabetes, and hypertension (Cl  roux et al., 1999). In individuals who are in high risk groups and those affected with heart disease, further treatments are available; including drug treatments with pharmacological agents. For example, individuals with hypertension are given antihypertensive agents including; ACE inhibitors, diuretics, beta blockers and calcium channel blockers (Leeman, 2010). The action of these drugs help to reduce the pressure in arteries thereby providing

great therapeutic advantages for individuals suffering from hypertension (Leeman, 2010).

Percutaneous catheter interventions (PCI) are used as a treatment modality in various heart diseases, for example balloon coronary angioplasty, stenting, valvatomies, closure of various intra-cardiac shunts, and more recently percutaneous valve replacement. Cardiac surgery with cardiopulmonary bypass (open heart surgery) is often required in treating patients with valve disease and coronary artery syndromes that are not amenable to PCI. As a result, significant numbers of patients undergo open heart surgery every year. In the United States more than half a million surgeries are done annually (US Department of Health, 2010). Most open heart surgeries require the interruption of blood flow to the myocardium and administration of a cardioplegic solution that rapidly arrests the heart (Hans et al., 2006). This is a crucial step in the operation as it provides the surgeon with an enhanced exposure to execute delicate surgical procedures. However, interruption of myocardial blood flow results in a period of ischemia. When blood flow is returned to the ischemic tissue during reperfusion, so called ischemia-reperfusion injury can occur (Piper et al., 1998).

Myocardial ischemia-reperfusion injury is thought to be mediated by intracellular Ca^{2+} overload during reperfusion. As a result most research work is focused on the development of drugs and strategies that will regulate intracellular Ca^{2+} and prevent Ca^{2+} overload. Recent studies have shown evidence that a newly developed drug, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-

ethoxyaniline (SEA0400) blocks the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) and provides enhanced protection against ischemia-reperfusion injury in ventricular cardiomyocytes (Takahashi et al., 2003) and hearts (Motegi et al., 2007). However this drug has not yet been tested in a model of cardioplegic myocardial protection.

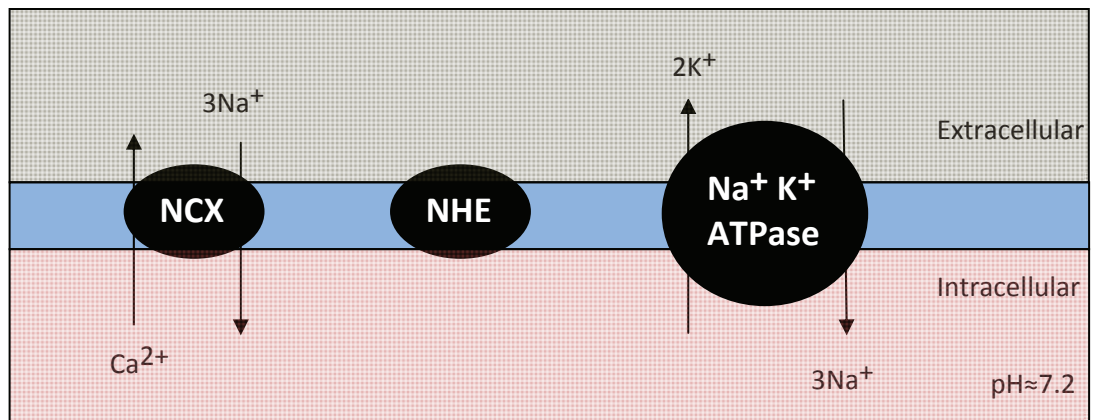
The overall objective of this study was to examine the NCX blocker SEA0400 as a cardioplegia additive to enhance myocardial protection and improve recovery of ventricular function after a period of ischemic cardioplegic arrest. The work presented in this thesis examined the effect of the drug in two models of cardioplegic arrest. The first was a whole heart model where left ventricular myocardial function and myocardial injury were analyzed. The second was an isolated ventricular myocyte model where contractions and intracellular calcium concentrations were evaluated. The following introduction will discuss the role of ischemia-reperfusion injury, cardioplegia, and potential strategies to improve myocardial function during reperfusion following cardioplegic arrest.

Ischemia Reperfusion Injury:

Most open heart surgeries include the interruption of blood flow for a limited period of time (Vinten-Johansen et al., 2000). Although this is a crucial step in the operation, it exposes the myocardium to a period of ischemia. Ischemia reperfusion injury is the damage sustained by the tissue due to an interruption of oxygenated blood supply (Javadov et al., 2009). When the oxygen

supply is interrupted, a variety of biochemical changes happen in the myocyte. Initially, the cellular metabolism of the individual cells is shifted from aerobic to anaerobic respiration. This results in the development of intracellular acidosis and a reduction of ATP synthesis. The increased acidosis activates the Na^+/H^+ exchanger (NHE) where H^+ ions are removed from the cell in exchange of Na^+ ion influx (Mentzer et al., 2003). Under non-ischemic conditions the intracellular Na^+ ions are transported out of the cell through the Na^+/K^+ ATPase. However during ischemia, due to the reduced availability of ATP, the Na^+/K^+ ATPase is not active to balance the intracellular Na^+ influx (Javadov et al., 2009). Consequently the NCX activates reverse mode where Na^+ ions are transported out of the cell in exchange for Ca^{2+} ion influx (Karmazyn et al., 2001). This results in an elevation of intracellular Ca^{2+} levels. Due to the reduced ATP synthesis the sarcolemma Ca^{2+} ATPase is not capable of removing the Ca^{2+} out of the cell (Javadov et al., 2009). In addition, the sarcoplasmic reticulum (SR) Ca^{2+} ATPase is incapable of sequestering the increased Ca^{2+} levels in the SR (Javadov et al., 2009). As a result, the myocardial tissue develops intracellular Ca^{2+} overload. Figure #1 represents the biochemical changes of major intracellular ions in a cell experiencing ischemia in comparison to a normal cell. This Ca^{2+} overload that is present during ischemia and early reperfusion is responsible for the myocardial dysfunction observed in reperfusion (Hendrikx et al., 1994).

A) Non-ischemia



B) Ischemia

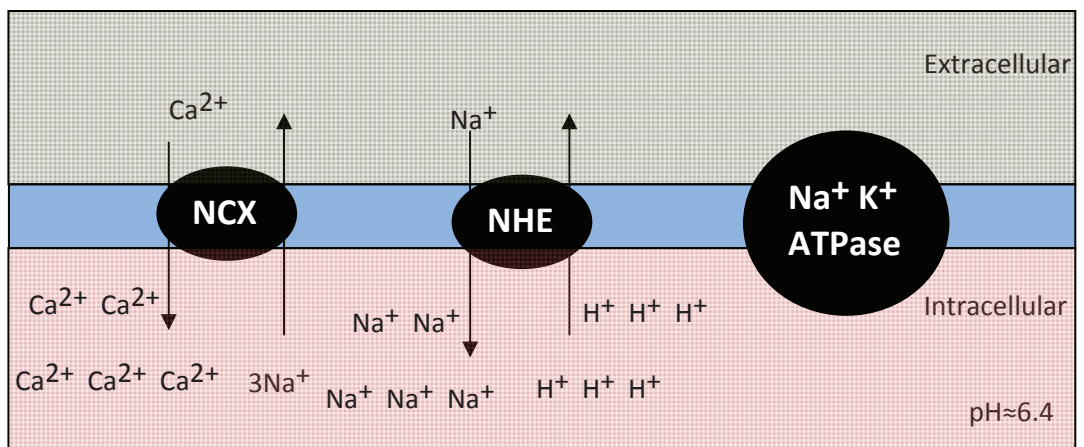


Figure #1: Representative diagram illustrating the changes in sarcolemma Na^+ transporter activity during A) non-ischemic conditions and B) ischemic conditions. During non-ischemic conditions the Na^+/K^+ ATPase transports 3Na^+ ions out of the cell for every 2K^+ ions pumped into the cell. The sodium balance is maintained through the ATPase. The NCX is active in forward mode where Ca^{2+} leaves the cell in exchange for Na^+ ions that enter the cell. However, during ischemia the cells are in a state of anaerobic respiration where acid is produced and ATP synthesis is reduced. Notice the drop in intracellular pH when exposed to ischemia. The acidic intracellular environment activates the NHE where H^+ ions are removed from the cell in exchange of Na^+ ions that enter the cell. Due to the reduced availability of ATP the Na^+/K^+ ATPase is not active to balance the intracellular Na^+ influx. As a result the NCX reverse mode is activated and Na^+ ions are transported out of the cell in exchange of Ca^{2+} ions that enter the cell.

Cardioplegia:

Most open heart surgeries include the interruption of blood flow for a limited period of time (Vinten-Johansen et al., 2000). Although this is a crucial step in the operation, it exposes the myocardium to a period of ischemia. One of the main causes of mortality and poor outcome in heart surgery is due to the inadequate protection of the myocardium leading to increased reperfusion injury (Piper et al., 1998; Weman et al., 2000). Various myocardial protective strategies have been used throughout the evolution of heart surgery. Today however, cardioplegia is used as the standard myocardial protection strategy (Vinten-Johansen et al., 2000). It has been used to protect the heart muscle from ischemia-reperfusion injury during surgery for several decades (Vinten-Johansen et al., 2000). Cardioplegic solutions contain a variety of chemical agents that are designed to arrest the heart rapidly in diastole and provide protection against ischemia reperfusion injury (Vinten-Johansen et al., 2000). Cardioplegia arrests and protects the heart by lowering the oxygen demand of the myocardium (Buckberg et al., 1977). This is made possible through the inhibition of the myocardial electrical - mechanical activity. Cardioplegia usually contains a very high potassium concentration, along with a variety of constituents that can vary slightly. Potassium is the key element of most cardioplegia due to its ability to depolarize the cell membrane and prevent the initiation of any myocardial action potentials. Consequently contractions are prevented resulting in a flaccid myocardium with very low oxygen demand. Cardioplegia is delivered during

surgery in one of three ways. The most common method requires cross-clamping of the aorta and insertion of the cardioplegic catheter in the aorta proximal to the cross-clamp. Once the catheter is positioned into the aorta the cardioplegia volume is delivered (antegrade). The next method of cardioplegic delivery is retrograde delivery. The cardioplegia is delivered to the heart through the coronary sinus. The third method of cardioplegia delivery is used in certain surgical cases in which the aorta needs to be opened for example in cases of aortic valve replacement/ repair. In this procedure the cardioplegia is delivered directly into the coronary arteries via cannulation of the coronary ostia (Braathen et al., 2010). There are many cardioplegic solutions used in the world today. They can be grouped in two main categories crystalloid cardioplegia and blood cardioplegia. The following section will review the benefit, effectiveness and differences between these main cardioplegic categories.

Crystalloid cardioplegia

Cold crystalloid cardioplegia protects the myocardium by hypothermia and electromechanical arrest, both of which lower the oxygen demand and increase the tolerance to ischemia (Gay, 1975). The crystalloid cardioplegia typically contains up to 30 mmol/L concentration of potassium to arrest the heart (Cohn, 2008). Sodium and calcium concentrations are present but at a very low concentration when compared to the extracellular fluid. In addition, osmotically active substances such as mannitol, and buffers such as bicarbonate are

included (Dunphy et al., 1999). The myocardial protection observed in clinics that use cold crystalloid cardioplegia show excellent results (Ledingham et al., 1987).

Cold Blood cardioplegia

Although cold crystalloid cardioplegia offers good results in clinics that use these solutions, there is another type of cardioplegia that is used widely throughout the world, known as cold blood cardioplegia. It is usually prepared by combining blood obtained from the patient with a crystalloid solution consisting primarily of potassium chloride (Cohn, 2008). The benefit of blood cardioplegia is that blood contains red blood cells that contain haemoglobin and thus carries more oxygen than crystalloid cardioplegia. Blood provides a superior buffer medium. It has osmotic properties, contains electrolytes, and maintains physiological pH (Cohn, 2008). In addition, blood contains endogenous antioxidants with free radical scavengers (Follette et al., 1978). In clinics where cold blood cardioplegia is used, successful outcomes are observed. Studies demonstrate that cold blood cardioplegia is an effective way to provide excellent myocardial protection (Hendrikx et al., 1999; Ovrum et al., 2010). Likewise, other studies have shown that crystalloid cardioplegia can be just as cardio protective (Guyton et al., 1985), cost-effective, and provide a bloodless operative field. Every solution has its unique advantages.

Warm blood cardioplegia

Another cardioplegic solution is warm blood cardioplegia. The hypothermia present in the cold blood or crystalloid cardioplegia only reduces myocardial O₂ consumption slightly. The main ingredient in any cardioplegia that drops the oxygen consumption of the heart is the potassium. By depolarization the membrane of the heart, contractions are prevented dropping the O₂ demand of the heart to nearly 10% of baseline values (Skarysz et al., 2006). Thus, some centers use warm blood cardioplegia solution. In a study where warm blood cardioplegia was compared to cold blood cardioplegia in coronary artery bypass graft (CABG) procedures, there was an increased cardiac troponin-I (Tn-I) release in the cold blood cardioplegia group (Skarysz et al., 2006). Warm blood cardioplegia might be a good way to protect the heart by preventing deleterious effects of hypothermia. However, without hypothermia the cardioplegia must be run continuously or more frequently, which poses a technical challenge with the use of warm blood cardioplegia. There are many cardioplegic solutions used today. They vary slightly in their compositions and the optimal cardioplegic composition and temperature is still a matter of debate.

Potential myocardial protection strategies:

There has always been an interest in finding ways to improve myocardial function during reperfusion after cardiac surgery. These strategies and

techniques vary in their procedure and application, but all share the same goal to prevent myocardial damage and improve myocardial function following surgery. The aim is to reduce the reperfusion injury sustained during open heart surgery and cardioplegic arrest. Among these widely studied strategies is ischemic preconditioning, and administration of pharmacological agents that block channels that play a role in Ca^{2+} elevation and Ca^{2+} overload during reperfusion. Prominent examples are the NHE, and NCX. The following will discuss these various strategies in further detail.

Ischemic preconditioning:

Ischemic preconditioning is a potential strategy for improving cardiac function and open heart surgery outcomes. Ischemic preconditioning refers to the procedure where the heart is exposed to a very short period of ischemia and reperfusion, followed by a lengthened period of ischemia (Murry et al., 1986). This procedure allows the myocardium to be more tolerant to ischemia-reperfusion injury. In a clinical study of ischemic preconditioning, 34 out of 70 patients were randomly selected to receive ischemic preconditioning before cardioplegic arrest and compared to the other 36 patients who were not exposed to ischemic preconditioning (Illes et al., 1998). The group exposed to ischemic preconditioning showed improved cardiac index after surgery in comparison to preoperative values. On the other hand the cardiac index in the control group decreased significantly. 13 patients in the control group required administration of inotropic agents, whereas no patients in the ischemic preconditioning group

required the use of inotropes (Illes et al., 1998). These results suggest that ischemic preconditioning can be very important in reducing myocardial ischemia-reperfusion injury and can improve heart function post cardiac surgery (Illes et al., 1998). Although ischemic preconditioning is a viable myocardial protection strategy, another approach widely researched is the administration of pharmacological agents that block channels that play a role in Ca^{2+} elevation and Ca^{2+} overload during reperfusion.

Pharmacological agents targeting Ca^{2+} overload:

Pharmacological drugs can be administered to help reduce the ischemia-reperfusion injury associated with the use of cardioplegia. Two drug groups that are potential targets are the NHE blockers and the NCX blockers. These channels play a role in Ca^{2+} elevation and Ca^{2+} overload during ischemia and reperfusion, therefore targeting them maybe an effective strategy to help reduce ischemia-reperfusion damage.

NHE Inhibitor

The sarcolemma NHE is an electro-neutral exchanger that extrudes one proton in exchange for one Na^+ ion under normal conditions. There are five isoforms of the NHE located on the cell membrane of various cell types. NHE1 is widely distributed in the cardiac tissue (Karmazyn et al., 2001). It is responsible

for mediating a number of physiological functions including intracellular pH, and cell volume (Vila-Petroff et al., 2010; Sharma et al., 2000).

During ischemia the cells shift to anaerobic respiration due to the lack of oxygen. Consequently there is a build-up of intracellular acid and the intracellular pH drops (Karmazyn et al., 2001). The acidosis induced by a shift to anaerobic metabolism during ischemia-reperfusion activates the NHE. The NHE correlates with internal pH, the exchanger activity is maximized at low intracellular pH. The NHE transports H^+ out of the cell and Na^+ ions in to the cell to help restore pH (Karmazyn et al., 2001). Consequently the intracellular Na^+ level is elevated by the activations of the NHE. This elevated Na^+ concentration activates the NCX reverse mode where Na^+ ions move in the direction of their electrochemical gradient to the exterior of the cell. In exchange for Na^+ efflux, Ca^{2+} is transported into the cell developing Ca^{2+} overload and subsequent cell injury (Xiao & Allen, 2003; Cross et al., 1995).

The elevated intracellular Na^+ concentration not only activates the NCX reverse mode, but also facilitates the absorption of fluid that results in myocardial edema. Administering NHE blockers may reduce myocardial edema that develops during early reperfusion (Tritto et al., 1998). Using a pharmacological NHE blocker can prevent the intracellular influx of Na^+ ions during ischemia. As a result the interior Na^+ concentration will remain relatively constant and the NCX will not activate reverse mode. This will prevent Ca^{2+} entry of via the NCX and

Ca^{2+} overload should not develop. This phenomenon has led to the development of many NHE inhibitors. Yet the issue of selectivity and specificity of pharmacological drugs remain a concern.

In 1995 a compound called HOE694 (cariporide) was examined as a NHE inhibitor. The drug was administered in various models to assess the selectivity and effect of the drug. The results showed that cariporide inhibited sodium influx through the NHE (Scholz et al., 1995). The cellular edema that developed consequent to intracellular acidosis and Na^+ entry was also reduced. The recovery of pH in cardiomyocytes exposed to ischemia was delayed. In the ischemia-reperfusion model of isolated working rat hearts, cariporide was shown to reduce ischemia-reperfusion injury and improve myocardial function during reperfusion. The compound was also administered intravenously and orally in anesthetised rats undergoing coronary artery ligation, and showed no negative systemic effect (Scholz et al., 1995). This data provided evidence for the selectivity of cariporide and the potential therapeutic effects of this approach for patients undergoing cardiac surgery.

Subsequently a clinical trial called the EXPEDITION study was conducted. The aim of the study was to assess the benefits of cariporide in preventing myocardial infarction or death in patients undergoing CABG (Mentzer et al., 2008). A number (5770) of high-risk patients undergoing CABG were randomly selected to receive: 180mg/h preoperative doses, followed by 40mg/h in the next 24hrs, followed by 20mg/h in the next 24hrs or placebo. Death or myocardial

infarction was assessed once after 5 days and again after 6 months. The data collected at 5 days showed that death or myocardial infarction was 20.3% in the placebo group in comparison to 16.6% in the cariporide treated group (Mentzer et al., 2008). These results confirmed the potential benefit of cariporide in preventing ischemia reperfusion injury. However when mortality was assessed, the control group had a mortality of 1.5%, in comparison to the cariporide group, which had a significant higher mortality rate of 2.2% ($p=0.02$). The increased mortality was not due to myocardial infarction. Cerebro-vascular strokes were the cause of mortality (Mentzer et al., 2008). This was a side effect possibly due to systemic delivery of the drug. The results from the EXPEDITION trial led to the arrest of cariporide development as a myocardial protective agent. Although NHE inhibitors are a potential myocardial protection strategy, the development of more specific NHE pharmacological blockers that specifically target myocardial tissue is required.

NCX Inhibitor

Another major sarcolemmal exchanger responsible for the regulation of Ca^{2+} concentration during contraction and relaxation is the NCX. It is the main pathway responsible for the transport of Ca^{2+} out of the cell following a contraction cycle (Barrrientos et al., 2009). However, during ischemia the cycle is reversed and the transporter operates in reverse mode which facilitates the build-up of Ca^{2+} overload during reperfusion (Cross et al., 1995). Administering a

pharmacological NCX blocker can prevent the influx of Ca^{2+} and prevent Ca^{2+} overload. Various NCX blockers are available; however their selectivity for NCX current remains a concern. 2-(2-(4-(4-nitrobenzyloxy)phenyl)ethyl)-isothiurea methanesulfonate (KB-R7943) is a common well known inhibitor (Barrrientos et al., 2009). KB-R7943 provides protection against ischemia and reperfusion injury when used in low concentrations (Macdonald and Howlett, 2008). KB-R7943 blocks the NCX and reduces systolic and diastolic Ca^{2+} overload during reperfusion (An et al., 2006). Although KB-R7943 provides improved myocardial protection it is not highly selective to NCX (Iwamoto et al., 2007). Currently, many experiments are examining the effect of more selective inhibitors such as SEA0400 (Iwamoto T et al., 2007).

SEA0400

The compound SEA0400 is a synthetically developed NCX inhibitor (Matsuda et al., 2001). Although it blocks the NCX current, it also shares some of KB-R7943's unselective effects including L-type Ca^{2+} current inhibition (Iwamoto T et al., 2007). Early studies were performed to compare the effect and selectivity of SEA0400 in comparison to KB-R7943. In cultured neurons, astrocytes, and microglia, SEA0400 inhibited the NCX at a much lower concentration than KB-R7943. The IC_{50} of SEA0400 was 5 to 33 nM, vs 2 to 4 μM for KB-R7943 (Matsuda et al., 2001). Delivering 3 μM of KB-R7943 blocked other various channels including the NHE. However with 3 μM SEA0400 there was no inhibition of the NHE. These results showed SEA0400 to be a more selective and potent

inhibitor in comparison to KB-R7943 (Matsuda et al., 2001). In another study conducted using isolated guinea-pig ventricular myocytes the effect of SEA0400 on myocardial ionic currents was examined and compared to the effect of KB-R7943. The results showed that 1 μ M SEA0400 inhibited more than 80% of the NCX current. On the other hand 10 μ M of KB-R7943 was required to achieve over 80% NCX current inhibition (Tanaka et al., 2002). At a concentration of 10 μ M KB-R7943 other myocardial ionic currents were affected and inhibited by more than 50%. Among them were the L-type calcium current, sodium current, and inward rectifying potassium current. In contrast 1 μ M of SEA0400 did not affect any of these other currents (Tanaka et al., 2002). It appears that SEA0400 at 1 μ M preferentially blocks the NCX current, yet at higher concentrations the L-type Ca²⁺ current is inhibited by the compound (Birinyi et al., 2005). These results confirmed the potency and selectivity of SEA0400 over KB-R7943 and provided evidence for the role of SEA0400 in the myocardial tissue.

