Influence of Cardiovascular Risk factors on Remote Ischemic Preconditioning

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INFLUENCE OF CARDIOVASCULAR RISK FACTORS ON REMOTE ISCHEMIC PRECONDITIONING

BY

TIFFANY TRACHTE

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INFLUENCE OF CARDIOVASCULAR RISK FACTORS ON REMOTE ISCHEMIC PRECONDITIONING

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Nutrition, Exercise, and Food Science and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

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ABBREVIATIONS

Akt     Protein kinase B
ATP     Adenosine triphosphate
BH₄     Tetrahydrobiopterin
BMI     Body mass index
Ca²⁺    Calcium
CVD     Cardiovascular disease
DBP     Diastolic blood pressure
DNA     Deoxyribonucleic acid
ERK1/2  Extracellular signal regulated kinase
FMD     Flow mediated dilation
GSK-3β  Glycogen synthase kinase
HDL     High density lipoprotein
H⁺      Hydrogen
iNOS    Nitric oxide synthase
IRB     Institutional review board
IL-6    Interleukin 6
IL-8    Interleukin 8
IPC     Ischemic preconditioning
IR      Ischemic reperfusion
IV      Independent variable
K_ATP   Adenosine triphosphate potassium sensitive channel
LDL     Low density lipoprotein
JAK-STAT3 Janus kinase signal transducer and activator of transcription
MTP     Mitochondrial permeability transition pore
Na⁺     Sodium
NF-κB   Nuclear factor-kappa B
K⁺      Potassium
PK⁺     Protein kinase
RHI     Reactive hyperemia index
RISK    Reperfusion injury salvage kinase
ROS     Reactive oxygen species
rIPC    Remote ischemic preconditioning
SAFE    Survivor activating factor enhancement
SBP     Systolic blood pressure
TCA     Tricarboxylic acid cycle
TGF-β   Transforming growth factor beta
TNF-α   Tumor necrosis factor
VO₂max  Maximum oxygen uptake
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ABSTRACT
INFLUENCE OF CARDIOVASCULAR RISK FACTORS ON REMOTE ISCHEMIC PRECONDITIONING
TIFFANY TRACHTE
2016

A powerful therapy against microvascular endothelial ischemia-reperfusion injury is remote ischemic preconditioning (rIPC), which triggers tissue protection by exposing a limb to small cycles of vascular occlusion. Animal models indicate that CVD risk factors reduce the protective benefits of ischemic conditioning. However, there are no human studies investigating how a burden of risk factors interferes with rIPC to prevent endothelial injury. The purpose of the present study was to determine the influence of risk factor burden on the capacity of rIPC to prevent endothelial reperfusion injury in humans.

Twenty-two (age: 45±14 yr., BMI: 31±8 kg/m²) sedentary adults (12 lower burden: ≤2 risk factors; 10 raised burden: 3-5 risk factors) were studied. Digital arterial tonometry (EndoPAT 2000, Itamar Medical Inc.) was used to assess microvascular endothelial vasodilation during reactive hyperemia before and after 65 min of left arm reperfusion injury (20 min brachial artery ischemia followed by 45 min reperfusion) that was preceded by rIPC (right arm: 3X5 min ischemia/reperfusion). All subjects provided written informed consent according to the Internal Review Board guidelines at South Dakota State University.

Repeated measures ANOVA was used to assess group differences between the reactive hyperemia index (RHI) before and after reperfusion injury. Statistical significance was set at P<0.05.
In lower burden subjects, rIPC was able to prevent endothelial reperfusion injury. The RHI following endothelial injury increased from baseline by 23% (from 2.1±0.4 to 2.5±0.5, P=0.072). In contrast, the RHI (baseline: 2.3±0.9) was significantly reduced by 25% despite induction of protection by rIPC (1.8±0.7) in the raised burden subjects (P=0.05). Between groups, the RHI after rIPC and endothelial injury was significantly different (P=0.008).

Microvascular endothelial injury was prevented when preceded by rIPC in the lower burden subjects. Remote IPC failed to protect against endothelial injury in the raised risk burden subjects. CVD risk factors appear to disrupt the protective properties of ischemic conditioning in humans.
CHAPTER 1

INTRODUCTION

Myocardial ischemia-reperfusion (IR) injury occurs in the heart’s muscular wall tissue. Ischemia occurs when blood flow is blocked from tissue or an organ and reperfusion occurs when blood flow is restored. Injury to the tissue or organ can occur by both ischemia and/or reperfusion of the tissue/organ. The absence of blood prevents the tissue from receiving the necessary nutrients and oxygen to keep the tissue alive, while reperfusion causes injury due to the tissue/organs inability to deal with the sudden influx of blood, oxygen and nutrients. The causes of IR-injury are a direct result of vascular surgery, organ transplantation, and organ resection. Three consequences of myocardial IR-injury are myocardial stunning, microvascular dysfunction/"no-reflow", and lethal reperfusion/cellular death.¹ Myocardial stunning is abnormal functioning of the heart, present for approximately 2-14 days, and is reversible. Microvascular dysfunction or “no-reflow” phenomenon happens when the ischemic tissue or organ is unable to reperfuse.² The death of cardiomyocytes is considered lethal reperfusion injury and is not reversible. Ischemia between 1-5 minutes may cause rapid heart rate or fibrillation, 5-20 minutes may result in myocardial stunning and beyond 20 minutes will result in tissue death.³ With respect to cardiovascular diseases (CVD), many people will undergo cardiac surgery to revascularize ischemic blood vessels. While these procedures are required for patient survival and to reduce complications from ischemia, many will experience further damage to the heart leading to death and disability.¹ Although a patient greatly benefits from the
effects of cardiac surgery, the benefits are reduced because of myocardial ischemia reperfusion injury.¹

A large body of evidence has identified a promising new strategy to exploit the inherent cardioprotective properties of the heart. Cardioprotection against ischemia reperfusion can be accentuated by conditioning the heart to become more tolerant to an ischemic episode, a property known as ischemic preconditioning (IPC).⁴ Ischemic preconditioning is a type of conditioning stimulus applied before the onset of sustained ischemia.⁵ The conditioning stimulus can be applied directly to the target organ/tissue or it can be applied remotely, a process termed remote ischemic preconditioning (rIPC). Remote ischemic preconditioning, which involves subjecting a distant organ to brief, sub-lethal bouts of ischemia followed by brief reperfusion, protects the target organ against IR-injury.⁶ Experimental models have shown that preconditioning, either local or remote, can benefit many organs from the damage caused by IR-injury including the heart, kidney, brain, lung, and skeletal muscle.⁷ Currently, it is not entirely clear how conditioning strategies protect against IR-injury, but several studies suggest that multiple factors, such as protein kinases or signal transduction pathways, contribute to the protective benefits.⁷ However, despite these cardioprotective benefits, rIPC has not been translated into widespread clinical use. Many clinical trials are underway to determine the clinical efficacy of rIPC to protect the heart against IR-injury that inevitably accompanies common coronary revascularization procedures.⁸ Preliminary findings of these studies largely suggest a substantial clinical benefit of preconditioning to protect the
heart, but there are factors that appear to reduce its efficacy, particularly in the context of clustered cardiovascular risk factors.

Cardiovascular risk factors include obesity, hypertension, insulin resistance, hyperglycemia, dyslipidemia, and aging. It is believed that the benefits of ischemic conditioning are decreased if a person possesses one or more of these risk factors. Preclinical studies have administered rIPC on animal subjects that were modified to obtain cardiovascular risk factors. These studies found that the benefits of rIPC were decreased. One study has been completed, on humans, which determined the correlation between aging and rIPC. The study’s results found that the benefits of rIPC on endothelial IR-injury were reduced. Collectively, animal data and one human study has consistently shown that the benefits of rIPC to protect against IR-injury are substantially reduced. It is currently unknown if similar findings occur in humans with other common CVD risk factors. If so, the clinical potential of ischemic conditioning strategies to protect the heart during coronary revascularization procedures may be adversely affected in the context of increased cardiovascular risk factors.

**Statement of the Problem**

While preclinical animal models of hypertension and hypercholesterolemia have shown reduced efficacy of IPC to protect against myocardial IR-injury, these findings have not been translated to humans. Increased CVD burden in clinical populations may lower the protective benefits of IPC which may adversely impact its clinical application to protect against IR-injury of the revascularized
heart. With the exception of advancing age, there are no studies investigating ischemic conditioning strategies on humans with clustered cardiovascular risk factors.

**Specific Aim 1:** To determine the influence of increased cardiovascular risk burden on the capacity of rIPC to protect against endothelial IR-injury.

**H₀:** Elevated cardiovascular risk will diminish the capacity of rIPC to resist endothelial IR-injury.

**Independent Variables**
- Lower burden vs. Raised burden
- Sham protocol vs. Experimental (rIPC) protocol

**Dependent Variables**
- Reactive Hyperemia Index (RHI)

**Delimitations:** The study will be delimited to apparently healthy men and women of all ethnic and racial backgrounds, between 20-79 years of age, from Brookings County, South Dakota.
Limitations: We will not directly assess smooth muscle relaxation in this study, which does not eliminate the potential for impaired microvascular reactive hyperemia to be caused by reduced smooth muscle function. Separating the subjects into two risk factor groups is another limitation because of the inability to account for the effects caused by graded progression of multiple cardiovascular risk factors. Also, there are a lot of people who do not yet have CVD but are taking medications to treat their risk factors, particularly high blood pressure and cholesterol medications. We will not be studying these people which limits the application of our findings to groups not on treatment.

Definition of terms

Myocardial ischemia-reperfusion injury – the process of restoring blood flow (reperfusion) to the myocardium which was not receiving blood flow (ischemia) causing cellular dysfunction, damage or death.¹

Endothelial ischemia-reperfusion injury – the process of restoring blood flow (reperfusion) to endothelial tissue which was not receiving blood flow (ischemia) causing cellular dysfunction, damage or death.

Ischemic conditioning – exposing an organ or tissue to repeated bouts of ischemia, intermittent with reperfusion, with the desire to condition and protect the exposed area to future ischemic episodes.
Ischemic preconditioning – ischemic conditioning completed prior to an ischemic episode.

Remote ischemic preconditioning – ischemic conditioning completed on a body part located remotely from targeted tissue or organ.

