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Cardiovascular Risk, Remote Ischemic Postconditioning, and Endothelial Ischemia-Reperfusion Injury

Brian A. Hemenway
South Dakota State University

CARDIOVASCULAR RISK, REMOTE ISCHEMIC POSTCONDITIONING, AND ENDOTHELIAL ISCHEMIA-REPERFUSION INJURY

BY

BRIAN A. HEMENWAY

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

Gary P. Van Guilder, Ph.D.
Thesis Advisor Date

Matthew Vukovich, Ph.D.
Head, Department of Health and Nutritional Sciences Date

Dean, Graduate School Date

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ABBREVIATIONS

ACC	American College of Cardiology
ACSM	American College of Sports Medicine
ADP	Adenosine diphosphate
AHA	American Heart Association
Akt	Protein kinase B
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BMI	Body mass index
Ca^{2+}	Calcium ion
CABG	Coronary artery bypass grafting
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
ERK1/2	Extracellular signal regulated kinase
FRS	Framingham risk score
GSK-3β	Glycogen synthase kinase
H^+	Hydrogen ion
H_0	Null hypothesis
HDL	High density lipoprotein
HR	Heart rate
IR	Ischemia-reperfusion
JAK	Janus kinase
K^+	Potassium ion
LDL	Low density lipoprotein
MetS	Metabolic syndrome
mPTP	Mitochondrial permeability transition pore
Na^+	Sodium ion
Na^+/Ca^{2+}	Sodium/calcium exchanger
Na^+/H^+	Sodium/hydrogen exchanger
NADPH	Nicotinamide adenine dinucleotide phosphate
PAT	Pulse amplitude tonometry
PCI	Percutaneous coronary intervention
PI3K	Phosphatidylinositol 3-kinase
PTCA	Percutaneous transluminal coronary angioplasty
RHI	Reactive hyperemia index
rIpost	Remote ischemic postconditioning
RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
SAFE	Survivor activating factor enhancement
SBP	Systolic blood pressure
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
STAT-3	Signal transducer and activator of transcription 3
STEMI	ST-elevation myocardial infarction
SWOP	Second window of protection
TNFα	Tumor necrosis factor

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ABSTRACT

CARDIOVASCULAR RISK, REMOTE ISCHEMIC POSTCONDITIONING, AND ENDOTHELIAL ISCHEMIA-REPERFUSION INJURY

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Remote ischemic postconditioning (rIpost) is a potent vasculoprotective stimulus that is thought to reduce reperfusion injury associated with heart disease. However, certain animal models of cardiometabolic risk factors such as dyslipidemia, diabetes, and hypertension suggest the beneficial effects of rIpost to lessen reperfusion injury may be diminished. These findings have not been studied in clinically relevant humans. The aim of this study was to determine whether the protective effects of rIpost are reduced in the context of raised cardiometabolic risk in humans.

Seven participants with raised cardiometabolic risk burden (age: 57±7 years; BMI: 31±4 $kg \cdot m^2$) participated in this 2X2 randomized cross-over design study. Raised cardiometabolic risk was established by the presence of 2 or more major risk factors and a 10-year Framingham risk score ≥10%. Microvascular endothelial function was measured using peripheral arterial tonometry (EndoPAT 2000, Itamar Medical Inc.) before and after 20 minutes of left arm, brachial artery ischemia, with and without the induction of rIpost (three, 5-minute bouts of right arm ischemia and reperfusion at 220 mmHg). Remote Ipost procedures were initiated immediately at the onset of reperfusion injury. Microvascular endothelial function was also assessed during a sham trial consisting of mock occlusions at the brachial artery (three, 5-minute inflations at 20 mmHg).

Five of the seven participants completed both the sham and rIpost trials. In these individuals, the reactive hyperemia index (RHI) at baseline during the sham trial was 1.26±0.36 and increased 38% following endothelial injury to 1.74±0.23 (p=0.108). Similarly, the RHI increased 24% from 1.35±0.46 to 1.67±0.4 (p=0.358) during the rIpost trial. Reactive hyperemia index following endothelial injury at 30-second intervals was also not affected by rIpost (p=0.27). There was a trend for the rIpost response following endothelial injury to be inversely related to the total number of risk factors (R^2=0.53, p=0.04) 10-year Framingham risk score (R^2=0.27, p=0.19), and the ACC/AHA ASCVD risk score (R^2=0.30, p=0.16), particularly during the most potent window of reactive hyperemia.

The ability of rIpost to reduce microvascular endothelial injury was not different compared with the sham trial, but tended to be worse in participants with elevated cardiometabolic risk factors.

INTRODUCTION

Microvascular reperfusion injury is the inevitable result following an extended period of ischemia occurring within an organ or tissue. Upon prolonged occlusion, the tissue deprived of direct blood supply eventually experiences necrosis, or infarct. The infarct may begin to develop after only 30 minutes of restricted blood flow, and the reintroduction of oxygenated blood to the site of damage causes the infarct size to grow even larger; a phenomenon referred to as ischemia-reperfusion (IR) injury. [1] Reperfusion injury not only increases an individual's chance of re-hospitalization, but also increases their risk of mortality. Although there still remains speculation of each specific mechanism occurring during IR-injury, it is understood that a rapid ionic shift and pH fluctuation serve as the main culprit. The ischemic phase causes a build-up of hydrogen ions coupled with ATP depletion, while the reperfusion itself worsens cell function by increasing oxidative stress, activating neutrophil accumulation, and stimulating inflammatory cytokines, which collectively impair mitochondrial function. [2-5]

Reperfusion injury is of great concern as a decline of active lifestyles coupled with unhealthy diets have taken a drastic toll on cardiovascular health, overall resulting in increased heart surgeries to treat acute myocardial infarctions. [6-8] Due to an increased prevalence of patients at high risk, coronary revascularization surgeries have become more complicated over the years resulting in an increased incidence of surgical and post-operative complications. [9] The most common forms of cardiovascular surgeries that result in IR-injury consist of acute ST-elevation myocardial infarction (STEMI), coronary artery bypass grafting (CABG), and percutaneous transluminal coronary angioplasty

(PTCA). Currently, IR-injury is untreatable; however, there are many clinical trials of cardioprotective strategies to help limit the harm caused by IR-injury.

Ischemic conditioning is the most powerful cardioprotective strategy identified to date. Ischemic conditioning consists of interspersed bouts of ischemia and reperfusion that are mild enough in duration and frequency to avoid damaging tissues. This methodology of cardioprotection triggers a hormetic response that may potentially reduce the overall extent of IR-injury, along with stimulating protection in all major organs and tissues including the heart, brain, kidneys, lungs, and skeletal muscle. The three main types of ischemic conditioning are: *pre-* (conditioning prior to ischemic injury), *per-* (conditioning during ischemic injury), and *post-* (conditioning after ischemic injury). These strategies are currently being investigated in several small clinical trials to determine if they can reduce cellular injury caused by ischemia and reperfusion. Pre- and perconditioning, although proven very efficacious, are less practical as we are unable to predict when an ischemic injury-causing event will occur, and thus unable to provide conditioning in a timely manner. [10] On the other hand, postconditioning has worthy therapeutic efficacy as it is implemented at the onset of reperfusion, immediately after ischemic episode is cleared, and has shown excellent clinical utility in emergency situations. Local postconditioning (occurring at the site of injury), has been shown to reduce IR-injury, and overall infarct size when applied during surgeries such as coronary angioplasty. [11] Remote conditioning (occurring distal to the site of injury) is typically applied on the upper arms or legs, and provides distant-site protection to organs and tissues experiencing IR-injury. Despite its practicality and ease of use, remote postconditioning has yet to be translated to a widespread clinical setting.

Individuals requiring coronary revascularization surgeries are typically plagued with a number health risks referred to as cardiovascular disease risk factors. Cardiovascular disease (CVD), or a cluster of risk factors including: age, family history, smoking, physical inactivity, obesity, hypertension, dyslipidemia, and pre-diabetes, is an ever-growing culprit of morbidity and mortality. [12] These risk factors have detrimental effects on the vascular system, and increase the likelihood of myocardial infarction, stroke, heart failure, and diabetes. An increased prevalence of risk factors reduces the benefit of CVD treatments and/or surgeries, and intensifies IR-injury by weakening the vascular endothelium. Certain risk factors, such as aging, hyperlipidemia, hypertension, and diabetes, have all been shown to reduce the effects of ischemic *pre*conditioning and cause greater vulnerability to vascular IR-injury in specific animal models. [13,14] However, very limited data are available with respect to the correlation between risk factors and the benefit of ischemic *post*conditioning. Furthermore, these findings have not ascertained in humans who have a chronically elevated burden of risk factors, proving an underlying issue translating findings from pre-clinical animal models to humans. [15]

Statement of Problem

Remote ischemic postconditioning (rIpost) is a promising experimental strategy used to reduce endothelial IR-injury. However, while animal models suggest that CVD risk factors may potentially reduce the protective benefits, data in human populations are limited. This study aims to provide a base of clinical knowledge of the correlation between CVD risk factors and rIpost, deemed advantageous to research and medical communities alike.

Specific Aim – The purpose of this quantitative experimental research study is to determine how cardiovascular risk factors affect the vasculoprotective capacity of rIpost and its ability to protect against endothelial IR-injury in humans.

H_0 – An elevated burden of risk factors will exacerbate IR-injury and diminish the protective capacity of rIpost in the instance of endothelial IR-injury.

Independent Variable(s)

- Low burden risk vs. Raised burden risk
- Experimental (rIpost) protocol vs. Sham protocol

Dependent Variable(s)

- Microvascular endothelial vasodilator function (reactive hyperemia)

Delimitations – Due to geographical location, participants will be recruited from Brookings County, South Dakota and the immediate surrounding area, allowing for all racial and ethnic backgrounds. All recruited participants must be classified as physically inactive. Participants may not have CVD or metabolic disease (with the exception of diabetes), or history of CVD as classified by the ACSM. [12,16,17] Participants may currently be taking medications if they are able to withhold their prescriptions the day of each trial.

Limitations – The greatest limiting factor remains that participants are required to be sedentary *and* free of CVD. Additionally, smooth muscle relaxation is not being measured during this study, which should be taken into account when determining the reactive hyperemic response.

Definition of Terms

Ischemia-reperfusion injury (IR-injury) – Tissue or vascular injury occurring with the sequential act of prolonged ischemia followed by the rapid restoration of blood flow, causing cellular/endothelial dysfunction in the form of reperfusion arrhythmias, myocardial stunning, and potential necrosis.

Reactive hyperemia – Provisional over-shoot of blood flow, resulting from an excess build-up of vasodilating metabolites during an extended length of ischemia, immediately occurring during reperfusion.

Ischemic conditioning – Tissue/organ and vascular protective mechanism resulting from regulated, brief bouts of ischemia and reperfusion that stimulate cellular protection pathways, which have the potential to limit and/or reduce the prevalence of cellular dysfunction, infarct size, and necrosis.

Remote ischemic postconditioning (rIpost) – Ischemic conditioning applied remotely (distal to the target organ), immediately at the onset of reperfusion within the previously ischemic organ or tissue.

Vascular endothelium – The innermost monolayer of a blood vessel in direct contact with blood itself, modulating the secretion of vasodilating, vasoconstricting, and anti-thrombogenic factors. Thus, the vessel's ability to vasodilate and constrict proves a reliable measure of endothelial function.

REVIEW OF LITERATURE

The following review of literature will attempt to provide an in-depth synopsis of IR-injury (focusing on the endothelium and myocardium), mechanisms/cell biology of damage-causing events during ischemia and reperfusion, and protection afforded by ischemic conditioning through its ability to combat reperfusion injury. Current clinical trials in animals and humans will be reviewed, while taking into account cardiometabolic risk, and providing insight toward clinical implications and/or complications of implementing rIpost with humans presenting a cluster of risk factors.

Clinical Relevance of IR-Injury

During an ischemic event, such as a heart attack or stroke, the transport of blood is blocked as some form of occlusion (e.g., plaque) prevents normal continual blood flow. Without constant blood flow, the tissue beyond the blockage will quickly become deprived of oxygen and nutrients, which begins the cascade of adverse events throughout the body. Eventually, if ischemia is prolonged beyond 15 minutes, the tissue will begin to die and the organ experiences necrosis. The site of necrosis may also be referred to as infarct, referencing the specific location and size of dead tissue within the organ (immediate hypoxic tissue) and its surrounding area. Certain biomarkers such as lactate dehydrogenase, creatine kinase-MB, and cardiac troponin and are often used to assess this type of myocardial damage. Researchers have indicated that these biomarkers are naturally elevated during cardiac revascularization surgeries, and are exacerbated in high-risk patients suffering extensive IR-injury. [18,19] Originally it had been believed that the sole cause of tissue damage resulting from an ischemic event was simply due to ischemia,

or lack of oxygen; however, it has since been recognized that reperfusion also induces supplementary damage and can further intensify infarct size. [20,21] Reperfusion, while vital for survival, poses a major drawback by stimulating damage severe enough to expand the original infarct size upwards of 50-percent of the post-ischemic size. [22] If severe enough, reperfusion injury may even lead to remote damage in other organs and tissues within the body that were never initially subjected to ischemia. Additional damage to the vascular endothelium resulting from IR-injury reduces its capacity of vasocontrol in the form of blunting endothelial-derived relaxing factors, nutrient delivery, and elimination of harmful waste. [23] Though it is most detrimental within the heart, many other organs, such as the brain, lungs, intestines, kidneys, and skeletal muscle are also largely affected by IR-injury.

