THE CONTRIBUTION OF BLOOD COMPONENTS TO MYOCARDIAL ISCHEMIA REPERFUSION INJURY AND THE BENEFITS OF VITAMIN E IN THE DIABETIC HEART

by

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DEDICATION

This work is dedicated to my wife Joyce who has stuck with me through thick and thin. She has always been and will always be my inspiration.
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ABSTRACT

Type 2 diabetes is a major risk factor in cardiovascular disease especially ischemic heart disease. The American Diabetes Association advocates that all diabetics are at risk for coronary myocardial infarctions (MI). Restoration of blood flow to the area of the myocardium that has undergone ischemia is the most important therapeutic goal in treating MI. However, restoration of blood to the myocardium contributes to further damage to the ischemic area. This phenomenon is known as ischemia-reperfusion (I-R) injury. Previous studies have found that experimentally induced diabetic animals are more susceptible to I-R injury compared to non-diabetic animals. One reason why diabetic hearts may be more susceptible to I-R injury may be due to the increased oxidative stress in diabetics. Diabetics also suffer from increased blood coagulation possibly due to increased thrombin activity. Thrombin, independent of its role in coagulation, may be contributing to additional myocardial damage following ischemia.

This dissertation describes three experiments: First, determine if a non-thrombogenic dose of thrombin impairs cardiac recovery and compliance in isolated non-diabetic rat hearts using a red cell perfusate following 30 minutes of global ischemia. Second, determine if type 2 diabetic rats exhibit increased platelet microparticle formation (PMP), increased thrombin activity and increased coagulability. Third, determine if MI’s were larger in diabetic rat hearts compared to non-diabetics and if vitamin E reduced MI size and improve cardiac function following coronary artery occlusion.

We found that a non-thrombogenic thrombin dose significantly reduced recovery from global I-R and reduced myocardial compliance. Thrombin may act as a contributing
factor in cardiovascular pathology independent of its role in coagulation. We found that thrombin production is increased 30 fold in type 2 diabetic rats compared to non-diabetic rats and PMP production is significantly increased in diabetics possibly resulting in a hypercoagulable state. Finally, we show that MI's are significantly larger in type 2 diabetic hearts compared to non-diabetics. Treatment with Vitamin E significantly reduced MI size and improved left ventricular function in treated diabetic rats compared to non-treated rats. These results help to explain the increased cardiovascular injury seen in the diabetic heart. Vitamin E may be a useful adjuvant to help diabetics reduce oxidative stress, minimizing cardiac damage done by oxidation compounds and activated blood coagulation components in the diabetic heart.
Chapter 1

BACKGROUND AND LITERATURE REVIEW

1.1 Introduction

This dissertation was an effort to better understand the pathophysiology of heart disease and specifically to understand why diabetics suffer from heart and vascular disease to a greater degree than the general population. Indeed cardiovascular complications are the most frequent cause of mortality and morbidity in diabetic patients (1). During the course of my graduate education at the University of Arizona my research began with a study of cardiac function using an isolated heart model to measure the recovery of contractile function (inotropy) and compliance, (lusotropy), following ischemia. During discussions with cardiovascular researchers and cardiac surgeons it became evident that the extracorporeal circuitry used to sustain the patient during transplantation, coronary bypass, and other invasive cardiac procedures causes the patient's blood to have extensive contact with the polymer surfaces in the bypass circuit, causing a marked procoagulant effect. During the bypass procedure extensive activation of the coagulation cascade in the blood results in extensive amounts of coagulation factors being activated including thrombin, the central plasma protein involved in activation of the coagulation cascade (2,3). Previous studies found that thrombin generation is increased during cardiac procedures with no clear explanation of the mechanism. Anti-coagulants are routinely given to prevent coagulation. However, anti-coagulants routinely used, such as low-molecular weight heparin, do not completely abolish the activation of the coagulation cascade or thrombin generation. Increasing
evidence suggests that thrombin, in addition to its coagulation function, contributes to myocardial contractile dysfunction. Researchers, using isolated adult rat ventricular cardiac myocytes, found that thrombin acts as a pro-arrhythmogenic factor during myocardial reperfusion (4). Thrombin exerts these effects by binding to specific myocardial cell surface receptors coupled via G-proteins to intracellular pathways such as inositol phosphate release and activation of Na⁺/H⁺ exchange and release of lysophatidylcholine (5). In addition to its direct myocardial effects, thrombin stimulates platelets to adhere to endothelium. During vascular damage platelets aggregate to form the initial platelet plug and release pro-inflammatory mediators. Platelets also release mediators of vascular smooth muscle tone such as adenosine diphosphate (ADP) and calcium (6). During invasive cardiac procedures, a combination of excessive thrombin activation and platelet aggregation can be a lethal combination for a patient already compromised by cardiac disease. A subset of patients that are particularly susceptible to these mechanisms described above are long-term diabetics. Why long-term diabetics are more susceptible to cardiovascular complications is not entirely clear. We do know that diabetics have a higher incidence of ischemic heart disease than non-diabetics. Diabetes is now considered one of the four major risk factors for cardiovascular disease, particularly coronary heart disease. In addition, the severity of ischemic heart disease is greater in diabetics than non-diabetics. In addition, diabetics also have episodes of ischemic attacks that may not be detected (silent heart attacks) when additional myocardial damage may be more lethal. Consequently, diabetics are less apt to survive a subsequent myocardial infarction than non-diabetics (7).
The following sections contain a synopsis of the background and current research on the pathophysiology of ischemia-reperfusion injury and how thrombin influences myocardial recovery after a period of ischemia. The next section reviews how platelets and constituent platelet microparticles (PMP's) are released when platelets become activated. PMP's have been implicated to enhance coagulability in a number of cardiovascular conditions such as cardiopulmonary bypass, stroke and diabetes (8,9). The first two parts of this dissertation explores why thrombin, platelets and platelet microparticles may be a contributing factor to the cardiovascular pathology seen in the diabetic heart.

The third section of this dissertation explores the effects of vitamin E on reducing the size of myocardial infarction after a thirty-minute ischemic period. Peroxidation of membrane phospholipids induced by oxygen free radicals is thought to be a major mechanism of ischemia-reperfusion injury in cardiac tissue leading to considerable structural and functional damage. Protective effects of antioxidants like dismutase, catalase and other free radical scavengers have supported the involvement of free oxygen radicals in I-R injury. A number of clinical trials found that free radical scavengers such as vitamins E and C are protective in the primary and secondary prevention of atherosclerosis, suggesting the potential in the prevention of coronary heart disease. In addition, several in vivo studies found vitamins E and C may help protect cardiac tissue against oxidative damage in ischemia-reperfusion models. A logical extension of this research was to investigate if vitamin E is beneficial in reducing the extent of myocardial damage and improves functional recovery after ischemia-reperfusion injury in a type 2 diabetic animal model. Several investigators found that diabetics have elevated levels of oxidative
stress within the vasculature and myocardium (10-15). Therefore, vitamin E administered prior to an ischemic event may function to protect the diabetic myocardium after an ischemic-reperfusion event and reduce the damage following reperfusion. We found that vitamin E reduces the overall size myocardial infarction after a 30 minute ischemic period.

Vitamin E is composed of a group of 8 related tocopherols derived from chromonols (Polyphenols) and naturally occurs in plants especially in seeds and leafy green vegetables. Alpha-tocopherol (α-tocopherol) is the most abundant of the tocopherols (16). Herbert Evans first described vitamin E as an “infertility agent” in 1922. When Evans fed pregnant rats a vitamin E deficient diet, the rats reabsorbed their fetuses at a certain stage of gestation. When the compound was reintroduced into the diet, pregnant rats continued to normal gestation and birth (17). The compound was named tocopherol-derived from the Greek “tokos” (childbirth) and “pherein” (to bear). Vitamin E is the major lipid-soluble chain breaking antioxidant in humans and animals, and has the largest role in protecting cell membranes. Vitamin E is also the most abundant antioxidant in low-density lipoproteins (LDLs). Antioxidants convert free radicals to relatively stable compounds and stop or prevent the chain reaction of free radical damage. The most important free radical molecules in biological systems are the superoxide anion (O₂⁻), the hydroxyl radical (HO⁻) and the peroxide radical (OH⁻) derived from hydrogen peroxide (H₂O₂). Hydrogen peroxide (H₂O₂) is not a true free radical. However, hydrogen peroxide is unstable and likely to be converted to the hydroxyl radical (OH⁻), the most potent
oxidizing agent known. The hydroxyl radical is converted by antioxidants to water (18,19). Vitamin E is very efficient in quenching the hydroxyl radical.

The following sections in this background review attempt to put into perspective the mechanisms that contribute to I-R injury in the non-diabetic heart and some of the contributing factors that cause the diabetic heart to have greater damage following I-R injury. Additionally some factors behind why diabetics exhibit increased thrombin activity and platelet activation are summarized. Finally, a brief history and epidemiological discussion on diabetes is presented to give the reader a perspective as to why diabetics are at increased risk for cardiovascular disease and the scope of the problem of diabetes has become as a major health care concern in the U.S.
1.2 Ischemia-reperfusion injury

Myocardial ischemia occurs when tissue is deprived partially or totally of blood flow (20). Restoration of blood to the previously ischemic heart is prudent but usually leads to increased injury as evidenced by a greater infarcted area than would occur with ischemia alone. This phenomenon has been well described by many researchers and is called ischemia-reperfusion injury (21-23). Researchers noted a paradox when oxygen (carried by blood) was restored to the myocardium. They observed cardiac enzyme release and myocardial ultra structural changes indicating further myocardial injury (24). The mechanisms of reperfusion injury have been an active topic of research for over 30 years. The precise mechanisms are as yet unknown but a number of laboratories have contributed to the further understanding of myocardial-reperfusion injury.

Myocardial reperfusion injury demonstrates some common features that have been described and characterized; (1) arrhythmias (2) contractile dysfunction (3) increased myocardial infarct size and (4) vascular injury (25,26). Ventricular arrhythmias can occur as a result of disruption to sarcoplasmic calcium and sodium-potassium ATPase pump function to decreased calcium efflux from the cytosol back into the sarcoplasmic reticulum. This leads to increased calcium influx into the cytosol leading calcium overload within the myocytes during reperfusion (27,28). Myocyte contractile dysfunction occurs during an ischemic event and during reperfusion in addition to the disruption of calcium homeostasis within the cardiac myocyte a decline in ATP, the energy source for the myocyte can contribute to the decline in myocyte function.
Prolonged ischemia without proper restoration of blood to the hypoxic myocardium can result in myocyte death within the compromised myocardium. However, if blood flow is restored to the myocardium in a timely fashion a contractile dysfunction may occur either temporarily or permanently. The temporary contractile dysfunction is termed “stunning”. Myocardial stunning may occur as a result of free radical damage and impaired calcium resequestration (29-31). Lethal reperfusion injury may occur during the reperfusion period if restoration of blood and oxygen is not accomplished in a timely fashion (32,33). Generally, a 30-minute period of ischemia will result in myocardial necrosis. Anoxia, during the ischemic period allows calcium to rise within the myocytes due to the loss of high-energy phosphates required for proper calcium resequestration and membrane pump function. In addition free radical production occurs due to lipid peroxidation and release of radicals from damaged mitochondria (20). Restoration of oxygen results in further elevation of calcium along with continued free radical production from myocardial cellular constituents and white blood cells (PMN’s). Finally, vascular dysfunction contributes to reperfusion injury; increased vascular permeability due to disrupted membrane function, changes in endothelial-derived products including a decrease in nitric oxide (which quenches superoxide anion, generated during reperfusion); increased leukocyte adhesion to endothelial cells and increased platelet aggregation (34,35).
1.3 Calcium overload and free radical generation causes increased oxidative stress during ischemia-reperfusion

Although calcium overload and free radical generation contribute to ischemia reperfusion injury, the interrelationship between these two consequences of I-R injury is not entirely clear. Do calcium overload and free radical generation occur independently or do they occur interdependently? Oxidative stress modifies proteins and phospholipids resulting in lipid peroxidation and protein thiol oxidation. These reactions alter lipid membrane fluidity, permeability and the functional properties of membrane proteins (36). Oxidative stress alters cellular functions such as depressing sarcoplasmic calcium and sodium-potassium ATPase functions which then lead to a decrease in calcium removal from the cardiac contractile proteins and increase in cytosolic calcium further elevating calcium levels within the cytosol. These altered transport properties can result in myocardial contractile dysfunction in the form of lowered contractility and compliance, which further damages the myocardial function during reperfusion (37-39).
1.4 Diabetic oxidative stress and ischemia-reperfusion injury

Free radical production is increased during myocardial ischemia and increased further during reperfusion (20,36,40-42). Also diabetes increases oxidative stress within the vasculature and myocardium (10-15). Oxidant balance in the heart has a very important role in protecting the heart and in maintaining normal cardiac contractile performance (43). The amount of available endogenous antioxidants is sufficient to protect the heart from oxidant production that occurs normally. However, antioxidant reserves may be inadequate under pathological situations such as inflammation, ischemia-reperfusion, and diabetes. During pathological conditions, antioxidant levels can be inadequate and oxidant injury can occur, compromising cardiac performance. The normally functioning heart supplies adequate cardiac output to meet the blood flow requirements of the peripheral tissues both at rest and at varying levels of tissue activity. Compromising cardiac function during overwhelming oxidative stress can lead to ischemia and injury of other organs in the body. The heart requires a continuous supply of high-energy phosphate compounds to support its constant contractile activity and mitochondrial "leak" of oxygen free radicals in the ischemic condition is a potential problem. The heart must balance oxidant production with available antioxidant stores under varying levels of activity and under different pathological conditions (i.e. impaired blood flow to segments of the heart due to atherosclerosis, and diabetes). The heart cell has developed very effective antioxidant, mechanisms. These mechanisms include superoxide dismutase, catalase, glutathione and vitamin E in lipid membranes, which are also found in most other tissues. During diabetes the stores of available antioxidants may be depleted or
overwhelmed by oxidative stress which may compromise myocardial function. One example of reduced anti-oxidant protection occurring during ischemic events is myocardial infarction due to coronary atherosclerotic plaque. The atherosclerotic plaque may be formed in part by increased oxidant damage to coronary endothelial surface (i.e. foam cell formation). The diabetic ischemic myocardium may not have sufficient anti-oxidant protection due to decreased anti-oxidants, increased oxidative stress or both. The result of reduced anti-oxidant protection in myocardial tissue that is subjected to I-R may result in an increased myocardial area that eventually becomes infarcted.

1.5 The role of thrombin in ischemia-reperfusion injury

Additional factors may contribute to myocardial ischemia-reperfusion injury undergoing a cardiovascular crisis such as angina, heart attack or already damaged heart repaired during coronary bypass/stent placement and angioplasty. During open-heart surgery an extensive amount of thrombin is produced by the body as blood is diverted through the extracorporeal heart-lung machine. Contact with the polymer surfaces in the bypass circuit causes a marked procoagulant response causing increased thrombin activation (44).

Thrombin is a key protein in the coagulation cascade. Prothrombin is the inactive precursor to thrombin, which acts as a serine protease. Generated at the site of vascular injury such as acute myocardial infarction, or coronary vessel occlusion, thrombin has several cellular and tissue regulatory functions including stimulating endothelial
prostacyclin production (45), acting as a potent chemoattractant for platelets, monocytes (46) and a mitogen for lymphocytes (47,48,48). In the setting of myocardial ischemia, thrombin is pro-arrhythmogenic during reperfusion (3). Thrombin is thought to exert these effects by binding to specific cell surface receptors. A thrombin-receptor complex coupled via G-proteins to intracellular pathways such as inositol phosphate release and with activation of Na+/H+ exchange and release of lysophatidylcholine leads to alterations in ventricular function (5).