This generated more interest in the drug and its potential role in ischemia reperfusion injuries. During ischemia and reperfusion Ca²⁺ overload develops in part due to the reverse mode of the NCX (Takahashi et al., 2003). Thus delivering the drug to the myocardial tissue should inhibit the NCX current, minimize Ca²⁺ overload, reduce myocardial damage, and improve reperfusion function. A recent ischemia reperfusion study was performed on isolated mouse ventricular cardiomyocytes to examine the effect of SEA0400 on ischemia reperfusion injury. The ventricular myocytes were patch clamped to measure

current and Ca^{2+} levels were measured through fura-2 fluorescence. During ischemia the NCX inward current was reduced from $(-0.04^{+/-}0.01 \text{ nA}$ to 0 nA at -100 mV) and the outward current was reduced from $(0.23^{+/-}0.08 \text{ nA}$ to $0.11^{+/-}0.03 \text{ nA}$ at $+50 \text{ mV}$) (Wang et al., 2007). During reperfusion the NCX current was restored and the inward current remained low in comparison to pre-ischemic current; from $(-0.04 \pm 0.01 \text{ nA}$ to $-0.07 \pm 0.03 \text{ nA}$ at -100 mV). However the outward current was dramatically elevated in magnitude compared to pre-ischemia values; from $(0.23 \pm 0.03 \text{ nA}$ to $0.49 \pm 0.12 \text{ nA}$ at $+50 \text{ mV}$) (Wang et al., 2007). The intracellular Ca^{2+} levels during ischemia were greater than pre-ischemic values represented by $138^{+/-}7\%$ of fluorescence. But during early reperfusion the intracellular Ca^{2+} levels were greatest at $210^{+/-}11\%$ fluorescence (Wang et al., 2007). When SEA0400 was delivered to the ventricular myocytes, the NCX inward and outward currents were blocked and the increase of Ca^{2+} during ischemia and reperfusion was inhibited (Wang et al., 2007). These results provide evidence for the potential use of SEA0400 in ischemia-reperfusion studies. Its ability to minimize Ca^{2+} overload and consequently minimize myocardial damage provides superior therapeutic advantage against ischemia reperfusion injury.

SEA0400 was also evaluated in whole heart models of ischemia and reperfusion. Rat hearts were extracted from the animal and mounted on a Langendorff perfusion apparatus. The hearts were perfused at a constant pressure for 15min. After stabilization the perfusion pressure was dropped

substantially to simulate ischemia. Following 60min of simulated ischemia the perfusion pressure was restored to pre-ischemic values for a reperfusion period of 60min. SEA0400 was delivered in the perfusion buffer during the first 10min of reperfusion. The results showed that hearts exposed to 1 μ M SEA0400 during reperfusion exhibited improved myocardial function (Takahashi et al., 2003). The cardio-protective effects of SEA0400 observed in ischemia-reperfusion studies are a result of the potent and selective inhibition of the NCX. The myocardial damage associated with elevated intracellular Ca²⁺ levels is absent due to the inhibition of NCX, this leads to improved myocardial recovery during reperfusion (Motegi et al., 2007). There have been many studies evaluating the effect of SEA0400 on ischemia reperfusion injury, and these studies suggest potential myocardial protective effects with SEA0400 delivery.

A Novel Myocardial Protection Strategy

Although many myocardial protection strategies have been examined, not all have been successful. For example in the cariporide study, although lab experiments showed great therapeutic advantage with cariporide against ischemia reperfusion injury, when it was delivered in a clinical trial harmful lethal side effects were witnessed and the strategy was aborted (Mentzer et al., 2008). Systemic side effects due to intravenous delivery are associated with a great level of risk due to lack of highly selective inhibitors. Thus, the strategy of incorporating cariporide systemically was unsuccessful. A better strategy to improve the ischemic reperfusion injury associated with cardiac surgery may be to deliver these blockers only to the myocardium. This can be achieved by

delivering the drug as an additive to cardioplegia. This approach ensures that the drug will be present during the ischemic period where the biochemical changes associated with ischemia-reperfusion injury begin. Also the drug will stay in the coronary circulation with minimal systemic exposure. This ensures that any side effects will be minimized. Furthermore, a drug that affects a later step in the biochemical pathway may minimize unwanted side effects. For example when using a NHE inhibitor, the pH and H⁺ ion balance are initially disturbed, which is followed by disruption of the Na⁺ balance that prevents the influx of Ca²⁺. The prevention of Ca²⁺ overload is achieved but ionic balance and pathways that may be responsible for hemostasis are disturbed (Karmazyn et al., 2001). On the other hand, the NCX inhibitor affects one of the later processes in the chain and interruption of biochemical activity is reduced in comparison to NHE inhibition. Furthermore, the time of delivery is also important; it may be superior to administer the drug during the cardioplegia to ensure the availability of the inhibitor at the start of ischemia. Therefore the myocardial protection strategy proposed here is to incorporate SEA0400 in the cardioplegia and deliver it to the myocardium exclusively. Although SEA0400 has been examined in previous ischemia reperfusion studies and showed potential benefit (Motegi et al., 2007); SEA0400 has yet to be examined as a cardioplegic additive. In this thesis a model of cardioplegic arrest and reperfusion will be used to evaluate the effect of SEA0400 as a cardioplegic additive.

Objectives and Hypothesis:

Ischemia and reperfusion injury is mediated primarily by an elevation of intracellular Ca^{2+} concentration. Ca^{2+} enters the cell through the reverse mode of the NCX during ischemia and early reperfusion (Xiao et al., 2003). Pharmacological inhibition of the NCX is a successful strategy in preventing Ca^{2+} overload and results in improved myocardial protection (Matsuda et al., 2001). Previous ischemia reperfusion studies showed enhanced myocardial protection and reduced myocardial damage with the use of the NCX blocker SEA0400 (Takahashi et al., 2003). However, the effect of SEA0400 has not been previously examined in a cardioplegic arrest model. In theory, incorporating SEA0400 as part of a cardioplegic protection strategy should yield similar or superior results to ischemia-reperfusion models. *The overall objective of this study was to evaluate the NCX blocker SEA0400 as a cardioplegia additive to enhance myocardial protection and improve recovery after a period of cardioplegic arrest. It is hypothesised that the use of SEA0400 as a cardioplegic additive will result in improved myocardial protection and improved contractile recovery during reperfusion. The specific objectives of this study are summarized below:*

- 1) Determine whether left ventricular function after cardioplegic arrest is improved when SEA0400 is delivered as a cardioplegic additive using a whole heart model.** The first objective was to establish a cardioplegic arrest model with the ability to measure left ventricle function.

- 2) **Determine whether SEA0400 prevents myocardial damage.** Myocardial tissue will be examined to evaluate the efficacy of SEA0400 and the physical changes associated with its delivery.
- 3) **Determine the effect of SEA0400 in cardioplegia on reperfusion arrhythmia.** Measuring the impact of SEA0400 on the electrical activity of the heart.
- 4) **Determine the effect of SEA0400 as a cardioplegic additive on individual ventricular cardiomyocytes.** Investigating the effect of SEA0400 in a cellular cardioplegic model will provide valuable information in determining the physiological actions on the cellular level and lead to a better understanding of the role of SEA0400 as a cardio-protection agent.

CHAPTER II: METHODS

Animals:

The animals used in the experimental protocols were Fisher 344 rats. All rats were adult males (≈ 3 months; ≈ 300 g). The rats were purchased from Charles River Laboratories (Saint-Constant, QC) and housed at Dalhousie University's Carleton Animal Care Facility for a minimum of 24hrs before experimental use. At the facility rats received free access to food and water and were housed in pairs (2 rats/cage). Protocols were conducted in accordance to the Canadian Council on Animal Care guidelines and were approved by Dalhousie University Committee on Laboratory Animal care.

Isolated whole heart protocol:

Heart Isolation

The rats were removed from the housing room to the weighing room at the Carlton Animal Care Facility and given 15 min to settle. Heparin was injected intraperitoneally at 1000 U/300g. After 5min the rats were anaesthetized with sodium pentobarbital (160 mg/kg intraperitoneal; CDMV; Saint-Hyacinthe, QC). The rats were asleep within 5-10 min. During this period the rats were brought from the Animal Carlton Care Facility and placed in the laboratory.

The animal was placed on the surgical table and the chest was opened by parasternal incision. The entire heart-lung mass was explanted and immediately placed in an ice-cold saline petri-dish bath ($\approx 4^{\circ}\text{C}$). The ascending aorta was quickly identified and dissected from the surrounding tissue and lung mass. The

heart was then removed from the cold saline petri-dish bath and attached to an appropriately-sized aortic cannula on the Langendorff perfusion apparatus (AD Instruments Inc., Colorado Springs, CO). The cannula was inserted into the aorta carefully to avoid interruption of coronary artery perfusion by inserting the cannula too far into the aorta. The cannula was secured to the ascending aorta with 3-0 silk. Retrograde coronary vascular perfusion was established with warm (37°C), oxygenated Krebs-Henseleit solution at a flow rate of 10ml/min (Table #1).

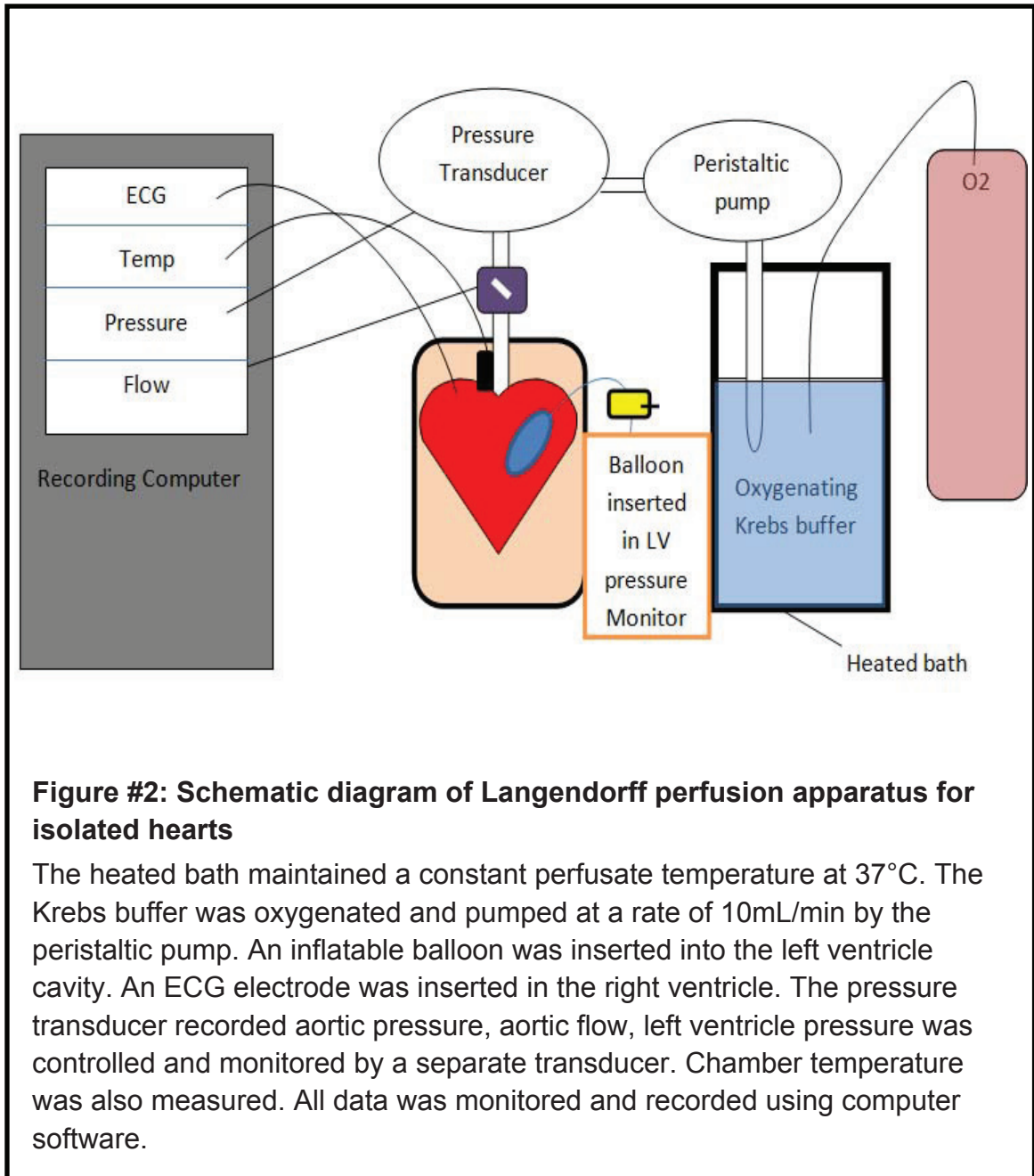
Table #1: Composition of Krebs-Henseleit buffer solution

Compound	Concentration (mM)
NaCl	118.5
NaHCO ₃	25
KH ₂ PO ₄	1.2
KCl	4.7
Glucose	11
MgSO ₄	1.2
CaCl ₂	2.5
EDTA	0.5
pH 7.4 Bubble with 95% O ₂ /5%CO ₂	

Langendorff perfusion setup

After isolation, the heart was attached to the Langendorff apparatus and perfused with oxygenated Krebs-Henseleit buffer for 5-10 min warmed to 37°C. During this time period any excess tissue mass was removed and heart rate was stabilized. Next an inflatable balloon connected to a pressure transducer was inserted into the left ventricle, and microelectrodes were inserted in the right ventricle. Figure #2 illustrates the setup of the Langendorff apparatus. After insertion of the balloon the heart was perfused for a stabilization period of 20 min. This was followed by hemodynamic measurements. Baseline ventricular function data was obtained by incrementally increasing the volume in the balloon and measuring the pressure generated by the left ventricle. These measurements generate pressure volume curves that provide quantifiable data of left ventricle function (description provided below under measurements section). After recording hemodynamic measurements, perfusion was stopped and a cardioplegic solution was delivered at a dose of 20mL/kg body weight. The infusion rate was adjusted so that aortic pressure was maintained less than 80 mmHg. After delivery of cardioplegia, the heart was arrested and exposed to 21°C room temperature. Following 45min of cardioplegic arrest, the myocardium was reperfused with warm oxygenated Krebs's solution. Reperfusion flow rate was initiated by gradually increasing the aortic flow from 0mL/min to 10 mL/min, 2ml increments were added every 30 seconds. After 20 min of reperfusion, ventricular

hemodynamic measurements were repeated. Finally the myocardium was removed from the apparatus and placed in a cold saline petri-dish.



Cardioplegic Solution

Cold blood cardioplegia was used to arrest the myocardium. The cardioplegia was prepared by obtaining the base solution from the QEII hospital (Halifax, Canada). Sodium bicarbonate and magnesium sulfate were added to the cardioplegia. Cardioplegia was then mixed with blood in a ratio of 1:4 respectively. Blood was collected from the experimental rat (300 g). After mixing the base solution with blood, the cardioplegic solution was oxygenated (gently bubbled with O₂) and placed in an ice bath (≈4°C). The approximate final ionic concentration of the cardioplegia is listed in table #2. Cardioplegic solution was delivered at a dose of 20mL/kg body weight at the beginning of ischemia.

Table #2: Base ionic composition of myocardial cardioplegic solutions

	Ionic concentration* (mM)
Na ⁺	136
K ⁺	18.8
Mg ²⁺	5
Ca ²⁺	1
Bubble with 95% O ₂ /5%CO ₂	
SEA0400 Cardioplegia included additional 1μM SEA0400 (dissolved in DMSO)	
Control (Standard) Cardioplegia did not included SEA0400	

*Approximate final ionic concentration after mixing base solution to blood in a ratio of 1:4 respectively.

Myocardial Troponin, ATP, and Mitochondria sampling

At the beginning of reperfusion the first 50ml of coronary sinus effluent was discarded. Then (10ml) was collected and stored in -70°C freezer to be used on a later date to measure the myocardial troponin release. After Langendorff experiments were completed for all hearts from each group, troponin-I release was analyzed. The coronary sinus effluent was examined using a high sensitivity ELISA for determination of cardiac troponin-I (Life Diagnostics, Inc., Cat. No. 2010-2-HSP). Frozen samples were collected from the freezer and thawed. All samples were analyzed with the ELISA assay at the same time. The procedures outlined in the ELISA kit were followed.

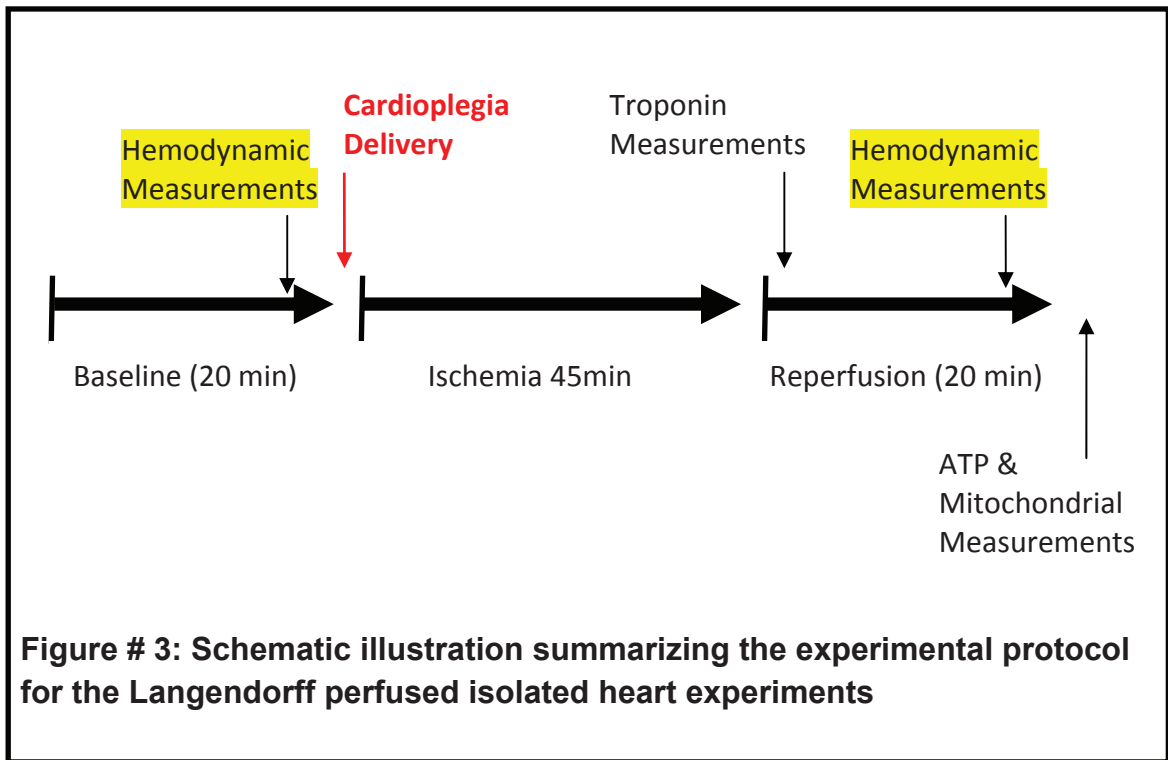
At the end of the Langendorff protocol the heart was removed and sliced in half across the left ventricle. One half was frozen in the -70°C freezer to be used on a later date to measure the myocardial ATP levels. After Langendorff experiments were completed for all hearts from each group, ATP levels were analyzed. The frozen half-heart samples were pulverized into smaller pieces and thawed before being used in an ATP assay. The ENLITEN ATP assay system bioluminescence detection kit for myocardial ATP measurement was used (Promega Corporation part#TB267, USA). The procedures outlined in the assay kit were followed.

The other half of the myocardium was subject to a series of centrifuge cycles to isolate myocardial mitochondria. The mitochondria were sent to the Electron Microscopy lab (Tupper Building, Halifax, Nova Scotia) to obtain EM

images of myocardial mitochondria. The measurements and image magnifications collected from samples are outlined in the following protocol (Li et al., 1992).

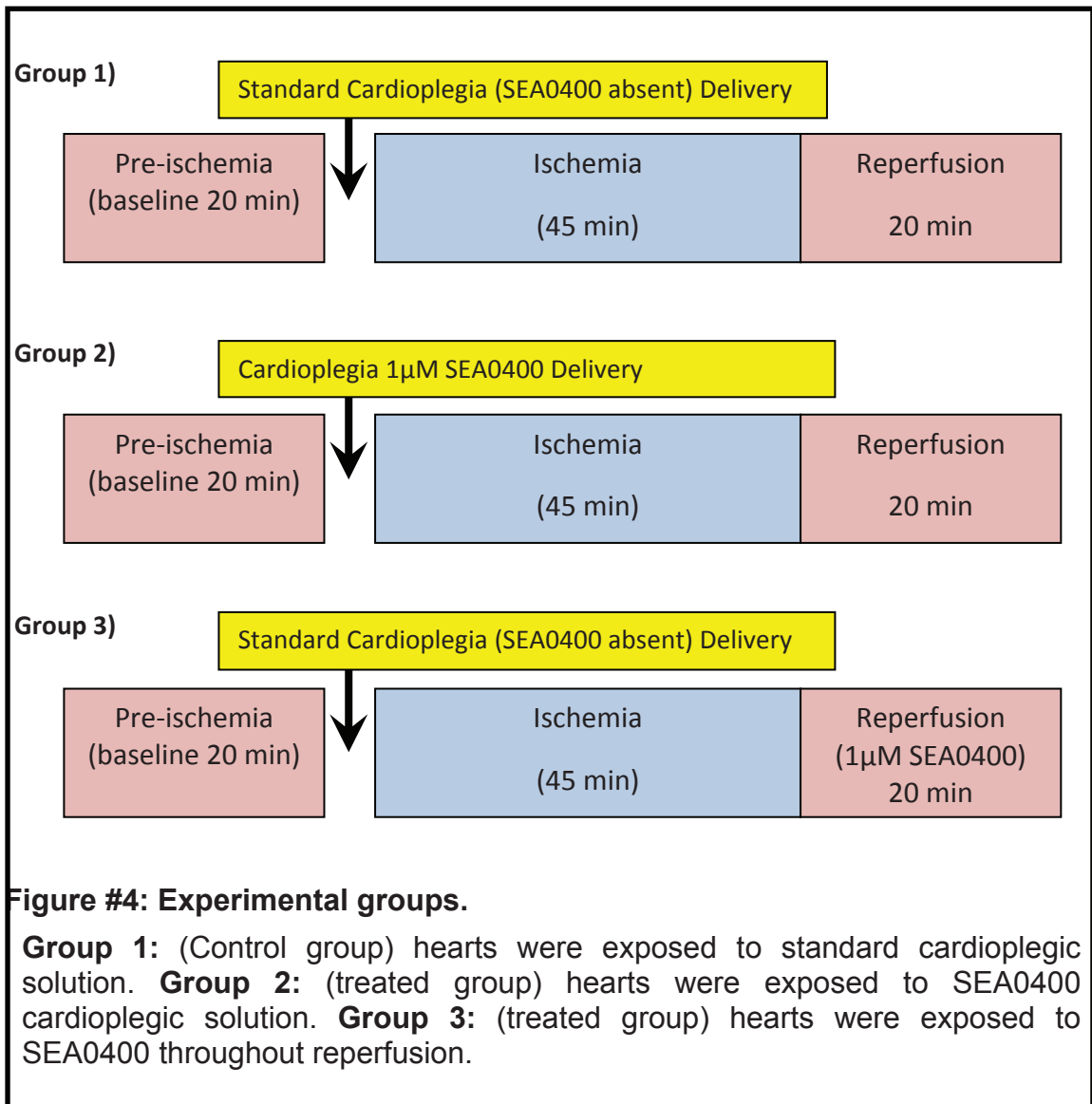
Experimental Protocol

Rat hearts were isolated and attached to the Langendorff apparatus. After 20min of perfusion with oxygenated Kreb's buffer, the myocardial hemodynamic measurements were recorded. This was followed by cardioplegia delivery and the interruption of perfusate flow. After 45min of ischemia the hearts were reperfused for 20min with oxygenated Kreb's buffer. At the beginning of reperfusion the first 50ml of coronary sinus effluent was discarded. This next 10ml of coronary sinus effluent was collected to measure troponin release. After 20min of reperfusion the hemodynamic measurements were repeated and recorded. At the end of the protocol the myocardium was removed and sliced in half. One half was used to analyse ATP levels and the other half was used to generate EM (Electron Microscope) myocardial mitochondria images. Throughout the entire protocol the electrical activity was recorded. Figure #3 represents an illustration of the experimental protocol



Experimental Groups

Hearts were selected at random to participate in one of the following experimental groups: 1) Group I (Control group) received the standard cardioplegic solution with DMSO. 2) Group II (treated group) received 1 μ M SEA0400 (dissolved in DMSO) in the cardioplegic solution. 3) Group III (treated group) received the standard cardioplegic solution but were exposed to 1 μ M SEA0400 throughout reperfusion. The experimental protocol for the various groups is outlined below in Figure #4.