Ischemia-reperfusion injury continues to prevail as the culprit of a paradoxical dilemma where that which is necessary for survival comes at the cost of compromising other organs and tissues. The mechanisms have been thoroughly studied through the clinical induction of IR-injury using many animal models. The instance of injury occurs locally, where stroke victims, for example, experience neuronal damage through cerebral ischemic injury followed by rapid reperfusion, altogether compromising the permeability of the blood-brain barrier and promoting harmful leakage. [24] However, IR-injury may also be remotely transferred throughout the body, which has been demonstrated in clinical situations such as pulmonary transplantation, where blood-free surgical procedures expose a patient to IR-injury, directly leading toward graft dysfunction and a decreased rate of survival. [25]

An array of pre-clinical studies have demonstrated the detriment of IR-injury in lungs, myocardial tissue, endothelial cells, skeletal muscle, and more. While assessing protective strategies against pulmonary IR-injury in rat models, Dorsa and colleagues (2015) noted extensive damage to the lungs after 30 minutes of occlusion at the abdominal aortic artery, followed by 60 minutes of reperfusion. [26] The remote IR-injury led to moderate-to-severe edema in the alveolar septa, as well as moderate congestion, and excessive neutrophil accumulation. [26,27] Similarly, Hirano et al., (2016) triggered remote IR-injury in the lungs of mice after after occluding the superior mesenteric artery for 45 minutes. Ensuing reperfusion, injury quickly transferred to acute respiratory distress syndrome within the following 4 hours. [28] With aims of studying ischemic conditioning models on myocardial and endothelial IR-injury, Zhao and colleagues (2003) occluded the left anterior descending artery of canine myocardium for 60 minutes, followed by 3 hours of reperfusion. Locally, they noted that approximately 25% of left ventricular mass was subjected to risk of injury, in which nearly the entire mass experienced necrosis. It was noted that both creatine kinase activity as well as subendocardial tissue edema drastically increased following reperfusion. Endothelial dysfunction was determined through the increased activity of myeloperoxidase and polymorphonuclear neutrophil (PMN) accumulation, as well as a blunted vaso-relaxation response to acetylcholine. [29] In this instance, both myocardial and endothelial injury mimic that which is expected from cardiac revascularization surgeries. In similar surgical settings, researchers continue to battle with renal IR-injury and attempt to alleviate harm caused from hepatic transplantation and other renal issues causing acute kidney injury and increased likelihood of prolonged hospitalization. [30,31]

As opposed to the aforementioned organs, skeletal muscle can endure much longer episodes of ischemia before permanent damage is initiated. Pottecher and colleagues (2016) assessed local skeletal muscle IR-injury in the gastrocnemius muscle of aged Wistar rats after exposing them to 3 hours of ischemia followed by 2 hours of reperfusion. [32] Rats experienced a significant reduction in maximal mitochondrial oxidative capacity, along with a drastic decrease in calcium retention capacity, which was used as an indicator of apoptosis and early mitochondrial failure. Wang and colleagues (2016) observed skeletal muscle IR-injury by subjecting rats to 3 hours of bilateral ischemia followed by 6 hours of reperfusion in hind limbs. [33] At the local site of IR-injury within the gastrocnemius, they noted skeletal muscle degeneration, necrosis, interstitial vessel hemorrhaging, and edema. It was conferred that injury remotely transferred to the lungs, showing capillary congestion from neutrophil penetration, ruined alveolar structures, and interstitial edema.

However, IR-injury most commonly affects the heart as a primary outcome from surgeries performed on patients suffering from coronary heart disease, such as STEMI, CABG, percutaneous coronary intervention (PCI), and others using thrombotic drugs. These interventions, while essential in reestablishing coronary blood flow to ischemic myocardial tissue, have become much more complicated as patients suffer from a cluster of cardiometabolic risk factors that reduce procedure effectiveness. [9] Patients suffering CABG induced IR-injury are more likely to endure re-hospitalization and premature mortality if they present with diabetes, obesity, and/or metabolic syndrome.

Clinical Repercussions of IR-Injury

Extreme benefits of reperfusion are quickly diminished through injury by induced clinical barriers such as myocardial stunning, reperfusion arrhythmias, cardiac and/or endothelial dysfunction, and no-reflow complications. [22] Cellular necrosis and apoptosis are very common during the reperfusion phase following prolonged ischemia. Ischemia-reperfusion injury may become lethal if the damage is severe enough, or the events are too accelerated for the body to withstand.

Myocardial Stunning

While myocardial stunning is generally a temporary issue resulting from IR-injury, it is the first of many complications. Following ischemia, myocardial stunning is known to diminish the contractile properties of the immediately reperfused cells, which stuns and impairs left ventricular ejection fraction. This has been known to occur following 5-15 minutes of ischemia. [34] The decreased responsiveness among the stunned cells derives from ATP depletion during ischemia, rapid alteration of metabolites, and increased cytotoxic injury of reactive oxygen species (ROS), further discussed in mechanisms of IR-injury. Fortunately, myocardial stunning is reversible and occurs in the absence of cell death. It has been postulated that myocardial stunning may actually act as a protective mechanism allowing the cells to withstand greater a fluctuation of oxygen and nutrient delivery. [35]

Reperfusion Arrhythmias

Following ischemia, the heart may be subject to rapid, irregular rhythms referred to as reperfusion arrhythmias. A study performed by Yamazaki and colleagues (1986) demonstrated that the occurrence of reperfusion arrhythmias in canine myocardium was

significantly greater when the occlusion was abruptly cleared, as opposed to staged reperfusion which allowed partial intracoronary reflow for two hours following ischemia. [36] Reperfusion arrhythmias are often observed after thrombolytic therapy and cardiac surgery, and may occur after only 15-20 minutes of myocardial ischemia, but are most often considered clinically non-significant. [34,37] Due to oxygen-derived free radicals (ROS) and rapid alteration in intracellular ionic/metabolite concentrations (specifically Ca^{2+} overload), the myocardial cells experience a great variability in electrical activity and electrophysiological changes. [36,37] Irregular electrical activity within the cardiac muscle is known to lead toward ventricular fibrillation and even ventricular tachycardia.

Cardiac and Endothelial (Dys)function

The body's ability to efficiently maintain vascular tone, regulate inflammation and clotting agents, and control substances passed through to the surrounding layers is referred to as endothelial function. The endothelium, being the immediate vessel layer located in contact with the blood, is primarily responsible for producing and releasing vasoactive and anti-thrombogenic substances that preserve vasodilator capacity and control constriction. A healthy endothelium is extremely plastic and flexible, responding quickly and efficiently to signaling molecules traveling throughout the blood. Being culprit of many elements, the endothelium's vasocontrol diminishes drastically over time. Endothelial dysfunction consists of increased endothelial permeability, decreased vasodilating properties, increased vasoconstriction, and subsequent loss of control. Cardiovascular disease risk factors are directly related to blood vessel health, as the greater burden or risk factors trends toward a higher prevalence of endothelial dysfunction, atherosclerosis, and atherothrombotic events. [38-40] More specifically, risk

factors, such as obesity, are known to preserve nitric oxide synthesis and impair vasodilator function. [41] According to Cai and Harrison (2000), there is a positive correlation between risk factors and increased oxidative stress in the form of an increased prevalence of ROS. [42] Through the increased production of ROS, the bioavailability of nitric oxide, arguably the most potent vasodilator, rapidly declines and begins a vicious cycle further enhancing dysfunction.

No-reflow Phenomenon

Reperfusion following prolonged ischemia (>20 minutes) may cause microvascular damage severe enough to prevent continual blood flow to the affected area. This is not an issue for myocytes and/or tissues that have already experienced necrosis, but could indeed threaten areas experiencing myocardial stunning, which are reliant upon blood flow for restoration, thus pushing them to the extent of necrosis. [34] No-reflow is the undesirable outcome of severe platelet-leukocyte coagulation, increased interstitial fluid, and prolonged vasoconstriction. [37] While an acidic environment causes leukocytes to become more rigid, they accumulate within the vessel lumen and form a blockade. The increased interstitial fluid causes additional pressure pushing inward on the vessel walls, eventually causing a collapse of the vessels structure. [35] Not only will this phenomenon jeopardize myocytes experiencing myocardial stunning, but will further exacerbate the myocardial stunning by prolonging microvascular ischemia.

Mechanisms of IR-Injury

Ischemia-reperfusion injury has been extensively studied providing a solid foundation of interaction between the two phases of injury. Many abnormalities occur

within the cell and throughout the surrounding area during both phases of ischemia and reperfusion. To date, researchers have pinpointed key mechanisms such as a rapid fluctuation of pH, calcium overload, the oxygen paradox, cellular inflammation, and mitochondrial dysfunction (Figure 1). [22,35] The culmination of cardiovascular disease risk factors further enhances endothelial dysfunction leading to more severe injury that is systemically transferred throughout the body to remote organs and tissues.

Ischemic Induced Anaerobic Metabolism – ATP Depletion

During ischemia, an inadequate supply of oxygen causes the cells to rapidly convert to anaerobic metabolism in a last ditch effort for continued ATP production. As a result of decreased ATP production, there is a drastic increase in ADP, AMP, and P_i. The lack of blood flow causes a disruption in the transport of glucose and fatty acids, delaying the production of pyruvate and Acetyl-CoA, which in turn hinders proper function of the Krebs cycle, and ultimately the electron transport chain. If prolonged, the endothelial cells and cardiomyocytes will die without a source of viable energy.

Fluctuation in pH

Anaerobic metabolism and ATP depletion cause the cells to become extremely acidic. During ischemia, the cell initiates a cascade of ionic imbalances with efforts to buffer the acidity. The rapid shift to anaerobic metabolism causes the decrease in intracellular pH through an overproduction and accumulation of H^+. The acidic environment is combatted by the excretion of excess H^+ into the cytosol through the Na^+/H^+ exchanger, which thus triggers the uptake of residual Na^+ into the cell overall disrupting the Na^+ gradient. [22,35,43] At the onset of reperfusion there is a rapid correction in

pH, posing as a source of hypercontracture, along with promoting deleterious effects on the mitochondria and a washout of lactic acid into the blood stream. [22]

Calcium Overload

Calcium overload has been considered one of the main damaging factors during both ischemic and reperfusion injury. During ischemia, cellular depletion of ATP causes an inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) as well as the Na^{+}/Ca^{2+} exchanger, overall preventing Ca^{2+} efflux. With reperfusion, Ca^{2+} continues to overload the cell as an accumulation of ROS leads to sarcoplasmic reticulum dysfunction, inhibiting the essential up-take of Ca^{2+}. [22] This can be particularly harmful due to the activation potential of enzymatic proteases, which may ultimately cause hypercontracture and cellular apoptosis. [43,44] The infiltration of macrophages and neutrophils cause increased inflammation, damaging the surrounding tissue. The Ca^{2+} continues to overload the mitochondria during the reperfusion phase, leading to the detrimental opening of the mitochondrial permeability transition pore (mPTP). [35,45]

Aerobic ATP Production – Oxidative Stress During Reperfusion

While reperfusion is necessary for the restoration of aerobic metabolism/ATP production, it also causes many complications through increased oxidative stress via the overwhelming increase in ROS. Key sources of ROS have been identified as xanthine oxidase, NADPH oxidase, reenergized mitochondria, lipoxygenase, and nitric oxide synthase. The hypoxic stages of ischemia and early reperfusion cause ROS to become even more reactive. Increased ROS may lead to the accumulation of procoagulant proteins such as tumor necrosis factor (TNFα) and transforming growth factor (TGF-β). The accumulation of ROS has also directly been linked to the alteration and denaturation

of lipid membranes and protein structures, along with breaking the strands of deoxyribonucleic acid (DNA). This damage may lead to cellular necrosis through the opening of the mPTP. [5,46] Damage may also be transferred systemically, promoting the adherence of PMN on the endothelial lining of remote organs/tissues. [35]

Opening of the Mitochondrial Permeability Transition Pore (mPTP)

Located within the mitochondrial inner membrane, the mPTP is activated during stressful ischemic events such as myocardial infarction. With great susceptibility, the mPTP is forced open during reperfusion via pH correction, Ca^{2+} overload, and the overwhelming accumulation of ROS. Research has pinpointed this protein pore as the main culprit of the inhibition of ATP production through the excessive influx of H^{+} upon opening. [35,47] Through the transport of K^{+} and P_i, the mitochondrial matrix may osmotically swell upwards of 20-40% its original size, potentially leading to mitochondrial rupture. [35,48] The swelling takes place in an 'all or none' manner, where mitochondrial Ca^{2+} overload causes depolarization, overall increasing the mPTP Ca^{2+} sensitivity and triggering a cascade of additional pore openings within the same mitochondria. [48,49] Certain cardioprotective strategies, such as ischemic conditioning, aim to inhibit the mPTP pore opening, overall eliciting a greater reduction of damage caused via IR-injury and preserving the capacity of ATP production.