Endothelial function – when paracrine factors are in homeostasis and maintain normal vascular tone, blood fluidity, and limit vascular inflammation and smooth muscle cell proliferation.¹⁰
CHAPTER 2

LITERATURE REVIEW

The following literature review will discuss the clinical implications and causes of IR-injury with particular reference to the myocardium and vascular endothelium, the potential of ischemic conditioning to protect against reperfusion injury and proposed mechanisms to explain its cardioprotective benefits, and lastly, the impact of cardiovascular risk factors on the severity of IR-injury and how elevated CVD risk affects rIPC in humans.

Clinical Implications of Ischemia-Reperfusion Injury

While prompt reperfusion of ischemic tissue is critical to preserve function and cell viability, it can increase the degree of injury beyond that attributed to ischemia alone. A major clinical problem, IR-injury affects all major organs, especially the brain, heart, liver and lungs.\textsuperscript{11} It can also induce localized damage to specific cells including the endothelial cells in vascular tissue, epithelial cells in the gastrointestinal region, and glomerular cells in the kidney.\textsuperscript{2,11} Ischemia – defined as diminished delivery of oxygen and nutrients to meet the metabolic demands of the cellular environment – can be caused by a variety of factors. The most prevalent of which is due to coronary artery disease that results in vessel occlusion in the heart. Indeed, the clinical consequences of ischemia manifest as myocardial infarction and stroke, which are the top leading causes of premature death worldwide. Acute vascular occlusion leading to ischemia requires quick restoration of blood flow via pharmacological and surgical intervention. In this
manner, IR-injury is a major limitation of current revascularization procedures, particularly percutaneous coronary angioplasty, coronary artery bypass graft surgeries, and even in heart transplantation. Although reperfusion is absolutely necessary to resupply the tissue with blood, it contributes to more extensive damage. Ultimately, IR-injury can lead to a heightened systemic inflammatory response, which, if unmanaged clinically can lead to multiple organ dysfunction, particularly in the heart which is often characterized by arrhythmias, myocardial stunning, and in severe cases premature mortality. Collectively, the mechanisms that attribute to IR-injury are varied and complex with implications that manifest differently in each individual person resulting in short term or fatal consequences. Risk factors such as diabetes, hypertension, and hypercholesterolemia, can may make a person more vulnerable to IR-injury.

Based on the duration of ischemia, a variety of different clinical consequences can arise, particularly reperfusion arrhythmias, myocardial stunning, and endothelial dysfunction.

Reperfusion arrhythmias

The first clinical consequence of IR-injury, (and the least significant problem) in the heart is the development of myocardial arrhythmias, which often occur following 1-5 minutes of ischemia. Upon reperfusion, the homeostasis of the cellular environment is altered, causing abnormal ionic reactions with the previously ischemic environment. Some of these that have been shown to alter the electrical stability of the heart include reactive oxygen species (ROS),
increased Ca\(^{2+}\), and decreased pH contributing to reperfusion arrhythmias.\(^{15}\) Frequent premature ventricular contractions within 90 minutes after reperfusion therapy, episodes of ventricular tachycardia, atrioventricular blocks are examples of reperfusion arrhythmias.\(^{16}\) It is thought that these electrical disturbances do not affect cardiac cell contractile performance and do not contribute to cell death. However, because the myocardium does not regenerate, these arrhythmias persist and may contribute to increased use of pharmacotherapy and hospitalization.\(^{17}\)

**Myocardial Stunning**

A more severe clinical problem linked to IR-injury is known as myocardial stunning. Myocardial stunning is defined as reversible injury to cardiac myocytes that produces diminished contractile performance in the absence of cell death. It typically occurs when the duration of ischemia is between 5-20 minutes.\(^{17}\) This clinical problem occurs because of damage to cardiomyocytes during IR-injury which results in left ventricular contractile dysfunction without cell injury.\(^{14}\) Myocardial stunning is generally a temporary condition and usually not fatal but it is a clinical concern because of the possibility of delaying reperfusion therapy.\(^{17}\)

**Endothelial Dysfunction**

Reperfusion leads to intravascular inflammation and severe endothelial dysfunction.\(^{18}\) There is a rise in production of the vasoconstrictor endothelin-1 and increased ROS that results in increased vasoconstriction and reduced blood
flow. Endothelial dysfunction also disturbs the balance between coagulation and anti-coagulation factors in the blood. Polymorphonuclear neutrophils are transported to the intravascular space from the interstitial space during ischemia, and such responses may contribute significantly to tissue damage during subsequent reperfusion.\(^\text{19}\) Moreover, Kharbanda and colleagues were one of first to show that 20 minutes of forearm ischemia impaired endothelium-mediated vasodilation. In 14 healthy men, blood flow responses to acetylcholine were blunted by \(~50\%\) following endothelial IR-injury.\(^\text{5}\) Furthermore, an experimental study done Pedersen and colleagues showed that endothelial IR-injury significantly diminishes tissue-type plasminogen activator (t-PA) release, a well-established marker of vascular endothelial health and function, in healthy men.\(^\text{20}\) From this evidence, it is important to recognize that IR-injury is ubiquitous and affects all cell types in the environment exposed to it. As described below under ‘Mechanisms of IR-injury’, the aforementioned clinical implications of IR-injury arise from reductions in adenosine triphosphate (ATP), microvascular spasm/plugging/disturbance, increased oxidant stress, abnormal Ca\(^{2+}\) homeostasis, and mechanical failure/contractile protein structure.\(^\text{17}\) In the following section, I will discuss the mechanisms that lead to IR-injury resulting in the clinical consequences described above.

**Mechanisms of IR-Injury**

The mechanisms thought to cause IR-injury are complex and not completely understood. However, a large body of evidence from experimental
and clinical trials indicates that ATP depletion, calcium overload, oxidant stress, inflammation and coagulation are the main mechanisms of IR-injury. Each mechanism contributes uniquely to the debilitating aspects of IR-injury, as well as partaking in a vicious cycle amplifying each mechanism’s contributions to IR-injury and exacerbating the conditions.

**ATP Depletion**

The heart almost exclusively relies on ATP production from oxidative phosphorylation as a result of glycolysis and beta-oxidation. During ischemia, oxidative phosphorylation ceases and the myocardial cell will resort for a very short period of time to anaerobic glycolysis. While the cellular environment’s pH has decreased because increased production and decreased removal of H\(^+\) ions, the availability of oxygen and nutrients have diminished. Without blood to transfer nutrients and oxygen, glucose or fatty acids cannot be catabolized to pyruvate which prevents acetyl-CoA from proceeding through the TCA cycle. Mitochondria within the affected cells, including endothelial cells and cardiomyocytes, are unable to resynthesize ATP and creatine phosphate resulting in further destructive consequences and cellular death caused by an overload of calcium.\(^{14,21}\)

**Calcium Overload**

A major contributor of IR-injury results from calcium overload. During ischemia, myocardial pH drops because of increase anaerobic glycolysis that
produces greater concentrations of H\(^+\). As a result, the decrease in pH activates the Na\(^+\)/H\(^+\) sarcolemma exchanger to increase removal of H\(^+\) from inside the cell to bring pH back to normal. Meanwhile, because of the drop in cellular ATP described above, the Na\(^+\)/K\(^+\)-ATPase is inhibited preventing the removal of Na\(^+\) from the cell. In turn, the Na\(^+\)/Ca\(^{2+}\) exchanger, which normally extrudes Ca\(^{2+}\) from the cell, is reversed. Consequently, the Ca\(^{2+}\) concentration rises inside the cell contributing to cellular dysfunction and calcium overload.\(^{11}\) For example, a higher concentration of Ca\(^{2+}\) will activate protease enzymes that disrupts cell membrane integrity, and in myocardial cells contributes to hypercontracture, necrosis, and apoptosis.\(^{22}\) Subsequently, activated neutrophils and macrophages infiltrate the cell and stimulate inflammation, eventually causing further damage to the surrounding tissue.\(^{21}\) In addition, immediately upon reperfusion the increase in Ca\(^{2+}\) also overloads the mitochondria. This in turn opens the mitochondrial permeability transition pore (MTP) which uncouples oxidative phosphorylation leading to further ATP depletion and cellular death.\(^{11}\)

**Oxidative Stress**

While ATP depletion and calcium overload drive the pathology of ischemia, rapid restoration of blood flow during reperfusion considerably increases the delivery of substrates to resume aerobic metabolism contributing to accelerated production of ROS. Oxidative stress is a major contributor to IR-injury and occurs when an imbalance in the cellular environment exists between the ROS and antioxidants.\(^{23}\) Superoxide anion, hydroxyl radicals, hypochlorous
acid, hydrogen peroxide, and peroxynitrite, are the major sources of ROS formed macromolecules.\textsuperscript{14} For example, ROS damages the molecular structure of proteins, lipids, deoxyribonucleic acid (DNA) and disrupts cell membrane permeability. In addition, ROS stimulate expression of inflammatory cytokines and alter Ca\textsuperscript{2+} control in the mitochondria by opening the MTP.\textsuperscript{11,21} Taken together, it is clear from many experimental studies that increased generation of toxic levels of ROS plays an important role in the pathogenesis of reperfusion injury.

\textit{Inflammation/Coagulation}

One main consequence of accelerated production of ROS during reperfusion is that they stimulate the expression of proinflammatory and procoagulant proteins; particularly tumor necrosis factor (TNF-\(\alpha\)), interleukin (IL-6 and IL-8), transforming growth factor beta (TGF-\(\beta\)) and nuclear factor-kappa B (NF-\(\kappa\)B). Proinflammatory proteins, such as TNF-\(\alpha\), signal for leukocytes while further expression of inflammatory genes create adhesive endothelial cells. When NF-\(\kappa\)B is activated, further expression of adhesion molecules and proinflammatory proteins attract monocytes, neutrophils and lymphocytes. Interleukin-6 and IL-8, contribute to the initial interaction between leukocytes and the endothelial cells as well as intracellular adhesion. The inflammation location is primed for leukocyte and platelet adhesion, which can eventually lead vascular dysfunction.\textsuperscript{23}
In summary, IR-injury, especially in the heart and vasculature, is a major problem of coronary artery disease and undermines its treatment efficacy. While extensive experimental investigations targeting potential pharmacological and non-pharmacological approaches to limit IR-injury are underway, to date there is no effective treatment for it. However, a promising strategy to prevent or reduce the adverse clinical consequences of IR-injury is to precondition the cells to become more tolerant against a sustained ischemic bout. The following section will detail the mechanisms of ischemic preconditioning and its capacity to reduce injury caused by ischemia and reperfusion.