Isolated Cardiomyocyte Protocol:

Ventricular cardiomyocyte Isolation

The rats were brought from the Carlton Animal Care Facility and placed in the laboratory. After 30min the rats were weighed, heparinized (1000 U/300g) and anaesthetized with sodium pentobarbital (160 mg/kg intraperitoneal; CDMV; Saint-Hyacinthe, QC). Next, the animal was placed on the surgical table and the chest was opened by parasternal incision. The aorta was identified and a silk was placed around the aorta. The aorta was cut and affixed to the perfusion apparatus cannula with a double knot silk suture. The heart was perfused retrogradely (18-20ml/min) through the aorta with oxygenated isolation solution (Table #3) for 5-10min. Perfusion was controlled by a peristaltic pump and solutions warmed to 37°C with a circulating bath.

Table #3: Composition of solution used in the isolated cardiomyocyte experiment

Composition of rat myocyte isolation solution	
Compound	Concentration (mM)
NaCl	135
KCl	4
HEPES	10
MgSO ₄	1.2
KH ₂ PO ₄	1.2
Glucose	12
CaCl ₂	0.05
pH 7.4 with NaOH	
Composition of high K ⁺ (KB) solution	
KOH	80
KCl	30
MgSO ₄ *7H ₂ O	3
KH ₂ PO ₄	30
L-Glutamic Acid	50
Taurine	20
EGTA (ethyleneglycoltetraacetic acid)	0.5
HEPES (hydroxyethyl piperazine ethanesulfonic acid)	10
Glucose	10
pH 7.4 with KOH	
Composition of Tyrode's buffer solution	
NaCl	129
NaHCO ₃	20
NaH ₂ PO ₄	0.9
KCl	4
Glucose	5.5
MgSO ₄	0.5
CaCl ₂	2.5
pH 7.2 Bubble with 95% O ₂ /5%CO ₂	

Next the heart was perfused with an enzymatic isolation solution that contained similar components to the isolation buffer with the addition of active enzymes. The enzymatic isolation solution contained the enzymes collagenase type 2 (240 U/mg, Worthington Biochemical Corporation, Lakewood, NJ), protease dispase II at 0.1 mg/mL (Roche Diagnostics, Laval QC), and trypsin (Sigma–Aldrich, Oakville, Ontario, Canada; 0.02 mg/mL). A low pressure gauge was attached to the perfusion apparatus and used to monitor perfusion pressure. After 20 min of perfusion with the enzymatic buffer there was a drop in pressure, indicating the end of enzymatic digestion. Next, the ventricles were separated from the heart and cut into smaller pieces. The minced ventricle tissue was stored at room temperature in a beaker containing high potassium buffer solution (Table #3). The suspension was filtered through 225 µm polyethylene mesh to remove large tissue particles. Experiments were performed only on rod-shaped myocytes with visible striations that had no obvious membrane damage.

Experimental protocol

An aliquot of isolated myocyte suspension (1ml) was transferred into a (2ml) test tube and mixed with 5µM of Fura-2 acetoxymethyl ester (fura-2 AM, Invitrogen, Burlington ON). The myocyte suspension was then transferred to an experimental chamber mounted on the stage of an inverted microscope (ECLIPSE TE2000; Nikon Canada, Mississauga, Ontario, Canada). The experimental chamber was a custom made plexiglass bath with a glass coverslip

base. Solutions were fed through one end of the experimental chamber at a rate of 6ml/min and allowed to drip out the opposite end of the bath. The myocytes were field stimulated with platinum electrodes connected to a stimulator (Harvard Apparatus, Saint-Laurent, QC) triggered by Clampfit software (Molecular Devices, Sunnyvale, CA) at 4Hz. Contractions were recorded using a video monitor and edge detector (model 105; Crescent Electronics, Sandy, Utah). Intracellular Ca^{2+} levels were calculated using fura-2 emission ratios. The cells were superfused with oxygenated Krebs's buffer before ischemia and during reperfusion. During ischemia the cells were superfused with cardioplegic solution. A stop valve ensured that at a given time the cells were exposed to only one type of solution. Figure #5 represents the perfusion apparatus setup. During the cardioplegic arrest period the myocytes were stimulated at various times.

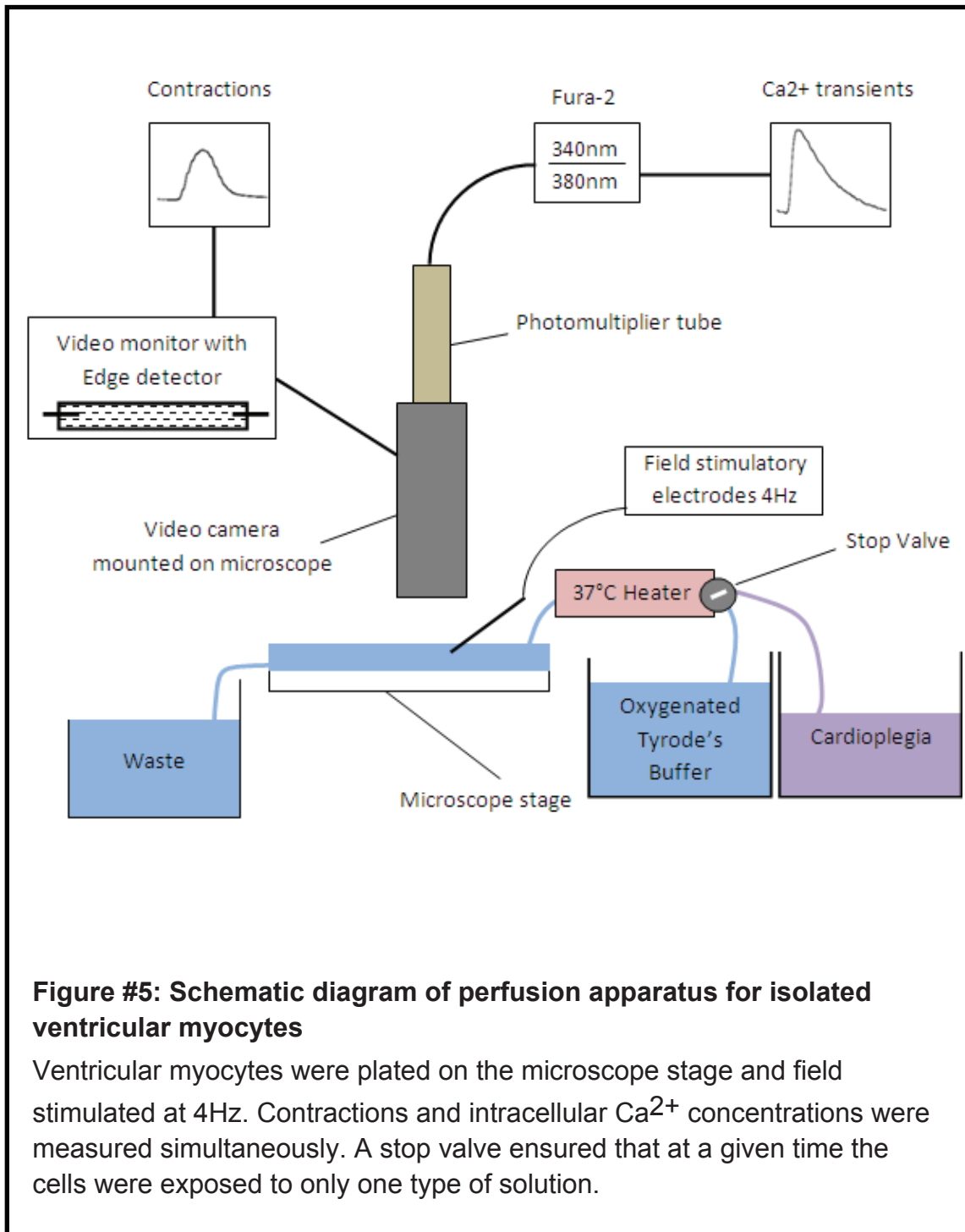


Figure #5: Schematic diagram of perfusion apparatus for isolated ventricular myocytes

Ventricular myocytes were plated on the microscope stage and field stimulated at 4Hz. Contractions and intracellular Ca²⁺ concentrations were measured simultaneously. A stop valve ensured that at a given time the cells were exposed to only one type of solution.

Cell shortening and intracellular Ca^{2+} were measured simultaneously throughout the experiment in 5min intervals. All myocytes were initially superfused with oxygenated Tyrode's solution (Table #3) for a 10min baseline period. Cardiomyocytes were then exposed to a cardioplegic solution (Table #4), which consisted of perfusion buffer formulated to mimic the whole heart experiment cardioplegia solution (Table #5). During the cardioplegic arrest period (45min), a 90% N_2 /10% CO_2 gas flow was directed over the top of the chamber to exclude atmospheric oxygen. After ischemia the myocytes were reperfused for 40min by reintroduction of oxygenated Tyrode's buffer and the removal of the 90% N_2 /10% CO_2 gas.

Table #4: Composition of isolated cardiomyocyte cardioplegic solution

Compound	Concentration (mM)
NaCl	83.52
NaHCO_3	33.30
NaH_2PO_4	0.72
Nagluconate	18.46
KCl	18.42
Glucose	4.4
MgSO_4	4.61
CaCl_2	1.0
pH 6.8 (Bubble with 90% N_2 /10% CO_2)	
SEA0400 Cardioplegia included additional 1μM SEA0400 (dissolved in DMSO)	
Control (Standard) Cardioplegia did not included SEA0400	

Table #5: Base ionic composition of cardioplegic solutions

Ionic concentration (mM)	Isolated Heart*	Isolated Cardiomyocyte**
Na ⁺	136	136
K ⁺	18.8	18.42
Mg ²⁺	5	4.61
Ca ²⁺	1	1.0
Bubble with 95% O ₂ /5%CO ₂		
SEA0400 Cardioplegia included additional 1μM SEA0400 (dissolved in DMSO)		
Control (Standard) Cardioplegia did not included SEA0400		

*Approximate final ionic concentration after mixing base solution to blood in a ratio of 1:4 respectively.

**Cardioplegia used in isolated cells does not contain blood.

Experimental Groups

Cardiomyocytes were selected at random to participate in the following experimental groups:

Experiment # 1:

To determine the effect of SEA0400 in a cardioplegic model three groups were examined:

1) Group I (Control group): Myocytes were superfused with Tyrode's solution for 10 minutes, followed by 45 minutes of simulated ischemia where cells were perfused with standard cardioplegia solution, followed by 40 minutes of reperfusion.

2) Group II (treated group): Myocytes were superfused with Tyrode's solution for 10 minutes, followed by 45 minutes of simulated ischemia where cells were perfused with 1 μ M SEA0400 cardioplegia solution, followed by 40 minutes of reperfusion.

3) Group III (Time control): Myocytes were superfused with Tyrode's solution for the entire duration of 85 minutes.

Experiment # 2:

To examine the effect of SEA0400 in a non-cardioplegic model:

The cardiomyocytes were first superfused with Tyrode's solution for 10 minutes, followed by 35 minutes of perfusion with Tyrode's solution enhanced with 1 μ M SEA0400.

The experimental protocol for the various groups is outlined below in Figure #6.

Experiment #1

Group 1)

Pre-ischemia (baseline 10 min)	Standard Cardioplegia (SEA0400 absent) (45 min)	Reperfusion (40 min)
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Group 2)

Pre-ischemia (baseline 10 min)	Cardioplegia 1 μ M SEA0400 (45 min)	Reperfusion (40 min)
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Group 3)

Time Control (95 min)

Group 1: (Control group) myocytes were exposed to standard cardioplegic solution. Group 2: (Treated group) myocytes were exposed to SEA0400 cardioplegic solution. Group 3: (Time control) myocytes were exposed to Tyrode's buffer throughout.

Experiment #2

Tyrode's Buffer (10 min)	Tyrode's Buffer with (1 μ M) SEA0400 (35 min)
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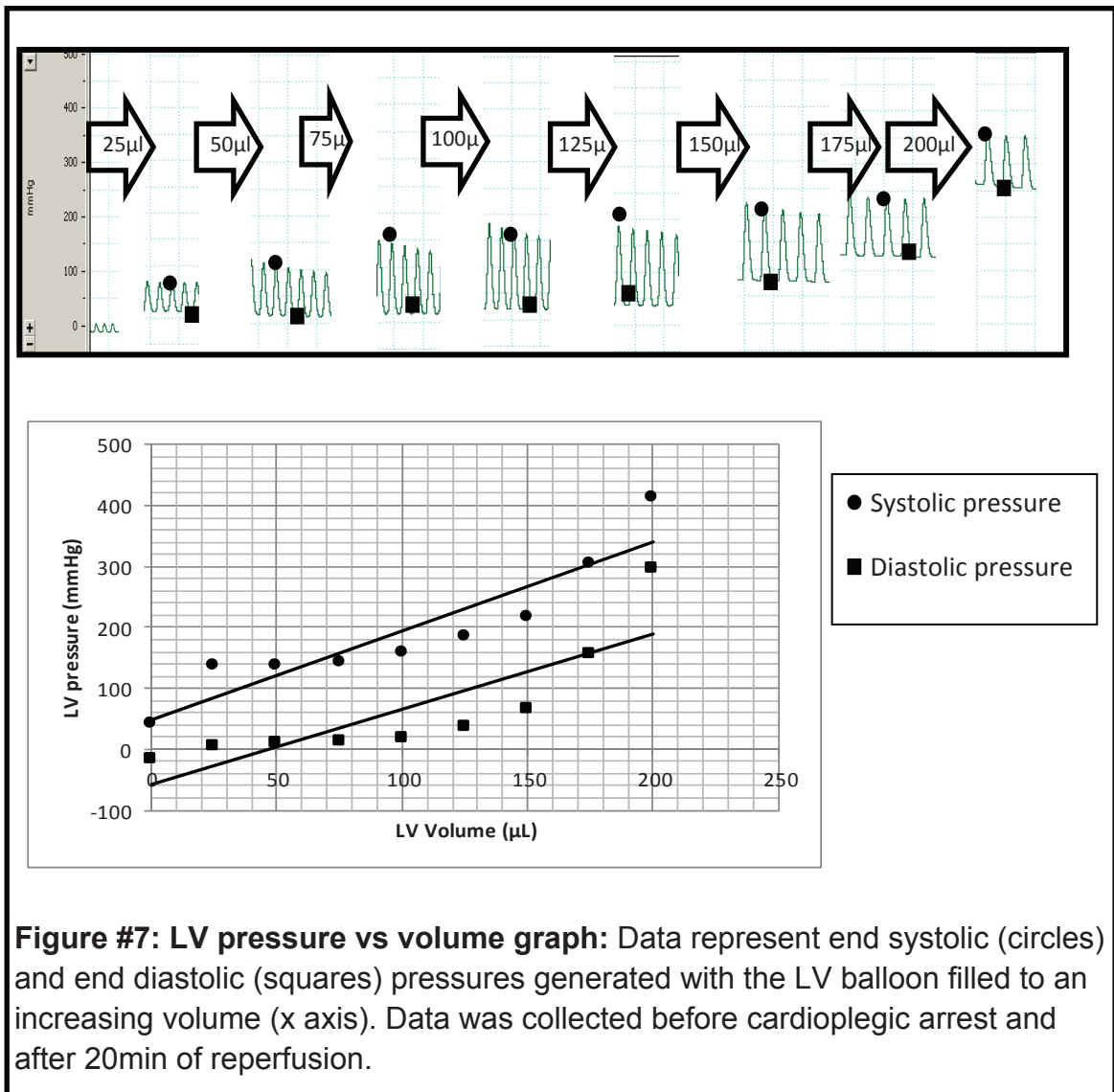
Exp#2: myocytes were superfused with Tyrode's solution for 10 minutes, followed by 35 minutes of 1 μ M SEA0400 Tyrode's solution.

Figure #6: Experimental groups.

Measurements

Hemodynamic data collection from Langendorff.

The Figure #7 illustrates the LV pressure vs volume curves. Data points for the curves were obtained from LV generated pressures in response to increased volume injected into the balloon.



The LV work: (difference between the area under the systolic and diastolic pressure volume curve)

Coronary vascular resistance (CVR): was derived from constant perfusion flow (10 ml/min) and mean perfusion pressure (MAP) with the following formula.

$$CVR \text{ (mmHg} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}) = \text{MAP (mmHg)} / 10 \text{ ml/min}$$

Isolated cardiomyocyte calculation:

Video images of experimental myocytes were relayed to a monitor and coupled to a video edge detector (Model# 105; Crescent Electronics, Sandy, UT). Cellular contractions were measured by tracking both edges of each experimental myocyte with the video edge detector. Recordings of approximately ten seconds in duration were taken every five minutes. The contractions from approximately 10 seconds of recording were averaged and recorded with Clampfit 8.2 (Molecular Devices, Sunnyvale, CA). Contraction amplitudes were measured by calculating the difference between the diastolic and systolic cell length.

Intracellular Ca^{2+} concentrations were measured using the Ca^{2+} sensitive fluorescent indicator fura-2. A stock solution of Fura-2 was prepared in anhydrous DMSO. Myocyte suspension (1 ml) was mixed with fura-2 before transfer to the light-tight experimental chamber. Once plated in the experimental chamber the cells were allowed 20min to settle and absorb the membrane permeable fura-2 dye. The concentration of fura-2 used was 5 μM . The ratio

emission at 510nm following excitation at 340nm and 380nm was used to calculate intracellular Ca^{2+} concentrations. Background fluorescence values were subtracted from the recordings made at each wavelength throughout the experiment. Emission ratios were converted to intracellular Ca^{2+} concentrations with an in vitro calibration curve. The Ca^{2+} concentrations were determined by using a calibration curve of e_{340}/e_{380} at known Ca^{2+} concentrations (O'Brien et al., 2008).

Ca^{2+} transient amplitudes were defined as the difference between diastolic Ca^{2+} and peak systolic Ca^{2+} . Both cell length and intracellular Ca^{2+} were measured in clampfit 8.2 (Molecular Devices Corp., Sunnyvale, CA). Trains of contractions or Ca^{2+} transients of approximately 5-10 seconds were averaged prior to measurement to control for variability induced by signal noise in the recordings. To facilitate comparisons between groups, data were normalized to control recordings at beginning of the experiments.

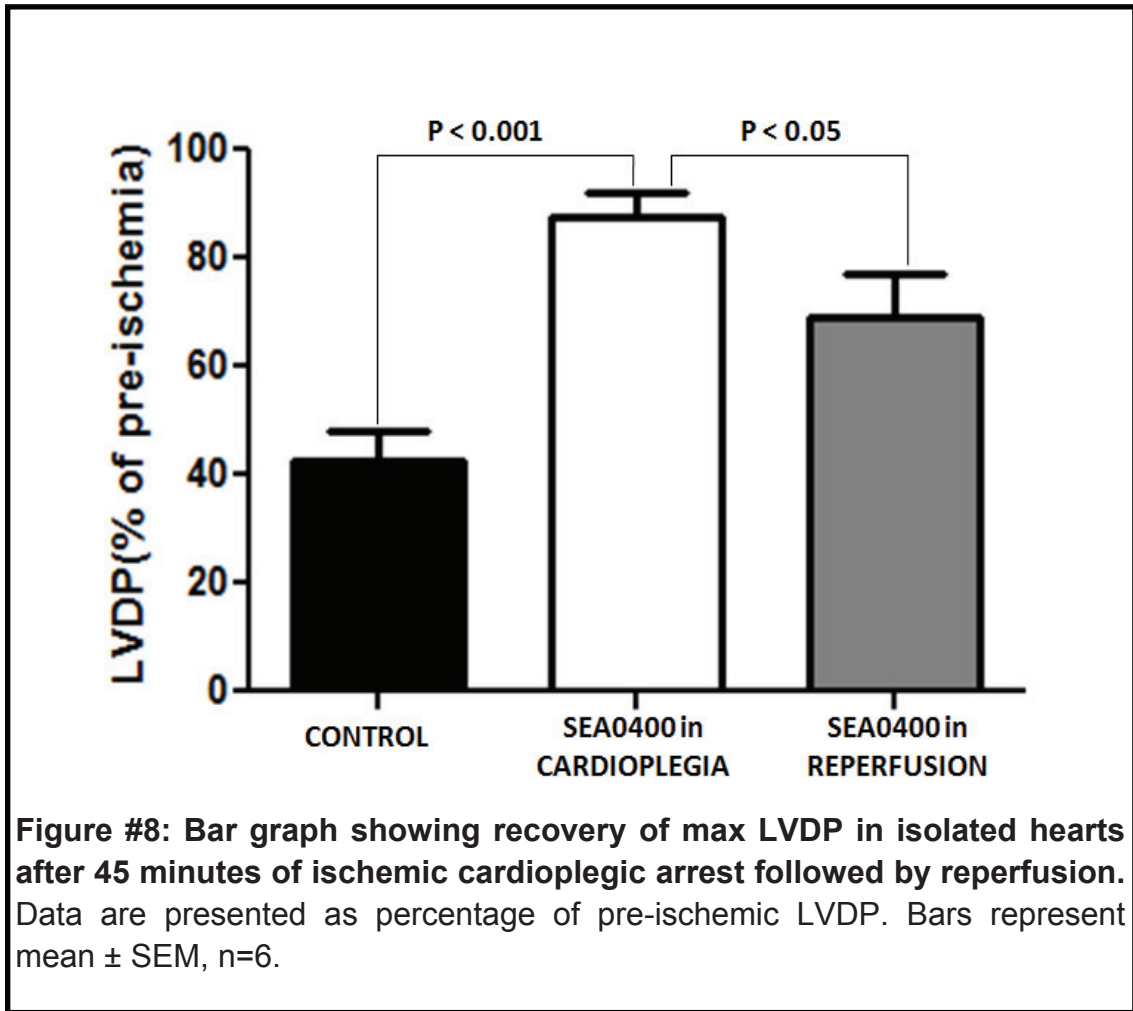
Statistical Analysis

All data are presented as means \pm S.E.M. Statistical analyses were performed using GraphPad Prism 5.0 and SigmaStat 3.1(Systat Software Inc, San Jose CA). Differences between groups were analyzed with one/two-way ANOVA. Differences were considered statistically significant if $p < 0.05$. All figures were constructed in GraphPad Prism 5.0 and Sigmaplot 8.0(Systat Software Inc, San Jose CA)