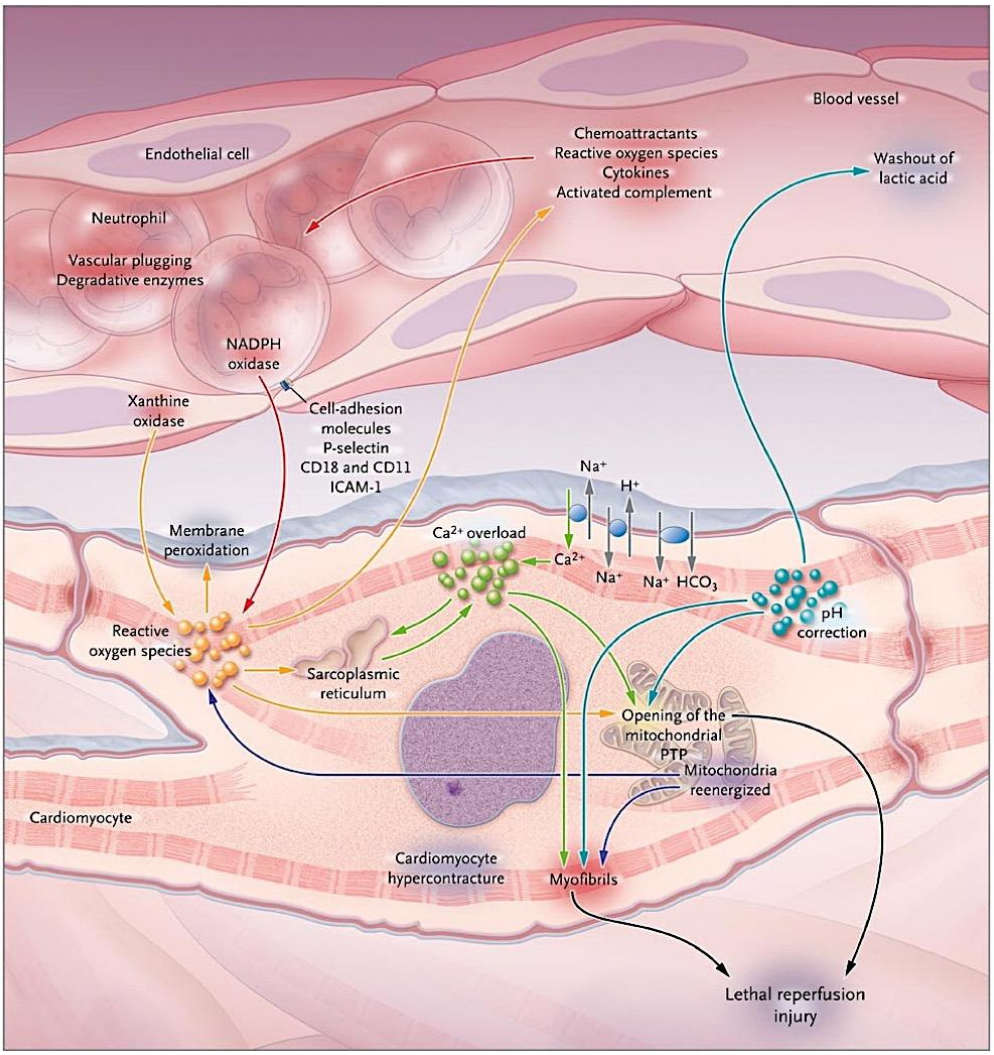

Figure 1. Representation of biomolecular changes occurring during IR-injury in the cardiomyocyte and endothelial cells as discussed in *Mechanisms of IR-Injury*. Image modified from Yellon & Hausenloy (2007). [22]

Ischemic Conditioning

Ischemic conditioning activates a hormetic response through short regulated bouts of ischemia and reperfusion, used to protect tissues/organs and the vascular system by stimulating cellular protection programs aimed at reducing cell dysfunction, infarct size, and necrosis. As previously mentioned, there are three different times to administer ischemic conditioning: pre- (prior to an event), per- (during an event), and post- (after an

event, at the onset of reperfusion). All three types of ischemic conditioning may be applied either locally (at the site of the organ or tissue experiencing IR-injury) or remotely (distal from the organ or tissue experiencing IR-injury). The foundation of ischemic conditioning relies on promoting a reactive hyperemic response, where short bouts of ischemia cause a build up of vasodilating metabolites, followed by an overshoot of blood flow at the moment of reperfusion. When repeated multiple times, the tissue/organ is able to trigger a protective response, which is transferred through the vascular system throughout the body. There has yet to be a standardized protocol for any form of ischemic conditioning, but the trend in literature typically consists of three, 5-minute bouts of ischemia followed by 5-minute bouts of reperfusion (3X5), or four, 3-minute bouts of ischemia followed by 3-minute bouts of reperfusion (4X3). All forms of ischemic conditioning provide two windows of protection. [50] An early phase, or classic phase, of protection begins only minutes after the completion of ischemic conditioning, lasting approximately 2 to 4 hours. [50,51] Although the early phase of protection is short-lived, it is very potent and has been linked with a great degree of infarct size reduction. A second window of protection (SWOP) occurs between 12 and 72 hours after ischemic conditioning, greatly expanding the overall length of protection, but occurring with a lower degree of infarct size reduction. [50,52,53]

Protective Effects of rIpost

Particular cardioprotective strategies have been adapted in efforts of reducing the detriment caused via IR-injury. Ischemic postconditioning, being one of the more practical methods, eliminates the dilemma of predicting the exact moment of an ischemic

event as it is applied immediately at the onset of reperfusion following the prolonged ischemia. Remotely applied distal from the compromised organ, rIpost is a simple, non-invasive method of cardioprotection that shows great clinical therapeutic efficacy.

Mechanisms of Ischemic Postconditioning to Improve Endothelial Function

Endothelial dysfunction, resulting from IR-injury, proves to be one of the most detrimental factors for the body to overcome. As the endothelium is responsible for vascular tone and remodeling, endothelial dysfunction has been directly related to a more severe degree of infarction. [54] Remote Ipost has been shown to attenuate endothelial dysfunction through both neural and humoral influences (Figure 2). The neural theory begins with the activation of G-protein coupled receptors, which are known to initiate the action of intracellular signaling pathways. [55] At this time, the activation of adenosine, bradykinin, and opioid receptors stimulate the process of amplified vasodilation, and trigger the humoral pathway. The humoral action activates downstream signaling cascades of phosphatidylinositol 3-kinase (PI3K), protein kinase, and extracellular signal-related kinase1/2 (ERK1/2), to be transferred throughout the body. Remote Ipost has also been shown to aid in the slowed restoration of pH, which was subsequently lowered during the ischemic phase of IR-injury. Research indicates rIpost has the potential to reduce infarct size approximately 36% when compared to a control group. [11]

The two key pathways that have been directly linked to cardioprotection resulting from rIpost are the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE). The RISK pathway, activated by ROS and protein kinase C, activates PI3K, an enzyme involved in many cellular functions such as cell survival and intercellular trafficking, and ERK1/2, which is a protein kinase involved in meiosis and

mitosis. Together, PI3K and ERK1/2 phosphorylate downstream targets such as glycogen synthase kinase-3β (GSK-3β), which ultimately deters the opening of the mPTP. [56] The SAFE pathway is initiated by increased levels of TNFα during the ischemic phase, which transphosphorylates adjacent Janus kinase (JAK) allowing it to transduce signals from the cell cytosol to the nucleus, ultimately activating the signal transducer and activator of transcription (STAT-3) pathway. [56] It has been suggested the RISK and SAFE pathways have some form of cross communication, and if either pathway is not activated during conditioning, cardioprotection will be diminished. [57,58] With the end goal of preventing the mPTP from opening, rIpost preserves mitochondrial function and allows cellular aerobic metabolism to proceed.

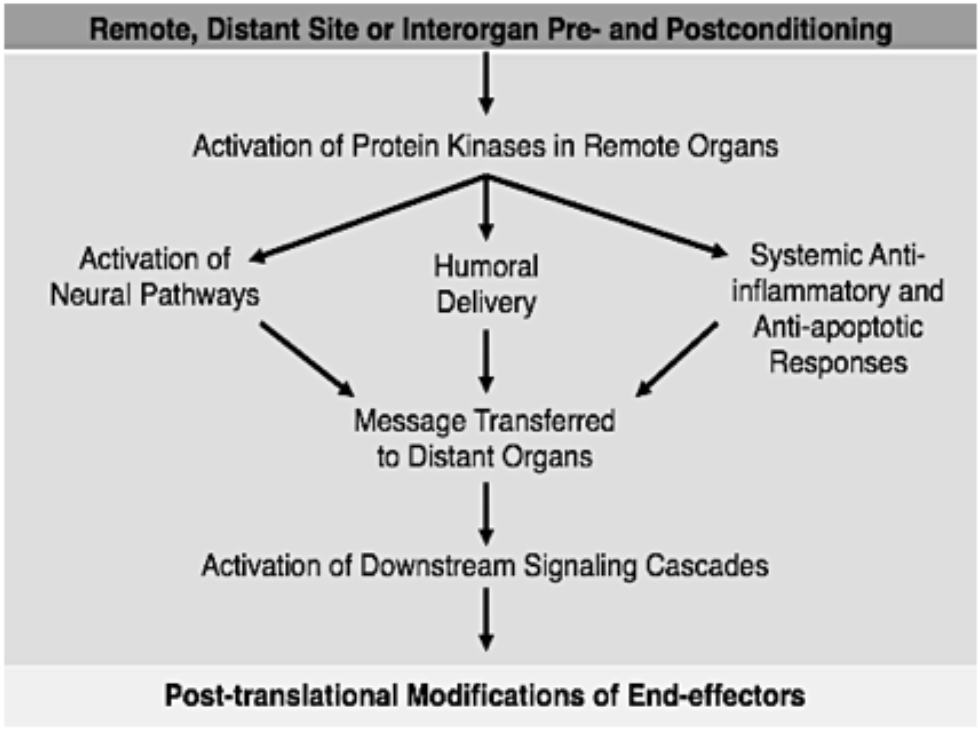

Figure 2. Mechanistic flowchart of remote ischemic pre- and postconditioning from the application of conditioning stimuli to the post-translational modification of end-effectors. Image modified from Krenz et al., (2013). [43]

CVD Risk Factors and Ischemic Conditioning

Many animal models, and few human studies, have been conducted over the years looking for a correlation between the incidence of cardiometabolic risk factors and the perceived benefit from ischemic conditioning. Although most literature documents ischemic *pre*conditioning (utilizing very similar mechanisms) on animal models with induced risk factors, a trend in the data makes it quite clear that the protective benefit may be greatly diminished. One example of the very few human studies compared young men (20-25 years) to older men (68-79 years) in efforts of demonstrating the effects of advanced age on remote ischemic *pre*conditioning. In this study the conditioning stimuli was performed for three, 5-minute cycles, thereafter IR-injury was induced on the opposite arm. Using flow mediated dilation of the brachial artery, researchers noted that endothelial function in older subjects decreased nearly twice the extent of that seen in younger subjects, and showed a much longer time to full restoration. Researchers concluded that advanced age eliminated the ability of ischemic preconditioning to attenuate endothelial dysfunction. [14] This particular study serves as a nice foundation and first step in linking risk factors with the reduced benefit of conditioning in humans.

With regards to ischemic postconditioning, select studies have assessed risk factors such as diabetes mellitus, hypertension, and hypercholesterolemia. Utilizing clinically induced diabetic Wistar rats, efficacy of postconditioning has been assessed through changes in infarct size. Badalzadeh et al., (2015) determined the infarct size of the diabetic rat hearts was completely unaffected by their postconditioning treatment when compared to the healthy control group. Researchers explain that the diabetic state further increases oxidative stress, thereby inactivating the RISK pathway, and more

specifically the ability to phosphorylate GSK-3β, which further enhances cell damage by allowing the active protein kinase to induce apoptosis. [59] A very similar mechanism was also reported in spontaneously hypertensive rats by Wagner and colleagues (2013). Two different postconditioning methods were implemented in two groups of rats subjected to either 20 or 30 minutes of ischemia. [60] The results concluded that myocardial hypertrophy, cardiac remodeling resulting from the overload of spontaneous hypertension, hindered the capacity of cardioprotection. Like the previous study with diabetes, these researchers suggested that hypertension prevented the phosphorylation of GSK-3β, whereas the non-hypertensive control group experienced a 2.1 fold increase in the phosphorylation of GSK-3β, which aided in preventing the opening of the mPTP. [60] Additionally, in the case of clinically induced hypercholesterolemic male Wistar rats, Wu and colleagues (2015) also reported no perceived cardioprotection from rIpost. [61] These specific rats were fed a cholesterol rich diet for 8-weeks, at which point IR-injury was induced and rIpost was completed. Researchers concluded that hypercholesterolemia inactivated the RISK pathway by blocking the phosphorylation of protein kinase B (Akt) and ERK1/2, overall abolishing the effects of rIpost. [61] While these pre-clinical trials demonstrate a large disconnect between cardioprotective ischemic conditioning and cardiometabolic risk factors, the literature lacks necessary evidence in human models.