Recently, investigators reported that thrombin can cause cardiac dysfunction when administered at low concentrations (0.03-1.0 units/ml of blood). These dysfunctions include reduction of ventricular contraction, decreased coronary perfusion, and induced arrhythmias (49-51). Investigators using isolated cardiac myocytes reported that thrombin has either a negative effect on contractile function (4) or a positive inotropic effect (52) using thrombin or thrombin receptor agonist peptide. Thrombin’s effect on the myocardium was postulated to be by activating the thrombin receptor. Studies have confirmed the thrombin receptor on coronary endothelium and isolated cardiac myocytes (3,3,51,51,52).

In addition to direct myocardial effects, thrombin may be an important factor in the coagulopathies commonly encountered in diabetics, the role that thrombin plays in diabetic coagulopathies has not been well elucidated. Various investigators have reported that thrombin production is increased in diabetes (53,54,54,55,55). The study by Ceriello et al. demonstrated that type 2 human diabetics with hyperglycemia have elevated thrombin levels compared to age-matched normal people or glycemic-controlled
diabetics. They concluded that uncontrolled blood glucose in diabetics contributed to the increased activation of thrombin (56). Another feature of the abnormal hemostasis seen in diabetes is increased platelet activation allowing the formation of the prothrombinase complex leading to increased thrombin formation, thrombin, in turn, converts fibrinogen to fibrin. The prothrombinase complex is composed of platelet phospholipids, phosphatidylinositol, phosphatidylserine, Ca\(^{2+}\), factors Va and Xa, and prothrombin. This interrelationship of thrombin activity and platelet activation has not been explained fully.

1.6 Thrombin, platelets and caspases in diabetes

Platelets function to protect the integrity of the vascular wall and initiate platelet plugs during early vessel injury. Platelets accumulate at the site of vascular damage and release the contents of platelet \(\alpha\)-granules, which contain pro-inflammatory substances such as thromboxane \(A_2\), and serotonin. Platelet granules also release mediators of vascular smooth muscle tone such as adenosine diphosphate (ADP) and calcium (6). A subset of platelet activation responses that is especially important for thrombus formation include exposure of phosphatidylserine and release of platelet microparticles (PMP), which increase procoagulant surface area (57). There are similarities of platelet activation processes to events occurring in nucleated cells undergoing apoptosis suggesting a role for caspases, the major effector enzymes of apoptosis. Diabetics demonstrate a number of altered vascular-abnormalities including endothelial cell dysfunction, coagulopathies (including thrombosis and platelet dysfunction). There is an association between platelet function and activation of thrombin in the coagulation cascade. However, the
mechanisms underlying the hypercoagulable state in diabetics are still unknown. In chapter 3 we report that diabetic rats have increased PMP's compared to non-diabetic rats. In addition, diabetic rats also have a significant increase in thrombin and caspase activities compared to non-diabetics. The combination of increased thrombin activity, increased PMP formation and increased caspase activity may contribute to the increased coagulation observed in diabetic blood. These findings demonstrate novel functions of caspases and provide a new insight for understanding diabetic hypercoagulopathies.
1.7 History and public health significance of diabetes

The following quote is an early description of diabetes:

"Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature as in dropsy. The course is a common one, namely the kidneys and bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease then is chronic, and it takes a long period to form, but the patient is short-lived if the constitution of the disease be completely established; for the melting is rapid, the death speedy. Moreover, life is disgusting and painful; thirst unquenchable; excessive drinking, which however is disproportionate to the large quantity of urine for more urine is passed; and one cannot stop them either from drinking or making water.

The Greek physician, Aretaeus the Cappadocian, wrote this passage sometime in the second or third century AD. The text is from "Source Book of Medical History," published in 1942 by Dorothy Clark (58). This is by no means is this the first description of diabetes; the Egyptians described a prescription for frequent urination, the most common symptom of diabetes, on an Egyptian papyrus dating back to 1500 B.C. However, Aretaeus of Cappadocia first coined the condition "diabetes," which is Greek for "siphon," since his description of patients with diabetes urinated frequently. Later in his passage Aretaeus describes the pathophysiology of diabetes:

"...The affection of diabetes is a species of dropsy, both in cause and condition, differing only in the place by which the humour runs. For, indeed, in ascites the receptacle is the peritoneum, and it has no outlet, but remains there and accumulates. But in diabetes, the flow of the humour from the affected part and the melting are the same, but the deflection is determined to the kidneys and the bladder; and in dropsical cases this is the outlet when the disease takes a favorable turn; and it is good when it proves a solution to the cause, and not merely a lightening of the burden."
In the latter disease the thirst is greater; for the fluid running off dries the body."

Aretaeus then goes on to cite the treatment for this ancient disease:

"...but the water used as drink is to be boiled with autumn fruit. The food is to be milk, and with it cereals, starch, groats of spelt, gruels. Astringent wines to give tone to the stomach, and these but little diluted, in order to dissipate and clear away the other humours; for thirst is engendered by saltish things."

Diabetes has been a disease that has been prevalent throughout human history and one of the earliest diseases that medical researchers have tried to understand and treat. In 1889, two European medical researchers, von Mering and Minowski, removed the pancreas of two dogs and immediately upon recovery the dogs began to urinate frequently and in large volumes (polyuria). The urine, they discovered, was sweet to the taste and contained sugar. The researchers realized the dogs had developed diabetes. Upon re-implantation of a portion of the pancreas subcutaneously back into the pancreatectomized dogs, the polyuria stopped and the hyperglycemia ceased. Von Mering and Minowski realized from this set of experiments that diabetes was a pancreatic disease and crucial to the metabolism of carbohydrates. In 1910, Sharpey-Shafer of Edinburgh suggested a single chemical was missing from the pancreas in diabetic people. He proposed calling this chemical "insulin." Real progress in treating diabetes began in 1921 when J.J.R McCleod, Frederick G. Banting, and a medical student, Charles H. Best at the University of Toronto, took fluid from animal pancreases, purified it, and injected it into Leonard Thompson, an 11-year-old boy suffering from severe diabetes. Leonard was barely alive and weighed only 75 pounds. But after injections of the fluid, his hyperglycemia subsided, he was able to eat a more normal diet, gained weight, and lived to adulthood
(juvenile diabetics prior to this experiment rarely lived to adulthood). The purified ingredient in the pancreatic fluid was insulin. Banting, Best, McCleod and a biochemist named J.B. Collip, who helped purify the insulin, won the Nobel Prize for medicine in 1922. Insulin was one of the first proteins to be crystallized in pure form, in 1926. The crystalline form allowed researchers to study its structure with x-ray crystallography and approximate its three-dimensional shape. In 1955, insulin became the first protein to be fully sequenced. That work resulted in a 1959 Nobel Prize for Frederick Sanger (59).

Even with recent advances in combination therapies, as well as recombinant technology allowing for more effective glucose control, there is still no cure for chronic diabetes. Diabetes (especially type 2) has become a major health care concern in the U.S. and worldwide. Diabetes currently affects an estimated 16 million people nation-wide with greater than 750,000 new cases diagnosed each year. The number of new cases of diabetes and number of existing cases are increasing in the U.S. The increase in cases of diabetes is not due to the aging of the U.S. population, but may be due to a change in eating and exercise habits resulting in a more sedentary lifestyle and as a result a larger overweight and unhealthier population. Trends also show that minority and elderly populations are disproportionately affected by diabetes. Diabetes remains a leading cause of mortality, and the number of deaths is increasing. Increased death rates are seen in all ages and races, and the highest rates are seen among minority populations and older Americans (1). In 1996, diabetes was the 7th leading cause of death in the United States. When leading causes of death were examined by race, diabetes ranked as the 7th leading cause among whites, Chinese, and Filipinos. Diabetes is the 6th leading cause of death among
black and Japanese; the 5th leading cause among ethnic Hawaiians; and the 4th leading cause among Native Americans. Diabetes ranked higher as a leading cause of death among women than among men and was the 4th leading cause of death among women who were black, Native American, or Filipino. Hispanics in the U.S followed the same trends of diabetes; diabetes ranked as the 6th leading cause of death overall and as the 4th leading cause of death among women. The CDC reported that the age-adjusted death rates for diabetes as the underlying cause of death and diabetes listed as cause of death were higher among Native Americans, blacks, and Americans of Hispanic origin than among whites (7).

1.8 Diabetes and Cardiovascular Disease

In the United States diabetics have a 2-4 higher rate of heart attacks than the general population. The incidence of coronary heart disease is 55% in diabetics compared to 2.4% in the general population. The disease is not only more common, but also more severe. It involves more vessels and the occlusion also extends more distally into the vessels. And 3 coronary vessel disease is more common in diabetics. The incidence of left main coronary artery involvement is 13% compared to 6% in the general population. It affects young patients and diabetes takes away the protection offered to the premenopausal women against coronary artery heart diseases. The incidence of angina is increased 60% in men and 90% increased in women; myocardial infarction is increased 50% in men and increased 150% in women; sudden cardiac death is increased 50% in men and increased 300% in women compared to the general U.S. population (1).
While deaths from other complications of diabetes are declining the mortality from cardiovascular disease in diabetics is increasing. The mortality in acute myocardial infarction in diabetics is two to four times higher than the general population (7). In addition, related complications such as cardiac arrhythmias, heart block, congestive heart failure and post infarction angina are increased in diabetics compared to non-diabetics (60,61). A June 2001 press release the National Institutes of Health stated; “If you have diabetes, you have the same high risk of having a heart attack as someone who has already had their first attack,” and stated further; “Even worse, your chances of dying from your first heart attack are the same as someone without diabetes who has had a second heart attack (62). Diabetes has become a major financial burden on the U.S. health care budget and crosses over all ethnic and socio-economic barriers. Without primary prevention the diabetes epidemic will continue to grow. Even worse, diabetes is projected to become one of the world’s main disablers and killers within the next twenty-five years (7). This makes research on finding treatments for diabetes and diabetes related illnesses a national and international health care priority.
Chapter 2

THROMBIN AMPLIFIES REPERFUSION INJURY FOLLOWING MYOCARDIAL ISCHEMIA

2.1 Abstract

Background: Previous studies report that thrombin decreases cardiac contractility and causes arrhythmias. However, the effects of thrombin on the recovery of ventricular function following ischemia have not been investigated. The present study examined if thrombin at a non-thrombogenic dose, impairs the recovery of ventricular function and reduces cardiac compliance following myocardial ischemia.

Methods: Isolated rat hearts were perfused at constant pressure for 30 minutes and then subjected to 30 minutes of normothermic, global ischemia. The hearts were then reperfused with a red blood cell perfusate (K2RBC), or diluted whole blood (DWB) or thrombin-treated DWB (0.5 Units/ml) for the first five minutes of reperfusion. Reperfusion was then continued with K2RBC. Cardiac pump function was assessed using an intraventricular balloon. Ventricular function, systolic and diastolic pressures, coronary perfusion pressure and coronary blood flow were measured after 5, 20 and 35 minutes of reperfusion. After 30 minutes of reperfusion, ventricular function curves were recorded for each heart.

Results: We found that after 35 minutes of reperfusion, the function of the K2RBC reperfused hearts recovered to 64% of pre-ischemia values. The function of the DWB reperfused hearts recovered to 45% of the pre-ischemia value. The DWB-thrombin reperfused hearts recovered to only 24% of the pre-ischemia value (p<0.05). After 35
minutes of reperfusion, the coronary blood flow decreased 8.6%, in the K2RBC group, 24% in the DWB group and 47% in the DWB-thrombin group. Also, the DWB-thrombin hearts demonstrated a significant reduction in diastolic compliance compared to either the K2RBC or DWB hearts (p<0.05).

Conclusions: The findings indicate that a low concentration of thrombin amplifies myocardial ischemia-reperfusion injury.

Key Words: thrombin, ischemia, reperfusion, myocardium, compliance
2.2 Introduction

Thrombin is produced in several pathophysiological conditions, including acute myocardial infarction, coronary angioplasty and coronary bypass surgery. Generated at the site of vascular injury, activated thrombin has several cellular and tissue regulatory functions, including stimulation of endothelial prostacyclin production (45), potent chemoattraction for platelets and monocytes (46) and is a mitogen signal for lymphocytes (47,48).

Thrombin is the central plasma protein involved in activation of the coagulation cascade (2,3). During open-heart surgery, an extensive amount of thrombin is produced as blood is diverted through the extracorporeal heart-lung machine (44). Contact with the polymer surfaces in the bypass circuit causes a marked procoagulant response. Anti-coagulants (heparin with or without aprotinin) are administered to counter this effect. These anti-coagulants inhibit the effects of thrombin on the coagulation cascade; however, they do not inhibit thrombin production (63,64).

In addition to its central role in coagulation, recent cardiac studies suggest that low concentrations of thrombin have direct effects on contractile dysfunction and arrhythmias (4,5,49,52,65). These studies examined either isolated, cultured cardiac myocytes exposed to thrombin in the setting of hypoxia-reoxygenation (4,65) or isolated whole hearts perfused with thrombin (5,49). The effects of thrombin in the pathogenesis of ischemia-reperfusion injury is not known.

The aim of this study was to determine if a low concentration, non-thrombogenic dose of thrombin, affects the recovery of myocardial function and cardiac compliance
following ischemia. We found that when ischemic hearts were reperfused with a low concentration of thrombin in the blood, there were significant decreases in the recovery of ventricular function, in coronary blood flow and in cardiac compliance. These results suggest a role of thrombin in the pathogenesis of reperfusion injury following ischemia.
2.3 Methods

Isolated Heart Preparation

All experiments in this study were performed in accordance with animal care guidelines approved by the institutional animal and care use committee and the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society. The model used was a modified Langendorff heart preparation. The details of this model were described previously (66, 67). Briefly, male, adult Sprague-Dawley rats (body weights 450-550 g) were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and tracheostomised. Each animal was ventilated with a small animal respirator (Harvard Apparatus, model #683), and a medial sternotomy was performed to expose the great vessels of the heart. Loose ligatures were placed around the right innominate artery and ascending aorta. Heparin (200 U) was then injected into the right atrium. Ligatures around the subclavian and common carotids were tied and a catheter (No 20 Jelco) was inserted into the innominate artery. The catheter was advanced toward the heart until the tip extended just into the aorta. The catheter was secured by tying two ligatures around the innominate artery. The hearts were arrested by infusing a cardioplegic solution (Plegisol-Abbott Labs) at approx. 3 ml/min, at less than 80-mmHg pressure into the innominate artery. The hearts were kept cold with ice packed in the thoracic cavity until the hearts were excised. The hearts were excised and an intraventricular balloon was inserted through the left atrium into the left ventricle. The balloon catheter was secured with a purse string suture on the left atrium. The innominate catheter was then attached to the perfusion circuit. Coronary flow was initiated as a small
hole was cut into the right atrium and as the aortic ligature was tied to insure that the perfusate flow was retrograde to the coronary circulation. The hearts were placed in a 37°C bath and perfused at a constant pressure to achieve an initial flow of 4 ml/min. The perfusate used was a red cell rich solution, referred to as K2RBC (66). The perfusate consisted of 5mM Krebs-Henseleit bicarbonate buffer plus 2% albumin and washed red blood cells to a packed cell volume of 20%. The perfusate was not recirculated.