CHAPTER III: RESULTS

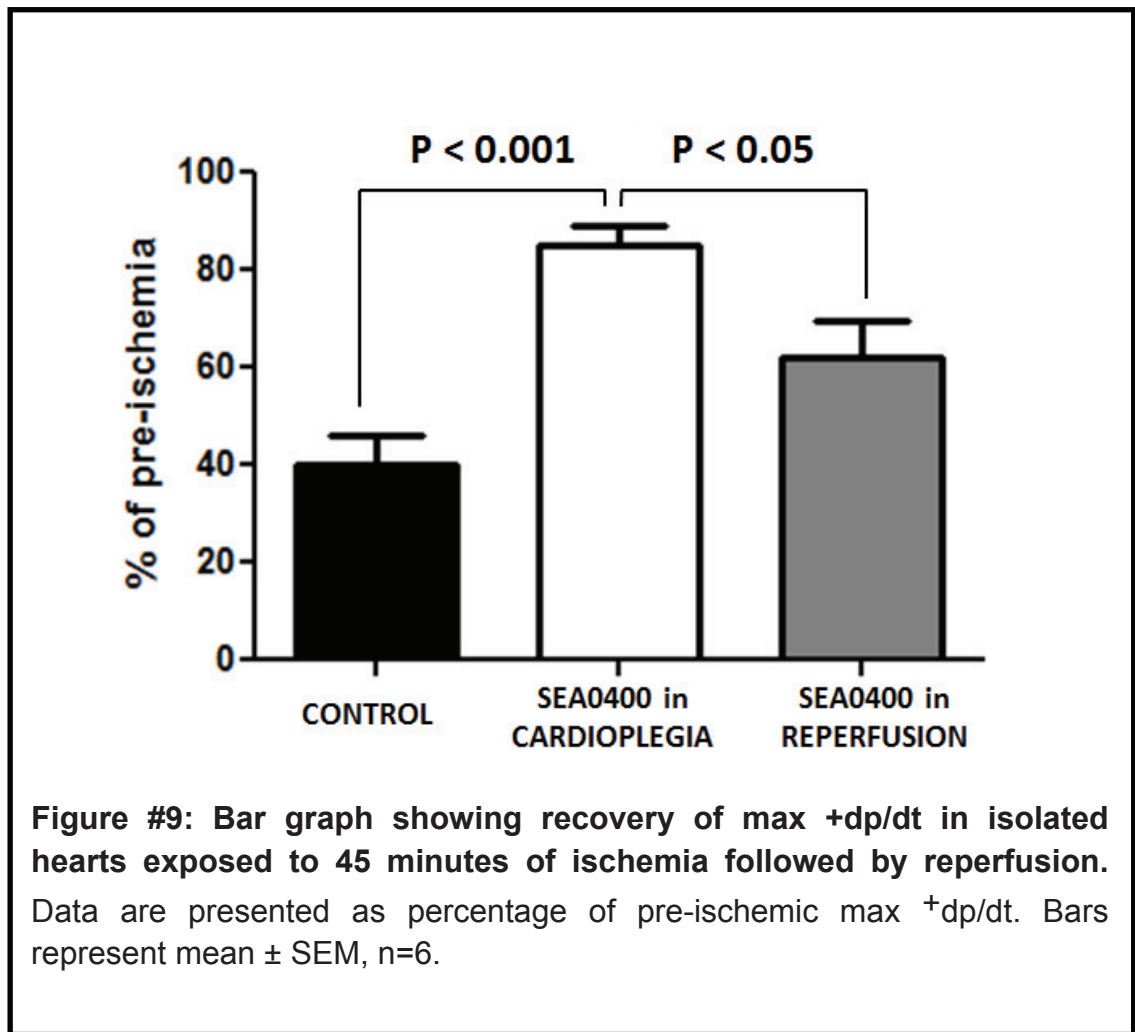
Left Ventricle (LV) contractile recovery in isolated hearts after a 45min period of cardioplegic arrest.

In the isolated heart experiments, various hemodynamic measurements were assessed to evaluate the contractile function of the LV. Hemodynamic measurements were recorded before ischemic arrest and repeated again after reperfusion. The recordings obtained after 20min of reperfusion were compared to pre-ischemic control values, and presented as a percentage of pre-ischemia values. The first hemodynamic measurement analyzed among the various groups was maximum (max) left ventricle developed pressure (LVDP). Figure #8 shows the max LVDP recovery during reperfusion in isolated hearts exposed to 45 minutes of ischemic cardioplegic arrest followed by 20min of reperfusion. The max LVDP during reperfusion recovered to $41 \pm 4\%$ of pre-ischemia in the control group where the hearts were protected using standard cardioplegia. When cardioplegia containing SEA0400 was used, max LVDP during reperfusion recovered to $88 \pm 2\%$ of pre-ischemia ($P < 0.001$ vs. control). In the final group where SEA0400 was delivered during reperfusion, max LVDP recovered to $72 \pm 8\%$ of pre-ischemia ($P < 0.05$ vs. control).



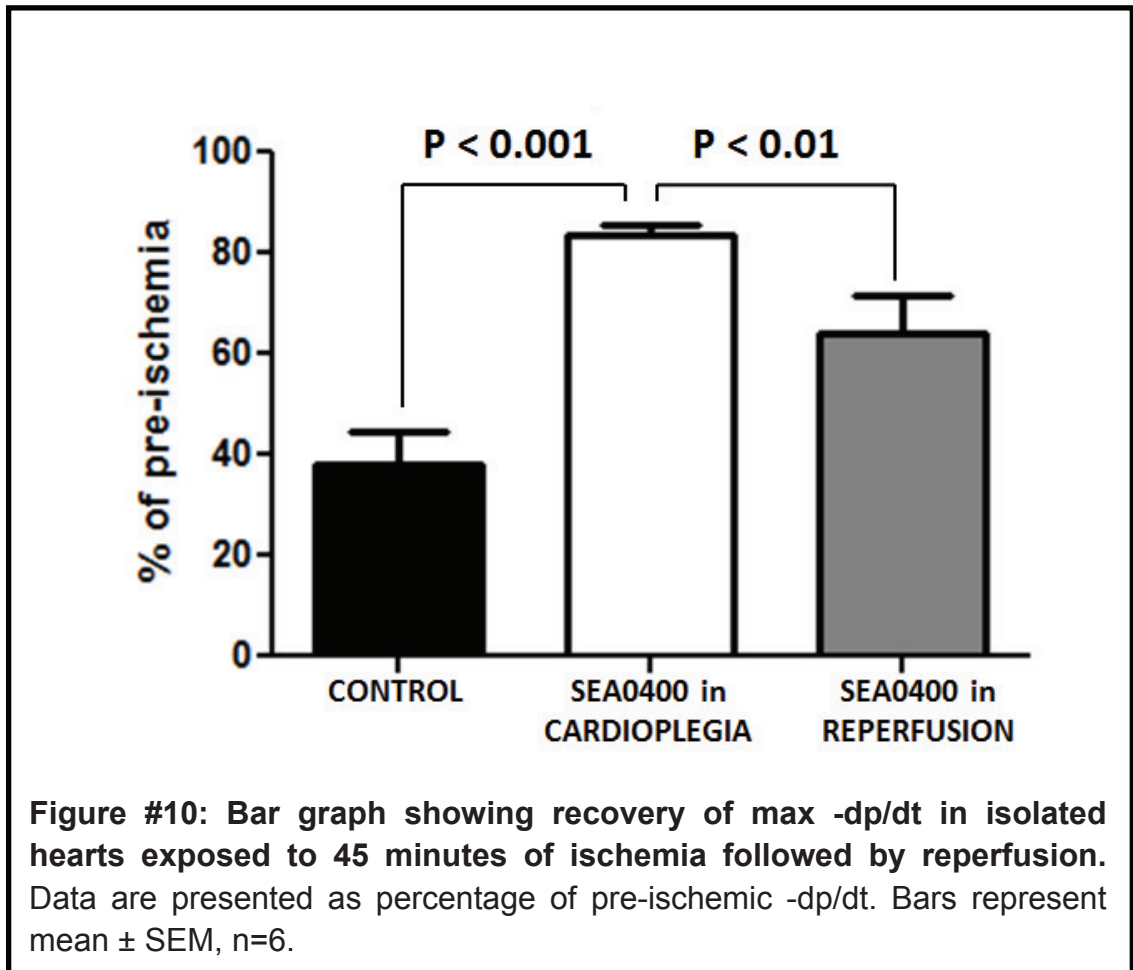
The next hemodynamic measurement analyzed among the various groups was the instantaneous changes in pressure. During a heartbeat, the heart cycles between systolic contraction and diastolic relaxation. The systolic function (contraction) of the LV was evaluated by measuring the maximal generated instantaneous pressure ($^+dp/dt$). The diastolic function (relaxation) of the LV was evaluated by measuring the maximal instantaneous drop in pressure. Figure #9 shows the max $^+dp/dt$ preservation in isolated hearts exposed to 45 minutes of ischemic cardioplegic arrest followed by 20min of reperfusion. The max $^+dp/dt$

after reperfusion recovered to $39 \pm 4\%$ of pre-ischemia in the control group where standard cardioplegia was delivered. When cardioplegia containing SEA0400 was delivered, the max $^{+}dp/dt$ during reperfusion recovered to $87 \pm 2\%$ of pre-ischemia ($P < 0.001$ vs. control). In the final group where SEA0400 was delivered during reperfusion, the max $^{+}dp/dt$ during reperfusion recovered to $65 \pm 8\%$ of pre-ischemia ($P < 0.05$ vs. control).

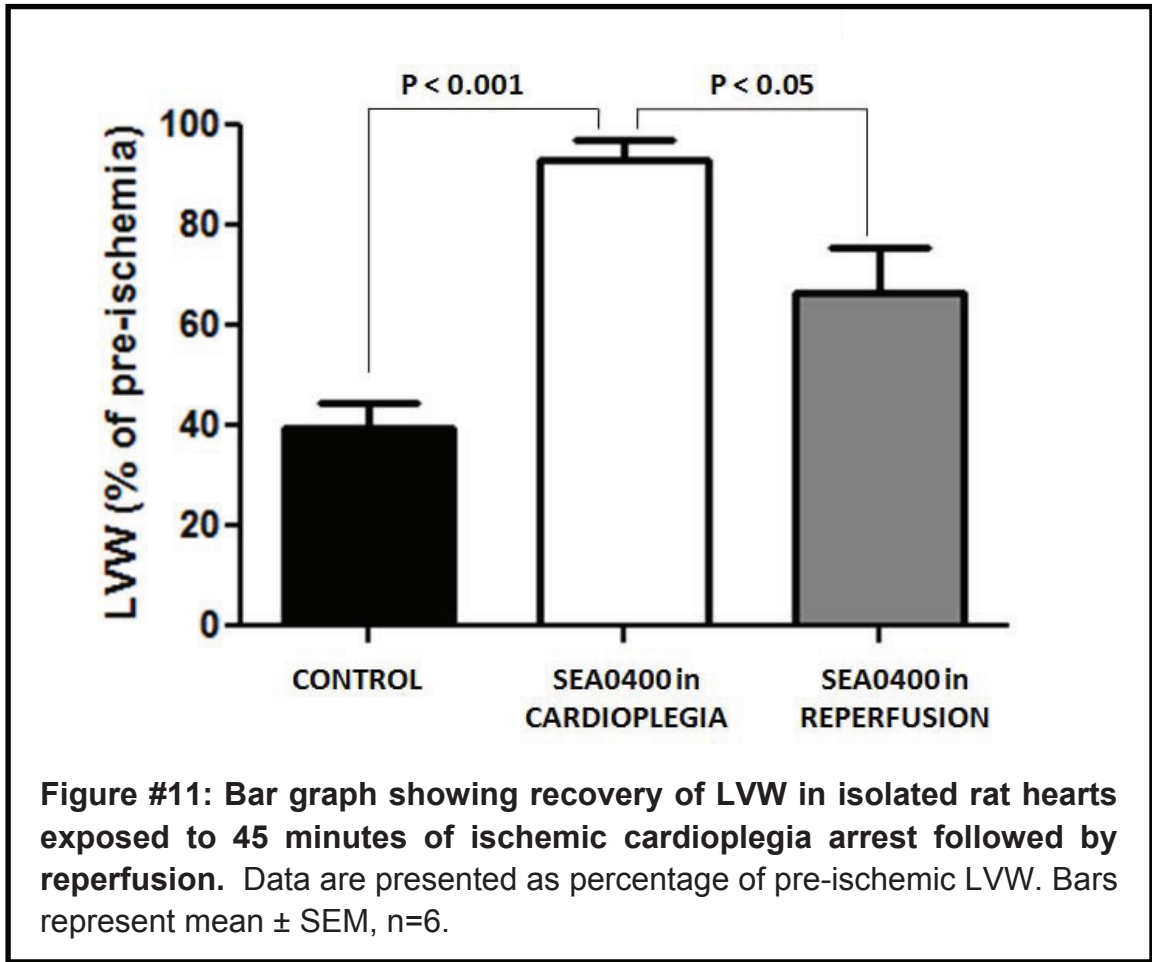


The diastolic function (relaxation) of the LV was evaluated by measuring the maximal negative instantaneous change in pressure $-dp/dt$. Figure #10 shows

the max -dp/dt preservation in isolated myocardium exposed to 45 minutes of ischemic cardioplegic arrest followed by 20min of reperfusion. The max -dp/dt during reperfusion recovered to $39 \pm 4\%$ of pre-ischemia in the control group where standard cardioplegia was delivered. When cardioplegia containing SEA0400 was delivered, the max -dp/dt during reperfusion recovered to $85 \pm 2\%$ of pre-ischemia ($P < 0.001$ vs. control). In the final group where SEA0400 was delivered during reperfusion, the max -dp/dt after reperfusion recovered to $65 \pm 8\%$ of pre-ischemia ($P < 0.05$ vs. control).

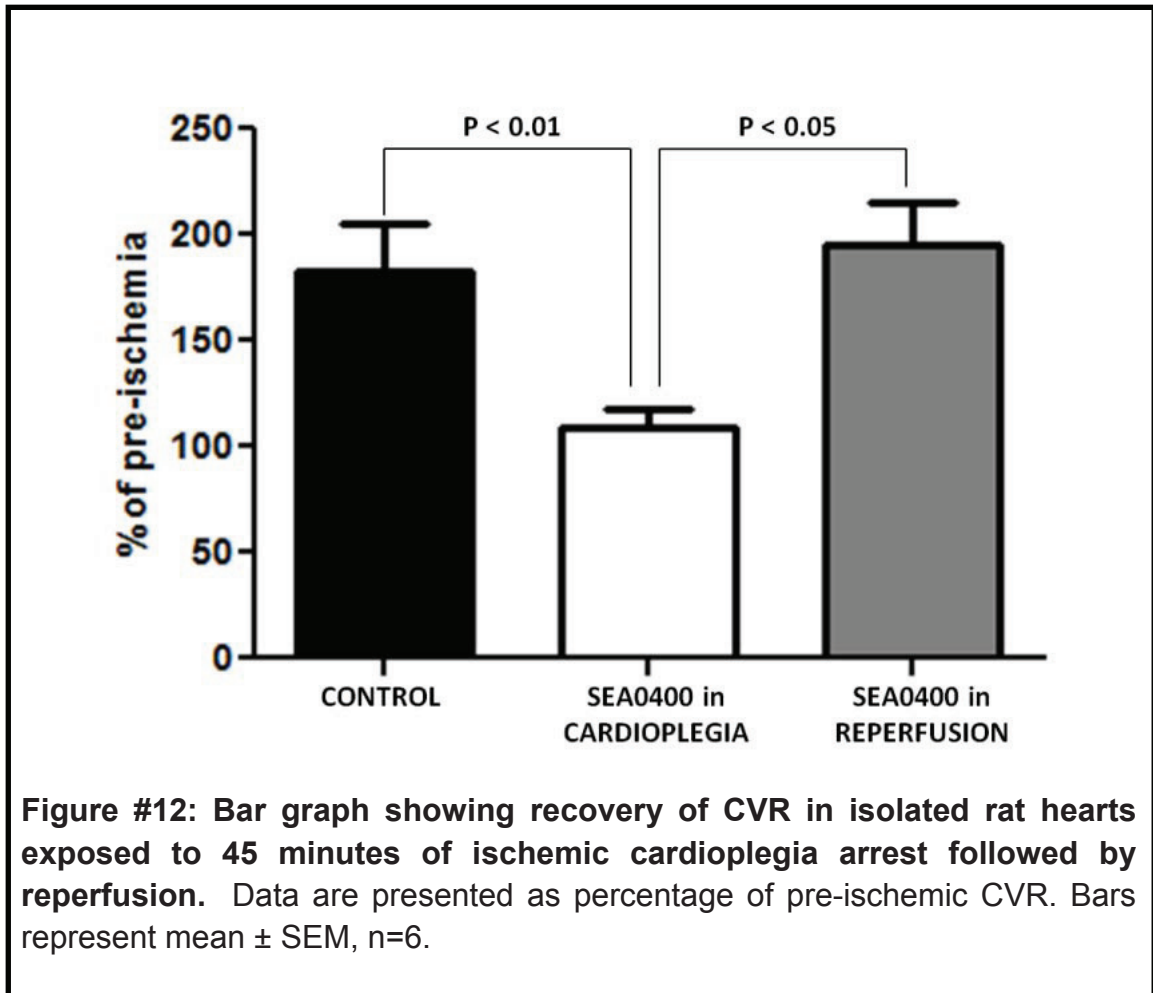


The next hemodynamic measurement analyzed among the various groups was the left ventricle work (LVW). The LVW was determined through generation of volume vs pressure curves. The area between the systolic and diastolic curves represented the LVW. Figure #11 shows recovery of LVW in isolated hearts exposed to 45 minutes of ischemic cardioplegic arrest followed by 20min of reperfusion. The LVW during reperfusion recovered to $39 \pm 4\%$ of pre-ischemia in the control group where standard cardioplegia was delivered. When cardioplegia containing SEA0400 was used, the LVW after reperfusion recovered to $90 \pm 2\%$ of pre-ischemia ($P < 0.001$ vs. control). In the final group where SEA0400 was delivered during reperfusion, LVW after reperfusion recovered to $68 \pm 8\%$ of pre-ischemia ($P < 0.05$ vs. control).



The final hemodynamic measurement analyzed among the various groups was coronary vascular resistance (CVR). Figure #12 shows the preservation of CVR in isolated hearts exposed to 45 minutes of ischemic cardioplegic arrest followed by 20min of reperfusion. The CVR during reperfusion increased dramatically to $181 \pm 19\%$ of pre-ischemia in the control group where standard cardioplegia was delivered. When cardioplegia containing SEA0400 was delivered, the CVR during reperfusion was only $110 \pm 7\%$ of pre-ischemia

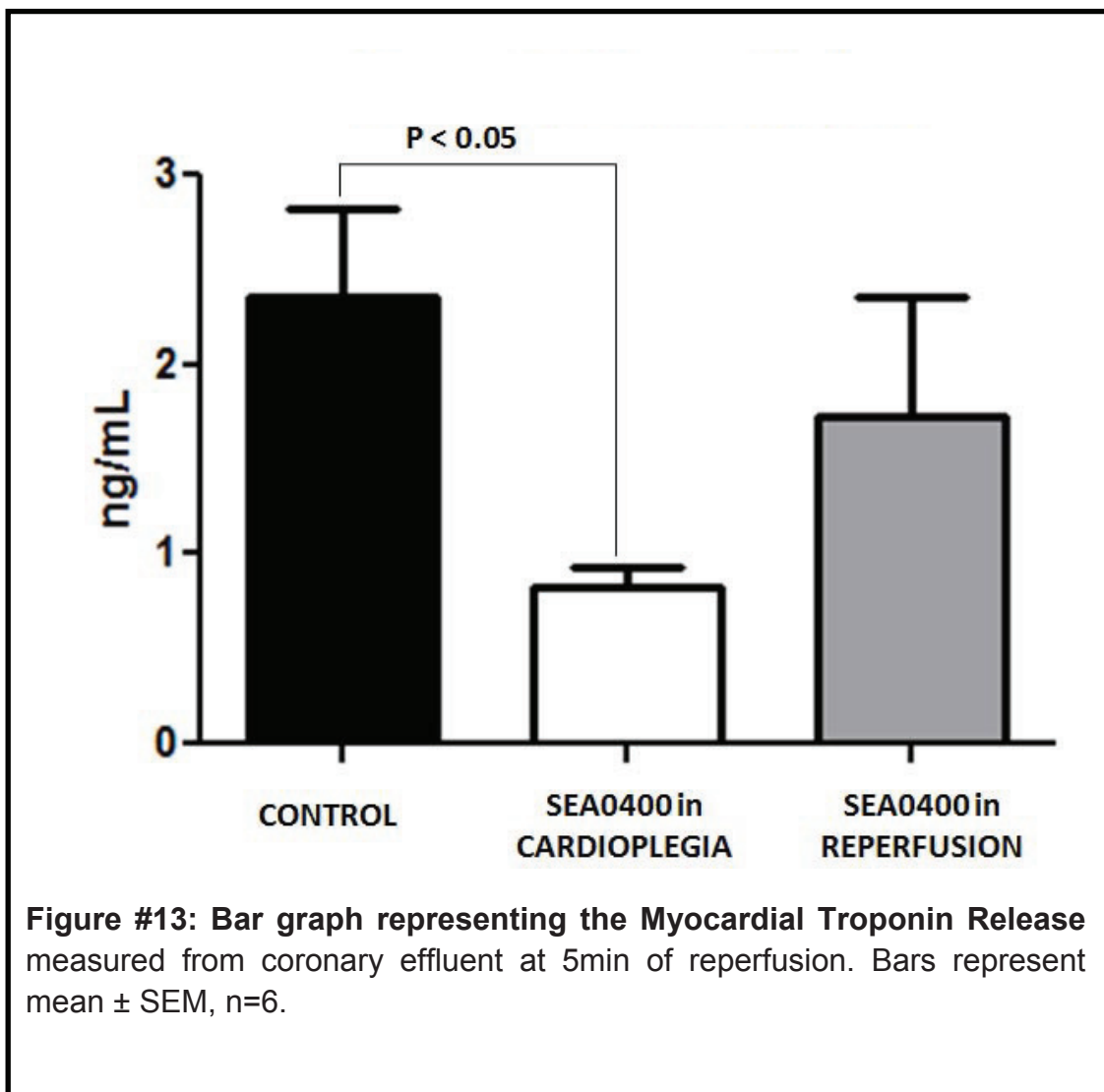
($P < 0.01$ vs. control). In the final group where SEA0400 was delivered throughout reperfusion, the CVR increased to $192 \pm 20\%$ of pre-ischemia.