Transferring Data to Human, Clinical Applications

The most drastic IR-injury occurs in individuals with a cluster of cardiometabolic risk factors as the endothelium is already compromised prior to the ischemic event. The previously discussed animal models had only utilized one acute, clinically-induced risk

factor per study as opposed to a more realistic cluster of risk factors expected in a human sample. Due largely in part to the Western diet being greatly over-proportioned and high in fat and sugar, along with increased physical inactivity, the prevalence of cardiometabolic risk factors are vastly increasing. [6,7] The unfortunate truth is that the patients who will most likely require some form of cardioprotection are the ones who suffer from significant disease burdens. In the majority of animal models, the induced ischemia is caused via an external occlusion of an otherwise healthy artery; whereas in real-life situations, humans will experience an internal blockage resulting from an unhealthy artery. [15] By way of nature, these risk factors build upon one another over the course of decades, making animal models truly non-replicable. To date, no studies have observed the effects of ischemic postconditioning on humans with a cluster of risk factors. This particular study aims to be the first to observe the response of rIpost in individuals suffering from a cluster of cardiometabolic risk factors.

Conclusion

Ischemia-reperfusion injury is a detrimental phenomenon that has been culprit of severe medical complications for many years. While certain cardioprotective mechanisms, such as rIpost, have been studied, there remains a large gap in the literature preventing researchers from translating data to real-life scenarios. Individuals plagued with multiple CVD risk factors are ultimately the ones who will be treated for cardiac-related events and subjected to IR-injury. In order to successfully implement ischemic conditioning into a clinical setting, we must better understand its underlying relation with CVD risk factors.

MATERIAL AND METHODS

The present study was completed at the Vascular Protection Research Laboratory through the College of Education and Human Sciences at South Dakota State University. Research had been approved by the Institutional Review Board #IRB-1507003-EXP.

Specific Aim – This quantitative experimental research study aimed to determine the effects of CVD risk factors on the ability of rIpost to protect against endothelial IR-injury in humans. With reference to the literature previously cited, it is hypothesized that an elevated burden of CVD risk factors will further the damage of endothelial IR-injury and greatly reduce the potential benefit of rIpost.

Participant Eligibility Screening – Ten participants (8 males; ages 23-66 years) enrolled in the study, having been recruited via flyers and letters of invitation (Appendices A & B). One participant had withdrawn from the study prior to the first session for unaffiliated reasons. Eligible participants were thoroughly explained each procedure and any potential risk as listed on the informed consent (Appendix C). Prior to the first session, each participant completed a 'PAR-Q & You' health history questionnaire (Appendix D) providing pertinent medical information. During the first session, baseline measurements were collected for use of determining each individual's CVD risk classification (Appendices E-H).

Inclusion Criteria

- Men and women, ages 20 - 79 years
- All races and ethnicities
- Physically inactive – Less than 30 minutes of physical activity 3 days•week^{-1} for the past 3 months
- No history of cardiovascular, pulmonary, and/or metabolic disease (exception of diabetes; n=1)
- Non-smoking

Exclusion Criteria

- Physically active – Achieving a minimum of 30 minutes of physical activity 3 days•week^{-1} for the past 3 months
- Known cardiovascular, pulmonary, and/or metabolic disease (exception of diabetes; n=1)
- Smoking, or having quit smoking in the past 6 months

Identification of the Burden of Cardiometabolic Risk

Cardiometabolic risk was assessed using the American Heart Association's risk classification of participants' current health and family history as recommended by the American College of Sports Medicine. [12,62] Participants were classified into either a raised burden group (≥2 risk factors and FRS ≥10%) or a low burden group (<2 risk factors) using the following guidelines:

- Age – Men ≥45 years of age & women ≥55 years of age
- Family History – Myocardial infarction, coronary revascularization, or sudden death before 55 years in father or other first-degree male relative, or 65 years in mother or other first-degree female relative
- Physically Inactive – Not having participated in a minimum of 30 minutes of moderate intensity physical activity at least 3 days•week^{-1} for the past 30 days
- Obesity – BMI ≥30 kg•m^2 *or* waist circumference >102 cm for men and >88 cm for women
- Hypertension – Systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg
- Dyslipidemia –
    - LDL-cholesterol ≥3.36 mmol•L^{-1}
    - HDL-cholesterol <1.03 mmol•L^{-1}

      (Negative risk factor if HDL ≥1.55 mmol•L^{-1})
    - Total cholesterol ≥5.17 mmol•L^{-1}
- Prediabetes – Impaired fasting blood glucose ≥5.56 mmol•L^{-1}

Baseline Measurements and CVD Risk Stratification

Anthropometric and Baseline Measurements

Auscultatory resting systolic and diastolic blood pressure and pulse rate were assessed using a GE Healthcare Carescape V100 monitor (GE Healthcare., Chicago, IL, USA) following 5 minutes of seated quiet rest. Body mass was measured to the nearest 0.1 kg using the Cosmed BOD POD integrated digital scale, and percent body fat was

assessed using air displacement plethysmography (BOD POD, Cosmed USA Inc., Chicago, IL, USA). Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Abdominal obesity was measured to the nearest 0.1 cm using the circumference at the narrowest region of the abdomen between the peak of the iliac crest and xiphoid process. Hip obesity was measured at the greatest circumference of the buttocks – both measurements taken while participants were standing erect with feet together. Participants completed an 8-minute Ebbeling walk test to assess their level of cardiorespiratory fitness with a predicted VO_2max (Appendix H).

Metabolic Measurements

Following an overnight fast, a 40μL sample of blood was collected from the index finger using a 21 G, 0.81 mm Fisherbrand lancet and capillary tube. Samples were analyzed using an Alere Cholestech LDX analyzer (Alere Inc., Waltham, MA, USA), assessing a full lipid profile consisting of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, and impaired fasting blood glucose.

CVD Risk Stratification

Thorough analysis of each PAR-Q document, as well as anthropometric and metabolic measurements provided the necessary information to quantify absolute number of risk factors. Participants were given one positive risk factor with reference to each criteria they met in the guidelines listed above. A negative risk factor was given for those who exceeded the guidelines for HDL-cholesterol. Online Framingham risk score (FRS) and ACC/AHA ASCVD risk calculators were used to assess the participant's likelihood of developing CVD in the next 10-years.

Experimental Design

A 2X2 randomized cross-over design was used to assess two independent variables. The first of which was risk burden, where participants were classified into either a low- or raised-burden group. The second independent variable served as the randomization of the order they received the sham (20 mmHg) and rIpost (220 mmHg) protocols.

Measurement of Microvascular Endothelial Function

Endothelial function can be measured multiple different ways among many different capillary beds. The two preferred methods of measurement are by determining vasorelaxing/dilating properties of an artery or capillary bed when prompted with pharmacological or flow-mediated vasodilation (reactive hyperemia). For the purpose of measuring endothelial function using reactive hyperemia as the stimulus, the brachial and conduit arteries and finger microvasculature are typically utilized. Using finger plethysmography has become more popular as the arm opposite of the stimulus (reactive hyperemia) may be used as a control. [63] Endothelial dysfunction was measured using a non-invasive, FDA-approved technology known as EndoPAT (pulse arterial tonometry), which utilizes fingertip sensors that measure changes in microvascular pressure created from arterial blood volume (EndoPAT 2000, Itamar Medica Inc., Caesarea, Israel).

During Phase 1, the participants lay supine in a bed while fingertip sensors were placed on each index finger, making sure the tip of the finger reached the back of the sensor. Each sensor would inflate to 10 mmHg below the individual's diastolic blood pressure, which was manually entered into the program. After 5 minutes of recording

baseline data of microvascular function, a rapid-inflation blood pressure cuff (Hokanson AG101/E20, D. E. Hokanson Inc., Bellevue, WA, USA) placed on the upper left arm was inflated to 220 mmHg for 5 minutes, occluding blood flow beyond the site of the cuff. The blood pressure cuff was the deflated following the 5 minutes of forearm occlusion, where an additional 5 minutes of data were collected to assess reactive hyperemia.

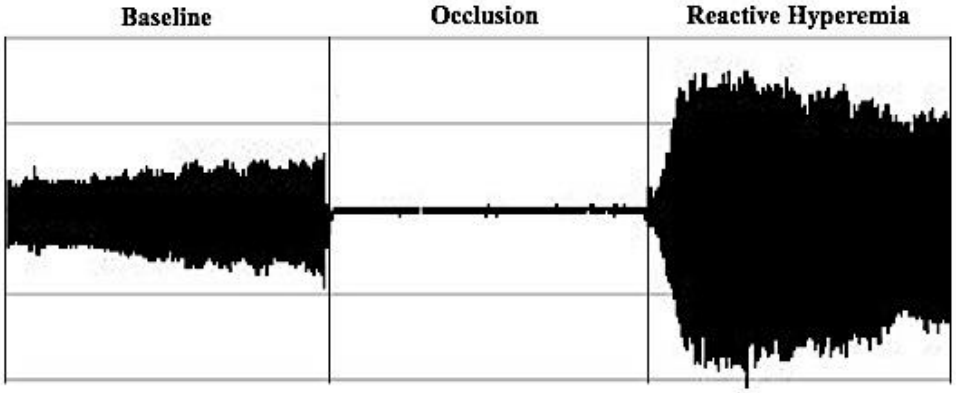

Figure 3. Representative tracing of baseline, occlusion, and reactive hyperemic phases measured in a low risk burden participant using PAT to assess microvascular endothelial function. Each block represents a 5-minute increment.

The total baseline assessment of microvascular endothelial function lasted approximately 15 minutes. Following baseline assessment, a 15-minute washout period was implemented, where participants were free to stand up and move around allowing the body to return to homeostasis prior to the induction of IR-injury and rIpost/sham.

Beginning Phase 2, participants were instructed to lay supine in a bed while a rapid-inflation blood pressure cuff on the upper left arm was inflated at 220 mmHg for 20 consecutive minutes. Immediately at the onset of left arm reperfusion, either an rIpost (220 mmHg) or sham (20 mmHg) protocol was implemented on the right arm consisting of a regulated cycle of three, 5-minute bouts of ischemia/reperfusion. Remote Ipost procedure was completed according to common protocols cited in the 2015 meta-analysis

from Le Page and colleagues. [64] Following the last 5-minute reperfusion period of rIpost/sham, Phase 3 was implemented to measure the reactive hyperemic response of the experimental protocols, mirroring the identical processes of Phase 1, while using new fingertip sensors (Figures 3 & 4; Appendix I).

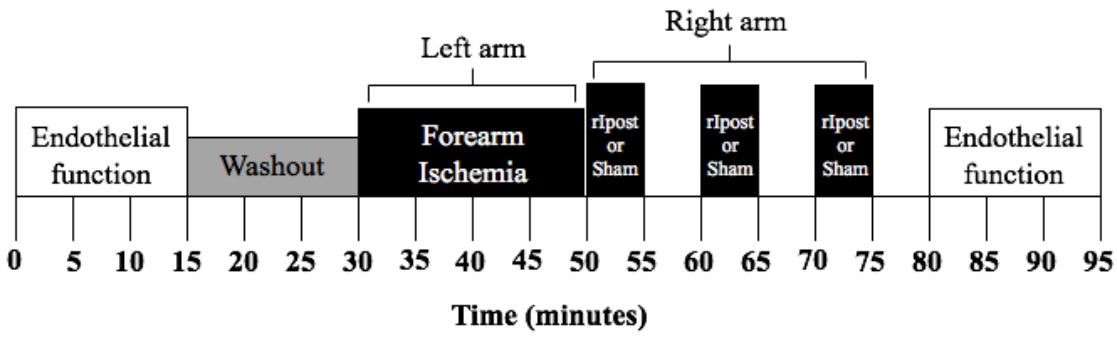

Figure 4. Representation of the rIpost (220 mmHg) and sham (20 mmHg) protocols. Endothelial function was measured using EndoPAT 2000.

Statistical Analysis

Data was analyzed using IBM SPSS Statistics Version 23.0 (Armonk, NY: IBM Corp). Reactive hyperemia index was calculated manually using EndoPAT 2000 software, as the ratio of reactive hyperemia post- IR-injury to pre- IR-injury of the occluded arm relative to the control arm. One-way ANOVA was used to determine significance in participant characteristics. Paired samples T-tests were run, analyzing heart rate and blood pressure over the course of each trial. Repeated measures ANOVA was used to determine differences in the RHI at 30-second intervals in the raised burden group. Bivariate correlations were used to determine the relationship between risk factors, FRS, ACC/AHA, and RHI at 90-120 seconds. Statistical significance was set at $p<0.05$, and data represent mean±SD unless otherwise noted. Any missing data was not used for analysis, although all attempts were made to retrieve missing data prior to analysis.

RESULTS

Participant Data

Ten participants enrolled in the study, however, one dropped out prior to the first session leaving us with a sample of nine individuals (7 males, 2 females; ages 23 – 66 years; 7 raised burden, 2 low burden). The two women (1 raised burden, 1 low burden) in the study were both pre-menopausal. Due to the size of the control group (n=2), the low burden characteristics will not be listed in a table. However, the two low burden participants (1 male, 1 female; age: 32±13 years; BMI: 23.5±0.4 $kg \cdot m^2$) were healthy individuals with normotensive blood pressure (SBP: 113±14 mmHg, DBP: 67±9 mmHg) and very low FRS and ACC/AHA risk scores (2.37±2.6 % and 0.6±0.7 %, respectively). Of the total sample of nine, only five participants completed both the sham and rIpost protocols due to limited supplies, all of whom were classified in the raised burden group. Table 1 lists the means and standard deviations of each variable measured for those in the raised burden group.

Table 1. Sample demographics within the raised burden group.