**The Protective Effects of Ischemic Preconditioning**

Although there are many implications of IR-injury there are various therapeutic methods that have shown benefits to decrease the impact and size of injury; one of these methods is IPC. This was first observed as a result of an original experiment by Murry et al., who showed a reduction in myocardial infarct size in canine hearts after the application of a preconditioning stimulus via occlusion of the circumflex coronary artery.\(^{24}\) Because of this study, the concept of IPC was introduced by these authors in 1986. In this experiment, the circumflex coronary artery was exposed to four 5 minute occlusions, each separated by 5 minutes of reperfusion, which was then followed by continuous ischemia for 40 minutes. They observed a 25% reduction in the myocardial infarct size with the IPC stimulus. This study laid the groundwork for many subsequent studies showing the protective benefits of preconditioning a tissue
locally and remotely. As mentioned previously, most of the damage during IR-injury occurs during reperfusion and not ischemia. For quite some time it was believed that ischemia was responsible for all of the damage and it is now understood this is not the case, although the duration of ischemia does determine the severity of injury. Bouts of 2-3 low dose ischemic periods, with equal periods of reperfusion between each ischemic bout, can induce cardioprotective benefits against future IR-injury events by decreasing the neutrophil accumulation and apoptosis in turn decreasing the size of myocardial infarction. The use of IPC therapy prior to a long term ischemic episode, such as coronary artery bypass surgeries, is associated with reduced markers of myocardial cell death.

Local IPC entails the occlusion of tissue or an organ directly at the source, for instance cross clamping of the aorta prior to coronary artery bypass surgery, which is very invasive and impractical. However, compared to local IPC, rIPC, which was first proposed in 1993 by Pryklenk et al., provides greater benefits since it is less invasive than local IPC. They showed that small episodes of ischemia in one vascular bed protected a different area of the myocardium in the canine to sustained coronary artery occlusion. These investigators suggested that preconditioning was mediated by some unknown factors that were released and transported from the preconditioned area to the tissue experiencing IR-injury. An experiment done by McClanahan et al. also in 1993, showed a reduction in myocardial infarct size in the rabbit when a preconditioning stimulus was applied to the kidneys. In this manner, rIPC was used for inducing protection...
of a distant organ through a peripheral limb or internal organ. Birnbaum et al. showed that cardioprotection against IR-injury was triggered via lower limb skeletal muscle IPC. Indeed, they showed that occluding blood flow in the gastrocnemius muscle resulted in a reduction of myocardial infarct size by 65% in rabbit heart. Similarly, Oxman et al. demonstrated that application of rIPC to the hind limb of rats using a tourniquet prevented reperfusion arrhythmias. The occlusion of a limb simply requires a tourniquet or inflatable cuff and is beneficial for patients undergoing any type of surgery. This method's protocol is similar to local IPC in that three 5 minute ischemic periods, intermittent with 5 minute reperfusion periods transpire; however rIPC occurs on a limb distal from the target organ. The resultant mechanisms are unclear but it is believed that the mechanisms are paralleled with mechanisms produced by local IPC; but include additional signal transduction pathways. A few theories exist relating to how rIPC is accomplished; for instance, the neural theory suggests that endogenous substance produced in the ischemic limb activates a local afferent pathway which activates an efferent pathway. The humoral theory proposes that the remote limb releases substances into the blood carrying it to other organs triggering intracellular pathways that mediate protection. Finally, the inflammatory suppression theory suggests that rIPC suppresses apoptosis and inflammation in cells reducing the systemic inflammatory response and buffers against ROS.
Impact of Cardiovascular Risk Factors on IR-injury and Preconditioning

It is believed that the cardioprotective benefits of ischemia preconditioning are reduced in the presence of cardiovascular risk factors. Unfortunately, a majority of the population who are in need of IPC possess one or more cardiovascular risk factors. Currently, there are numerous animal studies that predominately show that risk factors diminish the protective effects of ischemic conditioning. However, some of these studies show conflicting findings. Today, there is only one human study that tested local ischemic preconditioning in the presence of one cardiovascular risk factor (aging). The following paragraphs will describe the literature, to date, on the effects of cardiovascular risk factors on ischemic preconditioning. Of the studies that follow, it is important to note that they only investigated local ischemic conditioning; no studies have investigated remote ischemic conditioning.

Hyperlipidemia/Hypercholesterolemia

Many animal studies have been completed in regards to how hypercholesterolemia impacts the effects of IR-injury on the myocardium. Studies conducted on animals with experimentally-induced hypercholesterolemia have found negative responses to IR-injury; for instance, these animals experienced greater infarction size, increases in troponin T release, or greater amounts of cellular apoptosis. These studies confirm that hypercholesterolemia or hyperlipidemia show a noteworthy magnification of myocardial IR-injury. However, some animal studies have found opposing results, either
hypercholesterolemia has no effect on the outcome of IR-injury or it may make the heart more resilient. For example, studies have found reduced myocardial infarct size, reductions in cardiac contractile function, or cardiac functional parameters were not impaired.\textsuperscript{37-39} Mixed results require more studies in order to determine what mechanisms are responsible for infarctions and how hypercholesterolemia impacts infarction size and IR-injury.

In terms of IPC, it has been found that cardioprotective benefits are lost when an animal or person has hypercholesterolemia. Most studies, completed on animals, concluded that the presence of hypercholesterolemia increased the size of myocardial infarction.\textsuperscript{39,40} Two studies completed on humans found that increases in plasma cholesterol and LDL levels reduced the efficacy of ischemic preconditioning.\textsuperscript{41,42} Administering effective therapy cannot be done until it is determined how cardiovascular risk factors impact ischemia reperfusion therapy and whether or not ischemic preconditioning is effective in these groups.

\textit{Hypertension}

Much like hypercholesterolemia, hypertension exacerbates the negative effects caused by myocardial/endothelial IR-injury. Studies have found that, because of hypertension, the myocardium is at greater risk of electrophysiological disturbances and sustaining injury after IR-injury.\textsuperscript{32} Animal studies have found that the myocardium experiences earlier rigor contracture, depressed contractile function, and depressed lactate dehydrogenase.\textsuperscript{43-46} The increased sensitivity of the myocardium created by hypertension is believed to be
attributed to the mechanisms of IR-injury discussed previously, such as changes to ATP production, the mitochondria, glycolytic metabolism, pH level, and increased ROS/decreased antioxidants.\textsuperscript{47-51} Much like hypercholesterolemia, hypertension decreases the benefits of ischemic preconditioning.

\textit{Diabetes}

Diabetes is yet another cardiovascular risk factor that impacts the severity of myocardial IR-injury, as well as decreases the benefits of IPC. Along with the previous cardiovascular risk factors mentioned, a person with type I or type II diabetes will experience an increase of myocardial infarction after IR-injury. Interestingly, some studies conducted on animals found that animal hearts were more resistant to IR-injury for the first couple of weeks after the animal was induced with diabetes. However, these benefits decreased dramatically after two weeks.\textsuperscript{52} As expected, many clinical studies have found debilitating and deadly impacts caused by diabetes in animals as well as humans. Studies have shown increases in mortality for diabetic people and even greater occurrences of mortality with women.\textsuperscript{53-55} Much like other cardiovascular risk factors, people with type I or type II diabetes will not receive the traditional benefits of IPC; such as reduced infarction size, arrhythmias, or contractile dysfunction.\textsuperscript{56}
**Aging**

Aging is yet another cardiovascular risk factor that is unfortunately untreatable and unavoidable. While many preclinical animal models clearly show reduced efficacy of ischemic conditioning strategies and more severe myocardial IR-injury in aged hearts \(^{57,58}\), only one human study has been completed that has tested how IPC affected IR-injury between young and older men. \(^9\) The study consisted of 15 healthy and recreationally active young (20-25 years) and older (68-77 years) men who completed two sessions that were randomized to a sham or experimental protocol. During each session, the subjects completed a local preconditioning protocol that consisted of three 5 minute bouts of ischemia, at 20 mmHg (sham) or 200 mmHg (experimental), intermittent with 5 minutes of reperfusion. Upon completion of local IPC, the subjects underwent 20 minutes of IR-injury. Ischemic preconditioning and IR-injury protocols were completed on participants’ right upper arm and flow mediated dilation (FMD) measurements were attained on the right hand. Flow mediated dilation measurements were completed before and after local IPC and IR-injury to determine how IPC affected IR-injury. The results showed that the young men benefited from IPC while the older group did not. The young men’s FMD after IR-injury resulted in a 2% decrease from baseline measurements, whereas their FMD did not decrease following IR-injury preceded by local IPC. The older men experienced a significant decrease in FMD after completion of each protocol; which supports the belief that local IPC benefits are reduced in the older population or presence
of cardiovascular risk factors. As the authors of the study noted, the older population is the group who would typically need IPC.\textsuperscript{59}

**Literature Review Summary**

Clinically, IR-injury is a common and unavoidable occurrence that results in further injury to the affected tissue or organ. Ischemic reperfusion injury can affect all organs and tissue, including the endothelial layer of the vasculature and triggers a variety of mechanisms that lead to further cell damage; ultimately leading to cellular death or possibly morbidity. Adenosine triphosphate depletion, Ca\textsuperscript{2+} overload, oxidative stress, and coagulation generate an unsteady environment that attribute to multiple consequences of IR-injury, such as arrhythmias or myocardial stunning. A variety of therapies have been studied, such as IPC which has been found to diminish infarction size, arrhythmia occurrence, and stunning. Unfortunately, by and large, preclinical animal models with experimentally-induced cardiovascular risk factors appear to aggravate IR-injury as well as diminish the benefits typically provided by IPC. However, there are very limited human studies on this issue, which underscores the urgent need to identify how these risk factors alter endothelial protection in people who are a greater risk of IR-injury.
CHAPTER 3

METHODS

The present study was conducted by Ms. Tiffany Trachte, a graduate student in the Department of Health & Nutritional Sciences at South Dakota State University under the mentorship of Dr. Gary Van Guilder. This section will outline the methodology for the present study.

Specific Aim: To determine the influence of increased cardiovascular risk burden on the capacity of rIPC to protect against endothelial IR-injury.