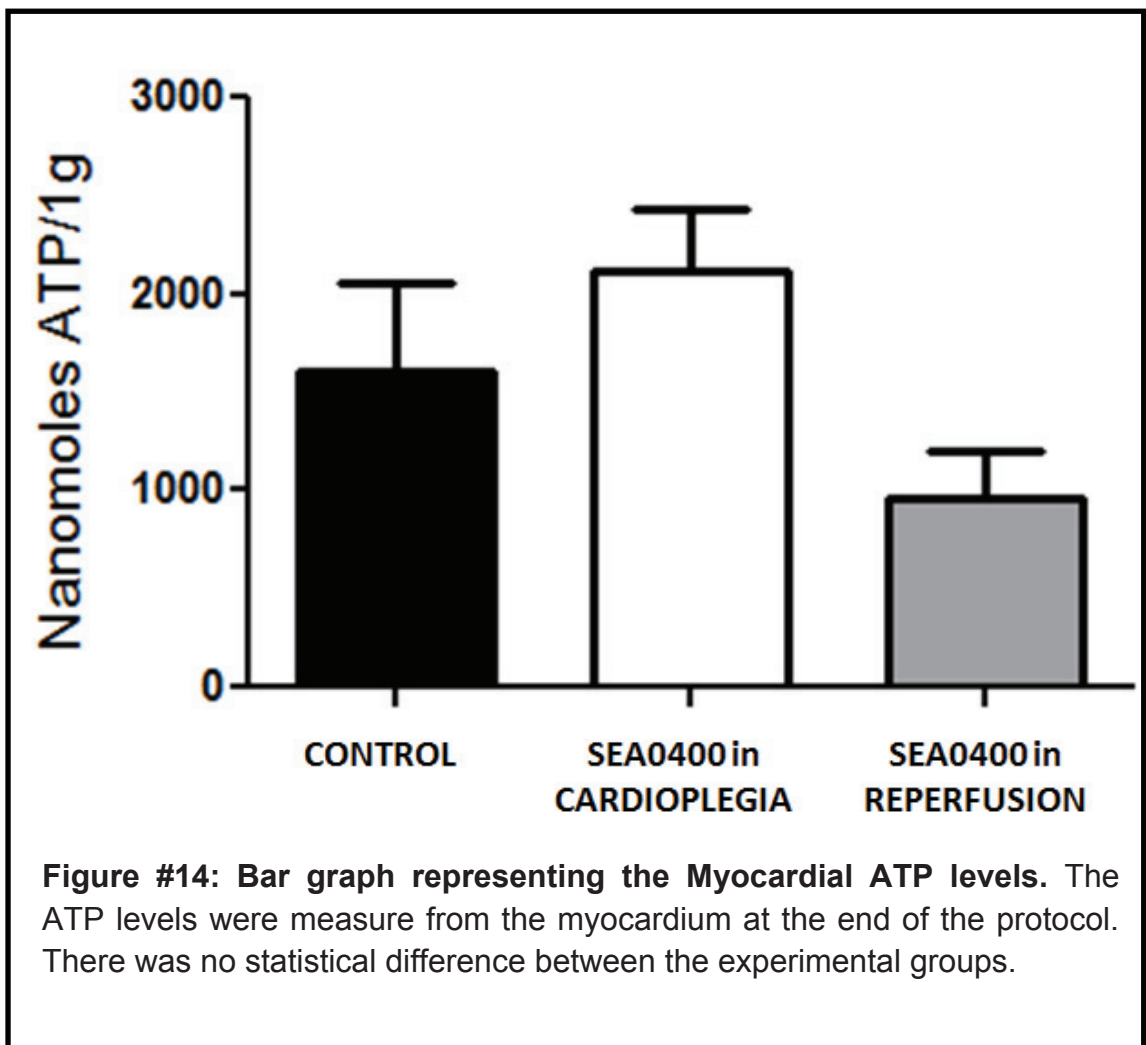
Bio-molecular effects in isolated hearts in response to a 45min period of cardioplegic arrest.

To evaluate the effect of SEA0400 on myocardial injury during cardioplegic arrest and reperfusion, we measured values of Tn-I release from the coronary sinus effluent during reperfusion. Figure #13 shows the amount of troponin

release measured from coronary sinus effluent during reperfusion. The greatest amount of troponin release was observed in the control group where the standard cardioplegia was delivered, 2.4 ± 0.4 ng/ml. In contrast the least amount of troponin release was observed in the group exposed to cardioplegia containing SEA0400, 0.9 ± 0.1 ng/ml ($P < 0.05$ vs control). When SEA0400 was delivered during reperfusion troponin release was similar to control.

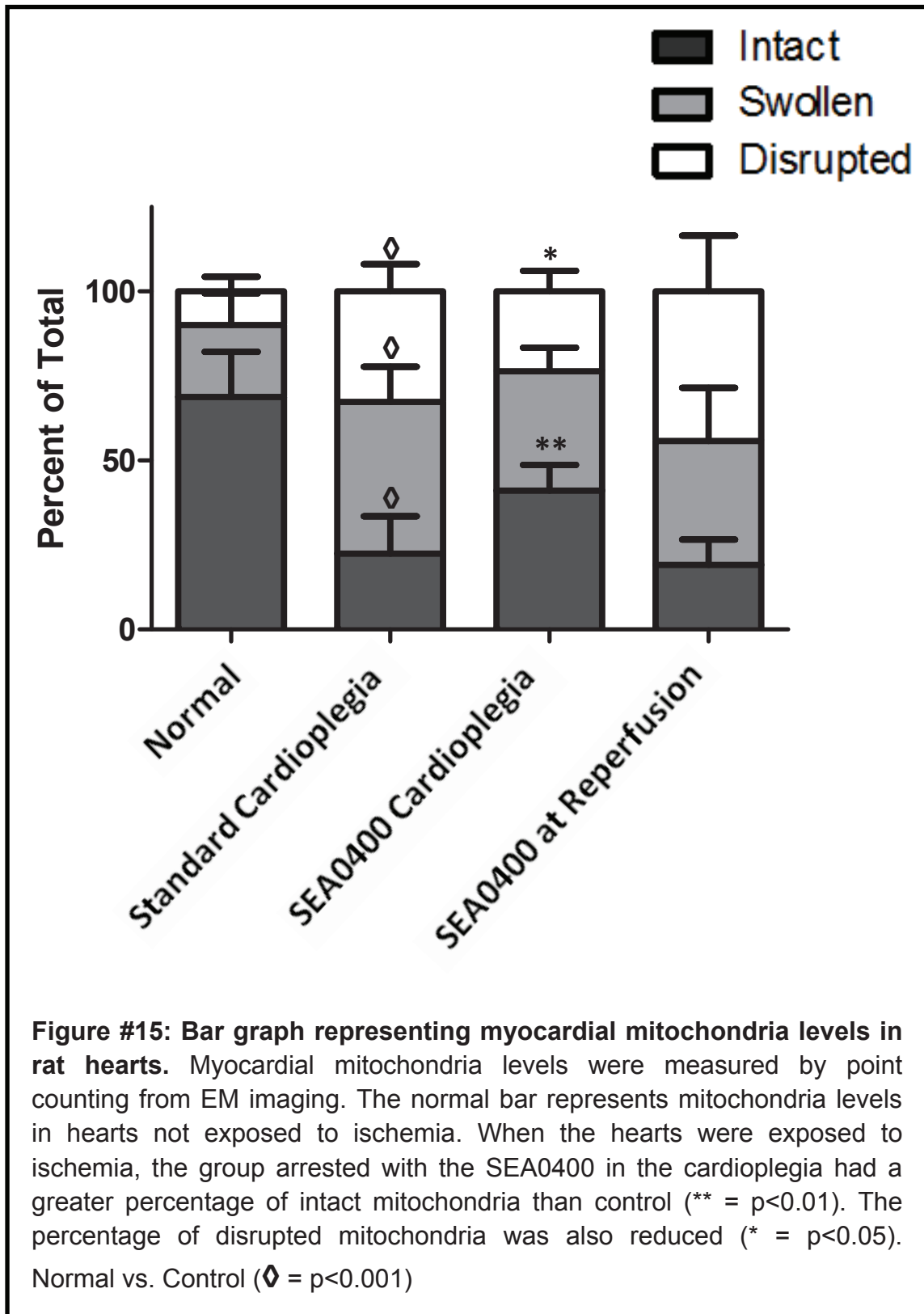


We evaluated myocardial energy status by measuring myocardial ATP levels at the end of the experiment. In hearts arrested with standard cardioplegia ATP levels were 1700 ± 310 nm/g. In hearts arrested with cardioplegia containing SEA0400 there was a small but not significant increase in ATP levels 2000 ± 270 nm/g. Figure #14 shows the amounts of myocardial ATP measured at the end of reperfusion. There was no significant difference in ATP levels among the various groups.



Myocardial Mitochondrial Morphology

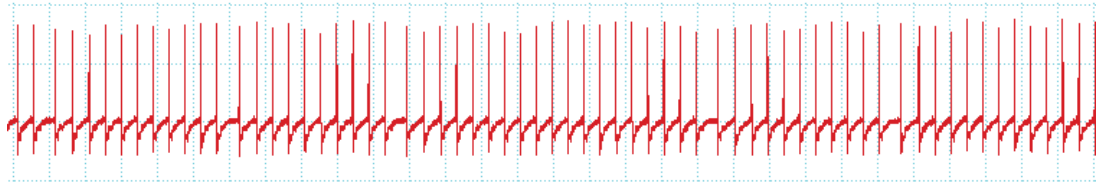
Myocardial mitochondria structural morphology and numbers were analyzed. Figure #15 shows the levels of mitochondria based on three different morphological structures, intact, disrupted, and swollen. The data from the three treated groups is presented as a percentage of total mitochondria with reference to a normal group (hearts not exposed to ischemia). The percentage of swollen mitochondria was similar among the three treated groups. The percentage of disrupted mitochondria varied among the groups. The percentage of disrupted mitochondria was greatest in the group exposed to SEA0400 during reperfusion ($44\% \pm 17$). In comparison the group exposed to standard cardioplegia had a lower number of disrupted mitochondria ($33\% \pm 8$). The group exposed to cardioplegia containing SEA0400 had the lowest amount of disrupted mitochondria among the treated groups ($23\% \pm 6$). The number of intact mitochondria was greatest in the group exposed to SEA0400 in the cardioplegia ($41\% \pm 7$). In comparison intact mitochondria levels were reduced in the group exposed to standard cardioplegia ($22\% \pm 11$). Similarly the group exposed to SEA0400 during reperfusion had a reduced number of intact mitochondria ($19\% \pm 7$).



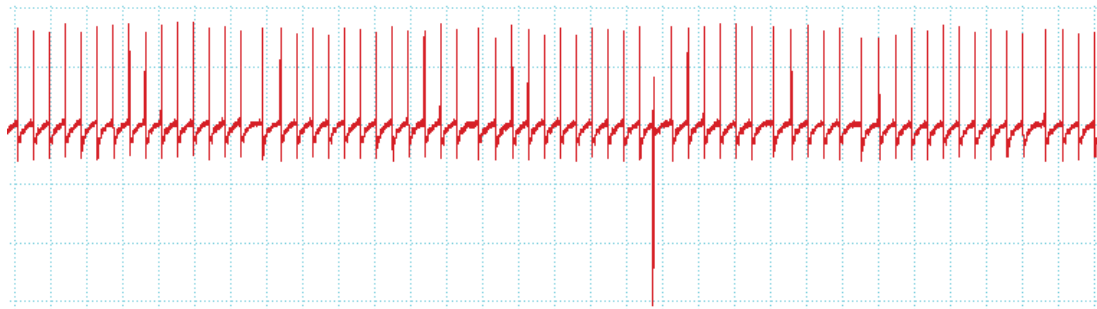
Arrhythmias observed during reperfusion in isolated hearts in response to a 45min period of cardioplegic arrest.

The electric activity of the heart was recorded throughout the protocol to monitor and assess the generation of arrhythmias during reperfusion. Figure #16 shows representative ECG traces from Langendoff apparatus. The reperfusion time period before the onset of arrhythmia was recorded. Figure #17 shows the time in minutes before the onset of arrhythmia. In the control group, reperfusion arrhythmia began after 1.6 ± 0.1 min of reperfusion. In contrast, when SEA0400 was delivered during the cardioplegia, reperfusion arrhythmia onset was delayed to 5.6 ± 1.5 min of reperfusion. Delivering the SEA0400 throughout reperfusion did not delay the onset of arrhythmia.

A)



B)



C)

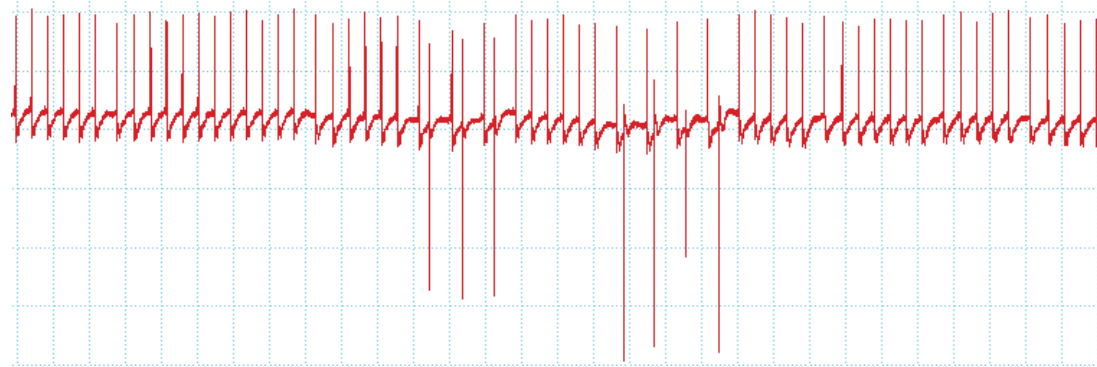


Figure #16: Representative ECG traces of heart rhythm from Langendorff apparatus during early reperfusion. A) Normal rhythm B) premature ventricular contraction (PVC) C) runs of ventricular bigeminy

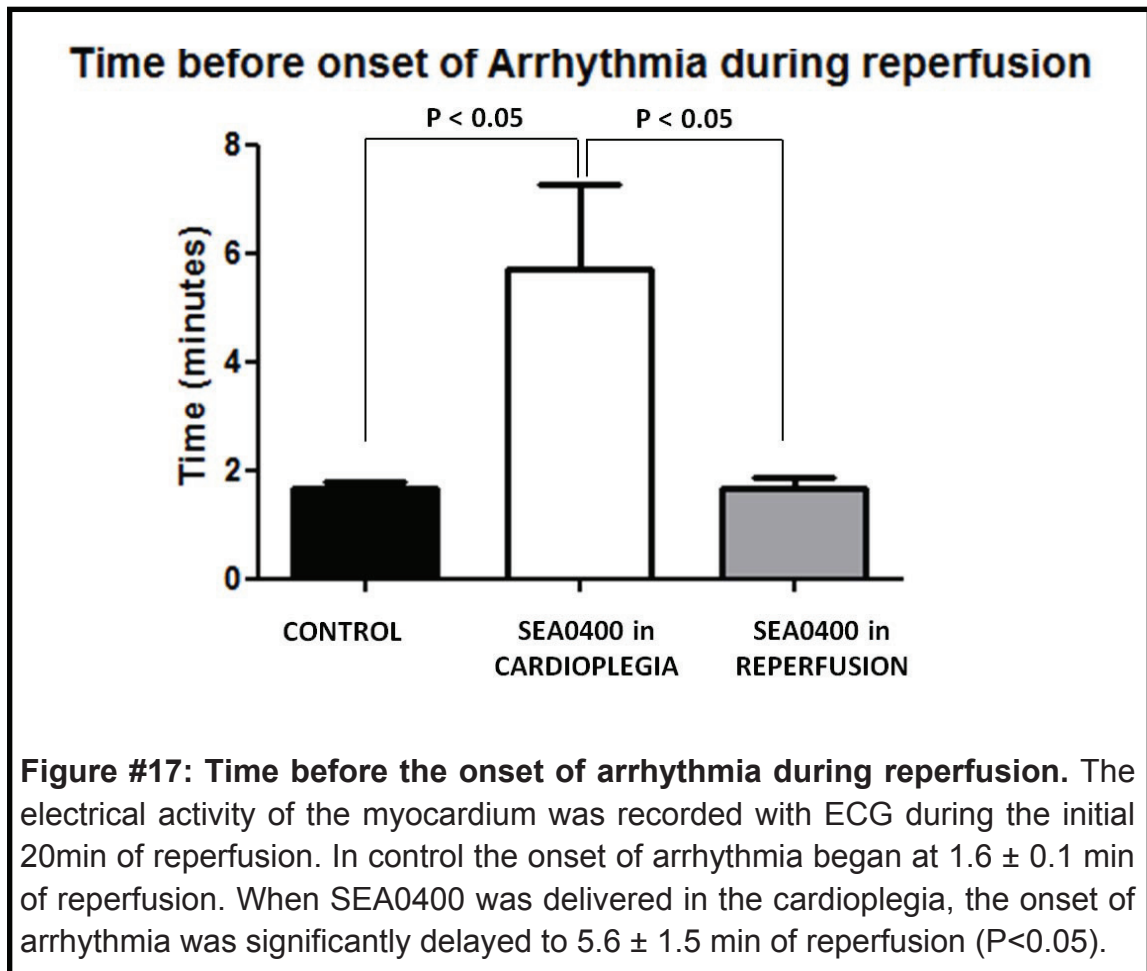


Figure #18 shows the time during reperfusion when arrhythmia stopped. There was no difference among the groups in the time arrhythmias stopped. All arrhythmias throughout the various groups disappeared after 18min of reperfusion.

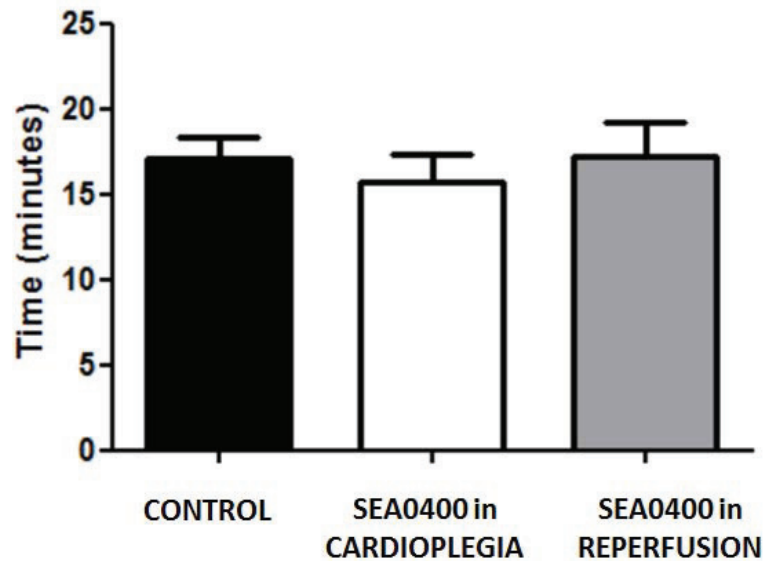


Figure #18: The time at which arrhythmias stopped during reperfusion. In all groups arrhythmias were absent after 18 min of reperfusion.

Recovery of isolated ventricular myocytes exposed to SEA0400.

Experiment #1:

In the isolated cardiomyocyte experiments, contractions and intracellular Ca^{2+} concentrations were measured to assess and evaluate the benefit of SEA0400 as a cardioplegic additive. The first measurement analyzed among the various groups was myocyte contraction. Figure #19 shows the representative

recordings of contraction amplitude during baseline (pre-ischemia) and during reperfusion (post-ischemia) for ventricular myocytes exposed to cardioplegic solution with or without SEA0400.