*Evaluation of Ventricular Pump Function*

Ventricular function was evaluated before and after ischemia with the intraventricular balloon catheter system (68). The catheter was connected to a blood pressure transducer (model P23XL, Spectramed) and recorder (Gould Electronics-Windograf). The balloon was filled with saline (approx. 250μl) to achieve a left ventricular end diastolic pressure (LVEDP) of 5 mmHg. After 15 minutes of perfusion, the hearts were paced at 250 bpm (Grass Stimulator, Model SD90). Ventricular function was assessed at constant heart rate and balloon volume as left ventricular developed pressure (LVDP=LVESP-LVEDP), +dP/dt, and −dP/dt. Ventricular recovery from ischemia was determined by comparing pre-ischemic cardiac function values with reperfusion function values, at the same heart rate and balloon volume. Coronary vascular resistance was calculated by dividing the perfusion pressure by the coronary flow per gram of heart weight.
Preparation of Diluted Whole Blood (DWB)

Whole rat blood was obtained from a donor animal and prepared immediately before use. Donor rats (450-600 gm) were anesthetized with sodium pentobarbital (50 mg/kg) and 16-18 ml of arterial blood were withdrawn into a heparinized syringe via cardiac puncture. The blood was then diluted 1:1 with Krebs bicarbonate solution and gently mixed. The DWB contained 50% of normal plasma and 50% of the normal leukocyte and platelet concentrations. A small aliquot of DWB was taken to measure pH, PO2 and PCO2 using a blood gas analyzer (Radiometer Model ABL330). Hematocrit and white cell counts were measured with a hematology analyzer (Serono Model 9018). The DWB-thrombin perfusate was prepared in a manner similar to DWB except thrombin (Thrombin, Topical-Gentrac 1000 Units) was reconstituted to 20 units/ml in sterile saline and added to the DWB (0.5 units/ml).

In-Vitro Test of the Effect of Thrombin on Platelet Aggregation

A separate series of tests were performed to determine if the dose of thrombin (0.5U/ml), used in this study, caused a thrombogenic response in the perfusate. Thrombin was added to DWB at various concentrations and the amount of platelet aggregation was measured. Thrombin was added to DWB at the following concentrations: 0.01, 0.1, 0.5, 1.0, 5.0, and 10.0 units/ml of DWB. The aliquots were then gently mixed and examined for platelet aggregation by measuring platelet counts with a hematology analyzer (Serono Model 9018) and by making hematology slides. The slides were viewed under a light microscope and scanned for the presence of microthrombi.
**Experimental protocol**

After the heart was connected to the perfusion circuit, the heart was perfused for 15 minutes, and then paced at 250 bpm for 15 minutes. Pre-ischemia measures of ventricular function, coronary flow and perfusion pressure was made after 30 minutes of control perfusion. Coronary flow was then completely stopped for 30 minutes. During ischemia the hearts were maintained at 37°C during the ischemic period by maintaining the bath temperature at 37°C. At the conclusion of the ischemic period, the blood flow was restored. A typical recording of the ventricular function tracing the ischemic period is shown in Figure 2.1. The beginning of the ischemic period starts on the left hand side of the figure (ISCHEM) then traces ventricular contracture during the ischemic period. The beginning of the reperfusion period is indicated at R0. After the 35-minute reperfusion period, Starling function curves were recorded.

**Cardiac Compliance**

After 35 minutes of reperfusion ventricular function curves were produced. Figure 2.2 shows a typical ventricular function curve generated by adding 50 µl increments of saline to the ventricular balloon while recording intraventricular pressure. Diastolic and systolic pressures were recorded for each balloon volume increment. To evaluate cardiac compliance, LVEDP vs. volume curves were plotted and the slope analyzed. In this case, an increase in slope is indicative of a decrease in cardiac compliance (69).
Ventricular function tracing during 30 minute ischemia period. Lower tracing is intraventricular balloon pressure. During ischemia ventricular pressure rises due to depletion of myocyte ATP stores (15).

**Figure 2.1**

Compliance curve. Curves are generated by adding 50μl increments of saline to intraventricular balloon. Tracings give diastolic (lower value) and systolic (upper value) pressures at each incremental step.

**Figure 2.2**
**Experimental Groups**

Three groups of isolated rat hearts were examined. In Group I (n=6), the hearts were perfused with K2RBC throughout the reperfusion period. In Group II (n=30) the hearts were initially reperfused with DWB for five minutes then perfused with K2RBC for the remaining 30 minutes. In Group III (n=9), the hearts were initially reperfused with DWB-containing 0.5 units/ml thrombin, then with K2RBC. No attempt was made to pace the hearts for the first 15 minutes of reperfusion. Ventricular pump function, coronary blood flow, perfusion pressure and heart rate were measured after 5, 20 and 35 minutes of reperfusion.

**Statistics and Data Analysis**

The results were expressed as means ± SEM. The ventricular pressure-volume data for cardiac compliance measurements were best fit by linear regression analysis. Comparisons among groups were performed with Repeated Measurement Analysis of Variance (RANOVA) using Statistical Package for the Social Sciences (SPSS 10.0) software. P values less than 0.05 were considered statistically significant.
2.4 Results

Recovery of Ventricular Function

Figure 2.3 summarizes the results of the recovery of ventricular function in terms of contractility (+dP/dt). After five minutes of reperfusion, while unpaced, Group I hearts recovered 121% of pre-ischemia values. The hearts were unpaced during the first fifteen minutes of reperfusion. Ventricular contraction is heart rate dependent and group one hearts developed increased ventricular contraction due to the hearts being unpaced. Group II hearts recovered 64% from pre-ischemic values. Group III hearts recovered 38% from pre-ischemia values. After 20 minutes of reperfusion, while paced, Group I hearts recovered 66% from pre-ischemia. Group II hearts recovered 49% from pre-ischemia. Group III hearts recovered 29% from pre-ischemia. After 35 minutes of reperfusion Group I hearts recovered 64% from pre-ischemia. Group II hearts recovered 45% from pre-ischemia. Group III hearts recovered 24% from pre-ischemia. The recovery of +dP/dt for Group III hearts was significantly less than the recovery of Groups I and II (p<0.05). In two experiments, both from Group II, the hearts were fibrillating at R5 and the +dP/dt measurements were not included in the R5 value. These results indicate that the non-thrombogenic dose of thrombin had significant negative influence on left ventricular recovery following ischemia.

Coronary Blood Flow

Figure 2.4 summarizes the results of the coronary blood flow measurements before and after ischemia. The coronary flows before ischemia was similar for the three groups. In
Group I, there was no decrease in coronary blood flow after 5 minutes of reperfusion. At R5, Group II flow decreased 16% and Group III flow decreased 34%. At R20, flow decreased 4% in Group I. Group II flow did not decrease further from the 5-minute measurement. Group III flow did not decrease further from the 5-minute measurement. At R35, Group I flow decreased 8.6%, Group II flow decreased 24% and Group III flow decreased 47%. Coronary flow in Group III decreased significantly (p<0.05) than Group I and II hearts after 5, 20 and 35 minutes of reperfusion. Thrombin reduced the coronary blood flow by 50% in the heart perfused with the DWB-thrombin perfusate compared to the DWB-only isolated hearts.

**Coronary vascular resistance**

Figure 2.5 gives the results for coronary vascular resistance (CVR). The pre-ischemic values of coronary vascular resistance were similar for all groups. At R5, Group I resistance increased 4.7% from pre-ischemia, Group II resistance increased 53.5% and Group III resistance increased 95%. At R20, Group I resistance increased 7.3%, Group II resistance did not increase significantly and Group III resistance increased 174%. At R35 minutes Group I resistance increased 21%. Group II resistance increased 117% and Group III resistance increased 223%. Group III resistance increased significantly (p<0.05) compared to Group I. These results indicate that thrombin is significantly increasing CVR in the myocardium during reperfusion period.

**Cardiac Compliance**
Ventricular function curves were generated late in reperfusion by adding saline in 50 μl increments to the ventricular balloon while recording balloon pressures. The LVEDP was then plotted versus balloon volume and the resulting LVEDP-volume curves were best fit with a linear least squares regression line. The effects of thrombin on cardiac compliance in the setting of ischemia-reperfusion were examined, by comparing the slopes of the best-fit pressure-volume data for the K2RBC hearts (n=6), DWB hearts (n=29) and DWB-thrombin hearts (n=9). A positive upward shift in slope in the LVEDP vs. balloon volume curve is indicative of increased cardiac stiffness or decreased compliance (69). Figure 2.6 gives the mean values for the K2RBC, DWB and DWB-thrombin reperfused hearts. The slope for the DWB-thrombin hearts was significantly greater than the K2RBC and DWB-only hearts. The DWB-thrombin hearts were less compliant, indicating that a relatively low dose of thrombin caused significant diastolic dysfunction in this model.

**Thrombin Effect on Platelet Aggregation**

The *in-vitro* hematology results demonstrated no significant reduction in platelet counts for the 0.05, 0.1, 0.5, and 1.0 thrombin unit/ml DWB (Figure 2.7). However, the 5.0 Unit/ml thrombin dose caused a significant decrease in platelet count (p<0.03). The decrease in the platelet count is most likely due to platelet aggregation. The physiological concentration used in the ischemia-reperfusion experiments (0.5 units/ml DWB) was ten-fold less than the dose that caused platelet aggregation. Thus, it is unlikely that a 0.5U/ml thrombin caused cardiac dysfunction via microthrombi formation. No significant thrombi
or platelet aggregates were viewed on blood slides with DWB-thrombin dosed at 0.01, 0.1, 0.5, and 1.0 units. Thrombi and platelet aggregates were viewed under microscopy in the 5.0 unit slides. The 10.0 unit thrombin dose caused a solid clot in the blood tube in two of the four experiments. The concentration of thrombin that was tested (0.5 U) did not cause significant platelet aggregation or thrombus formation in vitro.
Figure 2.3 Effects of ischemia-reperfusion on recovery of myocardial contractility.

+\text{dP/dt} is a direct measurement of myocardial contractility. Hearts were not paced during the first 15 minutes of reperfusion.
Percent Ventricular Recovery from Ischemia

Effects of ischemia reperfusion on recovery of myocardial contractility. \(+dP/dt\) is a direct measure of myocardial contractility. Hearts were not paced for the first 15 minutes of reperfusion.

\( * p<0.05 \) vs. K2RBC group.
\( ** p<0.05 \) vs. K2RBC group.
\( # p<0.05 \) vs. DWB group.

Figure 2.3
Figure 2.4 Coronary Flow for the pre-ischemic and reperfusion periods. Group I hearts decreased 8.6 % after 35 minutes of reperfusion group II decreased 24% and group III decreased 47% during reperfusion.
Coronary Flow

Figure 2.4
Figure 2.5 Coronary Vascular resistance increased during reperfusion. Group I hearts resistance increased 21%, group II hearts increased 108% and group III increased 216% during reperfusion.
Coronary Vascular Resistance

Figure 2.5

- Group I: K2RBC n=6 # - no significant change in flow after 35 minutes of reperfusion (p>0.05)
- Group II: DWB n=29
- Group III: DWB w/thrombin n=8 , * p<0.05 vs. K2RBC
Figure 2.6 Best fitted regression lines plotted for Group I (K2RBC), Group II (DWB) and Group III (DWB+Thrombin) compliance curves. Slope for regression lines computed with SigmaPlot regression analysis Slope for group I equals 0.25, group 2 equals 0.25, group III equals 0.49. A positive slope indicates a decrease in cardiac diastolic compliance.
Figure 2.7 Thrombin was added to DWB at various concentrations. Platelet counts were then measured and compared to DWB control. No significant decrease in platelet counts were noted until 5.0 Units/ml DWB was added. 4 experiments were performed with thrombin concentration dose done in triplicate.
Figure 7. Thrombin was added to DWB at various concentrations. Platelet counts were then measured and compared to DWB control. No significant decrease (p<0.03) in platelet counts were noted until 5.0 U thrombin/ml DWB was added. 4 experiments were performed with each thrombin concentration dose done in triplicate.
2.5 Discussion

The present study tested the hypothesis that thrombin aggravates ischemia-reperfusion injury in the heart. The results indicate that a low physiological, non-thrombogenic dose of thrombin caused a significant reduction in the recovery of ventricular function and compliance compared to hearts reperfused without thrombin in the perfusate. Thus, during ischemia-reperfusion episodes, such as myocardial infarction, cardiopulmonary bypass or balloon angioplasty generation of thrombin may play a significant role in reducing cardiac recovery following ischemia.

In the setting of myocardial ischemia, thrombin is pro-arrhythmogenic during reperfusion (3). Thrombin is thought to cause arrhythmias by binding to a specific surface thrombin receptor. Thrombin activates the thrombin receptor by cleaving an extracellular domain exposing a tethered ligand. The activated receptor is coupled to a G-protein complex, activating Phospholipase C (PLC) and the intracellular pathways, inositol 1,4,5-triphosphate and diacylglycerol. Investigators have postulated that activation of these pathways leads to alterations in Na+/H+ exchange, release of lysophatidylcholine and mobilization of intracellular calcium stores leading to alterations in ventricular function (5,51,65).
**Thrombin Amplifies Reperfusion Injury following Ischemia**

Although the effects of thrombin on cardiac conduction have been studied, the effects on pump function in ischemia-reperfusion have not been fully investigated. As Figure 2.3 indicates, we found that thrombin (0.5U/ml) significantly reduced ventricular functional recovery following ischemia. Thrombin addition during reperfusion caused a significant reduction in coronary blood following ischemia (Figure 2.4). There was no significant difference in coronary flow in the K2RBC perfused group from pre-ischemia to R35 (p>0.05). However, the reduction in flow was significantly different in the DWB and DWB-thrombin groups between pre-ischemia and R35 (p<0.05). Additionally, coronary flow was significantly less in the DWB-thrombin perfused hearts compared to the DWB-only and K2RBC perfused hearts (p<0.05).

As Figure 2.5 indicates, the coronary vascular resistance was increased significantly when thrombin was added to the perfusate. No significant increase in resistance was observed in Group I at R35 (p>0.05) from pre-ischemia. The resistance for Group III at R35 was significantly more than Group I hearts (p<0.05). Reperfusing hearts with DWB increased vascular resistance during the reperfusion period. However, the addition of a low physiologic, non-thrombogenic dose of thrombin in DWB significantly increased (p<0.05) the vascular resistance without a thrombogenic response in the coronary vasculature. Our *in vitro* experiments using incremental doses of thrombin in DWB perfusate demonstrated that the level of thrombin (0.5 units/ml) in the perfusate did not induce either thrombi or microthrombi formation. In fact, not until levels 10 times greater
then the levels used in our reperfusion experiment (5.0 units/ml) and higher did thrombi formation appear.

Thrombin Decreased Cardiac Compliance Following Ischemia

In addition to decreased contractility, a significant finding in this study is that thrombin, caused a significant decrease in cardiac compliance following ischemia. Figure 2.7 compares the K2RBC, DWB with the DWB-thrombin post-ischemic compliance curves. The best fit regression lines for the K2RBC (Group I), DWB (Group II) and DWB-thrombin (Group III) groups demonstrate that DWB-thrombin hearts exhibited a significant increase in post-ischemic left ventricular end diastolic pressure, indicating increased “cardiac stiffness” or decreased compliance. Cardiac compliance or distensibility is the ratio of change in ventricular volume to change in ventricular diastolic pressure (ΔV/ΔP).

Elevation of ventricular end diastolic pressure is one of the hallmarks of left ventricular dysfunction. A compliant heart can relax more effectively and a decrease in ventricular compliance can result in delayed left ventricular relaxation, which may limit diastolic filling and elevate filling pressure (70). Reduced ventricular compliance is a pathological condition found in left ventricular injury resulting from myocardial hypoxia, hypertrophy, fibrosis and infiltrative diseases (69,70). Even when anticoagulants are administered to prevent activation of the coagulation cascade, production of thrombin occurs (63,64) and may play an important role in reducing ventricular recovery and ventricular compliance.
in cardiac myocytes following ischemia-reperfusion. Additionally, the method employed to measure cardiac compliance following ischemia-reperfusion may be a novel tool to measure changes in ventricular distensibility following ischemia-reperfusion.