Subjects and Eligibility Screening: Twenty-two age-matched (12 women/10 men) adults between the ages of 20-79 years were studied [11 with lower risk burden, 11 with raised burden]. Sample size was established based on a recent human study in adults with advanced age that demonstrated significantly reduced protection of local arm IPC in 15 older men and women. Research subjects who expressed interest in volunteering for the study were screened for eligibility using a medical history form. Screening was conducted either on the phone, via email, or in person. Individuals that met eligibility requirements underwent the informed consent process and baseline measurements to determine the presence or absence of cardiovascular risk factors and 10-year cardiovascular disease risk score (described below). Inclusion and exclusion criteria are shown below.
Inclusion Criteria

- Any race or ethnicity
- Men and women between the ages of 20-79 years
- Apparently healthy with no history or evidence of cardiovascular, pulmonary or metabolic disease
- Non-medicated and not taking any vitamin supplements or energy drinks
- Non-smoking
- Sedentary

Exclusion Criteria

- History or evidence of cardiovascular, pulmonary or metabolic disease
- Participating in 3 or more days of ≥30 minutes of aerobic exercise for the past 3 months
- Currently pregnant, recent pregnancy, or women taking oral contraceptives
- Infected or wounded index fingers

Criteria to Identify Cardiovascular Risk Burden: Cardiovascular disease risk factors were established according to the ethnic-specific International Diabetes Federation definition and risk classification established by the American Heart Association. Research subjects were categorized into a raised risk burden or a lower risk burden group.
**Raised risk burden:** Subjects in the raised risk burden group will demonstrate with ≥3 of the following risk factors:

- Family history (myocardial infarction, coronary revascularization, or sudden death before 55 year in father or other male first-degree relative or before 65 year in mother or other female first-degree relative
- Ethnic-specific BMI classification of obese or abdominal obesity (defined as waist circumference with ethnic-specific criteria)
- Dyslipidemia (defined by one of the following):
  - total cholesterol ≥200 mg/dl;
  - fasting triglycerides ≥150 mg/dl;
  - high-density lipoprotein cholesterol <40 mg/dl in men or <50 mg/dl in women;
  - low-density lipoprotein cholesterol ≥130 mg/dl
- Systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg;
- Prediabetes as defined by a fasting blood glucose ≥100 mg/dl

**Lower risk burden:** Subjects in the lower risk burden group will demonstrate with sedentary status and less than ≤2 other risk factors.
Baseline Measurements to Determine Cardiovascular Risk Factors

Metabolic measurements

Following an overnight fast, a small drop of blood was taken from the index finger and analyzed for fasting cholesterol and blood sugar concentrations using the Cholestech LDX System (Alere Inc., Waltham, MA, USA). Thereafter, resting blood pressure, body height, weight, waist circumference, and percent body fat was completed using established procedures.\textsuperscript{62}

Blood Pressure measurements

Non-dominant arm auscultatory resting systolic and diastolic blood pressure was measured using an appropriately sized stethoscope and sphygmomanometer (Diagnostic 700 Series, American Diagnostic Corp, Hauppauge, NY) following 5 minutes of seated quiet rest using standard procedures. Resting blood pressure measurements were performed twice and separated by 3 minutes and averaged. Resting heart rate was measured using a 60 second radial pulse count.

Anthropometric measurements

Standing height and body weight was measured with a digital scale (Seca 876 digital scale, Seca Corporation, Hamburg, Germany). Abdominal waist circumference was assessed with a Gulick tape measured at the smallest part of the abdomen, above the umbilicus and below the xiphoid process to the nearest
0.1 cm at the end of normal expiration using standard procedures. Waist measurements were completed twice and averaged. Percent body fat was estimated by air displacement plethysmography (BODPOD, COSMED USA Inc., Illinois). Body mass index (BMI) was calculated as weight (kg) divided by height (m²).

**Determination of 10-year Cardiovascular Disease Risk**

Traditional cardiovascular risk factors (age, sex, total cholesterol, HDL-cholesterol, smoking status, and systolic blood pressure) were applied to the 10-year Framingham risk score to estimate future CVD risk. We utilized the ACC/AHA 2013 Cardiovascular Risk Calculator, which predicts a first atherosclerotic vascular event including a myocardial infarction and stroke.

**Experimental Design:** The experiment utilized a 2×2 factorial randomized crossover design. There were two independent variables (IV), each with two levels. IV1 was the risk burden group (low burden versus raised burden). IV2 was the conditioning group (rIPC versus a sham control).

**Experimental Procedures (rIPC or sham):** Following eligibility screening, informed consent procedures, and enrollment, 22 subjects were randomized to participate in two sessions of microvascular endothelial function in response to endothelial IR-injury in the absence and presence of rIPC (figures 1 and 2). These sessions occurred at least 1-2 weeks apart.
**Measurement of Microvascular Endothelial Function**

Endothelial function, an essential component of cardiovascular health, was measured with a non-invasive technique known as digital pulse arterial tonometry (EndoPAT 2000, Itamar Medical Inc.), an FDA–approved technique consisting of an index fingertip sensor probe that detects microvascular volume changes with each pulse. This volume change can be detected to provide an indication of reactive hyperemia, which is a well-established marker of endothelial vasodilator function. The amplitude of each arterial pulse can be quantified and analyzed by an automated, proprietary computer algorithm and tracked over time during the experiment. This test was administered in a supine position in a comfortable, quiet room. It consists of three, 5-minute phases:

**Phase 1: baseline**

**Phase 2: Short-term occlusion of blood flow**

**Phase 3: Rapid return of blood flow (reactive hyperemia)**

The amplitude of changes in finger volume is proportional to arterial dilation, a well-established predictor of endothelial function. An air-filled finger cuff was positioned on the index finger of each hand and set by the computer to inflate to 10 mmHg below diastolic blood pressure (about 70 mmHg). The finger cuff contains sensors that detect changes in finger volume with each heartbeat pulse. After 5 minutes of baseline data acquisition, a blood pressure cuff was
inflated on the left upper arm to 220 mmHg for 5 minutes to occlude blood flow to the arm and hand. After 5 minutes of arm occlusion, the blood pressure cuff was deflated and measurement of the change in fingertip volume was assessed for 5 minutes. The total duration of the assessment of microvascular endothelial function was 15-20 minutes.

After baseline assessment of endothelial function and subsequent to a 15 minute washout period, participants underwent either the rIPC or the sham trial. A high pressure cuff placed on the right upper arm was inflated to 220 mmHg (EC20 rapid cuff inflator, DE Hokanson, Inc., Bellevue, WA) for 5 minutes, followed by 5 minutes of reperfusion to induce rIPC. This procedure was repeated 3 times. Thereafter, the left arm underwent endothelial IR-injury by inflating the high pressure cuff to 220 mmHg for 20 minutes, followed by 40 minutes of reperfusion. Assessment of endothelial function was repeated following 40 minutes of reperfusion. The total time to perform this experiment was approximately 3 hours. The sham control was identical to the rIPC trial except the pressure cuff inflated to 15 mmHg only to avoid preconditioning.

**Timeline:** Upon IRB approval, enrollment and data collection procedures began in earnest in September 2015 and continued through the spring 2016 semester. All data collection procedures were completed by March 2016.
Statistical Analyses

The RHI was expressed as the post-to-pre occlusion PAT signal ratio in the index finger following 5 min of left brachial artery ischemia, relative to the same ratio in the contralateral non-occluded control arm, corrected for baseline vascular tone of the occluded arm. Subject characteristics were determined by one-way ANOVA. The Mann-Whitney U test was used to determine differences in the number of risk factors between groups. Data was checked for normality and spread. Measures of central tendency were used to calculate baseline subject data. The primary outcome was the difference in remote IPC-mediated endothelial protection between lower and raised risk burden groups. Repeated measures analysis of variance was used to determine differences in endothelial function before and after ischemic preconditioning. Non-parametric statistics was used if data did not meet assumptions of parametric analysis. Statistical significance was set at P<0.05. Missing data was coded with ‘-9’ and not used for analyses. Attempts were made to obtain missing data prior to data analysis. Linear regression was used to establish correlations between risk factors and the RHI response in the total sample. Data are means± SD unless otherwise noted. Data were analyzed with IBM SPSS Statistics version 23 (Armonk, NY).
CHAPTER 4

RESULTS

Twenty-two subjects (12 women, 10 men) volunteered to participate in this study between September 11, 2015 and March 1, 2016. Upon determination of cardiovascular risk factor classification, subjects were categorized into lower burden (≤2 risk factors) and raised burden risk groups (≥3 risk factors). Due to conflicting schedules and/or time constraints two of the subjects withdrew from the study. Among the remaining 20 subjects, 13 completed both the sham and remote ischemic preconditioning sessions while 7 subjects completed only the remote ischemic preconditioning session. A majority of the subjects were comfortable with the rIPC and IR-injury procedures. Upon completion of IR-injury, typical statements made by subjects were their forearms and hands felt as if “they were wearing a glove”, “like extreme pins and needles”, or “heavy”.

Table 1 displays means with standard deviation of subject characteristics in the lower burden and raised burden risk groups. Age was similar in mean and range; the age of the 3 men and 9 women in the lower burden group ranged from 25-59 years; while the 7 men and 3 women in the raised burden group ranged from 25-68 years. Body mass (P=0.029), waist circumference (P=0.011), and blood pressure (P=0.001) were greater in the raised burden subjects. Each subject within the lower burden group had systolic and diastolic blood pressure measurements within the normal range, whereas blood pressure in the raised burden group were prehypertensive or hypertensive. Triglycerides were the only lipids to show a significant difference (P=0.039) between groups. Interestingly,
the fitness tests resulted in a statistically significant difference (P=0.044) in which
the raised burden subjects measured a higher mean VO_{2\text{max}} compared to the
lower burden subjects. 10-year Framingham risk score (P=0.12) and the
ACC/AHA CVD risk factor score (P=0.06) were higher in the raised burden
subjects.

**Table 1. Subject characteristics**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lower Risk Burden (n=12)</th>
<th>Raised Risk Burden (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td>45 ± 13</td>
<td>47 ± 16</td>
</tr>
<tr>
<td>Sex, M/W</td>
<td>3 / 9</td>
<td>7 / 3</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>78 ± 14</td>
<td>103 ± 33*</td>
</tr>
<tr>
<td>BMI, kg/m^2</td>
<td>27 ± 4</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>38 ± 8</td>
<td>38 ± 11</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>84 ± 8</td>
<td>107 ± 26*</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>108 ± 8</td>
<td>130 ± 18*</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>66 ± 9</td>
<td>84 ± 14*</td>
</tr>
<tr>
<td>Total-cholesterol, mmol/L</td>
<td>4.5 ± 0.9</td>
<td>4.9 ± 1.4</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>2.6 ± 0.8</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.6 ± 0.5</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.8 ± 0.4</td>
<td>1.5 ± 0.9*</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.9 ± 0.4</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>VO_{2\text{max}}, ml/kg/min</td>
<td>34 ± 3</td>
<td>41 ± 9*</td>
</tr>
<tr>
<td>10-year Framingham Risk Score, %</td>
<td>0.59 ± 0.2</td>
<td>2.6 ± 4.1</td>
</tr>
<tr>
<td>ACC/AHA CVD RF score, %</td>
<td>0.64 ± 0.5</td>
<td>3.0 ± 3.9</td>
</tr>
</tbody>
</table>

Values are means ± SD. M, men; W, women; BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VO_{2\text{max}}, maximal oxygen uptake; *P<0.05

Table 2, below, displays the number of subjects within each
cardiovascular risk factor category. As expected, the number of risk factors,
particularly obesity, elevated blood pressure, high LDL, and elevated triglycerides
were significantly higher in the raised burden group. The most prevalent risk
factor in the raised burden group was obesity.
Table 2. Risk factor distribution within each group.