Variable	**Raised Risk Burden** (n=7)
Age, yrs	57±7
Sex, M/F	6/1
Body Mass, kg	101.1±17.5
BMI, $kg \cdot m^2$	31.1±4.4
Body Fat, %	31.9±8.9
Waist Circumference, cm	105.6±9.7
Systolic BP, mmHg	125±10
Diastolic BP, mmHg	79±7
Total Cholesterol, $mmol \cdot L^{-1}$	5.39±1.67
LDL-cholesterol, $mmol \cdot L^{-1}$	3.71±1.85
HDL-cholesterol, $mmol \cdot L^{-1}$	1.1±0.36
Triglycerides, $mmol \cdot L^{-1}$	1.26±0.58
Blood Glucose, $mmol \cdot L^{-1}$	5.63±0.50
VO_2max, $mL \cdot kg \cdot min^{-1}$	35.5±2.91
10-year Framingham Risk Score, %	18.5±12.8
ACC/AHA ASCVD Score, %	10.3±7.5

Data represent mean±SD. M, male; F, female; BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VO_2max, maximal oxygen consumption. ACC/AHA ASCVD, American College of Cardiology/American Heart Association Atherosclerotic Cardiovascular Disease.

With an average BMI of 31.1±4.4 $kg \cdot m^2$, the sample was classified obese with a risky high level of body fat (31.9±8.9 %), and excess abdominal obesity with waist circumference exceeding 102 cm. An average VO_2max of 35.5±2.91 $mL \cdot kg \cdot min^{-1}$, classified the raised burden group on the "fair/poor" borderline fitness category for maximal aerobic power, although two participants were unable to complete the Ebbeling walk test due to lower leg injury. [12] This sample was also, on average, classified with dyslipidemia and prediabetes, evidenced by elevated levels of total cholesterol, LDL-cholesterol, and blood glucose. Two participants were currently taking blood pressure and diabetes medication, one of whom was also taking cholesterol medication, however, every participant withheld all prescriptions/medications the morning of each session.

CVD Risk and Overall Burden

Table 2 depicts the percentage of participants with each specific cardiovascular disease risk factor within the two groups. The low burden group exhibited no risk factors, while, as expected, the raised burden group were plagued with many. Nearly each raised burden participant (86%) received a positive risk factor for age, as well as five of the seven (71%) raised burden participants had received a positive risk factor for the classification of prediabetes. It is important to note all participants, both low and raised burden, were classified as sedentary even though this risk factor is not accounted for in Table 2. Participants taking medications for a specific risk factor were classified with that risk factor even if the medications maintained their values within normal ranges. [12]

Table 2. Specific risk factor distribution within each group.

	Age # (%)	**Family History** # (%)	**Obesity** # (%)	**Elevated BP** # (%)	**High Total Cholesterol** # (%)	**High LDL** # (%)	**Low HDL** # (%)	**Elevated Triglycerides** # (%)	**Prediabetes** # (%)
Total n=9	6 (67)	1 (11)	3 (33)	2 (22)	3 (33)	4 (44)	3 (33)	2 (22)	5 (56)
Low Burden n=2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Raised Burden n=7	6 (86)	1 (14)	3 (43)	2 (29)	3 (43)	4 (57)	3 (43)	2 (29)	5 (71)

BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. All participants were classified as sedentary (<30 minutes of moderate intensity physical activity, 3 days•week^{-1} for the past 30 days).

Table 3 shows the distribution of cardiovascular risk factors among the seven participants in the raised burden group. The majority of participants (57%) had a total of three risk factors, not including sedentary lifestyle. Two participants were classified with metabolic syndrome (MetS) according to the definition from The International Diabetes Federation. Criteria for MetS includes a BMI ≥30 $kg \cdot m^2$, and/or a waist circumference >102 cm for men and >88 cm for women, along with two or more of the following risk factors (or current specific treatment): raised triglycerides, reduced HDL-cholesterol, raised blood pressure, and raised impaired fasting blood glucose. [65]

Table 3. Distribution of cardiovascular risk factors in raised burden population.

Risk Factors, #	**Raised Burden Group,** # (%)
0	0 (0)
1	0 (0)
2	1 (14)
3	4 (57)
4	1 (14)
5	0 (0)
6	1 (14)

Risk factors include age (men ≥45 yr, women ≥55 yr); family history (first degree male <55 yrs, first degree female <65 yrs suffering from heart disease, prior heart attack, or sudden death); BMI ≥30 $kg \cdot m^2$; SBP ≥140 mmHg; DBP ≥90 mmHg; elevated total cholesterol ≥5.17 $mmol \cdot L^{-1}$; elevated LDL-cholesterol ≥3.36 $mmol \cdot L^{-1}$; low HDL-cholesterol (men <1.03 $mmol \cdot L^{-1}$, women <1.29 $mmol \cdot L^{-1}$); elevated triglycerides ≥1.69 $mmol \cdot L^{-1}$; elevated impaired fasting blood glucose ≥5.56 $mmol \cdot L^{-1}$. Physical inactivity is not included in this count. Data represent sum of risk factors (relative %).

Blood Pressure and Heart Rate (sham and rIpost)

Throughout each session of sham and rIpost, heart rate and blood pressure were measured at three different points: baseline (after 5 minutes of quiet rest), immediately

preceding left arm IR-injury (30 minutes), and after rIpost/before the final assessment of endothelial function (80 minutes) (refer to Figure 4 and Table 4). There was no statistical significance in the fluctuation of HR, SBP, and DBP between the three testing points within each risk burden group (all $p>0.05$), however as expected the raised burden group showed consistently higher values throughout the course of each trial.

Table 4. Changes in heart rate and blood pressure during the assessment of microvascular function with rIpost.

	Low Risk Burden (n=2)			**Raised Risk Burden** (n=7)		
	Baseline	**30 min**	**80 min**	**Baseline**	**30 min**	**80 min**
HR, bpm	55±7	50±0	50±2	60±8	59±8	60±9
SBP, mmHg	114±13	106±6	109±11	129±18	131±15	130±16
DBP, mmHg	66±11	65±5	61±6	73±7	73±6	74±9

Data represent mean±SD. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; (all $p>0.05$).

Assessment of Microvascular Endothelial Function (sham and rIpost)

The low burden group only received rIpost, and when comparing the average RHI during baseline assessment to the average RHI following IR-injury and rIpost, there were no statistically significant changes. Figure 5 shows the RHI, at 30-second intervals, was slightly higher in the first 90 seconds following rIpost, however it appears to decline more rapidly than the RHI at baseline (all $p>0.05$).

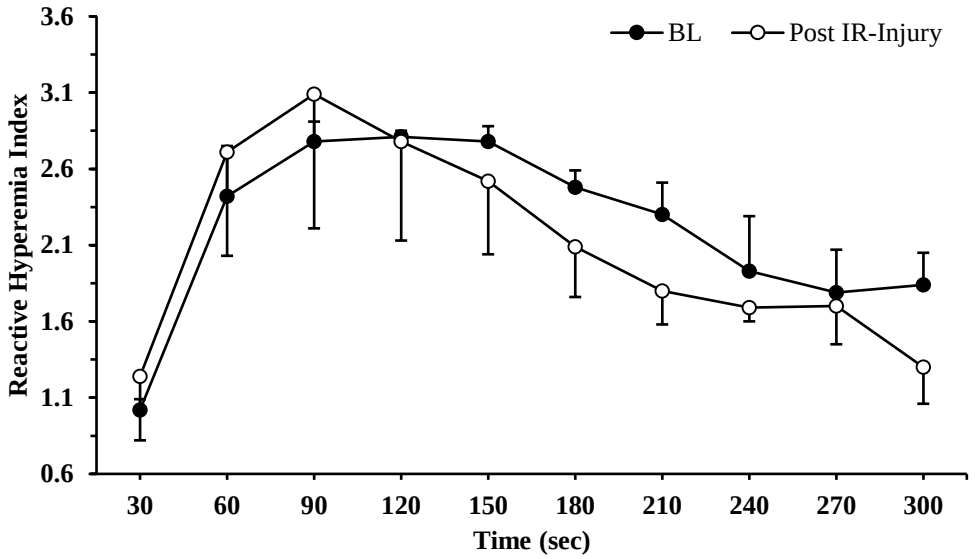

Figure 5. Reactive hyperemia index at 30-second intervals during baseline and following IR-injury during the rIpost protocol in the low burden group (n=2; all $p>0.05$).

With regards to the five raised burden participants who received both sham and rIpost protocols, endothelial function, as assessed by the RHI, showed a non-significant increase from baseline to post IR-injury in sham ($p=0.108$) and rIpost ($p=0.358$) (Figure 6).

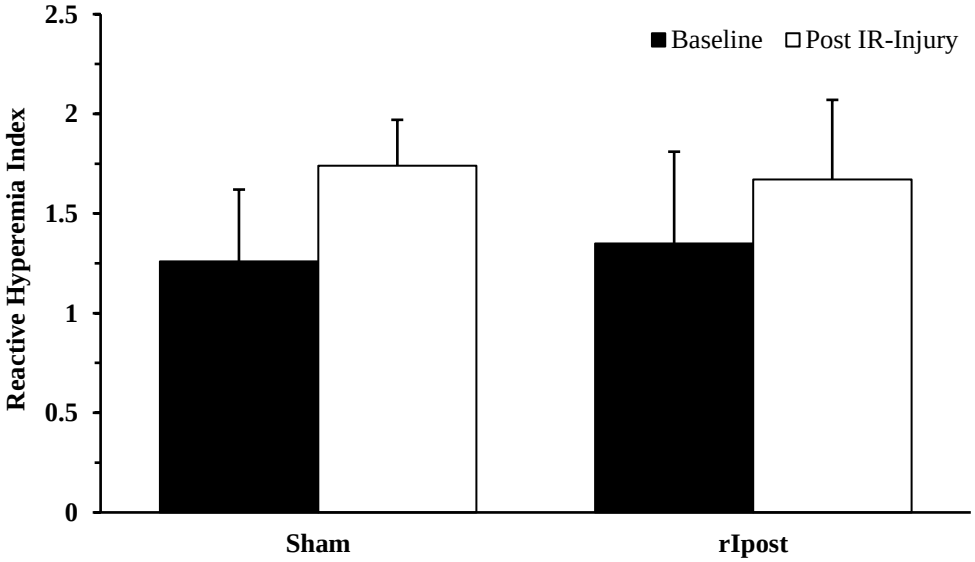

Figure 6. Change in reactive hyperemia index of the raised burden group (n=5) during the sham and rIpost protocols. The sham trial showed a 38.1% increase, while the rIpost trial showed a 23.7% increase (all $p>0.05$).

Due to a technical error with the computer program (EndoPAT 2000), we were unable to obtain data during the baseline assessment of the rIpost protocol for one raised burden participant. Therefore, we were only able to assess RHI at 30-second increments for four of the five raised burden participants who receive both the sham and rIpost protocols. Figure 7 depicts the average RHI at 30-second intervals during the baseline assessment of the sham and rIpost sessions for these four participants. There were no statistically significant differences between RHI during the baseline assessment of microvascular function in the sham and rIpost sessions (all $p>0.05$). The percent change in RHI from sham to rIpost over the course of the 300 seconds ranged from 12.99% (30 seconds) to 47% (240 seconds), with an average of 30.57% from 60-300 seconds.

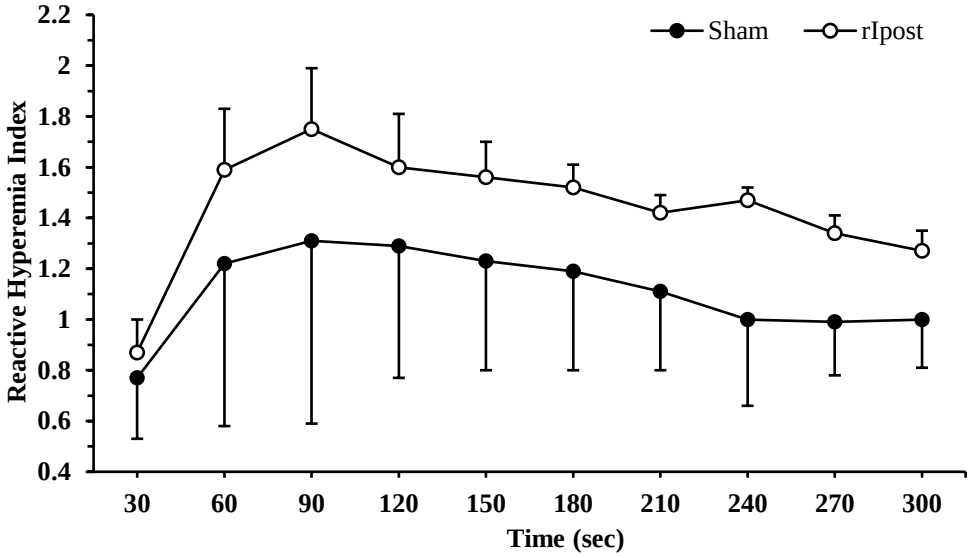

Figure 7. Reactive hyperemia index at 30-second intervals before IR-injury (baseline) with sham and rIpost trials in four raised burden participants (all $p>0.05$).

Similarly, the average RHI at 30-second intervals showed no statistically significant difference following 20 minutes of left arm IR-injury in the sham and rIpost protocols in these same four raised burden participants (Figure 8). The percent change in

RHI after IR-injury from sham to rIpost ranged from 0.93% (30 seconds) to 17.86% (180 seconds), with an average of 11.74% from 120-300 seconds.