Comments on the Model

Previous myocardial ischemia-reperfusion studies in our lab employed a constant coronary flow protocol (66). The model employed in this study utilized a constant coronary perfusion pressure protocol. Constant coronary perfusion pressure rather than constant coronary flow is a better model of the physiologic hemodynamics in vivo. In addition, coronary blood flow and myocardial performance are directly related to myocardial perfusion (71). This model indicates that perfusion with DWB during the initial reperfusion period reduced myocardial performance, coronary flow and increased coronary vascular resistance. The addition of thrombin to DWB perfusate further reduced the recovery of cardiac function, coronary flow and increased vascular resistance compared to K2RBC and DWB perfused hearts. Thus, during ischemic episodes such as myocardial infarction, coronary bypass or balloon angioplasty, thrombin generation may impair the recovery of ventricular function during reperfusion. Most isolated heart models use a buffered Krebs-Henseleit or Tyrodes perfusate (4,49,66,71-74). In this study the perfusate used was a red blood cell-Krebs solution perfused prior to the ischemic period, followed by diluted whole blood (DWB) with a non-thrombogenic amount of thrombin added during the first five minutes of reperfusion. Providing red cells in the perfusate markedly increases oxygen delivery to the heart (71). Reperfusion with DWB mimics the conditions found early in reperfusion. Previous studies using DWB or
whole blood indicate that leukocytes play a role in cardiac injury following ischemia (73,75).

Results from other studies indicate that low dose thrombin (1-2 U/ml), has direct positive or negative inotropic effects on cardiac myocytes (4,52). A study by Damiano et al. (49) used isolated guinea pig hearts perfused with a buffered Tyrode solution to illustrate the adverse effects of low concentrations of thrombin (0.03-1.0 U/ml) on ventricular function. However, these studies did not subject the isolated hearts to ischemia-reperfusion.

Krebs-Henseleit or Tyrode's perfusion does not allow blood cells and plasma constituents, such as leukocytes and thrombin, thus limiting the amount of functional recovery and damage. The Damiano et al. study demonstrated a reduction in left ventricular developed pressure, perfusion pressure increase and an increase in heart rate in a dose dependent manner with thrombin. This study used blood and plasma constituents, and more closely resembled the in vivo effects of thrombin in ischemia-reperfusion injury. Our results indicate that a non-thrombogenic dose of thrombin given over a limited reperfusion period causes significant reduction in recovery of left ventricular compliance and may interfere with left ventricular filling during diastole resulting in reduced ventricular contraction.
Conclusions and Implications

Over 300,000 patients undergo cardiopulmonary bypass surgery (CPB) each year in the U.S. Thrombin is generated at the CPB site and damages the reperfused myocardium, which may manifest as low cardiac function post-bypass (2). The findings in this study indicate that a non-thrombogenic concentration of thrombin significantly reduces cardiac performance and ventricular compliance during reperfusion following ischemia. Thrombin in fact may be affecting the cardiac myocyte and coronary microcirculation directly. Thrombin in the myocyte may be activating thrombin receptors by cleaving an extracellular domain exposing a tethered ligand. The activated receptor is coupled to a G-protein complex, activating Phospholipase C (PLC) and the intracellular pathways, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Investigators have postulated that activation of these pathways leads to Na+/H+ exchange, release of lysophatidylcholine and mobilization of intracellular calcium stores leading to alterations in ventricular function both resulting in compromised ventricular contractile, relaxation and altered endothelial function (8,51,65). Development of thrombin receptor antagonists may be beneficial in myocardial ischemia-reperfusion settings such as coronary bypass and angioplasty to help restore cardiac function, coronary perfusion and myocardial compliance.
Chapter 3

THROMBIN ACTIVITY AND PLATELET MICROPARTICLE FORMATION ARE INCREASED IN TYPE 2 DIABETIC PLATELETS: A POTENTIAL CORRELATION WITH CASPASE ACTIVATION

3.1 Abstract

Diabetics suffer from many complications including cardiac and vascular disease. A major contributor is an increase in blood coagulability that can amplify thrombosis. There is an association between thrombin activity and platelet function in thrombosis. Platelets provide an anchor for the attachment of the prothrombinase complex, which allows large quantities of thrombin to be formed. This process requires phosphatidylserine (PS) to move to the outer leaflet of the plasma membrane. This “flipping” of PS is reminiscent of a well-known step in apoptosis and may require activation of the same proteases, such as caspases. Another aspect of platelet activation similar to apoptosis is the release of numerous microparticles from the surface of the platelet. However, we do not know the underlying causes of the hypercoagulability observed in diabetes. The first aim of this study was to determine if thrombin activity is increased in type 2 diabetic rats. Increased thrombin activity may contribute to the hypercoagulability in diabetes. The second aim of this study was to determine if there was an increased quantity of PMPs in the blood of type 2 diabetic rats. The third aim of this study was to examine if caspases are increased in the platelets of type 2 diabetic rats. There was a significant increase in thrombin activity, the number of platelet microparticles and in caspase 3, 6, and 8 activity in the type 2 diabetic rats. The combination of increased thrombin activity, increased PMP
formation and increased caspase activity may contribute to the hypercoagulability of the diabetic blood.
3.2 Introduction

Diabetes is a growing problem in the United States and worldwide. Type 2 diabetes is the most prevalent form of diabetes, with 90% of all cases being this type (76). Two-thirds of people with diabetes mellitus die of cardiovascular disease due to a thrombotic event, with seventy percent due to cardiovascular complications and the remaining due to cerebrovascular events and peripheral vascular complications (77). The increased incidence of thrombosis in diabetes may be due to a hypercoagulable condition. The hypercoagulable state is evidenced by elevated levels of coagulation activation markers, such as prothrombin, fibrinogen, factor XI, and von Willebrand factor, and to decreased levels of protein C (78). Investigators have demonstrated that platelets are activated in diabetes mellitus. Sobol and Watala reviewed the role of platelets in diabetes-related vascular complications and conclude that diabetic subjects demonstrate both increased platelet adhesiveness and an exaggerated aggregation, with or without the addition of stimulating agents. These authors also state that the altered biophysical state of platelet membrane components in diabetes may be one of the major determinants of platelet hypersensitivity and hyperfunction (79). However, the intracellular mechanisms governing increased platelet activation, defined, as increased adhesiveness and aggregation, in type 2 diabetes are unclear. It is uncertain if there is an association between altered platelet intracellular mechanisms, thrombin activity and the hypercoagulable state in type 2 diabetes.
It is known that when platelets become activated, they express phosphatidylserine (PS) on the outer leaflet of the plasma membrane. Radziwon et al. reported that PS serves as an anchor on which the pro-thrombinase complex assembles, allowing prothrombin to be cleaved into the active form (57). When platelets become activated, they also release numerous platelet microparticles from the plasma membrane. PMPs have been implicated in the etiology of cardiovascular dysfunction in several conditions such as cardiopulmonary bypass, stroke, and diabetes (8,9). During cardiopulmonary bypass surgery, there is an increase in PMPs in the systemic blood, with the highest concentration of PMPs found at the time of reperfusion back into the systemic circulation (8). Interestingly, these cell changes that activated platelets demonstrate closely mirror those observed in apoptosis. Apoptosis, the programmed process of cell death, plays a very important role in many physiological and pathological conditions such as embryo and organ development, immune responses, tumor development and growth (80).

Due to similarities between apoptosis and platelet activation, including cell shrinkage, membrane blebbing and the formation of small, membrane bound particles (81), the question of whether the same proteases involved in apoptosis are involved in platelet activation requires further examination. Three groups of proteins have been implicated in mediating apoptosis: cell death receptors, Bcl-2 and related proteins, and caspases. In this study, we specifically aimed to determine whether there was a link between platelet activation and thrombin activity in diabetes, the first aim of this study was to determine whether there was an increased thrombin activity in type 2 diabetic rats. The second aim of this study was to determine if there is an increased quantity of PMPs in the blood from
type 2 diabetic rats. The third aim of this study examined whether caspases, which are known to be involved in apoptosis, are activated in the platelets of type 2 diabetic rats.

3.3 Methods

Diabetic Animal Model

Six type 2 diabetic rats (ZDF-fa/fa) and 4 age matched lean controls (ZDF-fa/-) were examined (Genetic Models Incorporated). The diabetic homozygous animals exhibit hypertension and express non-insulin dependent diabetes by approximately 16 weeks of age. In these experiments, the expression of diabetes was determined by blood glucose measurements (Figure 3.1). As an additional control, three Sprague-Dawley rats were also tested (SD control). All experiments were conducted according to the guidelines issued by the Institutional Animal Care and Use committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Blood Sampling

The rats were anesthetized using ether. Four ml of blood was obtained from each animal via cardiac puncture using citrated syringes (0.14 ml citrate-phosphate-dextrose solution, to each ml whole blood) (Sigma). Blood was then centrifuged at 50-x g for 30 minutes at 4° C to obtain platelet rich plasma (PRP). The PRP was measured with an automated cell counter (Serono 9018 CP) and was found to be 99% platelets. The PRP was diluted in sterile phosphate-buffered saline to obtain a final concentration of $10^7$ platelet/ml. A
subsample of the blood was centrifuged at 2500 x g for 25 minutes at 4° C to obtain platelet poor plasma (82).

**Measurement of Thrombin Activity**

Plasma was frozen in liquid nitrogen to preserve thrombin activity and stored at -80°C until the experiment was performed. Plasma thrombin activity was measured using H-D-Phenylalanyl-L-pipeocyl-L-arginine-p-nitroaniline dihydrochloride (S-2238, Chromogenix) (83). The assay principle is that thrombin catalyzes the splitting of p-nitroanaline (pNA) from the S-2238 substrate. The rate at which the pNA is released is measured photometrically. The reaction rate is linearly proportional to the amount of thrombin in the sample. Briefly, fifty microliters of plasma were mixed with prewarmed substrate and incubated at 37° for an hour. 300 microliters were then removed from each sample placed in a well of a 96-well plate and read at 405 nm on a microplate reader (Vmax, Molecular Devices) (57). Plasma from donor rats was kept frozen at -80°C until time of assay.

**Measurement of Platelet Microparticles**

A subsample of PRP was incubated with annexin binding buffer, (Pharmingen) for 10 minutes. PRP was then added to microcentrifuge tubes in which FITC-labeled antibodies to CD61 (The GPIIIa component of the GPIIb/IIIa protein constitutively expressed on the outer membrane of platelets) (20 ul, Pharmingen) had been added. The samples were incubated for an additional 10 minutes. At the end of the incubation period, samples were
diluted with 1 ml of ice-cold 1% paraformaldehyde, and stored on ice until data acquisition by flow cytometry.

**Measurement of Platelet Caspases**

Caspase 2, 3, 6, 8, and 9 activity was quantified using a colorimetric assay (Kit # KHZ1001, BioSource International Inc.). Briefly, platelets were lysed using the cell lysis buffer supplied. Fifty microliters of the lysate were aliquoted into a 96 well microplate. Fifty microliters of reaction buffer containing 10 mM DTT were then added to the sample wells. Five microliters of the 4mM VDVAD-pna (substrate for caspase-2), DEVD-pna (substrate for caspase-3), VEID-pna (substrate for caspase-6), IETD-pna (substrate for caspase-8), LEHD-pna (substrate for caspase-9), were added to the proper wells and the plate was then incubated at 37° C for 2 hours. The plate was then read at 405 nm on a microplate reader (Vmax, Molecular Devices).

**Data Analysis**

Both plasma thrombin activity and platelet caspase activity were compared among the diabetic, lean control, and the Sprague-Dawley control groups. Summary data were expressed as mean ± SEM. Comparisons among groups were made by ANOVA. P≤0.05 was considered statistically significant.
3.4 Results

Fasting Blood Glucose The results of the blood glucose comparisons between the type 2 diabetic rats and the age matched lean controls are summarized in Figure 3.1. We found the fasting blood glucose in the diabetic animals increased at 8 weeks and reached a plateau following 9 weeks at a blood glucose of 500 mg/dl. The lean controls maintained blood glucose levels below 100 mg/dl for the duration of the study.

Thrombin Activity Representative thrombin activity graphs for lean controls and thrombin activity are depicted in Figure 3.2. The results of the total endogenous thrombin potential assay for the lean controls and type 2 diabetic rats are summarized in Figure 3.3. We found a significant increase (30x) in thrombin activity in the type 2 diabetic rats compared to the age-matched lean controls and the SD controls (p<0.001).

Platelet Microparticle Formation The results of the platelet microparticle formation experiment for the lean controls and type 2 diabetic rats are summarized in Figure 3.4. We found a significant increase in the number of PMPs per platelet in the type 2 diabetic rat compared to the age-matched lean controls (p<0.001).

Caspase activity The results of the platelet caspase activity assay for the lean controls and type 2 diabetic rats are summarized in Figure 3.5. Caspase 2 activity was significantly increased in the diabetics as compared to the Sprague-Dawley controls (p
<0.04) but not compared to the lean controls (p < 0.4). Caspase 3 activity was significantly increased in the diabetics as compared to either the Sprague-Dawley or the lean controls (p < 0.03). Caspase 6 and 8 activity were also significantly greater in the diabetics as compared to either the Sprague-Dawley or the lean controls (p < 0.04 for caspase 6 and p < 0.02 caspase 8). The type 2 diabetic rats had significantly greater caspase 9 activity as compared to the Sprague-Dawley controls (p < 0.05) but not the lean controls (p < 0.1).

3.5 Discussion

Diabetes Mellitus is a growing public health problem in the United States and worldwide. In the U.S. alone, diabetes is the seventh leading cause of death (and the sixth leading cause of death by disease) (76). Diabetes is associated with many life-threatening complications such as kidney disease, neuropathies, cardiovascular and vascular diseases, and haemostatic problems. Many of these complications may be due to thrombotic events. Thrombin generation and activation may in part be due to increased caspase activity in platelets of diabetics (84). This caspase activity may be involved in chronic activation of the platelets resulting in a positive feedback loop, that is, thrombin activating platelets which allow for more anchoring of the prothrombinase complex, generating even greater amounts of thrombin, leading to this hypercoagulable state.
Thrombin activity

Thrombin plays a central role in the coagulation cascade. Its adequate production at the site of a vascular lesion is pivotal in arresting bleeding. However, in disease states such as diabetes mellitus, excess thrombin production or the enzymatic activity of thrombin may contribute to the hypercoagulability and arterial atherosclerosis. In the present study, we found that there was a remarkable 30-fold increase in thrombin activity in type 2 diabetic rats compared to controls. Several recent papers have described the interrelationship between phospholipid exposure on the plasma membrane and thrombin formation (85-87). Tedgui and Mallat found that tissue factor activity is highly dependent on the presence of PS on the outer leaflet of cellular membranes (85). They suggested that procoagulant microparticles may play a major role in blood thrombogenicity. Platelet microparticles are small, membrane bound fragments of platelets which are released when platelets become activated. The formation of platelet microparticles (PMPs) increases the surface area available for the prothrombinase complex to form, using exposed phosphatidylserine as an anchor (85-88). Since thrombin is able to activate platelets, and activated platelets release PMPs, this leads to a greater thrombin formation. This positive feedback loop could be one of the key reasons that diabetics are so hypercoagulable.
Platelet Microparticle Formation

An aim of this paper was to determine whether there was increased platelet activation, measured via platelet microparticle formation, in uncontrolled type 2 diabetes. We found a significant increase in the number of platelet microparticles per 1000 platelets in the diabetic rats.