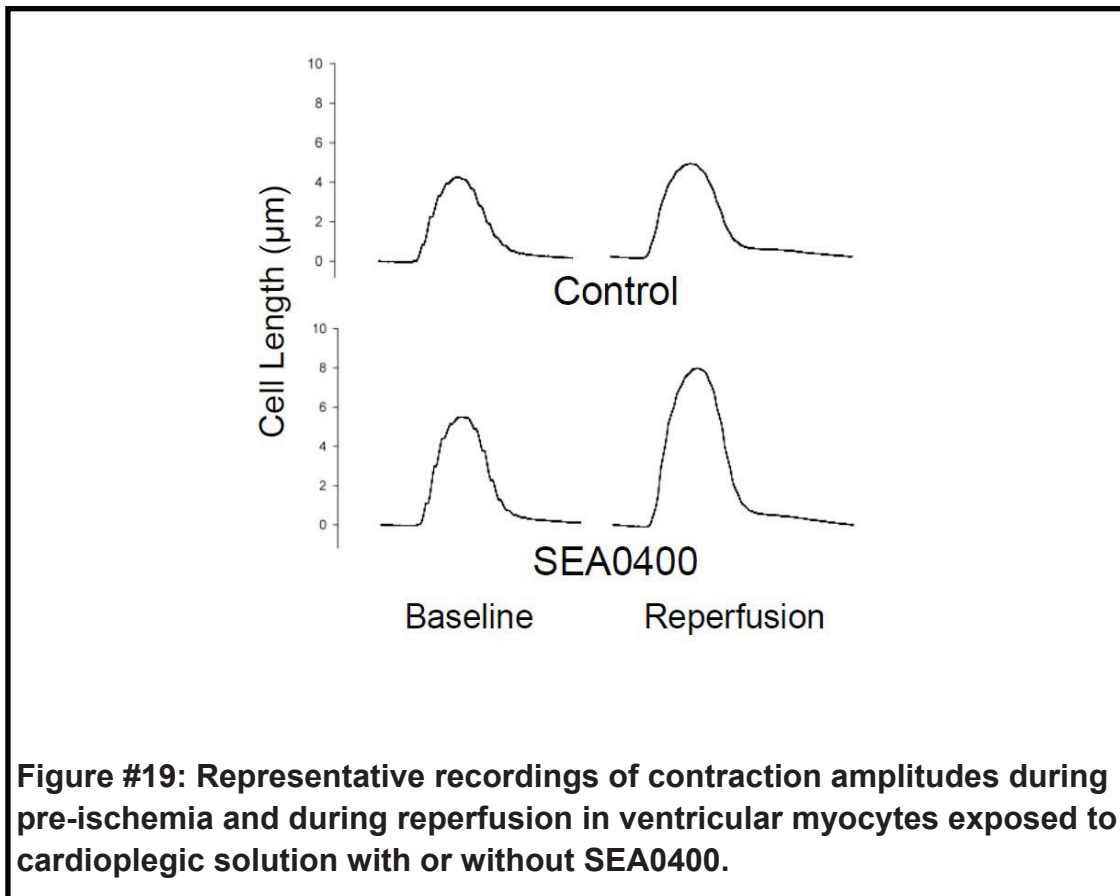
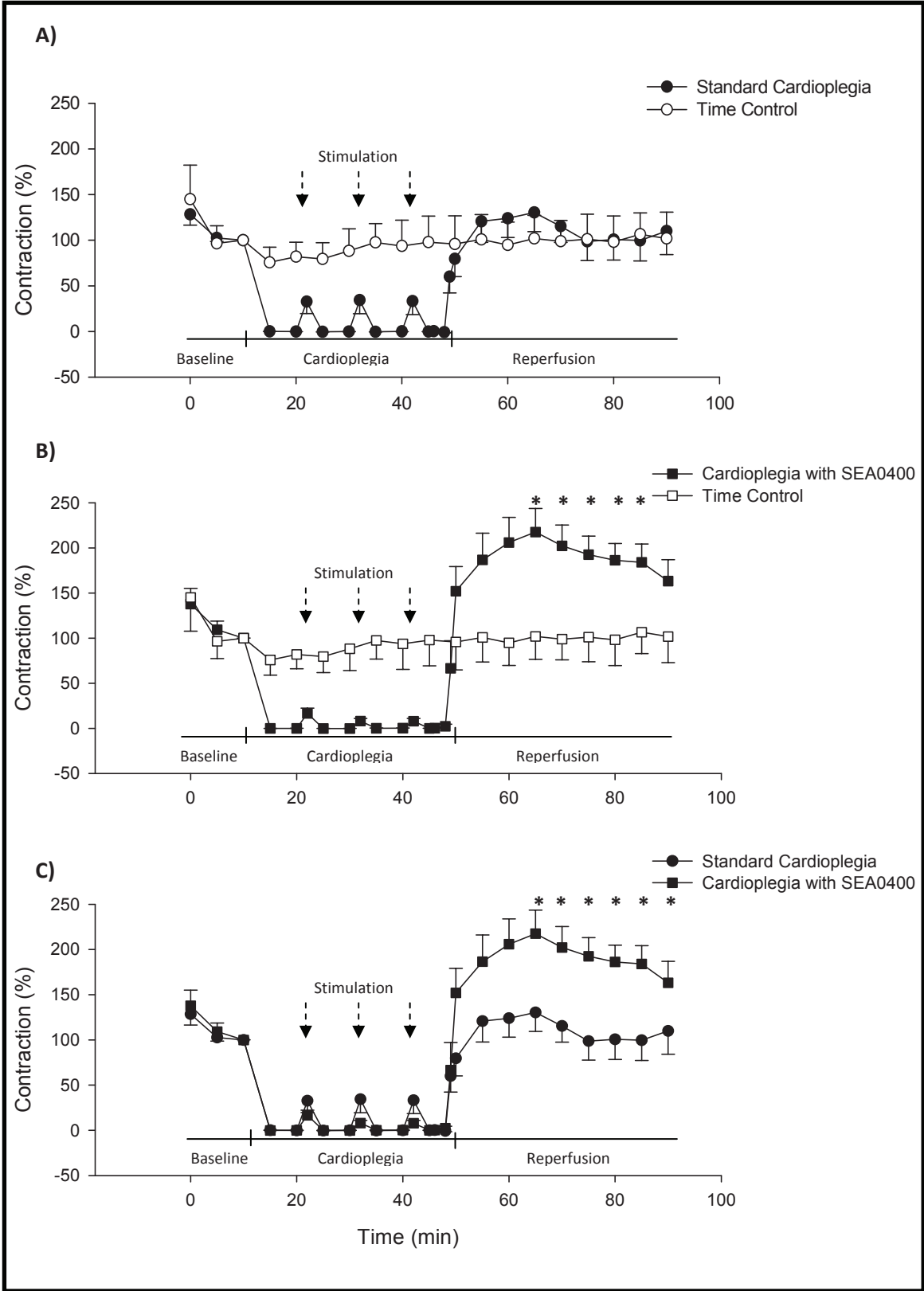


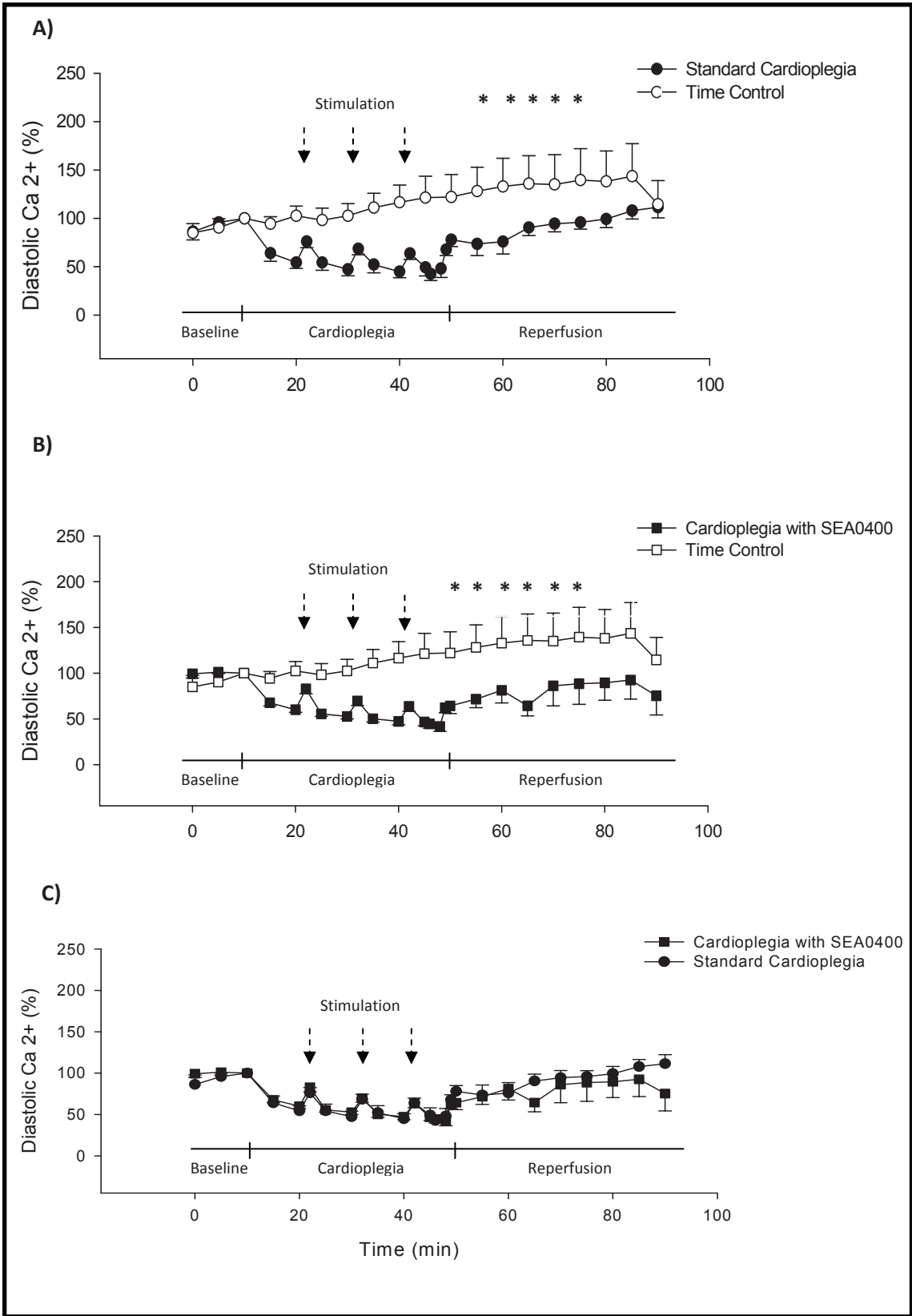
Figure #20 shows the contraction amplitudes of ventricular myocytes exposed to 45 minutes of ischemic cardioplegic arrest followed by 40min of reperfusion. Panel A compares the mean contraction amplitudes in cells that served as time controls and cells that were exposed to standard cardioplegia. Contraction amplitudes during reperfusion were similar in cells exposed to standard cardioplegia compared to time control. Panel B compares the mean contraction amplitudes in cells that served as time controls and cells that were exposed to cardioplegia containing SEA0400. Contraction amplitudes were significantly greater during reperfusion in cells exposed to SEA0400 cardioplegia compared to time control. Panel C compares contraction amplitudes in cells arrested with SEA0400 cardioplegia vs. cells arrested with standard cardioplegia. Contraction amplitudes were significantly greater during reperfusion in cells exposed to SEA0400 cardioplegia compared to standard cardioplegia.

Figure #20: Contraction amplitudes were significantly elevated during reperfusion in cells exposed to cardioplegic solution with SEA0400 (1 μ M). **A)** In ventricular myocytes exposed to standard cardioplegia, contraction amplitudes during reperfusion were similar to time control (n=9). **B)** In ventricular myocytes exposed to cardioplegia with SEA0400 (1 μ M) contraction amplitudes were significantly elevated during reperfusion relative to time control. **C)** Contraction amplitude in cells exposed to cardioplegia with SEA0400 were significantly elevated during reperfusion in comparison to cells exposed to standard cardioplegia without SEA0400. Data are normalized to contraction amplitudes immediately before cardioplegic arrest. * denotes $p < 0.05$. In response to electric stimulation (arrows) at various points during the cardioplegic arrest period, both cardioplegic solutions maintained depressed contractions in ventricular myocytes.



The next measurement analyzed among the various groups was the diastolic intracellular Ca^{2+} levels. Figure #21 shows the diastolic Ca^{2+} levels of ventricular myocytes exposed to 45 minutes of ischemia followed by 40min of reperfusion. Panel A compares the mean diastolic Ca^{2+} in cells that served as time controls and cells that were exposed to standard cardioplegia. Diastolic Ca^{2+} levels during reperfusion were depressed in cells exposed to standard cardioplegia compared to time control. Panel B compares the mean diastolic Ca^{2+} in cells that served as time controls and cells that were exposed to cardioplegia containing SEA0400. Diastolic Ca^{2+} levels during reperfusion were depressed during reperfusion in cells exposed to SEA0400 cardioplegia compared to time control.

Figure #21: Diastolic Ca^{2+} levels were significantly depressed during reperfusion in cells exposed to Cardioplegic solution with or without SEA0400 (1 μM). **A)** In ventricular myocytes exposed to standard cardioplegia, Diastolic Ca^{2+} levels during reperfusion were significantly depressed relative to time control (n=9) **B)** In ventricular myocytes exposed to cardioplegia with SEA0400(1 μM) Diastolic Ca^{2+} levels were significantly depressed during reperfusion. **C)** Diastolic Ca^{2+} levels were similar in cells exposed to cardioplegia with or without SEA0400 during reperfusion. Data are normalized to Diastolic Ca^{2+} levels at time=10mins. * denotes $p < 0.05$. In response to electric stimulation (arrows) at various points during the cardioplegic arrest period, both cardioplegic solutions maintained depressed diastolic Ca^{2+} levels in ventricular myocytes.



Panel C compares diastolic Ca^{2+} levels in cells arrested with SEA0400 cardioplegia vs. cells arrested with standard cardioplegia. Diastolic Ca^{2+} levels were not significantly greater during reperfusion in cells exposed to SEA0400 cardioplegia compared to standard cardioplegia.

The next measurement analyzed among the various groups was the Ca^{2+} transient amplitudes. Figure #22 shows the representative recordings of Ca^{2+} transient levels during baseline (pre-ischemia) and during reperfusion (post-ischemia) for ventricular myocytes exposed to cardioplegic solution with or without SEA0400.

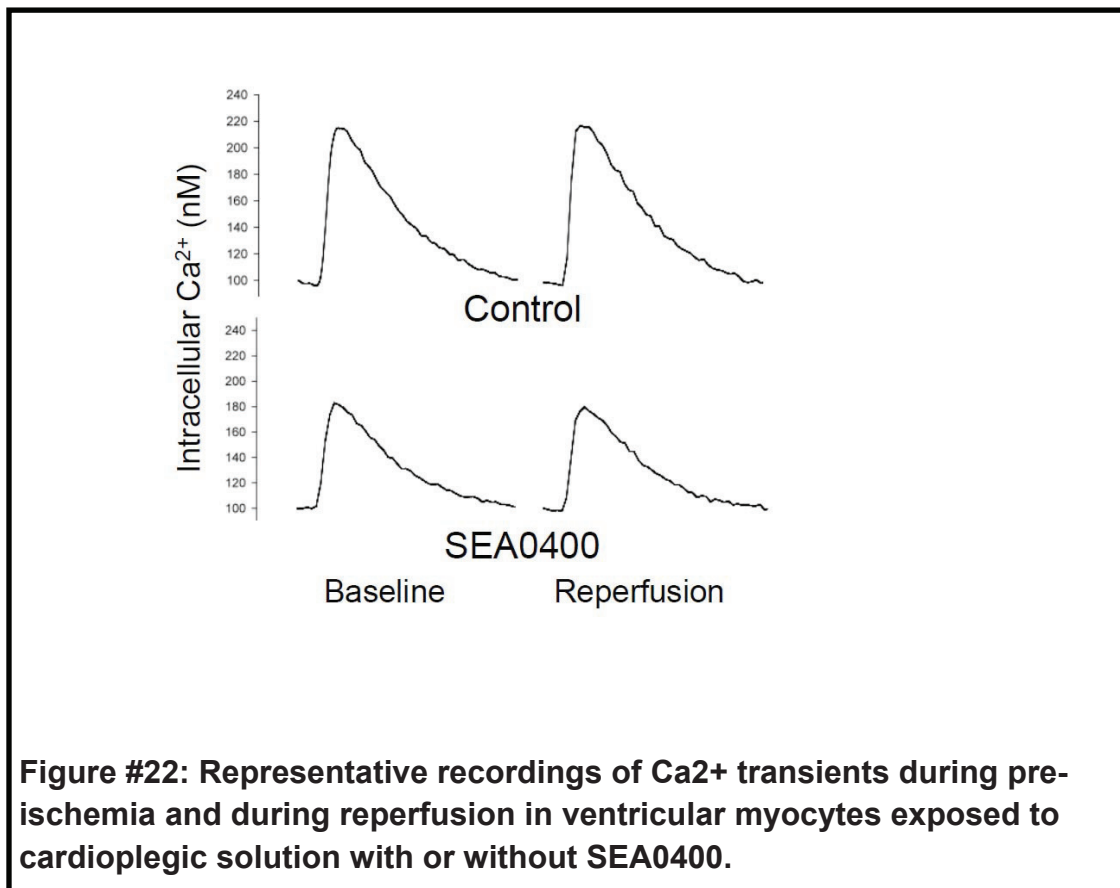
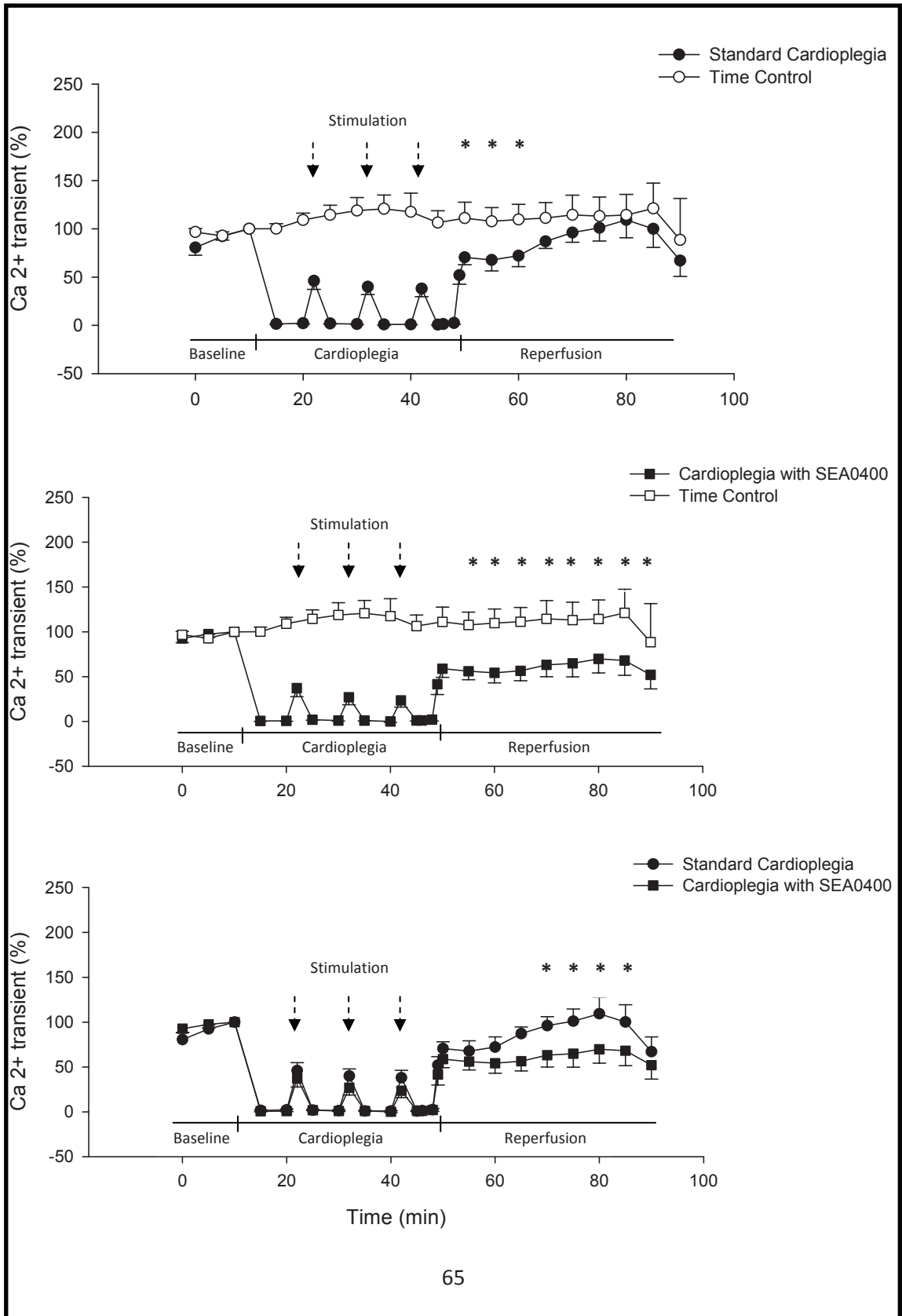


Figure #23 shows the Ca^{2+} transient amplitudes of ventricular myocytes exposed to 45 minutes of ischemia followed by 40min of reperfusion. Data were normalized to pre-ischemic contraction at time = 10min. Panel A compares the mean Ca^{2+} transient amplitudes in cells that served as time controls and cells that were exposed to standard cardioplegia. Ca^{2+} transient amplitudes during early reperfusion were depressed in cells exposed to standard cardioplegia compared to time control. Panel B compares the mean Ca^{2+} transient amplitudes in cells that served as time controls and cells that were exposed to cardioplegia containing SEA0400. Ca^{2+} transient amplitudes during reperfusion were depressed during reperfusion in cells exposed to SEA0400 cardioplegia compared to time control.

Figure #23: Ca^{2+} transient amplitudes were significantly depressed during reperfusion in cells exposed to Cardioplegic solution with SEA0400 (1 μM). **A)** In ventricular myocytes exposed to standard cardioplegia, Ca^{2+} transient amplitudes during reperfusion were depressed during early reperfusion. **B)** In ventricular myocytes exposed to cardioplegia with SEA0400(1 μM) Ca^{2+} transient amplitudes were significantly depressed during reperfusion relative to time control. **C)** Ca^{2+} transient amplitudes in cells exposed to cardioplegia with SEA0400 were significantly depressed during late reperfusion in comparison to cells exposed to standard cardioplegia without SEA0400. Data are normalized to Ca^{2+} transient amplitudes at time=10mins. * denotes $p<0.05$. In response to electric stimulation (arrows) at various points during the cardioplegic arrest period, both cardioplegic solutions maintained depressed transient Ca^{2+} levels in ventricular myocytes.



Panel C compares Ca^{2+} transient amplitudes in cells arrested with SEA0400 cardioplegia vs. cells arrested with standard cardioplegia. Ca^{2+} transient amplitudes were similar during early reperfusion. However during late reperfusion Ca^{2+} transient amplitudes were significantly greater in cells exposed to standard cardioplegia compared to SEA0400 cardioplegia.

Experiment #2:

Experiment #2 of the isolated myocytes experiments, evaluated the effect of SEA0400 on the ventricular myocyte in non-ischemic conditions. Contractions and intracellular Ca^{2+} concentrations were measured to assess and evaluate the effect of SEA0400. Figure #24 shows the contraction amplitudes of ventricular myocytes exposed to 10 minutes of Tyrode's solution followed by 35min of $1\mu\text{M}$ SEA0400 added to Tyrode's solution. There was no change in contraction amplitude with the delivery of SEA0400.

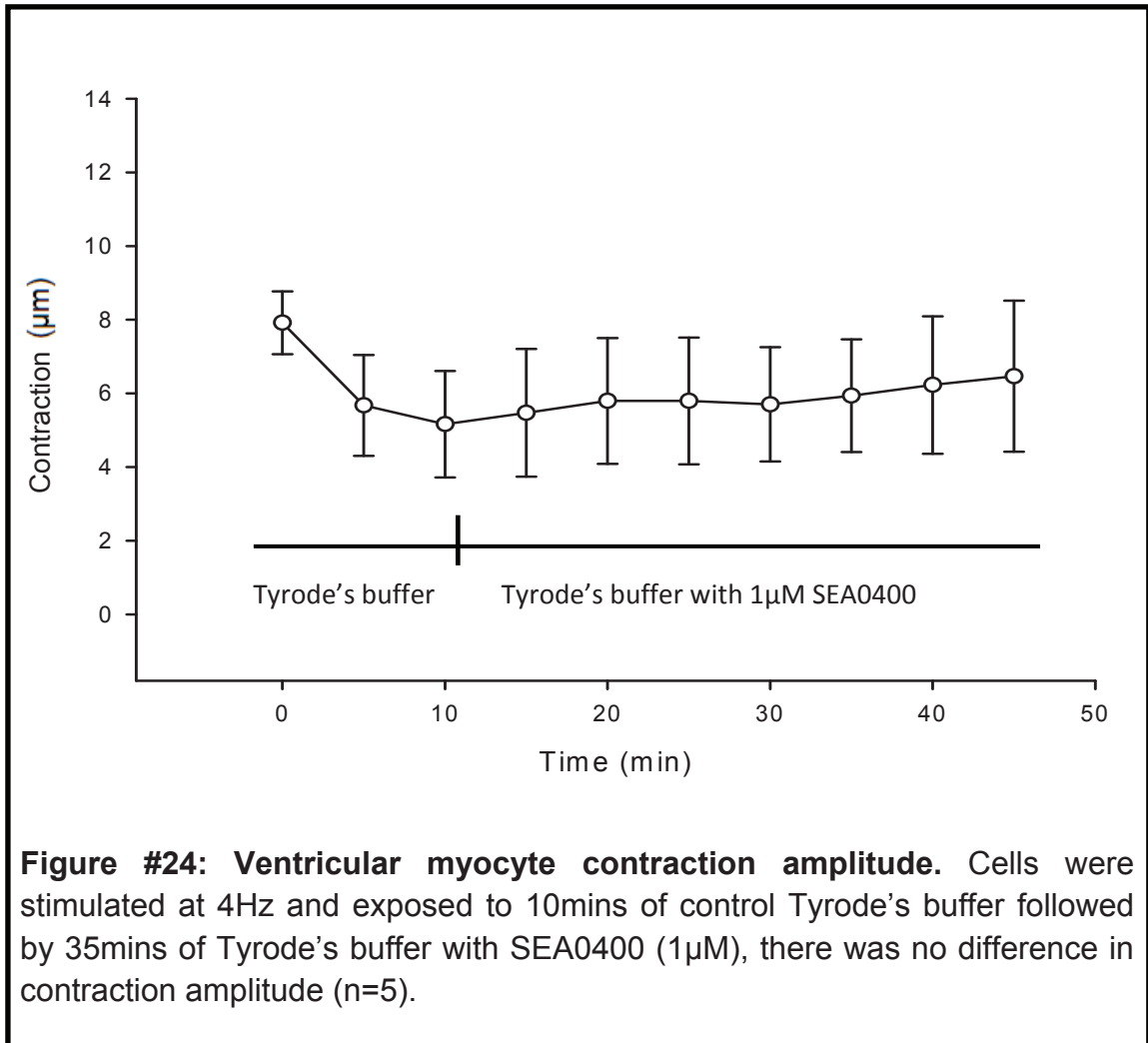


Figure #25 shows the diastolic Ca^{2+} levels of ventricular myocytes exposed to SEA0400. The diastolic Ca^{2+} levels gradually increased with addition of SEA0400 and reached statistical significance at time = 35min.