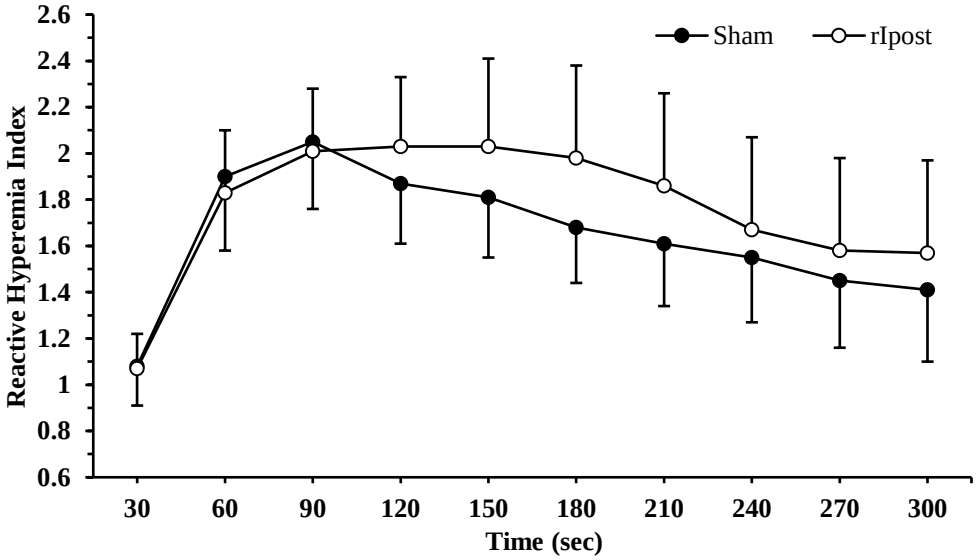

Figure 8. Reactive hyperemia index at 30-second intervals after IR-injury with sham and rIpost trials in four raised burden participants (all $p>0.05$).

CVD Risk and RHI at 90-120 Seconds of Hyperemia

When analyzing the reactive hyperemia index during the most potent time frame following rIpost, we observed an inverse relationship between the absolute number of risk factors. [66] As shown in Figure 9, data represent a strong negative correlation between peak reactive hyperemia and absolute number of risk factors (Figure 9-A, 90 seconds of hyperemia, $p=0.04$), as well as a negative correlation near statistical significance between RHI at 120 seconds of hyperemia and absolute number of risk factors (Figure 9-B, $p=0.061$). This data suggests that the greater number of risk factors is associated with a blunted reactive hyperemic capacity, despite attempts to induce rIpost.

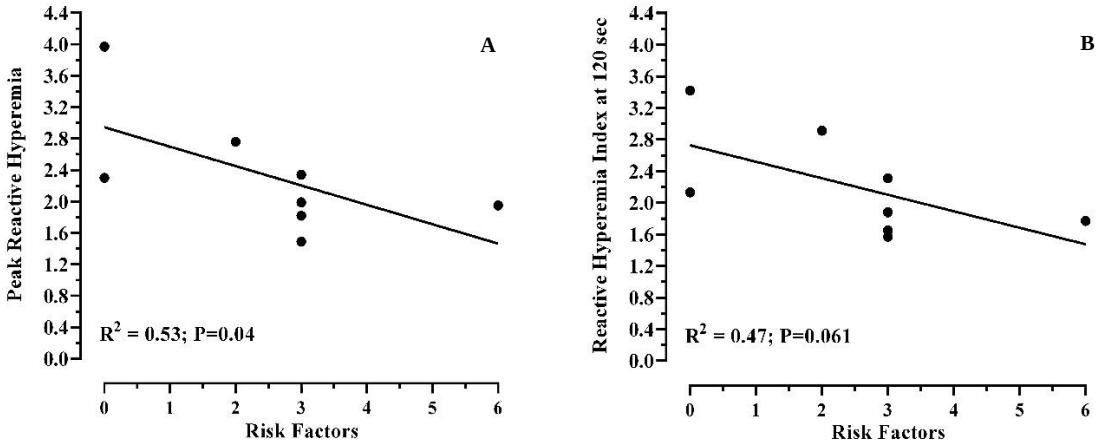

Figure 9. Correlations between total number of risk factors and **(A)** peak reactive hyperemia (90 seconds post-occlusion) and **(B)** reactive hyperemia at 120 seconds post-occlusion, both following endothelial IR-injury with rIpost. This data is missing one raised burden individual due to a technical error with EndoPAT 2000 (n=8).

While keeping focus on the most noteworthy window of RHI as suggested by Hamburg and colleagues (2008), there was also a non-significant negative correlation between peak RHI (90 seconds post-occlusion) with FRS (p=0.192) and the ACC/AHA risk score (p=0.16), shown in Figure 10-A. [66] Likewise, there was a non-significant negative correlation when comparing RHI at 120 seconds post-occlusion with FRS (p=0.238) and ACC/AHA risk score (p=0.193), as shown in Figure 10-B.

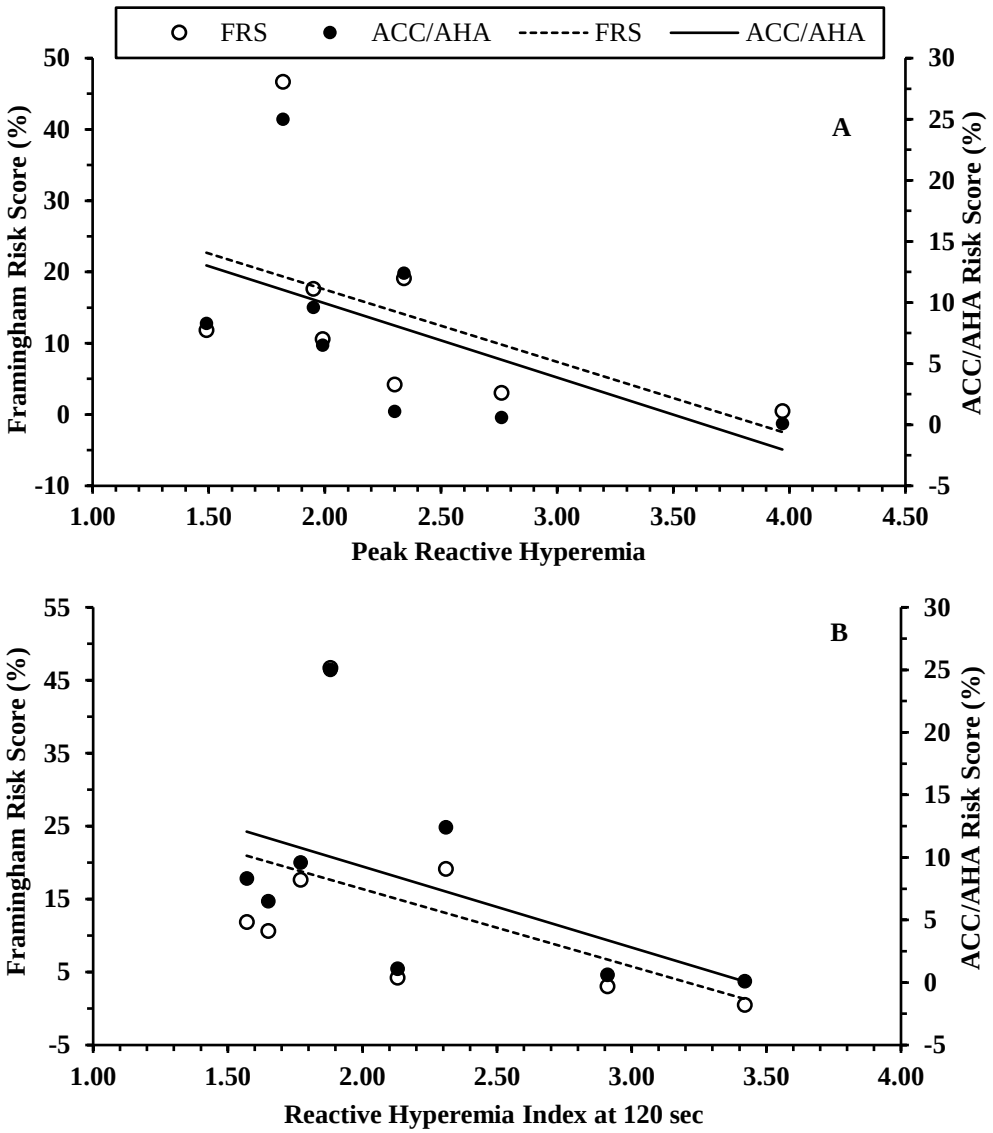

Figure 10. (A) Peak reactive hyperemia (90 seconds post-occlusion) correlated with Framingham risk score (R^2=0.27, *p*=0.192), and ACC/AHA ASCVD risk score (R^2=0.30, *p*=0.16). **(B)** RHI at 120 seconds post-occlusion correlated with Framingham risk score (R^2=0.22, *p*=0.238), and ACC/AHA ASCVD risk score (R^2=0.26, *p*=0.193).

DISCUSSION

Current data suggests there is an inverse relationship between CVD risk and the overall protection obtained through rIpost, supporting our hypothesis. Nine participants were split into two groups based on their absolute number of risk factors. The majority of the raised burden participants (≥2 risk factors) completed both sham and rIpost trials, while the low burden participants (<2 risk factors) were only able to complete the rIpost trial due to limited supplies. Remote Ipost was preformed for three, 5-minute cycles of ischemia and reperfusion at the right brachial artery, using a rapid-inflation cuff at 220 mmHg, immediately following 20 minutes of left arm ischemia. Vascular function was measured using EndoPAT 2000, before IR-injury and after sham and rIpost trials.

Main Findings

The main significant finding from this study occurs when directly comparing the absolute number of risk factors with peak reactive hyperemia at 90 seconds post-occlusion. The 90-120 second window of reactive hyperemia was deemed the most significant by Hamburg and colleagues (2008), where they observed the strongest relation between CVD risk factors and digital reactive hyperemic response. [66] Figure 9-A shows an inverse relationship between total number of risk factors and peak reactive hyperemia (R^2=0.53, p=0.04). This strong correlation suggests that those with a raised burden of risk factors do indeed experience a weakened hyperemic response when compared to those with fewer risk factors. The reactive hyperemic response in this case is directly associated with blunted protection from rIpost. This relationship continues through 120 seconds post-occlusion, (R^2=0.47, p=0.061) which may have likely reached statistical significance with a larger sample. Likewise, data shows an inverse relationship between both the

Framingham risk score and the ACC/AHA ASCVD risk score and RHI at 90 and 120 seconds post-occlusion (Figures 10-A and 10-B, $p>0.05$). This relationship suggests that as an individual's risk of developing cardiovascular disease within the next 10-years increases, the favorable vascular response to rIpost may be reduced. Framingham risk and ACC/AHA scores are directly impacted by CVD risk (age, blood pressure, total cholesterol, HDL-cholesterol, diabetes, and smoking status), therefore, we can make the assumption that risk factors are in part responsible for the influence of rIpost on RHI.

During the rIpost trial, the control group experienced an increase in RHI following IR-injury (Figure 5, $p>0.05$). Although this group only consisted of two participants, we still anticipated results indicating a more potent, longer lasting response to the postconditioning. On average, participants in the raised burden group experienced a slight increase in RHI following IR-injury during both the sham and rIpost protocols (Figure 6, $p>0.05$). We expected to see a decrease in RHI during the sham protocol as no conditioning was preformed and IR-injury was induced. Similarly, we noted an unexpected difference between the baseline measurements of endothelial function during the sham and rIpost protocols (Figure 7). This data only represents a portion of our raised burden sample (n=4), however it was expected that baseline values would remain similar between the two trials to ensure consistent measurements were achieved. Following IR-injury, these four raised burden participants showed no difference in RHI between the sham and rIpost trials for the first 90 seconds, however the RHI decline during the sham trial appeared to be more rapid compared to that in the rIpost trial (Figure 8, $p>0.05$). Data suggests that if the raised burden participants were receiving protection from rIpost, it may have had a latent activation occurring after the expected peak reactive hyperemia.

Mechanisms of Risk Factors and rIpost in Animal Models

The main objective of ischemic conditioning is to limit the damage caused from IR-injury. Individuals with CVD risk factors are prone to having weakened endothelial walls from increased vascular resistance, inflammation, and plaque build-up, overall leaving the vascular endothelium compromised and more susceptible to damage. Ischemic conditioning is a novel method of preventing damage, or greatly reducing damage of IR-injury, and has been studied in the remote form since remote ischemic *pre*conditioning was discovered in 1993. [67] There are still many unknowns pertaining to the specific mechanisms involved in remote ischemic conditioning, however we know it begins with the activation of neural and humoral pathways that promote cell survival programs in remote organs/tissues. Researchers have pinpointed two key cell survival programs known as the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways. Through the induction of staggered reperfusion, ischemic postconditioning is capable of releasing autacoid substances (e.g. adenosine, bradykinin, and opioids) that bind to G-protein receptor ligands and activate the RISK pathway, while TNFα activates SAFE. The activation of these pathways inhibit the opening of the mPTP, delay the abrupt restoration of intracellular pH, and activate anti-apoptotic and anti-necrotic pathways. [15,43] The inhibition of the mPTP is crucial, as its activation causes mitochondrial depolarization and the disruption of ATP production that leads to cellular necrosis.