<table>
<thead>
<tr>
<th></th>
<th>Aging #(%</th>
<th>Family history #(%</th>
<th>Obesity #(%</th>
<th>Elevated blood pressure #(%</th>
<th>High total cholesterol #(%</th>
<th>High LDL #(%</th>
<th>Low HDL #(%</th>
<th>Elevated triglycerides #(%</th>
<th>Prediabetes #(%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>10 (46)</td>
<td>5 (24)</td>
<td>15 (68)</td>
<td>6 (27)</td>
<td>6 (29)</td>
<td>6 (29)</td>
<td>6 (29)</td>
<td>4 (19)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Lower Burden</td>
<td>4 (33)</td>
<td>1 (9)</td>
<td>5 (23)</td>
<td>0 (0)</td>
<td>2 (18)</td>
<td>1 (9)</td>
<td>2 (18)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Raised Burden</td>
<td>6 (60)</td>
<td>4 (40)</td>
<td>10 (45)*</td>
<td>6 (100)*</td>
<td>4 (40)</td>
<td>5 (50)*</td>
<td>4 (40)</td>
<td>4 (40)*</td>
<td>1 (10)</td>
</tr>
</tbody>
</table>

LDL, low-density lipoprotein; HDL, high-density lipoprotein. *P<0.05 vs. low burden.
Table 3 shows the number of risk factors present in the combined sample and between lower and raised burden subjects. As expected, the raised burden group exhibited more risk factor clustering with 75% of the subjects having at least 3 risk factors.

**Table 3. Distribution of cardiovascular risk factors in the sample population.**

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Total # (%)</th>
<th>Lower Risk Burden # (%)</th>
<th>Raised Risk Burden # (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4 (18)</td>
<td>4 (33)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1</td>
<td>2 (9)</td>
<td>2 (17)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2</td>
<td>6 (27)</td>
<td>6 (50)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3</td>
<td>3 (14)</td>
<td>0 (0)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>4</td>
<td>5 (1)</td>
<td>0 (0)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>5</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>7</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>1 (10)</td>
</tr>
</tbody>
</table>

Risk factors included age (men ≥45 yr, women ≥55 yr); family history (first-degree male <55 years of age or first-degree female <65 years of age suffering from a heart attack, heart disease, or death); BMI ≥30 kg/m²; systolic blood pressure ≥130 mmHg and/or diastolic ≥85 mmHg; high total cholesterol ≥200 mg/dL; high LDL-cholesterol ≥130 mg/dL; low HDL-cholesterol (women <50 mg/dL, men <40 mg/dL); triglycerides ≥150 mg/dL and glucose ≥100 mg/dL. Data represent sum (relative %).

Table 4, below, displays the changes in heart rate and blood pressure measurements during the microvascular function protocol between the lower burden and raised burden risk groups. The lower burden group’s systolic and diastolic blood pressure measurements were consistent throughout the protocol. There was a significant main effect (P=0.028) of diastolic blood pressure across time in the raised burden group.
Table 4. Changes in heart rate and blood pressure during the microvascular function protocol with remote preconditioning.

<table>
<thead>
<tr>
<th></th>
<th>Lower Risk Burden</th>
<th>Raised Risk Burden</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>30 min</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>66±13</td>
<td>61±9</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>112±9</td>
<td>115±12</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>67±7</td>
<td>68±7</td>
</tr>
</tbody>
</table>

Data represent mean±SD. *P<0.05; the difference lies between rest and 30 min measurements with the 95 min measurement.
Figure 1 displays the reactive hyperemia index (RHI) at baseline and following endothelial IR-injury in the 13 (8 lower burden, 5 raised burden) subjects that completed the sham trial. Both groups displayed a small reduction in RHI following endothelial IR-injury, although this was not statistically significant.

Figure 1. Reactive hyperemia index at baseline and after endothelial IR-injury during sham session without rIPC.
Figure 2 displays RHI at baseline and with endothelial IR-injury in the presence of rIPC, for both lower and raised risk burden groups. There were no significant group differences in RHI at baseline. However, the RHI after endothelial IR-injury showed a significant difference (P=0.007) between the lower and raised burden groups. Following rIPC and endothelial IR-injury, the RHI for the lower burden group increased by 23% (P=0.072). On the other hand, despite rIPC the raised burden group experienced a 25% reduction (P=0.05).

Figure 2. Effects of rIPC on RHI at baseline and after endothelial IR-injury in the lower and raised burden groups. *P=0.05 vs. baseline in raised risk burden group. †P=0.007 vs. corresponding trial in lower burden.
Figure 3 displays the baseline pulse wave amplitude across 300 seconds during hyperemia between groups. There were no significant differences between groups throughout the 5 minutes of post-occlusion. However, there was on average a 25% decrease in reactive hyperemia across each 30 second interval in the raised burden group.

![Graph showing reactive hyperemia index at 30 second intervals before endothelial IR-injury between lower and raised burden groups.](image)

Figure 3. Reactive hyperemia index at 30 second intervals before endothelial IR-injury between lower and raised burden groups.
Figure 4 displays the RHI across 300 seconds following endothelial IR-injury with rIPC in both groups. While both groups demonstrated significant increases in reactive hyperemia across time, there was a marked difference between groups (P=0.028). Notably, between the time interval from 30-150 seconds, the reactive hyperemia response was, on average, 63% lower for the raised burden subjects following endothelial IR-injury despite remote preconditioning.

Figure 4. Reactive hyperemia index following endothelial IR-injury at 30 second intervals with remote ischemic preconditioning between the lower and raised burden groups. P<0.05 vs. raised burden.
Figure 5 displays the association between cardiovascular risk factors and the RHI in the presence of rIPC (left) and the percent change from baseline (right) after endothelial IR-injury. There was a significant correlation between the number of risk factors and the RHI after endothelial IR-injury with preconditioning, such that those with a greater risk burden demonstrated a reduced response with preconditioning.

Figure 5. Reactive hyperemia index and percent change from baseline, with rIPC, after endothelial IR-injury in association to number of cardiovascular risk factors.
CHAPTER 5

DISCUSSION

The main finding of this study supports the hypothesis that subjects with a greater risk factor burden did not demonstrate microvascular protection from endothelial injury with remote ischemic preconditioning compared to subjects with a lower risk factor burden. The novel results present the first direct evidence to demonstrate that subjects with raised CVD risk burden showed a reduced capacity of remote ischemic preconditioning to resist microvascular endothelial injury. These findings suggest that patients in need of coronary revascularization may not benefit from remote ischemic conditioning interventions if they present with clustered risk factors. Additional research is needed to identify strategies to recapture the cardioprotective effects of preconditioning in such patients.

To date, it is unclear how remote ischemic preconditioning provides cardioprotection but ongoing studies are researching the potential mechanisms. It is thought that a combination of neural and humoral pathways releasing bradykinin, adenosine, and opioids, which then circulate throughout the body produce a protective systemic effect against ischemic injury and reperfusion injury.\textsuperscript{66} It is believed that these factors in turn activate intracellular signaling pathways on the target organ which then initiate protection by upregulating cell survival proteins.\textsuperscript{66} Much effort has been completed to understand which mechanisms and signaling pathways are responsible for the protective elements of ischemic preconditioning. Two pathways have been proposed to be associated with remote ischemic preconditioning, the reperfusion injury salvage kinase
(RISK) pathway and the survivor activating factor enhancement (SAFE) pathway. Each pathway is trigged by either adenosine, bradykinin, opioids, or tumor necrosis factor (TNF-α). These effectors then bind to and activate their respective cellular receptors which initiates the RISK or SAFE pathways. The cascade from the RISK pathway (accumulation of protein kinases, activation of anti-apoptotic protein kinases (ERK1/2, Akt, PKC-E and PKG) overlaps with the activity from the SAFE pathway (activation of JAK-STAT3 cascade) to prevent opening of the mitochondrial permeability transition pore. Understanding the mechanisms aids in defining how the interaction of cardiovascular risk factors impact the pathways and reduce ischemic conditioning’s cardioprotection. However, as research is finding, animal models with hypercholesterolemia, diabetes, obesity, and hypertension show that protection from ischemic preconditioning after IR-injury, is markedly impaired.

For example, multiple studies have found that hypercholesterolemic animal models subjected to IR-injury did not benefit from bouts of rIPC established by an increase in infarct size versus the control models. In an effort to determine how hypercholesterolemia diminishes the effects of ischemic preconditioning Tang et al. (2005) investigated hypercholesterolemic rats who were subjected to 6x4 minute bouts of rIPC. They found that the myocardial infarct size was greater in the hypercholesterolemic rats versus the normocholesterolemic rats demonstrating reduced rIPC ability. Notably, they related the lower levels of tetrahydrobiopterin (BH₄) in the hypercholesterolemic rats to the reduced capacity of rIPC. This is significant given that BH₄ is an
essential factor for nitric oxide synthase (iNOS) which is linked to the pro-survival pathways inducing rIPC.\textsuperscript{71}

In addition, animal models genetically altered with diabetes have shown similar results, the incapacity to benefit from rIPC.\textsuperscript{72,73} Rana et al. (2015) linked diabetes to the disruption of numerous cell survival pathways. For example the over-activation of glycogen synthase kinase (GSK-3β) can lead to the opening of the mitochondrial permeability transition pore increasing permeability of the cell and resulting in swelling and cell death.\textsuperscript{66,74}

Furthermore, evidence exists that advanced aging is associated with the attenuation of ischemic preconditioning in human and animal models.\textsuperscript{59,75,76} Studies completed by Takayama et al. (2001) and Tani et al. (2001) found that middle and advanced aged rats had decreased activation of protein kinase C compared to young rats.\textsuperscript{75,76} Protein kinase C (PKC) is important in that it is an anti-apoptotic protein kinase that activates during reperfusion as part of RISK pathway. It is responsible for the opening of the mitochondrial K$_{ATP}$ which facilitates the beneficial effects of ischemic preconditioning.