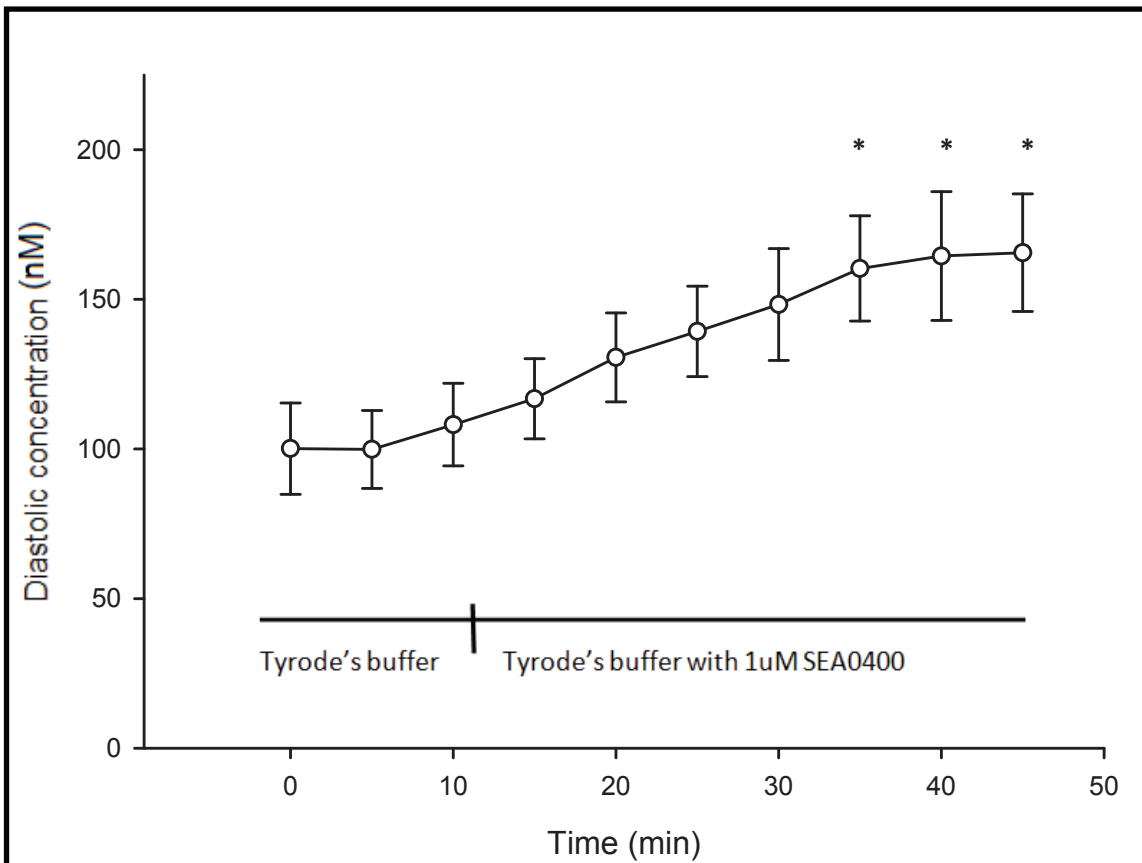


Figure #25: Ventricular myocyte diastolic Ca²⁺ concentration. In young adult cells stimulated at 4Hz and exposed to 15mins of control Tyrode's buffer followed by 30mins of Trode's buffer with SEA0400 (1 μ M), there was a significant elevation in intracellular diastolic Ca²⁺ levels, after 35min of perfusion (*=P<0.05).

Figure #26 shows the systolic Ca²⁺ levels of ventricular myocytes exposed to 10 minutes of Tyrode's solution followed by 35min of 1 μ M SEA0400 Tyrode's solution. The systolic Ca²⁺ levels were significantly elevated at 30min of

perfusion. The systolic Ca^{2+} levels gradually increased with addition of SEA0400 and reached statistical significance at time = 35min.

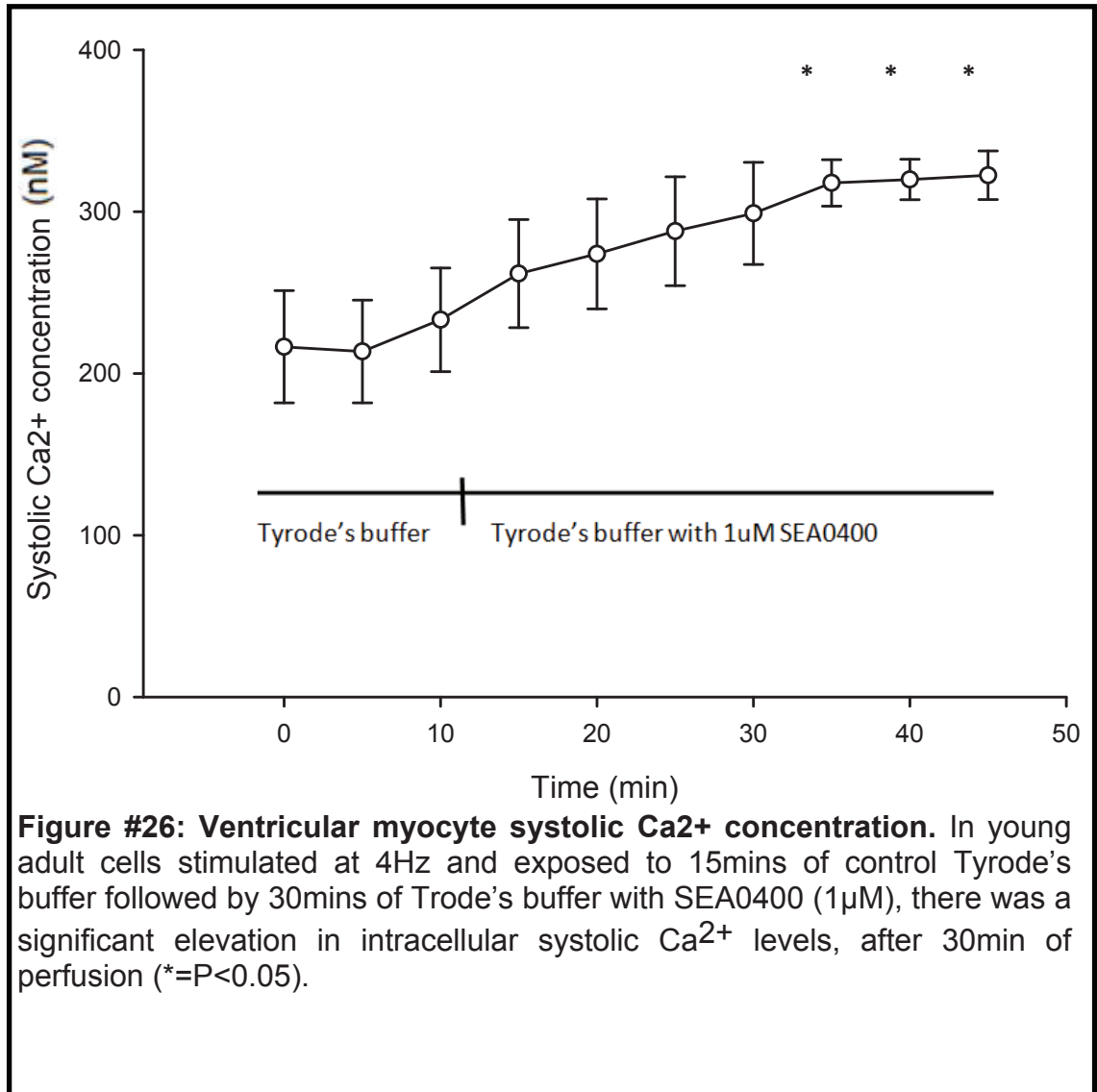


Figure #26: Ventricular myocyte systolic Ca^{2+} concentration. In young adult cells stimulated at 4Hz and exposed to 15mins of control Tyrode's buffer followed by 30mins of Tyrode's buffer with SEA0400 (1 μM), there was a significant elevation in intracellular systolic Ca^{2+} levels, after 30min of perfusion (*= $P < 0.05$).

Finally intracellular Ca^{2+} transient was measured. Figure #27 shows the intracellular Ca^{2+} transient levels of ventricular myocytes exposed to 1 μM SEA0400. The intracellular Ca^{2+} transient were significantly elevated at 35min of

perfusion. The intracellular Ca^{2+} transient levels began to increase at 15min but did not reach significance except at 30min.

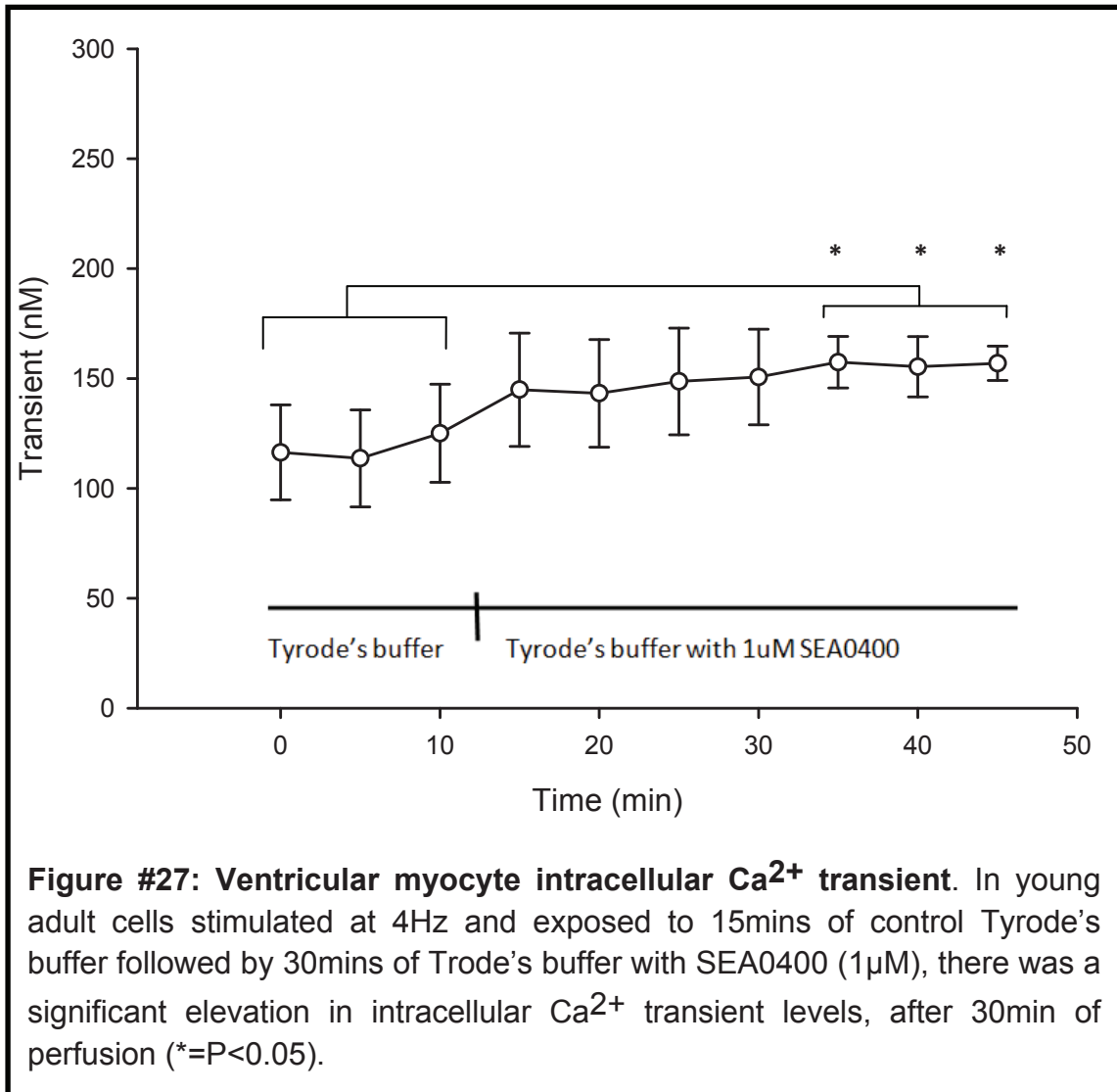


Figure #27: Ventricular myocyte intracellular Ca^{2+} transient. In young adult cells stimulated at 4Hz and exposed to 15mins of control Tyrode's buffer followed by 30mins of Trode's buffer with SEA0400 (1 μM), there was a significant elevation in intracellular Ca^{2+} transient levels, after 30min of perfusion (*= $P < 0.05$).

CHAPTER IV: DISCUSSION

Overview:

The overall aim of this study was to examine the Na⁺/Ca²⁺ exchange blocker SEA0400 as a cardioplegia additive to enhance myocardial protection and improve recovery after a period of cardioplegic arrest. The specific objectives of this study were: 1) to determine whether heart function after cardioplegic arrest is improved when SEA0400 is delivered as a cardioplegic additive using a whole heart model, 2) determine whether SEA0400 prevents myocardial damage, 3) determine the effect of SEA0400 on reperfusion arrhythmias, 4) determine the effect of SEA0400 as a cardioplegic additive on individual ventricular cardiomyocytes in an attempt to understand the specific mechanism of action of the drug.

Effects of SEA0400 on heart function in an isolated whole heart model of cardioplegic arrest and reperfusion

The first goal of this study was to adapt a whole heart model to measure heart function and determine whether SEA0400 provides myocardial protective effects when used in a model of cardioplegic arrest. The results of this study showed that there was improved recovery of heart function after an episode of ischemia in hearts protected with cardioplegia containing SEA0400, as indicated

by the improved preservation of LVW, LVDP, +/- dp/dt, and CVR. Hearts exposed to SEA0400 during reperfusion also showed improved heart function recovery. However arresting the hearts with cardioplegia containing SEA0400 provided maximum preservation.

SEA0400 has previously been shown to provide protective and beneficial effects when administered to the myocardium in ischemia and reperfusion models (Motegi et al., 2007). Studies were performed on isolated rat cardiomyocytes, and showed a reduction in cell death during reperfusion when SEA0400 was added to the perfusion buffer (Takahashi et al., 2003). SEA0400 was also evaluated in whole heart models of ischemia and reperfusion. Hearts treated with 1 μ M SEA0400 buffer during reperfusion exhibit improved myocardial function (Takahashi et al., 2003). The cardio-protective effects of SEA0400 observed in the ischemia-reperfusion studies is a result of the potent and selective inhibition of the NCX. The myocardial damage associated with elevated intracellular Ca²⁺ levels is absent due to the inhibition of NCX by SEA0400; this results in improved myocardial recovery during reperfusion (Motegi et al., 2007). Although there have been many studies evaluating the effect of SEA0400 on ischemia reperfusion injury, SEA0400 was not examined as a cardioplegic additive. This study was the first to analyse the efficacy of the drug on heart function in a cardioplegic model.

The results of this study showed that there was improved recovery of heart function after an episode of ischemia in hearts protected with cardioplegia

containing SEA0400. These findings correlate with previous ischemia and reperfusion studies which showed improved contractility with exposure to SEA0400 (Feng et al., 2006). During ischemia and early reperfusion as a consequence of the elevated intracellular Na^+ concentration, the NCX operates in reverse mode. Na^+ ions are exported from the cell in exchange for Ca^{2+} influx. This eventually leads to Ca^{2+} overload and myocardial injury (Xiao et al., 2003). However by exposing the myocardium to SEA0400, the reverse mode of NCX is blocked, preventing Ca^{2+} overload and minimizing myocardial injury (Birinyi et al., 2008). Delivering the drug during cardioplegia maximized myocardial contractile recovery. The NCX was blocked during the ischemic period preventing the activation of NCX reverse mode. In contrast control hearts contained active NCX that may have contributed to Ca^{2+} overload and myocardial dysfunction during reperfusion. In the last group of this study, SEA0400 was delivered only during reperfusion. In these hearts, the NCX reverse mode may have been active throughout the 45min ischemic period, facilitating the entry of injurious quantities of Ca^{2+} . Furthermore, during late reperfusion the NCX functions in forward mode to remove Ca^{2+} from the cell (Blaustein & Lederer, 1999). Having the SEA0400 present during reperfusion appears to block the NCX forward mode and increase build-up of intracellular Ca^{2+} (Lee & Hryshko, 2004). As a result delivering the drug in the cardioplegia provided maximum preservation of heart function following a 45min period of cardioplegic arrest. Data from previous ischemia reperfusion studies confirm that SEA0400 blocks the NCX reverse mode during

ischemia and prevents Ca^{2+} overload (Birinyi et al., 2005; Lee & Hryshko, 2004), to minimize ischemia-reperfusion injury.

Effects of SEA0400 on structural integrity of the myocardium

There are many changes that happen simultaneously in a heart exposed to ischemia. Although hemodynamic measurements provide physiological data about heart function, there are various molecular changes that occur and provide data about myocardial structural components to evaluate the efficacy of SEA0400. Therefore the second goal of this study was to determine whether SEA0400 affected structural components of the myocardium.

1) Troponin

The biomarker of choice for myocardial infarct detection is troponin I (Klug et al., 2011). Troponin is an important structural component of the myocyte. When the myocyte is damaged troponin is washed out and can be detected in the serum (Xiong et al., 2010). In this study, all hearts showed levels of troponin I release as a consequence of the 45min cardioplegic arrest period. However, troponin I levels were significantly higher in the control group where the standard cardioplegia was used. In contrast, when SEA0400 was delivered in the cardioplegia, troponin release was significantly reduced. This result suggests that myocardial damage was much lower when SEA0400 was used in the

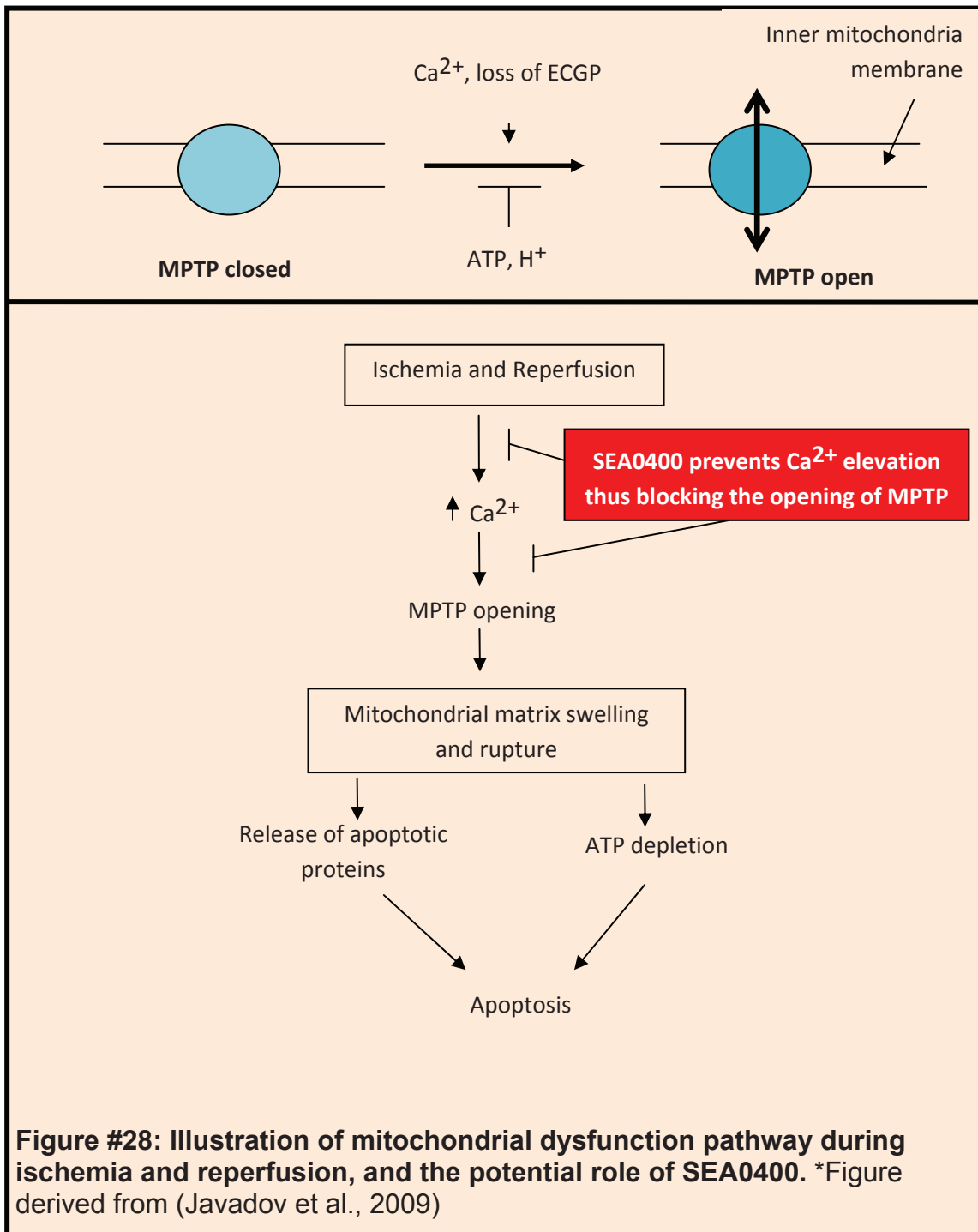
cardioplegia. The myocardium protection was far superior with the use of SEA0400 in cardioplegia.

II) Myocardial ATP

The increase in intracellular Ca^{2+} levels during reperfusion leads to cellular dysfunction through multiple pathways. One of the major pathways that lead to irreversible cell injury is the accumulation of Ca^{2+} in the mitochondria during periods of ischemia (Piper et al., 1994). A previous study performed on isolated myocardial tissue and ventricular myocytes showed that SEA0400 attenuated the decrease of ATP levels during ischemia (Namekata et al., 2006). During experimental ischemia, an increase in mitochondrial Ca^{2+} was observed in parallel to an increase in cytoplasmic Ca^{2+} . However, in the presence of SEA0400 both cytoplasmic and mitochondrial Ca^{2+} were reduced, suggesting that inhibition of the NCX can reduce Ca^{2+} concentrations in both the cytoplasm and mitochondria (Namekata et al., 2006).