Caspase activation

An objective of this paper was to determine if caspases were activated in the platelets of uncontrolled type 2 diabetic rats. Caspases are a family of 14 enzymatic proteins which have cysteine protease activity and that cleave their substrates after aspartate residues (89). Caspases can be further classified by their place in the process of apoptosis. A caspase initiator is a caspase that is directly activated by death receptors, and these caspases regulate downstream caspase activity. Prime examples of initiator caspases include caspase-8, -9, and -10 (90). Caspase executioners are those that are activated by the caspase initiators. These caspases then activate other substrates important in the process of programmed cell death and include caspase-3 and -7 (84). We found a significant increase in caspase -3, -6, -8 and -9 activity in the platelets from these Type 2 diabetic rats. Caspases -2, -3, -6, -8, and -9 are considered to promote pathways to apoptosis (89). This observation is based on observations that, in vitro, these caspases cut proteins whose cleavage is associated with apoptosis (89). The role of caspase activation
in activated platelets is unclear. Li et al. demonstrated an increase in caspase 3 activity as well as the amount of the biologically active p17 subunit of active caspase 3 which coincided with apoptotic morphology in platelets stored for 5 days under standard blood banking conditions (84). However, Wolf et al. reported that although human platelets contain caspase-9 and caspase-3, there was no evidence that these caspases were involved in platelet activation or the apoptotic phenotype those platelets demonstrated (91). It is possible that the caspase activation we demonstrated in the diabetic platelets causes PS to flip from the inner to the outer leaflet of the plasma membrane, leading to the increased thrombin activity that was observed (Figure 3.6).

In conclusion, thrombin activity is increased in the plasma from type 2 diabetic rats which is one possibility of why diabetics are prone to thrombotic events, leading to morbidity and mortality. Also, this is the first report of caspases other than caspase 3 being activated in platelets. This activation of both initiator and executioner caspases perhaps demonstrates an organization of platelet activation that is more similar to apoptosis than previously thought.

It is not known whether increased caspase activity is required for platelet activation and subsequent aggregation or whether other aspects of platelet activation lead to caspase activity. By determining mechanisms of platelet activation and its role in thrombin generation, we can elucidate novel ways to treat the hypercoagulability seen in diabetics.
Figure 3.1 Fasting blood glucose levels for lean controls and ZDF-\(fa/fa\) rats
Blood Glucose Lean Controls vs. Type 2 Diabetics

![Blood Glucose Graph](image)

- **Blood Glucose (mg/dl)**
- **Age (weeks)**

**Legend:**
- ● Gmi-ZDF fa/fa Type 2 Diabetics \(n=6\)
- ■ Gmi-ZDF fa/ Lean Controls \(n=4\)

**Figure 3.1**
Figure 3.2 Representative examples of 10 minute kinetic thrombin activity graphs. Upper graph is lean control and lower graph is ZDF-\textit{fa}/\textit{fa} diabetic.
Figure 3.2
Figure 3.3 Bar graph of total thrombin activity computed from 10 minute kinetic thrombin activity graph.
Figure 3.3
Figure 3.4 Bar graph of platelet microparticles lean controls vs. ZDF-fa/fa diabetics.

Platelet microparticles expressed as PMP per 1000 platelets.
Figure 3.4

Lean Controls    ZDF Diabetics
Total Thrombin Activity
Figure 3.5 Caspase activity in platelets for S-D controls, lean controls and ZDF-fa/fa diabetics. Caspase 2 activity was significantly increased in the diabetics as compared to the Sprague-Dawley controls (p < 0.04). Caspase 3, 6 and 8 activity was significantly increased in the diabetics as compared to S-D or lean controls (p < 0.03). The type 2 diabetic rats had significantly greater caspase 9 activity as compared to the Sprague-Dawley controls (p < 0.05) but not the lean controls (p < 0.1).
Caspases in Platelets

Figure 3.5
DIABETES:
- Increased Glucose
- Increased Insulin
- Increased Lipids

Pro-Caspase

Caspase

(+) Phosphatidylinerine Flipping

Increased Thrombin

Increased Adhesion & Aggregation

INCREASED HYPERCOAGULABILITY
Chapter 4

VITAMIN E REDUCES MYOCARDIAL INFARCT SIZE AND IMPROVES VENTRICULAR RECOVERY IN A TYPE 2 DIABETIC RAT MODEL

4.1 Abstract

There are many severe complications of type 2 diabetes, but ischemic events, primarily heart attacks and strokes, have emerged as the primary cause of death in this patient population. The pathophysiology underlying the severity of ischemic events in diabetes is poorly understood, but the diabetic heart may be more susceptible to ischemia-reperfusion (I-R) injury due to increased oxidative stress in the heart and in the blood perfusing the coronary vasculature. Increased reactive oxygen species (ROS) generation in neutrophils (PMNs), sequestered in the heart under ischemic conditions, may contribute significantly to oxidative injury. If so, then treatment with anti-oxidants may protect the heart by reducing the production of oxygen free radicals under ischemic conditions.

The purpose of this study was to first determine if myocardial injury due to ischemia-reperfusion was increased in the type 2 diabetic heart. Our second aim was to determine if chronic treatment with Vitamin E would attenuate ischemic injury and improve ventricular recovery in treated diabetic rats. Lean controls and type 2 diabetic rats (ZDF-ffa, GMI) were subjected to a left anterior descending coronary artery occlusion-reperfusion protocol. Infarct size was determined as well as the recovery of ventricular function using a Millar catheter system. Blood samples were taken from both groups
prior to ischemia to examine the level of chronic PMN activation. Four groups were studied. Group 1: Non-diabetic, untreated (ND), Group 2: Non-diabetic, Vit E treated (ND-E), Group 3: type 2 diabetic, untreated (D) and Group 4: type 2 diabetic treated (D-E). We found that, compared to controls, after 90 minutes of reperfusion, the recovery of ventricular function (+dP/dt) was somewhat depressed in the diabetic group (ND = 87.4% ±11.7 SEM, D = 68.1% ± 5.5, P=NS), however, the recovery of the diabetics treated with vitamin E was improved significantly (D-E = 97.4% ± 10.3). In addition the infarct size, expressed as the ratio of the necrotic area to the area at risk, was significantly increased in the diabetic group, but was reduced by vitamin E treatment (ND = 43.0% ± 1.9 SEM, D = 57.0% ± 3.9, D-E = 33.9% ± 5.1, P<0.05). Vitamin E treatment also significantly reduced the chronic increase in PMN ROS observed in the diabetic group (P<0.05) prior to ischemia. These results indicate that myocardial ischemic injury is increased in the type 2 diabetic hearts and that treatment with vitamin E may attenuate the severity of ischemic events in this emergent disease.

**Key Words:** diabetes, vitamin E, myocardial infarction, ischemia, reperfusion
4.2 Introduction

Diabetes has become a major health care concern in the U.S. While deaths from other complications of diabetes are declining, the mortality from cardiovascular disease in diabetics is increasing. The mortality in acute myocardial infarction in diabetics is two to four times greater than the general population (7). In addition, related complications such as cardiac arrhythmias, heart block, congestive heart failure and post infarction angina are increased in diabetics compared to non-diabetics (60,61). In a June 2001 press release the National Institutes of Health stated; “If you have diabetes, you have the same high risk of having a heart attack as someone who has already had their first attack,” and stated further; “Even worse, your chances of dying from your first heart attack are the same as someone without diabetes who has had a second heart attack (62).

The pathophysiology associated with the severity of cardiovascular disease in diabetes is not fully understood. However research has demonstrated that diabetic hyperglycemia increases free radical production and oxidative stress within the diabetic heart and coronary vasculature (10-15) that may be contributing to the increased cardiovascular complications observed in diabetes. Increased oxidative stress may also increase the inflammatory response that contributes to myocardial damage during ischemia-reperfusion (I-R) injury (92). Therefore, anti-oxidant therapy may be beneficial in reducing oxidative stress, within the diabetic heart, and help reduce myocardial damage following an ischemic injury.
In addition to oxidative damage, diabetic hyperglycemia may exert its adverse effects by activating the diacylglycerol-protein kinase C (DAG-PKC) pathway (93). Researchers have demonstrated that PKC activation and subsequent lipid membrane translocation is increased in diabetic complications in the retina (94) aorta (95) and the heart (96). However, the intracellular downstream targets of PKC activation are still largely unknown. Myocardial PKC activation is an active area of research in I/R injury (97-100), cardiac preconditioning (101) and diabetes (96,102,103). Specifically, the epsilon isoform of PKC (PKC-ε) has been implicated in the pathophysiology of the diabetic myocardium (102,104,105).

Vitamin E may be an effective treatment in reducing oxidative stress, lipid peroxidation and reducing PKC activation within the diabetic myocardium (103,106,107). Vitamin E is the most potent lipid anti-oxidant known and vitamin E treatment may offer a particular advantage by reducing cardiovascular injuries associated with increased oxidative stress in type 2 diabetes. There have been a number of recent clinical studies that investigated the efficacy of vitamin E in reducing cardiovascular disease. One of these studies, the 2000 Heart Outcome Prevention Evaluation (HOPE) reported no benefits of vitamin E therapy in reducing myocardial infarction (108). However, the 1996 Cambridge Heart Anti-oxidant Study (CHAOS) reported a significant reduction of non-fatal MI following vitamin E therapy (109). In both of these studies there was a diabetic subgroup. The diabetic subgroups followed the trend in each of the studies; either demonstrates no benefit (HOPE) or a significant reduction in MI (CHAOS). Conflicting
results from these clinical trials argues for further investigation of vitamin E therapy in reducing cardiovascular complications in diabetics.

The purpose of this study was to first determine if myocardial injury due to I-R injury was increased in type 2 diabetic hearts. Secondly, we aimed to determine if chronic treatment with vitamin E attenuated myocardial ischemic injury in diabetic rats. The last aim was to determine if vitamin E treatment would reduce PMN ROS generation, which may contribute to the blood component of I-R injury in type 2 diabetic rats.
4.3 Material and Methods

Animal model of diabetes

All experiments in this study were performed in accordance with animal care guidelines approved by the institutional care and use committee and the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society. Male Zucker diabetic fatty (ZDF fa/ fa) rats and their aged matched lean litter mates (ZDF fa/ -) were obtained from Genetic Models Incorporated (GMI- Indianapolis, Indiana) at 6 weeks of age. Housing was under controlled conditions of light (12 hr light-dark) and temperature (22-24 °C). Rats were fed Purina 5008, a standard rodent diet, ad libitum. This model for Non-insulin dependent diabetes mellitus (NIDDM) begins to develop hyperglycemia and insulin resistance at about seven weeks of age and glucose levels (fed) typically reach 500 mg/dl by 10 to 11 weeks of age (110). Overt diabetes develops at approximately 12 weeks with elevated insulin levels and elevated blood glucose levels. After 12 weeks of age insulin levels begin to decrease because the pancreatic Beta cells cease to respond to the glucose stimulus. The loss of insulin responsiveness has been associated with the loss of GLUT 2 transporters in the pancreas (111). The aged matched lean litter mates have the same genetics as the obese rats except they do not develop obesity and are phenotypically the same as other lean control rats.
Experimental groups

Diabetic rats and the lean controls were divided into four experimental groups: (1) lean, aged matched, rats treated with castor oil vehicle only (n=7); (2) diabetic rats, 12-14 weeks old, treated with castor oil vehicle only (n=7); (3) lean, aged matched, rats treated intraperitoneally (i.p.) with 50mg/kg d-alpha-tocopherol in castor oil vehicle every other day for two weeks (n=6); and (4) diabetic 12-14 week old rats treated intraperitoneally (i.p) with 50mg/kg d-alpha-tocopherol administered every other day for two weeks (n=7) (112).

Coronary Occlusion

Procedures

All experimental groups underwent ligation of the left anterior descending coronary artery (LAD) to induce myocardial infarction. Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg); additional injections of anesthetic were administered as needed to insure a deep plane of anesthesia. Rats were placed on a heated pad to maintain normal body temperatures. (36.5-38.0 °C) A polyethylene catheter (PE-10) was inserted and secured into the right femoral artery to allow blood sampling (700 ul) for blood gas analysis and for leukocyte analysis. Blood samples were taken pre-thoracotomy, post thoracotomy and at 15, 45 and 90 minutes of reperfusion. A midline skin incision was performed from the mental process of the mandible to the manubrium of the thoracic inlet. The neck muscles were gently dissected to expose the trachea and the rat was
tracheotomized. Dissection of the neck muscles adjacent to the trachea were bluntly
dissected to expose a 1 cm segment of the right carotid sheath and the vagus nerve was
dissected away from the carotid artery. The right carotid artery was then clamped and a
small incision bisecting, but not severing, the carotid was performed. A 3.5 french Millar
pressure transducer catheter (SPR-249, Millar Instruments Inc-Houston TX) was
introduced and guided into the mid-left ventricle and secured. The Millar catheter was
coupled to Gould electronics Windowgraf® recorder for digitized pressure and ventricular
performance measurements. A “window” thoracotomy was performed by making a 3 cm
incision on the left side of the chest and dissecting the muscles between the 4th and 5th
ribs. After opening the chest wall the rats were ventilated with a small animal respirator
(Harvard Apparatus, model #683) using room air. Respiration was maintained at 2.25-
2.50 mls/stroke and a rate of 80-88 strokes/min to maintain physiologic pH, pO2, pCO2
values. The animals were ventilated for 15 to 20 minutes prior to occlusion to allow for
cardiac and respiratory stabilization (A detailed written description developed by the
author for coronary occlusion is contained in Appendix A1 and A2).

Myocardial performance measurements

Prior to occlusion, baseline heart rate, systolic, diastolic, +dP/dt, -dP/dt (rates of
ventricular contraction and relaxation, respectively) were measured. A 700 ul blood
sample was drawn for blood gas analysis of pre-ischemia post-window thoracotomy.
Ventilation was then adjusted to maintain physiologic pH, pCO₂, and pO₂, (Table 4.1). After stabilization, the ribs were gently spread to expose the left side of the heart and visualize the left anterior descending coronary artery (LAD). A 5.0 silk suture on a tapered needle was then placed around the LAD. The ends of the suture were threaded through a 4mm piece of PE-190 polyethylene tubing, which was then tightened and clamped to induce ischemia. Blanching of the myocardial tissue distal to the suture was observed to insure proper ligation of the LAD. The chest wall was then closed. After thirty minutes of ischemia the ligature was unclamped but left in place during the ninety-minute reperfusion period. During the reperfusion period, ventricular functional measurements (HR, Systole/Diastole, +dP/dt,-dP/dt) were recorded every fifteen minutes and 700 ul aliquots of blood were drawn from the femoral catheter at 15 (R15), forty-five (R45) and at ninety (R90) minutes of reperfusion.