In the presence of cardiovascular risk factors ischemic preconditioning does not elicit the same benefits compared to healthy control animals.\textsuperscript{77-79} Some studies have found conflicting results, possibly due to the preconditioning protocol used or other varying experimental conditions.\textsuperscript{66} Despite conflicting studies, cardiovascular risk factors are associated with altering or eliminating the activation of protective signaling pathways which in turn reduce cardioprotection elicited by remote ischemic preconditioning. Our results are in line with previous
studies that found cardiovascular risk factors have reduced or eliminated the protection of ischemic preconditioning.

Studies continue to show that remote ischemic preconditioning provides remarkable protection against ischemic reperfusion injury in the endothelial and myocardial tissue of healthy subjects.\textsuperscript{5,6,65,80} Experimental data indicate that risk factors activate enzymatic systems that reduce nitric oxide and increase reactive oxygen species.\textsuperscript{32} These alterations in the endothelium leads to platelet adhesion and aggregation, leukocyte adhesion and migration, and smooth muscle cell proliferation; damaging endothelial function. In the presence of one or more cardiovascular risk factors, exposing a vulnerable endothelium IR-injury exacerbates the injury by preventing nitric oxide production, and worsening inflammation and oxidative stress.\textsuperscript{81} In support of this, our findings show that subjects with greater number of risk factors demonstrated a worse response to rIPC.

Currently, most research investigating ischemic conditioning is completed with healthy animal models that do not translate well or mimic humans in clinical settings. This study is novel in that it is the first human study to investigate the microvasculature of subjects with clustered cardiovascular risk factors in response to IR-injury subjected to rIPC. The importance of translating preconditioning from animal models to humans is that most individuals who participate in clinical trials or settings possess a clustering of cardiovascular risk factors.\textsuperscript{66} Likewise, it is not yet understood how cardiovascular risk factors affect IPC treatments, particularly those that are invoked remotely with a limb.
Although this study was carefully prepared, there were some unavoidable limitations. First, due to supply issues, 7 subjects did not complete a sham trial which impacted results when looking at responses to endothelial ischemic injury without remote ischemic preconditioning. Second, the subjects were predominately white Americans and cannot be applied to other ethnic groups. Third, adjustments were made to the reperfusion period after ischemic injury. Previous research investigating conduit arteries, showed that 15 minutes of reperfusion diminished endothelial function. In the present study we learned that at least 40 minutes is needed to characterize reperfusion injury in the digital microvasculature. Our findings are not able to identify a specific risk factor associated with the reduced rIPC response.

Despite these limitations, this study raised a few questions for future studies. This study measured endothelial function within the first window of protection provided by ischemic preconditioning, when the benefits are the most robust but for a shorter time span. Future studies could investigate how the second window of protection, when benefits are weaker but occur for a longer period of time, affects IPC in the presence of cardiovascular risk factors. Also, medications were not included in this study therefore future research could investigate a combination of treatments; ischemic preconditioning with medication in the second window for instance. Another variation of this study would be altering the traditional rIPC 3x3 protocol to more or longer bouts of ischemia.
In conclusion, the results of this study are consistent with prior preclinical studies and one clinical aging study, showing that rIPC may be reduced in the presence of cardiovascular risk factors. Before this treatment can be applied in clinical settings research needs to continue in order to determine how clustered cardiovascular risk factors interact with remote ischemic preconditioning. Animal models are useful however they do not manifest the risk factors or comorbidities typically seen in patients with CVD. Research should continue in hopes of ascertaining the correct combination of preconditioning strategies that will be effective against cardiovascular risk factors, however an international study has found that 90% of CVD is caused by 9 risk factors that can be modified through diet and exercise. In this regard, more emphasis and efforts need to be made on educating the public about the importance of exercise and diet in order to prevent cardiovascular risk factors and diseases.
CHAPTER 6 - APPENDICES

APPENDIX A

Protect Your Heart and Blood Vessels – Participate in a Research Study

Contact Tiffany Trachte

tiffany.trachte@sdsstate.edu

(720) 254-3475

Who we want:
- Healthy adults
- 20-79 years old
- Not taking certain medications
- Non-smoking
- Not regularly active

What you get:
- Cholesterol and blood pressure screening
- Fitness and body composition assessment
- State-of-the-art vascular health screening

How long does this take?
- Three sessions
- Flexible scheduling over 1-2 months
- Requiring about 8 hours of your time

Where do I do this?
- Vascular Protection Laboratory
- Department of Health & Nutritional Sciences
- On the South Dakota State Campus

This research is directed by Dr. Gary Van Guilder of the Vascular Protection Laboratory at South Dakota State and has been approved by the SDSU Institutional Review Board #IRB-
Please read (listen to) the following information:

1. This is an invitation for you, ______________________________, to participate in a research project under the direction of Dr. Gary P. Van Guilder, Director of the Human Vascular Protection Laboratory in the Department of Health & Nutritional Sciences.

2. The project is entitled: **Influence of Heart Disease Risk Factors on Ischemic Conditioning – Experiment 1**

3. Ischemic conditioning is a process that helps your blood vessels withstand the physiological stress that occurs when blood flow is stopped to a tissue. The purpose of the project is to identify how risk factors for heart disease, such as obesity, high cholesterol, or aging, affect the ability of ischemic conditioning to protect against this stress.

4. If you consent to participate, you will undergo 3 research sessions over the course of 3 months for a total duration of about 8 hours. Each session is described below.

**Session 1**

During the first testing session, we will determine whether you have any cardiometabolic risk factors, measure your resting heart rate and blood pressure, body height, weight, waist circumference, and percent body fat. You will also undergo a fitness test on a treadmill. The time required for session 1 will be ~2 hours.

**Measurement of blood fats and sugars:** Following an overnight fast of at least 10 hours, we will take a small drop of blood from your index finger using a tiny finger prick to measure the amount of fats and sugar in your body. The finger prick will take only one second to complete. During this test, you may feel a small prick that may be somewhat uncomfortable. After we have obtained the drop of blood, we will clean and place a band aid on your finger and provide you with something to eat and drink.

**Measurement of resting heart rate and blood pressure:** Following the finger prick described above, you will be rest quietly for 5 minutes until we measure your heart rate
and blood pressure. Resting heart rate will be measured using a 60 second arterial pulse count. To measure your blood pressure, we will place an inflatable cuff around your upper arm. We will inflate the cuff to a high pressure. Then we will release the pressure slowly while we listen for the sound of your heartbeat. We will repeat this measurement at least twice.

**Measurement of body composition:** Thereafter, we will measure your height and body weight using a digital scale. Abdominal waist circumference will be assessed with a tape measured at the smallest part of the abdomen. Percent body fat and muscle mass will be estimated by air displacement plethysmography. During this procedure, you will be placed in a small enclosed chamber and instructed to sit quietly for 2 minutes. This procedure does not require you to do anything except sit quietly.

**Cardiorespiratory fitness**
If you are over the age of 35, you will undergo the ‘Ebbeling sub-maximal walk test’, an 8 minute brisk treadmill walk to determine your cardiorespiratory fitness. This walking test is suitable for low risk, apparently healthy, middle-aged adults. The first few minutes of this test will consist of placing a heart rate monitor and wrist watch on you to measure the intensity of the walk. We will then determine a brisk self-selected walking pace that equates to 50-75% of your estimated maximum heart rate. The first 4 minutes of the walking test will be flat at your self-selected pace. Then, we will increase the ramp of the treadmill to 4% grade and you will maintain the same walking speed for 4 more minutes. The test will be completed in 8-9 minutes and then we will cool you down for 3 minutes at a slow walking speed.

If you are under the age of 35, you will have the choice to undergo a maximum cardiorespiratory fitness test or to complete the walk test described above. The research team will discuss with you the test that best suits you. The maximum fitness test will measure the amount of oxygen your muscles use while you jog at a moderate to vigorous intensity on a treadmill to exhaustion. You will jog at self-selected brisk pace during this test. Every 2 minutes, we will increase the incline of the treadmill by 2% until you reach your maximum exercise capacity. You will reach your maximum capacity in about 10-12 minutes. During the exercise test, we will conduct a procedure known as open-circuit indirect calorimetry, which will allow us to measure your expired levels of oxygen and carbon dioxide, your heart rate, and your breathing rate. You will be equipped with via a mouthpiece and nose clip to collect your expired air. At the end of the test, we will reduce the speed of the treadmill to a brisk walk and reduce the incline to 0% for 3-5 minutes for a cool-down period. The criteria to identify whether you reach maximum capacity will be based on your heart rate and the amount of oxygen and carbon dioxide that you breathe out through the mouthpiece.
Sessions 2 and 3

On a separate testing day, you will be randomized to participate in either the remote preconditioning trial or a control trial first. Two weeks after you completed session 2, you will be scheduled to complete session 3, the other trial. Each of these trials, described below, will last about 3 hours. These trials will be separated by at least 14 days.

Each of these trials will consist of four parts over the course of 3 hours:

Part 1: measurement of blood vessel health
Part 2: remote ischemic preconditioning or control
Part 3: forearm reperfusion injury
Part 4: repeated measurements of blood vessel health

Part 1 - measurement of blood vessel health: One of the main functions of your blood vessels is to ensure that all organs and tissues are supplied with adequate blood flow to match metabolic demand. The vessel regulates blood flow by dilation (increase volume – increase supply) or constriction (decrease volume – decrease supply). With each heartbeat, a pulse is produced that increases the volume of blood delivered to your limbs. This volume change can be detected to provide an indication of blood vessel health. We will measure this volume change using a non-invasive procedure known as digital pulse arterial tonometry. This test will involve the use of a small fingertip cuff that detects small changes in index figure volume with each pulse beat. The amplitude of each arterial pulse can be quantified and analyzed by an automated, proprietary computer algorithm and tracked over time during the experiment. This test will be administered while you lie down in a comfortable, quiet room. It consists of three, 5-minute phases:

Phase 1: baseline
Phase 2: Short-term occlusion of blood flow Phase 3: Rapid return of blood flow (hyperemia)

An air-filled finger cuff will be positioned on your index finger of each hand and set by the computer to inflate to 10 mmHg below your diastolic blood pressure (about 70 mmHg). Because the finger cuff contains sensors that detect changes in finger volume, it is important that your fingernails be trimmed to obtain the best signal. We will provide nail clippers as needed. After 5 minutes of baseline data acquisition, a blood pressure cuff will be inflated on left upper arm to 220 mmHg for 5 minutes to occlude blood flow to your left hand. After 5 minutes of arm occlusion, the blood pressure cuff will be deflated and measurement of the change in fingertip volume will be assessed for 5 minutes. The total duration of this measurement will be 15-20 minutes. You will complete this procedure 4 times during sessions 2 and again during session 3.