Although the NCX is present on both the sarcolemma and the mitochondrial inner membrane (Brierley et al., 1994), there still is a debate whether NCX located in the mitochondria can serve as a Ca^{2+} influx pathway during ischemia (Griffiths, 1999). Mitochondrial dysfunction results from an elevation of mitochondrial Ca^{2+} that causes the opening of the mitochondrial permeability transition pore (MPTP) (Crompton & Costi, 1988; Xie & Yu, 2007). Consequently, key cofactors in mitochondrial metabolism and substrate oxidization are lost. This leads to the inability of the mitochondria to maintain the

electrochemical gradient of protons (ECGP) across the inner mitochondrial membrane. The electrochemical gradient is the energy-transduction intermediate between the proton-translocating ATP synthetase and the respiratory chain (Mitchell and Moyle, 1967). Therefore, the failure of the mitochondria to maintain the electrochemical gradient of protons leads to the arrest of ATP synthesis. A study conducted by Namekata et al, showed a loss of ECGP in cardiomyocytes exposed to a period of ischemia (Namekata et al., 2006). However, with SEA0400 treated myocardial preparations the time course of ECGP loss was delayed and the reduction in ATP content was significantly smaller. These results suggest that SEA0400 maintains mitochondrial integrity and function by reducing mitochondrial Ca^{2+} overload during ischemia, thereby preserving the ability of the mitochondria to produce ATP.



In the current study, myocardial ATP levels were analyzed to investigate whether SEA0400 had a direct effect on myocardial energy production when used in a cardioplegic model. The results showed no difference in myocardial ATP levels between the groups. Although there was a small elevation in ATP level in the group treated with SEA0400 cardioplegia, this effect was not statistically significant. This finding differs from results of previous studies which showed that SEA0400 had a direct effect on mitochondrial energy production (Motegi et al., 2007). SEA0400 was shown to improve mitochondrial preservation and maintain ATP content during ischemia (Namekata et al., 2006).

The difference between the results of the current study and previous studies can be due to differences in experimental models used. In previous studies the effect of SEA0400 was examined in ischemia reperfusion models. Rat hearts were subject to a period of ischemia followed by a period of reperfusion (Motegi et al., 2007). This type of model results in substantial injury to the heart primarily due to Ca^{2+} overload during reperfusion (Hendrikx et al., 1994). As a result, control groups typically have very poor heart function during reperfusion (Takahashi et al., 2003). When experimental SEA0400 treated groups are assessed in ischemia reperfusion models, they are compared to control groups which have sustained a severe injury which results in very poor function (Motegi et al., 2007). However, in this study a cardioplegic arrest model was used which differs from the typical ischemia reperfusion model. In the cardioplegic model a cardioplegic solution is administered to the coronary circulation. The solution

contained a variety of chemical agents that were designed to arrest the heart rapidly in diastole and provide protection against ischemia reperfusion injury (Vinten- Johansen et al., 2000). The key element of cardioplegia, potassium, depolarized the cell membrane and prevented the initiation of any myocardial action potentials; as a result contractions were prevented resulting in a flaccid myocardium with very low oxygen demand (Buckberg et al., 1977). In the present study, all groups were arrested with cardioplegia during the ischemic period. As a result, control groups had significant myocardial protection throughout the ischemic period. Myocardial ATP levels were not significantly different between hearts treated with SEA0400 cardioplegia compared to control hearts treated with standard cardioplegia. This does not invalidate the role of SEA0400 to improve mitochondrial preservation and maintain ATP content during ischemia (Namekata et al., 2006). Perhaps with a longer ischemic period the damage sustained in the control group will be greater and the ATP levels will be further reduced. In comparison, the effect of SEA0400 on myocardial ATP preservation may be more profound.

III) Myocardial Mitochondria morphology

The myocardial mitochondria morphology was analysed to further examine the effect of SEA0400 on myocardial energy metabolism. The mitochondria are the structural sites of energy production inside the cell (Javadov et al., 2009). Any disturbance in the structural integrity may contribute to impaired ATP productivity. The functional recovery of the myocardium during reperfusion has been

suggested to correlate with the structural integrity of the mitochondria (Edoute et al., 1983). Electron microscopy analyses of the mitochondria helps in understanding the ultrastructural changes such as swelling and cristae disintegration (Zhu et al., 2010) that occur secondary to opening of the MPTP (Javadov et al., 2009). The results of this experiment showed that all hearts exposed to ischemia had some degree of change in mitochondria structure. The percentage of intact mitochondria was reduced and the percentage of swollen and disrupted mitochondria was increased. Yet, the highest number of intact mitochondria was found in the group where SEA0400 was delivered in the cardioplegia. Also, levels of disrupted mitochondria were lowest in the SEA0400 group. This data further illustrates and supports the idea of the effectiveness of the SEA0400 in maintaining and preserving the myocardium. SEA0400 appears to reduce the build up of intracellular Ca^{2+} by blocking the NCX (Birinyi et al., 2005; Lee & Hryshko., 2004). This prevents the development of injurious Ca^{2+} overload and maintains the ECGP, preventing the opening of MPTP and destruction of the mitochondria. Similar results were observed in previous experiments where the SEA0400 significantly reduced mitochondrial damage in ischemia reperfusion models (Motegi et al., 2007).

Effects of SEA0400 on electrical activity of the myocardium

The next objective of this study was to analyse the effect of SEA0400 on reperfusion arrhythmias. One of the consequences of ischemia reperfusion injury is the initiation of arrhythmias during reperfusion (Pogwizd, 2003). Reperfusion

arrhythmias are not desirable and can be lethal if they continue beyond early reperfusion. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger is an important regulator of calcium handling in the myocyte but can also contribute to the development of arrhythmias. Under Ca^{2+} overload conditions during reperfusion, the NCX can contribute to arrhythmogenesis mediated by the activation of the transient inward current (Pogwizd, 2003). Consequently NCX inhibitors could provide a therapeutic approach to prevent reperfusion arrhythmia. Previously SEA0400 has been reported to reduce arrhythmogenic membrane oscillations (Fujiwara et al., 2008). One study found that SEA0400 in isolated hearts treated with global ischemia had increased reperfusion arrhythmias (Feng et al., 2006). However most studies have found that SEA0400 causes a reduction or no change in arrhythmias (Namekata et al., 2005; Takahashi et al., 2003; Nagasawa et al., 2005). The results of the present study showed that when cardioplegia was administered to the myocardium throughout the cardioplegic ischemic period of 45min, the onset of reperfusion arrhythmias was delayed. In contrast, in the control group reperfusion arrhythmias were present at start of reperfusion. The delay of reperfusion arrhythmias may be related to the period of time in which SEA0400 is washout out of the myocardium during reperfusion. No other differences were observed between SEA0400 and control for the other indices evaluated. Delivering SEA0400 in cardioplegia appears to be safe in regards to reperfusion arrhythmias. There was no evidence of increased arrhythmias with SEA0400 delivery, rather a delay in the onset of arrhythmias during reperfusion.

Summary

The isolated whole heart study examined the effect of SEA0400 delivery in a model of cardioplegic arrest. Delivering the SEA0400 in the cardioplegia showed improved LV function during reperfusion, lower Troponin I release, improved mitochondrial structural integrity, and delayed onset of arrhythmias during reperfusion. To better understand the mechanism of action of the drug and the reason behind the improved function observed in the whole heart model, studies in individual isolated ventricular myocytes were conducted.

Effects of SEA0400 as a cardioplegic additive on isolated individual ventricular myocytes

The next objective of this study was to elucidate the mechanism by which hearts arrested with cardioplegia containing SEA0400 have improved function and preservation against cardioplegic ischemic arrest. From our isolated whole heart model, the SEA0400 appears to block NCX during ischemia, preventing the influx of Ca^{2+} mediated by reverse mode activity of the pump (Xiao & Allen, 2003). In order to monitor the direction of Ca^{2+} ion movement and contractility we used an individual cardiomyocyte cardioplegic model. The results of this study showed that contractions were depressed during the ischemic period in both groups. However, during reperfusion the cells treated with SEA0400 cardioplegia had greater contraction than cells arrested with standard cardioplegia. This data

correlates with our whole heart model and further supports the ability of SEA0400 to enhance myocardial protection during ischemia arrest.

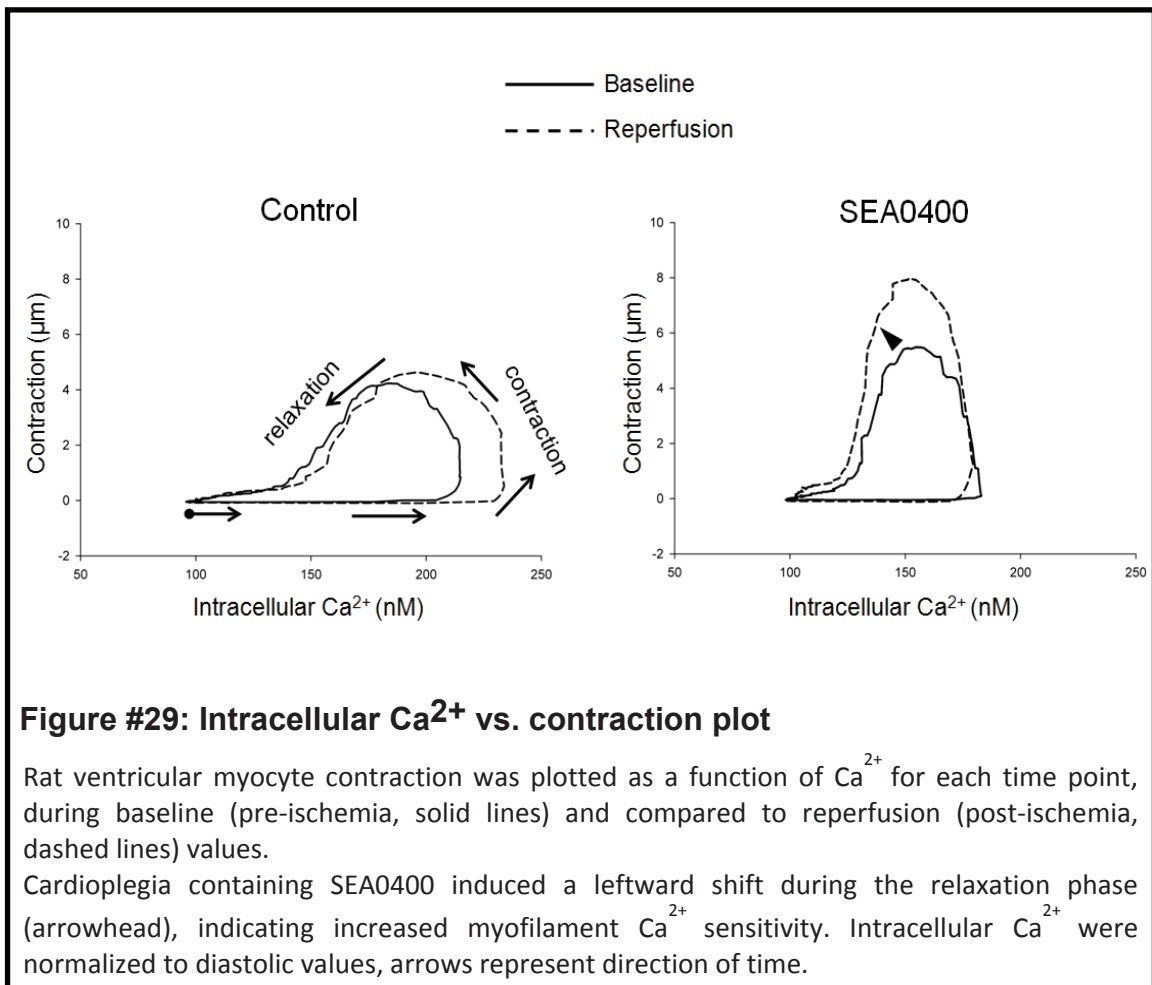
The results of this study showed no increase in intracellular diastolic Ca^{2+} throughout ischemia or reperfusion. Both SEA0400 cardioplegia arrested cells and standard cardioplegia arrested cells do not show elevation in intracellular Ca^{2+} concentrations. This observation contradicts the typical elevation of intracellular Ca^{2+} observed in ischemia reperfusion cardiomyocyte models (O'Brien et al., 2008). The Ca^{2+} concentrations are elevated during ischemia and reperfusion as a consequence of Ca^{2+} overload through NCX influx (Xiao & Allen, 2003). The absence of diastolic Ca^{2+} elevation during ischemia in our results is probably due to the characteristics of our model. In this study, a cardioplegic arrest model was used which differs from the typical ischemia reperfusion model. In the cardioplegic model the cardiomyocytes are superfused with a cardioplegic solution. The solution contains a variety of chemical agents that are designed to arrest and provide protection against ischemia reperfusion injury (Vinten-Johansen et al., 2000). As a result Ca^{2+} overload was not observed in our study, even in the absence of SEA0400.

Furthermore, differences between the whole heart model and isolated cell model, may explain why Ca^{2+} levels were not elevated during reperfusion in the myocyte model. The isolated heart model had cold blood cardioplegia delivered at 20cc/kg body weight. The whole heart was not paced, but rather the intrinsic

heart rate was maintained. Data was collected after 20minutes of stabilization both during baseline and reperfusion. However, in the individual ventricular myocyte model the cardioplegia was a warm crystalloid cardioplegia delivered at 37°C and perfused continuously during the ischemic period. Constant perfusion was required in order to achieve 45min of ischemia followed by reperfusion. The ventricular myocytes were paced at 4Hz and measurements were collected at every five minutes. These differences may be responsible for the absence of diastolic Ca^{2+} elevation.

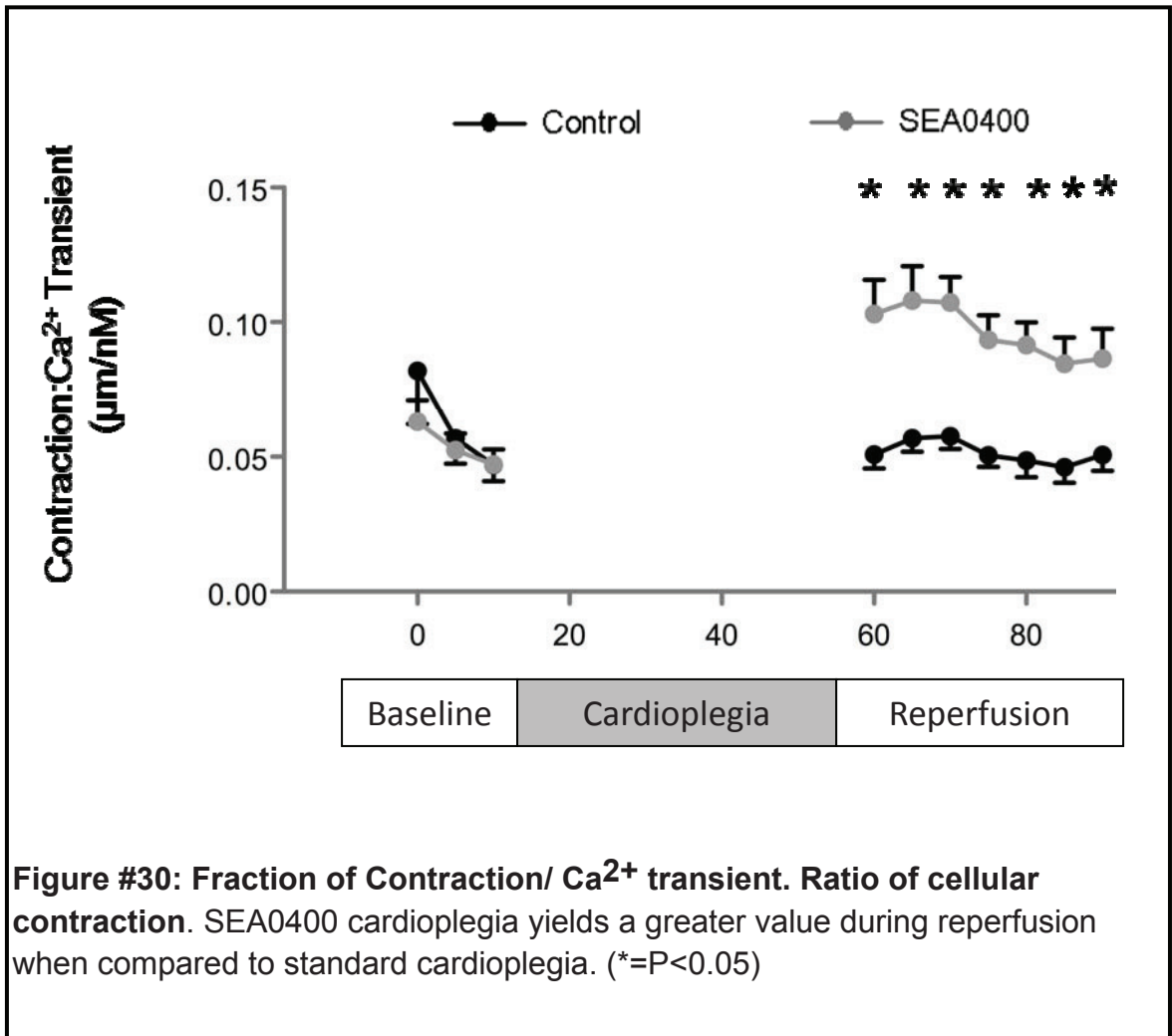
Improved myofilament Ca^{2+} sensitivity

The individual myocyte experiments showed that the Ca^{2+} levels among the SEA0400 cardioplegia and standard cardioplegia were similar. The contractions were significantly greater when SEA0400 was delivered as a cardioplegia additive. This suggests that there must be a difference in myofilament Ca^{2+} sensitivity between the two groups. Myocardial dysfunction during reperfusion can result from a decrease in myofilament Ca^{2+} sensitivity (Kusuoka et al., 1987). The contraction vs. Intracellular Ca^{2+} figure below represents the myofilament sensitivity to Ca^{2+} .



In the graph for the standard cardioplegia (left panel), the curve for post-ischemia shifts to the right indicating decreased myofilament Ca²⁺ sensitivity; for the same amount of contraction a higher value of Ca²⁺ is required. In contrast in the graph for SEA0400 cardioplegia (right panel) the post-ischemia plot has a leftward shift. This would suggest increased myofilament Ca²⁺ sensitivity. For the same amount of contraction a lower amount of Ca²⁺ is required in the presence

of SEA0400. Figure #30 represents the fraction of contraction/ Ca^{2+} transient compared between cardioplegia with or without SEA0400 during pre-ischemia (baseline) and during (reperfusion).



The primary action of SEA0400 is to block NCX and inhibit the exchange of Ca^{2+} for Na^{+} ions across the membrane (Matsuda et al., 2001). This action primarily leads to a reduction of intracellular Ca^{2+} which will affect the integrity of

the myocardium. By reducing the build up of intracellular Ca^{2+} , harmful effects such as protease activation and mitochondrial damage will be reduced (Hendrikx et al., 1994; Gulker et al., 1977; Buja, 2005). In addition, myofilament sensitivity to Ca^{2+} appears to increase after exposure to SEA0400. This suggests that SEA0400 may have an indirect effect on myofilament Ca^{2+} sensitivity. Ca^{2+} sensitizing agents such as EMD 57033, facilitate actin movement on myosin filaments and activate myofibrillar ATPase activity (Endoh, 2008). Although EMD57033 does not increase Ca^{2+} binding affinity, it may stabilize the Ca^{2+} bound conformation (Endoh, 2008). It is possible that SEA0400 could have a similar effect, although the exact mechanism is yet to be determined.

The effect of SEA0400 was also evaluated in a non-ischemic model of individual myocytes. The purpose of this experiment was to examine whether myofilament Ca^{2+} sensitivity improved with SEA0400 in a non-ischemic model. The cells were exposed to 30min of SEA0400 after 15min of standard buffer perfusion. The results of this experiment showed that SEA0400 alone caused an elevation of intracellular Ca^{2+} levels. This was an expected outcome, as a consequence of the NCX blocking effects of SEA0400 (Matsuda et al., 2001). The NCX during non-ischemic conditions operates in forward mode which is responsible for the removal of Ca^{2+} from the intracellular compartment of the cell in exchange for Na^+ entry (Xiao & Allen, 2003). However in spite of the elevation in intracellular Ca^{2+} , the contractions remained the same. This suggests that SEA0400 decreases Ca^{2+} sensitivity when administered without cardioplegia

under non-ischemic conditions. These results indicate that increased myofilament Ca^{2+} sensitivity by SEA0400 is specific to the cardioplegic arrest. Why the effect of SEA0400 on myofilament Ca^{2+} sensitivity differed between non-ischemia and cardioplegic arrest is not yet clear. However, this may be related to the myofilament changes that occur during ischemia (Kusuoka et al., 1987).

Summary

The isolated cardiomyocyte study examined the effect of SEA0400 delivery in a model of cardioplegic arrest. Delivering the SEA0400 in the cardioplegia showed improved contractility during reperfusion, without an increase in Ca^{2+} levels. This suggests that improved myofilament Ca^{2+} sensitivity can play a role in the improved LV function observed during reperfusion in the whole heart model.

Delivery of SEA0400 in cardioplegia

The data from this study provides evidence that SEA0400 delivery during cardioplegia is the most effective method of drug administration. 1) The results showed that delivering the drug during cardioplegia provides the most physiological therapeutic value. 2) Delivering the drug in cardioplegia prevents unwanted systemic effects by minimizing the delivery of the drug to the coronary circulation. 3) During ischemia increased intracellular Na^{+} concentration favours

the reverse mode of the NCX which leads to increased intracellular Ca^{2+} (Xiao & Allen, 2003). Therefore delivering the drug before the onset of ischemia is the most effective approach. 4) Although SEA0400 appears to preferentially block the reverse mode of the NCX, it can also block the forward mode of the NCX to some degree (Birinyi P et al., 2005). This can impede the removal of Ca^{2+} from the cytoplasm during reperfusion and result in poor heart function (Lee & Hryshko, 2004). Delivering the drug during reperfusion could impair recovery and lead to potential injurious increase in intracellular Ca^{2+} . Therefore SEA0400 delivery in cardioplegia is the most effective method and is a promising strategy to improve myocardial protection during cardiac surgery.

Future Steps

The goal of cardioplegic arrest is to protect the myocardium against ischemia and reperfusion injury (Vinten-Johansen et al., 2000). The optimum cardioplegic protection will result in maximum recovery of heart function during reperfusion. The results of this study have shown that incorporating SEA0400 in a cardioplegic solution provides better protection from ischemia and reperfusion injury when compared to standard cardioplegia alone. Although SEA0400 may have potential benefits as a cardioplegic additive, its mechanism of action is not completely known. Although we have tested an *in-vitro* model, this model needs to be refined to produce an increase in diastolic Ca^{2+} , perhaps by, cooling, lengthening the ischemic period or generating a bath with circulatory flow.

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