**Myocardial infarct size determination**

Following the ninety-minute reperfusion period the ligature was re-tightened and 10ml of 0.4% Trypan blue dye was injected via the femoral catheter (113). The blue dye stains the area of the myocardium unaffected by the occlusion. The ligated area does not stain and is the area of the myocardium defined as the “area at risk”. The heart was quickly excised from the chest cavity and placed into an organ matrix (Harvard Apparatus®) to maintain the architecture and allow for precise sectioning. A myocardial coronal section, 1.0-2.0mm thick, was sliced immediately distal to the ligated artery. The coronal section was sliced to ensure that the full-thickness coronal slice included ischemic myocardium.
The coronal section of myocardial tissue was then scanned with a high-resolution scanner (Hewlett-Packard-model 5370C). After scanning, the coronal section is placed in a 1% triphenyl tetrazolium chloride (TTC) and incubated at 37°C for 30 minutes (113). Following the 30-minute TTC incubation, the heart was placed in 10% buffered formalin solution for 24 hours. After 24 hours, the section was rescanned. The area at risk fraction was determined by scanning the coronal section at high resolution. The total area of the scanned myocardial section is computed in square millimeters. The un-dyed area (area-at-risk) was outlined using the scanning software and computed to square millimeters. Three measurements of total area and un-dyed areas were outlined and the mean was used for each calculated area. The area-at-risk was computed by dividing the area unstained by the blue dye (ligated region) by the total area to obtain an area at risk fraction. The TTC stained section was then scanned. The necrotic fraction was determined for the TTC section and the necrotic fraction was divided by the area-at-risk fraction to determine the percentage of the occluded myocardial tissue that consequently became infarcted during the occlusion period (Figure 4.1). During formalin fixation, myocardial slices desiccate and subsequently become smaller. The final necrotic/area-at-risk fraction measures the necrotic area after formalin fixation divided by the area-at-risk before formalin fixation. This allowed for the amount of desiccation following formalin fixation.

\textit{Ventricular arrhythmia determination}
Ventricular arrhythmias were defined as a period of unorganized ventricular function ranging from a short burst (3-10) of premature ventricular contractions (PVC) to sustained ventricular fibrillations requiring manual cardiac massage or electrical conversion. Two deaths occurred due to fatal arrhythmias (one lean vehicle and one diabetic vehicle). These were not included in the statistical analysis. The period of arrhythmic activity was measured in minutes and comparisons of arrhythmia activity between the groups were made.

**Neutrophil reactive oxygen species generation**

To access neutrophil reactive oxygen species generation, 2′7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR) was used. DCFH-DA diffuses into the neutrophil cytoplasm where it is deacetylated to 2′7′-dichlorofluorescin (DCFH) and trapped within the cell. In the presence of intracellular H2O2 DCFH is oxidized to dichlorofluorescein (DCF). DCF is a stable end product that fluoresces green and can be quantified using fluorescence-activated cell sorting (FACS). The results were expressed on a Total Fluorescence Index (TFI), the product of the percent cells positive for fluorescence and the mean channel fluorescence. Results were analyzed using WinMDI™ 2.8 software.

**Statistical analysis**

The results were expressed as means ± SEM. The heart rate, systolic and diastolic pressures, and +/-dP/dt and rate pressure product measurements were compared among the experimental groups using a general linear model Repeated Measurement Analysis of
Variance (RANOVA) using SPSS 10.0 software. A one-way analysis of variance (ANOVA) was used to compare pre-ischemic physiological data (body weight, function data), size of left ventricular infarction, arrhythmias and neutrophil ROS production in the experimental groups. Post Hoc tests using Tukey’s honestly significant difference was used to determine significance between each experimental group. P values less than 0.05 were considered statistically significant.
Perfused Area

Area at Risk no Blue Dye

Neg TTC Stain Pale Necrotic Area

LV-Left Ventricle
RV-Right Ventricle

Figure 4.1
Pre-Ischemia Data

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Body Wt (gm)</th>
<th>±SEM</th>
<th>Temp (°C)</th>
<th>±0.2</th>
<th>HR (bpm)</th>
<th>±16.6</th>
<th>Systolic BP (mmHg)</th>
<th>±8.4</th>
<th>Diastolic BP (mmHg)</th>
<th>±1.6</th>
<th>+dP/dt (mmHg/s)</th>
<th>±163</th>
<th>-dP/dt (mmHg/s)</th>
<th>±201</th>
<th>Rate Press Prod</th>
<th>±4187</th>
<th>Rate Press Prod</th>
<th>±HR*Dev Press</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Lean Controls</td>
<td>357.6</td>
<td>±7.3</td>
<td>37.4</td>
<td>±0.2</td>
<td>334.0</td>
<td>±16.6</td>
<td>116.4</td>
<td>±8.4</td>
<td>1.7</td>
<td>±1.6</td>
<td>2357</td>
<td>±163</td>
<td>1943</td>
<td>±201</td>
<td>38629</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Treated n=6</td>
<td>357.5</td>
<td>±9.6</td>
<td>37.5</td>
<td>±0.2</td>
<td>333.0</td>
<td>±6.7</td>
<td>117.0</td>
<td>±2.4</td>
<td>0.9</td>
<td>±0.8</td>
<td>±126</td>
<td>±146</td>
<td>±1226</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Diabetic</td>
<td>450.2*</td>
<td>±17.4</td>
<td>38.2</td>
<td>±0.3</td>
<td>333.0</td>
<td>±10.0</td>
<td>120.8</td>
<td>±8.3</td>
<td>1.2</td>
<td>±1.3</td>
<td>±124</td>
<td>±129</td>
<td>±3197</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>IV</td>
<td>Treated n=7</td>
<td>468.7*</td>
<td>±18.2</td>
<td>38.0</td>
<td>±0.2</td>
<td>336.0</td>
<td>±15.0</td>
<td>127.5</td>
<td>±8.3</td>
<td>0.5</td>
<td>±2.0</td>
<td>±107</td>
<td>±131</td>
<td>±2161</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

- p<0.05 Weights of Diabetics were significantly greater than weights of Lean Controls
- Arterial blood gasses were taken at pre-ischemia: pH in all groups were between 7.35 and 7.40, Oxygen saturation was between 92-95% for all groups.

*Table 4.1*
4.4 Results

**Ventricular arrhythmias during ischemia**

Analysis of left ventricular function during the ischemic period revealed that the diabetic groups (both treated and untreated) had longer periods of arrhythmias compared to the non-diabetic groups. Ventricular arrhythmias in the untreated lean group had a mean of 5.3 minutes (±1.0 SEM) compared to 2.2 minutes (±0.5 SEM) for the treated lean group during ischemia. Mean arrhythmia period in for the diabetic untreated diabetic group was 12.3 minutes (±1.7 SEM) compared to 7.4 minutes (±0.8) for the treated diabetics. Vitamin E treatment significantly decreased (p<0.05) ventricular arrhythmias in both the lean controls (untreated and treated) and the treated diabetic group compared to the untreated diabetic group (data not shown).

**Left ventricular function data**

The aim of this section were to determine if vitamin E treatment improved the recovery of cardiac function following 30 minutes of left ventricular regional ischemia. Table 4.1 summarizes the pre-ischemia baseline data for the four groups. The body weights were significantly greater in the diabetic groups compared to the lean control groups (p<0.05). Pre-ischemia physiological data were not significantly different among the groups. Figure 4.2 summarizes the heart rates for the four experimental groups measured at pre-ischemia and during the ninety-minute reperfusion period. A repeated measures analysis of variance (RANOVA) indicated a significant difference in heart rate over the entire
reperfusion period, between the untreated diabetic group and the treated lean group (p<0.05) but no significance between the lean untreated, diabetic treated and the diabetic vehicle groups. The significant reduction in heart rate in the untreated diabetic group was not due to anesthesia or experimental procedure (only three of the 27 rats were given supplemental anesthetic during the reperfusion period, none in the diabetic vehicle group). Our results indicate that although there was no overall significant improvement in heart rate with vitamin E, the untreated diabetic group had a lower overall heart rate recovery after 90 minutes of reperfusion.

Figure 4.3 summarizes the results of the systolic pressure measurements made during the study. The pre-ischemic systolic values were not significantly different between the groups. There was no significant difference in systolic pressures between the experimental groups over the ninety-minute reperfusion period. However, there was a trend for the treated groups to maintain systolic pressure at approximately the pre-ischemic values at the end of the reperfusion period: lean vitamin E mean systolic pressure at R90 was 105.9mmHg (91.0%), diabetic vitamin E was 110.0mmHg (88.73%). The untreated group’s mean systolic values declined at R90: lean vehicle 90.33mmHg (78.0%) and diabetic vehicle was 86.7mmHg (71.7%). Treatment with vitamin E may well improve systolic function during reperfusion following ischemia.

Figure 4.4 summarizes the results of left ventricular diastolic pressure measurements made during the study. No significant differences were noted among the experimental groups. Maintaining a low diastolic pressure during the reperfusion time is a sign of reduced ventricular impairment during the I-R period. The lean treated group maintained
near pre-ischemic values throughout the reperfusion period, while the untreated lean group had increased diastolic pressure during reperfusion, indicating more ventricular impairment. The diabetic groups showed similar increased diastolic pressure during the reperfusion period.

Figure 4.5 summarizes the results of ventricular contraction (+dP/dt) measurements made during the reperfusion period. Throughout the reperfusion period the treated groups (diabetic Vit E and lean Vit E) maintained a higher rate of ventricular contraction versus the non-treated groups (p<0.05). The untreated groups did not differ significantly throughout the reperfusion period. Figure 4.5 demonstrates that the treated diabetics had a significant improvement (p<0.05) in ventricular contractility compared to non-treated diabetics. There was also a significant improvement (p<0.05) in contractility in the lean treated group compared to the lean untreated group. There was no difference in contractility between the untreated groups (diabetic vs. lean). During the pre-ischemic period, the lean untreated group had lower (but not statistically different) contractility. Vitamin E treatment improved ventricular contraction in both treatment groups.

Figure 4.7 summarizes the normalized data during the reperfusion period as a percentage of pre-ischemia. The normalized results demonstrate that the contractility in the treated diabetic group improved significantly (P<0.05) compared to the untreated diabetic group, following ninety-minutes of reperfusion (treated diabetic=97.5%, untreated diabetics=68.1%). This figure indicated that untreated diabetic rats had reduced ventricular recovery following 90 minutes of reperfusion compared to non-diabetics.
Treatment with vitamin E had a positive effect in maintaining ventricular contraction in the treated groups compared to the untreated groups.

Figure 4.6 summarizes the rate of ventricular relaxation (-dP/dt) during the reperfusion period. No statistical difference was demonstrated in the pre-ischemic values. The diabetic vitamin E group maintained a statistically greater rate of ventricular relaxation compared to the vehicle treated diabetics and vehicle lean controls during reperfusion (p<0.05). The two untreated groups had lower overall ventricular relaxation compared to both treated groups and the treated diabetic group maintained the highest rate of ventricular relaxation during the reperfusion period (p<0.05). The results indicate that vitamin E improved ventricular relaxation during the reperfusion period.

Figure 4.8 summarizes the rate pressure product (RPP). RPP is a measure of myocardial work and oxygen consumption. There was no significant difference in the RPP at the pre-ischemic period between the experimental groups. The treated groups maintained a significantly higher rate pressure product (p<0.05) throughout the 90-minute reperfusion period compared to the untreated groups. There was no statistically significant difference in RPP between the lean vehicle and diabetic vehicle or the treated lean and treated diabetic groups. The treated groups maintained RPP throughout the reperfusion period and RPP recovered to near pre-ischemic values compared to the untreated groups: treated lean 98.7%, treated diabetic 98.0%, untreated lean 71.4%, untreated diabetic 53.5%. These results indicate that vitamin E significantly improved myocardial recovery, with better cardiac performance and increased oxygen consumption, following an ischemic event.
Heart Rate

Figure 4.2

Reperfusion Time

p<0.05 vs. lean treated group

Legend:
- ▲ Lean Vehicle n=7
- ○ Diabetic Vehicle n=7
- ■ Lean Vit E n=6
- ● Diabetic Vit E n=7

Beats per minute

Pre-ischemia R15 R30 R45 R60 R90
Systolic pressure

Figure 4.3
Diastolic pressure

![Diastolic pressure graph]

Figure 4.4
Positive Dp/dt
(Rate of Ventricular Contraction)

Reperfusion Time

+Dp/dt (mmHg/sec)

Pre-Ischemia R15 R30 R45 R60 R90

* p<0.05 treated groups are greater than untreated groups

Figure 4.5
Negative Dp/dt
(Rate of Ventricular Relaxation)

Figure 4.6

*p<0.05 significantly different then lean Vit E and diabetic vehicle

Lean Vehicle n=7
Diabetic Vehicle n=7
Lean Vit E n=6
Diabetic Vit E n=7
Percent Positive Dp/dt

Reperfusion Time

* p<0.05 vs. diabetic vehicle treated group

Figure 4.7
Rate Pressure Product
(DEV Pressure * Heart Rate)

Figure 4.8
**Myocardial Infarct Size**

The aim of this section was to determine if vitamin E treatment reduced myocardial infarct size following 30 minutes of left ventricular regional ischemia. Figure 4.9 summarizes the percentage of myocardium that subsequently became necrotic (infarct area) following reperfusion. The diabetic vehicle treated group had a significantly greater necrotic area than the other groups (p<0.05). The lean treated and untreated groups and the diabetic vitamin E groups did not differ significantly in necrotic area, though there was a reduction in overall mean necrotic area between the untreated and treated lean groups (43.0% ± 2.0% SEM vs. 31.2% ± 2.1% SEM). We found that type 2 diabetic hearts had larger infarct areas than the non-diabetics leans. We also found that treatment with vitamin E significantly reduced infarct size.

Table 4.2 summarizes the data for the sectioned area, area at risk, necrotic area and the fraction of the area at risk that became necrotic (infarcted area). The area at risk of the lean groups was nearly identical (lean vehicle-21.44± 4.26 mm², lean Vitamin E-21.26±5.75 mm²), while the diabetic treated and untreated area at risk was larger (29.23±5.33 mm² and 34.67±4.45 mm², respectively) but did not differ significantly. The diabetic vehicle treated had the overall largest infarct fraction (57.0%), while the lean vitamin E group had the smallest infarct fraction (31.2%). The treated diabetic had a greater reduction in infarct percentage (compared to the untreated diabetics) than the treated leans. The treated diabetic vs. untreated diabetic had a 43% reduction infarct region compared to 25% in the treated vs. untreated leans. Our results demonstrate that vitamin E treatment reduced myocardial infarct size following ischemia-reperfusion.
injury in both diabetic and non-diabetic rat hearts. There was a significantly larger overall reduction in infarct area in the diabetic treated groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>±SEM</th>
<th>Total heart blue dye area (mm²)</th>
<th>Area at risk (mm²)</th>
<th>Total Area (TTC) (mm²)</th>
<th>Necrotic area (mm²)</th>
<th>Necrotic/Area at risk (fraction)</th>
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<tr>
<td>I - Lean</td>
<td>98.28</td>
<td>2.35</td>
<td>21.44</td>
<td>90.50</td>
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<tr>
<td>II - Diabetic</td>
<td>106.91</td>
<td>4.11</td>
<td>34.67</td>
<td>101.27</td>
<td>18.81</td>
<td>0.5700*</td>
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<tr>
<td>III - Lean</td>
<td>102.37</td>
<td>4.84</td>
<td>21.26</td>
<td>93.07</td>
<td>6.11</td>
<td>0.3119</td>
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</tr>
<tr>
<td>Vitamin E n=6</td>
<td>5.75</td>
<td>5.39</td>
<td>1.90</td>
<td>0.021</td>
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<tr>
<td>IV - Diabetic</td>
<td>106.42</td>
<td>3.80</td>
<td>29.23</td>
<td>103.04</td>
<td>9.41</td>
<td>0.3394</td>
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<tr>
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<td>2.21</td>
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</tbody>
</table>

* p<0.05 vs. all other groups

Table 4.2
LAD Necrotic Fraction

* p<0.05 vs all other groups

Treatment groups

Figure 4.9
Pre-ischemia PMN ROS Production

Figure 4.10 summarizes pre-ischemia neutrophil reactive oxygen species (PMN ROS) production. The diabetic vehicle treated group had significantly greater ROS production compared to the untreated lean group prior to ischemia (p<0.05). The treated groups had significantly lower ROS production compared to the untreated diabetic group (p<0.05). There was no significant difference between the treated groups. These results indicate that diabetic neutrophils are chronically activated, as evidenced by significantly greater reactive oxygen species production, compared to non-diabetics. Treatment with vitamin E significantly reduced the ROS production in diabetic and non-diabetic rat neutrophils.
PMN ROS Pre-Ischemia Data

* p<0.05

Figure 4.10
4.5 Discussion

Diabetes has become a major health care concern in the U.S. and worldwide. Diabetes currently affects an estimated 16 million people in the U.S. with more than 750,000 new cases diagnosed each year. The vast majority (85-90%) of the diabetic population has non-insulin dependent diabetes (type 2). Diabetics have a 2-4 times higher rate of myocardial infarction than the general population. The 1999 CDC Surveillance Report states that 43% of all diabetes-related deaths were related to cardiovascular disease (ischemic heart disease, heart failure, cardiomyopathy or stroke). Ischemic heart disease (IHD) accounted for 58% of the deaths reported from cardiovascular disease (7). Insulin use, oral hypoglycemic drugs and tight blood glucose control have substantially decreased deaths from diabetic coma and infection. However, the deaths related to cardiovascular disease have increased.