Part 2 - remote ischemic preconditioning or control: Preconditioning your muscles has been shown to improve the function of your blood vessels and to protect the heart. It can
be caused by stopping blood flow to a tissue (e.g. muscle) for very short periods of time (i.e., a few minutes) and then letting blood flow back into the tissue for an equal amount of time. Stopping blood flow in this manner does not damage the tissue. You will undergo preconditioning of your right arm. A pressure cuff will be inflated around your right upper arm to a pressure similar to a blood pressure measurement while you rest on a hospital bed. Three, 5 minutes high-pressure inflations followed by three, 5 minute deflations will occur. We will measure your heart rate and blood pressure during the last minute of each 5 minute inflation-deflation cycle. The control trial will consist of the same procedure described here except the pressure cuff will be inflated to a low pressure to avoid stopping blood flow your arm.

**Part 3 - forearm reperfusion injury:** Immediately after part 2, your left arm will undergo reperfusion injury by inflating a high pressure cuff to 220 mmHg for 20 minutes, followed by 15 minutes of reperfusion. Because arm occlusion is sustained for a longer period during this part, you may experience greater discomfort and more intense feelings of numbness of the arm, hand and fingers. While most subjects do not report any pain, it is somewhat uncomfortable. During the 15 min reperfusion phase, a greater rush of blood in the arm will occur and you may feel ‘pins and needles’.

**Part 4 – repeated measurements of blood vessel health:** Following forearm injury, we will repeat the measurement of your blood vessel health 3 additional times in the final hour of the procedure. This measurement is the same as described above under ‘Part 1’.

5. Participation in this project is voluntary. You have the right to drop out at any time without penalty and you have the right not to participate in any aspect of the study. If you are a student, your grade for any class will not be affected if you volunteer for this project. You will not get extra credit by volunteering for this study. If you have any questions, you may contact Dr. Van Guilder at the phone number listed at the end of this form.

6. There are minimal risks to you if you volunteer for this project. First, blood pressure measurements involve inflating an upper arm cuff to a high pressure for 20 seconds. You may experience some mild discomfort during this test. Although obtaining a sample of whole blood (one drop, or about 40µl) via a small finger prick is safe and confers minimal physical risk, there is a chance that you will experience mild pain for a few seconds during the procedure. In addition, the finger prick may cause tiny bruising on the skin that should subside in a day. With respect to preconditioning your arm, the procedure is quick and poses no more than minimal risk to you. Blood flow to your right arm will be stopped for 5 minutes using a high pressure cuff. As a result, you will experience tight squeezing of the arm that may be mildly uncomfortable. Although, generally painless, you may experience mild numbness for 5 minutes. Lastly, when the blood pressure cuff is deflated, you will experience a rush of blood flow through the right arm. The skin will redden and feel warm with transient feelings of ‘pins and needles’ in the skin as blood flow returns to normal. These feelings should subside within 2-3 minutes.

In contrast, forearm reperfusion injury is induced by occluding blood flow to the left arm for 20 min, followed by 15 min of reperfusion. This protocol of prolonged arm ischemia
followed by reperfusion is a well-established method to show the protective benefits of preconditioning in human subjects. Because arm occlusion is sustained for a longer period, you may experience greater discomfort and more intense feelings of numbness of the arm, hand and fingers. While most subjects do not report any pain, it is somewhat uncomfortable. You may also feel that the arm is cold compared with the unaffected arm. Finally, the pressure cuff may cause minor bruising to the tissue of the upper arm. Placing the pressure cuff over a shirt sleeve will lessen the chance of bruising. During the 15 min reperfusion phase, a greater rush of blood in the left arm will occur. Similar to the blood vessel health test, the skin will redden and feel warm with transient feelings of ‘pins and needles’ as blood flow returns to normal. Some subjects report mild muscle twitching in the fingers and thumb during this phase.

7. By volunteering for this study, you will receive a state-of-the-art assessment of your vascular health and gain insight into the changes that occur in your blood vessels after a period of sustained arm occlusion. Moreover, you will be provided your respective cardiovascular risk factor information and your personal data regarding body composition and cardiorespiratory fitness at the conclusion of the study.

8. You will not be financially compensated by volunteering for the project. There is no financial cost to you for volunteering for this project.

9. Your data is strictly confidential. When the data and analysis are presented, you will not be linked to the data by your name, title or any other identifying item. To keep your identify protected we will assign you a unique alphanumeric code. This code will only be available to the research team. The code will link you to the study instead of your name. Your information will be kept confidential in a password-protected University computer. Copies of data forms will be stored in a locked filing cabinet in the office of Dr. Gary Van Guilder. All samples of your blood and DNA will coded using the alphanumeric code that we assign to you; they will not be linked to your name, address, or any other personal identifying information. All samples will be stored in a secure location in the Department of Health & Nutritional Sciences.

I have read the above and have had my questions answered. I agree to participate in the research project. I will receive a copy of this form for my information.

Participant's Signature _________________________________ Date __________

Project Director's Signature _______________________________ Date __________

If you have any questions regarding this study you may contact the Project Director, Dr. Gary Van Guilder, using the contact information presented below. If you have questions regarding your rights as a participant, you can contact the SDSU Research Compliance Coordinator at (605) 688-6975 or SDSU.IRB@sdstate.edu.

Project Director:
Gary P. Van Guilder, Ph.D.
Assistant Professor
Department of Health and Nutritional Sciences
South Dakota State University
Box 2203, Intramural 116
Brookings, SD 57007
Phone: 605-688-4082
Email: gary.vanguilder@sdstate.edu

This project has been approved by the SDSU Institutional Review Board, Approval No.: IRB-1507003-EXP
APPENDIX C

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

Yes  No
1. Have you ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?
2. Do you feel pain in your chest when you do physical activity?
3. In the past month, have you had chest pain when you were not doing physical activity?
4. Do you lose your balance because of dizziness or do you ever lose consciousness?
5. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?
6. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
7. Do you know of any other reason why you should not do physical activity?

If you answered YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

• You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.

• Find out which community programs are safe and helpful for you.

No to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:
• start becoming much more physically active — begin slowly and build up gradually. This is the safest and easiest way to go.
• take part in a fitness appraisal — this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

Delay becoming much more active:

• if you are not feeling well because of a temporary illness such as a cold or a fever — wait until you feel better; or
• if you are or may be pregnant — talk to your doctor before you start becoming more active.

Please note: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

Note: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

Name ___________________________ Date ___________________________

Signature of parent or guardian (for participants under the age of majority)
Witness ___________________________

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.
APPENDIX D

Vascular Protection Laboratory
Department of Health & Nutritional Sciences
Research Eligibility Screening Form

This Eligibility Screening Questionnaire is necessary to help us understand any potential risks associated with your eligibility in participating in a research study. All information you provide is personal and confidential.

GENERAL INFORMATION

<table>
<thead>
<tr>
<th>Name</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Address</td>
<td></td>
</tr>
<tr>
<td>City</td>
<td></td>
</tr>
<tr>
<td>State</td>
<td></td>
</tr>
<tr>
<td>Zip code</td>
<td></td>
</tr>
<tr>
<td>Phone (home)</td>
<td></td>
</tr>
<tr>
<td>Phone (work)</td>
<td></td>
</tr>
<tr>
<td>Email</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Hispanic Identity</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Emergency Contact Phone</td>
<td></td>
</tr>
</tbody>
</table>

MEDICAL DIAGNOSES

Do you have or have you ever had any of the following? Please check yes or no.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart attack</td>
<td></td>
<td></td>
<td>Pulmonary disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest pain</td>
<td></td>
<td></td>
<td>Heart valve problems</td>
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<td></td>
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<tr>
<td>Asthma</td>
<td></td>
<td></td>
<td>Heart murmur</td>
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<tr>
<td>Anemia</td>
<td></td>
<td></td>
<td>Rheumatic fever</td>
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<td></td>
</tr>
<tr>
<td>Cardiovascular surgery</td>
<td></td>
<td></td>
<td>Phlebitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Currently pregnant</td>
<td>Emboli (blood clot)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>--------------------</td>
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<tr>
<td>Emphysema</td>
<td>Coronary artery disease</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Diabetes (type 1 or 2)</td>
<td>Stroke</td>
<td></td>
<td></td>
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<tr>
<td>Cancer</td>
<td>Peripheral artery disease</td>
<td></td>
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<tr>
<td>Raynaud’s disease</td>
<td>Kidney disease</td>
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</tbody>
</table>

Please list any additional special conditions.

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
</table>

**Major Risk Factors**

<table>
<thead>
<tr>
<th>Major Risk Factors</th>
<th>Yes</th>
<th>No</th>
<th>Not sure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Are you a man over the age of 45 or a woman over the age of 55?</td>
<td></td>
<td></td>
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<tr>
<td>Are you postmenopausal?</td>
<td></td>
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<tr>
<td>Has your father or brother experienced a heart attack before age 55?</td>
<td></td>
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<tr>
<td>Has your mother or sister experienced a heart attack before the age of 65?</td>
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<tr>
<td>Has your doctor ever told you that you might have high blood pressure?</td>
<td></td>
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<tr>
<td>Is your cholesterol above 200 mg/dl?</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Do you have prediabetes (blood sugar ≥100 mg/dl)?</td>
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<tr>
<td>Are you physically inactive (less than 30 minutes of physical activity on at least 3 days/week)?</td>
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<tr>
<td>Do you currently smoke or have you quit smoking in the last 6 months?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you &gt; 20 pounds overweight?</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
- Cardiovascular diseases include cardiac, peripheral vascular or cerebrovascular disease
- Pulmonary diseases include chronic obstructive pulmonary disease, asthma, interstitial lung disease, or cystic fibrosis
- Metabolic diseases include Type 1 or 2 Diabetes mellitus, thyroid disorders, renal or liver disease

### SIGNS/SYMPTOMS SUGGESTIVE OF CARDIOVASCULAR AND PULMONARY DISEASE

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain discomfort (or angina equivalent) in the chest, neck, jaw, arms, or other areas that may be due to ischemia (decreased blood flow)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shortness of breath at rest or with mild exertion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness or fainting at rest or with mild exertion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orthopnea/paroxysmal nocturnal dyspnea (labored breathing at night or while sleeping)</td>
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<tr>
<td>Edema (excessive accumulation of tissue fluid usually in the ankles and lower legs)</td>
<td></td>
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</tr>
<tr>
<td>Palpitations or tachycardia (sudden rapid heartbeat)</td>
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<td></td>
</tr>
<tr>
<td>Intermittent claudication (lameness due to decreased blood flow)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Known heart murmur (abnormal heart sound)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unusual fatigue</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### MEDICATIONS

Please list any medications you are currently taking including prescriptions medications and over the counter medications, dietary supplements, vitamins, minerals, etc.