Much of the current literature pertaining to rIpost has been completed using animal models that are either healthy, or exhibit acute, clinically-induced risk factors. Zhang and colleagues (2017) studied the effects of rIpost, performed on the bilateral

femoral artery, on neuroprotection following cerebral IR-injury in rats. [68] These original findings demonstrate that rIpost up-regulated mRNA and protein levels of fibulin-5, a protein known for its role in vascular remodeling. Researchers noted a distinct decrease in infarct size with the rIpost rats, along with preventing leakage of the blood-brain barrier. They propose these mechanisms were triggered by the RISK pathway through the activation of the PI3K/Akt intracellular signaling. Similarly, another study pinpointing the RISK pathway noted an increased activation of GSK-3β dependent cell survival signaling as the mode of protection induced from hepatic rIpost. [69] Researchers exposed rats to IR-injury at the site of the coronary artery, while preforming hepatic rIpost at the onset of coronary reperfusion. The rIpost remotely increased phosphorylation of cardiac GSK-3β, which overall reduced tissue damage, prevented apoptosis, and allowed for restoration of cardiac function. However, while these two studies may show favorable outcomes with rIpost, the caveat is that both were completed using healthy rats. The pitfall is that clinicians are still not seeing the desired results when translating these models to humans. Ghaffari and colleagues (2017) studied rIpost with participants receiving thrombolysis after being admitted to the hospital with STEMI. [70] These participants, representing the typical candidate of revascularization surgery, were plagued with many risk factors such as hypertension, diabetes, dyslipidemia, smoking, etc. Remote Ipost was applied on the arm opposite of that which was used for the delivery of the thrombolytic agent, and began at the onset of thrombolysis therapy. Researchers noted the rIpost group experienced a greater elevation in ST-segment resolution coupled with no significant differences in ischemia and infarct size when compared to the control group. Not only was the infarct size not statistically different following rIpost, but the

elevated ST-segment resolution suggests this group had experienced comparable damage as the control group, proving no perceived cardioprotection – a common trend in data when attempting to translate ischemic conditioning to clinical settings.

Clinical Application of rIpost

There are many practical applications for rIpost that set it apart from its counterparts ischemic pre- and perconditioning. As postconditioning is to be applied at the immediate onset of reperfusion, it is much more clinically relevant with its ability to be implemented following an ischemic event. This data serves as a preliminary standpoint, indicating there may in fact be a link between cardiometabolic risk and the efficacy of rIpost. The large majority of current research have studied animal models with acute, clinically-induced risk factors such as hypertension, dyslipidemia, and diabetes mellitus; however, these models are unable to replicate the damage induced by years of living with these risk factors such as those in Ghaffari's study (2017). [70]

Study Limitations

While our study was meticulously planned, it had certain limitations we were unable to account for. Although not statistically significant, the increase in RHI following occlusion during the sham protocol suggests 20 minutes of ischemia followed by 30 minutes of reperfusion may not have been enough time to elicit injury measurable in the microvasculature of the index fingers. We should have noted a distinct decrease in vascular function/reactive hyperemia following reperfusion in the sham trial, therefore PAT may have been unable to measure IR-injury in the microvasculature of the index fingers following our set duration of reperfusion. As for the participants, the available budget prevented us from acquiring essential materials, and overall limited the number of

participants we were able to recruit. The sample predominantly consisted of males, and even though there was no ethnic delimitation set on the study, the sample we were able to recruit was primarily Caucasian due to our geographical location of Brookings, SD. The study was limited to sedentary individuals only, as the act of exercise works in a similar conditioning manner to that we were trying to study. This study aimed to represent the typical candidate for clinical rIpost, as many participants (8 of 9) were taking some form of medication ranging from birth control to hypertension and dyslipidemia medications, which may induce pharmacologic conditioning. It is recommended to control for specific medications in the future, as well as metabolic diseases such as diabetes.

Conclusion

This study aimed to determine a relationship between CVD risk factors and the perceived vasculoprotection induced from rIpost. Data suggests there is in fact an inverse relationship between the two variables, demonstrating that as the absolute number of risk factors increase, the reactive hyperemic response following rIpost decreases. A larger sample would be required for future studies, however this current data serves as a valuable starting point in the link between CVD risk and rIpost.

APPENDICES

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

Brian.Hemenway@sdstate.edu
(303) 507-3194

Brian.Hemenway@sdstate.edu
(303) 507-3194

Contact Brian Hemenway
Brian.Hemenway@sdstate.edu
(303) 507-3194

Brian.Hemenway@sdstate.edu
(303) 507-3194

Brian.Hemenway@sdstate.edu
(303) 507-3194

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 study 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-1507003-EXP

Brian.Hemenway@sdstate.edu
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(303) 507-3194

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(303) 507-3194

APPENDIX B

South Dakota State University

Department of Health and Nutritional Sciences

Vascular Protection Research Laboratory

116 Intramural Building
South Dakota State University
Brookings, SD 57007-1497
Phone: 605-688-4082
Gary.vanguilder@sdstate.edu

To whom it may concern,

Members of the Vascular Protection Research Laboratory at South Dakota State University are currently recruiting research volunteers to participate in a valuable study focused on cardiovascular health. We are trying to learn new ways to enable the tissues in our body to resist damage caused by a vascular event (e.g., heart attack, stroke, organ transplantation).

Without your support to volunteer in this study, we would not be able to learn new and effective ways to prevent and treat heart disease – a disease that has been the leading cause of death in the United States every year since 1908. As such, I would like to invite you to participate in this important study. If you are interested, please call or email **Mr. Brian Hemenway at the contact information listed at the end of this letter.** Mr. Hemenway will determine if you meet eligibility requirements and answer any questions you may have.

The study will consist of three scheduled sessions based on your availability. In the first session, you will complete an assessment of blood pressure, cholesterol and blood sugar values, body composition, and a short walking test. In the final two sessions, we will measure how well your arteries in your arm function in response to physiological stress. We are looking for non-smoking men and women of all ages and ethnicities (>20 yrs) who do not regularly exercise. You will be provided all of your health information with a clinical explanation as part of the study.

If you are interested, please call, email, or text Mr. Hemenway to determine if you are eligible to participate.

Thank you for your interest to improve cardiovascular health by volunteering for this study. On behalf of all the members of my research team, we are extremely grateful for your participation.

Sincerely,

Gary P. Van Guilder, Ph.D.
Assistant Professor
Director, Vascular Protection Research Laboratory
Department of Health and Nutritional Sciences
South Dakota State University
Phone: 715-450-6734
gary.vanguilder@sdstate.edu

Brian Hemenway | 303-507-3194 | brian.hemenway@sdstate.edu

APPENDIX C

Participant Informed Consent Form
Participation in a Research Project
South Dakota State University
Brookings, SD 57007

Department of Health and Nutritional Sciences
Project Director: Dr. Gary P. Van Guilder Phone No: 605-688-4082
E-mail: gary.vanguilder@sdstate.edu Date: 06/01/15

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 2**

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 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. At least one week 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 2-2.5 hours.

Each of these trials will consist of four parts over 2-2.5 hours:

Part 1: measurement of blood vessel health
Part 2: forearm reperfusion injury
Part 3: remote ischemic postconditioning or control
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 a computer to inflate to 10 mmHg below your diastolic blood pressure (about 70 mmHg). The finger cuff contains sensors that detect changes in finger volume. 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. Following 5 minutes of baseline data acquisition, a blood pressure cuff will be inflated on the 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 3.

Part 2 - 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 3 - remote ischemic postconditioning or control: Postconditioning 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 postconditioning of your right arm. A pressure cuff will be inflated around your right 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 4 – repeated measurements of blood vessel health: Following forearm injury, we will repeat the measurement of your blood vessel health one additional time in the final hour of the procedure. This is the same measurement 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 postconditioning 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 postconditioning 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 identity 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 D

Physical Activity Readiness Questionnaire - PAR-Q (revised 2002)

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. Has your doctor 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.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

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 _____

SIGNATURE _____ 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 E

Vascular Protection Research Laboratory
Department of Health and Nutritional Sciences
Eligibility Screening Questionnaire

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.

I. GENERAL INFORMATION

Name			
Address			
City			
State			
Zip code			
Phone (home)			
Phone (work)			
Email			
Age			
Sex			
Hispanic Identity			
Race			
Emergency Contact		Phone	

II. MEDICAL DIAGNOSES

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

Condition	Yes	No	Condition	Yes	No
Heart attack			Pulmonary disease		
Chest pain			Heart valve problems		
Asthma			Heart murmur		
Anemia			Rheumatic fever		
Cardiovascular surgery			Phlebitis		
Currently pregnant			Emboli (blood clot)		
Emphysema or COPD			Coronary artery disease		
Diabetes (type 1 or type 2)			Stroke		
Cancer			Peripheral artery disease		
Raynaud's disease			Kidney disease		

Please list any additional special conditions (e.g., recent injuries or surgeries, and muscle or bone pain).

III. Major Chronic Disease Risk Factors

Risk Factor	Yes	No	Not sure
Are you a man over the age of 45 or a woman over the age of 55?			
Are you postmenopausal?			
Has your father or brother experienced a heart attack before age 55?			
Has your mother or sister experienced a heart attack before the age of 65?			
Has your doctor ever told you that you might have high blood pressure?			
Is your cholesterol above 200 mg/dl?			
Do you have prediabetes (blood sugar ≥100 mg/dl)?			
Are you physically inactive (less than 30 minutes of physical activity on at least 3 days/week)?			
Do you currently smoke or have you quit smoking in the last 6 months?			
Are you > 20 pounds overweight?			

IV. Signs and Symptoms of Chronic Disease

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

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 includes Type 1 or 2 Diabetes mellitus, thyroid disorders, renal or liver disease

V. MEDICATIONS

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

Medication	Dose and frequency

VI. SUPPLEMENTS

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

Dietary supplement	Dose and frequency

APPENDIX F

Acute Infection and Inflammation Questionnaire

Subject ID: _____ **Research Tech:** _____

Please answer the following questions to the best of your knowledge.

Within the past 2 weeks have you experienced... *(Please check the severity of the symptoms you experience.)*

General Symptoms:				
• Fever	___ None	____ Mild	____ Moderate	____ Severe
• Chills	___ None	____ Mild	____ Moderate	____ Severe
• Muscle Pains	___ None	____ Mild	____ Moderate	____ Severe
• Headache	___ None	____ Mild	____ Moderate	____ Severe
Nasal Symptoms:				
• Watery Eyes	___ None	____ Mild	____ Moderate	____ Severe
• Runny Nose	___ None	____ Mild	____ Moderate	____ Severe
• Sneezing	___ None	____ Mild	____ Moderate	____ Severe
• Congestion	___ None	____ Mild	____ Moderate	____ Severe
Throat Symptoms:				
• Sore Throat	___ None	____ Mild	____ Moderate	____ Severe
• Inflamed Tonsils	___ None	____ Mild	____ Moderate	____ Severe
• Yellow/Green Sputum	___ None	____ Mild	____ Moderate	____ Severe
Chest Symptoms:				
• Cough	___ None	____ Mild	____ Moderate	____ Severe
• Chest Pain	___ None	____ Mild	____ Moderate	____ Severe
• Congestion	___ None	____ Mild	____ Moderate	____ Severe
• Shortness of Breathe	___ None	____ Mild	____ Moderate	____ Severe

Within the past 2 weeks have you experienced.... *(Please check yes or no.)*

Pneumonia	_____ Yes	_____ No
Influenza	_____ Yes	_____ No
Stomach Flue	_____ Yes	_____ No
Bronchitis	_____ Yes	_____ No
Strep Throat	_____ Yes	_____ No
Urinary Tract Infection	_____ Yes	_____ No
Hepatitis	_____ Yes	_____ No
Jaundice	_____ Yes	_____ No
Measles	_____ Yes	_____ No
Whooping Cough	_____ Yes	_____ No
Other	_____	

APPENDIX G

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

Subject ID:	**Tech:**	**Date:**

Variable	**Value**
Age, year	
Height, cm	
Weight, kg	
BMI, kg/m^2	
Obesity status	
Waist circumference, cm	
Hip circumference, cm	
WHR	
WHR status	
Body fat, %	
Resting heart rate, bpm	
Resting blood pressure 1, mmHg	/
Resting blood pressure 2, mmHg	/
Blood pressure classification	

APPENDIX H

Ebbeling Sub-Maximal Walk Test

The single stage (8 minutes) treadmill walking test is a submaximal aerobic fitness test that estimates VO_2max. 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_2max (mL/kg/min).