The three aims of the study were to first develop a model of ischemia-reperfusion-injury in a type 2 diabetic rat model and determine if myocardial injury is greater in diabetic rats compared to non-diabetic rats. Second to determine if chronic administration of vitamin E in type 2 diabetic rats would reduce myocardial infarct size and improve left ventricular function following I/R injury. Third, determine if vitamin E treatment would reduce neutrophil ROS production, thereby reducing the blood contribution (an oxidative stress contributor) to myocardial damage during I/R perfusion in this diabetic animal model.
**Vitamin E had a greater effect in reducing myocardial infarction in diabetic rats than non-diabetic rats.**

The results of this study indicate that the non-treated type 2 diabetic group (D 57%± 4.0 SEM) had a significantly larger (p<0.05) necrotic fraction following LAD occlusion compared to the non-diabetic untreated group (ND 43%±2.0). Vitamin E treatment reduced myocardial infarct fraction from 43%±2.0 in the non-diabetic untreated group (ND) to 31%±2.0 in the non-diabetic, treated group (ND-E), a 25 % reduction in infarct fraction. In the diabetic groups, vitamin E treatment reduced infarct fraction from 57%± 4.0, in the non-treated diabetics (D) to 34%±5.0 in the treated diabetics (D-E), a 43% reduction. Vitamin E treatment demonstrated a greater effect in reducing myocardial infarctions in vitamin E treated diabetics compared to non-treated diabetics.

Previous research into the sensitivity of the diabetic myocardium to ischemia-reperfusion (I/R) injury has produced conflicting results. Many previous myocardial ischemia-reperfusion animal models used experimentally induced diabetes using streptozotocin or alloxan. The results from these studies indicate that streptozotocin or alloxan induced diabetes made the heart less sensitive to ischemic injury (114-117) compared to non-diabetic hearts. In contrast, other studies have produced evidence that the diabetic heart is more sensitive to ischemic injury (118-121).

In one LAD study using a type 2 diabetic mouse model, Jones et al. demonstrated that non-diabetic controls and their diabetic counterparts (db/db) had similar areas at risk in the left ventricle, however, the diabetic mice had a greater area of necrosis than the non-diabetic mice - 56.3% vs. 27.2%, respectively (122). Our results demonstrate similar
results: non-treated type 2 diabetic group (D) had a 57.0% necrotic fraction while the non-treated non-diabetic group (ND) had a 43.0% necrotic fraction. From this study, we conclude that type 2 diabetic hearts are more sensitive to I/R injury following LAD occlusion then non-diabetic hearts.

Previous studies using vitamin E or vitamin E analogues resulted in reducing myocardial infarct size in mice, cats, rats and rabbits using the LAD occlusion model (27,98,123,124). These studies were important in establishing that vitamin E was effective in reducing myocardial infarct size in non-diabetic animal models. However, we know of no study that used the LAD occlusion to study the effects of chronic vitamin E treatment in a type 2 diabetic animal model.

*Vitamin E treatment improves myocardial function following I/R injury*

As the results indicate in figures 4.5 thru 4.7, chronic treatment with vitamin E improved left ventricular function and increased myocardial work (RPP-Figure 4.8). Many researchers have used an isolated heart model to measure ventricular function in diabetic and non-diabetic animal models *ex vivo*. Most isolated heart studies use either a Krebs-Henseleit or Tyrodes perfusion solution, which allows nominal ventricular function and 2-4 times the normal coronary flow (4,49,66,71-74,125). We used an LAD occlusion model that allows precise *in vivo* monitoring of ventricular function and more reliable reperfusion function data compared to the isolated heart model.
Anti-oxidants and diabetes

Research has provided evidence that free radical production is increased during myocardial ischemia and this increases further during the reperfusion period (20,36,40-42). It is also generally accepted that diabetes increases oxidative stress within the vasculature and myocardium (10-15). Oxidant balance in the heart has a very important role in protecting the heart and allowing normal cardiac contractile performance (43). The myocardial cell has developed very effective anti-oxidant defense mechanisms. These mechanisms include superoxide dismutase, catalase, glutathione, ubiquinone, vitamins C, A (beta-carotene) and vitamin E in nuclear and lipid membranes (36). However, the anti-oxidant reserves can be inadequate under pathological situations such as sepsis, inflammation, ischemia-reperfusion, and diabetes. During cardiac events in diabetics, the antioxidant levels may be depleted or overwhelmed by oxidative stress and may compromise myocardial function. One example of this reduced anti-oxidant protection is myocardial ischemia, such as myocardial infarct due to atherosclerotic plaque, which itself may be formed, in part, by increased oxidant damage to coronary endothelial surfaces (i.e. foam cell formation). The diabetic myocardium during ischemia may not have adequate anti-oxidant protection afforded normally due to decreased anti-oxidants levels, increased oxidative stress or both. The result of decreased anti-oxidant protection, in the diabetic heart, is that anoxia may increase myocardial infarction, compared to the non-diabetic heart. Our results indicate that reduced anti-oxidant levels
within the diabetic myocardium may greater injury (infarct size) and decreased ventricular recovery following I/R in the non-vitamin E treated diabetic group compared to the vitamin E treated diabetic and non-diabetic control groups. Vitamin E therapy offers an added benefit by protecting the diabetic heart from increased oxidant damage during myocardial ischemia.

**Vitamin E reduces arrhythmias during ischemia**

Vitamin E treatment has the added benefit of significantly reducing myocardial arrhythmias during ischemia. Previous research has demonstrated that the diabetic heart is more susceptible to ventricular arrhythmias (119,126,127) or in direct contradiction, has decreased sensitivity to arrhythmias (128-130). However, a number of clinical studies have investigated arrhythmias in diabetes and indicate that both the incidence and mortality associated with ventricular arrhythmias are increased in diabetics (7,130). There have been a number of studies that indicate that vitamin E or vitamin E analogues are cardioprotective in preventing ventricular arrhythmias during I/R injury in non-diabetic dogs (131) and rats (132,133). The benefit of antioxidants as anti-arrhythmia drugs has been investigated. Frolkis et al. demonstrated that anti-oxidants were effective in reducing cardiac arrhythmias in rats and rabbits (134). The study investigated the use of a number of anti-oxidants (including vitamin E) at pharmacological doses in reducing induced arrhythmias in animals. The mechanisms that increase the arrhythmogenicity in the myocardium heart include changes in lipid composition of the sarcoplasmic reticulum and changes in the phospholipid composition of the lipid membrane (134). These changes
resulted in myocardial alteration of calcium resequestration within the cardiac myocyte. Hyperglycemia associated with diabetes has also been associated with altered lipid membrane composition (93,102,106) similar to the alterations described in the Frolkis et al. study.

*Vitamin E reduces neutrophil ROS generation*

The results in Figure 4.10 indicate that the untreated diabetic group had a 152% increase in neutrophil ROS production compared to non-diabetics during the pre-ischemic period (p<0.05) Treatment with vitamin E reduced the ROS generation in both the diabetic and non-diabetic treated groups. Vitamin E therapy, in type 2 diabetics, may reduce myocardial infarct size by reducing PMN ROS production, thereby reducing the PMN contribution to diabetic myocardial I/R injury.

Researchers have demonstrated that activated white blood cells (neutrophils, monocytes and platelets) contribute both to the formation of atherosclerotic plaques (macrophage "foam cells") and to the myocardial damage due to reactive oxygen species released by neutrophils during reperfusion (135-137). Reactive oxygen species generation within the PMN may therefore play an important role in increasing the inflammatory response that contributes to infarct formation. Researchers have demonstrated that diabetes mellitus increases oxidative stress in tissues and leads to endothelial dysfunction (15,138). Other research has established that oxidized low-density lipoproteins (LDL) are potentially contributing to deposition of atherosclerotic plaques (and formation of foam cells within the plaques) in coronary arteries (139,140). Other researchers have demonstrated that
people in an insulin resistant condition (but not overtly diabetic), have higher levels of oxidized circulating LDL particles and circulating lipid peroxidation products. Research has also demonstrated that type 2 diabetics have higher circulating lipid peroxidation products compared to non-diabetics (135).

Our conclusions are that type 2 diabetic rats have a greater degree of sensitivity to I/R injury leading to larger myocardial infarct size and reduced ventricular function following I/R injury, compared to non-diabetic rats. The greater degree of injury demonstrated in type 2 diabetic rats may be a result of increased oxidative stress within the diabetic myocardium and increased neutrophil ROS production in the diabetic rats contributing to increased I/R injury. Vitamin E therapy may help to protect the diabetic myocardium from oxidative stress during I/R injury. In addition vitamin E may help to reduce the blood contribution to I/R injury by reducing ROS production in diabetic PMN's.

**Mechanisms of diabetic sensitivity to ischemia reperfusion-injury**

The favorable results with vitamin E in reducing myocardial infarct size may be due to a host of factors. These factors may contribute to diabetic individuals increased sensitivity and greater cardiovascular damage following an ischemic event. Some of the contributing factors may be due to increased oxidative stress within circulating blood leukocytes, diabetic myocardium, and endothelium. In fact, we demonstrated that diabetic rats have significantly greater amounts of reactive oxygen species within the neutrophil population (Figure 4.10) and treatment with vitamin E significantly reduced PMN ROS levels. Work
done in our lab previously demonstrated that ROS production within neutrophils was increased in experimentally induced type 1 diabetic rats. Following stimulation using the peptide fMLP, there was an even greater ROS production (12). Here we found that type 2 diabetic PMNs have higher ROS, which may be contributing to larger infarct sizes observed in the diabetics. In addition to increased ROS production, activated leukocytes and activated platelets may be more inclined to sequester within damaged myocardium following ischemia. Our lab and others have found that diabetic leukocytes may be sequestering within damaged areas in the myocytes and releasing ROS causing further damage (12, 152).

Another mechanism that may be involved diabetics greater sensitivity to ischemia is the adverse effects of activating the diacylglycerol-protein kinase C (DAG-PKC) pathway within the diabetic myocardium and vasculature. Several studies have reported increased DAG-PKC activity levels in retinal cells, aorta, heart and renal cells of diabetic rats and in these studies vitamin E was found to reduce the activation of DAG-PKC (94, 95, 103, 154,155,156,). Malhorta et al. found that the PKC-epsilon (ε) is a major isoform expressed in myocardiocytes after experimentally induced diabetes. After streptozotocin induced diabetes, 55% of PKC-ε was located in the cytosol and 45% was membrane bound compared to non-diabetic controls, which had 76% PKC-ε in the cytosolic region and 24% membrane bound- statistically significant difference. Additionally the other major isoform of PKC, PKC-δ found in the rat cardiac myocyte, did not express a change in localization in the diabetic rats (103). The downstream consequences of the localization of PKC was demonstrated in a five-fold increase in Troponin I (TnI)
phosphorylation. TnI is a regulatory component on the Troponin-Tropomyosin complex located on the actin thin filament contractile mechanism. TnI's role is to regulate the Myosin-ATPase cross bridging activity in the contractile apparatus. The consequence of TnI phosphorylation is associated with the loss of sensitivity to calcium in the myofibers. Phosphorylation of TnI may be a component of the systolic and diastolic dysfunction associated with diabetic cardiomyopathy. Vitamin E administered to both vascular cell cultures and organ tissues, reduced the activation of DAG-PKC and may be reducing vascular abnormalities seen in diabetic tissues (94, 103, 153).

The translocation sites and downstream effectors of PKC isoforms following stimulation by hormonal, growth factors, pathophysiological states (such as ischemia-reperfusion) and oxidative stresses induced by diabetes require further investigation. The sites of PKC-isoform targeting could determine the physiological and pathophysiological fate of the diabetic cardiac myocyte. Further investigation of phosphorylation sites of PKC-isoforms should be pursued. If the downstream phosphorylation sites are isoform specific then an insight on how PKC modulates protein function may lead to a better understanding of how and why diabetic cardiomyopathy occurs.

Lastly, peroxidation products, such as oxidised low-density lipoprotein (ox-LDL) has been implicated in contributing to athersclerosis, which damages the vascular endothelium (146). Oxidized low-density lipoprotein may be a common factor in the development of atheroma in hypertension, hypercholesterolaemia, and diabetes. LDLs impair endothelium dependent relaxation, which is mediated by nitric oxide, an endogenous vasodilator with antiatherogenic properties. Impaired endothelium dependent
relaxation may be a surrogate marker of later atherosclerosis, and may precede the formation of fatty streaks, the precursors of more advanced atherosclerotic plaques \((13,140)\). Endothelial dysfunction and increased concentrations of oxidized low-density lipoproteins were initially demonstrated in human subjects with hypertension and hypercholesterolemia, and have now been confirmed in people with diabetes \((14, 151)\). Vitamin E is lipophilic and, when incorporated into the low-density lipoprotein particle, inhibits its oxidation. Since vitamin E concentrations are reduced in some patients with diabetes and oxidative stress is enhanced \((15)\) dietary supplementation with vitamin E is a logical approach to reducing the incidence of atherogenic vascular complications associated with diabetes.

There is evidence suggesting the potential benefits of vitamin E in diabetes. First, the low-density lipoprotein of individuals with non-insulin or insulin dependent diabetes causes accumulation of cholesterol in cultured human intimal aortic cells \((147)\). This low-density lipoprotein is structurally different from that of healthy non-diabetic and contains a small, dense fraction that is easily oxidized \((148)\). Whether this is due to non-enzymatic glycosylation of lipoproteins, lipid content, or other changes within or outside the low density lipoprotein particle is not known. However, we do know that that Vitamin E delays the oxidation of all low-density lipoproteins in response to oxidative stress \((15)\). Second, recent work has shown that supplementation with oral vitamin E prevents abnormalities of endothelium dependent relaxation of the aorta and coronary arteries in streptozotocin induced type 1 diabetic rats \((149,150)\). The mechanism behind endothelial dysfunction in diabetes is incompletely understood but may involve decreased production
or increased inactivation of endothelium-derived nitric oxide. Oxidized low-density lipoprotein down regulates production of nitric oxide and may also interact with nitric oxide to reduce its bioavailability (149). Vitamin E may thus enhance release of nitric oxide and inhibit its breakdown, leading to greater bio-availability and improved endothelial function.
Chapter 5

SUMMARY AND CONCLUSIONS

The present study was undertaken to provide insight into the mechanisms underlying the severity of myocardial infarctions in type II diabetics. In summary, we found that thrombin, independent of its role in the coagulation cascade, may be a direct contributor to myocardial damage during I-R injury. In addition, we also found a 30-fold increase in thrombin activity in type 2 diabetic rats compared to lean controls. Next, we found platelets from type 2 diabetic rats have increased caspase and platelet microparticle (PMP) formation compared to lean controls and may contribute to the hypercoagulability of the diabetic blood. We also found that type 2 diabetic rats have significantly larger myocardial infarct size compared to their aged matched lean controls after I-R injury. We also found that vitamin E significantly reduced myocardial infarct size in type 2 diabetic rats and marginally improves ventricular function following I-R injury.