<table>
<thead>
<tr>
<th>Medication</th>
<th>Dose and frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Please list any dietary supplements you are currently taking including, not limited to vitamins, minerals, energy drinks, weight loss/weight gain supplements etc.

<table>
<thead>
<tr>
<th>Dietary supplement</th>
<th>Dose and frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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</tbody>
</table>
**APPENDIX E**

**Anthropometric and Hemodynamic Data Sheet**  
**Vascular Protection Laboratory**  
**South Dakota State University**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td></td>
</tr>
<tr>
<td>Height, cm</td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td></td>
</tr>
<tr>
<td>Obesity status</td>
<td></td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td></td>
</tr>
<tr>
<td>Hip circumference, cm</td>
<td></td>
</tr>
<tr>
<td>WHR</td>
<td></td>
</tr>
<tr>
<td>WHR status</td>
<td></td>
</tr>
<tr>
<td>Body fat, %</td>
<td></td>
</tr>
<tr>
<td>Resting heart rate, bpm</td>
<td></td>
</tr>
<tr>
<td>Resting blood pressure 1, mmHg</td>
<td>/</td>
</tr>
<tr>
<td>Resting blood pressure 2, mmHg</td>
<td>/</td>
</tr>
<tr>
<td>Blood pressure classification</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX F
Maximal Oxygen Consumption Test
Vascular Protection Laboratory
South Dakota State University

Treadmill Protocol Personnel Sheet

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age</th>
<th>Sex</th>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Personnel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic cart</td>
</tr>
<tr>
<td>RPE tech</td>
</tr>
<tr>
<td>Treadmill operator</td>
</tr>
<tr>
<td>Spotter 1</td>
</tr>
</tbody>
</table>

Equipment Needed
- Parvo Medics Metabolic cart
- Treadmill
- Heart Rate Monitor attached and working
- Rate of Perceived Exertion (RPE) chart
- Towels
- Gloves

Technician Responsibilities of a VO<sub>2</sub> Max Protocol
- Ensure metabolic cart is on for 30 minutes before calibration
- Prepare correct mouthpiece valves with hose and face mask
- Measure height and weight before setting-up subject
- Review medical history, risk classification, and ACSM indications for prior medical exam and medical supervision before the subject begins a test
- Ensure the client/subject has signed an informed consent form
- Instruct/coach subject about the protocol prior to warm-up and answer their questions, if any.
- Explain the RPE scale to the client
- Explain to the client how to handle the end of the test – straddle treadmill, light cycling on ergometer to facilitate cool-down
- Ensure client safety by carefully observing the client and monitor the test continuously
- Perform changes to workload according to protocol and communicate with client
- Measure and record heart rate, RPE, and symptoms on data sheet below.
- Encourage the client throughout the test
- Complete post-test cleaning of mouthpiece, hoses, and face mask
### Treadmill Maximum Oxygen Consumption Data Sheet

<table>
<thead>
<tr>
<th>Stage</th>
<th>Start Warm-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>Speed (mph)</td>
</tr>
<tr>
<td>Warm-up</td>
<td>-10 to -5</td>
</tr>
<tr>
<td>Set-up</td>
<td>-5 to 0</td>
</tr>
<tr>
<td><strong>Start Exercise</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>2</td>
<td>2-4</td>
</tr>
<tr>
<td>3</td>
<td>4-6</td>
</tr>
<tr>
<td>4</td>
<td>6-8</td>
</tr>
<tr>
<td>5</td>
<td>8-10</td>
</tr>
<tr>
<td>6</td>
<td>10-12</td>
</tr>
<tr>
<td>7*</td>
<td>12-14</td>
</tr>
<tr>
<td>8</td>
<td>14-16</td>
</tr>
<tr>
<td><strong>Start Recovery</strong></td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td>---</td>
</tr>
</tbody>
</table>

*Increase running velocity and proceed to stage only if subject has not reached maximum.

**Terminate the test when you observe:**

1. A plateau in oxygen consumption with increasing workload
2. A plateau in heart rate with increasing workload
3. A respiratory exchange ratio greater than 1.15
4. A heart rate within 10 beats of age predicted max heart rate
5. The subject can no longer stay on treadmill.
6. The subject requests to stop.

The goal is to finish the test within 12 minutes – so subject is not exhausted due to duration.
APPENDIX G

Ebbeling Sub-maximal Walk Test

The single stage (8 minutes) treadmill walking test is a submaximal aerobic fitness test that estimates VO$_2$\(_\text{max}\). It is suitable for low risk, apparently healthy, non-athletic adults 20-59 years of age. The walking pace required throughout the test also makes it appropriate for participants who experience problems such as knee pain when exercising at a jogging pace. The test can be administered to moderate sized groups of participants with low to moderate fitness levels and requires only a treadmill and a HR monitor.

Protocol

1. Calculate 85% of the client’s age-predicted max heart rate and record below.
2. Calculate 50-70% of his/her age-predicted max heart rate and record below.
3. Briefly explain the purpose of the test and how it is conducted to the client.
4. Obtain a stopwatch or timer. Instruct the participant straddle the treadmill.
5. When ready, instruct the client to warm up for 4 minutes while walking at 0% grade and a speed that corresponds to a heart rate between 50-70% of the age-predicted max. The recommended walking speed is from 3.4 to 4 mph, although these are highly variable.
6. Measure radial pulse counts the last 15 seconds of each minute. If heart rate is not between 50-70% of age-predicted max after the first minute, adjust the speed accordingly.
7. Following the 4-minute warm-up and after you have determined the appropriate walking speed, keep the participant at the same speed for an additional 4 minutes and while setting the grade to 5%.
8. Record steady-state heart rate from the average of the final 15 sec of the last two minutes at the 5% grade. (Note: to achieve steady-state, the heart rate from the last two minutes must not differ by more than 5 bpm. If the rate differs by more than 5 bpm, extend the test by an additional minute and record the steady-state heart rate for the new final two minutes. i.e., minutes 8 and 9).
9. At the end of the test, instruct the client to cool down at a slow walk and 0% grade for 2-5 min. Monitor and record the heart rate every minute.
10. Enter steady-state heart rate into the equation below to estimate VO$_2$\(_\text{max}\) (mL/kg/min).
## Data Collection Sheet for the Ebbeling Walking Test

<table>
<thead>
<tr>
<th>Name:</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting HR:</td>
<td>bpm</td>
</tr>
<tr>
<td>Resting BP:</td>
<td>mmHg</td>
</tr>
<tr>
<td>Age:</td>
<td>yrs</td>
</tr>
<tr>
<td>Gender M or F</td>
<td></td>
</tr>
<tr>
<td>Body Mass:</td>
<td>kg</td>
</tr>
<tr>
<td>85% predicted HRmax:</td>
<td>bpm</td>
</tr>
</tbody>
</table>

### Warm-up HR Training Zone:
- 50% predicted HRmax = bpm
- 70% predicted HRmax = bpm

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Speed (mph)</th>
<th>Grade (%)</th>
<th>HR (bpm)</th>
<th>RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>5</td>
<td></td>
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<td>7</td>
<td></td>
<td>5</td>
<td></td>
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<tr>
<td>8</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Workload

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Speed (mph)</th>
<th>Grade (%)</th>
<th>HR (bpm)</th>
<th>RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Recovery**

(reduce walking speed and set grade to 0%)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Speed (mph)</th>
<th>Grade (%)</th>
<th>HR (bpm)</th>
<th>RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 9th minute only required if HR during the 7th and 8th minute has not reached steady-state (within 5 bpm)

** An active recovery period of 2-5 minutes should immediately follow this test.
**Interpretation**

VO₂\text{max} is estimated using the following equation where:

- speed = mph
- HR = bpm
- age = years
- gender = 1 for males and 0 for females

\[ VO₂\text{ max} = 15.1 + (21.8 \times \text{speed}) - (0.327 \times \text{HR}) - (0.263 \times \text{speed} \times \text{age}) + (0.00504 \times \text{HR} \times \text{age}) + (5.98 \times \text{gender}) \]

**EXAMPLE**

Client is a 30-year-old male who walked at 3.6 mph at a grade of 5% with a steady-state HR of 159 bpm.

HR\text{max} = 190 bpm;
50\% \, HR\text{max} = 95 bpm;
70 \% \, HR\text{max} = 133 bpm:

**Estimated VO₂\text{max}**

\[ = 15.1 + (21.8 \times 3.6) - (0.327 \times 159) - (0.263 \times 3.6 \times 30) + (0.00504 \times 159 \times 30) + 5.98 \times (1) \]

\[ = 43.2 \, \text{mL/kg/min} \]
**APPENDIX H**

**Ischemic Preconditioning Protocol**  
Vascular Protection Laboratory

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time (min)</th>
<th>Protocol</th>
<th>Heart Rate, BPM</th>
<th>Blood Pressure,</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0</td>
<td>Resting</td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>1</td>
<td>30 min</td>
<td>Before rIpre/sham</td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>2</td>
<td>55 min</td>
<td>After rIpre/sham</td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>3</td>
<td>95 min</td>
<td>Immediately after Forearm IRI</td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>4</td>
<td>105 min</td>
<td>40 min after Reperfusion</td>
<td></td>
<td>/</td>
</tr>
</tbody>
</table>

**Cuff placement instructions:**
Remote preconditioning cuffs are placed on *right* arm. Forearm IRI cuff is placed on *left* arm. Place pressure cuffs snugly on the upper arms as high as possible.

**Ischemic preconditioning trial:**
Inflate pressure cuffs to 220 mmHg to cause ischemia for 5 minutes, followed by 5 minutes of cuff deflation. Repeat 3 times. Record heart rate and blood pressure before and after the preconditioning phase.

**Sham control trial:**
Inflate pressure cuffs to 15 mmHg to avoid ischemia for 5 minutes, followed by 5 minutes of cuff deflation. Repeat 3 times. Record heart rate and blood pressure before and after the sham phase.
REFERENCES


44. Peyton RB, Van Trigt P, Pellom GL, Jones RN, Sink JD, Wechsler AS. Improved tolerance to ischemia in hypertrophied myocardium by