Data Collection Sheet for the Ebbeling Walking Test

Name:	**Date:**
Resting HR:	**Resting BP:** mmHg
Age: yrs **Gender** M or F	**Body Mass:**
85% predicted HRmax: bpm	**Warm-up HR Training Zone:** **50% predicted HRmax =** bpm

Time (min)		**Speed (mph)**	**Grade (%)**	**HR (bp)**	**RPE**
Warm-up	1		0		
	2		0		
	3		0		
	4		0		
Workload	5		5		
	6		5		
	7		5		
	8		5		
	9*		5		
Recovery (reduce walking speed and set grade to 0%)**	1		0		
	2		0		
	3		0		
	4		0		
	5		0		

* 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

VO_2max is estimated using the following equation where:

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

VO_2 max = 15.1 + (21.8 x speed) – (0.327 x HR) – (0.263 x speed x age) + (0.00504 x HR x age) + (5.98 x 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.
HRmax = 190 bpm;
50% HRmax = 95 bpm;
70 % HRmax = 133 bpm:

Estimated VO_2max
= 15.1 + (21.8 x 3.6) - (0.327 x 159) - (0.263 x 3.6 x 30) +
(0.00504 x 159 x 30) + 5.98 (1)
= 43.2 mL/kg/min

APPENDIX I

Ischemic Postconditioning Protocol
Vascular Protection Research Laboratory

Subject ID:	**Tech:**	**Date:**	**Trial:**

Phase	**Time (min)**	**Protocol**	**Heart Rate, BPM**	**Blood Pressure, mmHg**
Baseline	0	Resting		**/**
1	30 min	Before rIpost/sham		**/**
2	80 min	30 min after reperfusion		**/**

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

Ischemic postconditioning trial:
Immediately following left arm reperfusion, inflate pressure cuff on right arm 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 postconditioning phase.

Sham control trial:
Immediately following left arm reperfusion, inflate pressure cuff on right arm to 20 mmHg for 5 minutes (simulating mock ischemic occlusions), followed by 5 minutes of cuff deflation – repeat 3 times. Record heart rate and blood pressure before and after the sham phase.

REFERENCES

1. Powers SK, Smuder AJ, Kavazis AN, Quindry JC. Mechanisms of exercise-induced cardioprotection. *Physiology (Bethesda).* 2014;29(1):27-38.
2. Ovize M, Baxter GF, Di Lisa F, et al. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovasc Res.* 2010;87(3):406-423.
3. Zaman J, Jeddi S, Daneshpour MS, Zarkesh M, Daneshian Z, Ghasemi A. Ischemic postconditioning provides cardioprotective and antiapoptotic effects against ischemia-reperfusion injury through iNOS inhibition in hyperthyroid rats. *Gene.* 2015;570(2):185-190.
4. Kaminski KA, Bonda TA, Korecki J, Musial WJ. Oxidative stress and neutrophil activation--the two keystones of ischemia/reperfusion injury. *Int J Cardiol.* 2002;86(1):41-59.
5. Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res.* 2006;70(2):181-190.
6. O'Keefe JH, Vogel R, Lavie CJ, Cordain L. Achieving hunter-gatherer fitness in the 21(st) century: back to the future. *Am J Med.* 2010;123(12):1082-1086.
7. Cordain L, Eaton SB, Sebastian A, et al. Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr.* 2005;81(2):341-354.
8. Lloyd-Jones DM, Leip EP, Larson MG, et al. Prediction of lifetime risk for cardiovascular disease by risk factor burden at 50 years of age. *Circ J.* 2006;113(6):791-798.
9. Hausenloy DJ, Boston-Griffiths E, Yellon DM. Cardioprotection during cardiac surgery. *Cardiovasc Res.* 2012;94(2):253-265.
10. Frasier CR, Moore RL, Brown DA. Exercise-induced cardiac preconditioning: how exercise protects your achy-breaky heart. *J Appl Physiol.* 2011;111(3):905-915.
11. Staat P, Rioufol G, Piot C, et al. Postconditioning the human heart. *Circ J.* 2005;112(14):2143-2148.
12. Lippincott W. *ACSM's Guidelines for Exercise Testing and Prescription. 9th ed.* Baltimore, MD: Wolters Kluwer Health; 2013.
13. Devan AE, Umpierre D, Harrison ML, et al. Endothelial ischemia-reperfusion injury in humans: association with age and habitual exercise. *Am J Physiol Heart Circ Physiol.* 2011;300(3):813-819.
14. van den Munckhof I, Riksen N, Seeger JP, et al. Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans. *Am J Physiol Heart Circ Physiol.* 2013;304(12):1727-1732.
15. McCafferty K, Forbes S, Thiemermann C, Yaqoob MM. The challenge of translating ischemic conditioning from animal models to humans: the role of comorbidities. *Dis Model Mech.* 2014;7(12):1321-1333.
16. Ferdinandy P, Schulz R, Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev.* 2007;59(4):418-458.

17. Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF, Schulz R. Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. *Pharmacol Rev.* 2014;66(4):1142-1174.
18. Otsuka T, Kawada T, Ibuki C, Seino Y. Association between high-sensitivity cardiac troponin T levels and the predicted cardiovascular risk in middle-aged men without overt cardiovascular disease. *Am Heart J.* 2010;159(6):972-978.
19. Kehl DW, Iqbal N, Fard A, Kipper BA, Landa AD, Maisel AS. Biomarkers in acute myocardial injury. *Transl Res.* 2012;159(4):252-264.
20. Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch Pathol.* 1960;70:68-78.
21. Jennings RB, Reimer KA. The cell biology of acute myocardial ischemia. *Annu Rev Med.* 1991;42:225-246.
22. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *New Engl J Med.* 2007;357(11):1121-1135.
23. Aggarwal S, Randhawa PK, Singh N, Jaggi AS. Preconditioning at a distance: Involvement of endothelial vasoactive substances in cardioprotection against ischemia-reperfusion injury. *Life Sci.* 2016;151:250-258.
24. Yao Y, Zhang B, Zhu H, et al. MG53 permeates through blood-brain barrier to protect ischemic brain injury. *Oncotarget.* 2016.
25. Del Sorbo L, Costamagna A, Muraca G, et al. Intratracheal Administration of Small Interfering RNA Targeting Fas Reduces Lung Ischemia-Reperfusion Injury. *Crit Care Med.* 2016.
26. Dorsa RC, Pontes JC, Antoniolli AC, et al. Effect of remote ischemic postconditioning in inflammatory changes of the lung parenchyma of rats submitted to ischemia and reperfusion. *Rev Bras Cir Cardiovasc.* 2015;30(3):353-359.
27. Greca FH, Goncalves NM, Souza Filho ZA, Noronha L, Silva RF, Rubin MR. The protective effect of methylene blue in lungs, small bowel and kidney after intestinal ischemia and reperfusion. *Acta Cir Bras.* 2008;23(2):149-156.
28. Hirano Y, Aziz M, Yang WL, Ochani M, Wang P. Neutralization of Osteopontin Ameliorates Acute Lung Injury Induced by Intestinal Ischemia-Reperfusion. *Shock.* 2016.
29. Zhao ZQ, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol.* 2003;285(2):579-588.
30. Okumi M, Tanabe K. Prevention and treatment of atypical hemolytic uremic syndrome after kidney transplantation. *Nephrology (Carlton).* 2016.
31. Barrera-Chimal J, Bobadilla NA, Jaisser F. Mineralocorticoid Receptor Antagonism: A Promising Therapeutic Approach to Treat Ischemic AKI. *Nephron.* 2016.
32. Pottecher J, Kindo M, Chamaraux-Tran TN, et al. Skeletal muscle ischemia-reperfusion injury and cyclosporine A in the aging rat. *Fundam Clin Pharmacol.* 2016.
33. Wang L, Shan Y, Ye Y, et al. COX-2 inhibition attenuates lung injury induced by skeletal muscle ischemia reperfusion in rats. *Int Immunopharmacol.* 2016;31:116-122.

34. Maxwell SR, Lip GY. Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options. *Int J Cardiol.* 1997;58(2):95-117.
35. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol.* 2012;298:229-317.
36. Yamazaki S, Fujibayashi Y, Rajagopalan RE, Meerbaum S, Corday E. Effects of staged versus sudden reperfusion after acute coronary occlusion in the dog. *J Am Coll Cardiol.* 1986;7(3):564-572.
37. Collard CD, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. *Anesthesiology.* 2001;94(6):1133-1138.
38. Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. *JAMA.* 1999;282(16):1523-1529.
39. Van Guilder GP, Hoetzer GL, Greiner JJ, Stauffer BL, DeSouza CA. Acute and chronic effects of vitamin C on endothelial fibrinolytic function in overweight and obese adult humans. *J Physiol-London.* 2008;586(14):3525-3535.
40. Van Guilder GP, Hoetzer GL, Smith DT, et al. Endothelial t-PA release is impaired in overweight and obese adults but can be improved with regular aerobic exercise. *Am J Physiol-Endoc M.* 2005;289(5):807-813.
41. Van Guilder, GP. *Vascular endothelial abnormalities associated with obesity.* (Dissertation). Colorado; University of Colorado; 2006.
42. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res.* 2000;87(10):840-844.
43. Krenz M, Baines C, Kalogeris T, Korthuis RJ. Cell survival programs and ischemia/reperfusion: Hormesis, preconditioning, and cardioprotection. In: Granger DN & Granger J, ed. *Colloquium series on integrated systems physiology: From molecule to function to disease*. Morgan & Claypool Life Sciences. 2013;5(3):1-122.
44. Murphy E, Steenbergen C. Ion transport and energetics during cell death and protection. *Physiology (Bethesda).* 2008;23:115-123.
45. Suleiman MS, Halestrap AP, Griffiths EJ. Mitochondria: a target for myocardial protection. *Pharmacol Ther.* 2001;89(1):29-46.
46. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol.* 2015;6:524-551.
47. Baines CP. The mitochondrial permeability transition pore and ischemia-reperfusion injury. *Basic Res Cardiol.* 2009;104(2):181-188.
48. Halestrap AP. A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. *Biochem Soc Trans.* 2010;38(4):841-860.
49. Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J Biol Chem.* 1992;267(13):8834-8839.
50. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev.* 2003;83(4):1113-1151.
51. Burckhartt B, Yang XM, Tsuchida A, Mullane KM, Downey JM, Cohen MV. Acadesine extends the window of protection afforded by ischaemic preconditioning in conscious rabbits. *Cardiovasc Res.* 1995;29(5):653-657.
52. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circ J.* 1993;88(3):1264-1272.

53. Baxter GF, Goma FM, Yellon DM. Characterisation of the infarct-limiting effect of delayed preconditioning: timecourse and dose-dependency studies in rabbit myocardium. *Basic Res Cardiol.* 1997;92(3):159-167.
54. Bhagat K. Endothelial function and myocardial infarction. *Cardiovasc Res.* 1998;39(2):312-317.
55. Hausenloy DJ. Signalling pathways in ischaemic postconditioning. *Thromb Haemost.* 2009;101(4):626-634.
56. Luna-Ortiz P, Torres JC, Pastelin G, Martinez-Rosas M. Myocardial postconditioning: anaesthetic considerations. *Arch Cardiol Mex.* 2011;81(1):33-46.
57. Lecour S, Suleman N, Deuchar GA, et al. Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). *Circ J.* 2005;112(25):3911-3918.
58. Gross ER, Hsu AK, Gross GJ. The JAK/STAT pathway is essential for opioid-induced cardioprotection: JAK2 as a mediator of STAT3, Akt, and GSK-3 beta. *Am J Physiol Heart Circ Physiol.* 2006;291(2):827-834.
59. Badalzadeh R, Mohammadi M, Yousefi B, Farajnia S, Najafi M, Mohammadi S. Involvement of Glycogen Synthase Kinase-3beta and Oxidation Status in the Loss of Cardioprotection by Postconditioning in Chronic Diabetic Male Rats. *Adv Pharm Bull.* 2015;5(3):321-327.
60. Wagner C, Ebner B, Tillack D, Strasser RH, Weinbrenner C. Cardioprotection by ischemic postconditioning is abrogated in hypertrophied myocardium of spontaneously hypertensive rats. *J Cardiovasc Pharmacol.* 2013;61(1):35-41.
61. Wu N, Zhang X, Jia P, Jia D. Hypercholesterolemia abrogates the protective effect of ischemic postconditioning by induction of apoptosis and impairment of activation of reperfusion injury salvage kinase pathway. *Biochem Biophys Res Commun.* 2015;458(1):148-153.
62. Goff DC, Jr., Lloyd-Jones DM, Bennett G, et al. 2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circ J.* 2014;129(25 Suppl 2):49-73.
63. Flammer AJ, Anderson T, Celermajer DS, et al. The assessment of endothelial function: from research into clinical practice. *Circ J.* 2012;126(6):753-767.
64. Le Page S, Bejan-Angoulvant T, Angoulvant D, Prunier F. Remote ischemic conditioning and cardioprotection: a systematic review and meta-analysis of randomized clinical trials. *Basic Res Cardiol.* 2015;110(2):11.
65. International Diabetes Federation. IDF worldwide definition of the metabolic syndrome. http://www.idf.org/metabolic-syndrome. Accessed March 21, 2017.
66. Hamburg NM, Keyes MJ, Larson MG, et al. Cross-sectional relations of digital vascular function to cardiovascular risk factors in the Framingham Heart Study. *Circ J.* 2008;117(19):2467-2474.
67. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circ J.* 1993;87(3):893-899.

68. Zhang W, Wang Y, Bi G. Limb remote ischemic postconditioning-induced elevation of fibulin-5 confers neuroprotection to rats with cerebral ischemia/reperfusion injury: activation of the AKT pathway. *Clin Exp Pharmacol Physiol.* 2017.
69. Yang S, Abbott GW, Gao WD, Liu J, Luo C, Hu Z. Involvement of Glycogen Synthase Kinase-3beta in Liver Ischemic Conditioning Induced Cardioprotection against Myocardial Ischemia and Reperfusion Injury in Rats. *J Appl Physiol (1985).* 2017.
70. Ghaffari S, Pourafkari L, Manzouri S, Nader ND. Effect of remote ischemic postconditioning during thrombolysis in STEMI. *Herz.* 2017.