In the past 10 years there has been much interest in nutritional antioxidant supplementation as a preventative strategy against myocardial ischemia-reperfusion (I-R) injury. The interest results from findings that reactive oxygen species (ROS) contribute significantly to myocardial I-R injury (20,36,40-42). These ROS products, such as superoxide anions, hydroxyl radicals, and peroxyl radicals formed during reperfusion, can lead to lipid peroxidation within the cardiac myocyte, resulting in decreased cardiac performance and increased damage to myocardial tissue. Type II diabetics may be more prone to oxidative stress because hyperglycemia depletes natural antioxidants and facilitates the production of free radicals. In addition, other factors such as homocysteine,
insulin resistance, and aging may contribute (141). Homocysteine, an amino acid formed during methionine metabolism (an essential amino acid) produces oxygen and sulfur-derived free radicals in the presence of trace metals and can promote oxidation of LDL cholesterol. Recent epidemiological studies found a positive association between increased plasma homocysteine and cardiovascular risk. Homocysteine may be a marker of increased oxidative stress in diabetics as well as non-diabetics.

A number of clinical trials have studied vitamin E's effectiveness in reducing heart disease (HOPE, CHAOS trials) (108,109), cancers (SELECT, ABTC prevention trials) (142) and vascular disease (143). However, limited information exists regarding the ability of antioxidant supplementation to protect the diabetic heart from I-R induced injury. This is unfortunate, given that compared with non-diabetic animals, myocardial I-R injury in type 1 diabetic animals results in a greater myocardial injury (53-55). This observation could be linked to the fact that diabetes is associated with reduced myocardial antioxidant protection (10-15).

Our results strengthen the argument for the administration of free radical therapy including the more biologically active form of Vitamin E, d-alpha tocopherol, in ameliorating the effects of free radical damage to the diabetic heart. Like the CHAOS and the ABTC studies finding a positive correlation between vitamin E supplementation and ischemic heart disease, we found that vitamin E is effective in protecting the diabetic myocardium following ischemia-reperfusion injury. Another aim of this was to measure the cardiac function before and after ischemia-reperfusion, comparing the untreated
diabetic rats with chronically treated vitamin E diabetics, and determine if any changes occurred in the functional aspects of the diabetic heart with vitamin E treatment. The pre-ischemic cardiac functional measurements were not significantly different between the four experimental groups (Chapter 4, Table 4.1). The diabetic groups had marginally better pre-ischemic functional measurements (+/- Dp/dt, rate pressure product) than the lean controls. However, after the LAD occlusion and 90-minute reperfusion period the heart rate, +Dp/dt, -Dp/dt and rate pressure product were significantly improved in the treated diabetic rats compared to the untreated diabetics, indicating that vitamin E treatment improved the recovery after ischemia. Figure 4.7 demonstrates that the diabetic vitamin E heart had the greatest +Dp/dt recovery (98%) following ischemia-reperfusion, compared to both the untreated diabetic (-78%) and both lean control groups (~93%) demonstrating greater improvement in contractility with the equivalent cardiac infarct size compared to the lean treated and untreated controls (Chapter 4 Table 4.2).

The hallmarks of diabetic cardiomyopathy include systolic and diastolic dysfunction, reduced cardiac compliance, myocardial fibrosis, and concentric hypertrophy leading to myocardial failure although no overt gross anatomic pathological changes are noted in the type II diabetic rat at this age the initial stages of adverse functional changes are taking place, especially after a myocardial compromise such as MI. In many heart diseases, including diabetic cardiomyopathy, diastolic dysfunction often precedes systolic dysfunction. Investigators have noted reduced ventricular filling and consequently impaired ejection during systole. What causes the pathophysiological changes in the diabetic myocardium is not yet fully understood. Even diabetics with tightly controlled
glycemic levels have been shown to have some of the myocardial changes attributed with diabetic cardiomyopathy.

The oxidative stress association is a rational explanation for some of the pathological changes seen in the diabetic myocardium. Oxidative stress may lead to free radical production of lipid membranes causing lipid peroxidation and changes in the nature and composition of the myocardial and vessel plasma membranes. These changes may activate intracellular signaling cascades (examples include MAP kinases, and protein kinase A and C), thereby causing activation of gene transcription factors and alter protein function. The target for these alterations may be the myocardial contractile proteins (actin-myosin), regulatory proteins (tropomyosin, troponin complex) or the structural proteins (titan, vinculin).

In addition to the direct effect within diabetic myocardial cells, vascular endothelial cells and lipid membrane protection, free radical production may be implicated in alterations of diabetic coagulation and platelet function. Indeed other studies have offered evidence that platelet activation is increased in diabetes. Frade et. al. found that platelets from patients with NIDDM aggregated maximally with the addition of very low concentration of ADP and arachadonic acid (144). Hughes et. al. demonstrated enhanced in vitro platelet hyperaggregation or increased platelet aggregates in more than a third of patients newly diagnosed type II diabetic patients (145)

Activated platelets release multiple chemical substances and proteins from their dense alpha-granules, in addition there may be an additional step, that is releasing platelet microparticles (PMPs) that act as additional signaling partners to adjacent structures
(endothelial cells, foam cells formation during atherosclerotic plaque formation) and increases catalytic surface area formation during prothrominase complex formation during the clotting cascade. The increase in PMP formation, thrombin activation and the subsequent increase in blood hypercoagulability becomes of greater interest when we demonstrated a 30 fold increase in thrombin activity in type II diabetic rats compared to their lean control litter mates. We also elucidated a potential mechanism for the greater degree of platelet activity by demonstrating a potential positive feed-back mechanism between increased platelet activation, PMP formation leading to greater thrombin activity and a hypercoagulable state. The counterbalancing effects of the fibrinolytic system of the clotting cascade, which may counterbalance the positive feed back system for a limited time, but eventually may not be able to curtail the continued increase in coagulation leading to increased thrombosis, vascular accidents/cardiovascular diseases and stroke, all hallmarks of diabetic cardiovascular disease. We also demonstrated for the first time, activation of initiator and executioner caspases in diabetic platelets. These caspase may play a role in the formation and activation of diabetic platelets and PMP formation. To our knowledge there has been no published studies on caspase activation, PMP formation and their role in diabetic coagulopathies. By determining the potential mechanism of platelet activation and the association with thrombin generation may be another key in developing therapies to manage hypercoagulopathies and ameliorate cardiovascular disease in diabetic patients.
Title: Preparation for Thoracic Window and Occlusion of Left Descending Coronary Artery in the Rat Heart.

Supplies:
1. 2"x2" gauze sponges
2. 4"x4" gauze sponges
3. Cotton tipped applicators
4. Size 2-0 silk sutures-3 10" lengths
5. Size 4-0 suture with tapered needle attached
6. Bench coat paper
7. Shallow pan
8. Rodent surgery board
9. 8" microwavable heating pad warmed to 37-40C
10. 5-1" strips of porous (white) cloth tape
11. Thermometer with rectal probe
12. 4-12" lengths of umbilical tape
13. 1-3cc syringe with blunt 23g x 1.5" needle
   with 12" length of PE-90 tubing
14. 1-threeway stopcock

Surgical Instruments:
1. 2 Small straight hemostats
2. 2 Small curved forceps
3. Curved small sharp jewelers forceps
4. Pair of Metzenbaum tissue scissors
5. Scalpel Handle #3
6. Scalpel Blade #10
7. Small Needle Holders
8. Brown-Adson tissue forceps
9. Rib Spreaders or 4" Gelpi Retractor
10. ½" Scoville-Lewis clamp, 2-1" bulldog clamps

Equipment:
1. Rodent ventilator (Harvard Apparatus Model 683) ensure that the ventilator is set
   with a ventilating rate of 80 strokes per minute and has a ventilating volume of
   approximately 2.25cc per stroke
2. Fiber optic light
3. Gould Window Graf with Diastolic/Systolic and Dp/dt transducers
4. Pressure (aneroid) gauge (Spectramed Model 23XL)
5. Millar catheter (SPR-249, Millar Instruments Inc-Houston TX)

**Prepared supplies:**

1. 15cc of 20 U/ml heparinized warmed PBS
2. 500ml wash bottle filled with PBS (pH7.4)
3. 18g catheter, 1-1/4" teflon tip (Critikon #4455) sleeved with a 1-1/4" piece of silicon tubing (Baxter #T5715-5) to serve as the trachea tube
Title: Procedure for Thoracic Window and Occlusion of Left Anterior Descending Coronary Artery in the Rat Heart.

Description: This procedure describes the surgical steps to catheterize the right common carotid artery for pressure monitoring and occlude the Left Descending Coronary Artery.

Supplies and Equipment:
1. Refer to the supplies list in SOP#AM17-XXX Preparation for Thoracic Window and Occlusion of Left Descending Coronary Artery in the Rat Heart.

Procedure:

1. Set up the surgical area with the supplies and equipment as stated in the procedures of SOP#AM17-XXX Preparation for Thoracic Window and Occlusion of Left Descending Coronary Artery in the Rat Heart.
2. Anesthetize and prepare the rat for surgery as stated in the procedures of SOP#AM17-001 Procedure for Administering Anesthesia to the Rat.

I. Tracheotomy

A. Using a scalpel, make a midline incision in the skin from the mandible to the thoracic inlet (About 1½”).
B. Using blunt tipped scissors, blunt dissect the underlying fascia and muscle to free the trachea. C. Using the tiny curved forceps separate the trachea from the surrounding tissue.
D. Again using the tiny curved forceps, pass a short length of 2-0 suture under (around) the trachea.
E. Slide the tips of a curved hemostat under the trachea, beside the suture, to elevate the trachea. Use the blunt scissors to cut an opening between the rings of cartilage in the trachea.
F. Gently insert the tracheal tube into the opening and tie securely with the 2-0 suture. Remove the curved hemostats.

II. Right Common Carotid Artery Catheterization
A. After tracheotomy, blunt dissect between the neck muscles (sternohyoid, sternomastoid mm.) down to the carotid sheath. Isolate 1 cm of carotid artery by dissecting away the vagus nerve and fascia sheath from the artery. Ensure artery is kept moist with warmed PBS (Figure 1).

B. A ligature is placed on the artery as cranial (anteriorly) as possible and a loose caudal (posterior) ligature is positioned approx 5-10mm away.

C. A small clamp (Scoville-Lewis clip) is placed on the moist distal part of the carotid artery to stop the flow of blood (figure 2).

D. Using a 23g needle, make a small puncture on the carotid artery between the ligatures creating a small flap to feed the catheter through.

E. Pick up the incised flap with a sharp tipped jewelers forceps and feed the 3.5 french Millar into the carotid artery using a blunt end small forceps. Release the clamp and gently feed the catheter into the carotid artery. Record a ten second strip of diastolic/systolic pressures. The advance the catheter down the artery into the left ventricle. When the catheter is in the left ventricle you will see a ventricular pressure wave (0-5mmHg diastolic pressure and normal 150-200mmHg systolic pressure). Record a 20 second chart strip and also record a digital section with a floppy disk formatted for the Gould Windowgraf®.

F. The posterior ligature is then tied round the artery and catheter. The free ends of the anterior ligature can further secure the catheter.

G. Close the skin flaps around the incision using a small 1” clamp to reduce the loss of body heat from the incision site. Continue to monitor the systolic/diastolic pressures.

H. Make sure that the respirator is operating properly and connect the end of the trachea tube to the respirator line. Secure the ventilating line to the surgical board with the piece of white cloth tape.
III. Left Thoracic Window

A. Make a skin incision on the left side of the rat between the 4 and 5 ribs, from just above the rat's axillary region to just below the sternum (approx 1½"), exposing the underlying musculature and being careful not to transect the axillary vein and artery (Figure 3).

B. Transect the underlying muscles down to the ribs. Make a stab incision in the intercostals muscles between the 4th and 5th ribs taking care not to incise a lung lobe.

C. Adjust the respirator to approx 2.25 ml and a rate of 75-80 respirations per minute. Adjust volume and rate to obtain an optimal blood gas for P\text{\textsubscript{1\text{O}}}\text{2}, P\text{\textsubscript{1\text{CO}}}\text{2}, pH, Sat O\text{2}.

D. Place the rib spreader or Gelpi retractor in the rib incision to expose the left side of the heart.

E. Visualize the left descending coronary artery between the left auricle and the pulmonary artery.

F. Grasp the 4-0-suture needle with the small needle holder and pierce the heart below the pulmonary artery and circumscribe around the left coronary artery coming out above the left auricle. Note: If the suture needle pierces into the ventricle or into the pulmonary artery severe bleeding will occur, do not proceed with experiment because the animal
will eventually bleed into the thoracic cavity and the animals pressures will diminish and will expire.

G. After successful ligation of the coronary artery use a 1/8" piece of Tygon tubing as an occluder. Tighten the suture with the occluder then clamp the occluder tight with a 1" Scoville clamp to prevent loosening of the ligature.

H. Place the ligature-occluder-clamp into the skin incision and close the incision site with a 1" bulldog clamp to prevent body heat loss and allow intrathoracic negative pressures to return.

I. Adjust respirator to obtain optimal respiration rate and tidal volume, check by drawing 0.3cc whole blood from catheter three-way-stopcock and measure in blood gas analyzer (ABL model 5). Check blood gasses periodically to ensure proper blood oxygenation.

After the occlusion period, reopen the thoracic incision and carefully remove the clamp from the occluder to start the reperfusion period. Re-close the thoracic incision and monitor for ventricular premature beats and ventricular fibrillation.

J. After the reperfusion period re-occlude the left coronary artery and inject 10cc of 0.4% Trypan Blue dye solution into the carotid artery catheter, slowly. This will stain the viable non-infarcted tissue and outline the infarcted area (Area-at-Risk).

K. After the dye injection quickly cut out the heart and place in a chilled plastic beaker with a 4X4” PBS soaked gauze sponge. Ensure the heart is kept chilled and moist.

L. Observe the heart and note viable tissue and infarcted tissue. Cut of the apex of the heart ensuring viable and infarcted areas are in the cut apex. Dissect off the infarcted area from the viable tissue and place in 1.5ml Eppendorff tubes; quickly snap freeze in liquid nitrogen, label and store in -70°C freezer.

M. Place the heart in the rat organ matrix then transect the left ventricle cutting a donut approximately 2mm thick with the area-at-risk and non-infarcted area visible (Figure 4).

N. Photograph scan the heart donut section, under the dissecting microscope, emphasizing the stained-nonstained regions (Figure 4). Another option is to scan the donut section on the HP scanner with Adobe Photoshop® scanning software and scan slice (2.5cm x 2.5cm. Ensure that heart slice is placed flat on scanner and scan with highest resolution possible (1200dpi. Save scan as .tiff file.

O. After the photograph place the donut slice into a 1% (w/v) triphenyl-tetrazolium-chloride (TTC) solution and allow slice to stain for 20-30 minutes at 37°C. After staining, place donut slice in 10ml 10% buffered formalin and allow to preserve for 24 hours then rescan or photograph under dissecting microscope to differentiate between area-at-risk and necrotic myocytes.
Figure 3
Figure 4
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